STUDIES ON MICROBIAL SPOILAGE

OF

ONIONS (*Allium cepa* L)

BY

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Abstract

Four micro-organisms were isolated from rotted onion bulbs, purple variety, purchased from markets in the Lagos State of Nigeria. Three of them were pathogenic; they produced soft rot when wound-inoculated into healthy onion bulbs. The micro-organisms were identified as Pseudomonas cepacia Buckholder, Candida utilis (Henneberg) Lodder and Kreger-Van Rig and Pseudomonas fluorescens Migula; the last being the most pathogenic of the three.

A study of the effect of infection on the nutritional quality of bulbs by the bacterial pathogens revealed a loss in total sugars and no appreciable change in amino acid and lipid contents of the bulbs. Infection by Candida utilis, however, resulted in an increase in total amino acids but an appreciable amount of loss of sugars and lipids.

Growth studies of P. fluorescens showed that it grew well on a variety of solid and liquid media at 29$\pm$2°C. It utilised glucose, fructose, sucrose, maltose, lactose, galactose, saccharose and mannitol; growth was best with glucose. It also utilised nitrogen sources including sodium nitrate, tyrosine, glycine, alanine, ammonium sulphate and asparagin. Glycine supported the greatest amount of growth. Of the different hydrogen ion concentrations, pH 2-12,

employed in the growth studies, growth was best at pH 6.

Severe rot occurred at room temperature (29± 2°C) and at high relative humidity (80 - 100%).

Pectic enzyme was produced by P.fluorescens in vivo and in vitro. Enzyme activity was highest at pH 5 in the six-day old culture filtrate while it was pH 4 with the extract from nine-day old rot. Activity of rot extract increased with increase in inoculum concentration. Studies using the thiobarbituric acid reaction indicated the presence of hydrolytic polygalacturonase in the filtrate.

Polyphenols were present in both healthy and infected tissues of the onion bulbs but in greater amount in the diseased tissue.

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Introduction and Literature Review

Onions, (*Allium cepa* . L), family *Liliaceae*, was originally a Middle East plant but is nowadays widely cultivated in many parts of America, Europe and Africa for its sharp-tasting, edible bulbs. The leaf bases (bulbs), which are consumed as vegetables, are cooked, pickled or eaten raw. They are important for their mineral and vitamin contents and they also add flavour to food (Jay, 1970).

Onions belong to the group of perishable tropical produce because the bulbs are often attacked by micro-organisms resulting in various spoilage diseases. The resultant losses from microbial spoilage occur in forms of reduction in the quantity and quality of the produce (Tomkins, 1951).

Quantitative pathogenic losses result from rapid and extensive breakdown of the host tissue by micro-organisms. The pattern of attack is usually an initial infection by one or a few specific pathogens followed by massive infection by a broader spectrum of non-specific biodeteriogens which are only weakly pathogenic or saprophytic on the dead or moribund tissue remaining from primary infection (Tomkins, 1951; Turner, 1959).

Apart from microbial spoilage, there are some other less important types of damage that cannot be ignored because they greatly influence the susceptibility of the crop to attack by micro-organisms. They are physiological and mechanical damages.

Spoilage of onions may arise from mechanical injuries sustained during handling of the crop. This problem is of particular interest in Nigeria since the bulbs are usually consumed far from site of production. Onions are known to grow very well in fairly sandy, well aerated soil in a dry environment. As a result, they are found mainly in the northern part of the country. The bulbs are found in the soil so they must be dug out at harvest. After harvest, they are dried, packed in sacks and transported to different parts of the country either by road or rail. Mechanical injury can take different forms and may arise at all stages in the history of the crop from harvest to operations such as drying, grading, packaging, transporting, storing, exposure in the market and finally in the home.

In addition, damage can be of physiological origin. According to Coursey and Booth (1972) ..... "since produce are alive, natural endogenous respiratory losses of dry matter together with transpiratory and wilting losses of water will always occur. Such changes not only affect the quality of the produce but also influence its susceptibility to microbial attack through changes in turgor". Microbial spoilage of onions leads to reduced yield and poor quality thereby reducing the market value of the crops.

Little was known about bacterial diseases of plants till the later part of the nineteenth century. The works of Burrill in 1883 represent the birth of science of bacterial phytopathology (Buckholder, 1948).

A number of pathogens have been associated with microbial spoilage of onions. Griffith (1887) isolated Bacterium allium Griffith from rotted onion bulbs. Delacroix (1905) demonstrated the pathogenicity of Bacillus cepivorous n. sp. on onions. The pathogen was isolated from rotted onion bulbs. Townsend (1904) assumed that a rot on sliced onion bulbs was caused by Bacillus aroidea while Takomoto (1921) later found the same organism causing rot of onions in Japan. Edelsztein-Kosowa (1933) in Poland described a bulb rot of onions caused by Bacillus cepae. Jones (1901) found that Erwinia carotovora caused a soft rot of onions and several investigations have proved its pathogenicity.

Buckholder (1942) found an onion pathogen and called it Pseudomonas allicola. Cother, Derbyshire and Brewer (1976) also found an organism that closely resembled P. allicola described by Stewart (1899) in the U.S.A. and in Bulgaria by Vitanov (1970).

Some fungal pathogens have been associated with rot of onion bulbs. Walker (1925) described three neck rot diseases of onions;

(1) the grey mold neck rot caused by Botrytis allii;

(2) the mycelial neck rot caused by Botrytis byssoidea n. s; and

(3) the small sclerotial neck rot caused by Botrytis squamosa n. s.

He found that of the three diseases the mycelial neck rot caused the greatest losses. The small sclerotic neck rot is found so far only on the white varieties and is confined more particularly to the dry outer scales of the bulbs. It is therefore the least destructive of the three diseases.

The fungus Macrosporium porri Ellis was first reported in the United States by Cooke and Ellis (1829) on leek (Allium porrum . L). Nolla (1927) described Alternaria allii on onions. Angell (1929) found that M. porri could cause bulb rot as well as purple foliage lesions. Teodoro (1923) attributed brown blotch infection on onions to Stemphylium botryosum Wallroth. He found that the fungus was a pathogen on onion foliage. However, Machacek (1929) found that the fungus could cause bulb rot. Similarly Young (1926) observed bulb membrane infection with M. parasiticum obtained from onion leaves.

Plant pathogens are known to get into their hosts through wounds and natural openings. Curtis, (1928)

(cited by Wood, 1967) observed that the mode of entry into plants by Sclerotinia cinerea which caused rot of stone fruit depended on the host species. It entered plums through stomata, apricots through stomata or the cuticle, peaches through hair sockets and cherries and nectarines directly through the cuticle. Stomata are the most important or only pathway of entry for many parasitic bacteria. Among those which enter in this way are Pseudomonas tabaci in tobacco; Erwinia amylovora in apple and pears; Xanthomonas phaseoli in Phaseolus spp and Xanthomonas malvacearum in cotton (Wood, 1967). Erwinia carotovora, a universal soft rot bacterium, as a rule requires a wound to enter a fleshy organ. Smith and Ramsey (1947) showed that potato tubers can be infected through their lenticels under wet conditions. Wounds found on plants can result from harvesting or post-harvest operations, from other organisms like nematodes, fungi, or insects; while some are created naturally. Natural wounds are surfaces exposed by abscission of leaves, floral and other parts of the leaves. In leaves, the abscission zone is the petiole. Other natural wounds are those which occur on emergence of laterals from main roots (Wood, 1967). This is the means of entry for the tobacco root rot fungus Thielaviopsis basicola (Conant, 1927 (cited by Wood, 1967)). Similarly, infection of wheat seedlings

by Cephalosporium gramineum , enters through wounds which result from breaking of the pericarp and coleorhiza as the root emerges on germination (Otieno, 1962 (cited by Wood, 1967)).

Plants usually contain some food substances that enable them to support growth of microbes. It is known that many storage tubers store starch as the predominant food material. It is usually an insoluble carbohydrate in form of minute grains. It is synthesized from glucose under the action of at least two enzymes - phosphorylase and Q - enzyme; while amylase is the chief enzyme responsible for its hydrolysis, converting it, first, to an intermediate product - maltose, which is finally hydrolysed to glucose by maltase. Ogundana (1969) found that the reserve carbohydrates found in freshly harvested yam tubers (except D. rotundata) were sucrose, maltose, glucose and fructose; while in D. rotundata, sucrose was the principal sugar.

Sucrose, a disaccharide, is the most transportable sugar in plants except - sugar cane (Saccharum officinarum Linn) where it is stored as food reserve. It is known that sucrose is stored as food reserve in carrot (Ricardo and Ap Rees, 1970). Its hydrolysis by the enzyme invertase gives rise to two important substrates for glycolysis - glucose and fructose (Turner, 1938).

Cother, Derbyshire and Brewer (1976) found glucose, fructose and sucrose as the only soluble sugars present in onions (Allium cepa).

The average water content of vegetables is about 88% with an average carbohydrate content of 8.6%, proteins 1.9%, fat 0.3% and ash 0.84%. Thus, vegetables contain enough nutrient to support the growth of molds, yeasts and bacteria and are consequently spoilt by most of these organisms. The high water content of vegetables favours growth of spoilage bacteria and the relatively low carbohydrate and fat contents suggest that much of this water is in available form (Jay, 1970).

During spoilage, organisms cause a series of biochemical and physiological changes which have a detrimental effect on the host. Jay (1970) claimed that the causative organisms responsible for bacterial soft rot in plants are able to break down pectin, giving rise to a soft, mushy, water-soaked appearance. The pectinase produced by these organisms is usually a protopectinase since the cementing substance of plants is a protopectin. He further observed that in acid medium, the micro-organisms tend to decarboxylate amino acids leaving amines which raise the pH towards neutrality. Complex cabohydrates such as cellulose are usually the last to be degraded. A limited number of micro-organisms can obtain their energy from fats

but do so only if a more readily useable energy source, such as sugar, is absent. First, the fat must be hydrolysed with the aid of lipase to glycerol and fatty acid which then can serve as an energy source for hydrolysing organism via β - oxidation processes.

One other characteristic feature of many phytopathogenic organisms is their ability to produce an array of enzymes capable of degrading the complex polysaccharide of plant cellwall and membrane constituents (Bateman and Millar, 1966; Wood, 1967; Albersheim, Neukom and Deuel, 1960; Wood, 1973; Porter, 1966; Tseng and Bateman, 1968). These enzymes are usually produced inductively and are extracellular, highly stable and are present in infected host tissue.

In most plant diseases, the pathogen penetrates or degrades the cellwalls of host tissue at some stage. How this is done is one of the most important problems in pathogenesis. Although, in some cases, penetration may be wholly mechanical; in others, it may be associated with some degradation of cellwall. This accompanying degradation may be caused by the action of enzymes secreted by the pathogen. Cellwalls of undifferentiated tissue have a complex structure and contain a wide variety of substances. Of the many enzymes which degrade the cellwalls, only those which degrade pectic substances and cellulose have been studied appreciably (Wood, 1967).

