# The isolation and characterisation of novel acidophilic thermoactinomyces isolates

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# Abstract.

Isolations from two pine forest soils (pH 3.5-4.5) yielded a total of 26 isolates from plates incubated at 50°C and identified as belonging to the genus *Thermoactinomyces*. Growth was carried out in shake flask and a fermenter. The isolates were confirmed as thermophilic, showing growth between 35 and 70°C with an optimum at 50/55°C, and as showing a degree of acidophily, with growth occurring between pH 4.5 and 6.0 when pH was controlled and between pH 4.5 and 7.0 without pH control. In both cases optimum growth occured at pH 5.0. Where growth occurred without pH control, a drop in culture pH occurred to between 4.2 and 4.9 and it appeared this drop was essential for growth to occur at pH 6.5 and 7.0. The pH range of these *Thermoactinomyces* isolates was found to differ considerably from *Thermoactinomyces* type and culture collection strains which characteristically showed growth only between pH 6.0 and 8.5. The pine forest isolates also differed from the type and culture collection strains in their colony morphology and in several physiological tests and it therefore appeared that these were novel *Thermoactinomyces* isolates. A range of extracellular enzyme activities were detected including protease, amylase, pullulanase, esterase and lipase. All were thermostable with protease, amylase and pullulanase activities showing half lives at 80°C of 10, 15 and 15min without calcium and 20, 120 and 100min with calcium, respectively. Esterase and lipase activities showed evidence of thermal activation and were completely stable at 90°C for up to 24h and 3h respectively. Both enzymes were totally denatured by treatment at 100°C for 10min. Amylase, pullulanase, esterase and lipase activities were found to be inducible and the possible presence of an amylase-pullulanase and an esterase-lipase complex was identified. Optimum enzyme activity occurred for protease at pH 5.5 and 70°C, amylase an pullulanase at pH 5.5 and 75°C, esterase at pH 6.0 and 65°C (the assay could not be conducted above 65°C) and lipase activity at pH 6.5 and 80°C respectively. Thermostability studies identified the possible presence of two proteases, one relatively thermostable to the other. This was supported by assays using specific chromogenic substrates which identified the thermostable enzyme as showing largely serine protease activity and the thermolabile enzyme as showing largely metalloprotease activity. A range of antibiotic screens identified gram positive activity only.

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2: MATERIALS AND METHODS

# CHAPTER 1

# INTRODUCTION

## 1.1: Actinomycetes.

Actinomycetes are a large and highly diverse group of Gram positive bacteria which exhibit morphologies ranging from coccus to permanent and highly differentiated mycelia (Goodfellow 1985). They inhabit a wide range of environments including soils, water and plant material such as composts and fodders etc. where they are saprophytes and play an important role in biodegradation and composting (Lacey 1973, Williams 1978, Al-Diwany & Cross 1978, Williams et al 1984, Williams 1985). They produce a number of extracellular enzymes important for this role including proteases, amylases, xylanases, cellulases, ligninases and esterases. They are also the largest producers of bioactive compounds, producing over two thirds of all naturally occurring antibiotics identified to date. Many of these products are produced by the genus *Streptomyces*. However, this does not necessarily mean that this genus is particularly active, although it is true that the genus produces a remarkable number and variety of products. In the past, attention has been concentrated on the more commonly isolated genera of the actinomycetes such as *Streptomyces* and *Micromonospora* (Cross 1982) and it is therefore no surprise that the majority of the products identified to date are derived from these genera. However, as the number of compounds identified increased, so the discovery of new products from these common genera became more and more difficult and as a result, attention has been broadened to include non-streptomycete genera such as *Nocardia*, *Actinomadura* and *Actinoplanes*, and the more recently discovered and classified genera such as the *Pseudonocardiaceae*. The targeting of new actinomycete genera has largely been made possible due to the

recent advances in taxonomic techniques, including chemotaxonomy and molecular techniques, which have supplied the information required for their selective isolation and accurate identification and classification (O'Donnell 1988, Goodfellow & O'Donnell 1989). The success of this shift in attention can be seen in the expansion in the number of products derived from non-streptomycete genera in the past 20 years. In 1974, the percentage of non-streptomycete derived products stood at approximately 5% of the 2000 or so products then identified, however this percentage has increased steadily so that by 1988 the figure had risen to approximately 21% of 5000 or so products (Table 1.1; Nisbet 1992, Okami & Hotta 1988 and Goodfellow & O'Donnell 1989). This does not mean that new products are not being isolated from streptomycetes, indeed Table 1.1 shows that the number of streptomycete derived products has continued to increase, thus indicating the continued usefulness of the genus. Due to the continued usefulness of the older genera, the largely unexplored potential of the newer genera as well as the continued isolation of new and novel species and genera, the future for new product discovery in the *Actinomycetales* appears good.

## 1.2: Microbial Products

The range of microbial products currently of commercial interest is extensive. Table 1.2 shows a brief list of some of these. The largest and most important area is that of pharmaceuticals with particular reference to the production of bioactive compounds.

**Table 1.1**

Number of antibiotics produced by selected actinomycete genera.

| Genus | Number of Antibiotics 1974 | 1980 | 1984 | 1988 |
|---|---|---|---|---|
| **Older genera** | | | | |
| *Streptomyces* | 1934 | 2784 | 3477 | 4876 |
| *Micromonospora* | 41 | 129 | 269 | 398 |
| *Nocardia* | 45 | 74 | 107 | 262 |
| *Actinomadura* | 0 | 16 | 51 | 164 |
| *Actinoplanes* | 6 | 40 | 95 | 146 |
| *Streptoverticillium* | 19 | 41 | 64 | 138 |
| *Streptosporangium* | 7 | 20 | 26 | 39 |
| *Dactylosporangium* | 0 | 4 | 19 | 31 |
| *Microbispora* | 4 | 6 | 6 | 10 |
| **Newer genera** | | | | |
| *Saccharopolyspora* | - | 4 | 33 | 44 |
| *Actinosynemma* | - | - | 5 | 14 |
| *Streptoalloteichus* | - | 3 | 4 | 12 |
| *Kitasatosporia* | - | - | - | 11 |
| *Kibdelosporangium* | - | - | - | 7 |

Table from Goodfellow & O'Donnell (1989).

**TABLE 1.2.**

List of commercially important microbial products.

| Sector | Product |
|---|---|
| Chemicals:-organic (bulk) | Organic acids (citric acid) |
| Chemicals:-organic (fine) | Enzymes (protease, amylase, lipase) |
| | Polymers (polysaccharides) |
| Chemicals:-inorganic | Metal leaching (Mining) |
| Energy | Production of ethanol from plant wastes |
| | Use of Biogas |
| Biodegradation | Breakdown of pollutants |
| | Sewage treatment |
| Food industry | Dairy products |
| | Beverages |
| | Bread products |
| | Food additives (colours+flavours) |
| | Amino acids |
| | Vitamins |
| | Starch products |
| | Glucose and high fructose syrups |
| Agriculture | Animal feedstuffs (single cell proteins) |
| | Biological control agents |
| | Nitrogen fixation innoculants |
| Pharmaceuticals | Antibiotics |
| | Regulatory proteins (insulin, interferon) |
| | Vaccines |
| | Enzyme inhibitors |

Data from Higgins (1985).

## 1.3: Bioactive Compounds

A bioactive compound can basically be defined as any compound that possesses biological activity. This loose definition therefore includes a wide range of compounds with a wide range of functions, for example antibiotics (ie: antibacterials, antifungals, antivirals, antitumours, anticancers, antihelminthics, acaricides, insecticides and herbicides), pigments, toxins, pheromones, hormones, enzyme inhibitors, immunomodulating agents, receptor agonists and antagonists, and specialised drugs such as anti-cholesterol and anti-inflammatories (Demain 1992, Nisbet 1992). These structures possess a wide range and diversity of chemical structures, for example alkaloids, aminoglycosides, anthracyclines, glycopeptides, *beta*-lactams, macrolides, nucleosides, peptides, polyenes, polyethers, polyketides, steroids, and tetracyclines (Okami & Hotta 1988). They are produced by a wide range of different organisms including bacteria, fungi, algae, plants and animals and they are generally synthesised during secondary metabolism, through pathways relating to the metabolism of sugars, shikimate, acetate/malonate, nucleosides and amino acids as well as via composite pathways (Okami & Hotta 1988). They are generally accumulated from late exponential phase to the stationary phase of growth and it appears that structural variation occurs in the later steps of the biosynthetic pathway. The later steps of the pathway therefore tend to vary even among strains producing compounds of the same group. Conversley however, the earlier steps in the pathway may be common among strains producing chemically related compounds.

Microbial producers include fungi, bacilli, pseudomonads and myxobacteria, however the most prolific producers are the actinomycetes, producing over two thirds, more than 4000, of the naturally occurring antibiotics identified to date (Okami & Hotta 1988). The search for and discovery of bioactive compounds is probably the largest and most important area of the pharmaceutical industry, however several problems exist in the continued discovery of new and novel products (Bu'lock 1982).

**1.4: The Search for Bioactive Compounds.**

The search for bioactive compounds generally involves three basic steps: (Okami & Hotta 1988), 1: isolation and cultivation of organisms from natural and man made environments, 2: screening of organisms for bioactivity, and 3: chemical characterisation and identification of antibiotics detected. It is therefore the first two stages which determine the product that is isolated and it is these two stages which determine the search and discovery of new and novel products. In the past, the search for bioactive compounds has largely been empirical with the isolation and screening of large numbers of organisms in the hope that new and novel products are eventually found (O'Donnell 1988). This has undoubtedly proved successful in the past, however with the large number of products currently identified, it is proving increasingly difficult to isolate new and novel products from this pool of organisms and an increasing amount of time and money is being wasted on the re-identification of known organisms and products. For this reason, attention is being turned towards new product discovery. There are essentially three methods which can be used in the isolation of new bioactive compounds, 1: genetic and

chemical manipulation of existing products to produce new derivatives or completely new compounds, 2: development of new screening systems targeted to identify new compounds, and 3: isolation and screening of new, previously unscreened organisms (Zahner et al 1982, Reichenbach et al 1988).

### 1.4.1: Genetic and Chemical Manipulation

Existing products can be chemically or genetically manipulated so that either new derivatives or totally new compounds are formed. Many methods are available (Okami & Hotta 1988), including directed biosynthesis, mutational biosynthesis, the bioconversion of foreign antibiotics, the use of mutants with blocked or altered biosynthetic pathways, cell fusion and in-vivo and in-vitro genetic manipulation. Directed biosynthesis is where specific compounds are supplied to a producing organism so that they are incorporated into the final structure of the product to produce a new variant. This procedure has worked for a wide variety of antibiotics, perhaps the most common being the penicillins. Mutational biosynthesis is where a producing organism, which is blocked at a specific point in the biosynthetic pathway, is supplied with analogues of the compound in which the organism is deficient so that new derivatives are produced. The low substrate specificity of many of the biosynthetic enzymes involved in antibiotic production can be used so that known products of one organisms can be converted to a new product by the biochemical pathway of another. For example the modification of kanamycin A and B by a gentamycin-producing strain of *Micromonospora* (Okami & Hotta 1988). Mutants that have a blocked or modified biosynthetic pathway may accumulate

novel intermediates not accumulated by the original strain and so go on to produce a novel product. For example the production of rifamycin P, Q and R by a mutant of a rifamycin B producing *Amycolatopsis* strain (Okami & Hotta 1988). Cell fusion creates a heterokaryon, mutant or recombinant clone, in the hope that new and novel products are produced. In vivo recombination, which can either be intraspecific or interspecific, involves the crossing of two producing organisms to create recombinants in the hope that new derivatives or products are produced as a result. In vitro manipulation involves the cloning of gene segments of one producing organism into another in the hope of producing new derivatives or products.

### 1.4.2: Development of New Screening Procedures

Organisms are screened in order to pick out any biologically active compounds. It is therefore obvious that only by developing new and novel screening procedures can new and novel products be identified. In the past, screening was largely concerned with detecting some kind of activity against a target organism, however this method of screening does not readily allow discrimination between new and already known compounds (Nolan & Cross 1988). The increase in information concerning the physiology and biochemistry of microorganisms, particularly of pathogens and the relationship they have with their host, has led to the development of far more sensitive and subtle screening procedures and to the development of more target directed screening where an important pathway, reaction or compound is targeted, for example the detection of pharmacologically active compounds such as inhibitors of specific target enzymes and

immunomodifying agents (Hamill 1982, Pechzynska-Czoch & Mordarski 1988, Umezawa 1988). With the increased subtlety and sensitivity of screening procedures, the range of targets has also rapidly expanded from including mainly anti-bacterial and fungal agents etc., to include screening for anti-cancer agents, anti-inflammatories and specialised drugs such as these for heart disease and high cholesterol. New screening procedures can therefore be developed either by searching for new targets, such as new pathways, enzymes or other important compounds, or by developing new and unusual screening tests in order to select for new or rare types of activity (Fleming et al 1982). An alternative approach is to alter the media in which the organisms are grown prior to screening. Organisms are usually grown in media which consist of carbon and nitrogen sources, and a salts medium containing phosphate, cations such as Ca, Mg and Na and trace elements such as Co, Cu, Fe, Mn and Zn. However, the nutritional make-up of a medium plays an important role in determining which antibiotics are produced (Okami & Hotta 1988) and thus by altering these constituents, new and novel activities could be identified. A similar approach is to alter the incubation conditions such as pH, aeration and temperature. These are often optimal for growth, however by altering these conditions new activities can be revealed. A further factor is the type of media used, for example broth, agar or solid state. Many actinomycetes for example show production on agar plates but not in liquid while many fungi show increased production on solid media.

### 1.4.3: Isolation and Screening of Novel Organisms

The screening of new and novel organisms represents potentially the most valuable source of novel bioactive compounds. It is widely accepted that not only does the number of species screened form only a relatively small proportion of the number of species that have been isolated, but also that the number of species isolated forms a very small proportion of the number of species that exist in a given environment. The potential for the isolation and screening of new and novel organisms is therefore enormous, however even modern isolation and screening procedures are often limited to the examination of organisms which are easy to isolate and cultivate and which are relatively fast growing. The result is an over-emphasis on aerobic, heterotrophic organisms which grow well at ambient temperature and neutral pH (Goodfellow & O'Donnell 1989). The basic approach in the isolation and screening of new and novel organisms is to introduce diversity and novelty into the screens in the hope that new and novel products are detected (Nolan & Cross 1988). One approach is to diversify into non-microbial organisms such as plants, insects and marine animals (Rinehart 1992, Waterman 1992). While it has long been known that bioactive compounds can be isolated from these areas, more concentrated studies are only now being attempted. For the isolation of new and novel microorganisms, two basic approaches exist, one is to target new or unusual environments, for example locations with unusual fauna and flora such as rain forests, Antarctica, Australia etc., or extreme environments such as hot springs, deep sea vents, soda lakes and acid soils etc., and the second is to target specific microorganisms or groups of microorganisms which have either

not been screened before or which show a novel or unusual physiology. For example the fungi are known to produce a wide variety of products and, like the actinomycetes, show extensive biodiversity and niche exploitation, common association with other organisms, and are abundant in numerous environments. Like the actinomycetes they therefore show a great potential for new product discovery, however, unlike the actinomycetes they still remain largely unexplored for bioactivity (Nisbet 1992). The actinomycetes themselves, although generally well studied, still possess numerous taxa which are new and relatively unexplored. The myxobacteria also show an unusual physiology and have also been found to produce numerous novel bioactive compounds (Reichenbach et al 1988) and yet have remained largely unexplored for the production of bioactive compounds. A particularly interesting area is that of organisms from extreme environments, for example thermophilic, acidophilic, alkalophilic, halophilic and barophilic organisms (Sharp & Munster 1986, Kelly & Deming 1988, Edwards 1990, Ingledew 1990, Cowan 1992a+c, Norris & Ingledew 1992, Adams 1993) and anaerobic microorganisms (Lowe et al 1993). Although the number of products identified from such organisms is small this is perhaps not surprising considering the lack of attention they have received. Of particular interest are the extremophilic actinomycetes such as thermophiles, acidophiles and alkalophiles which combine unusual physiology with the background of antibiotic production shown by other actinomycetes. The scope for the isolation of new and novel organisms is therefore enormous and selection of isolates can be made at five separate

stages (Williams & Wellington 1982, Wellington & Cross 1983, Cross 1982).

**(i): The selection of material from which isolates are to be obtained.**

The types of environment commonly used include soils, fodders and composts, dung, sediments, water, plant material and animals, most commonly from intestines. Each particular environment possesses its own specific properties such as organic and moisture content, pH, temperature, salinity, aeration etc., the exact make up of which determines the microbial flora it possesses. The choice of environment therefore determines to a large extent the type of organisms isolated. However, relatively little is known about the ecological distribution of many but the better known organisms such as the streptomycetes (Goodfellow & Simpson 1987) and thus it can prove difficult to predict what organisms are present in which environments. The choice of material may therefore simply involve an undirected approach whereby new environments are tested simply to see what turns up or it may involve a more directed approach whereby new environments are tested for specific organisms or groups of organisms. In the case of the extreme environments, one environment may select for organisms which are unusual in two different physiological areas, for example thermoacidophiles and thermohalophiles from acidic and saline hot springs, and alkalophilic halophiles from soda lakes. In each case the hope is that the new environment may possess novel organisms which may in turn yield novel metabolites (Nolan & Cross 1988).

(ii): The use of selective pre-treatments.

A variety of selective pre-treatments may be used in order to increase the likelihood of isolating the desired organisms. For example heat drying soil is used to reduce competing bacteria in the isolation of actinomycetes, filtration through 0.45um filters is used to concentrate cells from water and heating, for example pasteurisation, is used to select for organisms with endospores.

(iii): The use of selective media.

The selectivity of isolation media can be influenced by a wide range of factors including nutrient composition, the selective antibiotics used, and a range of physico-chemical conditions including pH, NaCl etc: and while this information is well established, selective isolation programmes have in the past, and to a certain extent still are today, been developed without regard for the physiological and biochemical properties of the target organisms. For example the different selective media and antibiotics used for the isolation of actinomycetes (El-Nakeeb & Lechevalier 1962, Lingappa & Lockwood 1962, Kuster & Williams 1964, Williams & Davies 1965, Orchard & Goodfellow 1974, Preobrazhenskaya et al 1978, Athalye et al 1981).
However, the recent advances in taxonomic studies have provided the necessary information concerning nutritional requirements, antibiotic tolerances and pH and salt requirements of organisms which is required for the design of selective media (Goodfellow & O'Donnell 1989). Physical selection methods can also sometimes be employed, for example the selective isolation of actinomycetes through the use of an appropriate agar medium

overlaid with a 0.22-0.45um-pore cellulose ester membrane which allows the actinomycete hyphae to penetrate to the underlying agar but prevents non-actinomycete bacteria from doing so (Hirsch & Christensen 1983).

**(iv): The use of incubation conditions.**

Selectivity can also be influenced by the incubation conditions used, for example aeration, temperature and length of incubation. Mesophilic organisms are generally isolated at temperatures between 25 and 37°C, however there is also scope to isolate psychrophilic organisms (below 15-20°C), thermophilic organisms (45-65°C) and extremely thermophilic/caldoactive eubacteria and archaebacteria (above 65°C). Incubation times are usually between 7 and 14 days. By increasing incubation times however, many of the slower growing organisms can be isolated. This may simply be because they are slow growing, or it may be that they can only grow once other competing organisms have died, or that the growth of other species modifies the medium in some way which allows them to grow, for example by supplying growth factors such as siderophores, or by removing toxic substances from the medium (Nolan & Cross 1988). By lowering the aeration, anaerobic or microaerophilic organisms, which have been largely ignored in the screening for bioactive compounds (Lowe et al 1993), can be selected.

**(v): Colony selection.**

This is often the most time consuming stage of the isolation procedure (Williams & Wellington 1982). Many criteria exist for isolate selection, for example, the members of specific targeted

taxa, isolates which show a specific physiology such as thermophiles, acidophiles etc.:, pigment producers, unusual looking isolates etc. Whatever the selection criteria, accurate identification of the isolates is essential in order that the desired isolates are selected and undesired isolates are not. The accurate identification of targeted isolates requires considerable taxonomic information including morphology, physiology and biochemistry, information which can now be supplied thanks to the recent advances in chemotaxonomic and molecular identification and classification techniques (Wellington & Cross 1982, Stackebrandt 1985, O'Donnell 1988, Goodfellow & O'Donnell 1989). Another factor which is important in the selection process is the definition of a novel organism. The criteria met by novel organisms vary greatly, for example at one extreme there is the isolation of organisms which form the basis for completely new taxa, such as the isolation of the archaebacteria *Sulfolobus acidocaldarius* (Brock et al 1972), *Methanothermus* (Stetter et al 1981) and *Thermoproteus* (Zillig et al 1981), while at the other extreme there is the isolation of organisms which differ from previously described species in little other than the ability to produce a novel secondary metabolite, for example the isolation of *Streptomyces sulfonofaciens* (Williams et al 1984). Amongst the new groups of organisms being targeted are the so called 'rare' organisms and those organisms which show a unique morphology and/or physiology, for example the extremophiles. The specialised physiology shown by the extremophiles can often be used in their isolation, for example the isolation of acidophiles from acid environments using acidified isolation plates.

Each stage of the isolation process therefore requires considerable information concerning the ecological distribution, nutrient and growth requirements, tolerances and information concerning classification so that the appropriate selective isolation procedure can be devised and the organisms accurately identified. The isolation of organisms from large numbers of environmental samples can be a time consuming, labour intensive and therefore costly process. A number of approaches have therefore been studied to reduce time. One is to screen environments directly for the targeted antibiotic activity so that an isolation is only carried out on those which show positive. A similar approach is to screen environments directly for the presence of the desired target organism, for example by the use of specific phage and/or DNA probes, again in order to select for isolation only those environments which are known to possess the desired organisms.

### 1.5: Screening for bioactivity.

There are many approaches to screening, at one extreme is the largely undirected approach whereby large numbers of unidentified isolates are screened. However, this can be a laborious, time-consuming and expensive procedure. To justify this, each isolate screened is subjected to as large a number of tests as possible (Nolan & Cross 1988). Isolates which prove positive in these screens can then be selected and put through a series of more directed screens in which more specific test systems are applied. At the other extreme is the more directed approach of screening specific novel organisms. Well studied organisms can also be subjected to new screening systems.

## 1.6: Microorganisms from Extreme Environments.

As already mentioned, microorganisms from extreme environments represent a large, diverse and largely unexplored group of organisms which often possess unique and unusual physiologies which make them of considerable importance in the search for new and novel products.

### 1.6.1: Thermophilic Bacteria

Thermophilic organisms characteristically show optimal growth at or above 50°C, although many are capable of growth below this temperature (Edwards 1990). Three groupings, based on temperature ranges for growth, have been proposed to separate organisms lying within this classification (Cowan 1992b): thermophiles, which grow between 30 and up to at least 60°C with an optimum of at least 50°C; extreme thermophiles, which grow between 40 and up to at least 70°C with an optimum of at least 65°C; and hyperthermophiles or ultrathermophiles which have no defined minimum growth temperature but which grow optimally above 70°C and up to 100°C and may grow up to 115-120°C. Environments of 40-70°C, from which members of the first two groups, thermophiles and extreme thermophiles, are found, are quite common and include self heating environments such as fodders and decomposing composts, and solar heated soils (Brock 1986). Thermophilic organisms isolated from these environments include fungi, aerobic and anaerobic spore formers, and actinomycetes. Hyperthermophiles are only found in limited environments where extremely high temperatures are encountered, often up to and sometimes above 100°C. Such

environments include geothermal hot springs and both shallow and deep sea vents (Brock 1986). As well as extremely high temperatures, these environments often impose additional physiological stresses (Edwards 1990). For example deep sea vents are subject to high pressures and thus organisms inhabiting them must be barophilic as well as extremely thermophilic, whereas organisms isolated from soda lakes must be halophilic as well as alkalophilic. Hot springs can be split into two basic types, those which are highly acidic, often with a pH of between 1.0 and 2.5, which are rich in sulphur and iron but otherwise poorly mineralised and whose buffering is based around sulfuric acid, and those which possess a pH of between 6.0 and 8.5 and which are highly mineralised and whose buffering is based around bicarbonate (Cowan 1992b). Organisms isolated from acid hot springs must therefore be thermoacidophiles whereas for the neutral hot springs, salinity can sometimes be quite high and organisms from these highly saline springs must be thermohalophiles. Table 1.3 lists some examples of thermophilic organisms. The maximum growth temperature for eubacteria is up to 75-80°C, above 80°C, archaebacteria predominate. However, thermophilic and hyperthermophilic organisms can be isolated from certain environments, such as hot springs, which often show a wide range of temperatures over its area. The ability of an organism to grow at high temperatures is dependent upon its physiology. Enzyme thermostability is known to be determined to a large extent by the amino acid sequence and composition. This affects the secondary and tertiary structure of the enzyme by influencing a range of forces including hydrogen bonding, hydrophobic bonding, ionic interactions, metal binding and

**Table 1.3:**
Examples of thermophilic archaebacteria and eubacteria.

| Organism | Temp. range (°C) | Temp. opt. (°C) |
|---|---|---|
| **Archaebacteria** | | |
| *Acidianus* spp. | 45-96 | 70-90 |
| *Sulfolobus* spp. | 60-90 | 70-85 |
| *Thermoplasma* spp. | 33-67 | 59-60 |
| *Pyrodictium* spp. | 82-110 | 105 |
| *Pyrococcus* spp. | 70-103 | 90-100 |
| *Thermofilum* spp. | 55-100 | 80-90 |
| *Thermoproteus* spp. | 80-97 | 85-88 |
| *Desulfurococcus* spp. | 85-90 | 85 |
| *Thermococcus celer* | 75-97 | 85-88 |
| *Methanococcus* spp. | 50-97 | 80-85 |
| *Pyrobaculum* spp. | 74-102 | 100 |
| **Eubacteria** | | |
| *Alicyclobacillus acidocaldarius* | 40-70 | 60-65 |
| *Bacillus tusciae* | 50-60 | 55 |
| *Alicyclobacillus acidoterrestris* | 35-55 | 42-53 |
| *Alicyclobacillus cycloheptanicus* | 42-53 | 48 |
| *Acidothermus cellulyticus* | 37-70 | 55 |
| *Thiobacillus* spp. | 35-55 | 50 |
| *Thermus* spp. | 45-79 | |
| *Thermotoga* spp. | 80 | |
| *Synechococcus lividus* | 55-74 | |
| *Bacillus stearothermophilus* | 40-80 | 55-65 |
| *Bacillus caldotenax* | | 80 |
| *Bacillus caldolyticus* | | 72 |
| *Clostridium thermocellum* | 40-68 | |
| *Clostridium thermohydrosulfuricum* | 40-78 | |
| *Acetomicrobium faecalis* | 70-73 | |
| *Thermoactinomyces* spp. | 35-70 | 50-60 |
| Thermophilic actinomycetes. | 35-65 | 45-55 |
| *Chloroflexus auraniacus* | 52-60 | |

disulphide bridges (Sharp & Munster 1986, Edwards 1990) and an increase in any or all of these forces can lead to increased thermostability. Relatively small alterations in the primary structure of an enzyme can therefore lead to more complex alterations in the tertiary structure which can in turn give rise to increased thermostability (Edwards 1990, Outtrup & Boyce 1990, Fogarty & Kelly 1990). Membrane structure is another important determinant in the thermostability of an organism. The cell membrane allows and controls transport in and out of the cell and is the site of action of many important biochemical reactions. Its integrity, ie: its fluidity, is therefore vitally important to the growth of the cell. However, the lipids with which it is composed are susceptible to changes in temperature, forming a rigid gel state when the temperature is too low and a liquid crystalline state when the temperature is too high. This, if increased above the cell's capacity to control it, will result in the breakdown of the membrane's integrity and its inability to function correctly. The fluidity of the membrane is determined by the fatty acids present, their length, degree of branching and degree of saturation, with increased thermostability being conferred by increased chain length, decreased branching and increased saturation. Normal straight chains therefore possess the highest thermostability, followed by iso-branched, ante-iso-branched and unsaturated chains with the lowest thermostability. Thermophilic eubacteria therefore contain higher proportions of long chain normal and/or iso-branched fatty acids (Edwards 1990). Eubacteria are also able to alter the chain length and degree of saturation of their fatty acids to suite changes in temperature within a certain range, normally between 30-40°C.

The thermoacidophilic bacilli, *Alicyclobacillus acidocaldarius*, *A.acidoterrestris* (Deinhard et al 1987a), *A.cycloheptanicus* (Deinhard et al 1987b), strain T-4 (Uchino & Katano 1981) and an unspecified *Bacillus* strain (Poralla & Konig 1983) contain the unusual fatty acid *w*-cyclohexane and the hopane glycolipids (Poralla et al 1980, Hippchen et al 1981, Poralla et al 1984), which are thought to play a role in the regulation of cytoplasmic pH as well as membrane thermostability. Archaebacteria are characterised by having isoprenoid alcohols as the major lipid component of their membranes. They contain only C20, which is a fully saturated phytanol, and C40, which is two phytanol molecules linked together. These are then linked to glycerol by way of the more stable ether linkages instead of the more labile ester linkages found in the eubacteria to form either a phytanyl glycerol diether or a dibiphytanyl diglycerol tetraether. Tetra- and diethers occur in varying proportions in both mesophilic and thermophilic archaebacteria with tetraethers predominating in the thermoacidophiles (Edwards 1990, Cowan 1992b). Diethers are able to form a lipid bilayer, the tetraethers a monolayer, particularly when cyclopentane rings are included. The cell wall of the archaebacteria is also thought to play an important role in their extreme thermostability. They are generally much simpler than eubacterial walls, consisting of a two-dimensional array of glycoprotein monomers, called an S-layer, which are thought to convey a considerable degree of chemical and thermal stability (Cowan 1992b). Another factor important in determining the thermostability of an organism is the presence of polyamines. Polyamines regulate nucleic acid synthesis, protein synthesis and cell division, and thermophilic bacteria contain polyamines of

greater chain length, for example more thermine, thermospermine and caldopentamine at high temperatures than low (Hamana & Matsuzaki 1987, Edwards 1990).

### 1.6.1.1: Uses of Thermophilic Bacteria.

Bioactive compounds have been identified from thermophilic organisms, although only in relatively small numbers. However, this may in part be due to the relatively little amount of attention they have received to date. The chief industrial importance of thermophilic microorganisms is in the production of thermostable enzymes, both intra and extracellular, which can be used at the high temperatures often required for the efficient running of many industrial processes and many enzymes from thermophilic organisms possess significant thermostabilities (Bragger et al 1989, Cowan 1992b, Adams 1993).

As yet however, thermophilic organisms have not superseded the main mesophilic producers (Bergquist & Morgan 1992) and only in a few cases has commercial production started. One reason for this is that many of the organisms tested do not produce the levels of enzyme required for commercial production, although this is perhaps not surprising considering the relatively early stages of study of growth and enzyme production of these organisms. Because of these relatively low levels of production, methods of increasing thermostability of mesophilic enzymes are also studied. One approach is protein engineering. Studies of thermostable enzymes have linked thermostability to the amino acid sequence and composition, usually to the presence or absence of a few key amino acids at key points. These changes have been found to affect the secondary and tertiary structure of the

enzyme by increasing hydrogen and hydrophobic bonding and ionised group interactions thus making the enzyme more stable under extreme conditions (Sharp & Munster 1986). For example, amylases from *Bacillus amyloliquefaciens*, *B.stearothermophilus* and *B.licheniformis* showed a 60% homology in their amino acid sequence (Fogarty & Kelly 1990) and yet showed considerable differences in their thermostability, with half-lives at 90°C of 2, 50 and 270min respectively. This was found to be due to the presence of increasing numbers of salt bridges involving specific lysine residues 385, 253 and 88 (Tomazic & Klibanov 1988a, 1988b). The thermostability of the amylase of *B.amyloliquefaciens* might therefore be considerably improved by inserting lysine at these points. Another example is that of the serine proteases from *B.amyloliquefaciens* and *B.licheniformis* which have had thermostability and stability at higher pH values increased through protein engineering (Outtrup & Boyce 1990). Another method to increase thermostability is by mutation. For example increased temperature activity and thermostability of amylase and pullulanase activity from a mesophilic *Bacillus cereus* isolate (optimum activity of 80°C) was obtained by mutation through UV irradiation (Bakshi et al 1992a+b). Stability can also be increased on a more temporary basis by a number of procedures, for example metals such as calcium often stabilise enzymes at high temperatures as can immobilisation. For enzymes such as lipases which act over a water oil interface, thermostability can be increased by lowering the water activity, for example porcine pancreatic lipase is almost immediately denatured at 100°C in aqueous phosphate buffer, but retained 50% activity after 50min and almost 100% activity after 60min at

100°C when the enzyme was used in 2M n-heptanol in tributyrin and the water content reduced to 0.8% and 0.015% respectively (Edwards 1990). However, it is considerably easier to obtain an inherently thermostable enzyme than to engineer one, particularly when the increase in thermostability of an engineered enzyme is often only slight. The potential for enzymes produced from thermophilic organisms is therefore great and can be illustrated by the commercial success of the production of DNA polymerase from *Thermus aquaticus*. Enzymes from thermophilic organisms however generally show reaction rates which are often no different to those shown by enzymes from mesophilic organisms (Ng & Kenealy 1986, Cowan 1992c), the advantage that enzymes from thermophiles have is that they can be used at higher temperatures than their mesophilic counterparts, where they often achieve the same rate of activity as enzymes from mesophilic organisms but show reduced rates of denaturation and so remain active for longer. The thermotolerance of an enzyme is often defined by the optimum temperature for activity, however this does not take into account that an enzyme may show optimum activity and be denaturing at the same temperature. A more accurate method therefore is the determination of the half life obtained at a fixed temperature (Ng & Kenealy 1986, Edwards 1990). There is also some evidence to suggest that enzymes from thermophilic organisms show increased resistance to other denaturants such as detergents and organic solvents, to chemical reactions which cause irreversible protein degradation, and to enzyme (proteolytic) attack (Cowan 1992c).

### 1.6.1.2: Thermophilic Actinomycetes

Thermophilic actinomycetes characteristically show growth in the range 30-60°C with an optimum at 50°C and thus do not classify either as extreme thermophiles or as hyperthermophiles. nine actinomycete genera contain thermophilic species, *Actinomadura*, *Excellospora*, *Microbispora*, *Micropolyspora*, *Pseudonocardia*, *Saccharomonospora*, *Saccharopolyspora*, *Thermomonospora* and *Streptomyces* (Greiner-Mai et al 1987). The genus *Thermoactinomyces* is not included in this list because of its close associations with the *Bacillaceae*. The monosporic thermophilic actinomycetes *Thermomonospora* and *Saccharomonospora* are typical and grow well in the range 30-55°C and may grow up to 60°C (Cross 1981) while the thermophilic streptomycetes grow well in the range 28-55°C and may grow up to 58°C (Goodfellow et al 1987), although the recently isolated *S.thermoautotrophicus* is capable of growth up to 65°C (Bell et al 1987). They are commonly isolated from heated environments including composts, manures and fodders as well as soils where they play a valuable role in decomposition.

### 1.6.1.3: *Thermoactinomyces*

Although still classified in the *Actinomycetales* (Lacey 1989), largely due to their filamentous morphology, recent studies using 16S ribosomal RNA oligonucleotide cataloguing (Stackebrandt & Woese 1981), DNA homology (Hirst et al 1991), enzyme homology (Smith et al 1984), combined taxonomic studies (McCarthy & Cross 1984), menaquinone composition (Collins et al 1982), phage typing (Kurtboke & Sivassithamparam 1993) and their formation of resistant endospores rather than the arthrospores produced by

other actinomycetes (Cross et al 1968a), have suggested that they should no longer be classified in the *Actinomycetales* but should be placed in the family *Bacillaceae*. The genus was first described in 1899 by Tsiklinsky and since then eight main species have been identified (Lacey 1971, Kurup 1975, Flockton & Cross 1975, Lacey 1989). All except one are thermophilic, growing in the range 30-65/70°C with optimum growth occurring between 50 and 55°C. The exception, *T.peptonophilus*, is mesophilic/thermotolerant. They therefore show a higher maximum temperature for growth than thermophilic actinomycetes which generally show growth up to 58-60°C. Following growth of a substrate mycelium, a well developed aerial mycelium is produced (Kretschmer 1984b) which is yellow in *T.dichotomicus* and white in all other species (Lacey 1989). In some species this aerial mycelium may be transient, rapidly autolysing after growth or nutrient exhaustion to leave only endospores (Kretschmer 1978, 1984a) while intercalary growth has been reported for *T.vulgaris* under conditions of slow growth (Kretschmer 1984c). Endospores are formed singly on both the aerial and substrate mycelia (Kretschmer 1978). They are either sessile or on unbranched sporophores (in the case of *T.dichotomicus* they are formed on dichotomously branched sporophores). They resist desiccation (Kalakoutskii & Agre 1973) and can therefore lie dormant in the environment for long periods of time (Nilsson & Renberg 1990). The developmental stages of sporulation in *T.vulgaris* and *T.sacchari* appear identical to those reported for the *Bacillacaea* (Cross et al 1971, Lacey & Vince 1971) and like *Bacillus* endospores, *Thermoactinomyces* endospores contain dipicolinic acid and are heat resistant,

characteristically showing $D_{100°}$ values of between 11 and 77 min (Attwell et al 1972, Lacey & Vince 1971, Cross et al 1968a, Kurup et al 1975, Lacey 1971). The endospores of the mesophilic *T.peptonophilus* contain lower amounts of dipicolinic acid and are less heat resistant (Lacey 1989). Spore germination can decrease following laboratory culturing (Agre et al 1972) and is affected by numerous factors including temperature (heat shock and cold shock), humidity and a variety of chemicals including amino acids and antibiotics (Agre et al 1972, Attwell et al 1972, Kalakoutski & Agre 1973, Attwell & Cross 1973, Foerster 1978). After germination, outgrowth of the germ tube is affected by $CO_2$ levels, with high $CO_2$/low $O_2$ levels stimulating outgrowth and low $CO_2$/high $O_2$ levels causing inhibition (Kretschmer & Jacob 1978, 1983, Ruttloff et al 1983). Cell walls of *Thermoactinomyces* species contain *meso*-diaminopimelic acid (*meso*-D.A.P), are wall chemotype III and sugar type C, ie: they contain no characteristic sugars or amino acids (Becker et al 1965, Lechevalier 1968, Lechevalier & Lechevalier 1970, Lechevalier et al 1971). They possess a mol% G+C ratio of around 52 to 54 (Goodfellow & Cross 1984) which is lower than the average for actinomycetes of around 70%+ but similar to *Bacillus* species. They characteristically possess menaquinones which are unsaturated with seven or nine isoprene units, MK-7 or MK-9 (Collins et al 1982, Kroppenstedt 1985) and polyamines (Hamana & Matsuzaki 1987). They also show a characteristic fatty acid profile which includes branched iso- and anti-iso-fatty acids but lack unsaturated oleic acid (Kroppenstedt & Kutzner 1978, Minnikin & O'Donnell 1984, Kroppenstedt 1985, O'Donnell et al 1985). The thermophilic species, but not the mesophilic

*T.peptonophilus*, are resistant to up to 25ug/ml of the antibiotic novobiocin, a factor which is used in their selective isolation (Cross 1968, 1981, Cross & Goodfellow 1973). Common habitats for *Thermoactinomyces* species include heated environments such as composts, manures, fodders, bagasse etc. (Cross 1981, Goodfellow & Cross 1984) as well as soils and sediments (Cross & Johnston 1971). Two species have been implicated in forms of allergic alveolitis, *T.vulgaris* in Farmers lung and *T.sacchari* in bagassosis (Lacey 1971). They have been shown to possess some anti-microbial activity, a strain of *T.thalpophilus* (formerly *T.antibioticus*) produced thermorubin, an agent active against Gram positive bacteria (Craveri et al 1964). They also produce a number of thermostable extracellular enzymes including the serine protease thermitase (Kleine 1982), alpha-amylases (Kuo & Hartman 1966, Obi & Odibo 1984a, Shimizu et al 1978), beta-amylase (Obo & Odibo 1984b), pullulanase (Odibo & Obi 1988), beta-glucosidase (Hagerdal et al 1979) and all type strains show esterase activity by the degradation of the tweens (Lacey 1989). *Thermoactinomyces* isolates have only been isolated from neutral environments on neutral isolation media and thus although no studies have been carried out on their pH response, it is likely that they are neutrophilic. However, an alkalophilic *Thermoactinomyces* isolate which only grows between pH 7.5 and 11.5 with optimum growth at pH 10.3-10.5, and which produces an alkalophilic thermostable protease, has recently been reported (Tsuchiya et al 1991, 1992) and this opens the possibility that *Thermoactinomyces* strains exist over a wide range of pH environments, possibly including acidophilic or acid tolerant strains.

### 1.6.2: Acidophilic Bacteria

Acidophilic microorganisms characteristically show optimal growth below pH 4.0 (generally between pH 2.0 and 4.0) and cannot grow at neutral pH. Some organisms which show a slightly higher pH optimum for growth of pH 4.5-5.0 and which also cannot grow at neutral pH are also considered acidophilic, however, many organisms are capable of growing at pH 4.0-5.0 but show optimal growth at neutral pH, these are classified as acid tolerant. Acidophilic organisms can be isolated from a wide range of different acid environments. Moderately acid environments, with a pH of between 3.0 and 5.0, are relatively common and include acidified lakes and soils, pine forest soils and acid peat bogs. More extreme acid environments with a pH of below pH 3.0 are often associated with mine waste sites and mining effluents and also include acid hot springs. Natural acidic environments are generally more common than alkaline ones since acidity often develops as a result of aerobic oxidation, for example the oxidation of hydrogen sulphide (common in geothermal areas, bogs and swamps etc.) to sulphuric acid (Sharp & Munster 1986). Table 1.4 shows examples of acidophilic bacteria. Because of the inorganic nature of most extremely acid environments such as geothermal springs and mine waste sites, most extreme acidophiles are capable of autotrophic growth by the oxidation of iron and/or sulphur. Heterotrophic acidophiles also exist however which exist on organic material fixed by autotrophic growth. Commonly isolated from extremely acid hot springs are the hyperthermophilic acidophilic archaebacteria such as *Sulfolobus* which are capable of both autotrophic and heterotrophic growth between pH 1.0-6.0 with an optimum at 1.5-2.0, and at 60-96°C

**Table 1.4**

Examples of acidophilic bacteria.

| Organism | pH range | Opt. pH |
|---|---|---|
| **Archaebacteria** | | |
| *Sulfolobus* spp. | 1.0-6.0 | 2.0-3.0 |
| *Acidianus* spp. | 1.0-6.0 | 1.5-2.0 |
| *Thermoplasma* spp. | 1.0-4.0 | 1.0-2.0 |
| *Desulfurolobus ambivalens* | | 2.5 |
| *Pyrodictium* spp. | | 5.5 |
| *Thermofilum* spp. | | 5.0 |
| *Thermoproteus tenax* | | 5.5 |
| *Thermococcus celer* | | 5.8 |
| **Eubacteria** | | |
| *Acidiphilium* spp. | 2.0-6.0 | 2.5-5.0 |
| *Thiobacillus* spp. | 2.0-4.5 | 3.5-5.5 |
| *Alicyclobacillus acidocaldarius* | 2.0-6.0 | 3.0-4.0 |
| *Bacillus tusciae* | 3.5-6.0 | 4.2-4.8 |
| *Alicyclobacillus acidoterrestris* | 2.5-6.0 | 3.5-4.5 |
| *Alicyclobacillus cycloheptanicus* | 3.0-5.5 | 3.5-4.5 |
| *Streptomyces* spp. | 3.5-6.5 | 4.5 |
| *Acidothermus cellulyticus* | 3.5-7.0 | 5.0 |
| *Bacillus naganoensis* | 4.1-6.0 | 4.7-5.5 |
| *Bacillus smithii* | | 5.7 |
| *Nitrobacter* sp. | 4.1-7.2 | 5.5 |

with an optimum at 70-90 (Brock et al 1972, Segerer et al 1986, Sharp & Munster 1986, Cowan 1992b). Also commonly isolated from acid hot springs, as well as solar and geothermally heated soils and acid mine wastes are four species of thermoacidophilic *Bacillus*. which grow heterotrophically between pH 2.0-6.0 with an optimum at pH 3.0-5.0 (Darland & Brock 1971, Bonjour & Aragno 1984, Deinhard et al 1987a+b) and the thermoacidophilic, cell-wall deficient archaebacteria, *Thermoplasma* species which grow heterotrophically in the pH range 1-4, with an optimum at pH 1-2 (Darland et al 1970, Segerer et al 1988). *Thiobacillus* and *Acidiphilium* species are mesophilic/thermotolerant acidophiles, although thermophilic *Thiobacillus* species have been reported (Williams & Hoare 1972). *Thiobacillus* species generally grow autotrophically in the pH range 2.5-4.5 with an optimum at pH 3.5-4.0 (Bryant et al 1983), although *T.acidophilous* has been reported as growing heterotrophically (Guay & Silver 1975) while *Acidiphilium* species grow heterotrophically at pH 2.0-6.0 with an optimum of pH 3.0 (Lobos et al 1986, Kishimoto & Tano 1986, Wichlacz et al 1986). They are both commonly isolated from acid sulphurous soils, the edges of acid springs where the temperature is at or below 50-55°C and mine waste sites and effluents. A less extreme thermoacidophile is the eubacterium *Acidothermus cellulyticus*, which grows heterotrophically at pH 3.5-7.0 and 37-65°C with an optimum at pH 5.0 and 55°C, has been isolated from acid hot springs (Mohagheghi et al 1986). Moderate acidophiles also include mesophilic actinomycetes, mainly streptomycetes, which grow at pH 3.5-6.5 with an optimum at pH 4.5, and which have been isolated from acid pine forest and peat soils and from acid mine waste sites (Khan & Williams 1975), and a number of

*Bacillus* species including *B.coagulans* Hammer 1915 which grows at pH 4.5, 5.7 and 7.7 and *B.smithii*, which grows at pH 5.7 but not at pH 4.5 and 7.7 (Nakamura et al 1988), and *B.naganoensis*, which grows at pH 4.1-6.0 with an optimum at pH 5.5 (Tomimura et al 1990). An acidophilic *Nitrobacter* strain has been isolated from an acid forest soil which showed growth at pH 4.1-7.2 with an optimum at pH 5.5 (Hankinson & Schmidt 1988).

Perhaps the greatest problem of acidophilic organisms is the maintenance of intracellular cytoplasmic pH. Acidophilic bacteria possess an internal cytoplasmic pH close to neutral while growing at pH values as low as pH 1 and 2. A large transmembrane pH gradient therefore exists between the cell cytoplasm, which has a relatively low concentration of $H^+$ ions, and its surroundings, which has a relatively high concentration of $H^+$ ions. This gradient would naturally lead to the influx of $H^+$ ions into the cell thus acidifying the cytoplasm. This is prevented by acidophilic organisms in two basic ways, the pumping of H+ ions out of the cell up the concentration gradient (a process which is therefore energy dependent) and the presence of a membrane reasonably impermeable to $H^+$ ions. Acid tolerant organisms are also thought to survive low pH values by possession of an impermeable membrane, however it is widely accepted that only obligate acidophiles possess the ability to export $H^+$ ions out of the cell against the concentration gradient. The H+ ion gradient also creates problems for the cell in the conservation of energy. In the chemiosmotic model, the energy for the oxidative phosphorylation of ADP to ATP is provided by the translocation of $H^+$ ions across a membrane by the creation of a membrane potential. This is outside positive in neutrophilic

organisms, however due to the H+ gradient the membrane potential for acidophiles is inside positive, the reverse of neutrophiles. Many reviews exist concerning internal pH control and energy conservation (Oshima et al 1977, Cobley & Cox 1983, Krulwich & Guffanti 1983, Guffanti et al 1984, Booth 1985, Michels & Bakker 1985, Goulbourne et al 1986, Krulwich & Guffanti 1986, Ingledew 1990, Norris & Ingledew 1992). The thermoacidophilic bacilli also possess the unusual fatty acid *w*-cycloheptane and hopanoids in their cell membrane and these are thought to play a role in the stability of the membrane at low pH and high temperature (Hippchen et al 1981, Poralla & Konig 1983, Poralla et al 1984, Deinhard et al 1987a+b). Evidence also suggests that acidophilic bacteria possess novel menaquinones and ubiquinones (Collins & Langworthy 1983).

### 1.6.2.1: Uses of Acidophilic Bacteria.

The autotrophic iron and sulphur oxidising acidophilic bacteria have been widely used by the mining and oil industries. They are used in conjunction with conventional extraction processes to speed up and improve the efficiency of extraction of metals such as copper and uranium and to extract metals such as gold from low grade ores where conventional processes could not be used. They are also used in the desulfurisation of coal and in the oil industry to enhance the efficiency of oil recovery (Norris & Ingledew 1992).

### 1.6.2.2: Acidophilic Actinomycetes

Actinomycetes from extreme environments show considerable potential for the production of novel products as they combine

unusual physiology with the proven background of antibiotic production shown by the actinomycetes. They have however remained largely unstudied, with the possible exception of the thermophilic actinomycetes. One group, the acidophilic actinomycetes, are the focus of this study. They are not obligate acidophiles as they do not show optimum growth below pH 4.0 and as a result they are not isolated from extremely acid environments. Previous studies have however shown acidophilic actinomycetes to differ quite considerably from neutrophilic actinomycetes not only in terms of their pH preferences but also in their basic physiology and biochemistry (Khan & Williams 1975). Preliminary studies carried out on acid soils such as from a pine forest (Corke & Chase 1964, Goodfellow et al 1968, Davies & Williams 1970, Williams & Mayfield 1971, Williams et al 1971, Hagedorn 1976) showed the actinomycete populations present differed from those of typical neutral soils. A further study (Khan & Williams 1975) isolated actinomycetes from a range of similar soils, all with similar pH values, ranging from 3.3 to 4.7, including pine and spruce forest soils, peat and acid mine waste sites, with media altered to pH 4.5. When grown in liquid culture, these isolates were found to grow optimally in the pH range 4.5 to 5.5 with variable growth at pH 3.5 and 6.5 (Khan & Williams 1975, Flowers & Williams 1977). They therefore differed considerably from the neutrophilic actinomycetes which typically show growth between pH 5.0 and 9.0 with an optimum at pH 7.0 (Flowers & Williams 1977, Williams et al 1984). A comparison of the starch hydrolysis of these isolates and that of neutrophilic strains (Flowers & Williams 1978) also indicated that the extracellular enzymes of these organisms may be active at low

pH. Amylolytic activity of the two acidophilic isolates tested occurred in the pH range 2.5 to 7.0 with an optimum between pH 4.0 and 4.5. This can be compared to the activity of the neutrophilic isolate tested, which occurred in the pH range 5.0 to 8.0 with an optimum between 6.0 and 6.5. The isolates also differed from the neutrophilic species in terms of their basic physiology and biochemistry, including nutritional requirements and tolerances (Khan & Williams 1975, Flowers & Williams 1976), and in terms of 5S RNA analysis (Park et al 1991). The acidophilic strains also showed a high degree of similarity, grouping together at the 85% similarity level and could therefore be grouped in a separate and quite distinct cluster from the neutrophilic species. All isolates were identified as belonging to the genus *Streptomyces* except three, two of which were *Micromonospora* and one a *Nocardia* strain (Khan & Williams 1975). These studies also showed acidophilic actinomycetes to be quite widespread in acid soils where it is thought they play an important role in decomposition (Khan & Williams 1975, Williams & Robinson 1981). The widespread presence of acidophilic actinomycetes in acid soils was confirmed by later isolations carried out by Lonsdale (1985) and Simpson (1987) which identified acidophilic *Streptomyces* from acid environments similar to those reported by Khan & Williams (1975). An acidophilic streptomycete which showed optimum growth in the pH range 5.0-5.5 and failed to grow above pH 6.5 has also been isolated from a soil which had become acidic (pH 5.0) by the use of nitrogenous fertilisers (Bromfield 1978), and a number of acidophilic streptomycetes capable of growth at pH 4.5-5.0 were

isolated and found to show glucose isomerase activity (Bok et al 1984).

## 1.7: Microbial Extracellular Enzymes

Microbially produced enzymes are another important area in the biotechnology industry. The sources of many important industrial enzymes are shown in Table 1.5. Relatively few genera are involved with members of the bacterial genus *Bacillus*, and the fungal genera *Aspergillus*, *Penicillium* and *Rhizopus*, together with some yeasts, predominating. Of particular importance for commercial production are enzymes which are stable and active under extreme conditions, for example temperature, pH solvents etc: which are often required for the optimum running of industrial processes.

### 1.7.1: Proteases

Proteases are the most widespread of all microbial extracellular enzymes. They can be grouped into three main classes, serine, metallo and acid proteases which generally have alkaline, neutral and acid profiles respectively (Priest 1984). They are widely used in detergents, in the food industry including brewing, baking and cheese processing, and in the leather industry (Priest 1984, Sharp & Munster 1986).

Serine proteases characteristically possess a serine residue at the active site. They are endopeptidases and show a strong activity combined with a low substrate specificity which allows them to attack a wide range of different protein substrates. They do not require metal ions and calcium is not generally required for stability. They generally show an alkaline pH optimum of

**Table 1.5.**
Sources of commercially important microbial enzymes.

| Enzyme | Producer |
|---|---|
| alpha-Amylase | *Aspergillus oryzae* |
| | *Bacillus amyloliquefaciens* |
| | *Bacillus licheniformis* |
| Cellulase | *Aspergillus* sp. |
| | *Trichoderma reesei* |
| Dextranase | *Penicillium* sp. |
| beta-Glucanase | *Aspergillus niger* |
| | *Bacillus amyloliquefaciens* |
| Glucoamylase | *Aspergillus niger* |
| | *Rhizopus* sp. |
| Glucose Isomerase | *Bacillus coagulans* |
| | *Actinoplanes missouriensis* |
| | *Streptomyces* spp. |
| Hemicellulase | *Aspergillus niger* |
| Invertase | *Aspergillus* sp. |
| | *Saccharomyces* sp. |
| Lipase | *Aspergillus* sp. |
| | *Candida cylindracea* |
| | *Mucor miehei* |
| | *Rhizopus* sp. |
| Pectinase | *Aspergillus niger* |
| Protease | *Bacillus amyloliquefaciens* |
| | *Bacillus licheniformis* |
| | *Bacillus stearothermophilus* |
| | *Aspergillus niger* |
| | *Aspergillus oryzae* |
| Pullulanase | *Bacillus* sp. |

Data from Priest (1984).

between pH 9.0 and 11.0 and the two main commercial enzymes, the subtilisins BPM' and Carlsberg, are produced by the neutrophilic *Bacillus amyloliquefaciens* and *Bacillus licheniformis* which show optimum activity at 50°C and pH 9.0 (Priest 1984, Outtrup & Boyce 1990). Serine proteases are commonly used in detergents where a high temperature and alkaline pH stability are required and one area where these have been found is from the alkalophilic members of the genus *Bacillus*, for example a serine protease from *Bacillus* AH-101 which showed optimum activity at pH 12-13 and 80°C (Takami et al 1989), and *Streptomyces*, for example a protease from an alkalophilic *Streptomyces* strain showed optimum activity at pH 12.3 and was also active at pH 13.0 (Nakanishi et al 1974). A thermostable serine protease, thermitase, produced by *Thermoactinomyces vulgaris* showed optimum protease activity at pH 7.5-9.5 and at 85°C using azocasein as the substrate, at pH 7.5-9.5 and 65-75°C using peptide nitroanilides, and showed esterolysis activity with an optimum at pH 7.5-9.5 and 60°C (Behnke et al 1982, Kleine 1982, Bromme & Kleine 1984). An alkaline protease, which showed optimum activity at pH 11.5-13.0 and 70°C without $Ca^{2+}$ and 80°C with $Ca^{2+}$, has also been identified from an alkalophilic *Thermoactinomyces* species (Tsuchiya et al 1992). Highly thermostable serine proteases have also been isolated from the thermophilic eubacterium *Thermus* and archaebacterium *Desulfurococcus* which showed optimal activity at pH 8.2 and 7.4, and half-lives of 5.5h at 85°C and 8.5min at 105°C, respectively (Coolbear et al 1988). Serine proteases have also been isolated from the archaebacteria *Pyrococcus furiosus* (pyrolysin) which showed optimal activity at

115°C and pH 6.5-10.0 (Eggen et al 1990) and from *Sulfolobus solfataricus* which showed optimal activity at pH 6.5-8.0 and 90°C (Burlini et al 1992). Metalloproteases characteristically contain a metal ion at the active site. They are endo-acting enzymes which generally show a neutral pH optimum, require calcium for stability and are inhibited by metal chelating agents such as E.D.T.A (Priest 1984). They are produced by numerous organisms including several species of *Bacillus*, for example *B.thermoproteolyticus* produces a metalloprotease thermolysin which is stable for 1h at 80°C (Sharp & Munster 1986) and *B.brevis* produces a metalloprotease which showed optimal activity at 60°C in the absence, and at 75°C in the presence of calcium (Paberit et al 1984). The amino acid sequence of this enzyme shows considerable homology to that of *B.amyloliquefaciens*, a far more thermolabile enzyme and it is thought that the difference in thermostabilities is due to the higher degree of hydrophobic bonds in thermolysin (Outtrup & Boyce 1990). A metallo-protease identified from a strain of *Streptomyces thermoviolaceous* showed optimum activity at pH 6.5-7.0 and a half-life at 70°C of 80min without calcium and 100min with calcium (James et al 1991). A neutral protease has also been reported from *Thermoactinomyces vulgaris* which showed optimal activity at 60-80°C and pH 7.0 (Klingenberg et al 1979). Highly thermostable neutral proteases have also been identified from the archaebacteria *Sulfolobus solfataricus* which showed optimal activity at pH 5.5-7.0 and 75-85°C (Hanner et al 1990, Colombo et al 1992) and from *Pyrococcus furiosus* which showed optimum activity at 115°C (Cowan 1992b+c, Adams 1993). Acid proteases are rare in bacteria but common in fungi.

They show optimum activity at low pH values, for example from *Aspergillus saito* which showed optimum activity at pH 3.0 (Tanaka et al 1977), and often contain an aspartic acid residues at the active site. They are used in cheese making and the main commercial producers include *Mucor* and *Aspergillus* strains, however they generally show low thermostability, for example 40-50°C for *Aspergillus saito* (Tanaka et al 1977). One exception is a protease from *Penicillium duponti* which showed optimum activity at 65°C and pH 4.5 (Emi et al 1976). More highly thermostable acid proteases have also been isolated from *Bacillus* novosp MN-32 which showed optimum activity at pH 3.0 and 70°C (Murao et al 1993) and from the archaeal *Sulfolobus acidocaldarius* which showed optimal activity against $C^{14}$ methylated bovine haemoglobin at pH 2.0 and 90°C (Lin & Tang 1990) and at pH 2.0 and 75°C against a range of peptide nitroanilides (Fusek et al 1990).

### 1.7.2: Amylases

Starch is made up of two components, amylose and amylopectin. Amylose is only poorly soluble in water and is a linear polymer containing approximately $10^3$ glucose residues which are linked by 1,4-alpha-bonds. Amylopectin is soluble in water and is a highly branched polymer containing approximately $10^4$ to $10^5$ glucose residues. It is made up of 1,4-alpha-linked glucose residues, 20-25 residues in length, which are linked to one another by 1,6-alpha-bonds. Amylopectin generally accounts for approximately 75-85% of starch. Most industrial starch-hydrolysing processes require high temperatures and thus the search for new enzymes has been targeted at obtaining enzymes

with increased thermostabilities. This can be achieved either by screening thermophilic organisms or by carrying out protein engineering, such as by site directed mutagenesis (Fogarty & Kelly 1990). Amylolytic activity is widespread and widely studied and for this reason it is also a useful test case in the comparison of the effects of pH and temperature on the hydrolytic activity of a range of different organisms.

(i): alpha-Amylase

alpha-Amylases (1,4-alpha-D-glucanohydrolase) are endo-attacking enzymes which attack the 1,4-alpha linkages in amylose and amylopectin to produce a range of oligosaccharides from the disaccharide maltose and the trisaccharide maltotriose to maltopentaose and maltohexaose. These oligosaccharides are of commercial importance in the chemical industry as research and clinical reagents, and in the food industry as additives and syrups. The importance of alpha-amylases which yield large percentages of specific oligosaccharides as their end product is therefore becoming increasingly important. They are produced largely by *Bacillus* species and fungi, with some from actinomycetes and other bacteria, and their pH and temperature optima varies considerably depending on the source. Amylases from mesophilic and thermotolerant organisms tend to show optimal activity between 40 and 60°C, while higher optima (70°C and above) are shown by enzymes from thermophilic organisms. Similarly, pH optima for activity tend to range from around pH 7.0 to pH 2.0. For the genus *Bacillus,* alpha-amylases generally range from being approximately neutral and showing relatively low temperature optima, for example *B.subtilis*, which showed

optimum activity at pH 6.0-7.0 and 50°C (Takasaki 1987), from *B.cereus*, which showed optimum activity at pH 6.0 and 55°C (Yoshigi et al 1985), from *B.circulans*, which showed optimum activity at pH 7.0-7.5 and 50°C (Takasaki 1983, Takasaki et al 1991) and at pH 6.0-6.5 and 60°C (Taniguchi et al 1983) and *B.megaterium*, which showed optimum activity at pH 7.0 and 60°C (Takasaki 1989), to thermostable and neutral, for example from *B.caldovelox* which was stable for 7min. at 90°C and pH 8.0 (Bealin-Kelly et al 1991) and a caldoactive *Bacillus* (Heinen & Heinen 1972), to acidic and thermostable, for example from *A.acidocaldarius*, which showed optimum activity at pH 2.0-3.5 and 70-75°C (Buonocore et al 1976, Uchino 1982, Uchino & Fukuda 1983, Kanno 1986). Other thermostable alpha-amylases are produced by *B.stearothermophilus* which showed optimum activity at 70°C and pH 5.3 (Outtrup & Norman 1984, Kim et al 1989) and *B.brevis* which showed optimum activity at pH 5.0-9.0 and 80°C (Tsvetkov & Emanuilova 1989). The main commercial enzymes, from *B.licheniformis* and *B.amyloliquefaciens*, showed optimum activity at 76 and 90°C and pH 9.0 (Saito 1973, Krishnan & chandra 1983) and pH 5.9-6.0 and 65-70°C (Fogarty 1983, Kochar & Dua 1990) respectively. Despite the prevalence of starch hydrolysis amongst actinomycetes, relatively few have been studied in any great detail with the exception of mesophilic streptomycetes, which produce very similar amylases that tend to show optimal activity at pH values at or just below neutral and relatively low temperatures of between 30 and 40°C (Fogarty 1983, Williams et al 1983), for example an amylase from *Streptomyces limosus* (Fairbairn et al 1985) showed optimum activity at pH 6.0-7.0 and 25-35°C and was unstable above 45°C.

An exception is an amylase from *S.hygroscopicus* which shows optimum activity at pH 5.5 and 50-55°C (Hidaka & Adachi 1980). Amylases from thermophilic actinomycetes are more thermostable, for example from *Thermomonospora curvata* which showed optimum activity at pH 5.5-6.0 and at 65°C (Glymph & Stutzenberger 1977, Collins et al 1993) and from *Streptomyces thermoviolaceous* which showed optimal activity at 55°C and pH 7.2 (Goldberg & Edwards 1990). Amylases have also been reported from two *Thermoactinomyces* strains, one showed optimum activity at pH 7.0 and 75°C (Obi & Odibo 1984a) and one at pH 5.0 and 70°C (Shimizu et al 1978). Other bacterial amylases have been reported from *Lactobacillus cellobiosus* (Sen & Chakrabarty 1986), optimum activity at pH 7.3 and 50°C, and *Pseudomonas stutzeri* (Sakano et al 1982a), optimum activity at pH 6.0-6.5 and 45°C. Fungal amylases tend to show relatively low pH optima between pH 4.0 and 5.0 and have largely been reported from mesophilic strains, for example *P.expansum* (Doyle et al 1989), *Aspergillus awamori* (Bhella & Altosaar 1985) and the commercially used *Aspergillus oryzae* (Priest 1984). They tend to show relatively low temperature optima of between 40 and 55°C although activity has been reported from the thermophilic fungus *Thermomyces lanuginosa*, formerly *Humicola lanuginosa* (Jensen et al 1988) and two amylases from *Aspergillus kawachii* have been shown to possess optimal activity at 70°C (Mikami et al 1987). Highly thermostable amylases have also been identified from extremely thermophilic archaebacteria, most notably from *Pyrococcus furiosus*, which showed activity in the range 40-120/130°C with an optimum at 100°C and pH 5.0 (Koch et al 1990), 100°C and pH 5.5 (Brown & Kelly 1993), 100°C and pH 6.5-

7.0 (Laderman et al 1993), and 110°C and pH 5.6 (Brown et al 1990), and from *Pyrococcus woesii* which showed optimal activity at 100°C and pH 5.5-6.0 (Koch et al 1991). Activity has also been identified for *Thermotoga maritima* which showed optimal activity at pH 5.0-6.0 and 90-95°C (Schumann et al 1991).

(ii): beta-Amylases

beta-Amylases (1,4-alpha-D-glucan maltohydrolase) occur widely in higher plants but have only recently been characterised from microorganisms. They are exo-acting enzymes which degrade amylose and amylopectin from the non-reducing end of the chain by hydrolysing the 1,4-alpha linkages to form maltose in the beta-form. They have been reported in the mesophilic *Bacillus* species *B.cereus* (Nanmori et al 1983) and *B.megaterium* (Takasaki 1989), and in the thermotolerant *Bacillus* species *B.circulans* (Fogarty & Kelly 1983). In each case, optimum activity occurred at pH 7.0 and at 40, 60 and 60°C respectively. A beta-amylase has also been reported from a thermophilic *Thermoactinomyces* species (Obi & Odibo 1988) which showed optimum activity at pH 7.0, but at only 60°C, and also for *Clostridium thermohydrosulfuricum* which showed optimum activity at 75°C and pH 5.5-6.0 (Hyun & Zeikus 1985a). They are of use in the brewing industry and for the production of high maltose syrups, however the relatively low temperature optima of known enzymes is a drawback to their widespread use (Priest 1984).

(iii): Glucoamylases

Glucoamylases are exo-acting and hydrolyse alpha-1,4 linkages to yield glucose. Hydrolysis of alpha-1,6 linkages also takes place but at a much reduced rate. They are rare in bacteria but common in fungi and have been produced commercially from *Aspergillus* and *Rhizopus*. However, fungal glucoamylases generally show relatively low temperature optima between 50°C and 60°C. A bacterial glucoamylase from *Clostridium thermohydrosulfuricum* shows much higher thermostability of 75°C at pH 4.0-6.0 (Hyun & Zeikus 1985b).

(iv): Pullulanases

Pullulanases are debranching enzymes which hydrolyse 1,6-alpha linkages such as those present in amylopectin and pullulan. The enzyme gains its name by its ability to hydrolyse the fungal polysaccharide pullulan which essentially consists of maltotriose units linked by 1,6-alpha bonds. The industrial use of pullulanase is assisting in the complete hydrolysis of starch to glucose. Pullulanase is commercially produced by a *Bacillus* strain. Pullulanases have also been found in a number of other bacteria including *Thermoactinomyces thalpophilus*, which showed optimum activity at pH 7.0 and 70°C (Odibo & Obi 1988), *Bacillus acidopullulyticus*, which showed optimum activity at pH 5.0 and 60°C (Schulein & Hojer-Pederson 1984, Jensen & Norman 1984, Kusano et al 1988), *Bacillus stearothermophilus*, which showed optimum activity at pH 6.0 and 65°C (Kuriki et al 1988a, 1988b), the thermophilic *Bacillus* strain 3183, which showed optimum activity at pH 6.0 and 75°C (Shen et al 1990) and *Clostridium thermohydrosulfuricum*, which showed optimum activity at 75°C

and pH 5.5-6.0 (Hyun & Zeikus 1985b). Pullulanase-amylase complexes, which show alpha-amylase activity (attacking alpha-1,4 linkages) and pullulanase activity (attacking alpha-1,6 linkages), have also been reported from *Bacillus subtilis* which showed optimum activity at pH 6.0-7.0 and 50-60°C (Takasaki 1987), from *Bacillus stearothermophilus* which showed optimum activity at pH 5.8 and 55°C (Suzuki & Imai 1985), from *Bacillus thermoamyloliquefaciens* which showed optimum activity at pH 6.2 and 63°C (Suzuki et al 1987) and from *Thermoactinomyces vulgaris* which showed optimum activity at pH 5.0 and 70°C (Shimizu et al 1978, Sakano et al 1982). The ability of the *T.vulgaris* enzyme complex to attack both alpha-1,4 and alpha-1,6 linkages was confirmed by its ability to attack numerous pullulan hydrolysates (Fukushima 1982, Sakano 1983, 1985). A highly thermostable pullulanase has also been isolated from the extremely thermophilic archaebacteria *Pyrococcus furiosus* and *Thermococcus littoralis* which showed optimum activity at 100°C and pH 5.6 (Brown et al 1990) and an amylopullulanase from the same isolates which showed optimal activity at 100°C and pH 5.5 (Brown & Kelly 1993).

### 1.7.3: Lipases and Esterases

Lipases can be defined as enzymes which hydrolyse triacylglycerols of animal fats and vegetable oils by acting over the oil-water interface. This definition therefore excludes those enzymes which act on water soluble esters, these are termed esterases (Antonian 1988, Jensen 1983, Harwood 1989). There are many tests for lipase and esterase activity including plate assays (Sierra 1957, Kouker & Jaeger 1987, Samad et al 1989)

and fluorometric assays (Dooijewaard-Kloosterziel & Wouters 1976, Roy 1980), however perhaps the commonest test for lipase activity utilises olive oil as both the growth and assay substrate and monitors the release of free fatty acids from the hydrolysis of olive oil (Omar et al 1986, Sztajer & Maliszewska 1988, Sugihara et al 1990, 1991) or triolein (Peled & Krenz 1981) by means of titration against a suitable alkali, while the commonest test for esterase activity utilises Tween 80 as the growth substrate and monitors the hydrolysis of para-nitrophenyl-palmitate, acetate or laurate (Winkler & Stuckman 1979, Heymann & Mentlein 1981) or alpha-napthyl-palmitate and acetate (Brahimi-Horn et al 1991) which produces a colour change. Assays, involving both plates and culture supernatant, have also been developed using tributyrin as the substrate (Lawrence et al 1967), however tributyrin can be degraded by both esterases and lipases (Sztajer & Maliszewska 1988, Rapp & Backhaus 1992)and so is an unsuitable assay for full characterisation. Some lipases also show esterase activity (Gowland et al 1987, Antonian 1988, Gilbert et al 1991, Shabtai & Daya-Mishne 1992) and there is often some confusion over the definition of lipase and esterase activity. Compared to proteases and amylases, lipases are of limited industrial use today being mainly used for flavour production in the cheese industry.. However, recent studies have identified some important applications, for example as fat digesters in detergents and in the production of organic chemicals (Harwood 1989, Godtfredsen 1990). These applications rely on several specific properties, for example under water restricted conditions hydrolysis is reduced thus allowing their use in the catalysis of a wide range of esterification and

transesterification reactions. Their use as catalysts in these reactions is broadened by the fact that they remain active in solvents and show a high specificity. Finally, they are often thermostable and thus can be used at the higher temperatures often demanded in industrial processes. The pH optima of most lipases and esterases is between pH 7.0 and 9.0 (Jensen 1983, Omar et al 1987, Antonian 1988, Yamamoto & Fujiwara 1988, Meghji et al 1990, Shabtai & Daya-Mishne 1992), however there are exceptions. A lipase from a *Bacillus* and *Pseudomonas* species showed optimal activity in the pH range 5.5 to 7.0 (Sugihara et al 1991, Iizumi et al 1989) and *Geotrichum candida* lipase II showed optimum activity at pH 6.0 (Sugihara et al 1990), *Aspergillus niger* lipase I and II showed optimal activity at pH 5.0-6.0 (Antonian 1988). Temperature optima of lipases vary considerably, fungal lipases often possess low temperature optima, for example lipase I and II from the mesophilic *Geotrichum candidum* show optimum activity at 30 and 40°C respectively, while lipase activity from *Thermomyces lanuginosa* (formerly *Humicola lanuginosa*) is optimal at 45°C (Omar et al 1987). Optimum lipase activity from the mesophilic *Bacillus* and *Pseudomonas* species (Sugihara et al 1991, Iizumi et al 1989, Yamamoto et al 1988) occurred at 60°C. Lipase and esterase activity has been identified in several thermophilic *Bacillus* species, probably *B.stearothermophilus* (Gowland et al 1987), and an intracellular esterase has been identified from *Sulfolobus acidocaldarius* which shows optimal activity at pH 7.5-8.5 and a half life of 1h 100°C (Sobek & Gorisch 1988), however no major studies have been carried out on lipases from thermophilic isolates which considering the importance of thermostable

enzymes from thermophilic organisms is perhaps an area that should be redressed.

## 1.8: Aims

The aims of this project were therefore as follows:

1: To isolate and identify acidophilic actinomycetes from natural acid environments, such as pine forests soils, from which they have been previously reported.

2: To optimise cultural conditions.

3: To characterise their response to pH and temperature, and to study growth physiology in both shake flask and the fermenter.

4: To characterise pH and temperature responses of extracellular enzymes.

5: To screen isolates for bioactivity.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1.1: The Isolation of Acidophilic Actinomycetes From Acid Soils

Soil samples were collected from Freshfields pine forest, Lancashire and dried at 45°C for 48h in an open Petri-dish and two different isolation procedures carried out.

A:) Spread Plate Method: - A 10 fold dilution was prepared by mixing 10g of dried soil with 100ml of distilled water and shaking at 300 rpm for 30min. Further ten fold dilutions were then prepared from this stock down to $10^{-5}$. A 0.1ml sample of each soil at each dilution was then spread onto agar plates which were incubated either for 10-28 days at 28°C or 7-14 days at 50°C.

B:) Dry Soil Method - A 10g sample of each pre-dried soil was put into a petri dish. A spreader, dipped into distilled water, was then put into the soil and streaked over a succession of five plates without returning to the soil. Plates were incubated either for 10-28 days at 28°C or 7-14 days at 50°C.

Soil pH was determined by adding approximately 10ml of distilled water to a 1g sub-sample of soil and shaking briefly by hand. Measurement of pH was made after 20min equilibration using a standard Corning electrode and pH meter. Several measurements were made and a mean calculated for each sample.

The isolation medium used was starch-casein agar (Kuster & Williams 1964), acidified to pH 4.5 by 5.45g/l $KH_2PO_4$ added aseptically, after autoclaving. The anti-fungal agent nystatin was added to all media to a final concentration of 100ug/ml. Rifampicin (5ug/ml) and tetracycline (4ug/ml) were also added to some of the media in order to select for *Actinomadura* strains (Athalye et al 1981) and *Nocardia* strains (Orchard & Goodfellow

1974) respectively. Colonies were removed from isolation plates using a wire loop and spread onto starch-casein agar plates at pH 4.5. Sub-culturing was carried out until at least three plates of each isolate had been obtained.

### 2.1.2: The Isolation of *Thermoactinomyces* isolates from acid soils

The selective isolation of *Thermoactinomyces* isolates was carried out from a range of acid soils collected in the Cheshire and Lancashire area. The main site was the pine forest at Delamere, Cheshire. Samples were dried at 45°C for 48h in an open Petri-dish. A 10 fold dilution was prepared by mixing 10g of dried soil with 100ml of distilled water and shaking at 300 rpm for 30min. Further ten fold dilutions were then prepared from this stock down to $10^{-2}$. A 0.1ml sample of each soil at each dilution was then spread onto agar plates which were incubated either for 7-14 days at 50°C. The isolation medium used was ME3 (see appendix), adjusted to pH 5.0 by the addition of HCl and NaOH prior to autoclaving. The medium also contained the anti-fungal agent nystatin (100ug/ml) and novobiocin (25ug/ml) which is selective for the genus *Thermoactinomyces* (Cross 1968).

### 2.2: Storage of Cultures

For short term storage, plates were sealed with parafilm and stored at 4°C. For long term storage, inoculum from these plates was used to prepare a spore-hyphal suspension in 30% (w/v) glycerol brain heart infusion (BHI) broth, adjusted to the appropriate growth pH using HCl/NaOH, which was then maintained in 2ml cryotubes (Nalgene) at -20°C.

## 2.3: Resuscitation of Cultures.

Isolates showing a drop in viability throughout culturing or storage, were put through the following resuscitation procedure. Three loopfuls of mycelium from a plate were inoculated into a bijou bottle containing 4ml of either brain heart infusion (BHI) broth plus 0.4% (w/v) glucose, or SV2a broth, at the appropriate growth pH of the organism concerned. Cultures were then given a cold shock by agitation at 4°C for 2h, followed by incubation at 50°C for 6-8h. The broth (2ml) was then spread onto each of two agar plates and incubated at 50°C for 24-48h.

## 2.4: Growth of Isolates

Growth of all isolates used in this study was measured by taking optical density readings at 550nm using a LKB spectrophotometer. Culture pH of media was adjusted as appropriate by the addition of HCl and NaOH prior to autoclaving. All shake flask studies were carried out in 250ml conical flasks containing 50ml of medium with shaking at 250 rpm.

## 2.5.1: Fermentation System

Laboratory scale batch fermentation experiments were carried out using an LH 500 series fermentation unit. A 1L vessel was used with a working volume of 800ml. Agitation was provided by a direct drive impeller fitted with two turbine blades operating at 600 rpm. Aeration was provided by an air pump delivering up to a maximum of 2.5L/min through a sterile air filter. Exhaust gas was passed through a water cooled condenser to reduce evaporation and then through a length of silicone tubing to bubble through a 2% stericol solution in order to maintain sterility.

Temperature control was provided by two systems. General control, which maintained the system 2-3 degrees below the required level by way of circulated heated water, from a controllable water bath, through a heating coil in the fermentation vessel and fine control, which was provided by a 100w electrical heater linked to a resistance thermistor. Culture pH was monitored and maintained by a pH electrode connected to a pH control unit which controlled the addition of acid (1M HCl) and alkali (1M NaOH) depending on upper and lower pH limits which could be set as required. The pH control unit was calibrated once the fermenter was set up by taking a sample of culture medium (before inoculation) from the vessel (see 2.5.3: Sampling) and measuring it's pH using a Corning electrode and pH meter. The reading on the pH control unit was then altered to the selected value using the appropriate controls on the unit. Upper and lower pH limits were set using similar controls. Organic antifoam 204 (Sigma) was added (2.5ml) to the medium prior to autoclaving while further sterile antifoam was added aseptically by injection with a sterile wide bore hypodermic needle and syringe through a self sealing rubber port when required during the fermentation.

### 2.5.2: Inoculum Preparation

Inocula for the fermenter were prepared by seeding two 250ml conical flasks containing 50ml of ME3 broth each with 2ml of a spore/hyphal suspension of the isolate, stored in glycerol-BHI solution at -20°C, and then incubating for 12-15h at 50°C. The two broths were then mixed together aseptically in a sterile 250ml side arm flask. The side arm of the flask was attached to an inoculation line into the fermentation vessel using a clipped

piece of sterile silicon tubing. The air supply was switched off, the clips on the inoculation line removed, and the inoculum passed into the fermenter vessel by gravity.

### 2.5.3: Sampling

Samples were removed from the fermenter vessel by way of a stainless steel sampling hood attached to a 25ml universal bottle with a universal screw thread. The hood was connected to a sampling line in the fermenter vessel using a clipped piece of sterile silicon tubing and a side arm on the hood was connected to a 20ml syringe by way of a sterile air filter. Sampling was carried out by removing the clip on the sampling line and drawing off the required volume of media from the vessel using the syringe. The sampling line was then clipped off, the universal bottle unscrewed, and a fresh, sterile universal fitted in it's place. A sample of culture medium was taken before inoculation as a blank in order to calibrate the spectrophotometer for later optical density readings. Further samples were then taken during the growth cycle as required.

## 2.6: Preparation of Dried Biomass

Cultures were incubated in the appropriate growth medium for 24h. They were then centrifuged at 4000 rpm for 20min and the supernatant discarded. The pellet was washed and resuspended twice in distilled water and the washed pellet was dried overnight on a vacuum centrifuge. The resulting dried biomass was stored at 4°C until required.

## 2.7: Detection of Endospores

The presence of refractile endospores was detected by microscopic analysis using an endospore stain. The method used was the Schaeffer-Fulton endospore stain (Doetsch 1981). One loopful of cell suspension was placed in a smear on a microscope slide. This was then heat fixed and placed over a steaming water bath. The slide was then flooded with 5% aqueous malachite green. Further additions of malachite green were made during heating to prevent drying of the stain. When the stain began to steam, the slides were left for 5min before being washed with water and stained for 2min with 5% aqueous safranin. The slide was washed with water and blotted dry prior to microscopy.

## 2.8: Determination of Spore Heat Resistance

Spore heat resistance was tested using a modification of the method described by Cross et al (1968a). Three loopfuls of biomass from a sporulating culture on SV2a agar plates were inoculated into 4ml of 250mM phosphate buffer (pH 5.0) in a bijou bottle and maintained at 100°C in a boiling water bath for between 10 and 90 min. The contents were then spread on two SV2a agar plates, incubated at the appropriate temperature for 24-48h and the colonies counted. The time at which the last colonies appeared was taken as being the maximum time at which the spores remained viable.

## 2.9: Detection of 2,6-Diaminopimelic Acid in Cell Walls

The detection of 2.6-diaminopimelic acid (D.A.P) was carried out using whole cell hydrolysates (Hasegawa et al 1983). Dried biomass (5mg) was hydrolysed by mixing with 0.1ml of 6M HCl in

a bijou bottle and autoclaving for 15 min at 121°C. After cooling, 5ul of the hydrolysate, along with 5ul of 0.01M DAP standard solution, was spotted onto a cellulose coated glass TLC plate (Merck 5718 Cellulose F). Single dimension ascending chromatography was then performed using the solvent system of methanol:pyridine:6M HCl:distilled water in the ratio 80:10:4:26 for 3h. Plates were dried and then developed by spraying with 0.5% ninhydrin in butan-1-ol (BDH) and heating at 100°C for 2-5 min.

## 2.10: Detection of whole Cell Sugars

The detection of whole cell sugars was carried out using whole cell hydrolysates (Meyertons et al 1988). Dried biomass (50mg) was hydrolysed by mixing with 1ml of 1M $H_2SO_4$ in a bijou bottle and heating at 100°C for 2h. After cooling, barium hydroxide was added using methyl red as an indicator until the mixture turned pink/salmon orange (pH 5.0-5.5). The mixture was then centrifuged at 6000 rpm and the supernatant removed into an eppendorf. This was then evaporated on a rotary evaporator and the remaining dried material dissolved in 0.4ml of distilled water and stored at -20°C until required. The assay was carried out by spotting 5-10ul of the hydrolysate, along with 5ul of the appropriate sugar standards, onto a Whatman K5 silica gel plate and developing 5 times using the solvent system aetonitrile:distilled water in the ratio 92.5:7.5. Chromatograms were run repeatedly for 45 min until the advancing phase was near the top of the plate. A 20 minute drying time was allowed in between each run. After the final run, and the subsequent drying

time, plates were sprayed with acid aniline phthalate (Merck) and heated at 100°C for 5-10 min.

## 2.11: Taxonomic Tests

Degradation of adenine, chitin, tyrosine, xanthine, hypoxanthine, and arbutin: The degradation of adenine, chitin and tyrosine (each 0.5% w/v), xanthine and hypoxanthine (each 0.4% w/v), and arbutin (0.1% w/v) was determined by the method of Gordon (1967). Isolates were grown on Nutrient agar (Oxoid) containing adenine, chitin and tyrosine (each 0.5% w/v), xanthine and hypoxanthine (each 0.4% w/v). Degradation was shown by the clearing of each substrate under and around colonies. For the degradation of tyrosine, the production of melanin was also looked for.

Degradation of Arbutin: The degradation of arbutin was determined by the method of Gordon (1967). Isolates were grown on nutrient agar containing 0.1% (w/v) of arbutin. Degradation was detected by a blackening of the medium.

Degradation of Esculin: The degradation of esculin (0.1% w/v) was determined by the method of Gordon (1966,1967). Isolates were grown on esculin broth containing (g/l) esculin 1, ferric citrate 0.5, peptone 10 and NaCl, 5. Degradation was detected by blackening of the medium.

Degradation of Casein: The degradation of casein was determined by the method of Gordon (1966). Isolates were grown on milk agar, made by mixing 5g of skimmed milk powder in 50ml of distilled water and 1g of agar in 50ml of distilled water after

autoclaving separately. Activity was detected by the presence of zones of clearing under and around colonies.

Hydrolysis of Tweens: The hydrolysis of tweens was determined by a modification of the method of Sierra (1957). Each tween was added (15 g/l) to a basal medium consisting of (g/l), soya peptone 15, NaCl 3, $CaCl_2.2H_2O$ 1, and agar 16. Culture pH was adjusted as appropriate by the addition of HCl and NaOH. Each isolate to be tested was streaked out and incubated at the appropriate temperature for between 24 and 48h. Esterase activity was determined by the presence of opaque bands around the colonies.

Degradation of Starch: Starch degradation was determined using both starch-casein agar and nutrient agar supplemented with 1% (w/v) of soluble starch. Amylase activity was detected by the presence of zones of clearing around the colonies.

Degradation of Cellulose: Cellulose degradation was determined by the method of Goodfellow & Pirouz (1982). A suspension of Whatman Standard Grade cellulose powder (1g) in 250ml of distilled water was ball-milled for 72h and then made up to 1 litre with 750ml of Gordon's (1967) basal medium. Cellulase activity was determined by the presence of clearance zones under and around the colonies.

Degradation of Xylan: The degradation of Xylan was determined by the method of Gordon (1967). Xylan (0.4% w/v) was added to

nutrient agar and xylanase activity was determined by the presence of zones of clearing around colonies.

Degradation of Olive Oil: The degradation of olive oil was determined by the plate assay method of Kouker & Jaeger (1987). Olive oil (15 g/l) was added to a basal medium consisting of (g/l), nutrient broth 16, NaCl 4, and agar 16. Culture pH was adjusted appropriately by the addition of HCl and NaOH prior to autoclaving. Once the medium had cooled after autoclaving, 10ml of a 0.001% (w/v) solution of rhodomine B was added. Lipolytic activity was determined by the presence of fluorescent halos around colonies under UV irradiation.

Degradation of Pullulan: The degradation of pullulan was determined by the method of Morgan et al (1979). Pullulan (0.3% w/v) was added to a basal medium consisting of (g/l), yeast extract 0.5, peptone 5, soya peptone 10, NaCl 3, and agar 16. A trace elements solution (Chen & Segal 1968) was also added. Culture pH was adjusted appropriately by the addition of HCl and NaOH prior to autoclaving. Pullulanase activity was determined by the presence of zones of clearing under and around colonies.

Tolerance to NaCl and Novobiocin: Tolerance to 1% (w/v) NaCl was tested using GYEA (see appendix) as the basal medium and tolerance to 50ug/ml novobiocin was determined using SV2a as the basal medium.

### 2.12.1: Preparation of Media for Antibiotic Screening

A range of different media in the form of broths, solid state and agars were used to grow up isolates for antibiotic screening. The formula for each medium used is given in Appendix. The broth media were prepared in 5ml volumes in shake tubes, the agar media were prepared in 4cm diameter Petri dishes and the solid state media were prepared in 100ml conical flasks, each as described individually in appendix.

### 2.12.2: Preparation of Seed Inoculum

Isolates (from 2ml spore-hyphal suspensions in glycerol-BHI at -20°C) were inoculated into 10ml of ME3 broth in shake tubes and incubated for 12-15h at 50°C and 250 rpm. This was then used to inoculate each of the test media, 0.5ml into each broth, 0.5ml onto each agar plate and 5ml into each flask of solid state media. The broths were then incubated with shaking at 250rpm, while the agars and solid state media were incubated statically, for 24, 48 and 72h at 50°C.

### 2.12.3: Methanol Extraction

Antibiotic activity was determined by carrying out a whole cell methanol extraction. An equal volume (5ml) of methanol was added to the broths, and to the agar plates, while 15ml was added to the flasks of solid state media. All methanol extracts were then left for 1h before being filtered under vacuum to remove large particles in the medium. For the broths, the complete methanol extract was filtered directly, for the agar media, the methanol was pipetted off the plate and filtered, and for the solid

state media, the solid particles were broken up and the liquid removed and filtered. All resulting filtrates were stored at 4°C.

### 2.12.4: Preparation of Test Organisms

A number of species were used as test organisms in order to assess antimicrobial activity. *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* were maintained on nutrient agar at 37°C, *Candida albicans* on yeast agar (see appendix) at 37°C. Plates were stored, sealed in parafilm at 4°C. *Mucor rouxii* and *Aspergillus niger* were maintained on slopes of malt yeast agar (see appendix) at 28°C. Suspensions were prepared by washing slopes of each organism with a glycerol-tween solution (15% w/v glycerol and 0.01% w/v tween 80). A viable count was carried out and the suspensions diluted to give a final concentration of $10^8$ cells/ml. Suspensions were stored at -20°C.

### 2.12.5: Preparation of Assay Plates

Two loopfuls of *S.aureus*, *B.subtilis* and *E.coli* grown on agar plates were incubated overnight in 50ml of nutrient broth in 250ml conical flasks at 37°C and 250rpm. The procedure was repeated for *C.albicans* except that MYGP replaced nutrient broth. 2.5ml of overnight cultures of *B.subtilis* and *E.coli* (diluted 1/100 in Ringers solution) were then added to 250ml of liquid nutrient agar (at 50°C) and poured into level 30cm by 30cm Autodata bioassay plates. An overnight culture of *S.aureus* (2.5ml) was mixed with 2ml of 2% (w/v) 2,4,6-triphenyltetrazolium chloride (TTC) and added to 250ml of liquid nutrient agar (at 50°C) and poured into Autodata bioassay plates as described above. The 2% TTC solution was added as a marker for growth of *S.aureus*, which

turns red where growth occurs. It was stored for up to 4 weeks in the dark at 4°C. An undiluted overnight culture of *C.albicans* (2.5ml) was added to 250ml of yeast agar (YA), at 50°C, and poured into Autodata bioassay plates as above. For *M.rouxii* and *A.niger*, a 10ml cell suspension was diluted 1/100 in Ringers solution and 2.5ml added to 250ml of liquid malt yeast agar (at 50°C) which was poured into Autodata bioassay plates as above. When the agar had set 8mm diameter wells were made in an 8 by 8 configuration in the agar using a sterile punch flamed in ethanol. Methanol extracts (200ul) were then pipetted into the wells and the plates incubated overnight at 37°C for *S.aureus*, *B.subtilis*, *E.coli* and *C.albicans*, and 28°C for *M.rouxii* and *A.niger*, using methanol controls.

### 2.12.6: Scoring Assay Plates

Antibiotic activity was measured by the size of the zones of inhibition. Zone size was read from the centre of the well to the limit of clearing.

## 2.13: Determination of Glucose

Glucose levels were determined by the o-toluidine assay (Sigma procedure 635). The assay was carried out by adding 5ml of o-toluidine to 0.1ml of suitably diluted culture supernatant or reaction mixture in a test tube. The test tube was then capped and placed in a boiling water bath for 5-10 min.