Studies by De Bary (1866) led to the concept that pectic enzymes may be involved in pathological manifestations induced in plant tissues by biotic agents. He demonstrated that a cellwall dissolving principle was contained in a fungus extract. He went further to show that the juice extracted from rotted carrot roots infected with Sclerotinia libertiana contained a thermolabile principle capable of dissolving cellwalls of plants. This was substantiated by the researches of Jones (1905, 1909, 1910). He described a pectinase produced by Bacillus carotovorous (Erwinia carotovora) capable of dissolving the middle lamella of plant cells and hence concluded that it was responsible for the soft rot of vegetables. Brown (1915) also demonstrated that treatment of young hyphae from Botrytis cinerea led to a loss of coherence between cells leading to collapse of cellwalls and even death of the protoplast. These findings have led to the fact that, today, pectic enzymes are now implicated routinely as a feature of host-pathogen interaction.

The involvement of pectic enzymes in the degradation of pectic constituents of cellwalls and of the middle lamella in plant tissues has been reported for such diverse types of diseases as soft rots, dry rots, wilts, blights and leafspots and for diverse pathogenic agents as fungi, bacteria and nematodes. Even virus-infected

tissues have been reported to undergo alterations in their pectic constituents (Bateman and Millar, 1966).

Pectic enzymes are classified according to three major criteria:

(a) the mechanism by which the α- 1, 4 glycosidic bond is split (i.e. hydrolytic or trans-eliminative cleavage). Albersheim, Neukom and Deuel (1960) found that the α- 1, 4 glycosidic bonds in pectin can be split by a trans-elimination of the proton from C_5 of the methyl galacturonate moiety to yield an unsaturated bond between C_4 and C_5 of the reaction product. The enzyme is a transeliminase or lyase. This enzyme was different from the hydrolytic polygalacturonases which were hitherto believed to be the only chain splitting enzymes. The products of hydrolytic and transeliminative reactions are distinguishable by their reaction with thiobarbituric acid (TBA) (Neukom, 1960; Ayers, Papavizas and Diem, 1966);

(b) enzyme's "preference" for a substrate (i.e. preference for pectin or pectic acid). Those that exhibit a distinct specificity for pectin rather than pectic

acid as substrate are called pectin methyl galacturonases. They show a high specificity towards methyl ester group of pectinic acid, saponifying this to yield methyl alcohol and pectinic acid of lower methoxyl content. Those that show preference for pectic acids are called polygalacturonases. They rupture the uronide chains hydrolytically at the glycosidic linkages to give shorter chains and, at the same time, they liberate reducing groups;

(c) the position in the pectic chain at which cleavage occurs (i.e. random or terminal point of attack). Pectic enzymes that attack their substrate in a terminal manner, splitting off only terminal monomeric products (galacturonic acid residues) are called exo-enzymes whereas the endo-enzymes attack randomly along the chain to release oligomers (Demain and Phaff, 1957).

Many plant pathogens are capable of producing pectic enzymes. In most instances, the enzymes are produced inductively rather than constitutively. Pectic substances are the main inducers of the enzymes

in culture media [redacted] (Fergus and Wharton, 1957; Singh and Wood, 1956). Kamal and Wood (1956) however showed that Verticillium dahliae produced pectic enzymes in the absence of pectic substances. The type of induced pectinase activity may be influenced by environmental condition. Damle (1952) clearly demonstrated that Botrytis cinerea is capable of producing different α- 1, 4 pectic degrading enzymes in vitro depending on the pH of the culture medium. The pectic enzymes produced on an acid medium had an optimum pH of 5.6 while the enzymes produced on an alkaline medium had an optimum pH 8.0.

Kaji (1958), Garibaldi and Bateman (1971) found that the abilities of enzymes to degrade model substrates are not necessarily indicative of their ability to degrade plant cellwall. Therefore the extent to which pectinases are responsible for tissue degradation in infected plants should be determined by a comparison of the amounts of pectic materials present in both diseased and healthy tissues. Some soft rot organisms produce pectinesterase in culture but others do not do so readily (Gaumann and Bohn, 1947; Echandi and Walker, 1957; Ceponis and Friedman, 1959; Echandi, Van Gundy and Walker, 1957). This does not imply that the enzyme does not participate in pathogenesis since most tissues of higher plants contain large amounts of pectinesterase (Wood, 1967).

Dimond and Waggoner (1953) described the conditions that must be met before specific toxins produced by a pathogen *in vivo* can be assumed to be responsible for symptoms of disease produced by the pathogen in a particular host. The conditions are

(a) reproducible separation from the sick plant;

(b) purification; and

(c) reproduction of at least a portion of the disease syndrome when the toxin is placed in a healthy plant.

Their arguments also apply to pectic enzymes. The most satisfactory proof that pectic enzymes play a part in the development of a disease would be by demonstrating that they are present and active in the plant tissue. Evidence of pectinase activity can sometimes be obtained microscopically because, in some diseases, the cells of invaded tissue separate along the line of the middle lamella. The middle lamella and other parts of the cellwall thereby lose their staining ability with reagents which colour pectic substances (Wood, 1967).

The pectic enzymes of plant pathogens are best known in relation to diseases in which a relatively large mass of parenchyma is rapidly invaded to give a mass of water-soaked tissue, generally soft in texture and with

little coherence (Wood, 1967). Cole and Wood (1961) investigated the effects of two soft rot pathogens, B. cinerea and Penicillium expansum, and two firm rot pathogens, S. fructigena and Pyrenochaeta furfuracea, on pectic substances in apple fruit. Analysis of rotted tissue revealed that each of the fungi caused some degradation of pectic substances but the greatest loss occurred in tissue infected with the soft rot pathogens which destroyed up to 48% of the pectic substances.

In many plant diseases, particularly the soft rot, tissue maceration is a characteristic symptom. This process involves the separation of cells from each other within a tissue system. The pectic enzymes that split the $\alpha - 1_4$ 4 bonds between the galacturonic acid moieties in the pectic fraction of the cellwall remain the only enzymes confirmed to cause plant tissue maceration. It has, however, been found that not all pectic enzymes have the ability to macerate plant tissue. A polygalacturonase has been reported from Clostridium felsineum which failed to macerate tissue (Kaji, 1958).

Pectic enzymes that cause maceration of plant tissue have been shown to cause injury and death of unplasmolysed plant cells (Brown, 1915; Tribe, 1955; Spalding 1969; Mount, Bateman and Basham, 1970; Wood, 1972). In some infections, degradation of host cellwalls and cellular

injury are associated with permeability increases detectable in early stages of pathogenesis (Friedman and Jaffa, 1960; Fox [illegible], Manners and Myers, 1972; Byrde et al, 1973). Polygalacturonate lyase from E. chrysanthemi purified to homogeneity or polygalacturonase from V. albo-atrum cause simultaneous maceration and death of cells in disks of potato tissue (Basham, 1974; Mussell, 1973). Injury of tissue treated with pectic enzymes is characterized by a rapid irreversible increase in permeability of affected cells. This appears to result from damage to plasmalemma (Tribe, 1955; Wood, 1972; Hall and Wood, 1973).

Although pectic enzymes are well known for their role in plant pathogenesis, they often meet with some resistance from some plants. Several mechanisms have been proposed to account for induced changes in resistance of plant tissues to degradation by pectic enzymes. These include

(a) induced changes in the pectic substances of the host;

(b) masking of pectic substances by non-pectic polymeric substances;

(c) pectic enzymes inactivation by phenolic or tannin constituents of the host; and

(d) repression of enzyme synthesis by sugars (Bateman and Miller, 1966).

There is evidence that the oxidation of phenols in certain varieties of apples produces high molecular weight, tannin-like substances which inactivate the enzymes responsible for maceration and so limit or prevent infection (Byrde , 1957). It had also been demonstrated that the products formed by oxidation of the leuco-anthocyanins of apples in some types of rot prevent the degradation of pectic substances which occur in other rots where, for unknown reasons, their oxidation is prevented (Cole, 1958).

From the effect of microbes and their mode of action on plants, it is apparent that plant produce would have the problem of spoilage in storage. The development of control measures for bacterial diseases of plants has not been as satisfactory as those for fungal diseases (Buckholder, 1948). Brown and Boyle (1945), however, found that Penicillin suppresses the growth of Agrobacterium tumefaciens and Erwinia carnegienna, the sensitivity being similar to that found with Staphylococcus aureus.

Jones and Mann (1963) suggested that before onion bulbs can be stored, it is necessary to dry the foliage especially in the neck region, as this shrivels on drying to form a seal which prevents ingress of pathogens. Robinson , Browne and Burton , (1975)

also suggested that commodities resistant to evaporation such as the onion may be stored at lower humidities, thus reducing the microbial hazards.

There is little information available about the nature and possible control measures against the extensive post-harvest deterioration of onions in Nigeria. The present study is therefore undertaken with the following objectives:-

(1) To isolate and identify the pathogens responsible for onion rot;

(2) To establish the relationship between the pathogens and the host;

(3) To study the effect of rot on nutritional value of the onion;

(4) To investigate the physiology of the pathogens;

(5) To investigate the mode of action of the pathogens; and

(6) To find adequate control measures against the disease.

MATERIALS AND METHODS

Collection of Samples:

The purple variety of onions (Allium cepa . L) was used for the study. The bulbs were obtained from local markets in Lagos area.

Incubation: The experiments were incubated at room temperature 29 ± 2°C unless otherwise stated.

Sterilization: Before the onions were used, the dry outer scales were peeled off and the bulbs surface-sterilized by cleaning with 70%(v/v) ethyl alcohol solution.

All glasswares were washed with detergent in tap water, rinsed in several changes of distilled water, dried and sterilized in the oven at 170°C for 4h

Hockey sticks were sterilized by dipping in 70%(v/v) ethanol and flamed over a bunsen flame. Inoculation loops were flamed till red hot and cooled. Scapels, needles and pairs of scissors were sterilized by first dipping in 70% (v/v) ethanol followed by flaming.

The commercial pectin used in the enzyme studies was added aseptically to the mineral solution already sterilized at 121°C for 10 minutes and then treated at 112°C for 10 minutes. This was to help retain the molecular identity of the pectic substances.

All media were sterilized by autoclaving at 121°C for 15 minutes, unless otherwise stated.

Media:

Solid media:

1. Motility medium - Glucose 2g.
Peptone 3g.
Yeast extract 2g.
Agar 5g.

The contents were steam dissolved in 1 litre of distilled water followed by sterilization at 115°C for 15 minutes.

2. Baird - Parker modification of Hugh and Leifson medium for Staphylococci and Micrococci

Tryptone	10g.
Yeast extract	1g.
Dextrose	10g.
Agar	2g.
Distilled water	1000ml

The mixture was steam dissolved and the pH adjusted to 7.2. Twenty millilitre of 0.2% (W/V) bromocresol purple were added and the mixture sterilized at 115°C for 10 minutes.

3. Hugh and Leifson medium:-

Peptone	2g.
Sodium chloride	5g.
Dipotassium hydrogen phosphate	0.3g.
Agar	3g.
Distilled water	1000ml

Mixture was heat dissolved and p^H adjusted to 7.1. Fifteen millilitre of 0.2% bromothymol blue were added together with sterilized dextrose to give a final 1% ($^W/_V$) concentration.

Nutrient agar:-

Peptone	5g.
Agar	15g.
Distilled water	1000ml

Mixture was heat dissolved and sterilized at 115°C for 15 minutes.

Gelatin Agar:-

Beef extract	3g.
Peptone	5g.
Agar	15g.
Distilled water	1000ml

Mixture was heat dissolved, and sterilized at 115°C for 15 minutes.

Blood agar:-

Blood agar base	40g.
Distilled water	1000ml

Mixture was sterilized, cooled to 45°C. Five per cent defibrinated blood ($^V/_V$) was added, mixed thoroughly and 20ml aliquots poured into sterile Petri-dishes.

Staphylococcus medium:

Tryptone	10g.
Yeast extract	2.5g.
Gelatin	30g.
Lactose	2g.
D - Mannitol	10g.
NaCl	7.5g.
Dipotassium phosphate	5g.
Agar	15g.
Distilled water	1000ml

Mixture was heat dissolved and sterilized at 121°C for 15 minutes.

Simmon citrate agar:-

Magnesium sulphate	0.2g.
Ammonium dihydrogen phosphate	1g.
Dipotassium phosphate	1g.
Sodium citrate	2g.
Sodium chloride	5g.
Bacto-Agar	15g.
Bacto-Bromothymol blue	0.08g.
Distilled water	1000ml

Mixture was heat dissolved and sterilized at 121°C for 15 minutes.

MacConkey agar:-

Peptone	17g.
Proteose peptone	3g.
Lactose	10g.
Bile salt	1.5g.
NaCl	5g.
Agar	13.5g.
Neutral red	0.03g.
Crystal violet	0.001g.
Distilled water	1000ml

Mixture was heat dissolved and autoclaved at 121°C for 15 minutes.

Deoxycholate citrate agar:-

Bacto - Peptone	10g.
Bacto - Lactose	10g.
Sodium deoxycholate	1g.
Sodium chloride	5g.
Dipotassium phosphate	2g.
Ferric citrate	1g.
Sodium citrate	1g.
Bacto-Agar	15g.
Distilled water	1000 ml
Bacto - Neutral red	0.03g.

Mixture was heated to boiling to dissolve.

Potato Dextrose agar:

Potatoes	200g.
Bacto - Dextrose	20g.
Bacto-Agar	15g.
Distilled water	1000ml

Mixture heated to dissolve and then autoclaved for 15 minutes at 121°C.

Yeast extract agar:

Yeast extract	3g.
Peptone	5g.
Agar	15g.
Distilled water	1000ml

Mixture was heated to dissolve and then autoclaved at 121°C for 15 minutes.

Levine EMB agar:

Bacto - Peptone	10g.
Bacto - Lactose	10g.
Dipotassium phosphate	2g.
Bacto - Agar	15g.
Bacto - Eosin Y	0.4g.
Bacto - Methylene blue	0.065g.
Distilled water	1000ml

Mixture was heated to dissolve and autoclaved at 121°C for 15 minutes.

Violet red bile agar:

Bacto - Yeast extract	3g.
Bacto - Peptone	7g.
Bacto - Bile salts No. 3	1.5g.
Bacto - Lactose	10g.
Sodium chloride	5g.
Bacto - Agar	15g.
Bacto - Neutral red	0.03g.
Bacto - Crystal violet	0.002g.
Distilled water	1000ml

Mixture was heated to boiling to dissolve the medium completely. It was not autoclaved.

Corn meal agar:

Corn meal extract	2g.
Agar	15g.
Distilled water	1000ml

Mixture was heated to dissolve and then autoclaved for 15 minutes at 121°C.

Brilliant green agar:

Bacto - Oxgall	20g.
Bacto - Peptone	10g.
Bacto - Lactose	10g.
Bacto - Brilliant green	0.0133g.
Distilled water	1000ml

Mixture heated to dissolve and then autoclaved at 121°C for 15 minutes..

Malt extract agar:

Maltose, Technical	12.75g.
Dextrin, Difco	2.75g.
Glycerol	2.35g.
Bacto - Peptone	0.78g.
Bacto - Agar	15g.
Distilled water	1000ml

Mixture was heated to dissolve and then autoclaved at 121°C for 15 minutes.

Onion extract agar:

Onions	200g.
Agar	30g.
Distilled water	1000ml

Fresh onions were diced and blended with distilled water in an electric blender. It was filtered by means of a vacuum filter pump. Agar was added to the filtrate and autoclaved for 10 minutes at 115°C. The pH of the medium was 6.3.

Tryptic Soy agar:

Bacto - Tryptone	15g.
Bacto - Soytone	5g.
Sodium chloride	5g.
Bacto-Agar	15g.
Distilled water	1000ml

Mixture was heated to dissolve and autoclaved for 15 minutes at 121°C.

Endo Agar:

Bacto - Peptone	10g.
Bacto - Lactose	10g.
Dipotassium phosphate	3.5g.
Bacto-Agar	15g.
Bacto - Basic Fuchsin	0.5g.
Sodium sulfite	2.5g.
Distilled water	1000ml

Mixture was heated to dissolve and autoclaved for 15 minutes at 121°C.

Czapek solution agar:

Saccharose - Difco	30g.
Sodium nitrate	2g.
Dipotassium phosphate	1g.
Magnesium sulphate	0.3g.
Potassium chloride	0.5g.
Ferrous sulphate	0.01g.
Bacto - Agar	15g.
Distilled water	1000ml

Mixture was heated to dissolve and then autoclaved at 121°C for 15 minutes.

Bismuth Sulfite agar:

Bacto-Beef extract	5g.
Bacto - peptone	10g.
Bacto - Dextrose	5g.
Disodium phosphate	4g.
Ferrous sulphate	0.3g.

Bismuth sulfite indicator	8g.
Bacto - Agar	20g.
Bacto - Brilliant green	0.025g.

Mixture was heated to dissolve for a few minutes in 1litre of distilled water.

Liquid media:

1. Nitrate broth:-

KNO_3	0.2mg.
Tryptone	10g.
NaCl	5g.
Distilled water	1000ml

Mixture was added into 5 ml tubes and sterilized at 115°C for 15 minutes.

2. Nutrient broth:-

Beef extract	3g.
Peptone	5g.
Distilled water	1000ml

Mixture was heat-dissolved and sterilized at 115°C for 15 minutes.

3. Peptone water:-

Tryptone	10g.
NaCl	5g.
Distilled water	1000ml

Mixture was steam dissolved and the pH adjusted to 7.2 at room temperature. It was then autoclaved at 115°C for 15 minutes.

4. Onion broth:-

Onions	20g.
Distilled water	1000ml

Onions were diced and blended in a waring blender containing distilled water. The mixture was filtered by means of a vacuum filter pump and the filtrate sterilized at 115°C for 10 minutes.

5. MR-VP medium:-

Peptone	5g.
Phosphate buffer	5g.
Dextrose	5g.

Mixture was sterilized at 121°C for 15 minutes.

6. Medium for sugars:-

Peptone	1g.
NaCl	2.5g.
Distilled water	500ml

The base was adjusted to pH 7:2 and sterilized at 121°C for 15 minutes. Five millilitre Durham tubes were put in MacConkey bottles. 10% sugar was added to the peptone base in the proportion of 10ml sugar to 100ml base. Mixture was poured in the bottles and sterilized at 110°C for 10 minutes after addition of a few drops of Andrade indicator.

7. Basal medium:-

$(NH_4)_2SO_4$	2.0g.
$MgSO_4 \cdot 7H_2O$	0.2g.
NaCl	5.0g.
$Ca(NO_3)_2 \cdot 2H_2O$	0.2g.
$FeSO_4$	0.01g.
K_2HPO_4	5.5g.
KH_2PO_4	0.5g.
Distilled water	1000ml

The mixture was steam dissolved and then sterilized for 15 minutes at 121°C.

Reagents:

1. Gram Stain:-

(a) Gram's crystal violet

Solution A -	Crystal violet (90% ($^{W}/_{v}$) dry wt content)	= 2g.
	Ethyl alcohol	= 20ml
Solution B -	Ammonium oxalate	0.8g.
	Distilled water	80ml

Solution A & B were mixed.

(b) Gram's iodine

Iodine	1g.
Potassium iodide	2g.
Distilled water	300ml

(c) 95% ($^{V}/_{v}$) ethyl alcohol (decolourizer)

(d) Safranin (counter stain)

Safranin (2.5% ($^{W}/_{v}$) in 95% ($^{V}/_{v}$)	
ethyl alcohol	= 10ml
Distilled water	=100ml

2. Methylene blue:-

Methylene blue (80% dye content)	0.3g.
Ethyl alcohol (95% ($^{V}/_{v}$)	30ml
Distilled water	100ml

One-third gramme methylene blue were dissolved in 30 ml 95% ($^{V}/_{v}$) ethyl alcohol in 100 ml distilled water.

3. Kovac's reagent:-

Five grammes of p - dimethyl Aminobenzaldehyde were dissolved in a mixture of 75 ml Amyl alcohol and 25 ml concentrated sulphuric acid.

4. Methyl red solution:

One-tenth gramme methyl red was dissolved in 300ml ethanol. The content was made up to 500ml with distilled water.

5. Molisch's reagent:-

Concentrated Sulphuric acid

α - Naphthol (5% ($^{W}/_{V}$) in ethanol was prepared fresh).

6. Fehling's solution A:

Thirty-five grammes of $CuSO_4 7H_2O$ were dissolved in distilled water and made up to 500ml.

Fehling's solution B:

One hundred and twenty grammes of potassium hydroxide and 173g of sodium potassium tartarate (Rochelle salt) were dissolved in distilled water and made up to 500ml.

7. Benedict's reagent:

One hundred and seventy-three grammes of sodium carbonate were dissolved in 800ml warm water. It was filtered through a fluted filter paper into a 1000ml measuring cylinder

and made up to 850ml with water. Also 17.3g of copper sulphate was dissolved in 100ml water and made up to 150ml. The first solution was poured into a 2-litre beaker and to it was slowly added the copper sulphate solution while stirring.

8. Barfoed's reagent:-

Thirteen and one third grammes of copper acetate were dissolved in 200ml of water and to that was added 1.8ml of glacial acetic acid.

9. Seliwanoff's reagent contained 0.05% ($^{W}/v$) resorcinol in 3 N HCl.

10. Iodine solution contained 0.005N iodine in 3% ($^{W}/v$) potassium iodide.

11. Anthrone reagent contained 0.2% ($^{W}/v$) anthrone in concentrated sulphuric acid.

12. Millon's reagent contained 15% ($^{W}/v$) of mercuric sulphate in 15% ($^{V}/v$) sulphuric acid.

13. Ninhydrin reagent:-

Four-fifth gramme of ninhydrin and 0.12g hydrindantin were dissolved in 30ml methyl cellosolve. To this was added 10ml acetate buffer, it was prepared fresh and stored in a brown bottle.

14. Buiret reagent:

Three grammes of copper sulphate ($CuSO_4.7H_2O$) and 9g sodium potassium tartarate were dissolved in 500ml of 0.2N sodium hydroxide. five grammes potassium iodide was added and made up to 1 litre with 0.2N sodium hydroxide.

15. Folin - Denis reagent:

To 750ml water (in a 2 litre round bottom flask) was added 100g sodium tungstate ($Na_2 WO_2 2H_2O$), 20g phosphomolybdic acid and 50ml O-phosphoric acid. The content was refluxed for 2h. After cooling, the content was poured into a one-litre standard flask and made up to the mark with distilled water.