The mixture was allowed to cool and the absorbance values read at 635nm against a similarly treated water and medium blank. The absorbance values were then read off against a calibration curve prepared using glucose in the concentration range 0-1mg in

order to obtain the concentration of glucose present in the sample.

## 2.14: Determination of Reducing Sugars

Reducing sugar levels were determined using the dinitrosalicylic acid (DNS) assay (Miller 1959). DNS reagent was prepared by dissolving 10g of dinitrosalicylic acid, 8g phenol, 2g sodium sulphite and 200g potassium sodium tartarate in 500ml of 4% (w/v) NaOH. The solution was stirred until clear, made up to 1L and stored in the dark at 4°C. The assay was carried out by adding 2ml of DNS reagent to 1ml of suitably diluted culture supernatant or reaction mixture in a test tube. The test tube was capped and placed in a boiling water bath for 5-10 min. The mixture was allowed to cool and the absorbance values were read at 540nm against a similarly treated water and medium blank. The absorbance values were then read off a calibration curve prepared using glucose in the concentration range 0-10mg in order to obtain the concentration of reducing sugars present in the sample and expressed as glucose equivalents.

## 2.15: Determination of Ammoniacal Nitrogen

Ammoniacal nitrogen levels were determined by the indolephenol assay (Wainwright & Pugh 1973). Phenol solution was prepared by dissolving 62.5g of phenol in a mixture of 25ml of ethanol and 18.5ml of acetone. Sodium hypochlorite solution was prepared by diluting 11.25ml of sodium hypochlorite in 88.75ml of distilled water to obtain a final concentration of 0.9% available chloride ions was obtained. Both of these solutions were stored in the dark at 4°C. Phenolate reagent was prepared directly before use

by mixing 20ml of 27% (w/v) NaOH with 20ml of phenol solution and diluting to a final volume of 100ml with distilled water. The assay was carried out by adding 0.5ml of suitably diluted culture supernatant to 1.75ml distilled water, 1.2ml sodium hypochlorite solution and 1.2ml phenolate reagent in a test tube and incubating at 25°C for 20 min in the dark. The absorbance values, which were taken at 630nm using a similarly treated water and medium blank, were then read off against a calibration curve prepared using ammonium sulphate in the concentration range 0-50mg in order to obtain the concentration of ammoniacal nitrogen present in the sample, expressed as ammonium sulphate equivalents.

## 2.16: Determination of Protease Activity

Two methods of protease determination were used.

Azocasein Assay: The azocasein assay (Jones et al 1988) was carried out by pre-heating 0.4ml of suitably diluted culture supernatant to 50°C in an eppendorf. 0.2ml of 5mg/ml azocasein solution (made up on the day) and 0.4ml of $KH_2PO_4$ buffer, pH 6.0, were then added and the mixture incubated at 50°C for 30 min. The reaction was stopped by the addition 0.4ml of 20% (w/v) trichloroacetic acid (TCA) and the reaction mixture allowed to stand for 2-3 min. The mixture was centrifuged at 13,500 rpm for 5 min, the supernatant removed into a cuvette and 0.4ml of 2M NaOH added to develop the colour. The absorbance values were read at 440nm against a similarly treated water and medium blank. One unit (U) of activity was defined as that required to cause a change in absorbance of 0.01. Activity was expressed in terms of Units/ml/h.

Chromogenic Assay: The chromogenic assay was carried out by monitoring the release of the chromogenic compound nitroanilide from the hydrolysis of an amino acid chain to give a colour reaction. The chromogenic substrates were dissolved in 50mM Tris/HCl buffer, pH 6.0, to a final concentration of 3mM. The assay was carried out by adding 0.1ml of undiluted culture supernatant to 0.9ml of the chromogenic solution in an eppendorf and incubating at 50°C for 10 min. The reaction was stopped by placing on ice and the absorbance values read at 405nm against a similarly treated water and medium blank. One unit (U) of activity was defined as that required to cause a change in absorbance of 0.01. Activity was expressed in terms of Units/ml/min.

## 2.17: Use of Protease Inhibitors

The effect of EDTA, a metalloprotease inhibitor and PMSF, a serine protease inhibitor was tested in both the azocasein and chromogenic assays. EDTA, in 1mM HCl and PMSF, in isopropanol were both prepared to a final concentration of 500mM. The assay was carried out by pre-incubating 990ul of undiluted supernatant with 10ul of 500mM EDTA or PMSF in an eppendorf at 50°C for 30 min. This mixture was suitably diluted and the azocasein or chromogenic assay carried out as described previously.

## 2.18: Determination of Amylase Activity

Amylase activity was determined by monitoring the release of reducing sugars from the hydrolysis of soluble starch (Obi & Odibo 1984). The assay was carried out by adding 0.5ml of 2% starch in 250mM $KH_2PO_4$ buffer, pH 6.0 to 0.5ml of suitably

diluted culture supernatant in an eppendorf and incubating at 50°C for 30 min. A reducing sugar assay using DNS was then carried out with activity measured as mg reducing sugar as glucose/ml$^{-1}$/h$^{-1}$.

## 2.19: Determination of Pullulanase Activity

Pullulanase activity was determined by measuring the release of reducing sugars from the hydrolysis of pullulan (Odibo & Obi 1988). The assay was carried out by adding 0.5ml of 0.5% (w/v) pullulan in 250mM $KH_2PO_4$ buffer, pH 6.0, to 0.5ml of suitably diluted culture supernatant in an eppendorf and incubating at 50°C for 30 min. A reducing sugar assay using DNS was then carried out with activity measured as mg reducing sugar as glucose/ml$^{-1}$/h$^{-1}$.

## 2.20: Determination of Esterase Activity

Esterase activity was determined by monitoring the release of para-nitrophenol (pNP) from the hydrolysis of para-nitrophenyl palmitate (Winkler & Stuckman 1979). The assay was carried out by adding 30mg of para-nitrophenol palmitate (pNP-palmitate) to 10ml isopropanol and heating gently to dissolve. Sodium deoxycholate (20.7mg) was then added to 10ml of 20mM $KH_2PO_4$ buffer, pH 6.0, and the two solutions mixed to produce the reagent mixture, 50ul of which was added to 250ul of suitably diluted culture supernatant in an eppendorf tube and incubated at 50°C for 30 min. 1M $Na_2CO_3$ (700ul) was then added to develop the colour and the absorbance values were read at 410nm against a similarly treated water and medium blank. The absorbance values were read off a calibration curve using pNP concentrations in the

range 0-0.2mM in order to obtain the concentration of free pNP present in the sample. Activity was defined as uMoles pNP released/min/ml.

## 2.21: Determination of Lipase Activity

Lipase activity was determined by monitoring the release of free fatty acids (FFA's) from the hydrolysis of olive oil, and its main constituent, triolein (Sztajer et al 1988 and Omar et al 1987). The assay was carried out by adding 1ml of culture supernatant to 4ml of 50mM Tris-HCl buffer, pH 6.5 and 5ml of an emulsion made up of either 10% triolein or olive oil in 2% polyvinyl alcohol (PVA) in a 100ml conical flask. The pH of the reaction mixture was then taken and the flask incubated at 50°C for 30 min with continual shaking at 250 rpm. The reaction was stopped by the addition of 20ml of ethanol-acetone mixture (1:1 v/v) and a titration carried out using 0.025M NaOH. One unit of lipase activity was defined as the amount that liberated 1uMole of free fatty acid per minute per ml, (uMol FFA/min/ml).

# CHAPTER 3

# THE ISOLATION, SELECTION AND PRELIMINARY CHARACTERISATION OF ACIDOPHILIC ACTINOMYCETES

# THE ISOLATION OF ACIDOPHILIC ACTINOMYCETES.

## 3.1: Introduction

The first step in the selective isolation of acidophilic actinomycetes is the selection of appropriate acid environments. Previous studies have isolated acidophilic actinomycetes from environments with pH values ranging between pH 3.2 and 4.7 (Jensen 1928, Williams et al 1971, Khan 1972, Khan & Williams 1975). Environments tested in these studies included mainly soils, such as peat and acid podzols (for example pine and spruce forest soils), and acidified man made environments such as mine waste sites (Table 3.1). One soil listed in these studies, a pine forest soil from Freshfields in Lancashire has been shown to yield quite large numbers of acidophilic actinomycetes (Williams et al 1971, Khan 1972, Khan & Williams 1975) and for this reason it was selected for use in this study. In addition, six volcanic soils collected in Iceland and 16 actinomycete strains putatively designated either acid tolerant or acidophilic were supplied by Glaxo Group Research.

## 3.2: Description of Freshfields Pine Forest Soil

The Freshfields pine forest soil has been comprehensively studied in the past for both it's physical and chemical properties, such as pH, moisture and organic content (Goodfellow et al 1968), as well as for the actinomycete populations it supports (Davies & Williams 1970, Williams & Mayfield 1971, Williams et al 1971, Khan 1972, Khan & Williams 1975). It is a developing podzol under *Pinus nigra*, and consists of an acidic surface litter ($A_0$) horizon overlaying a darkly stained, acidic ($A_1$) soil horizon which

**Table 3.1**

Environments that acidophilic actinomycetes have been isolated from.

| Soil Type | Plant Cover | pH | |
|---|---|---|---|
| Podzol | *Pinus sylvestris* | 3.6-4.2 | Delamere, Cheshire |
| Podzol | *Pinus sylvestris* | 3.8-4.0 | Storeton, Cheshire |
| Podzol | *Calluna vulgaris* | 3.5 | Thurstaston, Cheshire |
| Podzol | *Erica tetralix* | 3.5 | Thurstaston, Cheshire |
| Podzol | *Pinus nigra* | 4.7-6.9 | Freshfields, Lancashire |
| Peat | *Juncus effusus* | 4.0 | Moorhouse, Northumberland |
| Peat | *Eriophorum augustifolium* | 3.3 | Thurstaston, Cheshire |
| Peat | *Juncus effusus* | 4.0 | Thurstaston, Cheshire |
| Peat | *Sphagnum* spp. | 4.5 | Denbigh, Wales |
| Leaf Litter | *Abies* spp. | 4.5 | Conway, Wales |
| Coal mine waste | *Rubinia pseudocacia* | 3.2-4.2 | Wisconsin, USA |
| Coal mine waste | None | 3.5 | Maypole, Lancashire |
| Coal mine waste | None | 4.5 | Low Hall, Lancashire |
| Coal mine waste | *Deschampsia flexuosa* | 4.5 | Lancashire |
| Coal mine waste | *Deschampsia flexuosa* | 3.5-4.0 | Maypole, Lancashire |
| Lead mine waste | *Agrestris tenuis* | 4.5 | Goginan, Wales |
| Lead mine waste | *Calluna vulgaris* | 4.5 | Goginan, Wales |

Table constructed using data from Khan+Williams (1975).

in turn overlays an alkaline parent sand (C horizon). The $A_0$ horizon itself consists of three separate layers, a surface loose litter layer (L) followed by two layers of decomposing litter (F1 and F2-H). Table 3.2 shows the profile of the soil as determined by Williams et al (1971) and Khan (1972). Previous studies have yielded acidophilic actinomycetes from the acid ($A_1$) horizon and from the F1 and F2-H layers of the $A_0$ horizon. The surface loose litter (L) layer, which is also at a suitable pH for the isolation of acidophilic actinomycetes, has however yielded few isolates and this was thought to be due to lower moisture content (Goodfellow et al 1968, Khan 1972). Only the $A_1$ horizon and the F1 and F2-H layers of the $A_0$ horizon were therefore sampled in this study.

### 3.3: Isolation from the Freshfields soils

The surface litter layer was removed (approximately the top 4cm) and samples taken from the underlying F1 and F2-H layers and $A_1$ horizon (approximately the following 4-6cm). Care was taken to avoid the alkaline C horizon underneath. A total of eight soil samples were taken from four different sites around the forest and their pH was found to be between 3.69 and 4.06 (Table 3.3a). The isolation yielded a total of 26 thermophilic and 553 mesophilic isolates. All thermophilic isolates appeared to be similar while the mesophilic isolates could be putatively identified as streptomycetes and separated into five different morphological types. All mesophilic isolates were obtained from the starch-casein plates containing nystatin only, none were obtained from plates containing tetracycline or rifampicin.
The most effective soil dilutions for the isolation of mesophilic isolates were $10^{-1}$ and $10^{-2}$, the $10^{-3}$ and $10^{-4}$ dilution plates

**Table 3.2.**
The pH of the Freshfields soil in relation to depth.

| Horizon | Depth (cm) | Mean pH |
|---|---|---|
| $A_{(0)}$- L<br>F1<br>F2-H | 1-5 | 3.70 |
| $A_{(1)}$ | 6-10 | 4.20 |
| Upper C | 11-25 | 7.50 |
| Lower C | 26+ | 8.10 |

Table constructed using data from Williams et al (1971) and Khan (1972).

**Table 3.3a**

Description and average pH values of the Freshfields soil samples.

| Site | Mean pH (+/-S.D) |
|---|---|
| Top of ridge | 3.85 (+/-0.07) |
| Bottom of hollow | 3.67 (+/-0.09) |
| Under tree | 3.89 (+/-0.05) |
| Near edge of forest | 4.06 (+/-0.11) |

Each reading represents the average of eight readings.

**Table 3.3b**

Description and pH of Icelandic soils used in the isolation.

| soil No: | Site | pH |
|---|---|---|
| 3979 | soil under ice | 4.0 |
| 3981 | volcanic crater | 3.6 |
| 3983 | geiser | 3.2 |
| 3996 | geothermal pool | 3.0 |
| 3997 | geothermal pool | 3.0 |
| 4005 | soil near stream | 3.5 |

Data and description supplied by Glaxo Group Research.

yielded relatively few isolates. The thermophilic isolates were obtained only at the $10^{-1}$ and $10^{-2}$ dilutions. The spread plate method proved the most effective method of isolation for both the mesophilic and thermophilic isolates, the dry soil method yielded relatively few isolates, and a higher proportion of these were lost through contamination. Contamination at 28°C was largely due to the growth of competing fungi while at 50°C it was due to the growth of competing bacilli. Overall however, contamination was minimal, particularly at 50°C.

### 3.4: Isolation from the Icelandic Soils

The pH of the Icelandic soils was listed as being between 3.0 and 4.0 by Glaxo (Table 3.3b). Four of the six soils (numbers 3981, 3983, 3996 and 3997) yielded no actinomycete isolates at all. However, these were all inorganic sands which are generally unfavourable for actinomycete populations.

The two remaining soils, number 3979 (soil under ice, pH 4.0) and number 4005 (soil near stream, pH 3.5), yielded 19 and 11 mesophilic isolates respectively. No thermophilic isolates were obtained. These soils were both darkly stained and possessed some plant cover and thus showed a higher organic content. They were therefore more favourable for actinomycete populations. A total of 553 mesophilic and 26 thermophilic isolates were therefore obtained, as pure cultures, from the Freshfields soils and a total of 30 isolates were obtained from the Icelandic soils. The next step was to screen these isolates in liquid culture in order to select the acidophilic from the neutrophilic strains.

# THE SELECTION OF ACIDOPHILIC ACTINOMYCETES

## 3.5: Introduction

The ability of an actinomycete isolate to grow on agar at pH 4.5 does not necessarily classify it as acidophilic. Many actinomycetes are capable of growing on solid media at this pH by raising local pH in the agar, and thus classification on agar is unreliable. For this reason, all the soil isolates, including the 16 Glaxo isolates, were put through a preliminary pH screen in liquid media at pH 4.0, 5.0 and 7.0 in order to select strains which appeared acidophilic or which showed some degree of acid preference compared to the neutrophilic and acid tolerant strains. As a guide-line for selection, those strains which showed optimum growth at either pH 5.0 or pH 4.0, but none at pH 7.0 were termed acidophilic, those strains which showed optimum growth at pH 7.0 with little or no growth at pH 5.0 and none at pH 4.0 were termed neutrophilic, and those which showed optimum growth at pH 7.0, none at pH 4.0, but some at pH 5.0 were termed acid tolerant.

## 3.6: Preliminary pH Screen

Of the mesophilic isolates, 99 of the pine forest isolates and 5 of the Glaxo isolates showed optimum growth at pH 7.0 with no growth at pH 4.0 or 5.0. These isolates were thus termed neutrophilic. A further 156 pine forest isolates, 10 Icelandic isolates and 4 Glaxo isolates showed equal growth at pH 7.0 and pH 5.0 with none at pH 4.0. However, where growth occurred at pH 5.0, there was an increase in culture pH to 5.5 or above.

These were also therefore termed neutrophilic. The remaining 277 pine forest isolates, 20 Icelandic isolates and 7 Glaxo isolates showed optimum growth at pH 7, good growth at pH 5.0 and none at pH 4.0. Where growth occurred at pH 5.0, there was no increase in culture pH, which remained generally static around pH 5.0, and where growth occurred at pH 7.0, final culture pH remained above pH 6.5. These isolates were termed acid tolerant. The thermophilic isolates however, appeared to show some degree of acid preference. None of the thermophilic isolates showed significant growth at pH 4.0 ($O.D_{550}$ of between 0.15 and 0.40), all showed optimum growth at pH 5.0 ($O.D_{550}$ of between 1.4 and 1.7), and 12 showed some growth at pH 7.0 ($O.D_{550}$ of between 0.5 and 1.2). The remaining 14 showed no growth at pH 7.0. Moreover, for those isolates that did show growth at pH 7.0, a drop in culture pH to between 4.5 and 4.8 was observed . A final culture pH of between 4.5 and 4.8 was also observed after growth at pH 5.0. The thermophilic isolates (optimum growth at pH 5.0) therefore differed quite clearly from the mesophilic ones (optimum growth at pH 7.0). A drop in culture pH to below pH 5.0 was recorded for those thermophilic isolates that did grow at pH 7.0, compared with static or more usually, increased culture pH for the mesophilic isolates on the same medium. The increase in culture pH that occurred during growth of the mesophilic isolates was probably due to deamination as the medium SV2a is complex and highly proteinaceous. The drop in culture pH that occurred during growth of the thermophilic isolates was thought to be due to acids released from the metabolism of glucose. However, why deamination, as occurred with the mesophilic isolates, either did not take place, or did not give rise to an increase in culture pH, is

not known. As a result of the preliminary pH screen, all of the mesophilic isolates, including the Icelandic and Glaxo isolates were classified either as neutrophilic or at best, as acid tolerant, and so were rejected. However, as the 26 thermophilic isolates were found to show some degree of acid preference and so were selected for further study.

# PRELIMINARY pH AND TEMPERATURE CHARACTERISATION OF THERMOPHILIC ACTINOMYCETES

## 3.7: Introduction.

From the preliminary pH screen all the mesophilic isolates, including those supplied by Glaxo, were found to be neutrophilic or at best acid tolerant. Only the 26 thermophilic isolates obtained from the Freshfields soils were found to show any degree of acid preference. However, they could not be classified as acidophilic due to the limited data available from this screen. More information concerning their response to pH was required and for this reason each isolate was put through a more comprehensive pH screen, testing the growth of each isolate in liquid culture over a range of pH values between pH 3.0 and 8.0. Each isolate was also put through a comprehensive temperature screen whereby growth in liquid culture was tested over a range of temperatures between 30 and 70°C in order to determine the temperature profiles of the isolates.

## 3.8: The pH Profiles of the Thermophilic Isolates

The pH profile of each isolate was tested in SV2a broth over a range of pH values between pH 3.0 and pH 8.0. All 26 isolates were found to show very similar pH profiles with growth occurring either in the pH range 4.5 to 6.5 (for 14 of the isolates) or 4.5 to 7.0 (for the remaining 12 isolates).

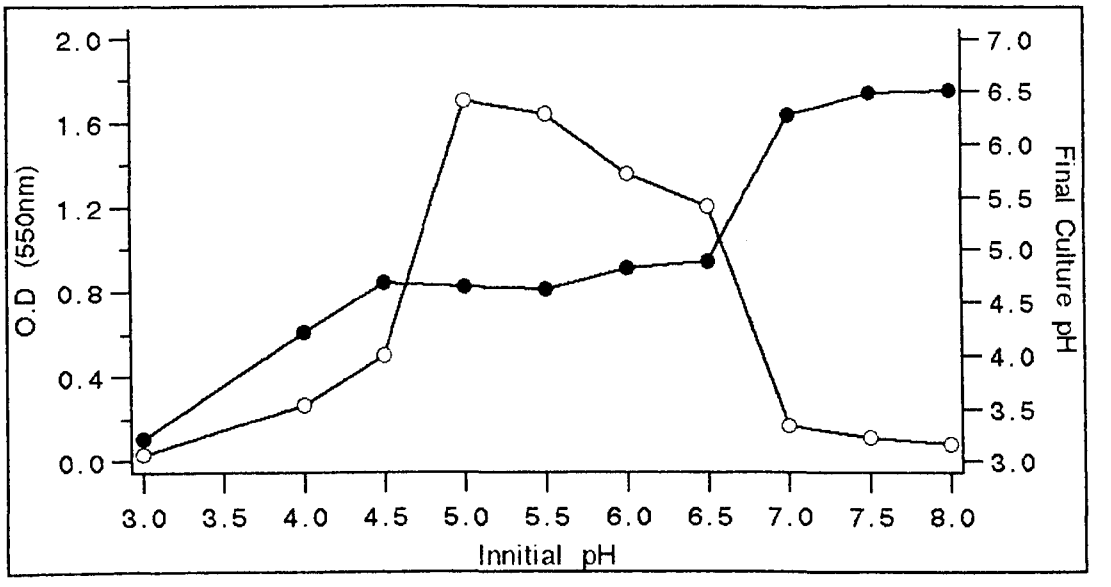
All showed optimum growth at pH 5.0, and when growth occurred above this level, a drop in culture pH to between pH 4.5 and 4.9 was observed. Because of this similarity, the profiles of all 26

isolates are not shown. Instead, one isolate representative of the two different pH profiles are shown (Fig 3.1a and b). When compared to the pH profiles of previously isolated acidophilic actinomycetes, for example strains termed *Actinomyces (Streptomyces) acidophilus* (Jensen 1928) which showed growth in liquid culture between pH 2.6 and 5.5 and streptomycetes isolated from Freshfields pine forest which showed optimum growth between pH 4.5 and 5.5 with variable growth at pH 3.5 and 6.5 (Khan & Williams 1975, Flowers & Williams 1976), the profiles of the pine forest isolates, although showing a general similarity, showed two crucial differences. First, the pH range for growth of the pine forest isolates extended up to pH 6.5/7.0 and second, generally poor growth occurred below pH 5.0, and these differences meant that the isolates could not be classified as acidophilic. However, the profiles differ considerably from those of acid tolerant and neutrophilic actinomycetes which characteristically show growth in the pH range 5.5 to 8.5 with optimum growth between pH 6.5 and 7.5 (Williams et al 1971, Khan & Williams 1975) and thus they cannot be classified as acid tolerant either. Their pH profiles are however similar to those of certain members of the genus *Bacillus*. As well as obligate acidophiles such as *Alicyclobacillus acidocaldarius*, *A.acidoterrestris*, *A.cycloheptanicus* and *Bacillus tusciae* which show growth in the pH range 2.0 to 6.0 with optimum growth between pH 3.5 and 4.5 (Darland & Brock 1971, Deinhard et al 1987a+b, Bonjour & Aragno 1984, there are also moderate acidophiles such as *B.naganoensis*, which shows growth in the pH range 4.1 to 6.0 with an optimum of pH 4.7 to 5.5 (Tomimura et al 1990), *B.smithii*, which shows growth at pH 5.7 but not at pH 4.5

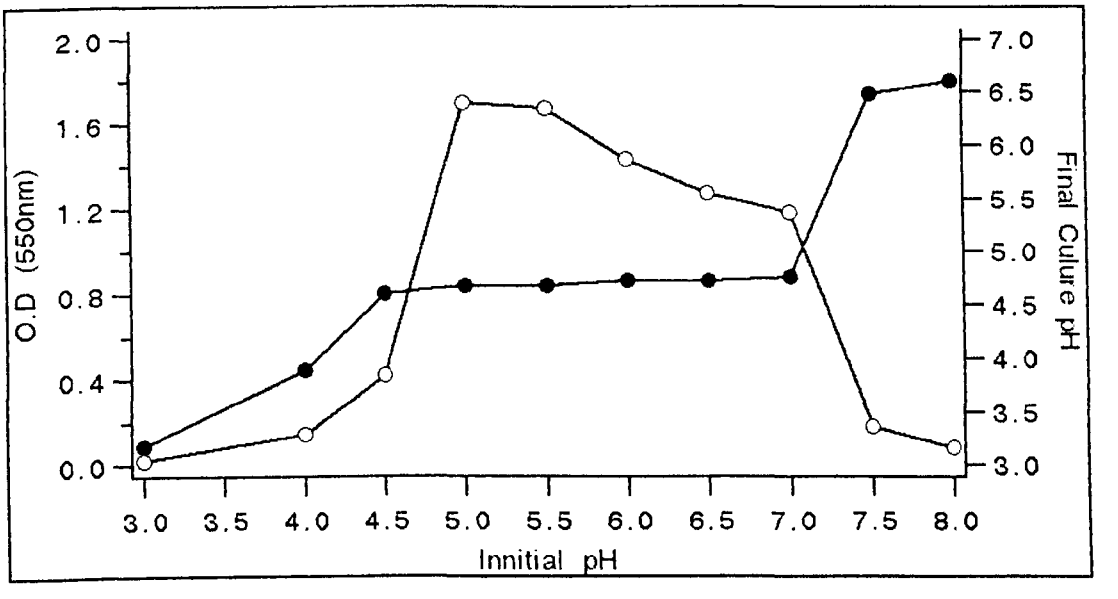
**Figure 3.1**

Final growth yield, as $O.D_{550}$ (open circles) and final culture pH (closed circles) plotted against a range of starting culture pH values for a:) isolate 17c, and b:) isolate 17a, grown in SV2a at 50°C. Data taken after 48h incubation. Each is typical of three replicates. Standard deviation never greater than 5% for pH data and 10% for growth data.

a:)


O.D (550nm)
2.0
1.6
1.2
0.8
0.4
0.0
Final Culture pH
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0
Innitial pH

b:)


O.D (550nm)
2.0
1.6
1.2
0.8
0.4
0.0
Final Culure pH
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0
Innitial pH

or at pH 7.7 (Nakamura et al 1988) and *B.coagulans*, which shows growth at pH 4.5, 5.7 and 7.7 (Nakamura et al 1988). Growth and culture pH were measured after 24, 48, 72 and 96h and plotted against time for isolate 17c and 17a at both pH 5.0 and 6.5 (Fig 3.2). At pH 5.0, both isolates showed significant growth after 24h and a maximum was reached after 48h. During this time culture pH decreased to pH 4.65 for isolate 17c and to pH 4.75 for isolate 17a. At pH 6.5, both isolates again showed significant growth after 24h with a maximum occurring after 48-72h. Growth after 24h was however slightly less effective than at pH 5.0 and it was also accompanied by a significant drop in culture pH from 6.5 to 5.08 for 17c and to 5.14 for isolate 17a. Further growth up to 48 and 72h was accompanied by smaller drops in culture pH until final values of pH 4.68 and 4.74 were reached for isolates 17c and 17a respectively. The same basic trend was shown by all the thermophilic isolates.

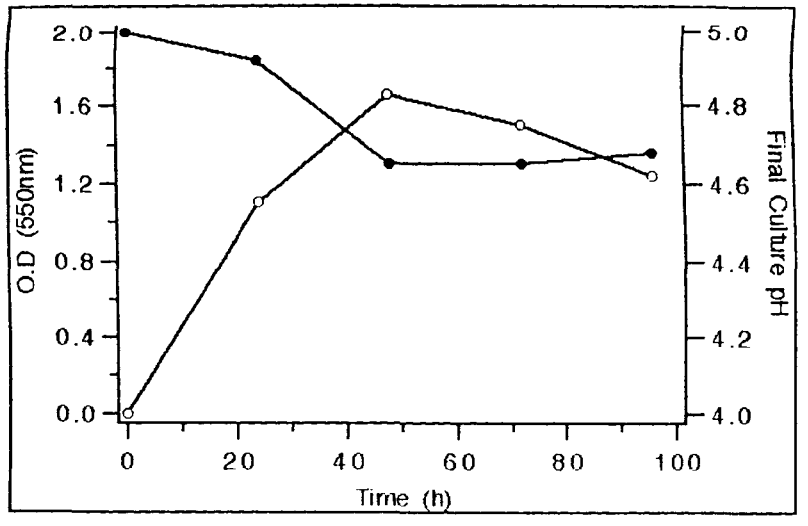
Two important points emerged from this data. First, the drop in culture pH appeared to occur as a direct result of growth, possibly due to the release of acids from the metabolism of glucose, and second, the drop in culture pH appeared to have no adverse affect on growth. These pose the questions, whether the drop in culture pH was a mechanism that allowed these organisms to grow at higher pH values, or was it simply a by-product of growth, and would the pH profile for growth of these isolates be the same if the starting pH was maintained through growth.

If the profile was the same then the isolates could be classified as acid tolerant. However, if the isolates could not grow at higher pH values that were controlled and kept constant, it would confirm them as acidophilic. These questions can however only

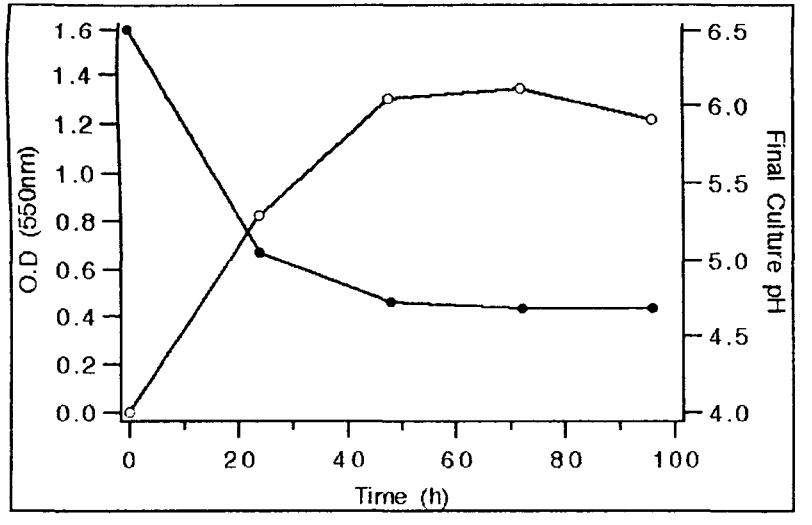
**Figure 3.2**

Final growth yield, as $O.D_{550}$ (open circles) and final culture pH (closed circles) plotted against incubation time for a:) isolate 17c-starting pH of 5.0, b:) isolate 17c-starting pH of 6.5, c:) isolate 17a-starting pH of 5.0 and d:) isolate 17a-starting pH of 6.5, grown in SV2a at 50°C. Data taken after 48h incubation. Each is typical of three replicates. Standard deviation never greater than 5% for pH data and 10% for growth data.

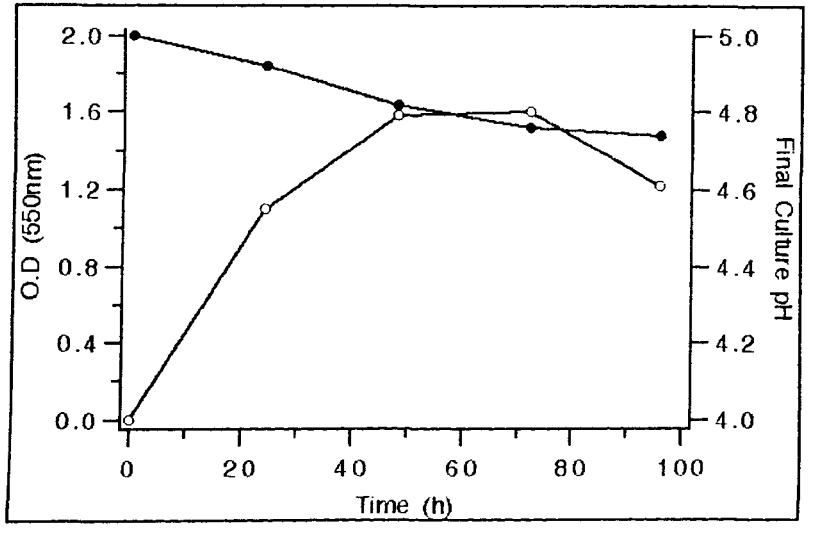
a:)


O.D (550nm)
Final Culture pH
Time (h)
0.0
0.4
0.8
1.2
1.6
2.0
4.0
4.2
4.4
4.6
4.8
5.0
0
20
40
60
80
100

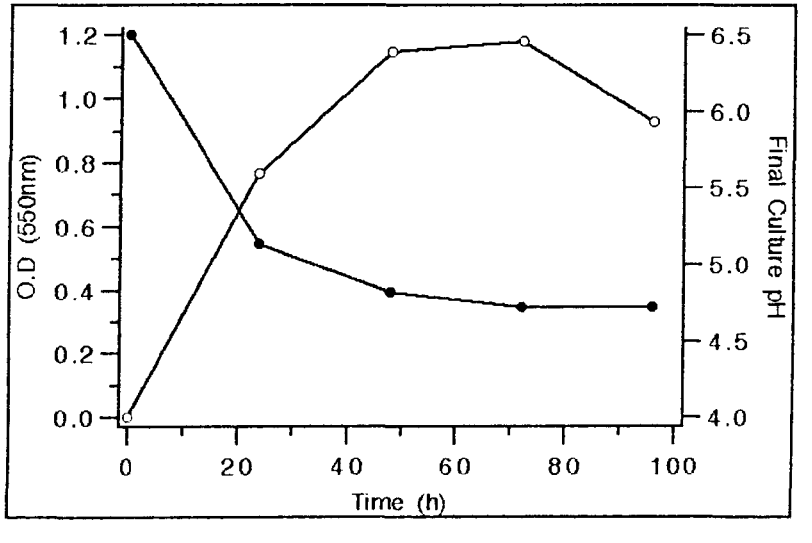
b:)


O.D (550nm)
Final Culture pH
Time (h)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
4.0
4.5
5.0
5.5
6.0
6.5
0
20
40
60
80
100

c:)


O.D (550nm)
Final Culture pH
Time (h)
0.0
0.4
0.8
1.2
1.6
2.0
4.0
4.2
4.4
4.6
4.8
5.0
0
20
40
60
80
100

d:)


O.D (550nm)
Final Culture pH
Time (h)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
4.0
4.5
5.0
5.5
6.0
6.5
0
20
40
60
80
100

accurately be answered by studying growth in a fermenter which can be used to grow cultures both with and without pH control and where pH is not controlled, change in culture pH can be constantly monitored.

## 3.9: The Temperature Profiles of the Thermophilic Isolates

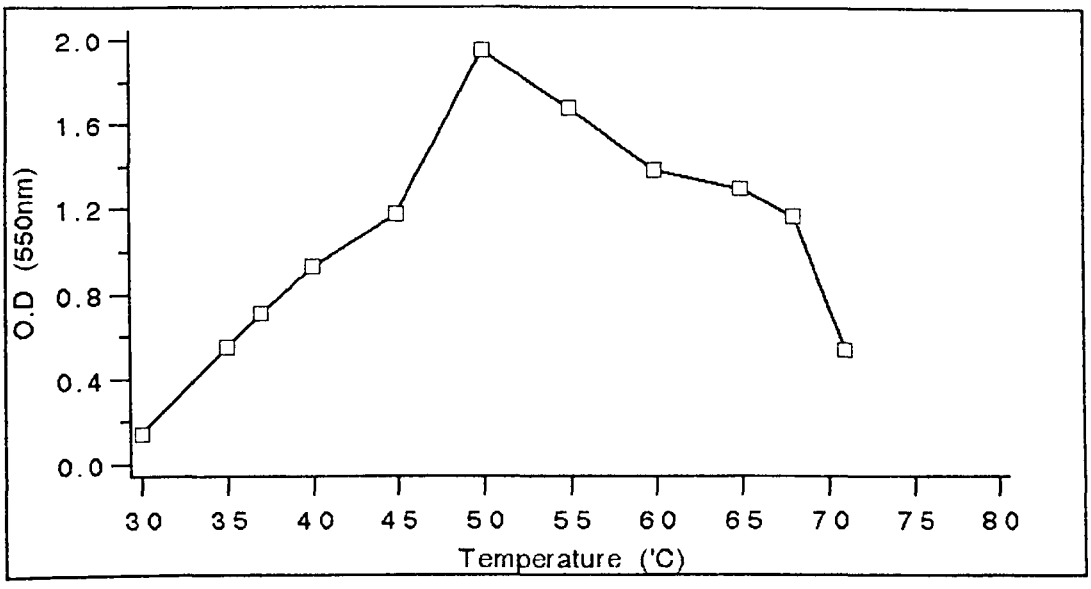
Growth of each isolate was tested in SV2a broth over a range of temperatures between 30 and 70°C. The data for the temperature profiles were taken after 48h growth as this was generally when maximum growth had occurred. The exception was for 68 and 71°C. At these temperatures growth reached a maximum after 24h and isolates had often lysed by 48h. Readings for these two temperatures were therefore taken after 24h incubation.

All the isolates showed very similar temperature profiles and so the same two representatives used in the pH screen (17c and 17a), were selected again and their temperature profiles are shown in Fig 3.3 (a and b). Growth for these, and all 26 isolates, occurred in the temperature range 35 to 71°C, with significantly better growth occurring above 40°C and optimum growth occurring between 50 and 55°C. The isolates could therefore be classified as thermophilic.

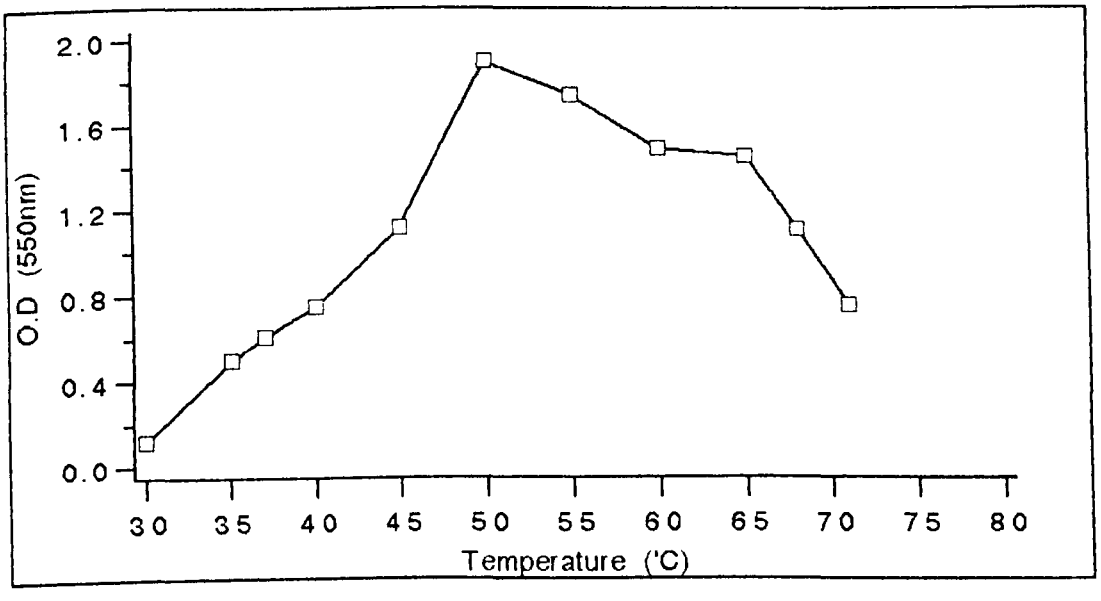
**Figure 3.3**

Final growth yields, as $O.D_{550}$ plotted against a range of incubation temperatures for a:) isolate 17c, and b:) isolate 17a, grown in SV2a broth at pH 5.0. Data taken after 48h incubation. Each is typical of three replicates. Standard deviation never greater than 10%.

a:)


O.D (550nm)
2.0
1.6
1.2
0.8
0.4
0.0
30 35 40 45 50 55 60 65 70 75 80
Temperature ('C)

b:)


O.D (550nm)
2.0
1.6
1.2
0.8
0.4
0.0
30 35 40 45 50 55 60 65 70 75 80
Temperature ('C)

# CHAPTER 4

# IDENTIFICATION OF THE THERMOPHILIC ISOLATES

## 4.1: Preliminary Identification

A preliminary identification carried out under a light microscope putatively identified each of the 26 thermophilic isolates as belonging to the genus *Thermoactinomyces* (Figure 4.1). Because this genus is very distinctive, it is fairly simple to identify and distinguish from actinomycete isolates using a few key tests, for example the presence of endospores, the presence of *meso*-D.A.P, the lack of characteristic cell wall sugars and amino acids and resistance to novobiocin. In order to confirm this initial classification therefore, a range of these determinative tests were carried out. The presence of refractile endospores had been noted during the microscopic studies and this was confirmed using a Schaeffer-Fulton endospore stain (Doetsch 1981). The endospores were then tested for their heat resistance and found to remain viable at 100°C for up to between 40 and 50 min (Table 4.1). This was comparable to the *Thermoactinomyces* type strains tested which were viable for between 30 and 50 min, and easily distinguished from the characteristic actinomycete arthrospores of the thermotolerant *S.thermoviolaceous* and the thermophilic streptomycete obtained from Glaxo's culture collection, which were destroyed after 10 min at 100°C. Each isolate was found to be resistant to novobiocin by testing for growth on SV2a agar containing 50ug/ml of novobiocin, to possess *meso*-DAP (Table 4.2) but no characteristic cell wall sugars (Table 4.3). These results, combined with the microscopic analysis, confirmed that all the thermophilic isolates belonged to the genus *Thermoactinomyces*.

Figure 4.1.

Photograph (*40 magnification) of *Thermoactinomyces* isolate 17c grown in ME3 medium at pH 5.0.

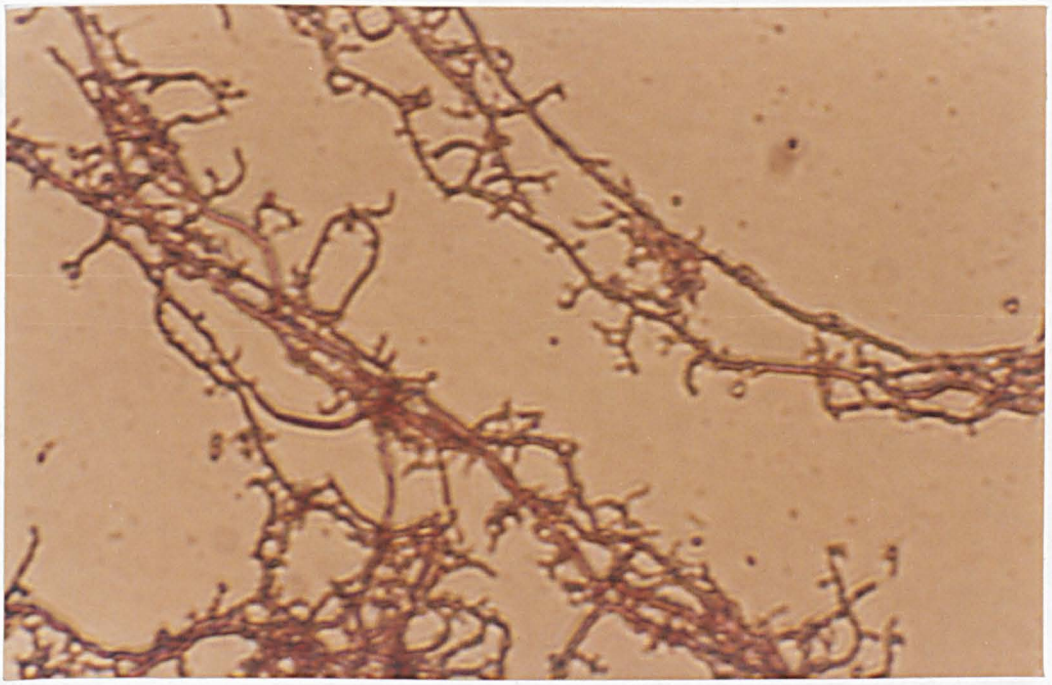


**Table 4.1**

Resistance of endospores of the *Thermactinomyces* isolates to 100°C with *Thermoactinomyces* type strains and arthrospores of the thermotolerant *Streptomyces thermoviolaceous* and a thermophilic streptomyete from Glaxo's culture collection as controls.

| | Time (min) | | | | | | |
|---|---|---|---|---|---|---|---|
| **Isolate** | 10 | 20 | 30 | 40 | 50 | 60 | 90 |
| 17c | + | + | + | + | - | - | - |
| 5b | + | + | + | + | + | - | - |
| 2b | + | + | + | + | - | - | - |
| 5e | + | + | + | + | + | - | - |
| 17a | + | + | + | + | + | - | - |
| | | | | | | | |
| *T.vulgaris* 2316c | + | + | + | + | - | - | - |
| *T.thalpophilus* A1502 | + | + | + | - | - | - | - |
| *T.sacchari* 2433c | + | + | + | + | + | - | - |
| *T.putidus* 2501c | + | + | + | + | - | - | - |
| | | | | | | | |
| *S.thermoviolaceous* | - | - | - | - | - | - | - |
| Thermophilic strep: | - | - | - | - | - | - | - |

**Table 4.2**

Pine forest isolates tested for the presence of 2,6-diaminopimelic acid (D.A.P), with *Thermoactinomyces* type and culture collection strains, and a Streptomyces strain as controls.

| Isolates | *meso*-D.A.P | LL-D.A.P |
|---|---|---|
| 1a | + | - |
| 2a | + | - |
| 2b | + | - |
| 3d | + | - |
| 4a | + | - |
| 5b | + | - |
| 5d | + | - |
| 5e | + | - |
| 8a | + | - |
| 15a | + | - |
| 17a | + | - |
| 17c | + | - |
| *T.vulgaris* 2316c | + | - |
| *T.thalpophilus* A1502 | + | - |
| *T.putidus* 2501c | + | - |
| *T.sacchari* 2433c | + | - |
| A1503 | + | - |
| A1506 | + | - |
| A1510 | + | - |
| A1513 | + | - |
| *Streptomyces* sp. | - | + |

**Table 4.3**

Pine forest isolates tested for the presence of cell wall sugars, with *Thermoactinomyces* type and culture collection strains as controls.

| Isolates | Rhamnose | Galactose | Mannose | Arabinose | Xylose |
|---|---|---|---|---|---|
| 1a | - | - | - | - | - |
| 2a | - | - | - | - | - |
| 2b | - | - | - | - | - |
| 3d | - | - | - | - | - |
| 4a | - | - | - | - | - |
| 5b | - | - | - | - | - |
| 5d | - | - | - | - | - |
| 5e | - | - | - | - | - |
| 8a | - | - | - | - | - |
| 15a | - | - | - | - | - |
| 17a | - | - | - | - | - |
| 17c | - | - | - | - | - |
| *T.vul.* 2316c | - | - | - | - | - |
| *T.thalp.* A1502 | - | - | - | - | - |
| *T.putidus* 2501c | - | - | - | - | - |
| *T.sac.* 2433c | - | - | - | - | - |
| A1503 | - | - | - | - | - |
| A1506 | - | - | - | - | - |
| A1510 | - | - | - | - | - |
| A1513 | - | - | - | - | - |
| *Strep.* sp. | - | + | - | + | - |

## 4.2: Comparison of the *Thermoactinomyces* Isolates with Type and Culture Collection Strains

Each isolate was identified as belonging to the genus *Thermoactinomyces*. However, it was not known whether these isolates were similar to known *Thermoactinomyces* species, or whether they were novel strains. Of particular importance was the pH response. *Thermoactinomyces* isolates had only ever been isolated from neutral soils and their pH profiles had never been tested. It was therefore not known whether the pH profiles shown by the pine forest isolates were unique, or whether they were common to all *Thermoactinomyces* isolates and for this reason the pH response of the *Thermoactinomyces* type and culture collection strains was tested and compared to that of the pine forest isolates. The pine forest isolates were also compared to the *Thermoactinomyces* type and culture collection strains in terms of their basic physiology and their colony morphology. Four type strains, *T.vulgaris* 2316c, *T.thalpophilus* A1502, *T.sacchari* 2433c and *T.putidus* 2501c, and a selection of unspecified *Thermoactinomyces* isolates were obtained from Glaxo's culture collection.

## 4.3: The Effects of pH on the Growth of *Thermoactinomyces* Type and Culture Collection Strains

The type and culture collection strains were grown up in SV2a broth at pH 5.0 and 7.0. All the type and culture collection strains tested showed growth at pH 7.0 but failed to grow at pH 5.0 (Table 4.4). Moreover, when growth occurred at pH 7.0 the culture pH only dropped to a minimum of 6.5 (data not shown). The type and culture collection strains were therefore

**Table 4.4**

*Thermoactinomyces* type and culture collection strains tested for growth at pH 5.0 and 7.0 in SV2a.

| **Isolate:** | **Growth at pH 5.0** | **Growth at pH 7.0** |
|---|---|---|
| *T.vulgaris* 2433c | - | + |
| *T.thalpophilus* A1502 | - | + |
| *T.putidus* 2501c | - | + |
| *T.sacchari* 2433c | - | + |
| A1501 | - | + |
| A1503 | - | + |
| A1504 | - | + |
| A1506 | - | + |
| A1508 | - | + |
| A1509 | - | + |
| A1510 | - | + |
| A1511 | - | + |
| A1512 | - | + |
| A1513 | - | + |
| A1515 | - | + |

neutrophilic and differed distinctly from the pine forest isolates in their response to pH.

## 4.4: The pH Profiles of the *Thermoactinomyces* Type Strains

The four type strains *T.vulgaris* 2316c, *T.thalpophilus* A1502, *T.sacchari* 2433c and *T.putidus* 2501c, were grown up in shake flask, in SV2a broth over a range of pH values between pH 5.0 and 9.0. Each of the four strains tested showed growth in the pH range 6.0 to 8.5 with optimum growth occurring at pH 7.0. These profiles were therefore obviously different to the pH profiles of the pine forest isolates (Fig 4.2). Moreover, when growth of the type strains occurred there was, at most, only a small drop in culture pH of around 0.2 to 0.4 pH units below that of the initial pH (data not shown).

## 4.5: Physiological Tests

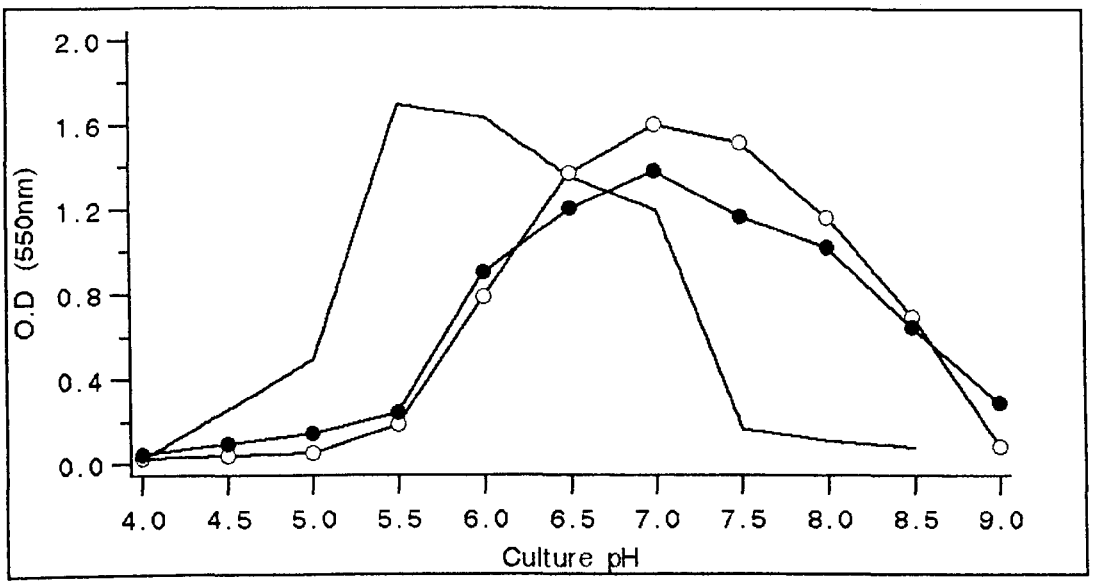
Each of the pine forest isolates was tested, along with the four type strains and a selection of culture collection strains, for a range of physiological factors designed to distinguish between the type strains (Lacey 1989).

The pine forest isolates all gave identical results for each test, thus indicating that they were all very similar, if not the same strain (Table 4.5a). They differed from all the type and culture collection strains in two tests, the ability to degrade adenine (pine forest isolates were positive, type strains negative) and the ability to degrade tween 20 (pine forest isolates were negative, type strains positive). As well as these two major differences, the pine forest isolates differed from each type strain in at least

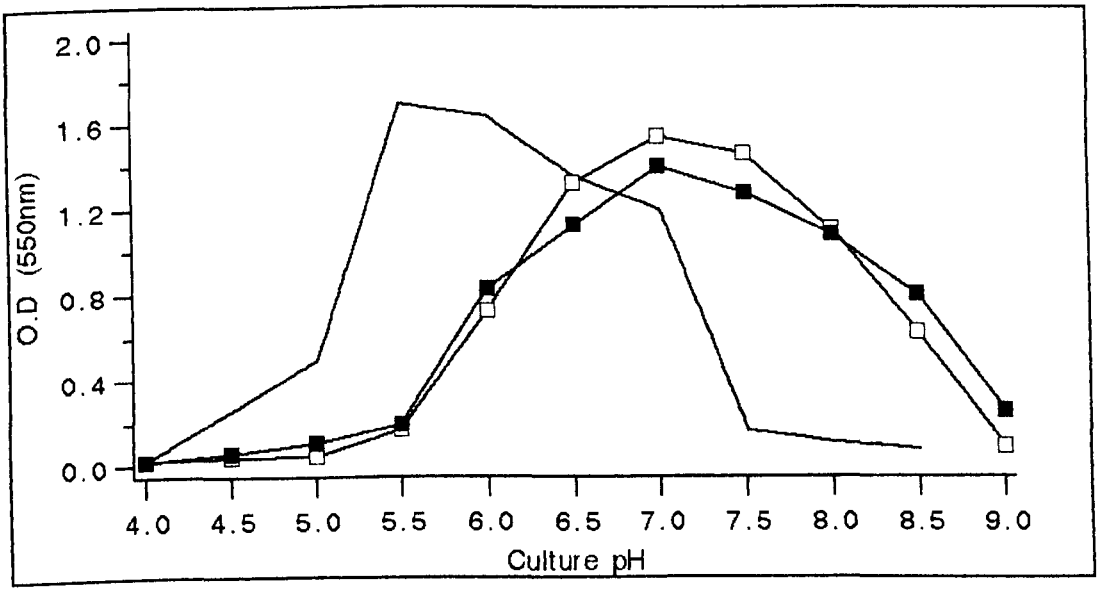
**Figure 4.2**

Growth yield, as $O.D_{550}$ of *T.vulgaris* 2316c (open circles), *T.thalpophilus* A1502 (open squares), *T.putidus* 2501c (closed circles) and *T.sacchari* 2433c (closed squares), grown in SV2a at 50°C and plotted against a range of culture pH values. Each is typical of three replicates. Standard deviation never greater than 10%. The pH profile of isolate 17c is shown in outline as a comparison.

a:)


2.0
1.6
1.2
0.8
0.4
0.0
O.D (550nm)
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
Culture pH

b:)


2.0
1.6
1.2
0.8
0.4
0.0
O.D (550nm)
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
Culture pH

one other test. For example they differed from *T.vulgaris* 2316c, A1503 and A1506 in one other test, from *T.sacchari* 2433c in 2 other tests, from A1510 in 3 other tests, from *T.putidus* 2501c and A1513 in 4 other tests and from *T.thalpophilus* in 5 other tests (Table 4.5a and b).

## 4.6: Colony Morphology

The colony morphology of the pine forest isolates was compared with that of the type and culture collection strains on a variety of different media. The pine forest isolates differed quite distinctly from the type and culture collection strains tested. *Thermoactinomyces* isolates classically show extensive aerial sporulation giving either a yellow (*T.dichotomicus*) or white (all other species) powdery appearance to the colony. The white powdery appearance was shown by all the type and culture collection strains tested (Fig 4.3), however the pine forest isolates showed no aerial sporulation on any of the media used and always gave brown mycelial colonies, which appeared after 12-24h incubation at 50°C (Fig 4.4). If cultures were incubated further, after approximately 72-96h cell lysis began to take place (Fig 4.5), a common occurrence amongst *Thermoactinomyces* strains, which was then followed by some degree of limited aerial sporulation. The pine forest isolates therefore differed quite significantly from the type and culture collection strains tested, in terms of their basic physiology, their colony morphology, and most importantly, in terms of their response to pH. The pine forest isolates therefore appeared to be novel *Thermoactinomyces* strains and showed a response to pH

**Table 4.5a**

Pine forest isolates tested for the ability to degrade a range of substrates, including tolerance to 1% NaCl and 25ug/ml novobiocin.

| **Substrate** | 1a | 2a | 2b | 3a | 3d | 4a | 5b | 5d | 5e | 8a | 15a | 17a | 17c |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Arbutin | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Casein | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Adenine | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Esculin | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Starch | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Tyrosine | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Xanthine | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Hypoxanthine | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Chitin | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Cellulose | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Xylan | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Tween 20 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Tween 40 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Tween 60 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Tween 80 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 1% NaCl | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Melanin prod: | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Novobiocin res: | + | + | + | + | + | + | + | + | + | + | + | + | + |

**Table 4.5b**

*Thermoactinomyces* type and culture collection strains tested for the ability to degrade a range of substrates, including tolerance to 1% NaCl and 25ug/ml novobiocin.

| **Substrate** | T.vul: 2316c | T.put: 2501c | T.sac: 2433c | T.thal: A1502 | A1503 | A1506 | A1510 | A1513 |
|---|---|---|---|---|---|---|---|---|
| Arbutin | + | + | + | - | + | + | + | - |
| Casein | + | + | + | + | + | + | + | + |
| Adenine | - | - | - | - | - | - | - | - |
| Esculin | + | + | + | - | + | + | - | - |
| Starch | - | - | - | + | - | + | + | + |
| Tyrosine | - | + | - | - | - | - | + | - |
| Xanthine | - | + | - | + | - | - | - | + |
| Hypoxanthine | - | - | - | + | - | - | - | - |
| Chitin | - | - | - | - | - | - | - | - |
| Cellulose | - | - | - | - | - | - | - | - |
| Xylan | - | - | - | - | - | - | + | - |
| Tween 20 | + | + | + | + | + | + | + | + |
| Tween 40 | + | + | + | + | + | + | + | + |
| Tween 60 | + | + | + | + | + | + | + | + |
| Tween 80 | + | + | + | + | + | + | + | + |
| 1% NaCl | + | + | - | - | + | - | + | - |
| Melanin prod: | - | + | - | - | - | - | - | - |
| Novobiocin res: | + | + | + | + | + | + | + | + |

Figure 4.3.

Photographs of *Thermoactinomyces* type strains, a): *T.vulgaris*, b): *T.thalpophilus*, c): *T.putidus* and d): *T.sacchari*.

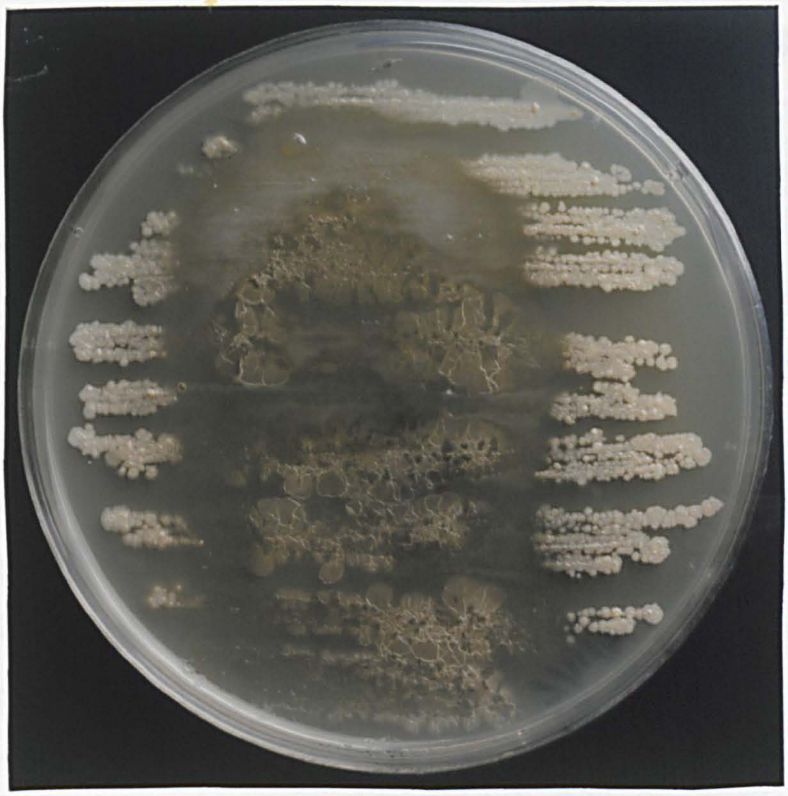
*T.vulgaris*, *T.thalpophilus* and *T.putidus* were grown on SV2a agar, *T.sacchari* was grown on CYC agar, both at pH 7.0.

Figure 4.4.

Photographs of *Thermoactinomyces* pine forest isolates a): 17c and b): 17a, grown on SV2a agar at pH 5.0 after 48h incubation.

Figure 4.5.

Photograph of *Thermoactinomyces* pine forest isolate 17c grown on SV2a agar at pH 5.0 after 96h incubation.



which was clearly different from that shown by *Thermoactinomyces* strains isolated from neutral soils.

## 4.7 Ecological Study

All 26 *Thermoactinomyces* isolates obtained in the initial study were from just one site, the Freshfields pine forest in Lancashire. In order therefore to further examine the ecological distribution of these isolates, a number of different acid and neutral soils were collected and a selective isolation for *Thermoactinomyces* carried out. A total of five different soils were collected (Table 4.6). However, the main soil used was from Delamere pine forest. Like the Freshfields soil this had been well studied in the past and shown to yield large numbers of acidophilic mesophilic actinomycetes. The Delamere soil was a mature podzol under *Pinus silvestris* and consisted of an acidic surface litter ($A_0$) horizon overlaying four identifiable, darkly stained, acidic soil horizons ($A_1$, $A_2$, $B_1$ and $B_2$) which were in turn laid on an alkaline parent sand (C horizon). Samples were collected from the four acidic soil horizons $A_1$ to $B_2$. These isolations, which were smaller in scale than for the Freshfields soils, yielded 5 isolates from the Delamere soils and 3 from the spruce forest soil. All eight were identified as *Thermoactinomyces* and were found to show the same pH profile, general morphology and basic biochemistry as the Freshfields isolates. No isolates were obtained from the neutral soils tested. It can therefore be concluded that the *Thermoactinomyces* isolates obtained are quite widespread in acid soils, albeit often present in low numbers, and that they are not present in neutral soils. Along with the pH profiles for growth, this supports the

proposition that they are actively growing in acid forest soils and that they are therefore active members of the microbial population present in these soils. It also therefore discounts the possibility that the isolates were not native to these soils but had originated in other environments and the isolations were picking up endospores which were lying dormant, not actively growing hyphae. The Isolation conditions of 50°C, pH 5.0 and in the presence of novobiocin and nystatin, were highly selective for the *Thermoactinomyces* isolates with the result that no competing organisms grew on the isolation plates.

**Table 4.6**

Soils used in the ecological study.

| Soil | Mean pH (+/- S.D) | Location |
|---|---|---|
| Pine forest | 4.05(+/-0.11) | Delamere, Cheshire |
| Pine forest | 4.22(+/-0.18) | Cheshire |
| Spruce forest | 4.08(+/-0.20) | Cheshire |
| Spruce forest | 4.20(+/-0.17) | Lancashire |
| Peat soil | 4.35(+/-0.16) | Lancashire |

# CHAPTER 5

# GROWTH STUDIES

## 5.1: Introduction

During the preliminary pH and temperature studies the *Thermoactinomyces* isolates had been maintained and grown on SV2a agar and broth. This proved to be an effective growth medium, giving optical density readings at 550nm between 1.4 and 1.8. However, in order to obtain the optimum growth medium a range of different media were investigated. A defined medium was also required so that the effect of nutritional factors on growth, enzyme and antibiotic production could be accurately studied. To this end a range of carbon and nitrogen sources were individually tested for their ability to support growth in the presence of a basal medium. The best three of each were then selected and combined with a basal salts medium to produce a range of defined media that were then compared so that the optimum could be found.

## 5.2: Growth in Defined Media

A range of carbon and nitrogen sources were tested for their ability to support growth. Each carbon source was added (15 g/l) to an SV2a basal medium consisting of (g/l): soya peptone, 15 and NaCl, 3. Each nitrogen source was added (1 and 3 g/l) to a basal medium consisting of (g/l): glucose, 15, NaCl, 3 and $KH_2PO_4$ 1. The three most effective carbon and nitrogen sources identified in this test were glucose, sucrose and glycerol, and ammonium sulphate, glutamate and proline, respectively (Data not shown). However, all nitrogen sources used gave relatively very poor growth ($O.D_{550}$ between 0.5 and 0.7), particularly when compared

to growth obtained using the complex nitrogen source soya peptone ($O.D_{550}$ between 1.5 and 1.9), and it therefore appeared that these organisms were unable to grow effectively on defined nitrogen sources. The selected carbon and nitrogen sources were then combined with a basal salts medium to produce nine different combinations (Table 5.1). Each carbon source was added to a final concentration of 0.1M while each nitrogen source was added to a final concentration of 0.02M. The salts medium was prepared by adding 0.5ml of 400mM $KH_2PO_4$, 0.5ml of 200mM $MgSO_4.7H_2O$, 0.5ml of 20mM $CaCl_2.2H_2O$ and 0.5ml of trace elements solution to 48ml of each medium aseptically after autoclaving. Trace elements solution contained (g/l) $ZnSO_4$ 0.861, $MnSO_4$ 0.223, $H_3BO_3$ 0.0618, $CuSO_4$ 0.125, $Na_2MoO_4$ 0.0484, $CoCl_2$ 0.0476, $FeSO_4$ 1.8, KI 0.083 and 1ml of 1M $H_2SO_4$.
All nine media supported only poor growth (Table 5.2), with the most effective being the three media containing ammonium sulphate. This was followed by the three media containing glutamate and then the three containing proline. The isolates were therefore unable to grow effectively on the defined media tested and this appeared to be due to an inability to utilise defined nitrogen sources.

## 5.3: Growth in Complex Media

In order to find the most effective growth medium A range of complex media and their various modifications were tested against SV2a (Table 5.3). The medium ME3 was found to be most effective, giving average optical density readings in the range 1.8 to 2.1, as opposed to SV2a which gave optical density readings in the range 1.4 to 1.8. Growth in ME3 was also generally faster

**Table 5.1.**

Make up of the nine defined media tested in the growth study.

| **Medium 1a** | **Medium 1b** | **Medium 1c** |
|---|---|---|
| glucose | glucose | glucose |
| proline | ammonium sulphate | glutamate |
| salts | salts | salts |
| **Medium 2a** | **Medium 2b** | **Medium 2c** |
| glycerol | glycerol | glycerol |
| proline | ammonium sulphate | glutamate |
| salts | salts | salts |
| **Medium 3a** | **Medium 3b** | **Medium 3c** |
| sucrose | sucrose | sucrose |
| proline | ammonium sulphate | glutamate |
| salts | salts | salts |

**Table 5.2.**

Growth, as $O.D_{550}$, of selected *Thermoactinomyces* pine forest isolates on each of the nine defined media, at 50°C and pH 5.0. All data is typical of at least two replicates and standard deviation was never greater than 20%.

| Isolate: | Media | | |
|---|---|---|---|
| | m1a | m2a | m3a |
| 1a | 0.325 | 0.280 | 0.295 |
| 2b | 0.320 | 0.310 | 0.322 |
| 4a | 0.537 | 0.510 | 0.509 |
| 5b | 0.385 | 0.325 | 0.390 |
| 5d | 0.350 | 0.315 | 0.390 |
| 8a | 0.508 | 0.368 | 0.525 |
| 14b | 0.385 | 0.330 | 0.348 |
| 17a | 0.468 | 0.374 | 0.338 |
| 17c | 0.406 | 0.366 | 0.375 |
| 18a | 0.396 | 0.365 | 0.381 |

| Isolate: | Media | | |
|---|---|---|---|
| | m1b | m2b | m3b |
| 1a | 0.421 | 0.393 | 0.382 |
| 2b | 0.380 | 0.364 | 0.346 |
| 4a | 0.595 | 0.501 | 0.607 |
| 5b | 0.465 | 0.390 | 0.415 |
| 5d | 0.532 | 0.380 | 0.535 |
| 8a | 0.505 | 0.475 | 0.495 |
| 14b | 0.345 | 0.311 | 0.294 |
| 17a | 0.507 | 0.420 | 0.486 |
| 17c | 0.510 | 0.488 | 0.430 |
| 18a | 0.413 | 0.390 | 0.403 |

| Isolate: | Media | | |
|---|---|---|---|
| | m1c | m2c | m3c |
| 1a | 0.450 | 0.435 | 0.480 |
| 2b | 0.530 | 0.490 | 0.540 |
| 4a | 0.410 | 0.440 | 0.451 |
| 5b | 0.424 | 0.500 | 0.480 |
| 5d | 0.420 | 0.416 | 0.470 |
| 8a | 0.505 | 0.455 | 0.500 |
| 14b | 0.395 | 0.351 | 0.375 |
| 17a | 0.420 | 0.420 | 0.470 |
| 17c | 0.484 | 0.445 | 0.507 |
| 18a | 0.480 | 0.390 | 0.425 |

**Table 5.3.**

Growth, as $O.D_{550}$, and resulting culture pH, of selected *Thermoactinomyces* pine forest isolates grown on a range of complex media, at pH 5.0 and 50°C. All data is typical of at least two replicates and standard deviation was never greater than 10%.

| | | MEDIA | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | MYA | | GYEA | | YEA | | CYC | |
| Isolate | T(h) | O.D | pH | O.D | pH | O.D | pH | O.D | pH |
| 2b | 24 | 0.708 | 4.91 | 0.788 | 4.95 | 0.722 | 4.94 | 0.440 | 4.97 |
| | 48 | 0.980 | 4.88 | 1.070 | 4.95 | 1.085 | 4.88 | 0.530 | 4.96 |
| 5b | 24 | 0.780 | 4.91 | 0.888 | 4.96 | 0.690 | 4.96 | 0.388 | 4.97 |
| | 48 | 1.032 | 4.84 | 1.125 | 4.88 | 1.027 | 4.84 | 0.462 | 4.96 |
| 5e | 24 | 0.750 | 4.90 | 0.712 | 4.95 | 0.780 | 4.91 | 0.502 | 4.95 |
| | 48 | 0.994 | 4.87 | 1.090 | 4.90 | 1.120 | 4.84 | 0.650 | 4.95 |
| 17a | 24 | 0.735 | 4.92 | 0.900 | 4.90 | 0.740 | 4.91 | 0.418 | 4.96 |
| | 48 | 0.962 | 4.87 | 1.138 | 4.84 | 1.060 | 4.83 | 0.580 | 4.92 |
| 17c | 24 | 0.852 | 4.89 | 0.995 | 4.91 | 0.804 | 4.95 | 0.450 | 4.97 |
| | 48 | 1.088 | 4.81 | 1.227 | 4.83 | 1.182 | 4.85 | 0.522 | 4.94 |

| | | MEDIA | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | GMY | | GMY1 | | ME1 | | ME2 | |
| Isolate | T(h) | O.D | pH | O.D | pH | O.D | pH | O.D | pH |
| 2b | 24 | 0.697 | 4.97 | 0.821 | 4.91 | 1.145 | 4.88 | 1.204 | 4.89 |
| | 48 | 1.087 | 4.92 | 1.193 | 4.87 | 1.322 | 4.82 | 1.380 | 4.82 |
| 5b | 24 | 0.796 | 4.96 | 0.900 | 4.92 | 1.052 | 4.87 | 1.160 | 4.88 |
| | 48 | 1.052 | 4.91 | 1.204 | 4.88 | 1.360 | 4.80 | 1.347 | 4.73 |
| 5e | 24 | 0.767 | 4.93 | 0.872 | 4.94 | 1.080 | 4.89 | 1.190 | 4.87 |
| | 48 | 1.088 | 4.88 | 1.176 | 4.90 | 1.400 | 4.81 | 1.408 | 4.79 |
| 17a | 24 | 0.805 | 4.97 | 0.808 | 4.95 | 1.146 | 4.85 | 1.145 | 4.83 |
| | 48 | 1.158 | 4.90 | 1.148 | 4.92 | 1.390 | 4.78 | 1.394 | 4.74 |
| 17c | 24 | 0.784 | 4.94 | 0.976 | 4.95 | 1.189 | 4.85 | 1.154 | 4.81 |
| | 48 | 1.035 | 4.89 | 1.250 | 4.91 | 1.426 | 4.75 | 1.386 | 4.69 |

| | | MEDIA | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ME3 | | ME4 | | ME5 | | ME6 | |
| Isolate | T(h) | O.D | pH | O.D | pH | O.D | pH | O.D | pH |
| 2b | 24 | 1.750 | 4.72 | 1.120 | 4.80 | 1.092 | 4.80 | 0.592 | 4.90 |
| | 48 | 2.058 | 4.34 | 1.320 | 4.76 | 1.270 | 4.72 | 0.870 | 4.84 |
| 5b | 24 | 1.860 | 4.50 | 1.088 | 4.82 | 1.128 | 4.81 | 0.628 | 4.91 |
| | 48 | 2.150 | 4.28 | 1.313 | 4.76 | 1.310 | 4.68 | 0.910 | 4.88 |
| 5e | 24 | 1.630 | 4.84 | 1.055 | 4.80 | 1.025 | 4.78 | 0.525 | 4.94 |
| | 48 | 1.980 | 4.50 | 1.345 | 4.74 | 1.235 | 4.72 | 0.835 | 4.88 |
| 17a | 24 | 1.745 | 4.82 | 1.122 | 4.83 | 1.085 | 4.85 | 0.585 | 4.92 |
| | 48 | 1.960 | 4.70 | 1.388 | 4.78 | 1.282 | 4.79 | 0.782 | 4.87 |
| 17c | 24 | 1.730 | 4.74 | 1.180 | 4.82 | 1.028 | 4.81 | 0.628 | 4.88 |
| | 48 | 2.188 | 4.35 | 1.356 | 4.78 | 1.305 | 4.80 | 0.805 | 4.85 |

| | | MEDIA | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | AM1a | | AM1b | | SV2a | | Sv2a1 | |
| Isolate | T(h) | O.D | pH | O.D | pH | O.D | pH | O.D | pH |
| 2b | 24 | 1.244 | 4.82 | 1.332 | 4.72 | 1.245 | 4.85 | 0.922 | 4.93 |
| | 48 | 1.588 | 4.54 | 1.635 | 4.50 | 1.590 | 4.73 | 1.188 | 4.88 |
| 5b | 24 | 1.188 | 4.80 | 1.268 | 4.75 | 1.280 | 4.83 | 0.910 | 4.92 |
| | 48 | 1.570 | 4.64 | 1.640 | 4.51 | 1.647 | 4.71 | 1.155 | 4.88 |
| 5e | 24 | 1.205 | 4.79 | 1.302 | 4.80 | 1.286 | 4.84 | 0.906 | 4.93 |
| | 48 | 1.482 | 4.71 | 1.633 | 4.63 | 1.623 | 4.72 | 1.210 | 4.88 |
| 17a | 24 | 1.174 | 4.82 | 1.257 | 4.78 | 1.276 | 4.82 | 0.905 | 4.93 |
| | 48 | 1.478 | 4.75 | 1.628 | 4.68 | 1.682 | 4.73 | 1.180 | 4.86 |
| 17c | 24 | 1.160 | 4.82 | 1.243 | 4.81 | 1.305 | 4.82 | 0.900 | 4.91 |
| | 48 | 1.475 | 4.75 | 1.622 | 4.74 | 1.701 | 4.68 | 1.202 | 4.85 |

| | MEDIA | | | | | |
|---|---|---|---|---|---|---|
| | SV2a2 | | SV2a3 | | SV2a4 | |
| Isolate | O.D | pH | O.D | pH | O.D | pH |
| 2a | 1.120 | 4.90 | 1.112 | 4.92 | 0.645 | 4.95 |
| | 1.366 | 4.80 | 1.353 | 4.87 | 0.988 | 4.90 |
| 5b | 1.184 | 4.86 | 1.124 | 4.89 | 0.587 | 4.94 |
| | 1.407 | 4.78 | 1.346 | 4.85 | 0.912 | 4.91 |
| 5e | 1.105 | 4.91 | 1.040 | 4.92 | 0.622 | 4.96 |
| | 1.315 | 4.83 | 1.287 | 4.87 | 0.890 | 4.93 |
| 17a | 1.095 | 4.89 | 1.202 | 4.95 | 0.640 | 4.94 |
| | 1.384 | 4.81 | 1.388 | 4.89 | 0.873 | 4.92 |
| 17c | 1.091 | 4.90 | 1.150 | 4.88 | 0.592 | 4.96 |
| | 1.306 | 4.81 | 1.370 | 4.82 | 0.880 | 4.91 |

than in SV2a, reaching far higher O.D readings after 24h than SV2a. The limiting factor for growth in all these media appeared to be the nitrogen source used, with soya peptone the most effective, often giving optical density readings double that of the same medium containing a different complex nitrogen source such as yeast extract or casein hydrolysate. None of the isolates proved able to grow significantly on the defined media used and so these media were rejected. The reason for this poor growth appeared to be due to the defined nitrogen sources used. ME3 was selected for use in all further growth experiments, replacing SV2a. Growth of the two selected isolates, 17a and 17c, was tested in ME3 over the pH range 4.5 to 7.0 (data not shown) and both isolates gave exactly the same profiles as for growth in SV2a. Growth occurred in the pH range 4.5 to 6.5/7.0 with optimum growth at pH 5.0, and a drop in culture pH to between 4.4 and 4.8 was observed where growth occurred at pH 5.0 and above. This drop in culture pH occurred in both media despite the release of between 350 and 400ug/ml in ME3 and between 400 and 500ug/ml in SV2a, of ammoniacal nitrogen (Table 5.5). One possible explanation of this is that the effect of the ammonium ions released is cancelled out by the release of acids from the metabolism of sugars present in the media.

## 5.4 Growth in Basal Medium

Each isolate was grown in SV2a basal medium, which contained (g/l) soya peptone 15 and NaCl 3, at pH 5.0 and 7.0 (Table 5.4). All isolates grew at pH 5.0, showing optimum $O.D_{550}$ readings of between 1.3 and 1.7 after 48h, but failed to show significant growth at pH 7.0. However, growth at pH 5.0 resulted in an

**Table 5.4.**

Growth, as $O.D_{550}$, and resulting culture pH, of selected *Thermoactinomyces* pine forest isolates grown in SV2a basal medium, at pH 5.0 and 7.0 and 50°C. All data is typical of at least two replicates and standard deviation was never greater than 20%.

| | | pH 5.0 | | pH 7.0 | |
|---|---|---|---|---|---|
| **Isolate** | **T(h)** | **O.D** | **Culture pH** | **O.D** | **Culture pH** |
| 1a | 24 | 0.944 | 4.81 | 0.190 | 6.40 |
| | 48 | 1.226 | 6.22 | 0.288 | 6.82 |
| 2b | 24 | 1.048 | 4.72 | 0.147 | 6.53 |
| | 48 | 1.447 | 5.53 | 0.257 | 7.02 |
| 5b | 24 | 1.210 | 4.66 | 0.065 | 6.88 |
| | 48 | 1.707 | 7.50 | 0.126 | 7.10 |
| 5d | 24 | 0.965 | 4.78 | 0.082 | 6.87 |
| | 48 | 1.510 | 7.40 | 0.126 | 7.22 |
| 5e | 24 | 1.050 | 4.81 | 0.105 | 6.78 |
| | 48 | 1.403 | 7.96 | 0.210 | 7.24 |
| 17a | 24 | 1.258 | 4.65 | 0.118 | 6.82 |
| | 48 | 1.524 | 7.44 | 0.306 | 6.85 |
| 17c | 24 | 1.128 | 4.77 | 0.088 | 6.90 |
| | 48 | 1.519 | 6.53 | 0.155 | 6.88 |
| 18a | 24 | 0.538 | 4.80 | 0.155 | 6.93 |
| | 48 | 1.625 | 6.85 | 0.212 | 7.05 |

**Table 5.5.**

Levels of ammoniacal nitrogen present in SV2a basal, ME3 and SV2a media after growth of selected *Thermoactinomyces* isolates in shake flask at pH 5.0 and 50°C. All data represents an average of at least three replicates and standard deviation was never greater than 5%.

**Basal medium**

| Isolates | Ammoniacal Nitrogen (ug/ml) |
|---|---|
| 2b | 1675 |
| 5b | 1590 |
| 5e | 1698 |
| 17a | 1730 |
| 17c | 1695 |
| Medium blank | 72.5 |

**SV2a medium**

| Isolates | Ammoniacal Nitrogen (ug/ml) |
|---|---|
| 2b | 472.5 |
| 5b | 480 |
| 5e | 455 |
| 17a | 495 |
| 17c | 488 |
| Medium blank | 67.5 |

**ME3 medium**

| Isolates | Ammoniacal Nitrogen (ug/ml) |
|---|---|
| 2b | 365 |
| 5b | 372.5 |
| 5e | 350 |
| 17a | 365 |
| 17c | 385 |
| Medium blank | 55.2 |

**Starch medium**

| Isolates | Ammoniacal Nitrogen (ug/ml) |
|---|---|
| 2b | 388 |
| 5b | 375 |
| 5e | 375 |
| 17a | 392.5 |
| 17c | 395 |
| Medium blank | 55 |

increase in culture pH, to between pH 5.5 and pH 8.0. Growth on the basal medium therefore differed considerably from growth on the complete SV2a and ME3 media which contained the sugars, where there was a drop in culture pH, and indicated that it was the metabolism of sugars that resulted in the drop in culture pH in the complete media. The increase in culture pH due to growth in the basal medium was caused by the release of ammoniacal nitrogen (Table 5.5) and was therefore probably as a result of deamination. The levels of ammoniacal nitrogen released in the basal medium were very much larger than in ME3 or SV2a, between 1500 and 1650ug/ml compared to between 300 and 400ug/ml. This increase in amount of ammoniacal nitrogen released, added to the fact that because there were no sugars present in the basal medium there was no release of acids as a result of their metabolism, explained the increase in culture pH. The reason why the levels of ammoniacal nitrogen were so much higher in the basal medium than ME3 or SV2a was probably because in the basal medium, soya peptone served as both nitrogen and carbon source and thus the isolates had to deaminate in order to obtain both carbon and nitrogen from the amino acids, hence giving rise to a greater degree of deamination and thus a greater amount of ammoniacal nitrogen released into the medium.

## 5.5: Growth in Basal Medium Containing Starch

Each isolate was grown in SV2a basal medium containing 15g/l of soluble starch, at pH 5.0 and 7.0 (Table 5.6). All isolates showed growth at pH 5.0 and 7.0, with optimum $O.D_{550}$ readings of between 1.4 and 1.7 after 48h at pH 5.0 and between 1.2 and 1.5 after 48h at pH 7.0. Growth was therefore better at pH 5.0 than

**Table 5.6.**

Growth, as $O.D_{550}$, and final culture pH, of *Thermoactinomyces* pine forest isolates grown in SV2a basal medium containing 15g/l of soluble starch. All data is typical of at least two replicates and standard deviation was never greater than 10%.

| | | pH 5.0 | | pH 7.0 | |
|---|---|---|---|---|---|
| **Isolates** | **T(h)** | **O.D** | **Final pH** | **O.D** | **Final pH** |
| 1a | 24 | 1.284 | 4.88 | 0.986 | 4.94 |
| | 48 | 1.785 | 5.22 | 1.347 | 5.44 |
| 2b | 24 | 1.218 | 4.76 | 0.890 | 5.03 |
| | 48 | 1.588 | 5.44 | 1.412 | 5.48 |
| 5b | 24 | 1.205 | 4.80 | 0.922 | 4.88 |
| | 48 | 1.573 | 5.28 | 1.387 | 5.35 |
| 5d | 24 | 1.216 | 4.82 | 0.788 | 5.20 |
| | 48 | 1.684 | 5.37 | 1.456 | 5.52 |
| 5e | 24 | 1.186 | 4.80 | 0.860 | 4.92 |
| | 48 | 1.377 | 5.21 | 1.245 | 5.20 |
| 17a | 24 | 1.190 | 4.76 | 1.020 | 5.01 |
| | 48 | 1.516 | 5.39 | 1.422 | 5.32 |
| 17c | 24 | 1.232 | 4.78 | 0.950 | 4.94 |
| | 48 | 1.664 | 5.28 | 1.385 | 5.48 |
| 18a | 24 | 1.192 | 4.86 | 0.795 | 5.03 |
| | 48 | 1.530 | 5.40 | 1.290 | 5.27 |

at pH 7.0. At pH 5.0, after an initial drop in culture pH after 24h to between 4.7 and 4.9, final culture pH then increased to between 5.2 and 5.5 after 48h. At pH 7.0, culture pH dropped to between 4.8 and 5.0 after 24h before increasing to between 5.2 and 5.5 after 48h. The drop in culture pH was probably due to the metabolism of sugars (ie: glucose) from the breakdown of starch. The following increase in culture pH was due to ammoniacal nitrogen released into the medium (Table 5.5) and was thus probably due to deamination. Approximately the same levels of ammoniacal nitrogen were found in this starch medium as in ME3 and yet a small increase in culture pH occurred in this medium which did not occur in ME3. A possible reason for this could be that a smaller degree of sugar metabolism occurred in this medium, which contained 1.5% starch as opposed to 1.0% glucose and 2.0% malt extract in ME3, with the result that less acids were produced to offset the ammoniacal nitrogen.

## 5.6: Growth in Basal Medium Containing Oils

Each isolate was grown in SV2a basal medium containing 15g/l of Tween 80, vegetable oil or olive oil, at pH 5.0 and 7.0. When grown on Tween 80 and vegetable oil (Table 5.7a and b), at pH 5.0 significant growth occurred, with $O.D_{550}$ readings in the range 1.4 to 1.9, however culture pH increased to between 7.0 and 8.7. Growth therefore differed to that which occurred when sugars and other carbon sources were present in the medium. At pH 7.0, growth was generally poorer, with $O.D_{550}$ readings of up to 0.9, and was characterised by a drop in culture pH after 24h, to between 5.7 and 6.4. This was then followed by better growth, with $O.D_{550}$ readings between 1.0 and 1.4, and an increase in

culture pH to between pH 7.0 and 8.5 after 48h. Thus two systems appeared to be operating at the two pH values. At pH 5.0, growth resulted in an increase in culture pH which appeared to have no effect on growth, while at pH 7.0, after an initial drop in culture pH good growth occurred which again resulted in an increase in culture pH. When grown on olive oil (Table 5.7c), at pH 5.0 growth was very good, with $O.D_{550}$ readings around 2.0 . Most commonly this resulted in an increase in culture pH to between 7.0 and 8.5, as for growth in tween 80 and vegetable oil, however occasionally a slight decrease occurred to below pH 5.0 after 24h and sometimes this would then increase up to above pH 7.0 after 48h (as for isolate 5e) or remain below pH 5.0 (as for isolate 5d). Similarly, sometimes culture pH would increase above pH 7.0 after 24h but then drop below pH 5.0 after 48h (as for isolate 17a). At pH 7.0, a similar pattern appeared. One possible explanation for the overall increase in culture pH during growth on the three substrates tested was that as negatively charged free fatty acids were assimilated, positive ions were released, possibly in the form of ammonium ions, thus raising culture pH. The initial drop in culture pH at pH 7.0 was probably due to metabolism of freely available sugars present in the soya peptone as the same result occurred in the basal medium, where soya peptone was the only carbon source present. Moreover, as optimum growth occurred after the drop in culture pH, it appeared that this drop was required for growth. Growth of the isolates in oils was therefore clearly different to growth in media where sugars were present. Growth on oils and in the basal medium therefore showed that the isolates are able to continue growing if culture pH increases, even to around pH 8.5.

However, it still appeared that a starting pH between 5 and 6 was required.

**Table 5.7a**

Growth, as $O.D_{550}$, and resulting culture pH, of selected *Thermoactinomyces* isolates grown on SV2a basal medium plus tween 80. All data is typical of at least two replicates and standard deviation was never greater than 20%.

| | | pH 5.0 | | pH 7.0 | |
|---|---|---|---|---|---|
| **Isolate** | **T(h)** | **O.D** | **Culture pH** | **O.D** | **Culture pH** |
| 1a | 24 | 0.850 | 7.12 | 0.308 | 6.15 |
| | 48 | 1.580 | 8.24 | 0.469 | 7.20 |
| 2b | 24 | 0.805 | 7.50 | 0.235 | 6.48 |
| | 48 | 1.520 | 8.21 | 0.262 | 6.80 |
| 5b | 24 | 1.120 | 8.10 | 0.480 | 5.70 |
| | 48 | 1.640 | 8.22 | 1.025 | 8.12 |
| 5d | 24 | 0.985 | 7.55 | 0.682 | 5.92 |
| | 48 | 1.610 | 8.21 | 1.312 | 8.21 |
| 5e | 24 | 0.925 | 7.36 | 0.495 | 5.72 |
| | 48 | 1.525 | 8.14 | 1.140 | 8.20 |
| 15a | 24 | 0.930 | 7.47 | 0.544 | 6.02 |
| | 48 | 1.690 | 8.20 | 1.195 | 8.13 |
| 17a | 24 | 1.130 | 7.88 | 0.465 | 6.09 |
| | 48 | 1.625 | 8.20 | 1.155 | 7.80 |
| 17c | 24 | 1.080 | 7.90 | 0.450 | 6.17 |
| | 48 | 1.510 | 8.19 | 1.120 | 7.92 |
| 18a | 24 | 0.828 | 7.56 | 0.435 | 6.22 |
| | 48 | 1.620 | 8.18 | 1.120 | 7.38 |

**Table 5.7b**

Growth, as $O.D_{550}$, and resulting culture pH, of selected *Thermoactinomyces* isolates grown on SV2a basal medium plus vegetable oil.

| | | pH 5.0 | | pH 7.0 | |
|---|---|---|---|---|---|
| **Isolate** | **T(h)** | **O.D** | **Culture pH** | **O.D** | **Culture pH** |
| 1a | 24 | 1.388 | 8.12 | 0.325 | 6.18 |
| | 48 | 1.675 | 8.58 | 1.245 | 8.42 |
| 2b | 24 | 1.347 | 8.20 | 0.840 | 6.12 |
| | 48 | 1.680 | 8.62 | 1.026 | 8.22 |
| 5b | 24 | 1.215 | 8.11 | 0.738 | 6.43 |
| | 48 | 1.710 | 8.77 | 0.963 | 8.50 |
| 5d | 24 | 1.330 | 8.14 | 0.485 | 6.08 |
| | 48 | 1.675 | 8.33 | 1.050 | 8.20 |
| 5e | 24 | 1.318 | 8.05 | 0.970 | 6.34 |
| | 48 | 1.580 | 8.53 | 1.012 | 8.20 |
| 15a | 24 | 0.956 | 8.02 | 0.276 | 5.94 |
| | 48 | 1.350 | 8.37 | 0.682 | 8.10 |
| 17a | 24 | 1.345 | 8.21 | 0.387 | 6.02 |
| | 48 | 1.650 | 8.64 | 0.940 | 8.11 |
| 17c | 24 | 1.405 | 8.17 | 0.368 | 5.88 |
| | 48 | 1.920 | 8.69 | 1.035 | 8.18 |
| 18a | 24 | 1.276 | 8.11 | 0.863 | 6.40 |
| | 48 | 1.584 | 8.43 | 1.004 | 8.35 |

**Table 5.7c**

Growth, as $O.D_{550}$, and resulting culture pH, of selected *Thermoactinomyces* isolates grown on SV2a basal medium plus olive oil.

| | | pH 5.0 | | pH 7.0 | |
|---|---|---|---|---|---|
| **Isolate** | **T(h)** | **O.D** | **Culture pH** | **O.D** | **Culture pH** |
| 2b | 24 | 2.146 | 7.30 | 1.572 | 6.88 |
| | 48 | 2.482 | 7.50 | 1.887 | 8.22 |
| 5b | 24 | 2.040 | 7.70 | 1.952 | 5.72 |
| | 48 | 0.786 | 8.43 | 2.060 | 7.85 |
| 5e | 24 | 2.240 | 4.84 | 1.870 | 7.00 |
| | 48 | 0.745 | 8.23 | 2.127 | 7.81 |
| 17a | 24 | 1.945 | 7.50 | 1.520 | 4.84 |
| | 48 | 2.128 | 4.47 | 2.283 | 7.53 |
| 17c | 24 | 1.988 | 7.52 | 1.285 | 6.06 |
| | 48 | 0.824 | 8.18 | 2.166 | 8.12 |

## 5.7: Introduction

Shake flask studies carried out in Chapter 3 to determine the pH range for growth of the pine forest isolates, showed that the isolates grew optimally in the pH range 5.0 to either 6.5 or 7.0, with optimum growth at pH 5.0 and only relatively poor growth at pH 4.5. Where growth occurred at pH 5.0 and above there was a drop in culture pH to between pH 4.4 and 4.8. This made an accurate assessment of their response to pH difficult in shake flask and for this reason the two selected isolates (17c and 17a), were grown in a fermenter over a range of pH values both with and without pH control.