16. Sodium carbonate (saturated solution)

To each 100ml of water was added 35g anhydrous sodium carbonate in a 200ml beaker. (The water was warmed to about 80^oC before addition of Na_2C0_3). Content was allowed to cool overnight. On the next day, the solution was seeded with crystals of anhydrous sodium carbonate until it became saturated. The content was filtered into another conical flask with glass wool.

17. Nessler's reagent: Thirty five grammes potassium iodide was dissolved in 100ml of water and 4% ($^{W}/v$) mercuric chloride added while stirring or shaking until a slight red precipitate remained (about 325ml required). A solution of 120g sodium hydroxide in 250ml water was introduced while stirring and made up to 1 litre. A little more mercuric chloride was added until there was a permanent turbidity. The mixture was allowed to stand for 24h and then the sediment decanted. The solution was kept in a dark coloured bottle.

Isolation of pathogens:

The dry outer scales of the diseased onions were removed, the bulbs were surface sterilized by cleaning with 70% ($^{V}/v$) ethyl alcohol. By means of a sterile scapel, slices (5 x 15mm) of the diseased tissue from the advancing edge of lesion were cut (Bradbury, 1970). They were washed in 0.1% ($^{W}/v$) solution of mercuric chloride and in several changes of sterile distilled water for fungal isolation (Pathak, 1974). Three slices were placed on potato dextrose agar (PDA) in Petri-dishes and incubated at room temperature for 48 hours (Clark and Lorbeer, 1973).

For Bacterial isolation, the slices were washed in several changes of distilled water and then crushed in 10ml sterile distilled water in a clean sterile Petri-dish. Three loopfuls of the suspension were streaked on dry onion agar plates using a sterile inoculating loop. This was done by gently streaking the needle on the agar surface in a zig-zag manner. The plates were incubated at room temperature for 48h (Bradbury, 1970). Some developing colonies were selected and transferred to fresh onion agar plates.

Purification and preparation of stock cultures

From a 24h-old culture was taken a loopful of the cell suspension by means of a sterile inoculating loop and suspended in 10ml sterile distilled water. A loopful of this suspension was streaked on dry onion agar plates and incubated for 48h. Developing colonies resembling the parent culture (Bradbury, 1970) were transferred to onion agar slants and covered with sterile paraffin. Some isolates were also freeze-dried and used as stock cultures.

Establishment of pathogenicity of the isolates

To ascertain that the isolated micro-organisms were responsible for infection, the isolates were used to infect healthy purple variety onion bulbs. The dry outer scales of the bulbs were removed before use. The bulbs were surface-sterilized by cleaning with 70% ($^{v}/v$) ethanol solution. The glass jars were sterilized by wiping with 70% ($^{v}/v$) alcohol.

Several loopfuls from a 24h-old culture were suspended in 10ml sterile distilled water till the solution was turbid. The optical density of the suspension (0.26) was taken using the 620nm filter with the Photo-electric colorimeter, moded AE-11 obtained from Erma Optical Works Ltd., Japan. Serial dilutions 10^{-1} to 10^{-8}, were made from the original suspension. One-tenth millilitre aliquots from 10^{-6}, 10^{-7} and 10^{-8} dilution tubes were plated in duplicates using the spreading plate method. This made it possible for the number of organisms in the tubes to be calculated. The plates were incubated for 48h at room temperature and the number of organisms counted.

With the aid of a sterile cork borer, three equally spaced holes of 0.5cm diameter were made on the bulbs. Bacterial suspension of 0.05ml portions from tubes containing original suspension, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions were inoculated into the marked areas.

Five onion bulbs were used for each dilution and placed in sterile glass jars. Four bulbs inoculated with 0.05ml sterile distilled water served as controls. All the bulbs were incubated at room temperature for one week. Development of rot was assessed as diameter of rotted tissue. Also the number of marked areas with 0.6cm diameter of rotted tissue were counted and calculated as percentages of the total number of areas inoculated. This was done for each dilution. Reading of the averages of diameter of rotted tissue were taken everyday for each dilution.

Pathogenicity tests were carried out on the white varieties of onions, garlic and shallots (a variety from Ghana).

Reisolation of the pathogens:

Small portions of the diseased tissues were cut and washed in two changes of sterile distilled water. They were crushed in 10ml

sterile distilled water and a loopful of the suspension streaked on potato dextrose agar plates and onion extract agar in duplicates and incubated at room temperature for 48h . The developing colonies were compared with those of the original isolates. This was done in conformity with Koch's postulates which stipulated that

(1) the suspected organism must be constantly present in the infected tissue and must be absent in healthy individual ;

(2) the isolated organism must be in pure cultures;

(3) the same disease must be reproducable in the host on re-inoculation into healthy individuals;

(4) the same organism must be re-isolated from the artificially infected hosts.

Mode of entry of pathogen into the host tissue

To determine the mode of entry of pathogen into the host tissue, six unwounded onion bulbs were surface sterilized. They were inoculated by rubbing the suspension of the organism on the fleshy leaves with a moist cotton wool.

The suspension was made by suspending loopfuls of the organisms from a 24h-old culture in about 10ml sterile distilled water. To a second batch of six bulbs, shallow cuts were made with a sterile cork borer and inoculated with the suspension of the organisms. Inoculated bulbs were placed in sterile glass jars and incubated at room temperature for two weeks. Two wounded and unwounded bulbs inoculated with sterile water served as controls.

Identification of the pathogens:

Each pathogen was identified to the generic level by means of the schemes of Shewan, Hobbs and Hodgkiss (1960), Skerman (1960), Sorokin and Cullimore (1969) and Bergey's Manual of Determinative Bacteriology, VIIIth edition (1974). The following characteristics were used to separate the isolates into the appropriate groups.

(a) Cultural characteristics: The shape and size of colony on nutrient agar, pigment production and optical properties (opague, transparent or translucent).

(b) Morphology of the cells.

(c) Motility.

(d) Biochemical tests: The following tests were made on the bacterial pathogens:

(1) Gram stain: Each pathogen was stained according to the Gram's stain technique (Collins and Lyne, 1976) and examined by means of the photo-microscope using an oil immersion objective. The reaction of each organism to stain, the form of the cells and cell grouping, were determined.

(2) A 24h-old Yeast culture was stained with methylene blue.

(3) Proteolytic activity of the pathogen: Nutrient gelatin was inoculated with a loopful of the organism and incubated at room temperature for 7 days. An uninoculated tube served as the control. After incubation all the tubes were placed in the refrigerator overnight. The inability of gelatin to solidify showed a positive result.

((4) Utilization of sugars as sources of carbon:

Peptone water containing either glucose, mannitol, sucrose or

lactose was inoculated with a loopful of each pathogen. Acid production was indicated by change in colour of the indicator contained in the liquid medium and gas production was observed in the inverted Durham tubes.

(5) Hugh and Leifson test (Oxidative/ Fermentative test)

Two tubes of the medium were heated for ten minutes in boiling water to expel the dissolved oxygen content, the tubes were cooled and inoculated with each of the pathogens. One tube was incubated aerobically and the other anaerobically by sealing the surface of the medium with 2ml of sterile vaseline to obtain anaerobic condition. Positive oxidative test was indicated by acid production in aerobic tube only, while positive fermentative test was indicated by acid production in anaerobic tubes. Acid production was indicated by a change in the colour of the medium from green to yellow.

(6) Staphylococci and Micrococci forms:

Baird - Parker modification of Hugh and Leifson's medium was used to

differentiate between Staphylococci and Micrococci. The method used was the same as for the Hugh and Leifson's test.

(7) Hydrogen Sulphide production: Tubes of nutrient broth were inoculated. A strip of paper impregnated with lead acetate solution was placed at the top of the tubes and held in place by a cotton plug. They were incubated at room temperature and examined for blackening of the paper.

(8) Indole production: A few drops of Kovac's reagent (prepared by dissolving 5g p-dimethyl aminobenzaldehyde in a mixture of 75ml of concentrated sulphuric acid) were added to the culture broth. A rose pink colouration indicated indole production.

(9) Methyl red test: MR -VP broth (Glucose - phosphate medium) was inoculated with each pathogen and incubated for 5 days at 30°C. A few drops of methyl - red solution (prepared by dissolving 0.1g of the dye in 300ml of ethanol and made up

to 500ml with distilled water) were added to the inoculated broth. Positive test was indicated by the red colour development.

(10) Voges - Proskaeur test (Baritt's method):

MR - VP medium (glucose - phosphate medium) was inoculated with each pathogen and incubated for 5 days at 30°C. Three millilitre of 5% ($^{V}/v$) alcoholic α- naphthol solution and 3ml of 40% ($^{W}/v$) potassium hydroxide solution were added to the incubated broth. Positive test was indicated by the appearance of a red colouration as the reagent oxidised acetyl methyl carbinol (acetoin) from dextrose to diacetyl.

(11) Citrate utilization: Solid Simmon's citrate agar was inoculated with each isolate using a straight sterile wire and incubated at 30°C for 3 days. Utilization of citrate was indicated by the medium changing from green to deep blue colour.

(12) Oxidase test (Kovac's, 1956):
Fresh cultures of each pathogen were rubbed on filter paper soaked with 1% (W/v) tetramethyl - p - phenylene diamine dichloride by means of a clean platinum loop. Development of a purple colour within 10 seconds was taken as positive.

(13) Catalase test: Onto the suspension of each pathogen on a slide, few drops of a mixture of equal volume of hydrogen peroxide (20 volumes) and 10% Tween (V/v) 80 in water were added. A positive test was indicated by production of gas bubbles.

(14) Nitratase test: To an 18h old culture of the isolates in peptone water containing 0.8% (W/v) potassium nitrate, 0.5ml of 0.8% (W/v) sulphanilic acid in 5/N acetic acid was added, the contents mixed and 0.5ml of 0.5% (W/v) naphthylamine in 5/N acetic acid was added. The contents of the tubes were shaken and the result read after two minutes. Pink colour development indicated positive result while no colour change, a negative result.

(15) Coagulase test: The slide coagulase test - One or two colonies of the organism was emulsified in a drop of water on a slide. A straight wire was dipped into human plasma and this used to stir the bacterial suspension. Formation of clots showed a positive result.

(16) Haemolysis on blood agar: The blood agar was inoculated and incubated at 37°C. Growth and discolouration of the medium indicated occurrence of haemolysis.

(17) Ammonia test: Culture was incubated in nutrient broth for 5 days. A small piece of filter paper was dipped in Nessler's reagent and placed on top of the culture tube. The tube was heated to 50-60°C. The filter paper turned brown or black if ammonia was present.

Analysis of food contents of both fresh and spoilt onions

1. Qualitative tests for carbohydrate:

   (a) Molisch's test: To 2ml onion extract was added two drops of Molisch's reagent. This was mixed and 2ml of concentrated sulphuric acid poured slowly down the side of the tube. The presence of a purple ring at the interphase showed the presence of a carbohydrate.

   (b) Starch test: To 2ml onion extract was added a drop of iodine and mixed. The appearance of a blue black colour showed the presence of starch.

   (c) Benedict's test: To 1ml onion extract was added 5ml of Benedict's reagent. It was thoroughly mixed and then boiled for 12 minutes. The appearance of a brick red precipitate showed the presence of reducing sugars..