## 5.8: The Effects of pH on Growth, Glucose Utilisation, Protease Production and Ammoniacal Nitrogen Levels of Isolates 17c and 17a grown in a Fermenter on ME3 Medium.

Isolates 17c and 17a were grown in a fermenter over a range of pH values between pH 4.5 and 7.0 using ME3 broth. Growth, protease activity, glucose and ammoniacal nitrogen levels were measured from the time of inoculation ($T_o$) every half an hour up to 10h. Samples were then taken after 12h, 16h and finally 24h.

### 5.8.1: Growth Without pH Control

The two isolates showed optimal growth without pH control in the range 4.75 to either 6.5 for isolate 17c or 7.0 for isolate 17a and wherever growth occurred in this range, the final culture pH dropped to between 4.4 and 4.75. Both isolates showed optimum

growth at pH 5.0 and relatively poor growth at pH 4.5. This data therefore supported the profiles obtained in shake flask, with the exception that in the fermenter, growth was tested and found also to be optimal at pH 4.75, thus extending the pH range for optimal growth of these isolates below pH 5.0. Table 5.8a shows that very similar fermentation data was observed for both isolates within the pH range for optimal growth (pH 4.75-6.5/7.0) and for this reason only growth at pH 5.0 and 6.5 for isolate 17c (Fig 5.1+5.3), and pH 5.0 and 7.0 for isolate 17a (Fig 5.2+5.4) is shown graphically. For both isolates maximum $O.D_{550}$ of between 1.70 and 2.14 occurred approximately 5-8h after $T_{(0)}$ and culture pH dropped to between 4.4 and 4.75 during exponential growth before it levelled off towards the end of exponential growth and the beginning of stationary phase (approximately 6-9h after $T_{(0)}$). This drop in culture pH was probably due to the release of hydrogen ions as a direct result of the metabolism of glucose. Glucose levels dropped gradually for the first 2-3h after which a sudden increase in levels was observed to between 13 and 15g/l. This occurred during the onset of exponential growth and was followed by a rapid drop over the next few hours and tailing off after approximately 16-24h leaving final glucose levels of between 1 and 3g/l after 24h. The sudden increase in glucose levels shortly after the onset of exponential growth may have been due to reducing sugars being released from the breakdown of the malt extract present in the medium and the sensitivity of the o-toluidine used in the assay to these reducing sugars. The continued decrease of glucose levels after exponential growth is due to the continued of growth of the isolates, no increase in $O.D_{550}$ was seen however due to an equal rate of lysis of old

**Table 5.8a**

Summary of growth data for isolates 17c grown in a fermenter in ME3 medium. Optical density readings were taken at 550nm and represent maximum growth.

| pH | O.D | Final pH | Glucose | Protease | Ammoniacal Nitrogen | T(d) |
|---|---|---|---|---|---|---|
| 4.5 uncontrolled | 0.84 | 4.65 | 7.3 | 425 | 122.5 | 2h 30m |
| 4.5 controlled | 0.92 | 4.50 | 7.2 | 470 | 142.5 | 2h 30m |
| 4.75 uncontrolled | 1.80 | 4.65 | 1.92 | 1945 | 372.5 | 1h 15m |
| 4.75 controlled | 1.73 | 4.75 | 2.12 | 1930 | 367.5 | 1h 45m |
| 5.0 uncontrolled | 2.14 | 4.38 | 1.35 | 2220 | 405 | 1h |
| 5.0 controlled | 1.92 | 5.00 | 1.97 | 2080 | 397.5 | 1h 15m |
| 6.0 uncontrolled | 1.84 | 4.62 | 1.42 | 2120 | 392.5 | 1h |
| 6.0 controlled | 1.68 | 6.00 | 1.24 | 1975 | 380 | 1h 30m |
| 6.5 uncontrolled | 1.80 | 4.67 | 1.81 | 2080 | 380 | 1h |
| 6.5 controlled | 0.42 | 6.50 | 9.1 | 102.5 | 77.5 | -- -- |
| 7.0 uncontrolled | 0.27 | 6.95 | 9.3 | 80 | 67.5 | -- -- |
| 7.0 controlled | 0.30 | 7.00 | 9.9 | 90 | 67.5 | -- -- |

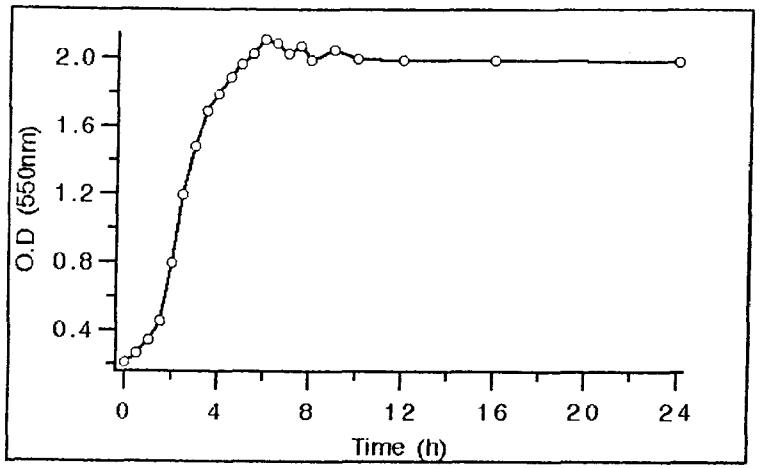
**Table 5.8b**

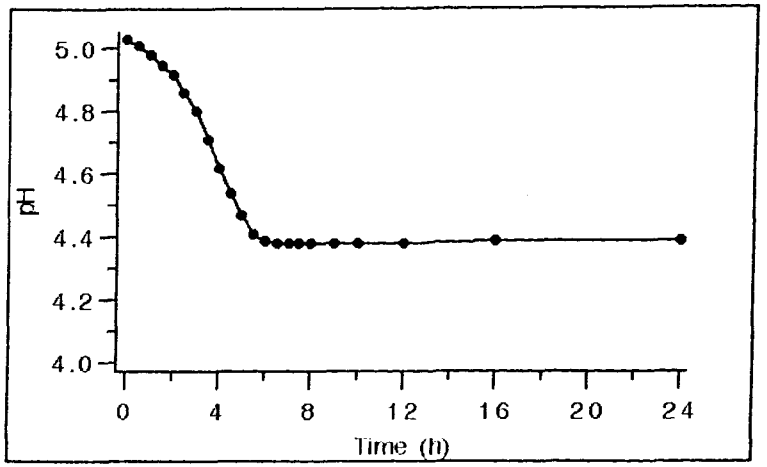
Summary of growth data for isolates 17a grown in a fermenter in ME3 medium. Optical density readings were taken at 550nm and represent maximum growth.

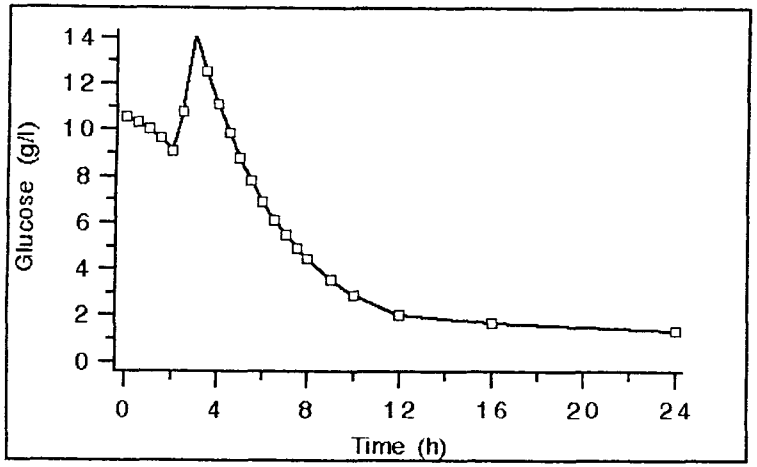
| pH | O.D | Final pH | Glucose | Protease | Ammoniacal Nitrogen | T(d) |
|---|---|---|---|---|---|---|
| 4.5 uncontrolled | 0.82 | 4.64 | 6.75 | 465 | 130 | 2h 15m |
| 4.5 controlled | 0.97 | 4.50 | 6.90 | 485 | 150 | 2h 15m |
| 4.75 uncontrolled | 1.70 | 4.64 | 2.21 | 1955 | 375 | 1h 30m |
| 4.75 controlled | 1.72 | 4.75 | 2.05 | 1935 | 377.5 | 1h 30m |
| 5.0 uncontrolled | 1.92 | 4.67 | 1.85 | 2095 | 395 | 1h |
| 5.0 controlled | 1.84 | 5.00 | 2.20 | 1965 | 365 | 1h 15m |
| 6.0 uncontrolled | 1.84 | 4.74 | 1.79 | 2080 | 382.5 | 1h |
| 6.0 controlled | 1.74 | 6.00 | 1.95 | 1975 | 370 | 1h 30m |
| 6.5 uncontrolled | 1.83 | 4.71 | 2.05 | 2025 | 390 | 1h |
| 6.5 controlled | 0.38 | 6.50 | 9.00 | 110 | 72.5 | -- -- |
| 7.0 uncontrolled | 1.69 | 4.72 | 1.78 | 1945 | 377.5 | 1h |
| 7.0 controlled | 0.38 | 7.00 | 9.20 | 115 | 67.5 | -- -- |

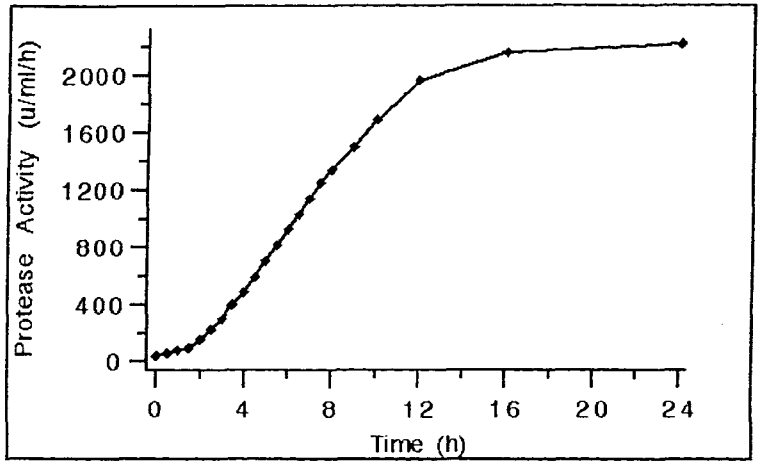
**Figure 5.1.**

Growth (open circles), culture pH (closed circles), glucose (open squares), protease activity (closed diamonds), and Ammoniacal nitrogen (closed triangles), for isolate 17c grown in the fermenter, without pH control, at pH 5.0 and 50°C. Each is typical of at least two replicates and error was never greater than 10%.


O.D (550nm)
2.0
1.6
1.2
0.8
0.4
0
4
8
12
16
20
24
Time (h)


pH
5.0
4.8
4.6
4.4
4.2
4.0
0
4
8
12
16
20
24
Time (h)


Glucose (g/l)
14
12
10
8
6
4
2
0
0
4
8
12
16
20
24
Time (h)


Protease Activity (u/ml/h)
2000
1600
1200
800
400
0
0
4
8
12
16
20
24
Time (h)

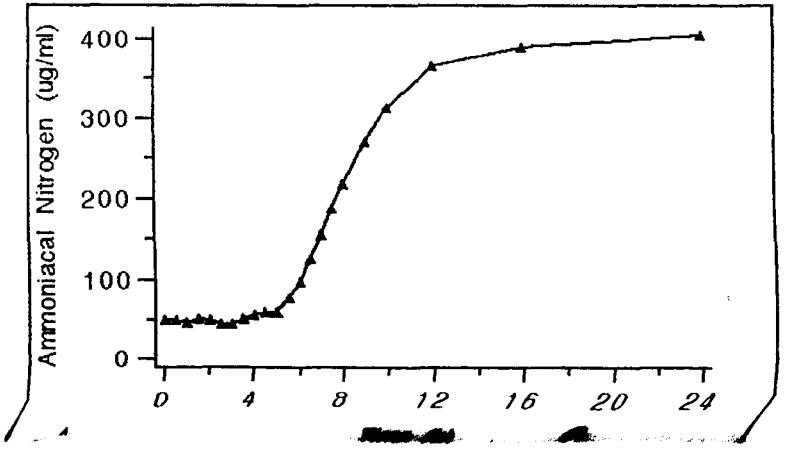
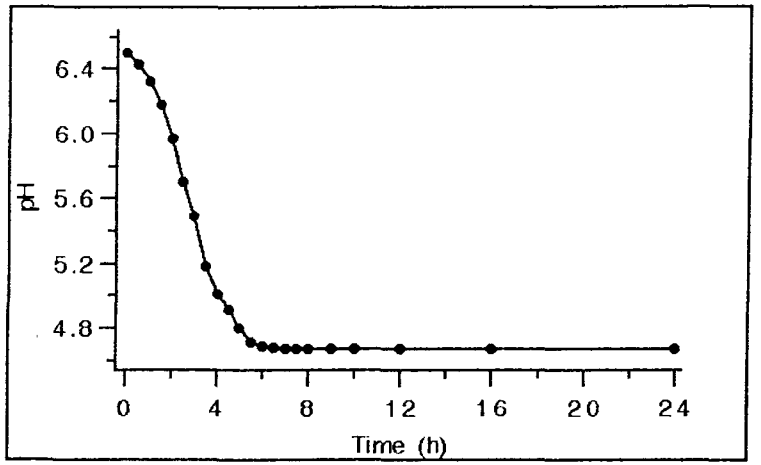
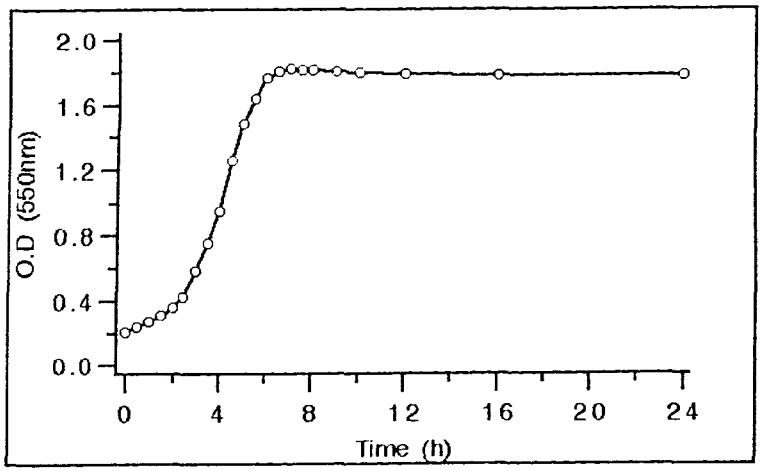
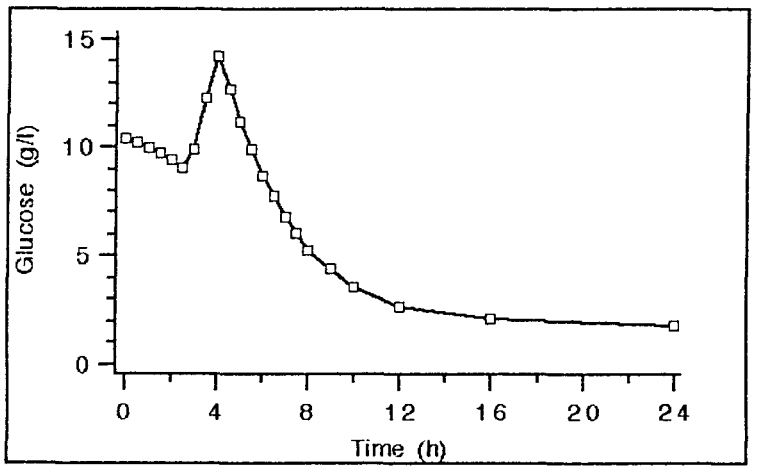

Ammoniacal Nitrogen (ug/ml)
400
300
200
100
0
0
4
8
12
16
20
24

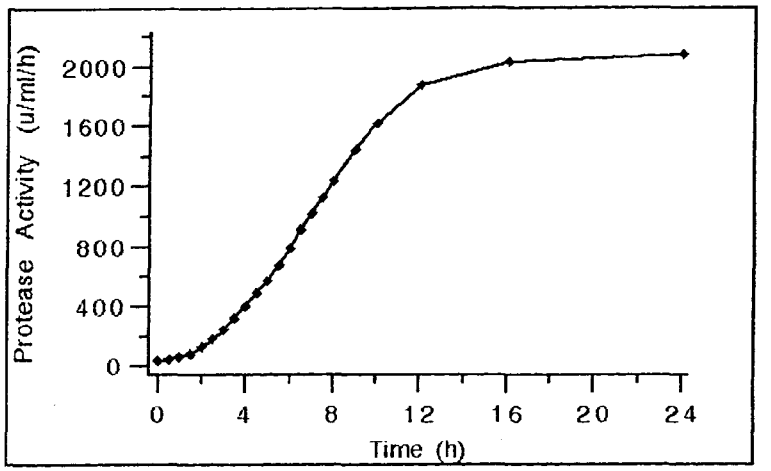
**Figure 5.2.**

Growth (open circles), culture pH (closed circles), glucose (open squares), protease activity (closed diamonds), and Ammoniacal nitrogen (closed triangles), for isolate 17c grown in the fermenter, without pH control, at pH 6.5 and 50°C. Each is typical of at least two replicates and error was never greater than 10%.


pH
6.4
6.0
5.6
5.2
4.8
0
4
8
12
16
20
24
Time (h)


O.D (550nm)
2.0
1.6
1.2
0.8
0.4
0.0
0
4
8
12
16
20
24
Time (h)


Glucose (g/l)
15
10
5
0
0
4
8
12
16
20
24
Time (h)


Protease Activity (u/ml/h)
2000
1600
1200
800
400
0
0
4
8
12
16
20
24
Time (h)

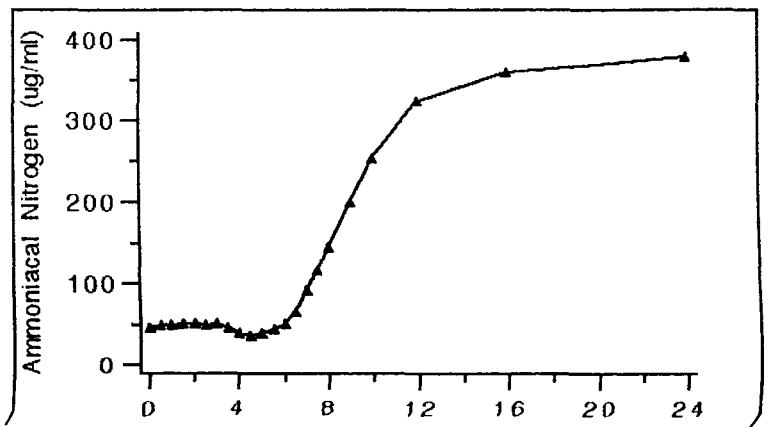
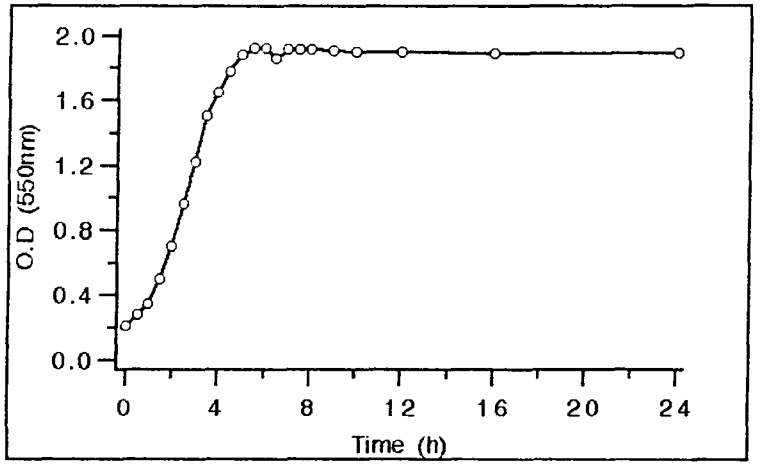
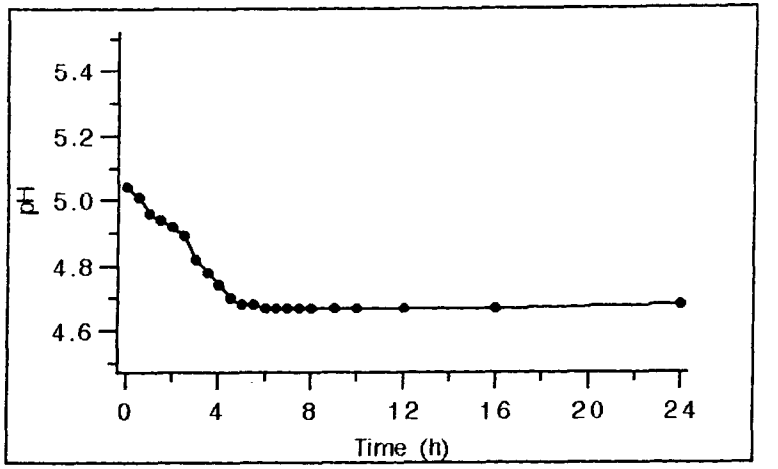
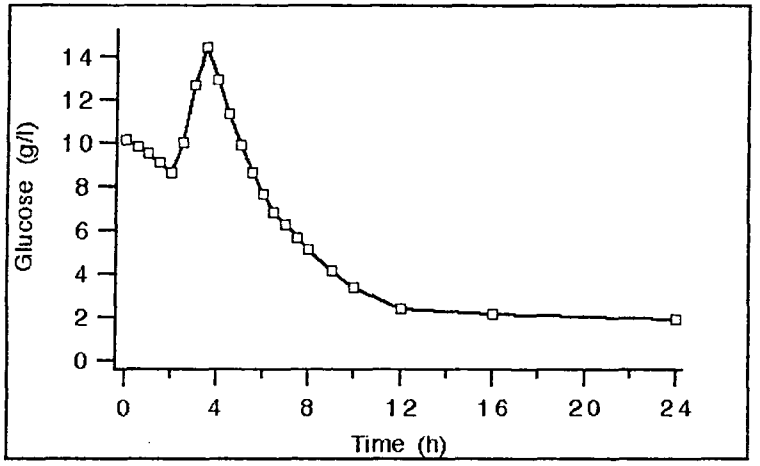

Ammoniacal Nitrogen (ug/ml)
400
300
200
100
0
0
4
8
12
16
20
24

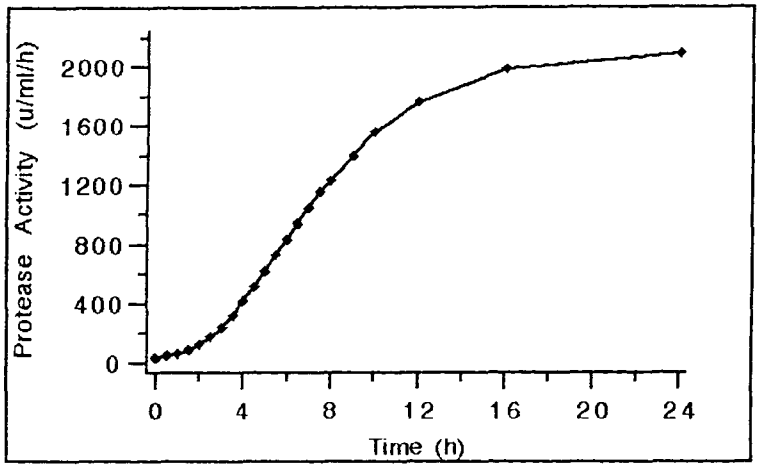
**Figure 5.3.**

Growth (open circles), culture pH (closed circles), glucose (open squares), protease activity (closed diamonds), and Ammoniacal nitrogen (closed triangles), for isolate 17a grown in the fermenter, without pH control, at pH 5.0 and 50°C. Each is typical of at least two replicates and error was never greater than 10%.


O.D (550nm)
2.0
1.6
1.2
0.8
0.4
0.0
0
4
8
12
16
20
24
Time (h)


pH
5.4
5.2
5.0
4.8
4.6
0
4
8
12
16
20
24
Time (h)


Glucose (g/l)
14
12
10
8
6
4
2
0
0
4
8
12
16
20
24
Time (h)


Protease Activity (u/ml/h)
2000
1600
1200
800
400
0
0
4
8
12
16
20
24
Time (h)

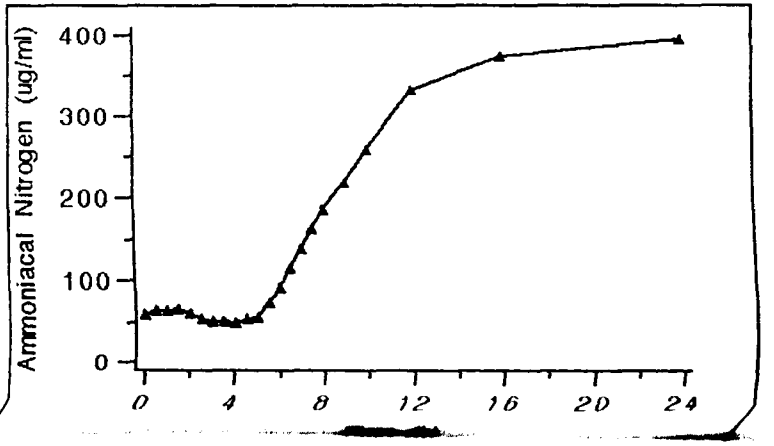
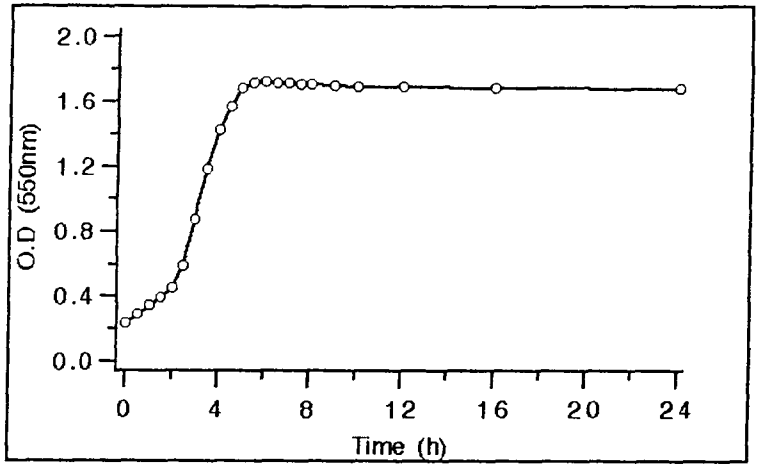
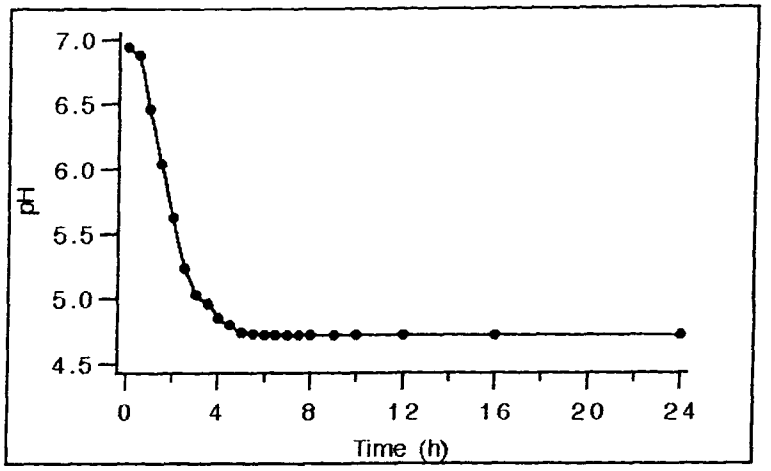
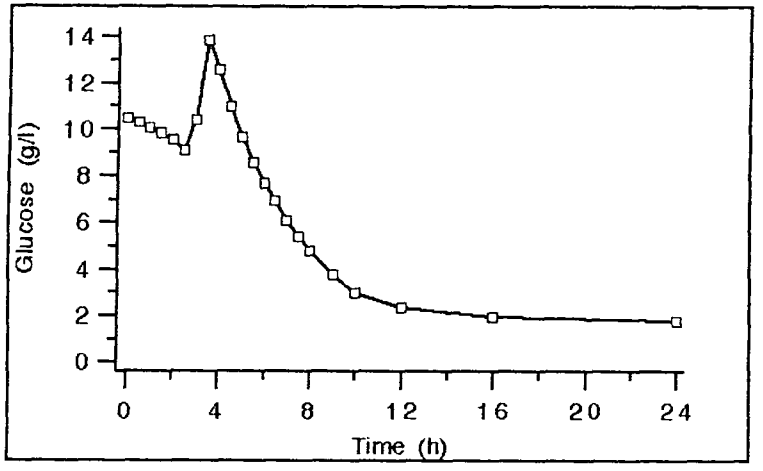

Ammoniacal Nitrogen (ug/ml)
400
300
200
100
0
0
4
8
12
16
20
24

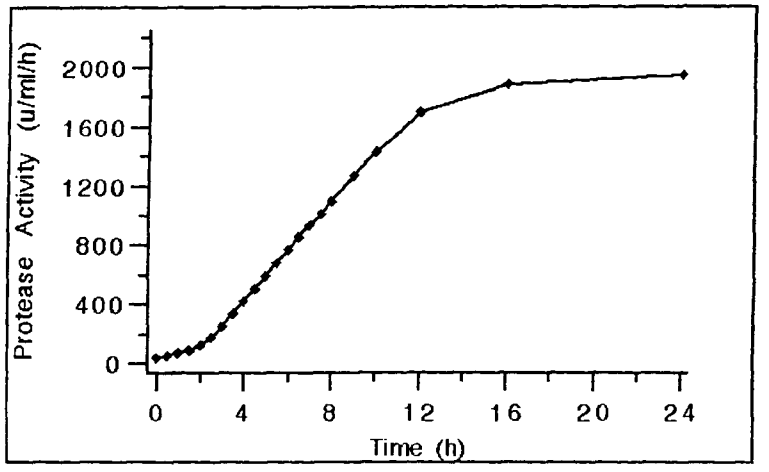
**Figure 5.4.**

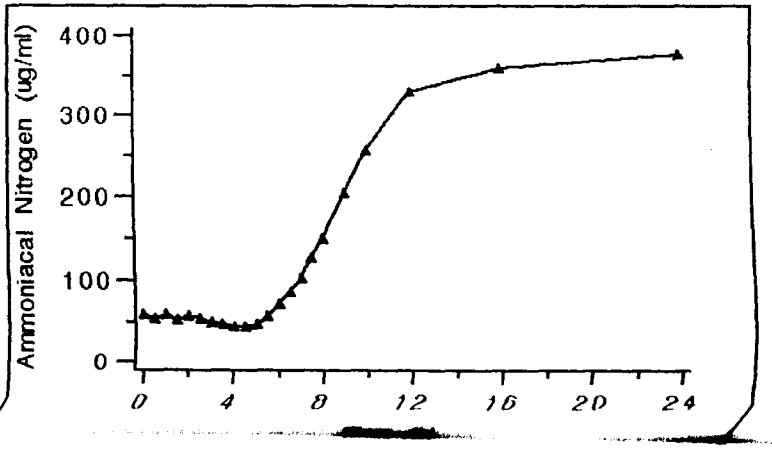
Growth (open circles), culture pH (closed circles), glucose (open squares), protease activity (closed diamonds), and Ammoniacal nitrogen (closed triangles), for isolate 17a grown in the fermenter, without pH control, at pH 7.0 and 50°C. Each is typical of at least two replicates and error was never greater than 10%.


O.D (550nm)
2.0
1.6
1.2
0.8
0.4
0.0
0
4
8
12
16
20
24
Time (h)


pH
7.0
6.5
6.0
5.5
5.0
4.5
0
4
8
12
16
20
24
Time (h)


Glucose (g/l)
14
12
10
8
6
4
2
0
0
4
8
12
16
20
24
Time (h)


Protease Activity (u/ml/h)
2000
1600
1200
800
400
0
0
4
8
12
16
20
24
Time (h)


Ammoniacal Nitrogen (ug/ml)
400
300
200
100
0
0
4
8
12
16
20
24

hyphae. Protease activity lagged growth and increased steadily until levelling off began to occur after 12h and maximum activity of between 1850 and 2250 was approached 16-24h after $T_{(0)}$. Ammoniacal nitrogen levels started to increase 6-8h after $T_{(0)}$ and approached a peak of between 350 and 400ug/ml after approximately 24h. The increase in ammoniacal nitrogen 6-8h after $T_{(0)}$ coincided approximately with the end of exponential growth and also with the point at which culture pH stopped decreasing. The levelling off of culture pH was therefore probably due to the release of ammoniacal nitrogen by deamination and it could be that this was a mechanism to prevent the culture pH from dropping below the minimum level required for growth to continue.

Despite these similarities, an important difference occurred in the growth most notably at pH 6.5/7.0. At these pH values, a noticeable lag phase occurred for 2-3h immediately after $T_{(0)}$ (Fig 5.3+5.4). A less prominent lag also occurred at pH 6.0 (data not shown) and all coincided with the drop culture pH. It therefore appeared that at these high pH values, particularly pH 6.5/7.0, a drop in culture pH was helpful to growth.

At pH 4.5 (data not shown), growth of both isolates was relatively poor with $O.D_{550}$ gradually increasing until a maximum of between 0.79 and 0.84 was reached after 5-7h. After this point, growth appeared to stop as glucose utilisation, protease and ammoniacal nitrogen production also largely ceased. As $O.D_{550}$ increased in the first few hours, so the culture pH dropped to between 4.1 and 4.3 and the point at which minimum pH was reached coincided almost exactly with the point at which growth stopped. It therefore appeared that as growth started,

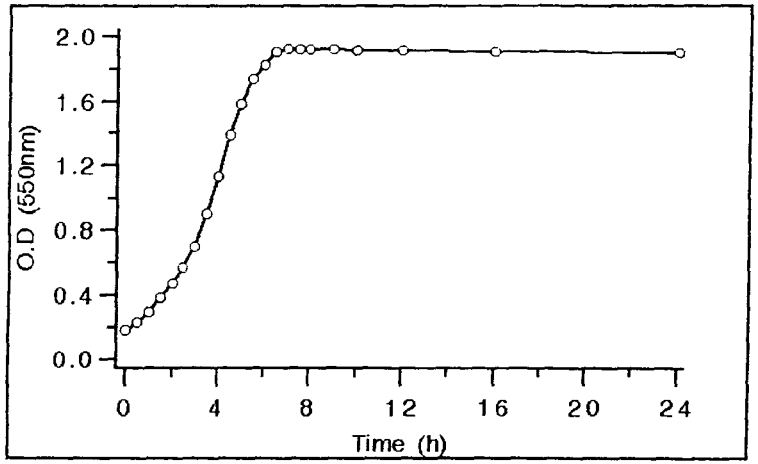
metabolism of glucose began and culture pH dropped. However, the pH very soon dropped below the minimum level required for growth of the isolates with the result that growth stopped. Growth completely failed to occur for isolate 17c at pH 7.0 (data not shown) and there was no change in culture pH and only negligible glucose utilisation, protease activity and ammoniacal nitrogen production.

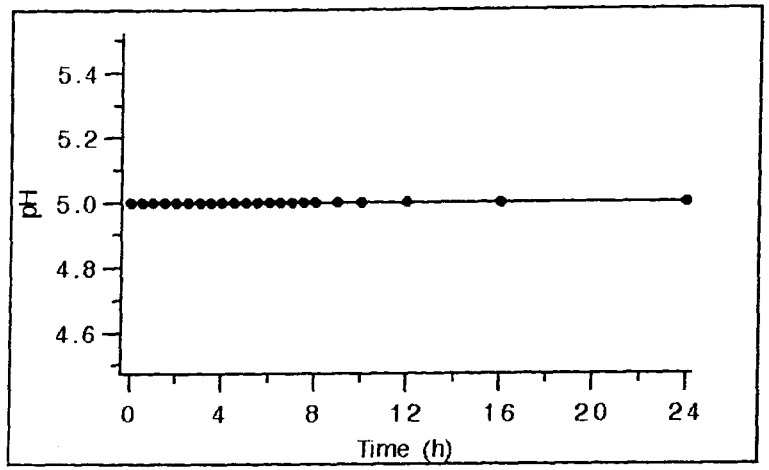
### 5.8.2: Growth With pH Control

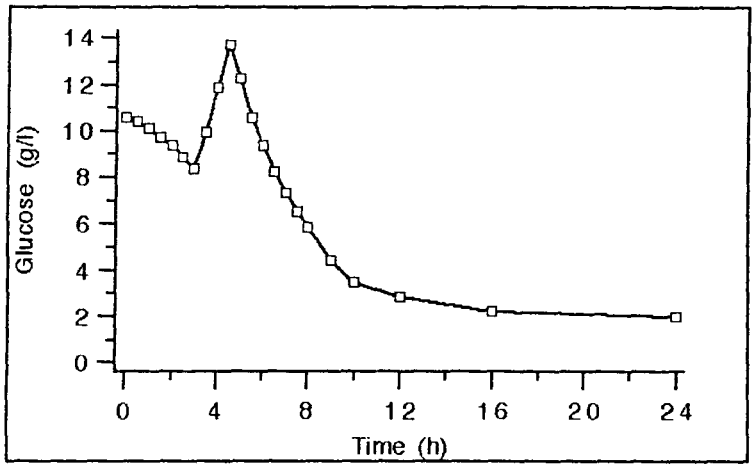
Both isolates showed growth with pH control in the pH range 4.75 to 6.0 with optimum growth at pH 5.0 and again only relatively poor growth at pH 4.5. The isolates were therefore unable to grow at pH 6.5 or 7.0 with pH control which they were able to do without pH control and it therefore appeared that the drop in culture pH was required for growth at these pH values. A summary of all fermentation data is shown in Table 5.8b. Within the pH range 4.75-6.0. Very similar data was obtained for both isolates at each pH and as a result, only the data for optimum growth at pH 5.0 is represented graphically for each isolate (Fig 5.5+5.6). Within this pH range, both isolates showed maximum $O.D_{550}$ readings of between 1.68 and 1.92 which occurred 6-8h after $T_{(0)}$, glucose levels dropped gradually for the first 2-3h until shortly after the onset of exponential growth when a sudden increase occurred to between 13 and 15g/l, which was then followed by a rapid drop over the next few hours gradually tailing off leaving final glucose levels of between 1.5 and 3g/l after 24h. As before, this could be due to the release of reducing sugars into the medium due to the breakdown of the malt extract in the medium and the sensitivity of the o-toluidine assay to these

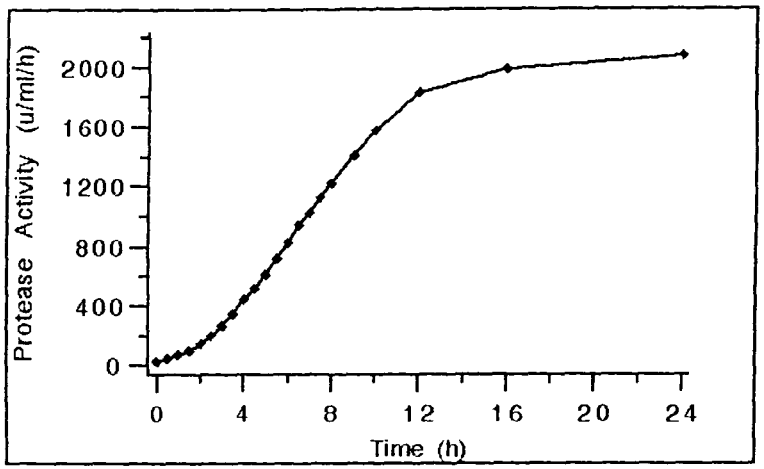
**Figure 5.5.**

Growth (open circles), culture pH (closed circles), glucose (open squares), protease activity (closed diamonds), and Ammoniacal nitrogen (closed triangles), for isolate 17c grown in the fermenter, with pH control, at pH 5.0 and 50°C. Each is typical of at least two replicates and error was never greater than 10%.


O.D (550nm)
2.0
1.6
1.2
0.8
0.4
0.0
0
4
8
12
16
20
24
Time (h)


pH
5.4
5.2
5.0
4.8
4.6
0
4
8
12
16
20
24
Time (h)


Glucose (g/l)
14
12
10
8
6
4
2
0
0
4
8
12
16
20
24
Time (h)


Protease Activity (u/ml/h)
2000
1600
1200
800
400
0
0
4
8
12
16
20
24
Time (h)

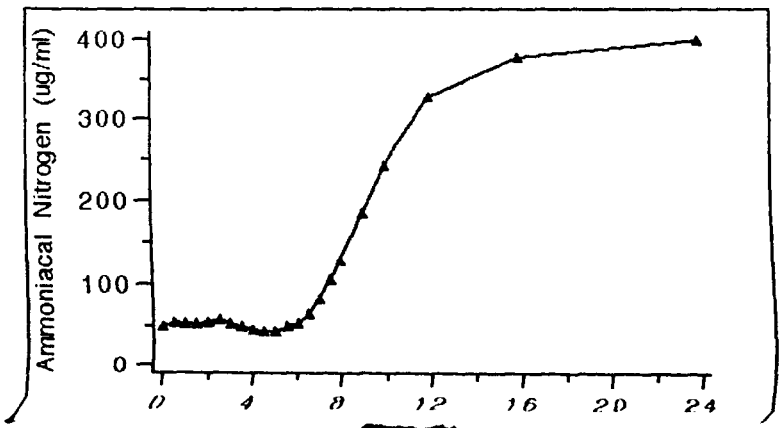
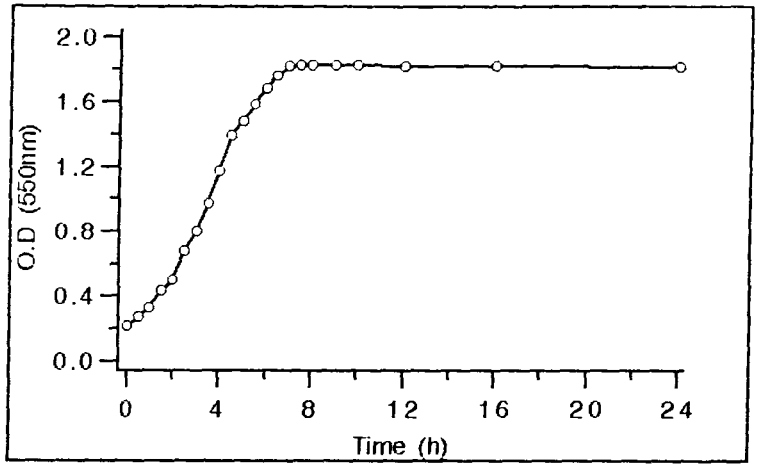
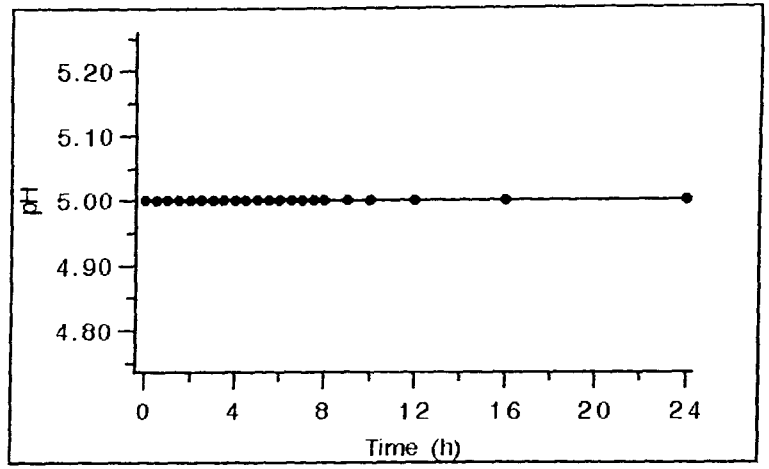
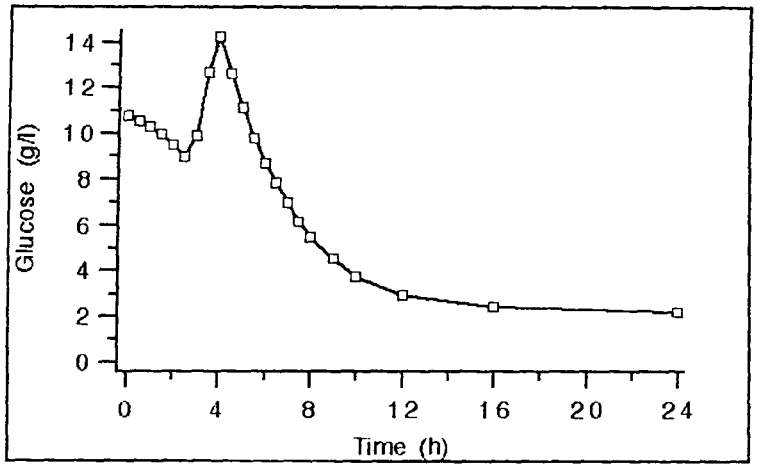

Ammoniacal Nitrogen (ug/ml)
400
300
200
100
0
0
4
8
12
16
20
24

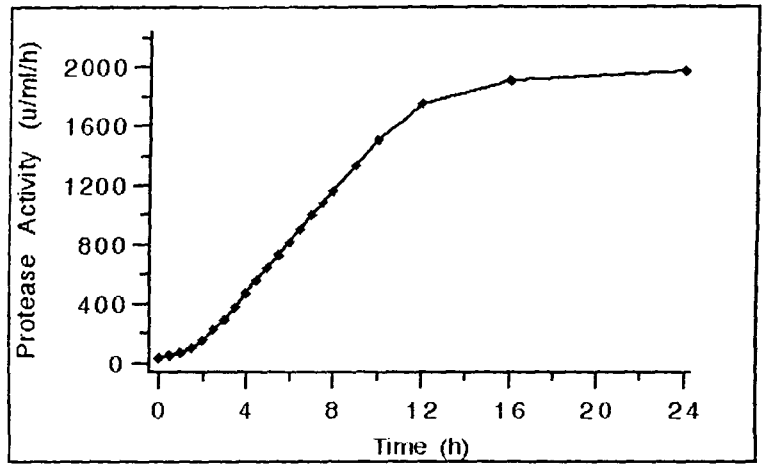
**Figure 5.6.**

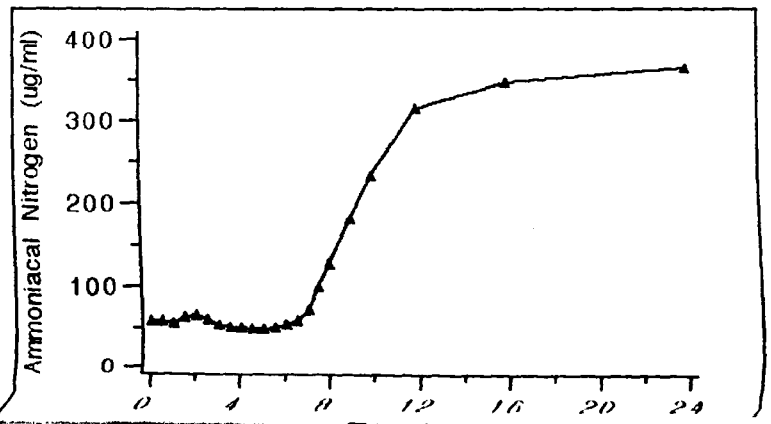
Growth (open circles), culture pH (closed circles), glucose (open squares), protease activity (closed diamonds), and Ammoniacal nitrogen (closed triangles), for isolate 17a grown in the fermenter, with pH control, at pH 5.0 and 50°C. Each is typical of at least two replicates and error was never greater than 10%.


O.D (550nm)
2.0
1.6
1.2
0.8
0.4
0.0
0
4
8
12
16
20
24
Time (h)


pH
5.20
5.10
5.00
4.90
4.80
0
4
8
12
16
20
24
Time (h)


Glucose (g/l)
14
12
10
8
6
4
2
0
0
4
8
12
16
20
24
Time (h)

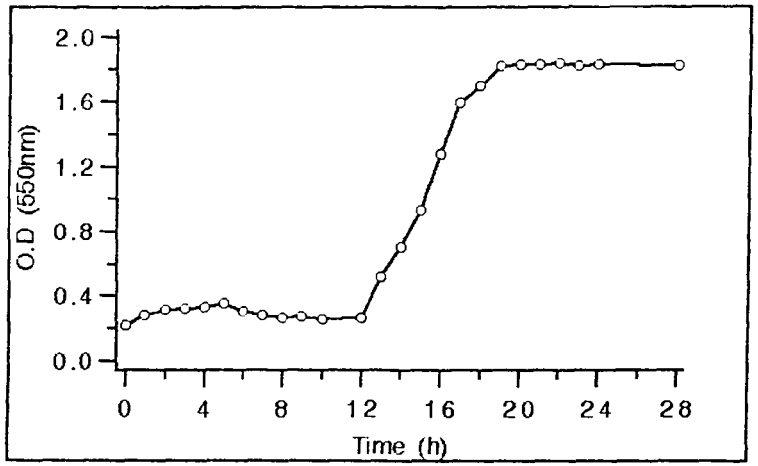

Protease Activity (u/ml/h)
2000
1600
1200
800
400
0
0
4
8
12
16
20
24
Time (h)

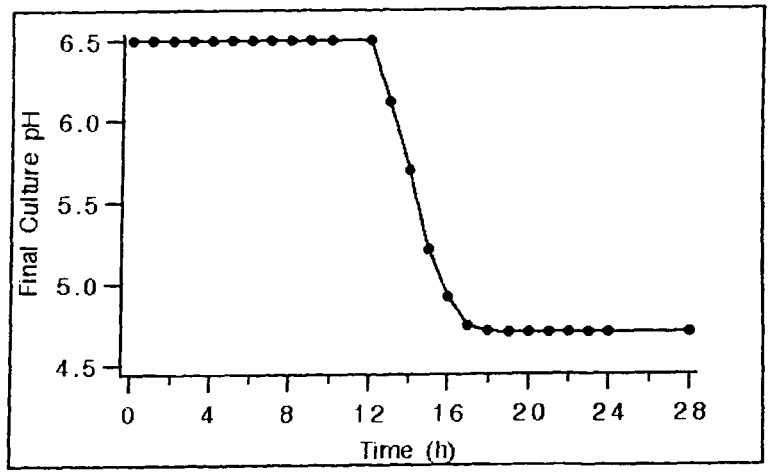

Ammoniacal Nitrogen (ug/ml)
400
300
200
100
0
0
4
8
12
16
20
24

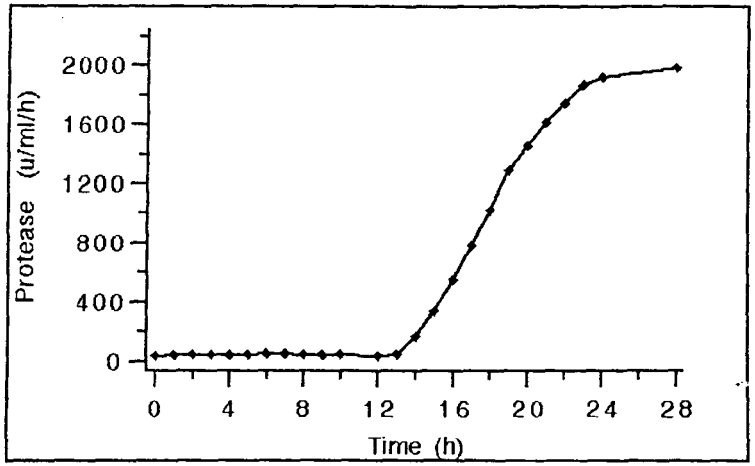
sugars. Protease activity lagged growth slightly and increased steadily before beginning to tail off approximately 12h after $T_{(0)}$ and approaching maximum activity of between 1850 and 2250u/ml/h after 16-24h. Ammoniacal nitrogen levels started to increase 6-8h after $T_{(0)}$ and approached a maximum of between 350 and 400ug/ml after approximately 24h. At pH 4.5, similar data was observed for growth without pH control. Maximum $O.D_{550}$ reached was between 0.9 and 1.0 and thus only slightly higher than where pH was not controlled. Similarly, glucose utilisation was incomplete (approximately 2.5 to 3.5 g/l utilised), no increase in glucose levels occurred after initiation of growth, hence no breakdown of malt extract occurred, and protease and ammoniacal nitrogen levels remained relatively low, between 400 and 500u/ml/h and 130 and 150ug/ml respectively. Growth of both isolates was therefore generally slightly better, both in terms of $O.D_{550}$ reached, and in the doubling times, when culture pH was not controlled. The inability of the isolates to grow with pH control at pH 6.5 was further tested by growing isolate 17c at pH 6.5 with pH control for 12h, then removing this control (Fig 5.7). While pH was controlled, no growth was recorded, however when this control was removed, growth started immediately and was accompanied by the characteristic drop in culture pH and increase in glucose utilisation, protease and ammoniacal nitrogen production. The growth of the isolates with pH control therefore compared favourably to previously isolated acidophilic actinomycetes which showed growth in the pH range 4.0 to 6.0 with an optimum at pH 4.5/5.0 and variable growth at pH 3.5 and 6.5 (Williams et al 1971 and Khan & Williams 1975) and thus classified the isolates as acidophilic. It

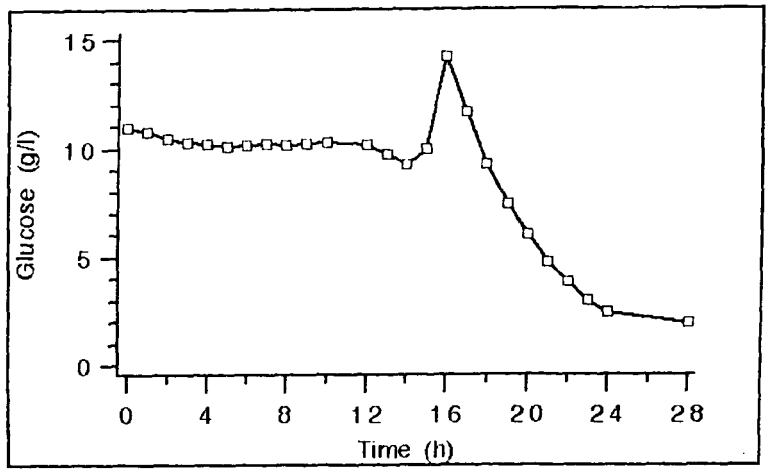
**Figure 5.7**

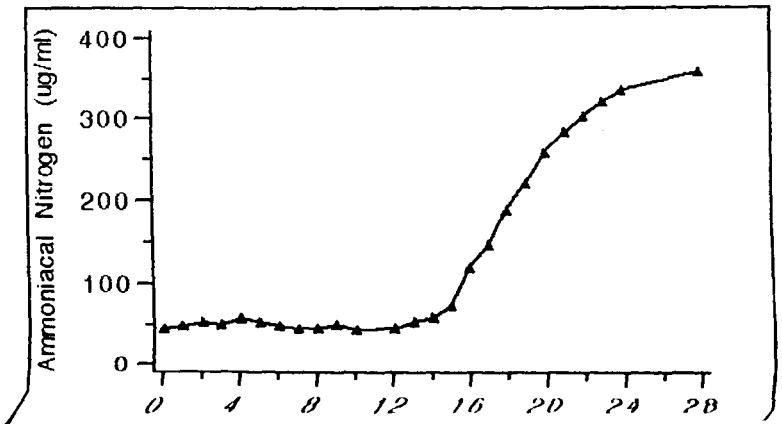
Growth (open circles), pH (closed circles), glucose (closed squares), protease (closed diamonds) and ammoniacal nitrogen (closed triangles) for isolate 17c grown in the fermenter with pH control at pH 6.5 and 50°C. At 12h, pH control was removed. Each is typical of at least two replicates and error was never greater than 10%.


O.D (550nm)
2.0
1.6
1.2
0.8
0.4
0.0
0
4
8
12
16
20
24
28
Time (h)


Final Culture pH
6.5
6.0
5.5
5.0
4.5
0
4
8
12
16
20
24
28
Time (h)


Protease (u/ml/h)
2000
1600
1200
800
400
0
0
4
8
12
16
20
24
28
Time (h)


Glucose (g/l)
15
10
5
0
0
4
8
12
16
20
24
28
Time (h)


Ammoniacal Nitrogen (ug/ml)
400
300
200
100
0
0
4
8
12
16
20
24
28

was also very similar to moderate acidophiles such as *B.naganoensis* which grows in the pH range 4.1 to 6.0 with an optimum at pH 4.7 to 5.5 (Tomimura et al 1990) and *Acidothermus cellulyticus* which grows in the pH range 3.7 to 7.0 with an optimum at pH 5.0 (Mohagheghi et al 1986).

## 5.9: Growth of Isolates 17c and 17a in SV2a Basal Medium in a fermenter

Isolates 17c and 17a were grown both with and without pH control, over a range of pH values between pH 4.0 and 6.5, in a fermenter using SV2a basal medium. Growth, protease activity, glucose and ammoniacal nitrogen levels were measured from the time of inoculation ($T_o$) every half an hour up to 10h. Samples were then taken after 12h, 16h and finally 24h.

### 5.9.1: Growth Without pH Control

Both isolates showed good growth without pH control in the pH range 4.5 to 6.0, with an optimum at pH 5.0. Little or no growth occurred at pH 4.0 and 6.5 and where growth occurred in the pH range 4.5 to 6.0, culture pH increased to between 6.5 and 7.5, in marked contrast to growth in the complete ME3 medium which gave rise to a drop in culture pH. A summary of all the fermentation data is shown in Table 5.9a.

Between pH 4.5 and 6.0, the fermentation data obtained for both isolates was very similar and as a result only the data for optimum growth at pH 5.0 is represented graphically for each isolate (Fig 5.8+5.9). Within this pH range, growth of both isolates started relatively slowly compared to growth in ME3 so that maximum $O.D_{550}$ of between 1.4 and 1.6 was reached 7 to 9h

**Table 5.9a**

Summary of growth data for isolates 17c grown in a fermenter in SV2a basal medium. Optical density readings were taken at 550nm and represent maximum growth.

| pH | O.D | Final pH | Protease | Ammoniacal Nitrogen | T(d) |
|---|---|---|---|---|---|
| 4.0 uncontrolled | 0.37 | 3.89 | 75 | 65.5 | -- -- |
| 4.5 uncontrolled | 1.49 | 6.55 | 1550 | 765 | 2h |
| 4.5 controlled | 0.82 | 4.50 | 355 | 180 | 2h 30min |
| 4.75 uncontrolled | 1.56 | 6.82 | 1675 | 745 | 2h |
| 4.75 controlled | 1.48 | 4.75 | 1600 | 740 | 2 15min |
| 5.0 uncontrolled | 1.55 | 7.20 | 1740 | 775 | 2h |
| 5.0 controlled | 1.55 | 5.00 | 1740 | 785 | 2h |
| 6.0 uncontrolled | 1.38 | 7.42 | 1500 | 610 | 2h 30m |
| 6.0 controlled | 1.50 | 6.00 | 1670 | 722.5 | 2h 15m |
| 6.5 uncontrolled | 0.43 | 6.87 | 90 | 70.5 | -- -- |
| 6.5 controlled | 0.22 | 6.50 | 72.5 | 67.5 | -- -- |

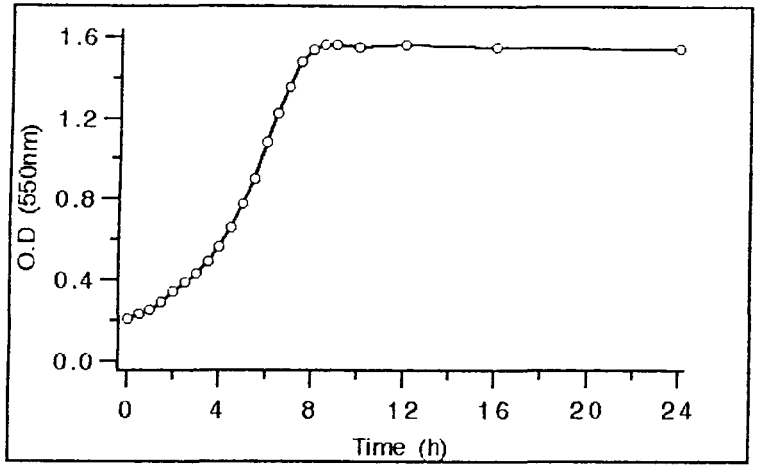
**Table 5.9b**

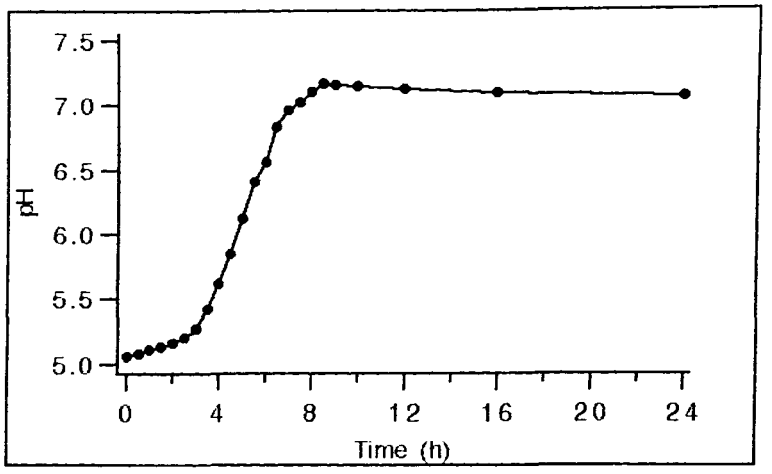
Summary of growth data for isolates 17a grown in a fermenter in SV2a basal medium. Optical density readings were taken at 550nm and represent maximum growth.

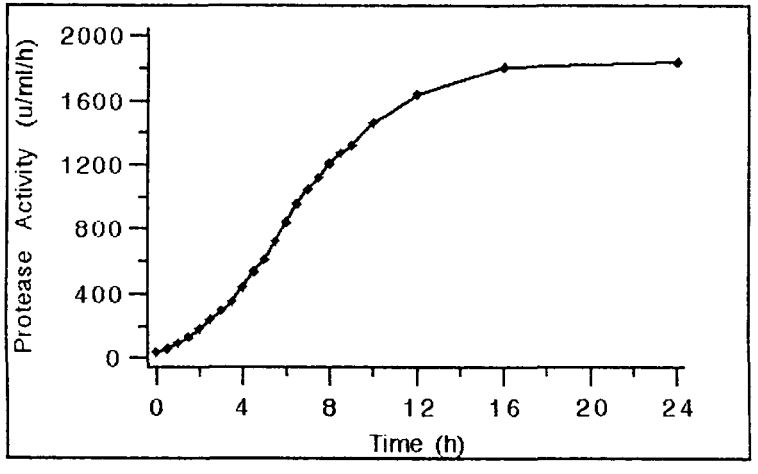
| pH | O.D | Final pH | Protease | Ammoniacal Nitrogen | T(d) |
|---|---|---|---|---|---|
| 4.0 uncontrolled | 0.35 | 4.11 | 75 | 65 | -- -- |
| 4.5 uncontrolled | 1.46 | 6.64 | 1675 | 715 | 2h 15m |
| 4.5 controlled | 0.84 | 4.50 | 300 | 195 | 2h 30m |
| 4.75 uncontrolled | 1.53 | 6.88 | 1755 | 740 | 2h 15m |
| 4.75 controlled | 1.46 | 4.75 | 1700 | 700 | 2h 15m |
| 5.0 uncontrolled | 1.52 | 7.13 | 1715 | 730 | 2h |
| 5.0 controlled | 1.52 | 5.00 | 1725 | 710 | 2h |
| 6.0 uncontrolled | 1.37 | 7.44 | 1505 | 625 | 2h 15m |
| 6.0 controlled | 1.41 | 6.00 | 1605 | 655 | 2h 30m |
| 6.5 uncontrolled | 0.45 | 6.81 | 90 | 77.5 | -- -- |
| 6.5 controlled | 0.34 | 6.50 | 75 | 65 | -- -- |

**Figure 5.8**

Growth (open circles), culture pH (closed circles), protease activity (closed diamonds), and ammoniacal nitrogen (closed triangles), for isolate 17c grown in a fermenter, without pH control at pH 5.0 and 50°C, in SV2a basal medium. Each is typical of at least two replicates and error was never greater than 10%.


O.D (550nm)
1.6
1.2
0.8
0.4
0.0
0
4
8
12
16
20
24
Time (h)


pH
7.5
7.0
6.5
6.0
5.5
5.0
0
4
8
12
16
20
24
Time (h)


Protease Activity (u/ml/h)
2000
1600
1200
800
400
0
0
4
8
12
16
20
24
Time (h)

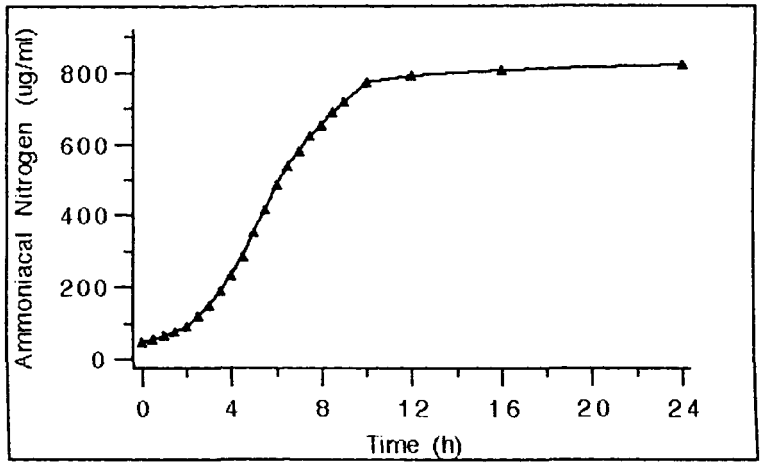
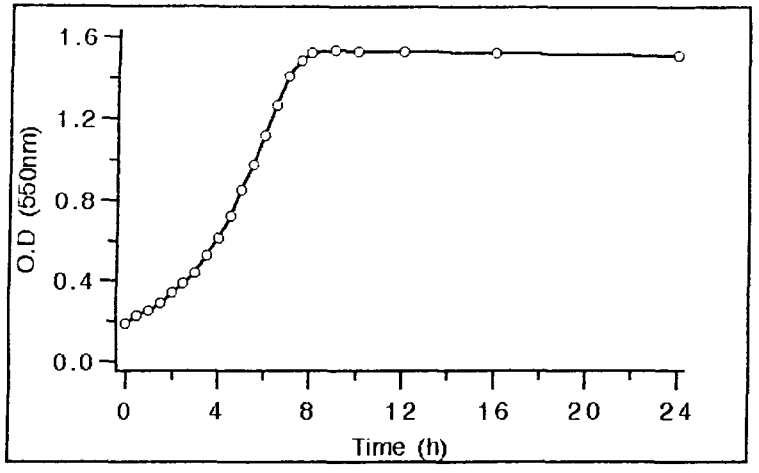
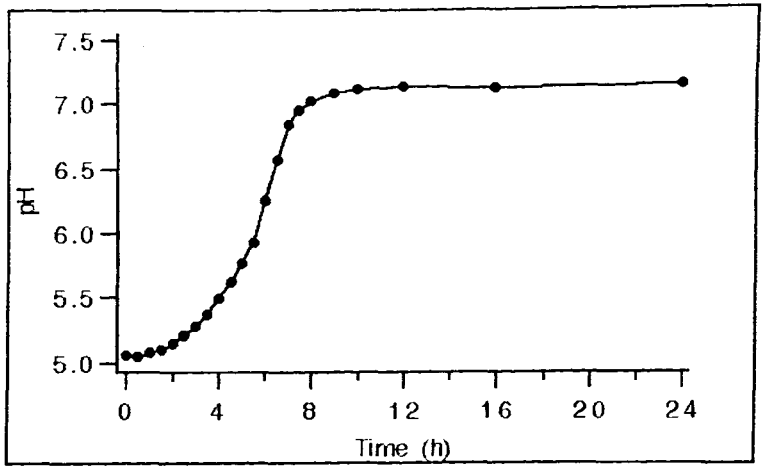
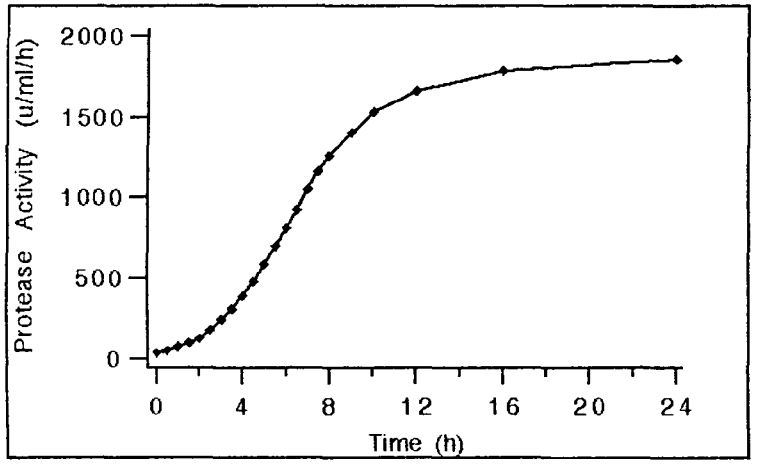

Ammoniacal Nitrogen (ug/ml)
800
600
400
200
0
0
4
8
12
16
20
24
Time (h)

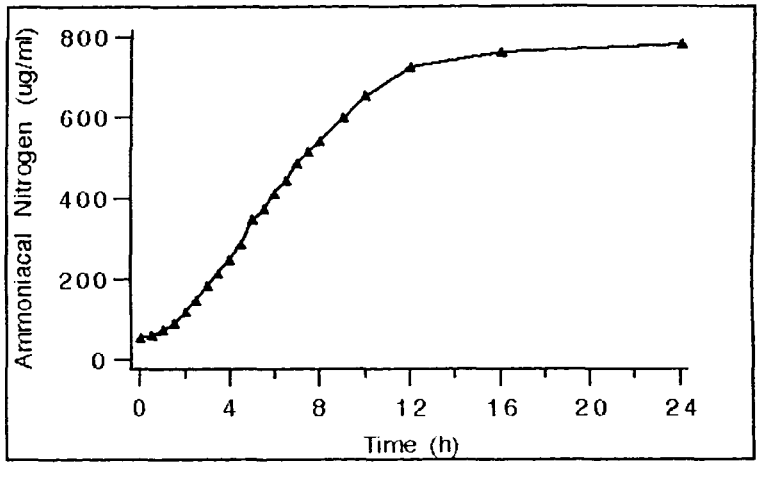
**Figure 5.9**

Growth (open circles), culture pH (closed circles), protease activity (closed diamonds), and ammoniacal nitrogen (closed triangles), for isolate 17a grown in a fermenter, without pH control at pH 5.0 and 50°C, in SV2a basal medium. Each is typical of at least two replicates and error was never greater than 10%.


O.D (550nm)
1.6
1.2
0.8
0.4
0.0
0
4
8
12
16
20
24
Time (h)


pH
7.5
7.0
6.5
6.0
5.5
5.0
0
4
8
12
16
20
24
Time (h)


Protease Activity (u/ml/h)
2000
1500
1000
500
0
0
4
8
12
16
20
24
Time (h)


Ammoniacal Nitrogen (ug/ml)
800
600
400
200
0
0
4
8
12
16
20
24
Time (h)

after $T_{(0)}$. This growth resulted in an increase in culture pH which started shortly after $T_{(0)}$ and approximated with an increase in ammoniacal nitrogen levels and protease activity both of which began almost immediately after $T_{(0)}$, much earlier than for growth in the complete ME3 medium. The increase in culture pH therefore appeared to be due to the release of large amounts of ammoniacal nitrogen from the deamination of amino acids in the soya peptone. The high levels of ammoniacal nitrogen released were probably due to a greater degree of deamination, taking place because both carbon and nitrogen had to be obtained from the soya peptone. This also confirmed the drop in culture pH which occurred as a result of growth in SV2a and ME3 was due to the metabolism of sugars. Culture pH reached a maximum of between 6.4 and 7.8 after 12-16h, protease activity approached between 1700 and 1950u/ml/h, slightly lower levels than reached in the complete ME3 medium, after 24h and ammoniacal nitrogen approached between 700 and 850ug/ml, approximately double that released in the ME3 medium, after 24h. There was little or no growth at pH 4.0 and 6.5 with the result that there were only negligible changes in $O.D_{550}$, protease activity, ammoniacal nitrogen and culture pH.

### 5.9.2: Growth With pH Control

Both isolates showed growth with pH control in the pH range 4.75 to 6.0, with an optimum at pH 5.0, only poor growth at pH 4.5 and little or no growth at 6.5. Both isolates were therefore able to grow well at pH 4.5 without pH control, which resulted in an increase in culture pH, but not with pH control. With pH control, the range for growth was the same as the range for growth in the

complete ME3 medium. A summary of all the fermentation data is shown in Table 5.9b. Very similar fermentation data was observed for both isolates within this pH range and as a result only the data for optimum growth at pH 5.0 is represented graphically for each isolate (Fig 5.10+5.11). Within this pH range, growth of both isolates started relatively slowly so that maximum $O.D_{550}$ of between 1.4 and 1.6 occurred 7-9h after $T_{(0)}$. Ammoniacal nitrogen levels and protease activity started to increase almost immediately after $T_{(0)}$, with protease activity approaching a maximum of between 1700 and 1950u/ml/h after 24h and ammoniacal nitrogen approaching between 700 and 850ug/ml after 24h. At pH 4.5, growth was gradual reaching an optimum $O.D_{550}$ of between 0.8 and 1.0, 5-8h after $T_{(0)}$, with no exponential phase being observed. As a result of this poor growth, corresponding protease activity and ammoniacal nitrogen levels were also low, reaching a maximum of between 200 and 250u/ml/h, 8-10h after $T_{(0)}$ for protease activity and between 150 and 200ug/ml, 10-12h after $T_{(0)}$, for ammoniacal nitrogen. No growth occurred at pH 4.0, 6.5 and 7.0 with the result that only negligible changes occurred in $O.D_{550}$, protease activity and ammoniacal nitrogen levels and thus data is not shown.

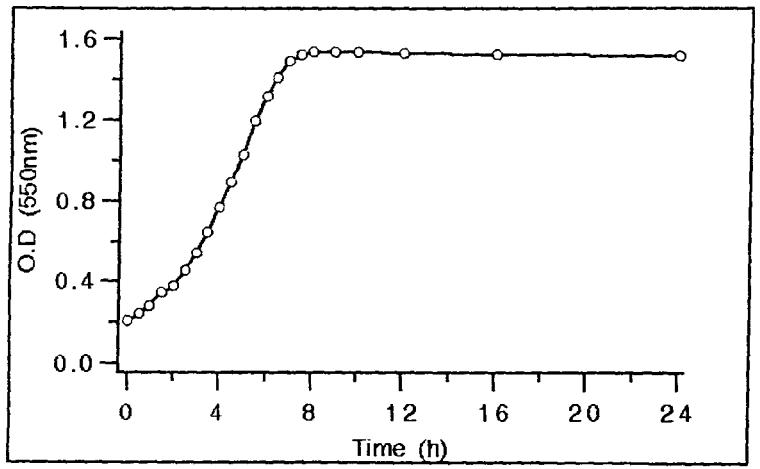
## 5.10: Morphology of Isolate 17c During Growth

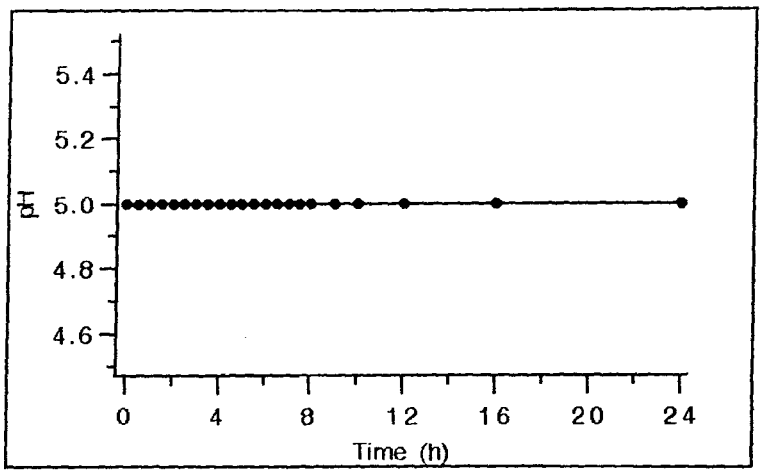
The morphology of isolate 17c was studied during growth under optimum conditions at pH 5.0 with no pH control. At $T_{(0)}$ the culture consisted of small groups, or single, largely unbranched hyphal strands (Fig 5.12a). Over the initial 6-8h, growth progressed with the development of increasingly densely packed and branched hyphal masses (Fig 5.12b+c). This was maintained

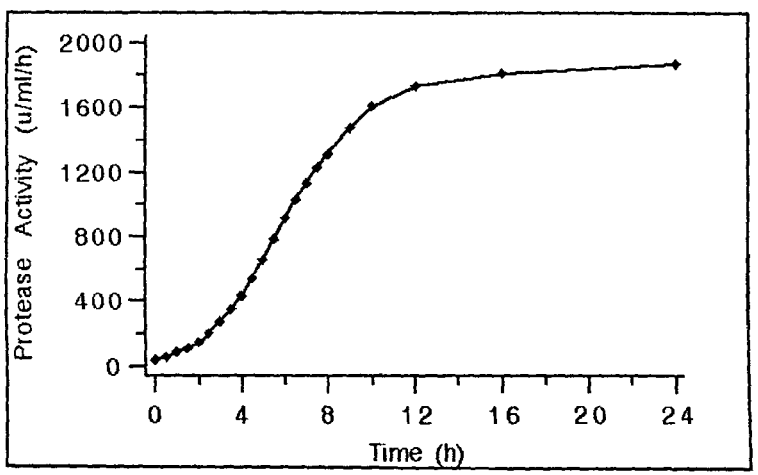
until approximately 40h when endospore production and hyphal lysis began to take place (Fig 5.12d) until almost total lysis had occurred by 56h leaving only hyphal fragments and endospores (Fig 5.12e).

**Figure 5.10**

Growth (open circles), culture pH (closed circles), protease activity (closed diamonds), and ammoniacal nitrogen (closed triangles), for isolate 17c grown in a fermenter, with pH control at pH 5.0 and 50°C, in SV2a basal medium. Each is typical of at least two replicates and error was never greater than 10%.


O.D (550nm)
1.6
1.2
0.8
0.4
0.0
0
4
8
12
16
20
24
Time (h)


pH
5.4
5.2
5.0
4.8
4.6
0
4
8
12
16
20
24
Time (h)


Protease Activity (u/ml/h)
2000
1600
1200
800
400
0
0
4
8
12
16
20
24
Time (h)

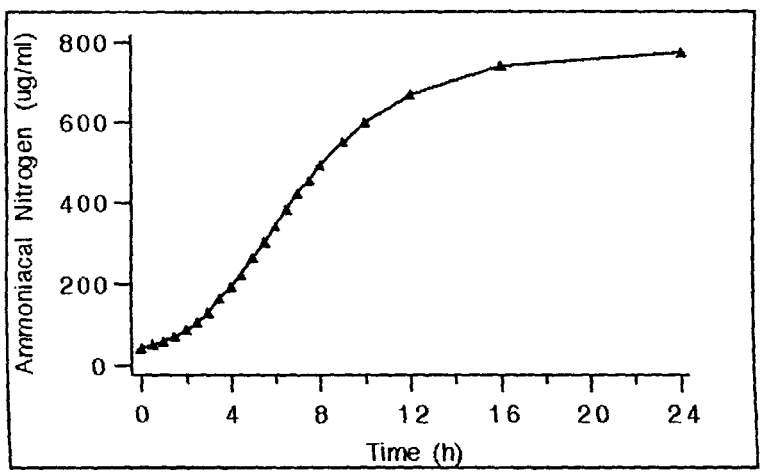
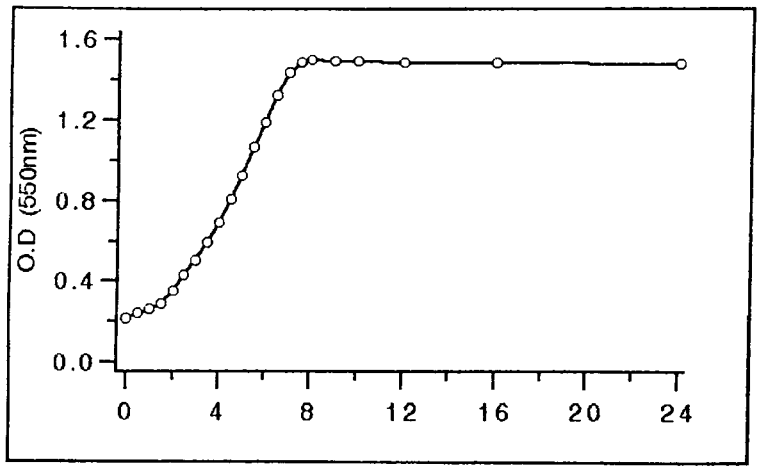
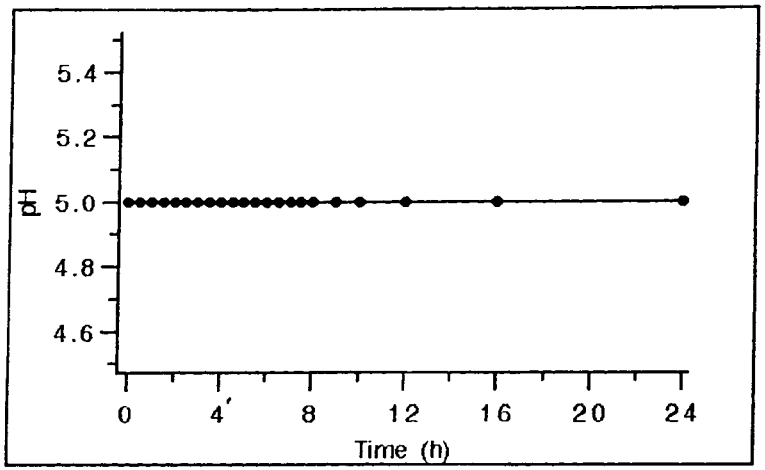
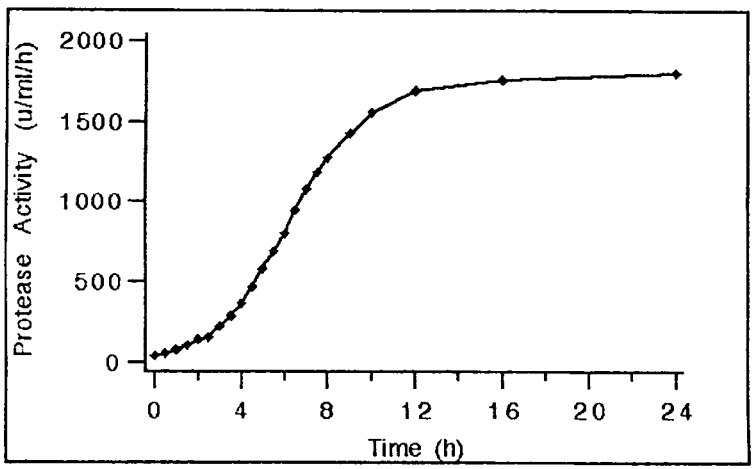

Ammoniacal Nitrogen (ug/ml)
800
600
400
200
0
0
4
8
12
16
20
24
Time (h)

**Figure 5.11**

Growth (open circles), culture pH (closed circles), protease activity (closed diamonds), and ammoniacal nitrogen (closed triangles), for isolate 17a grown in a fermenter, with pH control at pH 5.0 and 50°C, in SV2a basal medium. Each is typical of at least two replicates and error was never greater than 10%.


O.D (550nm)
1.6
1.2
0.8
0.4
0.0
0
4
8
12
16
20
24


pH
5.4
5.2
5.0
4.8
4.6
0
4
8
12
16
20
24
Time (h)


Protease Activity (u/ml/h)
2000
1500
1000
500
0
0
4
8
12
16
20
24
Time (h)

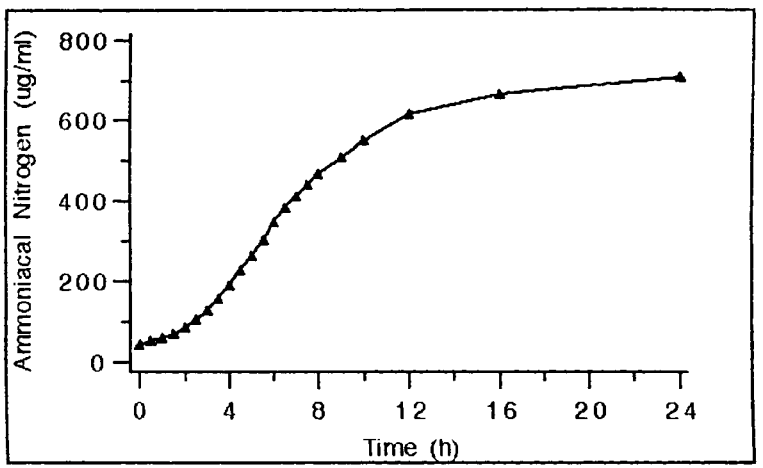

Ammoniacal Nitrogen (ug/ml)
800
600
400
200
0
0
4
8
12
16
20
24
Time (h)

Figure 5.12a-f

Isolate 17c grown in ME3 medium in a fermenter at pH 5.0, with no pH control. Figures 5.12a-d at *10 and figures 5.12e+f at *40 magnification.

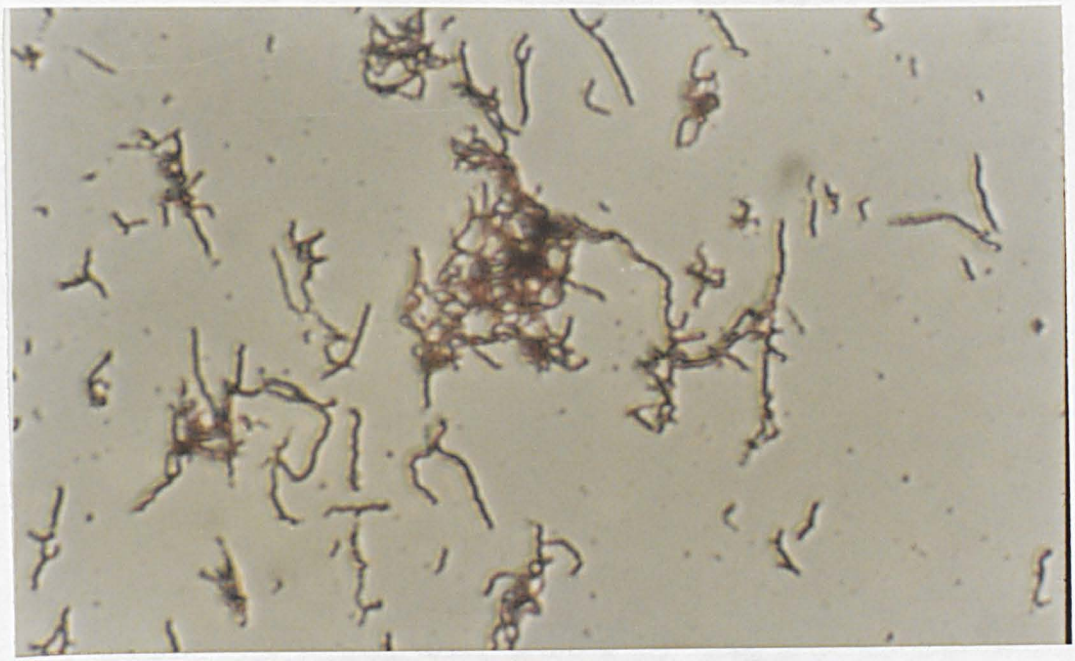


Figure 5.12a: Taken at Time = 0h.

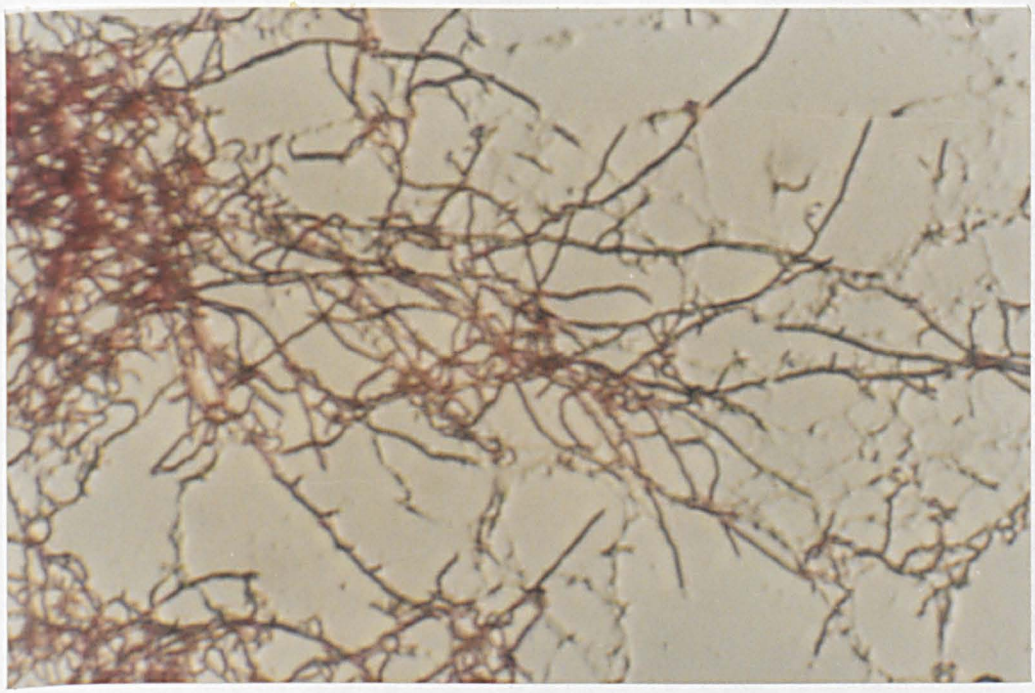


Figure 5.12b: Taken at Time = 3h.