   (d) Fehling's test:

      (i) To 1ml onion extract was added equal amounts of Fehling's A and B. The mixture was heated. The appearance of a brick red precipitate showed the presence of reducing sugars.

(ii) The precipitate was filtered. To the filtrate was added dilute hydrochloric acid, followed by sodium hydroxide and the Fehling's test carried out. The appearance of a brick red precipitate showed the presence of a non-reducing disaccharide which was hydrolysed to reducing sugars (monosaccharides).

(e) Barfoed's test: To 1ml onion extract was added 5ml of Barfoed's reagent. It was mixed and boiled for 10 minutes. The appearance of a red precipitate showed the presence of reducing sugars.

(f) Seliwanoff's test: To 1ml onion extract was added 5ml of seliwanoff's reagent. It was mixed and boiled for 1 minute. The appearance of a red recipitate showed the presence of fructose.

2. Analysis of sugars by Paper chromatography:

Twenty grammes of the onion bulb was removed from the first leaf base and blended in a waring blender with 100ml distilled water. It was filtered using a vacuum pump. Fifty millilitre of the filtrate was passed through 10cm column of ion exchange

resins, Amberlite IR-120 (H^+ cycle) and IRA-400 (OH^- cycle) for cation and anion removal respectively (Phillips and Pollard, 1953; White and Hess, 1956). The deionized sample was deproteinized by mixing 25ml with 50ml 0.3N $Ba(OH)_2$ followed by 50ml 5% (W/v) $ZnSO_4 \cdot 7H_2O$ (Somogyi, 1945).

The sugars in the deproteinized onion extract were separated using one way ascending paper chromatography. The treated sample and a 1% (W/v) solution of Glucose, Fructose, Sucrose, Maltose, Galactose, Lactose, Raffinose, Arabinose, Mannitol, Dulcitol, Sorbose, Rhamnose and Saccharose in distilled water were spotted at 2.5cm intervals on Whatman paper No.1. They were run in the solvent ethyl acetate - Pyridine - Water (8:2:1) for 16 hours.

The chromatograms were dried for 30 minutes at room temperature and developed in Aniline hydrogen phthalate solution (Patridge, 1948). For identification, the R_f values of known samples were compared to values of the unknown sugar extracts.

3. Total sugar determination:

The deionized and deproteinized onion extract was treated using the Anthrone's method

(Plummer, 1978). The colour of the sample was developed by adding 1ml of the sample to 4ml of Anthrone reagent (2g/l in conc H_2SO_4). The mixture was placed in a water bath for 10 minutes, cooled and the absorption read at 620nm against a reagent blank in the photoelectric Colorimeter Model AE-11 from Erma Optical Works Limited, Japan. The reagent blank contained 1ml distilled water and 4ml Anthrone reagent. Concentration of the sugars present was determined from the standard calibration curve previously obtained using serially diluted glucose solution as standard.

4. Qualitative test for proteins and Amino acids:

(a) Biuret test:

To 2ml onion extract was added 2ml of Biuret reagent and mixed. The appearance of a purple colouration showed the presence of peptide bonds (CO-NH).

(b) Millon's test:

To 2ml onion extract was added 3 drops of Millon's reagent. It was mixed and boiled. The appearance of a red precipitate showed the presence of hydroxyl group attached to benzene ring.

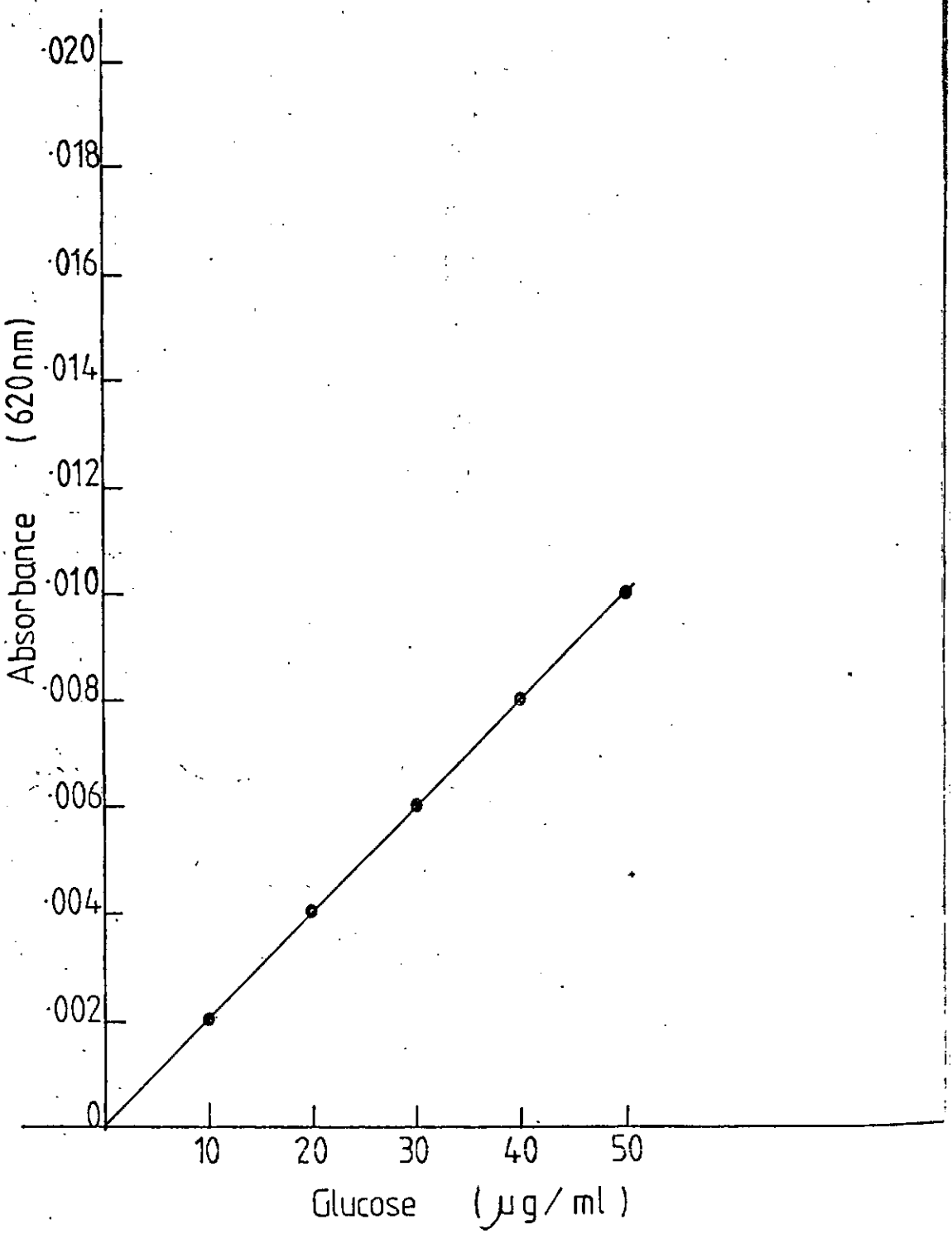
(c) Ninhydrin test:

To 2ml onion extract was added 0.2ml ninhydrin reagent. The mixture was placed in a boiling water bath for 10 minutes. The appearance of a purple colour showed the presence of peptide linkage.

Fig. A:

Calibration curve for glucose using the Anthrone's method. All values were read against water as the blank.

Fig. A


·020
·018
·016
·014
·012
·010
·008
·006
·004
·002
0
Absorbance (620nm)
10
20
30
40
50
Glucose (µg/ml)

5. Analysis of Amino acids by Paper chromatography:

The onion extract was used as the test sample. These and the reference Amino acids (Leucine, Tyrosine, Alanine, Glycine, Proline, Tryptophan, Valine, Cysteine and Threonine) were spotted on a Whatman Paper No. 1. One way ascending paper chromatography was used using n-butanol - acetic acid - water (12:3:5) as solvent for 4h. The chromatogram was dried for 15 minutes and amino acids were detected with Ninhydrin solution. It was dried in the oven at 100°C for 5 minutes. The amino acids appeared as purple spots. By comparison with the R_f values, the amino acids in the extract were identified.

6. Total amino acid determination:

The onion extract was treated using the Ninhydrin reaction to determine the total amino acid content. The colour was developed using the buffered Ninhydrin reagent containing 0.8g Ninhydrin in 30ml methyl cellosolve with 10ml acetate buffer(pH = 5.6). To 2ml onion extract was added 2ml buffered Ninhydrin reagent. The solution was boiled for 15 minutes in a water bath, cooled to room temperature and then mixed with 3ml 50% ($^{v}/_{v}$) ethanol. After 10 minutes,

the absorption in a colorimeter was read at 470nm against a reagent blank. The reagent blank contained distilled water instead of the onion extract and treated as explained above. The concentration of the amino acids was determined from the standard curve obtained by using serially diluted solution of glycine.

7. Qualitative test for lipids:

To 1ml onion solution was added 1ml water. The contents were mixed thoroughly, boiled for 5 minutes and cooled. It was divided into two portions, one portion was mixed with ether and the other with a few drops of Sudan III solution. The solubility of the mixture and the appearance of a red colour showed the presence of oils.

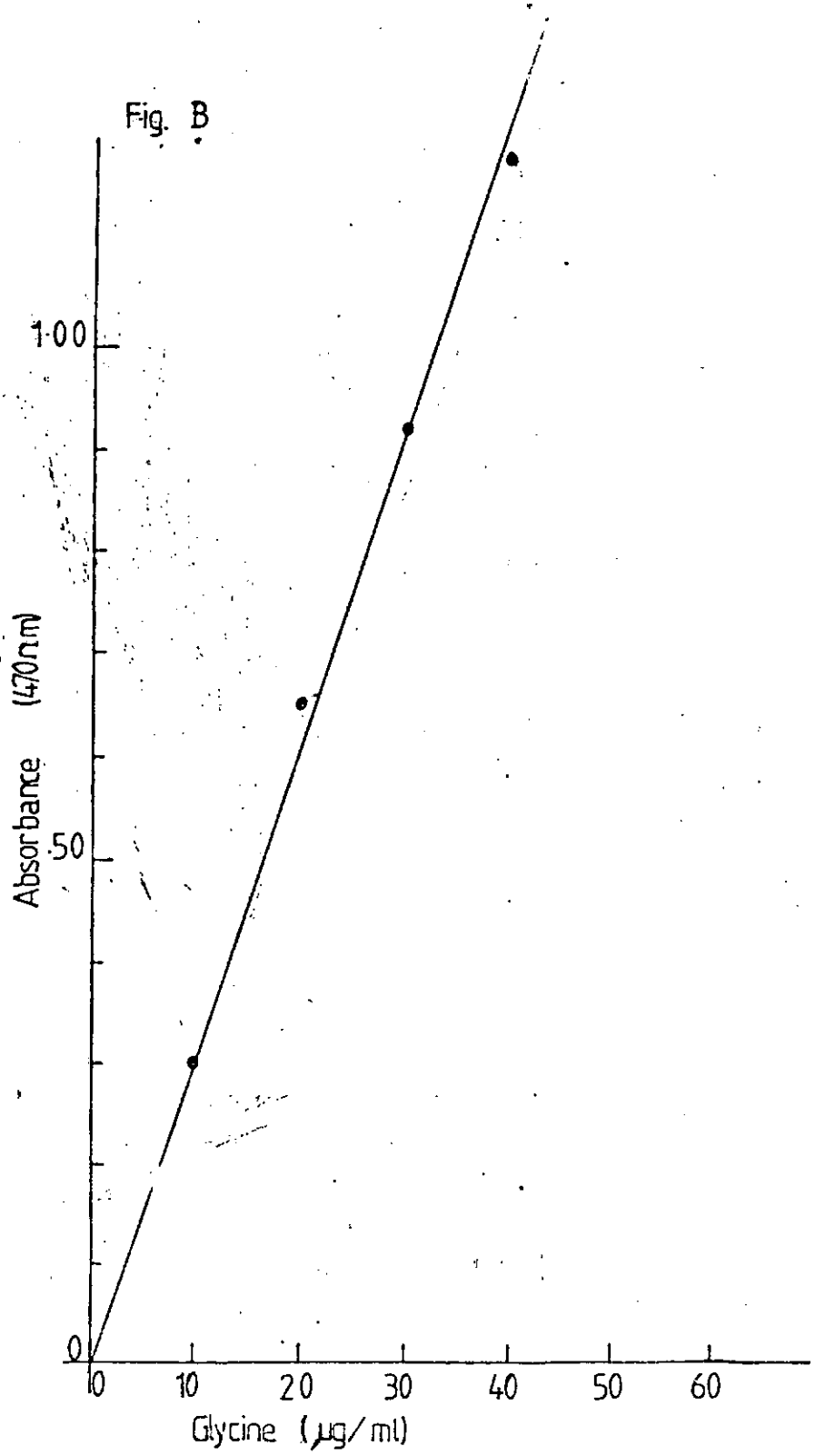
8. Total lipid content determination:

The amount of lipid was determined by an ether extraction method described by Block (1956). A known weight of the dry onion was transferred into a thimble and the total lipid extracted by means of diethylether for 24h in a solid/liquid soxhlet extractor. The extract was evaporated in a water bath and the residue weighed to give the amount of lipid in the sample. Each sample was run in duplicate.

Fig. B:

Calibration curve for glycine using the ninhydrin reaction. All values were read against water as the blank.

Fig. B



Calculation:

Weight of thimble = Ag

" " " + Sample = Bg

Weight of dried sample = B - A = Cg

Weight of residue (after extraction) = Dg

Weight of ether extracted lipid = C - D = Xg

% weight of lipid = $\frac{X}{C} \times 100$

Total water content determination:

The total water content was determined by drying a known weight of the sample to a constant weight at 80°C. The sample was re-weighed to get the difference in weights. Four readings were taken for each sample and the average found. The results were expressed in percentage.

If Ag is weight of sample and Bg is weight of sample dried, then % water = $\frac{A - B}{A} \times 100$

Effect of certain food substances on growth of the pathogens:

1. Lipolytic activity:

This was tested by inoculating three types of butterfat media. They were Butterfat + Nutrient agar, Butterfat + Agar and Butterfat + Basal medium. Duplicate plates were used for each pathogen. The plates were incubated at room temperature for 3-5 days. The surfaces of

the cultures were then flooded with saturated copper sulphate solution. The reagent was allowed to react with the medium for 15 minutes. Excess reagent was poured off and the plates allowed to stand for a further 10 minutes. Lipid hydrolysis was indicated by the fat globules becoming opaque and greenish-blue in colour.

2. Amylolytic activity:

Three types of starch media were used for the test. They were Starch + nutrient agar, Starch + Agar and Starch + Basal medium. All the media were inoculated and incubated at room temperature for 3 days. Duplicate plates were used for each pathogen. After incubation, the surfaces of the plates were flooded with Gram iodide solution. Hydrolysis of starch was shown by clear zones around the bacterial growth while unchanged starch retained blue black colouration.

3. Cellulolytic activity:

Three types of cellulose media were used. They were Cellulose + nutrient agar, cellulose + Agar and cellulose + Basal medium. Duplicate plates of each medium were inoculated and incubated at room temperature for one week.

Cellulolytic activity was indicated by the "clearing" of the medium by the pathogen.

4. Utilization of Pectin:

This was tested by inoculating plates of three types of pectin media with the pathogens and incubated at room temperature for 3 days. The different pectin media were Pectin + Nutrient agar, Pectin + Agar and Pectin + Basal medium. Growth on the plates indicated that Pectin was utilized.

5. Utilization of protein (Meat):

The ability of the pathogens to use meat for growth was tested by growing them on three types of meat media. The media were Meat + Nutrient agar, Meat + Agar and Meat + Basal medium. The inoculated plates were incubated at room temperature for three days. Growth on the plates showed that meat could be utilized.

Growth Studies of P.fluorescens

1. Growth of P.fluorescens on different solid media:

Different solid media were prepared. The media used are listed in table 5 of the result. They were each inoculated with same amount of cells/ml of suspension (9.2×10^7 cells). Duplicate plates were incubated at $4^{\circ}C$, $29 \pm 2^{\circ}C$, $37^{\circ}C$ and $44^{\circ}C$ for 3 days. The extent of growth was estimated by colony count.

2. Growth of P.fluorescens in liquid media:

Onion broth and nutrient broth were prepared and sterilized. Growth was studied on these media using the turbidimetric method.

The media were divided into 9ml portions in test tubes. A suspension of the organism in the appropriate broth (was) prepared and serial solutions made. The optical density of each dilution was read at 620nm on the Photoelectric Colorimeter model AE - 11. Using the plate count method, the number of organismsin each tube was determined. A graph of optical density against log of number of organism served as the standard curve.

For the growth studies, (1)1ml aliquots of the bacterial suspension were transferred into 9ml portions of the liquid media. Some of the

tubes were incubated at 4°C, some at 29 ± 2°C and others at 37°C. Duplicate readings of the optical density were taken at 2h intervals for 30h. With the help of the standard curve, the number of organisms present at each time was determined. Growth curves were plotted for each medium at different temperatures.

3. Effect of different carbon sources on growth:

The ability of P.fluorescens to utilize different carbon sources for growth was investigated. The carbon sources were galactose, lactose, maltose, mannitol, sucrose, sorbose, fructose and glucose. Equal weight of carbon (0.4g) in each sugar was incorporated into a basal medium (see under media). Growth was studied using the turbidimetric method and growth curves plotted for each sugar. Growth was also estimated using different concentrations of glucose.

4. Effect of different nitrogen sources on growth:

Eight nitrogen sources were incorporated singly into the basal medium. Equal weight (0.15g) of nitrogen was present in all the nitrogen sources which were alanine, glycine, tyrosine, sodium nitrate, ammonium sulphate and asparagin. Growth was determined using the

turbidimetric method and growth curves plotted for each nitrogen source. Growth was also estimated using different concentrations of glycine.

5. Influence of P^H on growth:

Onion broth was prepared and 50ml of it poured into a 250ml flask. The contents were adjusted to P^H 2, 4, 6, 8, 10 and 12 using 6 N HCl and 2 N NaOH with a pH meter before autoclaving. After cooling, the media were inoculated with a 24h-old culture and incubated at room temperature for 30h. Growth was studied using the turbidimetric method.

T-test analysis was used to find out the level of significance in the growth of P.fluorescens on these different media and at different temperatures.

Correlation coefficient r =

$$\frac{\Sigma xy - \frac{\Sigma x \, \Sigma y}{n}}{\sqrt{\left(\Sigma x^2 - \frac{(\Sigma x)^2}{n}\right)\left(\Sigma y^2 - \frac{(\Sigma y)^2}{n}\right)}}$$

where x and y are corresponding values used in the analysis and n = number of observation.

t - statistics = $r \frac{n-2}{\sqrt{1-r^2}}$ with n - 2 d.f

d.f. = degrees of freedom.

Growth of P.fluorescens on the onion bulb and recovery of cells from infected tissue:

Healthy onion bulbs were surface sterilized with 70% ($^{v}/_{v}$) ethanol. Small cuts were made on the first leaf base with a sterile cork borer of 0.5cm diameter. The bulbs were inoculated with suspension containing 3.2×10^{9} cells and incubated at room temperature for seven days. At 24h intervals, the diameter of rotted tissue was measured and the average of ten readings recorded. Also at 24h intervals, tissue of 2cm diameter was removed from the inoculated area with a sterile cork borer, blended in 10ml sterile distilled water and the amount of bacterial cells per gramme of tissue estimated using the dilution plate method. Bulbs inoculated with sterile distilled water were used as controls.

Influence of environmental conditions on the development of rot:

1. Effect of temperature: Five healthy onion bulbs were peeled and surface sterilized with 70% ($^{v}/_{v}$) ethanol. Five wounds were made on each bulb using a sterile cork borer on the first leaf base. On the wounds were inoculated 0.05ml aliquots containing 3.2×10^{9} number of cells. Control bulbs were inoculated with same

amount of sterile distilled water. After inoculation, the bulbs were placed in sterile glass jars and placed in incubators kept at 4°C, 29±2°C, 37°C and 44°C for one month. Development and extent of rot were observed.

2. Effect of relative humidity:

Six healthy onion bulbs were peeled and surface sterilized with 70% (v/v) ethanol. A wound was made on the outermost fleshy leaf base of each bulb with a cork borer. Into this wound was inoculated 0.05ml aliquot containing 3.2×10^9. The control bulbs were inoculated with same amount of sterile distilled water. Each bulb was placed on a glass ring inside sterile glass jar and placed in a relative humidity chamber. In the lower part of the chamber was placed 100ml of the appropriate saturated salt solution corresponding to 95, 90, 85, 80, 75, 70, 65, 60, 55 and 50% relative humidity at 29°C (Winston and Bates, 1960).

The extent of rot at various relative humidities was determined by measuring the radius of rot from point of inoculation.

3. Effect of anaerobic condition:

Suspension containing 3.2×10^9 number of cells of the bacterial pathogen was streaked on four dry nutrient agar and four onion agar plates. The plates were incubated for two days at room temperature in the anaerobic incubator in which nitrogen was the only available gas.

(b) Healthy onion bulbs were surface sterilized with 70% (v/v) ethanol. They were wounded with the cork borer and inoculated with 0.05ml suspension containing 3.2×10^9 cells. The bulbs were put in sterile glass jars and left opened in the anaerobic chamber. Control bulbs were inoculated with same amount of sterile distilled water and all the bulbs incubated for one week. Development of rot was observed.

Histology of Onion tissue inoculated with P.fluorescens:

Pieces from the bulbs containing healthy and diseased tissue were cut. The sections were frozen using carbon dioxide gas and then cut to 15 micron thickness using the freezing microtome. The cut sections were transferred to slides and tissue fixed with 90% (v/v)

ethanol for 5 minutes. The slides were again transferred to 70% ($^{v}/v$) ethanol for 5 minutes and stained with haematoxylin for 15 minutes. They were washed in tap water for about 15 minutes until they were blue in colour. They were then counterstained in eosin for 1-3 minutes, washed again in tap water for 1 second, dehydrated in 90% ($^{v}/v$) ethanol for 30 seconds and transferred to absolute ethanol for two changes of 5 minutes each. They were then cleaned in two changes of xylene for 5 minutes each and mounted in canada balsam. The slides were examined by means of the photomicroscope for appearance of the cells after and before infection.