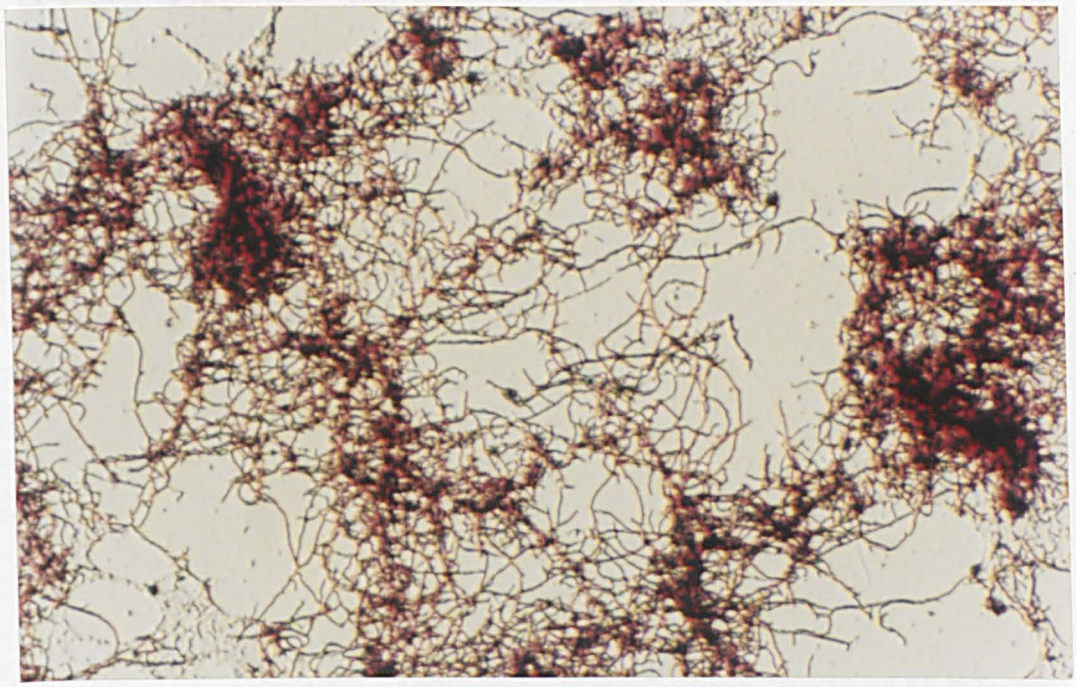


Figure 5.12c: Taken at Time = 8h.

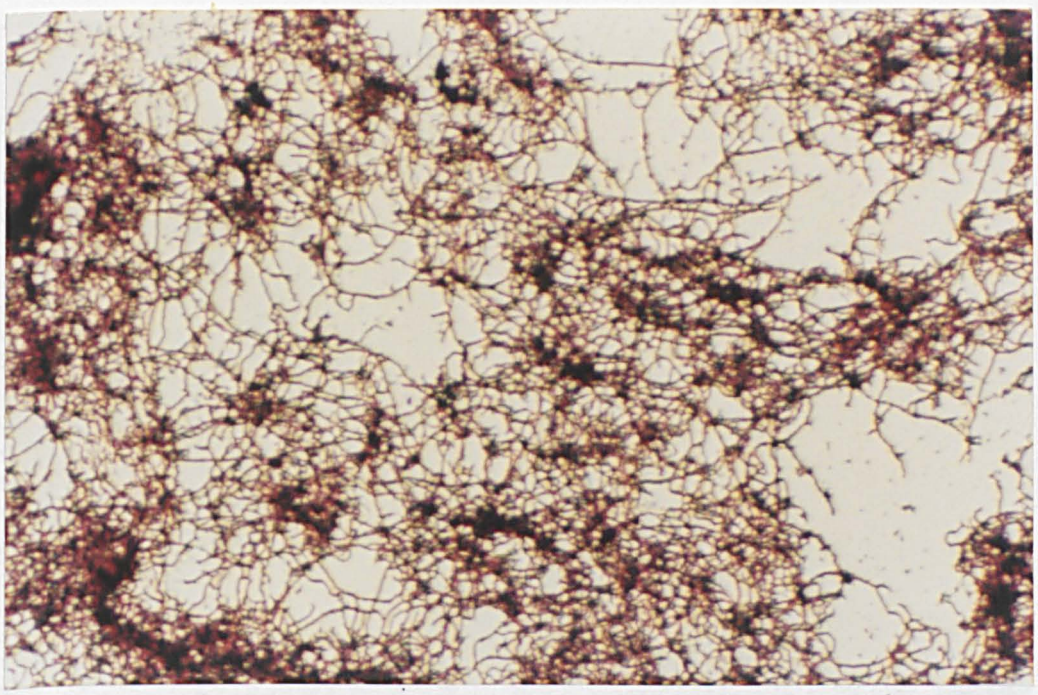


Figure 5.12d: Taken at Time = 15h.

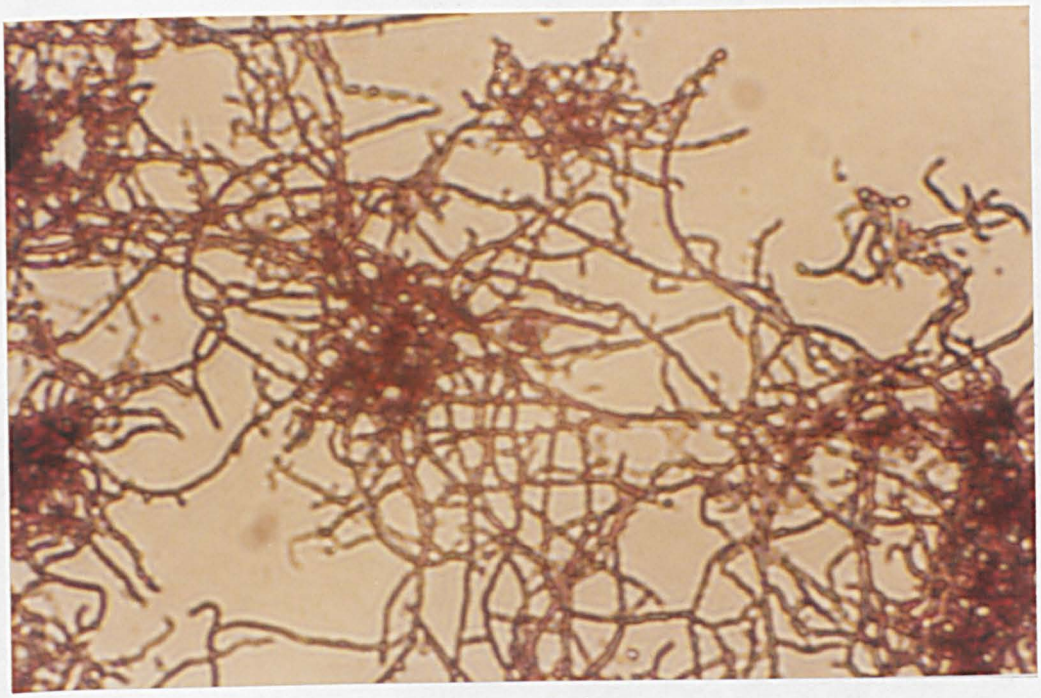


Figure 5.12e: Taken at Time = 42h

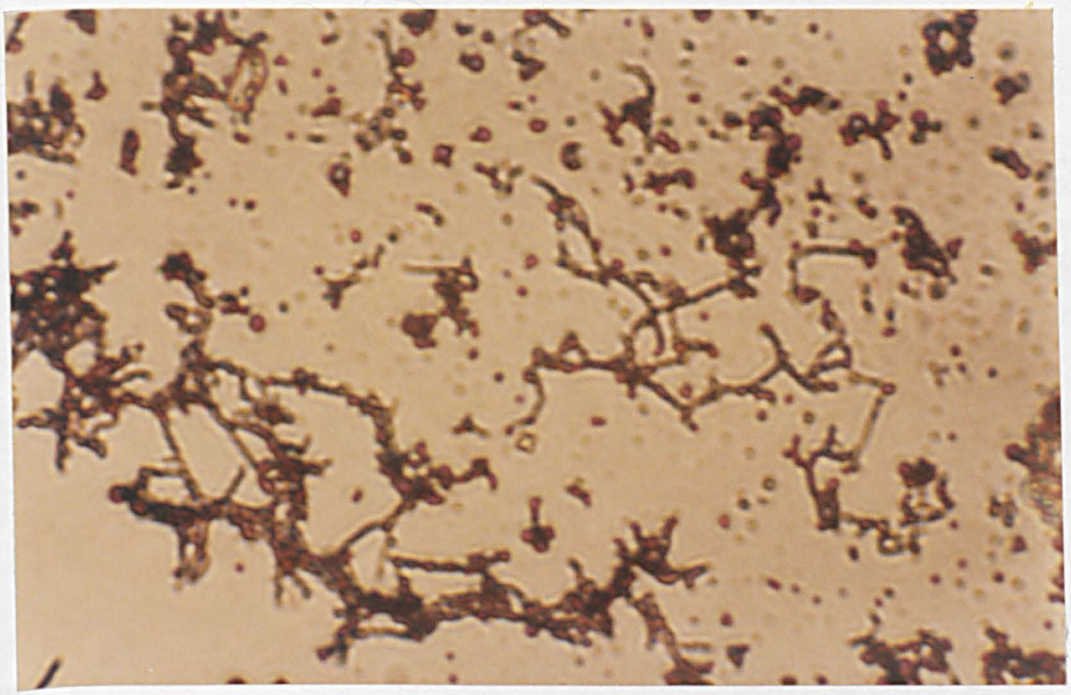


Figure 5.12f: Taken at Time = 58h

# CHAPTER 6

# ENZYME AND ANTIBIOTIC STUDIES

## 6.1: Introduction

From the fermentation studies carried out in chapter 5, the isolates were determined as acidophilic. Experiments were then carried out in order to characterise the extracellular enzyme products of the isolates and to determine antibiotic activity, if any.

## 6.2 Extracellular Enzyme Activities

Preliminary enzyme screens carried out on a selection of isolates identified a range of extracellular enzyme activities including amylase, pullulanase, esterase and lipase. The activities of each of these enzymes, as well as protease, were then tested over a range of pH and temperature values so that the resulting profiles could be determined and compared to the pH and temperature profiles for growth of the isolates. The thermostability of each enzyme was tested by maintaining culture supernatant at a suitable temperature, taking samples at regular intervals and assaying for activity as normal at 50°C. For each enzyme activity the supernatant was pre-incubated in the presence and absence of 5mM calcium chloride in order to determine if any of the enzyme activities were stabilised by calcium, and the time taken for activity to drop by 50% ($T_{(50)}$) was calculated.

## 6.3: The Effect of pH and Temperature on Protease Activity of Isolate 17c

(i) pH Activity: Activity occurred in the pH range 4.5 to 7.5 with an optimum at pH 5.5 (Fig 6.1a). This profile therefore backed up the pH profile for growth of the isolates and supported the theory

explaining the growth of the isolates without pH control at pH 6.5 and 7.0.

(ii): Temperature Activity: Activity occurred over a wide range of temperatures between 40 and 95°C, with optimal activity between 70 and 75°C (Fig 6.1b).

(iii): Thermostability: Protease activity was found to be relatively stable at 70°C retaining at least 70% activity after 3h (data not shown). Thermostability was therefore tested at 80°C (Fig 6.2). In the absence of calcium ions activity dropped very rapidly to approximately 50% ($T_{(50)}$) in the first 10 min. Activity then stabilised over the next 30 min before gradually decreasing over the following 140 min down to approximately 23%. This biphasic decrease could indicate the presence of more than one protease enzyme, one heat labile, breaking down quickly, giving rise to the initial drop, and one more thermostable giving rise to the gradual decrease over the next 170 min, or simply the presence of one enzyme which shows a gradual tailing off of activity. In the presence of calcium ions, the initial rapid drop was slowed so that $T_{(50)}$ occurred after 60 min. This was then followed by a gradual drop down to approximately 28%. Thus calcium chloride stabilised protease activity, particularly over the initial 30 min.

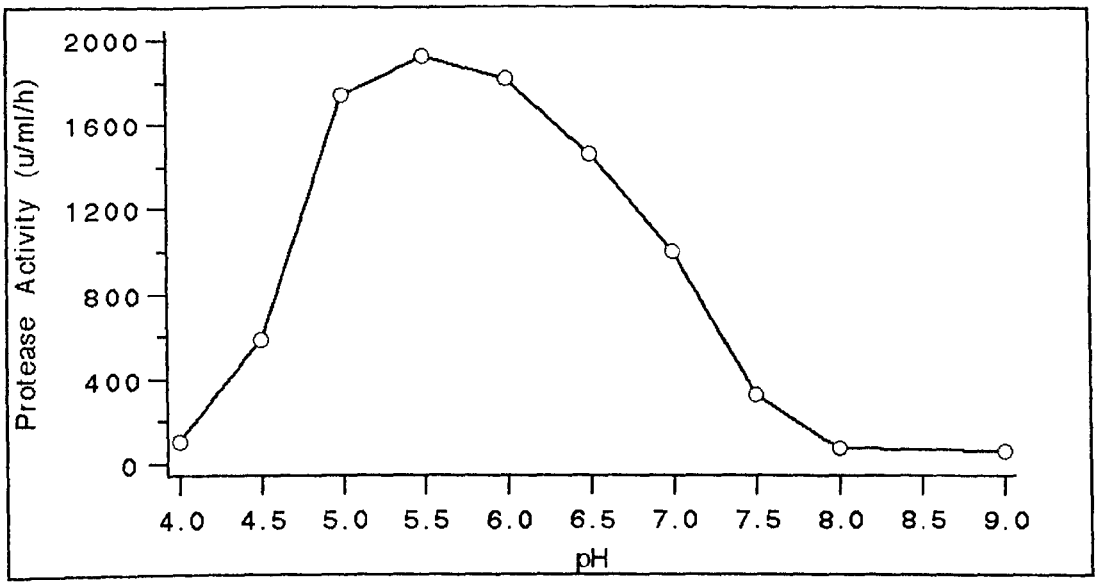
## 6.4: The Effect of pH and Temperature on Amylase Activity of Isolate 17c

(i): pH Activity: Activity occurred in the pH range 4.5 to 7.5 with an optimum at pH 5.5 (Fig 6.3a). The profile also therefore backed

**Figure 6.1**

The effect of a:) pH and b:) temperature on extracellular protease activity of isolate 17c grown in ME3 broth at pH 5.0 and 50°C. The pH profile was carried out at 50°C, the temperature profile at pH 5.5. Each is representative of three replicates and the standard deviation was never greater than 10%.

a:)


Protease Activity (u/ml/h)
2000
1600
1200
800
400
0
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
pH

b:)

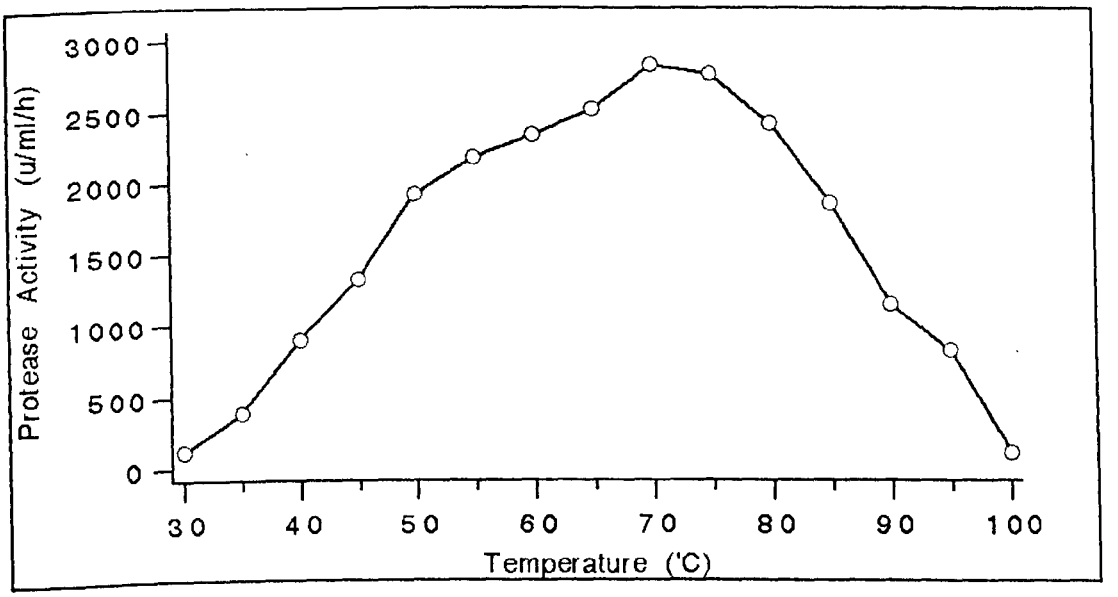
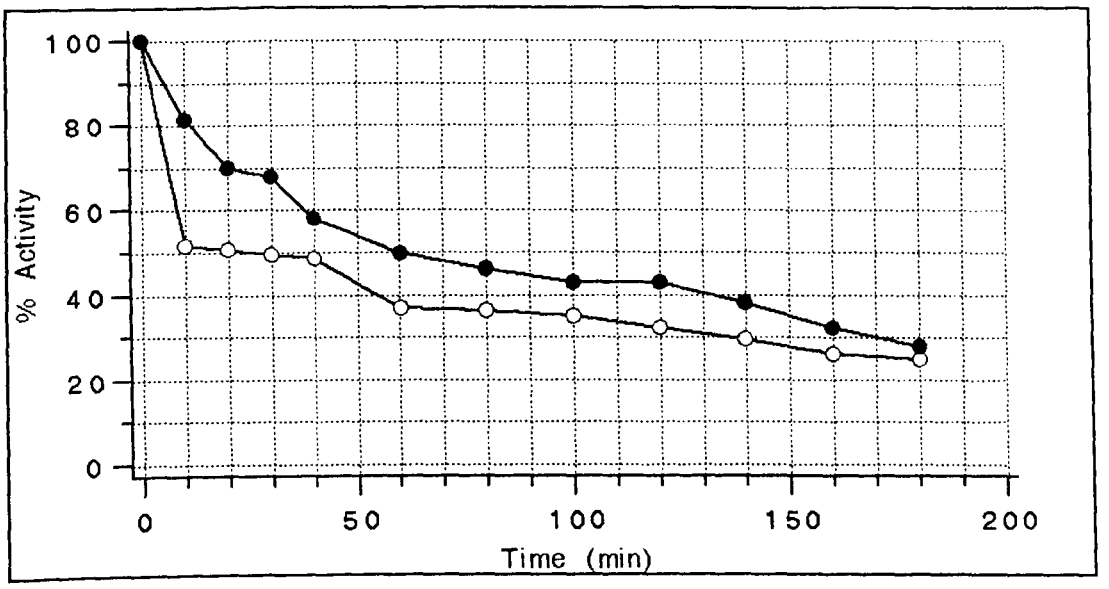

Protease Activity (u/ml/h)
3000
2500
2000
1500
1000
500
0
30
40
50
60
70
80
90
100
Temperature (°C)

**Figure 6.2**

Thermostability of protease activity of isolate 17c at 80°C with (closed circles) and without (open circles) 5mM $CaCl_2$. Each is representative of three replicates and the standard deviation was never greater than 10%.


100
80
60
40
20
0
% Activity
0
50
100
150
200
Time (min)

up the pH range for growth of the isolates and supported the theory explaining the growth of the isolates without pH control at pH 6.5 and 7.0.

(ii): Temperature Activity: Activity occurred over a wide range of temperatures from 45 to 95°C with optimal activity between 75 and 80°C (Fig 6.3b).

(iii): Thermostability: Amylase activity was found to be relatively stable at 70°C retaining at least 75% activity after 3h (data not shown). Thermostability was therefore tested at 80°C (Fig 6.4). In the absence of calcium ions $T_{(50)}$ was reached after 30 min, this was then followed by a more gradual decrease to around 20% after 180min. In the presence of calcium ions activity was stabilised and $T_{(50)}$ was not reached until after approximately 120 min. This was also then followed by a gradual decrease down to around 30% after 180min. Amylase activity therefore appeared more thermostable and showed a slightly narrower pH profile than protease activity.

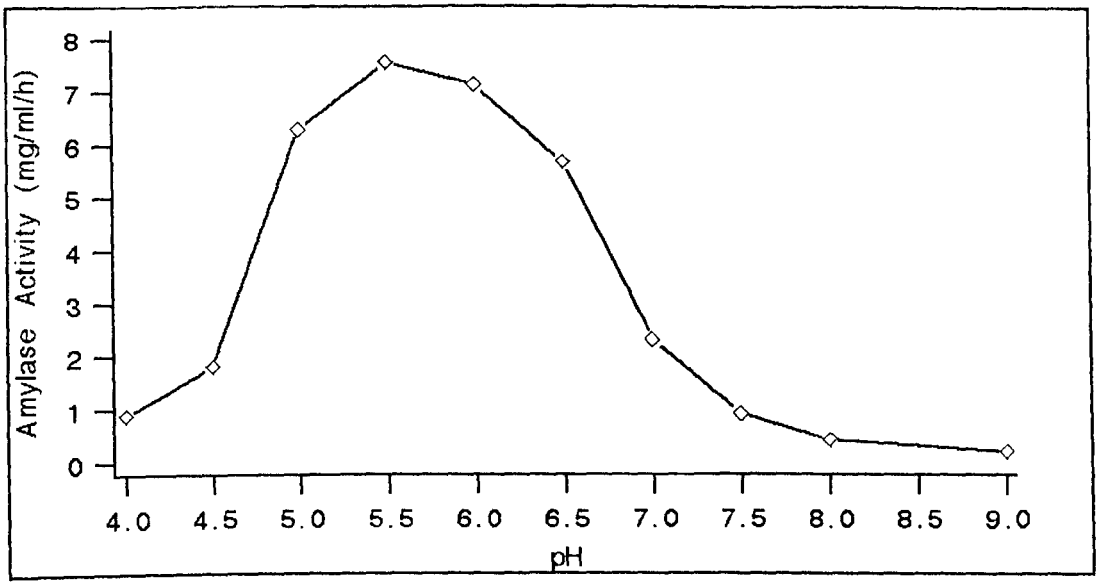
## 6.5: The Effect of pH and Temperature on Pullulanase Activity of Isolate 17c

(i) pH Activity: Activity occurred in the pH range 4.5 to 7.0 with an optimum at pH 5.5 (Fig 6.5a). This profile also backed up the pH profile for growth of the isolates and supported the theory explaining the growth of the isolates without pH control at pH 6.5 and 7.0.

**Figure 6.3**

The effect of a:) pH and b:) temperature on extracellular amylase activity of isolate 17c grown in ME3 basal medium containing 15g/l of soluble starch at pH 5.0 and 50°C. The pH profile was carried out at 50°C, the temperature profile at pH 5.5. Each is representative of three replicates and the standard deviation was never greater than 10%.

a:)


Amylase Activity (mg/ml/h)
0
1
2
3
4
5
6
7
8
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
pH

b:)

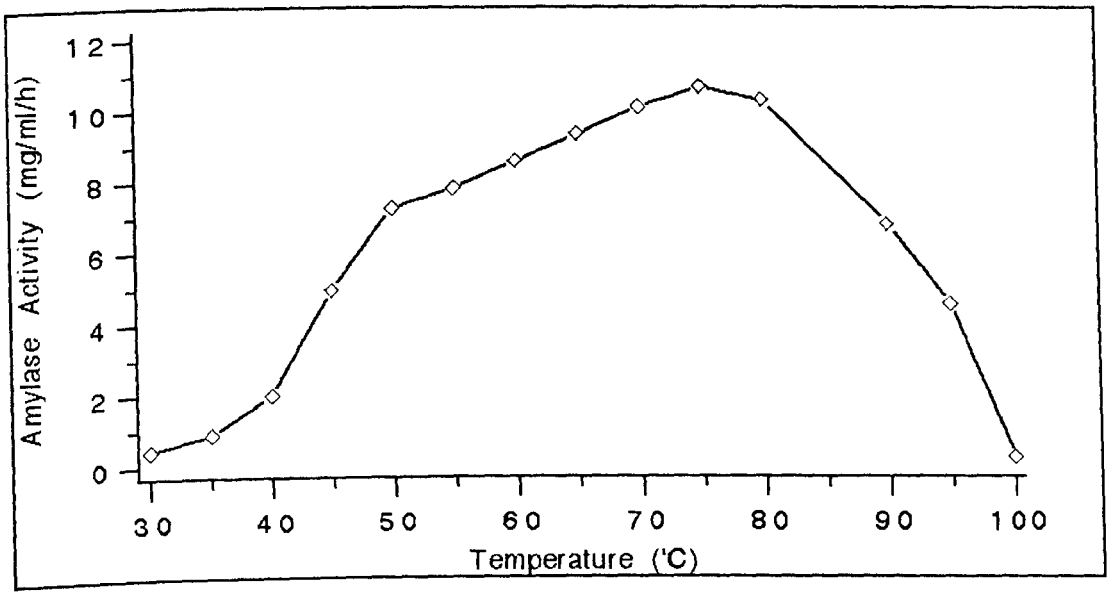
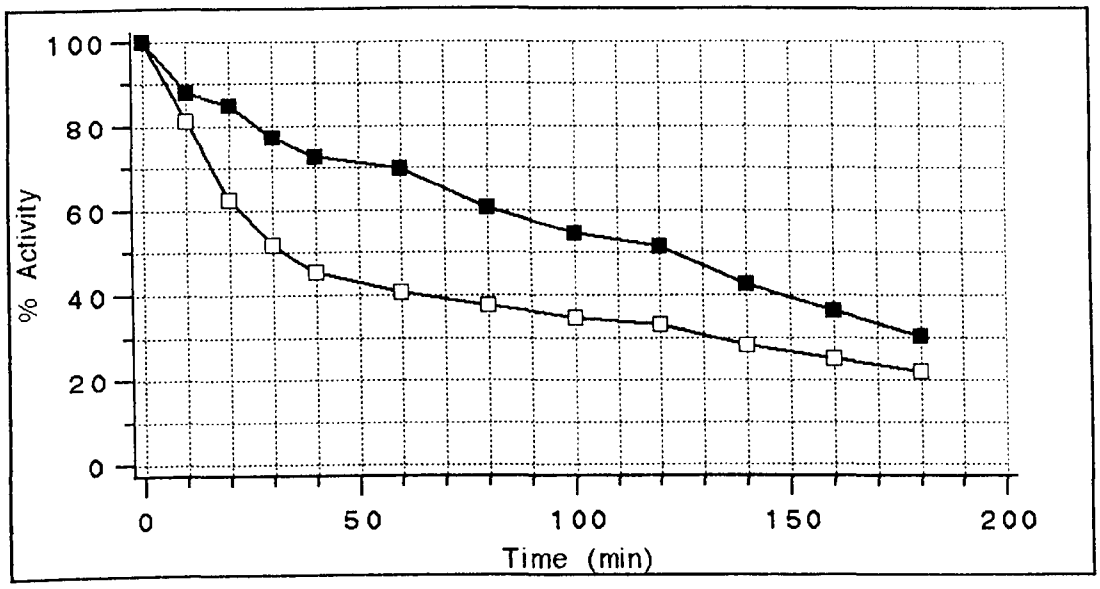

Amylase Activity (mg/ml/h)
0
2
4
6
8
10
12
30
40
50
60
70
80
90
100
Temperature (°C)

**Figure 6.4**

Thermostability of amylase activity of isolate 17c at 80°C with (closed circles) and without (open circles) 5mM $CaCl_2$. Each is representative of three replicates and the standard deviation was never greater than 10%.


100
80
60
40
20
0
% Activity
0
50
100
150
200
Time (min)

(ii): Temperature Activity: Activity occurred over a wide range of temperatures between 40 and 90°C with optimum activity between 75 and 80°C (Fig 6.5b).

(iii): Thermostability: Pullulanase activity was found to be relatively stable at 70°C retaining at least 75% activity after 3h (data not shown). Thermostabilty was therefore tested at 80°C (Fig 6.6). In the absence of calcium ions $T_{(50)}$ was reached after approximately 30min This was then followed by a gradual decrease in activity down to around 20% after 180min. In the presence of calcium ions, activity was stabilised and $T_{(50)}$ was reached after 100min, after which a gradual decrease occurred down to around 30% after 180min. Pullulanase activity therefore showed a similar thermostability and pH and temperature profile to the amylase activity.

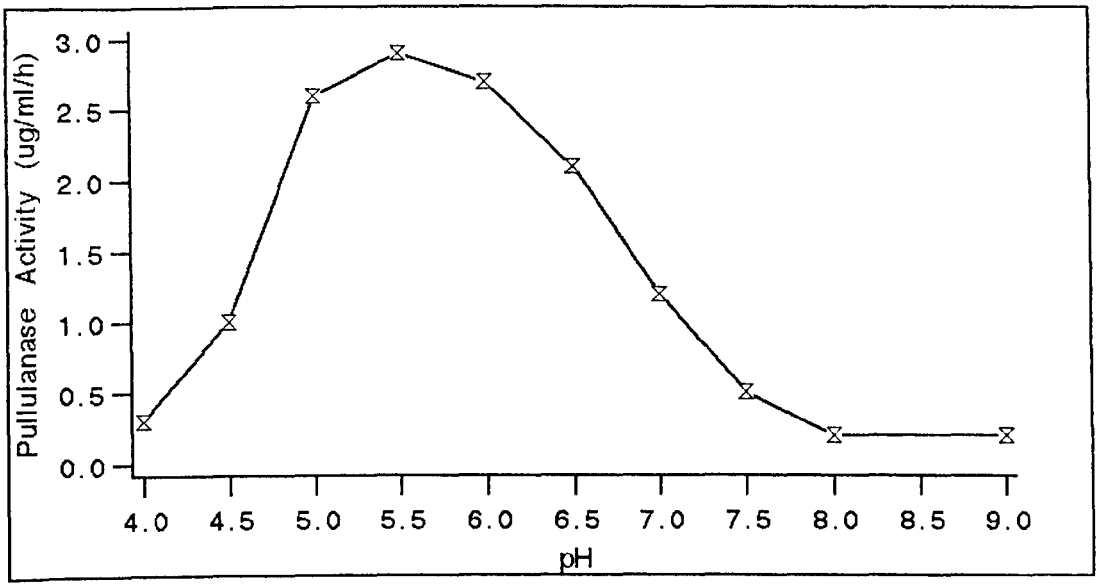
## 6.6: Amylase-Pullulanase Complex

Many isolates, including *Thermoactinomyces vulgaris* (Shimizu et al 1978, Sakano et al 1982), produce an amylase-pullulanase complex which hydrolyses both the alpha-1,4 and the alpha-1,6 linkages. This complex is therefore distinguished by the ability to hydrolyse both starch and pullulan. Isolate 17c showed both amylase and pullulanase activity when grown on SV2a basal medium containing either soluble starch or pullulan. However, neither amylase nor pullulanase activity was detected when either starch or pullulan was replaced by glucose thus indicating that the enzymes are inducible. This could either indicate the presence of an amylase-pullulanase complex, or that there are two enzymes both of which are induced by both starch and

**Figure 6.5**

The effect of a:) pH and b:) temperature on extracellular pullulanase activity of isolate 17c grown in ME3 basal medium containing 15g/l of soluble starch at pH 5.0 and 50°C. The pH profile was carried out at 50°C, the temperature profile at pH 5.5. Each is representative of three replicates and the standard deviation was never greater than 10%.

a:)


Pullulanase Activity (ug/ml/h)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
pH

b:)

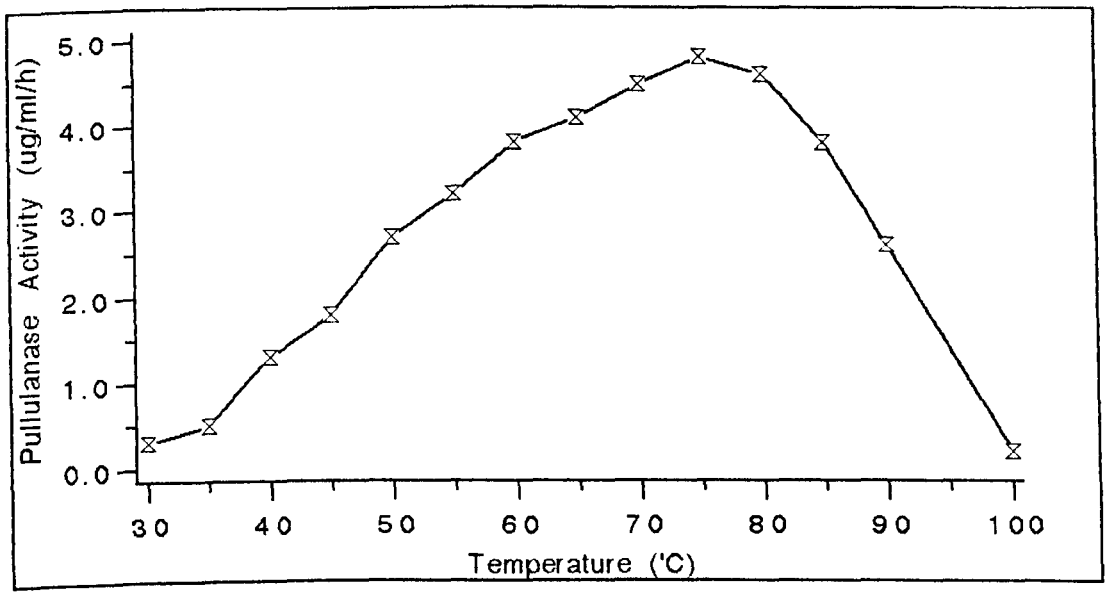
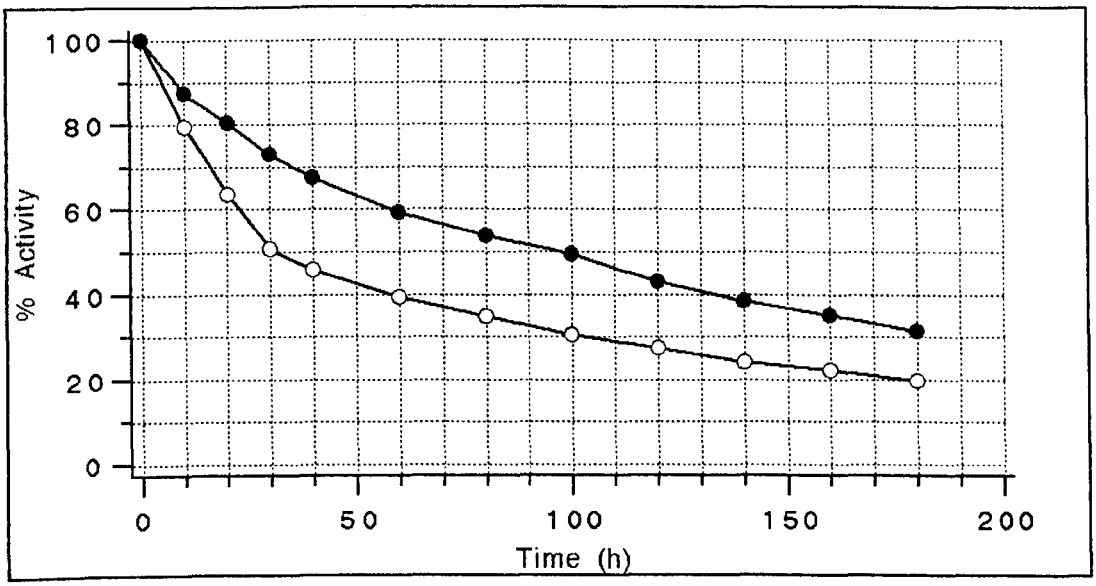

Pullulanase Activity (ug/ml/h)
5.0
4.0
3.0
2.0
1.0
0.0
30
40
50
60
70
80
90
100
Temperature (°C)

**Figure 6.6**

Thermostability of pullulanase activity of isolate 17c at 80°C with (closed circles) and without (open circles) 5mM $CaCl_2$. Each is representative of three replicates and the standard deviation was never greater than 10%.


100
80
60
40
20
0
% Activity
0
50
100
150
200
Time (h)

pullulan. The presence, either of a complex, or of two separate enzymes, can however only be determined by the purification of the enzyme(s) from the supernatant.

## 6.7: The Effect of pH and Temperature on Esterase Activity of Isolate 17c

Esterase activity could not be measured above pH 7.0 and 65°C, and thus only incomplete profiles are shown. The reason for this was that the substrate used in the assay (pNP-palmitate) was not stable above these levels.

(i) pH Activity: Esterase activity occurred from pH 5.0 to 7.0 (Fig 6.7a) and it appeared from the graph that a high degree of activity would continue at least up to pH 7.5 and 8.0. Optimum activity appeared to occur at pH 6.5. The pH profile for esterase activity therefore appeared to extend higher than the profiles of protease and amylase activity It also appeared to differ from other esterases which generally show optimum activity between pH 7 and 9 (Jensen 1983, Antonian 1988, Shabtai & Daya-Mishne 1992, Meghji et al 1990).

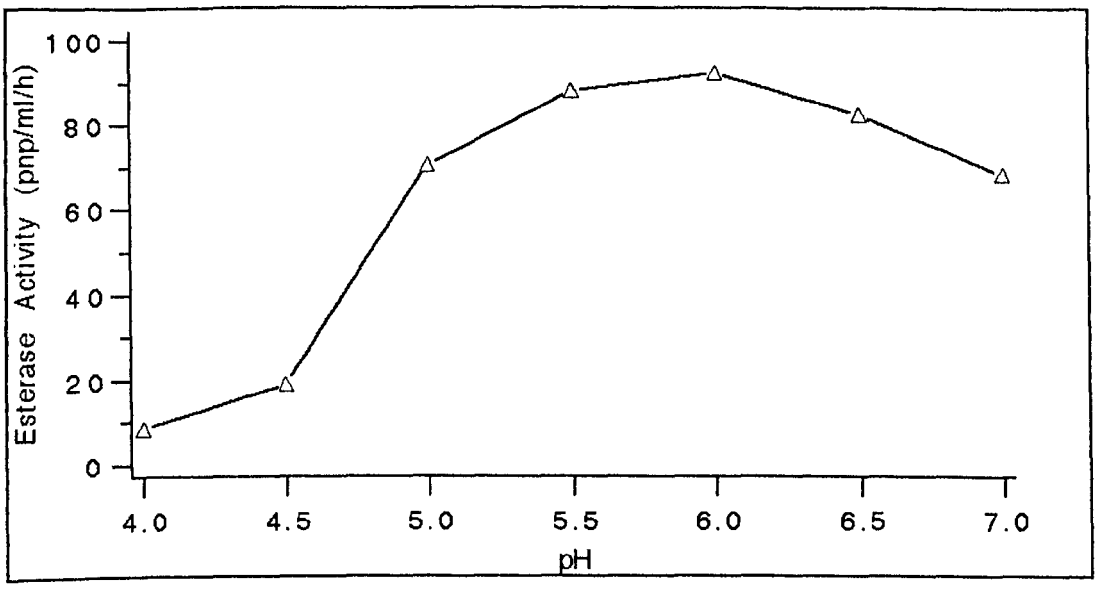
(ii): Temperature Activity: Esterase activity increased with temperature up to 65°C (Fig 6.7b) and thus appeared to show a similar profile to that of protease and amylase activity.

(iii): Thermostability: Thermostability was tested at 90°C (Fig 6.8a). The presence of calcium ions had no effect on activity, which in both cases increased sharply to between 150 and 180% after 10 min. An overall drop in activity then occurred down to

**Figure 6.7**

The effect of a:) pH and b:) temperature on extracellular esterase activity of isolate 17c grown in ME3 basal medium containing 15g/l of tween 80 at pH 5.0 and 50°C. The pH profile was carried out at 50°C, the temperature profile at pH 6.0. Each is representative of three replicates and the standard deviation was never grater than 10%.

a:)


Esterase Activity (pnp/ml/h)
100
80
60
40
20
0
4.0
4.5
5.0
5.5
6.0
6.5
7.0
pH

b:)

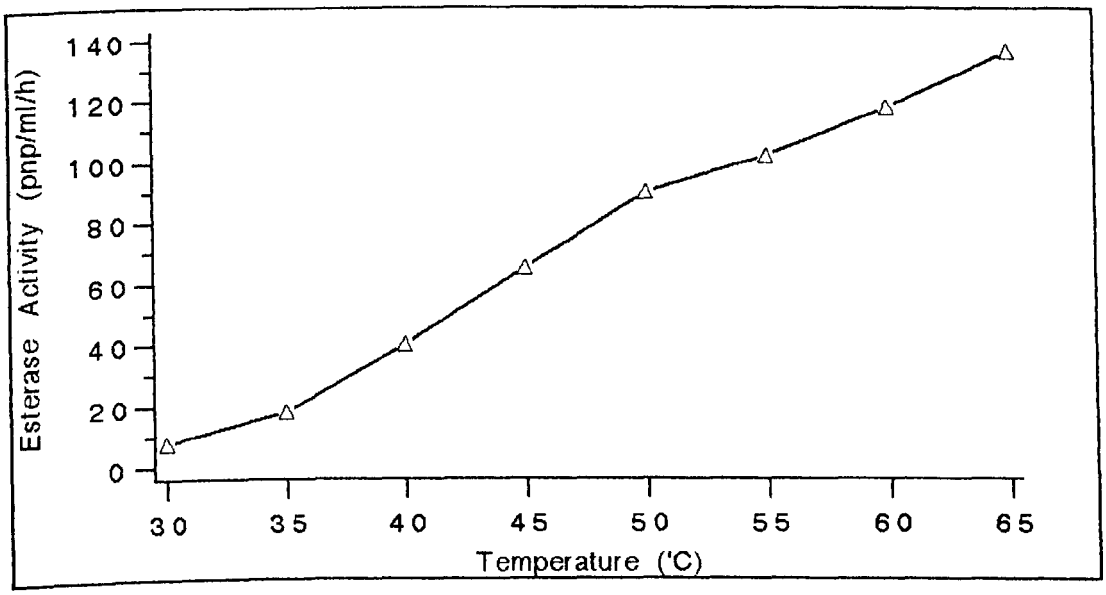
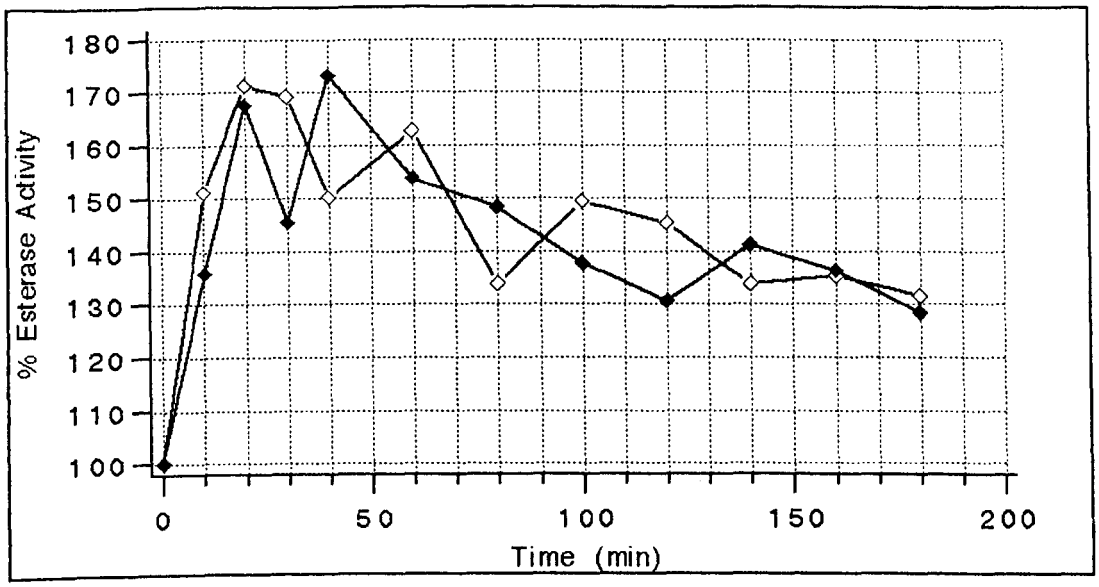
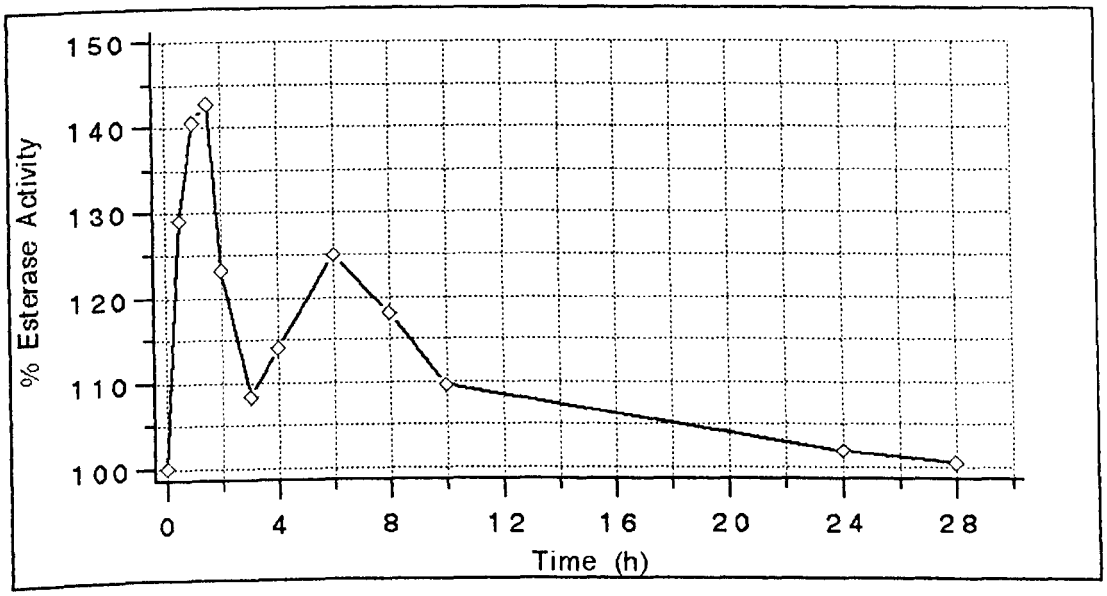

Esterase Activity (pnp/ml/h)
140
120
100
80
60
40
20
0
30
35
40
45
50
55
60
65
Temperature ('C)

**Figure 6.8**

Thermostability of esterase activity of isolate 17c at 90°C with (closed circles) and without (open circles) 5mM $CaCl_2$. Each is representative of three replicates and the standard deviation was never greater than 10%.