Pathogenicity of P.fluorescens on other vegetables

Vegetables such as cucumbers, carrots, garlic, potatoes and white variety onion were washed in tap water and surface sterilized with 70% ($^{v}/v$) ethanol. Shallow cuts were made on them with a sterile cork borer of 0.5cm diameter. They were inoculated with suspension containing 3.2×10^{9} cells and incubated at room temperature for 7 days. The vegetables inoculated with sterile distilled water served as controls.

Enzyme Studies

Growth media:

Two types of culture media were prepared. (a) Solid media (b) Shake cultures for enzyme induction in vitro.

A. Solid media: To an inorganic salt solution - basal medium (see under media p.29) was added pectin as the only carbon source and 2% ($^{W}/_{v}$) oxoid agar.

B. Shake culture: Medium for shake culture had the same constituents as the basal medium and the carbon source was pectin.

Pectic enzyme substrate: Citrus pectin was purified by washing before use. To 30g of powder was added 150ml 70% ($^{V}/_{v}$) ethanol containing 0.05N HCl. Mixture was stirred for 30 minutes using a magnetic stirrer and then filtered under reduced pressure. The residue was washed with several changes of 70% ($^{V}/_{v}$) ethanol until washing was free of chloride (tested with Ag NO_3). It was finally washed with 95% ethanol followed by absolute ethanol and then dried in an oven at 50°C wrapped in alluminium foil cups. This substrate was used to induce pectic enzyme synthesis in vitro. To overcome the difficulty of bringing the substrate into solution, it was slowly added

to water and stirred vigorously. Solution was made up at double strength and then mixed with equal volume of buffer to obtain the desired concentration and pH.

Buffers:

Mixtures of appropriate volumes of the following were used for desired pH values as described by Dawson, Elliot and Jones, 1959 (cited by Arinze, 1978).

Citric acid - Sodium citrate	pH 3.0 - 6.7
Na_2HPO_4 - NaH_2PO_4	pH 5.7 - 8.0
Tris (hydroxymethyl) Amino methane - HCl	pH 7.2 - 9.0

Inoculation of solid media:

A loopful of the bacterial suspension was streaked on dry agar plates. The plates were incubated at 30°C for four days. Appearance of colonies was observed everyday for four days.

Inoculation of shake cultures:

The medium was dispensed in 30ml aliquots in 150ml Erlenmeyer flasks. Inoculation was made with 0.05ml portion of bacterial suspension containing 3.2×10^9 cells using a sterile pipette. The flasks were incubated in a rotary shaker (60rpm) at 25°C for the appropriate number of days.

Culture filtrate for enzyme studies:

Shake cultures after 7 days incubation were filtered through millipore filter pore size 45μM. The filtrate was dialysed at 4°C for 24 hours using a visking cellulose tube (8" x 32" diameter) against distilled water (20ml filtrate : 500ml H_2O) at P^H = 7 before storing at -20°C.

Preparation of rotted tissue for enzyme studies:

(a) Tissue inoculation: Healthy onion bulbs were surface sterilized by washing with 70% ($^V/v$) ethanol. Discs (0.5cm diameter) were cut on the first leaf base of the bulbs with a cork borer. Aliquots of 0.05ml containing 3.2×10^9 cells were introduced into the cavity using a disposable syringe. The bulbs were placed in sterile glass jars previously cleaned with 70% ($^V/v$) ethanol. The jars were closed and incubated at room temperature for appropriate number of days.

(b) Extraction of rotted tissue for enzyme assay: Rotted tissue was removed, mixed with 0.1 M phosphate buffer P^H = 7.0 containing 0.2 M Nacl, 10^{-3} M dithiothreitol in the ratio of 1 : 10, tissue : buffer. Nacl was used to extract the protein from

tissue (Blackhurst and Wood, 1963 (cited by Arinze, 1978). Dithiothreitol was used to prevent oxidation by oxidative enzymes present in the tissue (Anderson, 1968 (cited by Arinze, 1978)). The tissue was homogenised in an omnimixer for 5 minutes at full speed, and filtered under vacuum. The sample was dialysed against distilled water with visking tubing for 24 hours at 4°C before storage at -20°C.

Partial Enzyme Purification

$(NH_4)_2S0_4$ precipitation:

Solid $(NH_4)_2SO_4$ was added to crude culture filtrate and rot extracts as a first step in the fractionation of proteins present in the solutions. $(NH_4)_2SO_4$ saturation levels of 40, 60, 80, 90 and 100% were obtained. The amount of salt added to achieve the desired saturation level was calculated from Tables (Green and Hughes, 1955). Addition of salt was carried out at 25°C in a water bath. The precipitate obtained at each saturation level was removed (after leaving to stand for 30 minutes) by centrifugation at 12,000g for 30 minutes at 25°C. The supernatant was used for the next higher saturation. The precipitate

at each level was dissolved in a small volume of water and dialysed against water at 4°C for 24 hours before storing at -20°C.

Enzyme assays

(i) Viscometry: This was the method routinely used to determine the chain-splitting action of culture filtrates and rot extracts. The reaction mixture consisted of 4ml 2% pectic substrate and 0.1M citrate buffer pH 5.0, 1ml water and 2ml enzyme extract. Enzyme activity was expressed in relative viscometric units defined as $\frac{1000}{t}$ when t = time in minutes for 50% loss in viscosity of the reaction mixture. Loss in activity was measured in a Cannon-Fenske viscometer size 200 at 26 ± 1°C in a water bath. Viscometers were calibrated against water, the flow time of which represented 100% viscosity reduction. Autoclaved enzyme was used as control.

(ii) Cup-plate assay: This technique was originally described by Dingle, Reid and Solomons (1953). One gramme pectin was dissolved in 50ml water and the pH adjusted to 5.0 by addition of few drops of 6HCl. An equal volume of 0.2M sodium citrate buffer pH 5

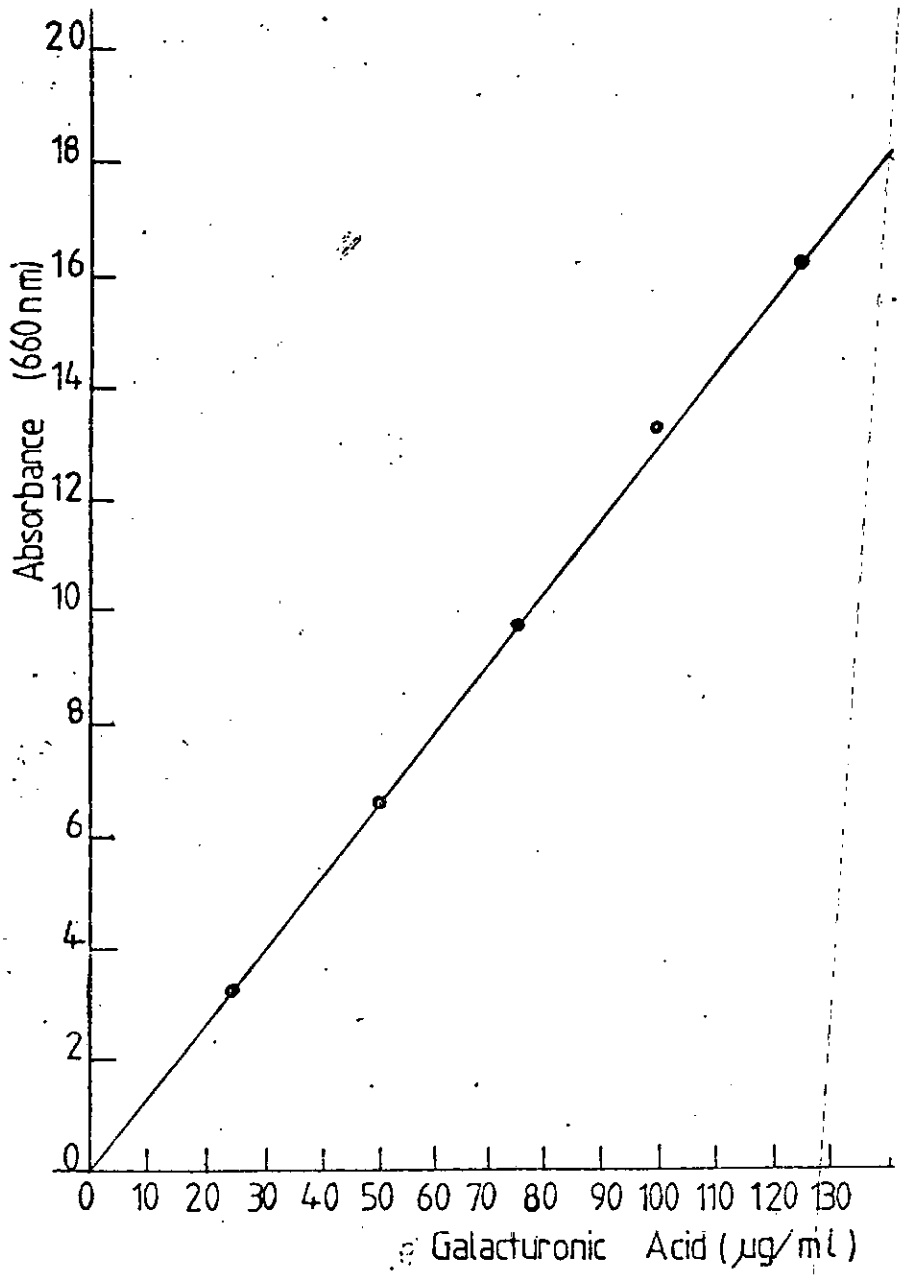
was added to yield a solution of 2% (w/v) pectin in 0.1M citrate buffer. One gramme oxoid agar was added and the mixture autoclaved for 10 minutes at 121°C. Twenty millilitre aliquots were transferred to sterile petri-dishes and allowed to set. Wells were cut out of the agar with a sterile cork borer (0.5cm) diameter and 0.02ml of enzyme sample pipetted into each well under sterile conditions. Plates were incubated at 25°C for 18 hours after which the agar surface was flooded with 5N HCl. Whenever there was enzyme activity, a white precipitate would form around the well within 5 minutes. The diameter of the precipitate around the well was used as a measure of enzyme activity.

(iii) Release of reducing group: The carbonyl reducing group at carbon 1 of galacturonic acid is normally involved in the formation of α- 1, 4 glycosidic linkage in pectic substances. When the linkage is split by hydrolysis, the reducing group is released and can be qualitatively and quantitatively analysed. The method of analysis here is based on the Nelson-Somogyi method (1944) which involves

Fig. C:

Calibration curve for galacturonic acid used as standard in the estimation of amount of reducing groups released using the Nelson-Somogyi method. All values were read against water as the blank.

Fig. C


Absorbance (660 nm)
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8
10
12
14
16
18
20
0 10 20 30 40 50 60 70 80 90 100 110 120 130
Galacturonic Acid (µg/ml)

oxidation of the reducing group by means of cupric ions. The reaction mixture consists of enzyme sample, pectic substrate (2% ($^{W}/_{V}$) in 0.1M citrate buffer pH 5:0) and water in the proportion 2 : 4 : 1. This was incubated in a water bath at 25 ± 1°C and at intervals of 5 minutes 1ml samples were removed, diluted to 10ml with distilled water and 0.2ml samples taken from this and estimated for reducing group content. The following reagents were made up:

(a) Copper reagent A:

25g Na_2CO_3 (anhydrous)
25g Potassium sodium tartrate
20g $NaHCO_3$
200g Na_2SO_4 (anhydrous)

} They were dissolved in 800ml water and made up 1 litre.