% Esterase Activity
180
170
160
150
140
130
120
110
100
0
50
100
150
200
Time (min)


% Esterase Activity
150
140
130
120
110
100
0
4
8
12
16
20
24
28
Time (h)

between 120 and 140% after 180 min. Activity never therefore dropped below 100% and thus the experiment was repeated with pre-incubation over a period of hours (Figure 6.8b). This showed a similar trend with a sharp increase in activity after 2h followed by a gradual decrease, this time down to between 90 and 100% after 28h. Boiling the supernatant in a water bath at 100°C however caused total loss of activity after 10 min. Activity therefore appeared to be extremely thermostable.

## 6.8: The Effect of pH and Temperature on Lipase Activity of Isolate 17c

(i) pH Activity: Activity occurred largely in the pH range 5.0 to 8.0 (Fig 6.9a) with an optimum at pH 6.5. Lipase activity therefore showed a wider pH profile which extended up to higher pH values than amylase and protease activity, and it's profile appeared similar to the predicted profile for esterase activity. It therefore differed from the profiles of other lipases which generally show similar pH optima to esterases with optimum activity between pH 7.0 and 9.0 (Jensen 1983, Antonian 1988).

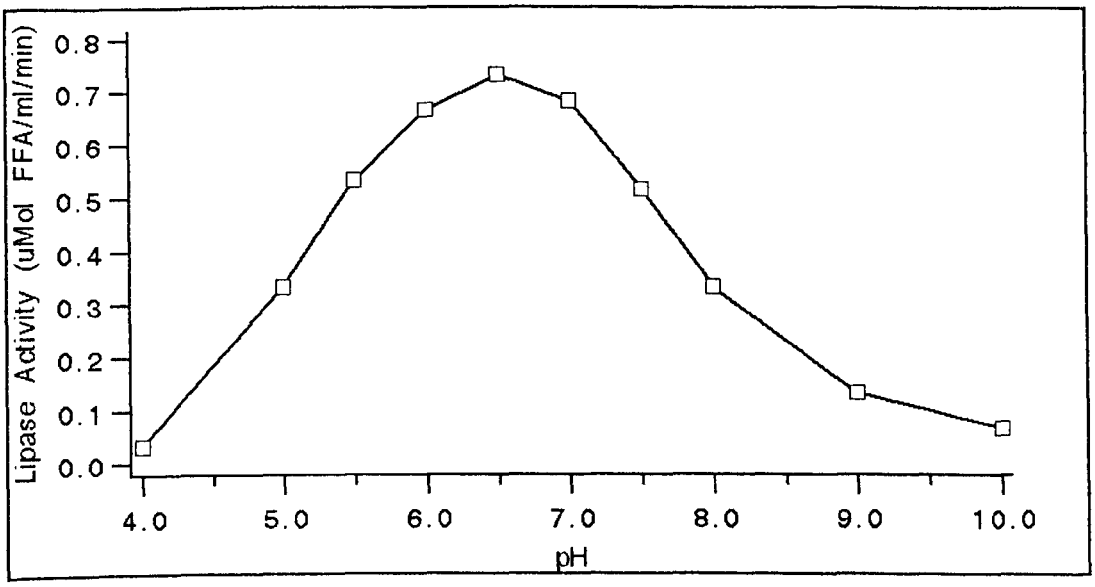
(ii): Temperature Activity: Activity occurred in a wide range of temperatures between 45 and 95°C with optimal activity between 65 and 85°C and an optimum at 80°C (Fig 6.9b).

(iii): Thermostability: Thermostability was tested at 90°C (Fig 6.10) and a similar response was shown to that for esterase activity. Activity increased sharply to between 120 and 140% after 10 min, followed by an overall drop in activity to between 95 and 100% after 180 min. Calcium ions again had no effect and

**Figure 6.9**

The effect of a:) pH and b:) temperature on extracellular lipase activity of isolate 17c grown in ME3 basal medium containing 15g/l of olive oil at pH 5.0 and 50°C. The pH profile was carried out at 50°C, the temperature profile at pH 6.0. Each is representative of three replicates and the standard deviation was never greater than 10%.

a:)


Lipase Activity (uMol FFA/ml/min)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
4.0
5.0
6.0
7.0
8.0
9.0
10.0
pH

b:)

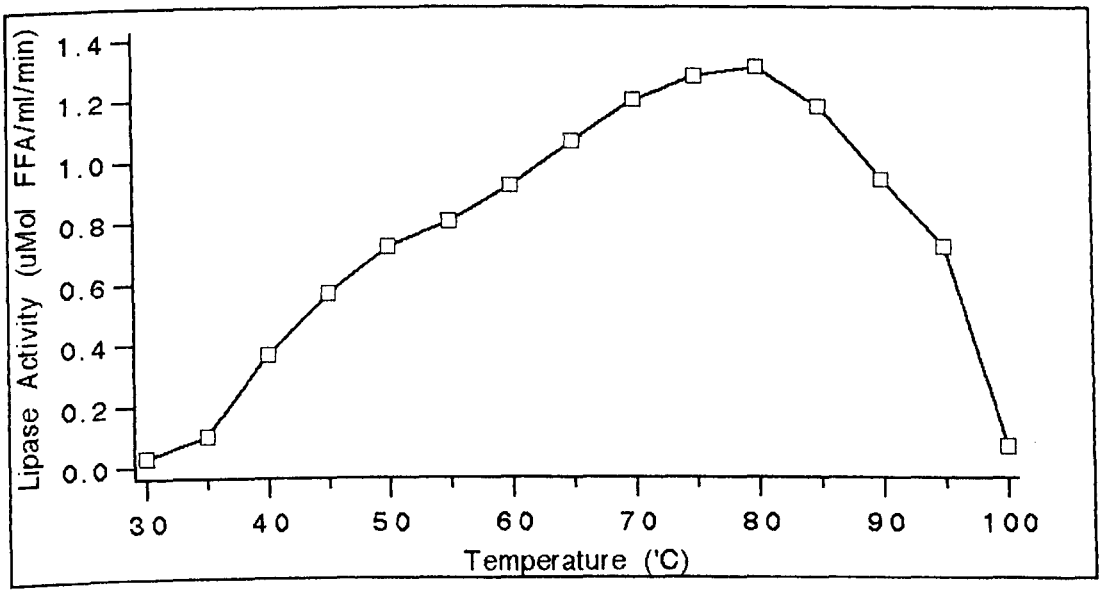
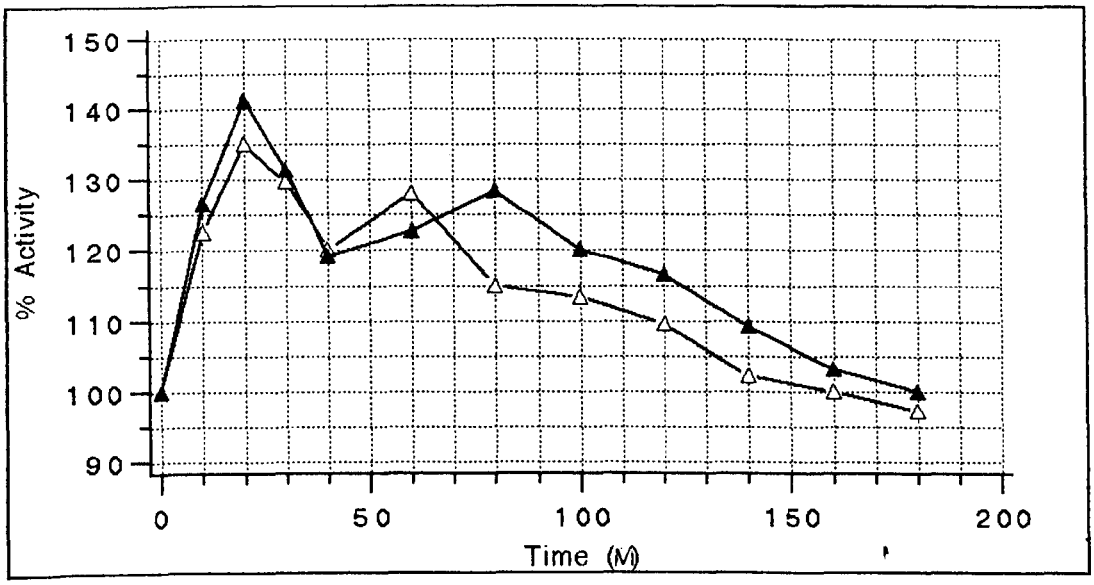

Lipase Activity (uMol FFA/ml/min)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
30
40
50
60
70
80
90
100
Temperature ('C)

**Figure 6.10**

Thermostability of lipase activity of isolate 17c at 90°C with (closed circles) and without (open circles) 5mM $CaCl_2$. Each is representative of three replicates and the standard deviation was never greater than 10%.


150
140
130
120
110
100
90
% Activity
0
50
100
150
200
Time (M)

boiling the enzyme in a water bath at 100°C caused total loss of activity after 10 min. Lipase and esterase activities therefore appeared very similar.

Such thermostability may in part be due to the strong internal hydrophobic interactions of esterases and lipases (Antonian 1988) holding the enzyme together at high temperatures and it may in part be due to the fact that only crude enzyme preparations were used in the assay and there may have been stabilising effects of the supernatant and substrate on the enzyme activity.

### 6.9: Esterase-Lipase Complex

There are separate assays for the measurement of esterase and lipase activity. Esterase assays commonly use Tween 80 as a growth substrate, lipase assays commonly use olive oil. Despite these different systems, both esterase and lipase activity has been detected after growth on either Tween 80 or olive oil (Gowland et al 1987, Antonian 1988). This may be due to the presence of a lipase-esterase complex. In this study, when isolate 17c was grown on Tween 80, the resulting supernatant showed not only esterase activity but also a small degree of lipase activity, and similarly when grown on olive oil, the supernatant not only showed lipase activity but also a small degree of esterase activity. This could either indicate the possible presence of an esterase-lipase complex or the presence pf two separate enzymes with a certain cross specificity. However, as with the amylase-pullulanase complex this can only be determined by activity gel electrophoresis. Neither esterase nor lipase activity was observed when either Tween 80 or olive

oil was replaced by glucose thus suggesting they are both inducible.

## 6.10: Thermal Activation of Esterase and Lipase Activities

The increase showed by esterase and lipase activities after maintenance at 90°C could have indicated thermal activation, as described for glucanase from *Thermomonospora curvata* (Stutzenberger & Lupo 1986). An enzyme is considered to be thermally active if, when heated to a temperature above that of its normal operating temperature it achieves a rate of activity greater than that shown at the normal operating temperature and a rate which is maintained even after the temperature is subsequently lowered. In order to test this hypothesis, culture supernatant was heated at 90°C for 20 min, assaying at 50°C every 10 min. The supernatant was then cooled to 4°C and activity assayed again at 50°C. The supernatant showed the characteristic increase in activity after 20 min at 90°C, but after cooling, activity had dropped to normal levels. Thus thermal activation was not taking place. Another possible explanation of the initial increase in activity was that other extracellular enzymes present in the supernatant normally inhibit lipase activity, however at 90°C these enzymes are quickly denatured thus allowing esterase and lipase activities to increase. A possible explanation of the extreme thermostability of both these enzymes compared to the growth temperature of the organisms and the thermostability of the protease, amylase and pullulanase activities is that these enzymes often possess hydrophobic active sites and are thus often tightly coiled structures which are

better able to resist high temperatures. For example, esterase and lipase enzymes of mesophilic organisms have often been shown to be thermostable (Sugihara et al 1991, Iizumi et al 1990).

### 6.11: Inhibitors of Protease Activity

The bi-phasic thermostability of protease activity (Fig 6.2) suggested that two enzymes may be produced, one relatively heat labile and one more heat stable. This was investigated using the metalloprotease inhibitor E.D.T.A and the serine protease inhibitor phenylmethylsulfonylfluoride (P.M.S.F). The presence of 5mM P.M.S.F and 5mM E.D.T.A in unheated supernatant caused 74-78% and 48-52% inhibition of activity respectively (Table 6.1a) while in supernatant that had been pre-incubated at 80°C for 20 min, inhibition was approximately the same at 73-74% (Table 6.1b). The number of enzymes present could not therefore be determined from this data as either two proteases, a serine and a metallo, or one protease, showing both serine and metallo properties,could be present and a further experiment was carried out using a chromogenic assay. A range of chromogenic substances containing the chromophore nitroanilide linked to a variety of specific amino acid chains were tested with cleaving of these chains from the chromophore by the enzyme causing a colour change. The compounds succinyl-alanine-alanine-proline-phenylalanine-nitroanilide (S.A.A.P.P-NA) and succinyl-alanine-alanine-proline-leucine-nitroanilide (S.A.A.P.L-NA) were selected as the only two to show activity (Table 6.2a). Using S.A.A.P.P-NA, 93% inhibition occurred in the presence of 5mM P.M.S.F, while only 6% inhibition occurred in the presence of 5mM E.D.T.A (Table 6.2b).

**Table 6.1a**

Inhibitors of protease activity using azocasein assay.

| | % Activity (+/-S.D) | % Inhibition |
|---|---|---|
| Supernatant | 100 | 0 |
| E.D.T.A | 52 (+/-2.0) | 48 |
| P.M.S.F | 24 (+/-2.0) | 76 |
| E.D.T.A + P.M.S.F | 2 (+/-1.0) | 98 |

**Table 6.1b**

Inhibitors of protease activity after pre-incubation at 80°C for 20 minutes using azocasein assay.

| | % Activity (+/-S.D) | % Inhibition |
|---|---|---|
| Supernatant | 100 | 0 |
| E.D.T.A | 28 (+/-1.4) | 72 |
| P.M.S.F | 27 (+/-1.9) | 73 |

**Table 6.2a**

Chromogenic substrates tested for activity.

| | |
|---|---|
| n-Succinyl-ala-ala-pro-phe-nitroanilide | +ve |
| n-Succinyl-ala-ala-ala-nitroanilide | -ve |
| n-Succinyl-ala-ala-val-ala-nitroanilide | -ve |
| n-Succinyl-ala-ala-pro-leu-nitroanilide | +ve |
| n-Succinyl-ala-ala-val-nitroanilide | -ve |

**Table 6.2b**

Inhibitors of protease activity using the chromogenic assay.

Using n-S.A.A.P.P-NA.

| | % Activity (+/-S.D) | % Inhibition |
|---|---|---|
| Supernatant | 100 | 0 |
| P.M.S.F | 3 (+/-0.5) | 97 |
| E.D.T.A | 94 (+/-2.0) | 6 |

Using n-S.A.A.P.L-NA.

| | % Activity (+/-S.D) | % Inhibition |
|---|---|---|
| Supernatant | 100 | 0 |
| P.M.S.F | 74 (+/-2.5) | 26 |
| E.D.T.A | 22 (+/-0.8) | 78 |

This substrate therefore appeared to register largely serine protease activity. Using S.A.A.P.L-NA, 26% inhibition occurred in the presence of 5mM P.M.S.F, while 78% inhibition occurred in the presence of 5mM E.D.T.A (Table 6.2b). This substrate therefore appeared to register largely metalloprotease activity. These results were therefore also not conclusive as again they could either indicate the presence of two proteases, one showing largely serine and one largely metallo protease activity, or of the presence of one protease, showing both serine and metallo activities. The only way to determine the number of proteases present would therefore be to carry out an activity gel.

## 6.12: Antibiotic Activity

A selection of the isolates were screened for activity against the test organisms *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Candida albicans, Mucor rouxii* and *Aspergillus niger* in a range of different media including broths, agars and solid states. No activity was shown against *E.coli, M.rouxii, A.niger* and *C.albicans* but some was shown against the gram positive organisms *S.aureus* (Table 6.3a) and *B.subtilis* (Table 6.3b). For media contents see appendix. Optimum antibiotic activity occurred in the solid state medium SM76 and the broth SM6. Both media contained a corn based product, SM76 consisted of cracked corn and a basal medium (Merck patent) in a 100ml conical flask and SM6 consisted of corn steep liquor and MD30E maltodextrin. Three broths showed slightly less activity, SM3, SM12 and SM13. SM3 consisted of glucose, MD30E maltodextrin, arkasoy soya flour and molasses (beet), SM12 consisted of glucose, arkasoy soya

**Table 6.2a**

Chromogenic substrates tested for activity.

| | |
|---|---|
| n-Succinyl-ala-ala-pro-phe-nitroanilide | +ve |
| n-Succinyl-ala-ala-ala-nitroanilide | -ve |
| n-Succinyl-ala-ala-val-ala-nitroanilide | -ve |
| n-Succinyl-ala-ala-pro-leu-nitroanilide | +ve |
| n-Succinyl-ala-ala-val-nitroanilide | -ve |

**Table 6.2b**

Inhibitors of protease activity using the chromogenic assay.

Using n-S.A.A.P.P-NA.

| | % Activity (+/-S.D) | % Inhibition |
|---|---|---|
| Supernatant | 100 | 0 |
| P.M.S.F | 3 (+/-0.5) | 97 |
| E.D.T.A | 94 (+/-2.0) | 6 |

Using n-S.A.A.P.L-NA.

| | % Activity (+/-S.D) | % Inhibition |
|---|---|---|
| Supernatant | 100 | 0 |
| P.M.S.F | 74 (+/-2.5) | 26 |
| E.D.T.A | 22 (+/-0.8) | 78 |

flour, peptone, lab lemco and yeast extract, and SM13 consisted of glucose, Glycerol solution, pharmedia, starch, CSL and peptone. No activity occurred in the other media tested including ME3, SM79, which consisted of vermiculite and 10ml of SM17 (AM1) broth, SM17 broth which consisted of glucose, glycerol, starch, arkasoy soya flour, peptone and yeast extract, SM2, which consisted of estol 1434 glyceryl trioleate and arkasoy soya flour, SM10, which was a defined medium, SM18 which consisted of glucose, starch, pharmedia and molasses (beet), and SM19 which consisted of tomato paste, oat flour and glucose. Activity was also absent on the three media tested in agar format, ME3, SM12 and SM18. Both ME3 and SM18 also failed to yield activity as broths, however, SM12 showed some activity. The reason for the lack of activity on agar is not known. Apart from the solid state medium SM76, activity was generally quite poor. One possible explanation for this is that SM76 was the only medium which gave rise to extensive sporulation, as seen by the white powdery growth over the surface of the substrate characteristic of other *Thermoactinomyces* strains, and it is well known that members of the genus *Bacillus* which produce secondary metabolites usually do so as they are undergoing sporulation. Moreover, it is also well known that filamentous organisms produce increased yields of antibiotic when large hyphal masses are allowed to develop, again something that occurred during growth on SM76, but which does not generally occur during growth in flask where hyphae tend to fragment, or on agar where lysis is often very rapid. A better approach for the screening of these isolates for bioactive compounds would therefore be to select media which gave rise to extensive sporulation and/or to extensive hyphal

networks. The only antibiotic derived from *Thermoactinomyces* to date has been the anti-gram positive compound Thermorubin (Craveri et al 1964) and it could be this that is being produced by the isolates. The isolates were also put through a range of more complex and extensive screens by Glaxo. These included 3 anti-bacterial, 3 anti-viral, 2 anti-fungal, 4 cardiovascular,1 anti-inflammatory and 2 anti-cancer screens. For each screen, the isolates were grown on a variety of selected screening media, however, all results proved negative.

**Table 6.3a**

Antibiotic activity of a selection of isolates grown on a variety of screen media and tested against *S.aureus*. Activity is measured in zones of inhibition (mm), measured from the edge of the well to the edge of the zone. All data represents an average of three replicates.

| | Liquid Media | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Isolate | SM2 | SM3 | SM6 | SM10 | SM12 | SM13 | SM17 | SM18 | SM19 | ME3 |
| 1a | --- | 3 | 6 | --- | 3 | 3 | --- | --- | --- | --- |
| 2a | --- | 2 | 5 | --- | 2 | 2 | --- | --- | --- | --- |
| 2b | --- | 3 | 6 | --- | 3 | 2 | --- | --- | --- | 2 |
| 5a | --- | 3 | 5 | --- | 2 | 2 | --- | --- | --- | --- |
| 5b | --- | 2 | 5 | --- | 2 | 2 | --- | --- | --- | --- |
| 5d | --- | 2 | 5 | --- | 2 | 3 | --- | --- | --- | --- |
| 5e | --- | 3 | 6 | --- | 3 | 3 | --- | --- | --- | --- |
| 15a | --- | 3 | 6 | --- | 2 | 2 | --- | --- | --- | 1 |
| 17a | --- | 2 | 6 | --- | 3 | 2 | --- | --- | --- | --- |
| 17c | --- | 2 | 4 | --- | 2 | 2 | --- | --- | --- | --- |
| 18a | --- | 3 | 6 | --- | 3 | 3 | --- | --- | --- | 1 |

| | Solid State Media | | Agars | | |
|---|---|---|---|---|---|
| Isolate | SM76 | SM79 | ME3 | SM12 | SM18 |
| 1a | 9 | --- | --- | --- | --- |
| 2a | 8 | --- | --- | --- | --- |
| 2a | 9 | --- | --- | --- | --- |
| 5a | 8 | --- | --- | --- | --- |
| 5b | 7 | --- | --- | --- | --- |
| 5d | 7 | --- | --- | --- | --- |
| 5e | 8 | --- | --- | --- | --- |
| 15a | 8 | --- | --- | --- | --- |
| 17a | 7 | --- | --- | --- | --- |
| 17c | 6 | --- | --- | --- | --- |
| 18a | 8 | --- | --- | --- | --- |

**Table 6.3b**

Antibiotic activity of a selection of isolates grown on a variety of screen media and tested against *B.subtilis*. Activity is measured in zones of inhibition (mm), measured from the edge of the well to the edge of the zone. All data represents an average of three replicates.

| | Solid Media | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Isolates | SM2 | SM3 | SM6 | SM10 | SM12 | SM13 | SM17 | SM18 | SM19 | ME3 |
| 1a | --- | 2 | 5 | --- | 2 | 2 | --- | --- | --- | --- |
| 2a | --- | 2 | 5 | --- | 2 | 2 | --- | --- | --- | --- |
| 2b | --- | 3 | 6 | --- | 2 | 2 | --- | --- | --- | --- |
| 5a | --- | 2 | 6 | --- | 2 | 2 | --- | --- | --- | --- |
| 5b | --- | 2 | 5 | --- | 1 | 2 | --- | --- | --- | --- |
| 5e | --- | 3 | 6 | --- | 2 | 2 | --- | --- | --- | --- |
| 15a | --- | 3 | 6 | --- | 2 | 3 | --- | --- | --- | --- |
| 17a | --- | 2 | 5 | --- | 2 | 1 | --- | --- | --- | --- |
| 17c | --- | 2 | 5 | --- | 2 | 2 | --- | --- | --- | --- |
| 18a | --- | 3 | 6 | --- | 3 | 2 | --- | --- | --- | --- |

| | Solid State Media | | Agars | | |
|---|---|---|---|---|---|
| Isolate | SM76 | SM79 | ME3 | SM12 | SM18 |
| 1a | 7 | --- | --- | --- | --- |
| 2a | 7 | --- | --- | --- | --- |
| 2b | 9 | --- | --- | --- | --- |
| 5a | 7 | --- | --- | --- | --- |
| 5b | 8 | --- | --- | --- | --- |
| 5d | 7 | --- | --- | --- | --- |
| 5e | 8 | --- | --- | --- | --- |
| 15a | 7 | --- | --- | --- | --- |
| 17a | 6 | --- | --- | --- | --- |
| 17c | 6 | --- | --- | --- | --- |
| 18a | 7 | --- | --- | --- | --- |

# CHAPTER 7

# DISCUSSION

## 7.1: Ecology.

None of the mesophilic isolates obtained in this study were shown to be acidophilic thus failing to support the findings of Khan & Williams (1975). There are two possible explanations for this. One is that the organisms isolated by Khan & Williams, which were noted to be difficult to isolate, were simply not successfully isolated in this study. However, this seems unlikely for a number of reasons. A total of three separate isolations were carried out at different times of the year to account for any seasonal variation, a large number of different locations were sampled from the site and a large number of different samples were collected from each location and the isolation procedure followed was exactly as described by Khan & Williams. The correct soil horizon was also used as it matched the description, depth and pH as described by Khan (1972). The second explanation is that the organisms were isolated but that they did not pass through the pH screens, however this also seems unlikely as their pH profile (optimum growth at pH 4.5-5.5 with varying growth at pH 3.5 and 6.5) was determined in buffered liquid media and would make them easily identifiable in the pH screens used. The isolation of *Thermoactinomyces* isolates from the pine forest soils was also surprising as these are not typical heated environments where thermophilic actinomycetes are commonly isolated. Perhaps the most likely explanation for their presence was that the layer of decaying litter between 4 and 8cm below the surface from which they were isolated became heated enough due to microbial activity, particularly from fungi which were in obvious abundance. Their isolation from the Freshfields site and their subsequent isolation, albeit in low numbers, from other acid

soils of similar pH indicates that, like the acidophilic mesophilic actinomycetes described by Khan & Williams, while they may be present in relatively low numbers, they are quite widespread in acid soils where they may play an important role in nutrient recycling. This and the failure to isolate these organisms from the neutral forest soils tested further indicates that they are actively growing members of the microbial population present in these acid soils and that they were not simply organisms from neutral soils lying dormant, which was a particular possibility given the longevity of endospores in the environment. This also further differentiated them from acid tolerant organisms, which may be found in acid environments but which thrive in neutral soils. With endspores showing such longevity in the environment the failure to isolate these organisms from neutral soils may appear strange as transfer between soils would be expected. However, one possible explanation for this could be the poor aerial sporulation of these isolates, although there is no evidence to suggest that the isolates also show poor sporulation in the environment.

## 7.2: Physiology.

The isolates were easily identified as members of the genus *Thermoactinomyces* by the presence of refractile endospores and characteristic branching hyphal structure. They were however shown to differ considerably from the *Thermoactinomyces* type and culture collection trains tested in terms of their pH profile (optimum growth at pH 5.0 for the pine forest isolates compared to pH 7.0 for all type and culture collection strains tested) but also in terms of their colony morphology (the pine forest isolates

showed no aerial sporulation and therefore did not show the characteristic white appearance on agar plates shown by the type and culture colection strains, but instead appeared as matt brown granular colonies) and in at least two of the basic physiological and biochemical tests carried out. This data also identified the isolates as being similar if not identical to each other and it therefore appears that they form a novel species of the genus. The *Thermoactinomyces* isolates identified in this study showed growth between pH 4.5 and 6.5/7.0 with an optimum at pH 5.0 where pH was not controlled, and between pH 4.5 and 6.0 with an optimum at pH 5.0 where pH was controlled. As has already been mentioned this profile is significantly different from the *Thermoactinomyces* type and culture collection strains tested, it also differs from that of other neutrophilic microorganisms in general which characteristically show growth between pH 6.0 and 8.5. However, the profile is also significantly different from true acidophiles such as *Alicyclobacillus* sp., *Thiobacillus* sp. and acidophilic archaea which characteristically show optimum growth between pH 2.0 and 4.5. The isolates cannot therefore be classified as either neutrophilic or acidophilic. Neither can they be classified as acid tolerant as this definition is that of an organism which can grow at pH 5.0 but which grows optimally at neutral pH values. The best descriptive term for these isolates is therefore that of moderately acidophilic, along with other microorganisms which show similar pH profiles, for example *Bacillus naganoensis* which shows optimum growth at pH 4.7 to 5.5 (Tomimura et al 1990), *Acidothermus cellulyticus* which shows optimum growth at pH 5.0 (Mohagheghi et al 1986) and a *Nitrobacter* strain which shows optimum growth at pH 5.5

(Hankinson & Schmidt 1988). When the isolates were grown without pH control in a complex medium containing sugars such as ME3 or SV2a, a drop in culture pH to between pH 4.3 and 4.9 was observed, and it appeared that at the higher pH values (pH 6.5 and pH 7.0) where growth could only occur when pH was not controlled, that this drop in culture pH was required for growth. This further supports the definition of these organisms as moderately acidophilic, however this is complicated by data obtained from growth in other types of media. For example when grown on SV2a basal medium, containing only soya peptone and NaCl, and no sugars, the isolates grew well at pH 5.0 and showed no growth at pH 7.0, but when growth occurred at pH 5.0 there was an increase in culture pH often up to pH 7.0-7.5. Similarly, when grown on Sv2a basal medium plus either Tween 80, vegetable oil or olive oil growth was good at pH 5.0 and yet there occurred an increase in culture pH often up to pH 8.0-8.5. When grown on these substrates at pH 7.0 however, growth also took place and there was observed an initial drop in culture pH after 24h down to between pH 5.0 and 6.0 followed by an increase up to pH 7.0-8.5 after 48h. When grown on SV2a basal medium plus starch with culture pH increasing up to pH 5.5 with growth at pH 5.0 and culture pH dropping to pH 5.0-5.5 when grown at pH 7.0. This wide range of pH changes suggests a wide range of metabolic processes taking place, although whether the cells can in some way utilise this to alter culture pH to suit themselves, or whether it simply occurs as a bi-product of metabolism is not clear. This action may serve no advantage or disadvantage to the cell, or it may be that they are able, to some small extent at least, to utilise its effects to manipulate the pH conditions. The

isolates can grow in the range pH 4.75-6.0 when culture pH is controlled but can only grow at higher pH values (pH 6.5-7.0) when the culture pH is not controlled thus suggesting that the drop in culture pH is in some way required for growth at these higher pH values. The method of this reduction of culture pH is probably as a result of $H^+$ ions released as a direct result of the metabolism of sugars, hence no pH drop and thus no growth occurred at pH 7.0 on SV2a basal medium containing no carbon source. Growth on SV2a basal medium, Tween 80, vegetable oil and olive oil also shows that the isolates are capable not only of withstanding but also of growing as culture pH increases so long as the starting pH is pH 5.0. At a starting pH of 7.0, growth occurred with a simultaneous drop in culture pH to within the optimum growth limits (pH 4.75-6.0).

A novel alkalophilic *Thermoactinomyces* isolate has also recently been isolated (Tsuchiya et al 1991, 1992) which showed optimum growth at pH 10.3 and this could point to existence of a wider diversity of *Thermoactinomyces* species present in a wider diversity of habitats than has been previously thought.

## 7.3: Enzymology.

The extracellular protease, amylase and pullulanase activity profiles for the pine forest isolates showed optima at pH 5.5. Activities previously detected from neutrophilic *Thermoactinomyces* species have generally showed optima at pH 7.0 (Kleine 1982, Obb & Odibo 1984a+b, Odibo & Obb 1988). The enzymes from the pine forest isolates therefore clearly differed from those of the majority of other *Thermoactinomyces* strains tested. There are however two important exceptions, amylase

activity reported from a *T.vulgaris* strain (Shimizu et al 1975) showed an optimum at pH 5.0, while amylase and pullulanase activity reported also from a *T.vulgaris* strain (Sakano et al 1982, 1983) showed an optimum at pH 4.5-5.0. These profiles are surprisingly low because the strains in question appeared to be neutrophilic (they were cultured at pH 7.0) and if this were the case would therefore be expected to show similarly extracellular enzyme activity to the other neutrophilic strains listed (Kleine 1982, Obb & Odibo 1984a+b and Odibo & Obb 1988). However, it could be that these two enzymes simply possess a low pH optimum for neutrophilic strains, or it could be that the strains were not neutrophilic.

The pH profiles of enzymes from neutrophilic bacteria in general varies considerably and this makes distinct comparisons between them and the pH profiles of the three enzymes from the pine forest isolates difficult. For example many enzymes from neutrophilic bacteria show optimal activity at pH 7.0, however many also show optimal activity at pH 6.0 and sometimes at pH 5.0. For example amylase activities of the closely related *Bacillaceae* have pH optima ranging from pH 5.0 to pH 8.0 (Taniguchi et al 1983, Takasaki 1983, 1987, 1989, Yoshigi et al 1985, David et al 1987, Takasaki et al 1991), while pullulanases have been isolated which show activity between pH 4.5 and pH 7.0 (Schulein & Hojer-Pederson 1984, Shen et al 1990, Kuriki et al 1988, Jensen & Norman 1984, Takasaki 1987, Bakshi et al 1992). Thus while it is true that the enzyme pH profiles of the pine forest isolates are different to many enzymes from neutrophilic bacteria this is not true across the board. There is however perhaps more significance in the pH profile of the protease

activity shown by the pine forest isolates. Neutral and serine proteases generally show pH optima of pH 7.0 and above (Priest 1984, Outtrup & Boyce 1990) whereas the pH profile shown by the pine forest isolates is more of what might be expected from an acid protease. However, it does not show as low a profile as proteases from acidophilic bacilli which showed optimal activity between pH 3.5 and 5.5 (Darland & Brock 1971, Bonjour & Aragno 1984, Deinhard et al 1987a+b).

The temperature profile of the protease, amylase and pullulanase enzymes, with optimum activity at 75-80°C, was similar to that shown for other *Thermoactinomyces* species (Shimizu et al 1978, Kleine 1982, Obb & Odibo 1984a, Odibo & Obb 1988), although higher than for the *B*-amylase reported by Obb & Odibo which was 60°C, and to the thermophilic bacilli, for example *A.acidocaldarius* (Buonocore et al 1976, Kanno 1986), *B.brevis* (Tsvetkov & Emanuilova 1989), *B.stearothermophilus* (Kim et al 1989) and *B.licheniformis* (Saito 1973).

The protease, amylase and pullulanase activities of the pine forest isolates showed considerable thermostability, with half lives at 80°C of between 5 and 30min without calcium and between 20 and 60min with calcium. These figures were similar to those shown by enzymes from other *Thermoactinomyces* strains, however comparisons between the thermostability of the enzymes from the pine forest isolates and other microorganisms is difficult as a range of different methods exist for its determination. For example amylase and pullulanase activity reported by Shimizu et al (1978) showed a half life at 70°C of over 120min, the *a*-amylase reported by Obb & Odibo (1984a) showed 74% activity after 30min at 70°C. Thermostability

analysis of protease activity indicated the possible presence of two enzymes, one thermostable and one thermolabile, however further characterisation using protease inhibitors and chromogenic substrates failed to conclusively support this, indeed this information and that of the temperature profile tended to support the presence of only one enzyme. The number of proteases present therefore remains undetermined but could easily be resolved by the use of non-denaturing gel electrophoresis followed by determination of enzyme activity *in-situ.* This would also answer the question of whether separate amylase and pullulanase, and esterase and lipase enzymes exist, or whether there exists one enzyme showing dual activity dor each.

The pH optima for esterase and lipase activities were significantly higher (pH 5.5-7.5) than those for protease, amylase and pullulanase activities (pH 5.0-6.0). However, they were also lower than has reported for esterases and lipases from other organisms which is generally between pH 7.0 and 9.0 (Jensen 1983, Omar et al 1987, Antonian 1988, Yamamoto & Fujiwara 1988, Meghji et al 1990, Shabtai-Mishne 1992). Temperature optima were similar, at 80°C, however, both esterase and lipase activities did show considerable thermostability, retaining at least 90% activity after 24h at 90°C, and only being denatured by boiling for 10min. This prolonged thermostability at very high temperatures may owe something to the strong internal hydrophobic interactions that esterases and lipases characteristically possess holding the structure together at high temperatures. It may also be in part due to the fact that only crude enzyme preparations were used and hence there may be a

stabilising effect of the supernatant and substrate. It is possible that both activities are the result of an esterase/lipase complex as esterase activity was also observed when isolates were grown on olive oil and similarly lipase activity was observed when isolates were grown on Tween 80. This would also explain the great similarities between the temperature and pH optima and thermostability of the two activities. However, the presence of two separate enzymes or of a complex can again only be determined by the use of a non-denaturing activity gel electrophoresis. As well as gel electrophoresis, a whole range of further studies need to be carried out on all the enzymes recorded including purification, calculation of structure and molecular weight, and further kinetic studies such as calculation of $K_m$, $K_{cat}$ values etc:.

## 7.4: Screening for Secondary metabolites.

Direct antibiotic activity was only detected against gram positive bacteria. No activity was detected against gram negative bacteria, fungi or yeasts, or in any of the more complex, target directed screens carried out by Glaxo. This apparent lack of activity is typical of *Thermoactinomyces* strains in general (activity has previously only been detected against gram positive bacteria (Craveri et al 1964)) and is therefore perhaps not surprising. However, only a handful of screens were carried out and further tests, including the development of novel screens, may reveal novel activity. One alternative is to use a wider range of solid state media. As mentioned in chapter 6, the medium which gave best results in the antibiotic screens was the solid state SM 76. This gave rise to full hyphal development and

sporulation as seen by significant white growth over the surface of the medium. The association between high antibiotic yield and the presence of large hyphal masses and/or sporulation is well known and this may explain the success of SM 76, as well as the weaker production in liquid media where hyphae were highly fragmented and sporulation was limited. Future experiments with a wider range of solid state media may therefore prove successful. Similarly, despite the scarcity of direct antibiotic activity, only a handful of target directed screens were carried out and with a broader range of these, the isolates may yet prove interesting.

# APPENDIX

## GROWTH MEDIA

| **Starch-Casein** | g/l |
|---|---|
| *(Kuster & Williams 1964)* | |
| Starch | 10 |
| Casein | 0.3 |
| $KNO_3$ | 2 |
| NaCl | 2 |
| $K_2HPO_4$ | 2 |
| $MgSO_4.7H_2O$ | 0.05 |
| $CaCO_3$ | 0.02 |
| $FeSO_4.7H_2O$ | 0.01 |

| **SV2a** | g/l |
|---|---|
| Glucose | 15 |
| Glycerol | 15 |
| Soya Peptone | 15 |
| NaCl | 3 |

| **ME3** | g/l |
|---|---|
| Glucose | 10 |
| Malt Extract | 20 |
| Soya Peptone | 15 |
| NaCl | 3 |

| **SV2a/ME3 basal** | g/l |
|---|---|
| Soya Peptone | 15 |
| NaCl | 3 |

| **YEA** | g/l |
|---|---|
| *(Corbaz et al 1963)* | |
| Glucose | 4 |
| Malt Extract | 10 |
| Yeast Extract | 4 |

| **GYEA** | g/l |
|---|---|
| *(Cross et al 1968b)* | |
| Glucose | 10 |
| Yeast Extract | 10 |
| $K_2HPO_4$ | 0.5 |

| **CYC** *(Cross+Attwell 1974)* | **g/l** |
|---|---|
| Czapek-Dox liquid medium powder | 33.4 |
| Yeast Extract | 2.0 |
| Vitamin free Casamino Acids | 6.0 |

| **GMY** | **g/l** |
|---|---|
| Glucose | 10 |
| Yeast Extract | 15 |
| Malt Extract | 10 |
| NaCl | 3 |

| **GMY1** | **g/l** |
|---|---|
| Glucose | 10 |
| Yeast Extract | 15 |
| Malt Extract | 20 |
| NaCl | 3 |

| **ME1** | **g/l** |
|---|---|
| Glucose | 4 |
| Malt Extract | 20 |
| Soya Peptone | 15 |
| NaCl | 3 |

| **ME2** | **g/l** |
|---|---|
| Glucose | 10 |
| Malt Extract | 20 |
| Peptone | 15 |
| NaCl | 3 |

| **ME3** | **g/l** |
|---|---|
| Glucose | 10 |
| Malt Extract | 20 |
| Soya Peptone | 15 |
| NaCl | 3 |

| **ME4** | **g/l** |
|---|---|
| Glucose | 10 |
| Malt Extract | 20 |
| Tryptone | 15 |
| NaCl | 3 |

| ME5 | g/l |
|---|---|
| Glucose | 10 |
| Malt Extract | 20 |
| Yeast Extract | 15 |
| NaCl | 3 |

| ME6 | g/l |
|---|---|
| Glucose | 10 |
| Malt Extract | 20 |
| Casein Hydrolysate | 15 |
| NaCl | 3 |

| AM1 | g/l |
|---|---|
| Glucose | 2 |
| Glycerol | 40 |
| Starch | 2 |
| Soya Peptone | 15 |
| NaCl | 3 |

| AM1a | g/l |
|---|---|
| Glucose | 5 |
| Glycerol | 20 |
| Starch | 5 |
| Soya Peptone | 15 |
| NaCl | 3 |

| AM1b | g/l |
|---|---|
| Glucose | 10 |
| Glycerol | 10 |
| Starch | 10 |
| Soya Peptone | 15 |
| NaCl | 3 |

| SV2a1 | g/l |
|---|---|
| Glucose | 15 |
| Glycerol | 15 |
| Yeast Extract | 15 |
| NaCl | 3 |

| SV2a2 | g/l |
|---|---|
| Glucose | 15 |
| Glycerol | 15 |
| Peptone | 15 |
| NaCl | 3 |

| SV2a3 | g/l |
|---|---|
| Glucose | 15 |
| Glycerol | 15 |
| Tryptone | 15 |
| NaCl | 3 |

| SV2a4 | g/l | YA | g/l |
|---|---|---|---|
| Glucose | 15 | Glucose | 40 |
| Glycerol | 15 | Yeast Extract | 5 |
| Casein Hydrolysate | 15 | Casein Hydrolysate | 5 |
| NaCl | 3 | Bactopeptone | 3 |

SCREENING MEDIA

| SM 2 | g/l |
|---|---|
| Arkasoy soya flour | 15.0 |
| Estol 1434 Glyceryl trioleate | 18.0 |
| Sodium Sulphate | 1.0 |
| $CoCl_2.6H_2O$ | 0.001 |

| SM3 | g/l | SM6 | g/l |
|---|---|---|---|
| Glucose | 5.0 | Corn steep liquor | 40.0 |
| MD30E Maltodextrin | 50.0 | MD30E Maltodextrin | 20.0 |
| Arkasoy soya flour | 25.0 | NaCl | 2.5 |
| Molasses (beet) | 3.0 | $MgSO_4$ | 0.5 |
| $K_2HPO_4$ | 0.25 | | |

| SM 10 | g/l |
|---|---|
| Glycerol | 23.0 |
| L-Proline | 11.5 |
| NaCl | 0.5 |
| $K_2HPO_4$ | 2.1 |
| $Na_2SO_4$ (anhydrous) | 0.28 |
| 0.02M $MgCl_2$ | 10ml |
| 0.02M $CaCl_2$ | 10ml |

| SM 12 | g/l |
|---|---|
| Arkasoy soya flour | 10.0 |
| Glucose | 50.0 |
| Peptone | 4.0 |
| Lab Lemco | 4.0 |
| Yeast extract | 1.0 |
| Nacl | 2.5 |

| SM 13 | g/l |
|---|---|
| Glucose | 20.0 |
| Glycerol | 10.0 |
| Pharmedia | 10.0 |
| Starch | 20.0 |
| CSL | 10.0 |
| Peptone | 5.0 |
| NaCl | 5.0 |

| SM 17 | g/l |
|---|---|
| Glucose | 2.0 |
| Glycerol | 40.0 |
| Starch | 2.0 |
| Arkasoy soya flour | 5.0 |
| Peptone | 5.0 |
| Yeast extract | 5.0 |
| NaCl | 5.0 |

| SM 18 | g/l |
|---|---|
| Glucose | 15.0 |
| Starch | 40.0 |
| Pharmedia | 25.0 |
| Molasses (beet) | 20.0 |

| SM 19 | g/l |
|---|---|
| Tomato paste | 40.0 |
| Oatflour (Avenaflo) | 15.0 |
| Glucose | 2.0 |

| SM 76 | per 100ml conical flask |
|---|---|
| Cracked corn | 10.0g |
| Basal liquid (Merck patent) | 10ml |

| SM 79 | per 100ml conical flask |
|---|---|
| Vermiculite | 1.2g |
| Basal liquid (SM 17) | 10ml |

SOLUTIONS

| Universal Buffer | g/l |
|---|---|
| *(Johnson & Lindsey1939)* | |
| Boric Acid | 1.769 |
| Citric Acid | 6.008 |
| $KH_2PO_4$ | 3.893 |
| Diethyl Barbituric Acid | 5.266 |

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