(b) Copper reagent B

15g $CuSO_4 \cdot 7H_2O$
2 drops of Conc H_2SO_4

} in 100ml H_2O

(c) Arsenomolybdate reagent:

25g Ammonium molybdate dissolved in 450ml H_2O

21ml Conc H_2SO_4 added

3g Sodium arsenate ($Na_2HASO_4.7H_2O$) in 25ml H_2O added

The solution was incubated at 37°C for 48 hours and stored at room temperature in brown glass bottle.

Twenty-five parts of copper reagent A and 1 part of reagent B were mixed to obtain the "cu reagent" just before the test was carried out. To the test sample (0.2ml) was added 0.8ml water and 1.0ml of "the reagent". The mixture was heated for 30 minutes in a boiling water bath and cooled rapidly in ice. One millilitre arsenomolybdate reagent was added and mixed thoroughly until CO_2 effervescence ceased. The intensity of the blue colour which developed was estimated against a reagent blank containing autoclaved enzyme at 660nm in a spectrophotometer. A standard curve was obtained with galacturonic acid (5-50 µg/ml) for calibration.

T.B.A. reaction:

This reaction was used to establish that the cleavage of the pectic chains was by hydrolysis and not by transelimination. Products of hydrolysis of pectic substances will react with thiobarbituric acid (TBA) to give a pinkish-orange complex which absorbs maximally at 515nm whereas the transeliminative complex would be cherry-pink in colour and absorb at 550nm.

The reaction mixtures were as described by viscometry in the same proportion. The mixture was incubated at 25°C in a water bath for one hour. Samples of 2.5ml were then removed and added to a mixture of 2.5ml 1N HCl and 5ml 0.04 M thiobarbituric acid in a test tube and mixed thoroughly. The test tube was capped, boiled in a water bath for 30 minutes, cooled and the colour produced read both at 515nm for PG activity and 550nm for transeliminase activity. Autoclaved enzyme sample was used for blank reading.

Macerating activity:

The ability of the enzyme samples to separate cells in tissue was determined using potato tissue and onion tissue. Long cores of tissue were cut from healthy tubers and bulbs by means of a cork borer and discs (0.5 x 0.05cm) were cut out from the cores by means of a hand microtome. The discs were suspended in several changes of 0.5 M citrate buffer pH = 5 to wash off the starch and other cytoplasmic materials from the surface of cut cells. They were quickly dried on tissue paper and transferred to an enzyme reaction mixture (6 discs/ml) consisting of

2ml enzyme sample

1ml 0.05 M citrate buffer pH_5

1ml water

This was incubated at 25°C in a water bath and at intervals of 20 minutes 3 discs were removed, washed in citrate buffer pH5 and macerating activity of the enzyme tested. This was estimated by testing loss in coherence of the discs, determined from the ease with which the discs were pulled apart with two dissecting needles.

Protein measurement:

The protein content of samples was determined conventionally by a colorimetric method described by Lowry, Rosebrough, Farr and Randall (1951).

Colorimetric method with Folin-Ciocalteu reagent:

This method was used mostly for crude protein preparations.

Reagents:

A. 2% ($^{w}/_{v}$) Na_2CO_3 in 0.1N NaOH

B. 1% ($^{w}/_{v}$) $CuSO_4.5H_2O$ solution.
2% ($^{w}/_{v}$) Potassium tartrate. Equal proportions mixed on day of use.

C. 50ml reagent A added to 1ml of reagent B; mixed on day of use.

D. Folin-Ciocalteu reagent (BDH, Poole) (Folin & Ciocalteu, 1927)

To 5ml of reagent C was added 1ml of protein sample at room temperature and left for 30 minutes. 0.5ml reagent D was added and after a further 30 minutes, the mixture was transferred to a cuvette and O.D at 750nm estimated in a spectrophotometer against a reagent blank. Preparations of crystalline bovine serum albumin (Koch-Light Ltd) containing between 10-100μg/m were used to obtain a calibration curve.

Effect of different carbon sources on enzyme production and activity:

Shake cultures were used. The carbon source was substituted for by glucose, galacturonic acid, pectin, sodium polypectate. In addition, pectin and sodium polypectate were substituted for by dextrose in potato dextrose medium. The cultures were incubated at room temperature for 7 days and enzyme activity assayed viscometrically.

Effect of days of incubation on enzyme activity

The shake cultures used had pectin as the only carbon source. The culture filtrates were assayed for enzyme activity everyday for 10 days.

Effect of different concentrations of pectin on enzyme activity:

The shake cultures used had pectin as the only carbon source. Different pectin concentrations of 1% ($^{W}/_{V}$), 2% ($^{W}/_{V}$), 3% ($^{W}/_{V}$) and 4% ($^{W}/_{V}$) were used. The cultures were incubated at room temperature for 7 days.

Effect of P^{H} on enzyme activity:

The shake cultures used had pectin as the only carbon source. The buffer solutions used for enzyme assay had P^{H} values of 4, 5, 6, 7, 8 and 9. To test for effect of Ca^{++} on enzyme activity 0.01 M $Cacl_2$ solution was substituted for water in the reaction mixture for assay at P^{H} 5 and 9. Enzyme assay was done viscometrically.

Determination of total phenols

(a) Extraction of total phenols:

An extract of tissue (healthy or rotted) was obtained by blending in a blender for 3 minutes at full speed (10g fresh weight/50ml methanol). The mixture was filtered under vacuum. The filtrate was shaken vigorously with same volume of petroleum ether in a separating funnel under a fume chamber. This was done four

times and the ether evaporated to 10ml in a rotary evaporator. This extract was finally shaken with same volume of petroleum ether before storage at +4°C.

(b) Phenol estimation:

The following reagents were used

(1) Folin-Denis reagent (BDH, Poole, England

(2) Saturated Na_2CO_3 solution

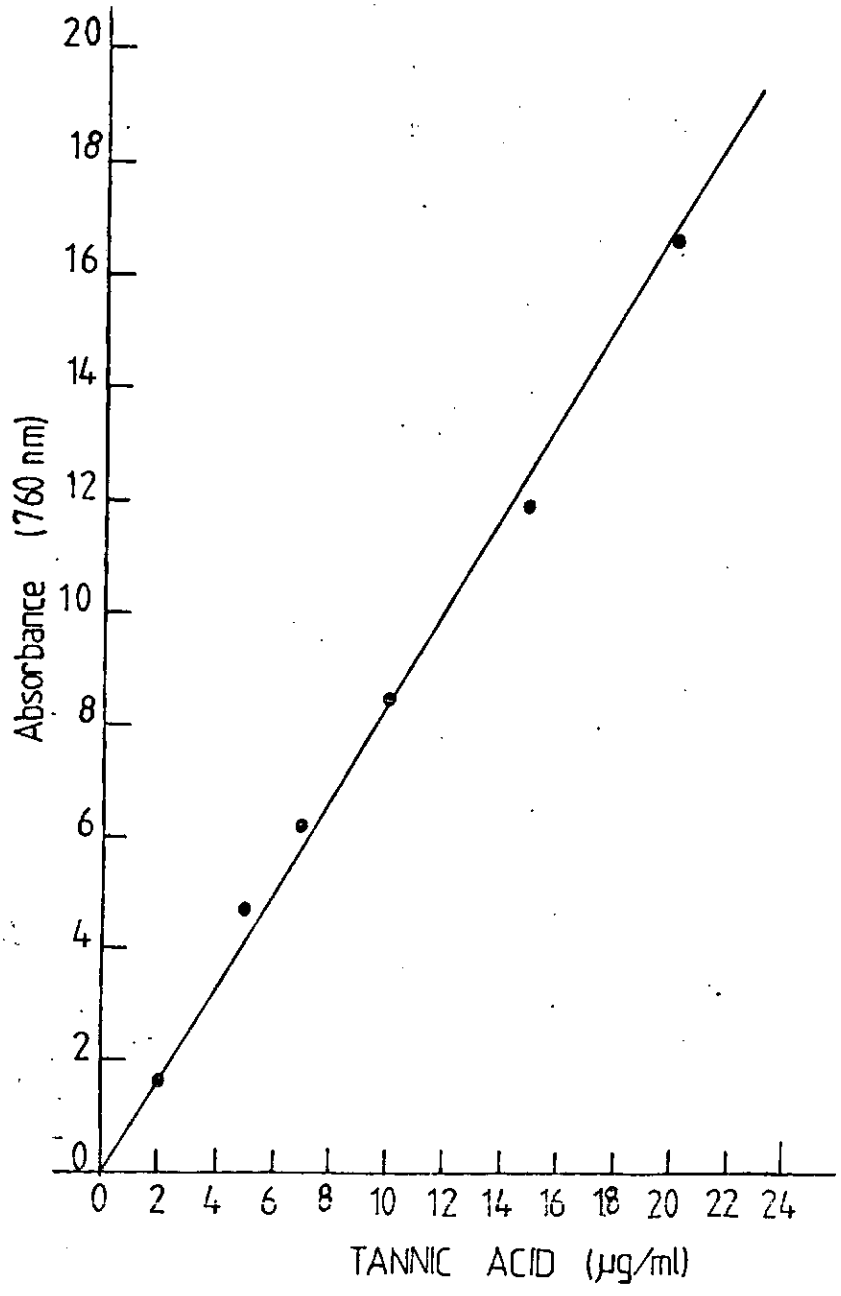
An aliquot (1ml) of the methanol extract was diluted with 7.5ml with distilled water in a test tube. One millilitre and 0.5ml F.D reagent were saturated Na_2CO_3 added to make up to 10ml. One hour later, the solution was centrifuged at 10,000g for 5 minutes to remove precipitate, filtered and the extinction value read at 760nm in a spectrophotometer against a reagent blank prepared with methanol.

A calibration curve was prepared using 10-100 µg tannic acid/ml and results expressed as µg tannic acid equivalent/g fresh weight.

Fig. D:

Calibration curve for tannic acid used as standard in phenol estimation using the Folin-Denis reagent. All values were read against methanol as the blank.

Fig. D


Absorbance (760 nm)
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0 2 4 6 8 10 12 14 16 18 20 22 24
TANNIC ACID (µg/ml)

Results

1. Causal organisms

Four micro-organisms were isolated from rotted onion bulbs obtained from local markets in Lagos (Plates1, 2, 3). Isolation was done on onion extract agar and potato dextrose agar. Two of the isolates were Gram-negative, non-spore forming rods, one a Gram-positive coccus and the fourth a yeast. These organisms were identified as:

B_1 = Staphylococcus sp

B_2 = Candida utilis (Henneberg) Lodder and Kregen-Van Rij

B_3 = Pseudomonas fluorescens Migula, 1895

B_4 = Pseudomonas cepacia Buckholder 1950

Table I shows the biochemical characteristics of the isolates.

The isolates were sent to the Commonwealth Mycological Institute, Kew, Surrey, England and to the National Foundation of Yeast Cultures, Brewing Industry, Research Foundation, Surrey, England for confirmation of identification.

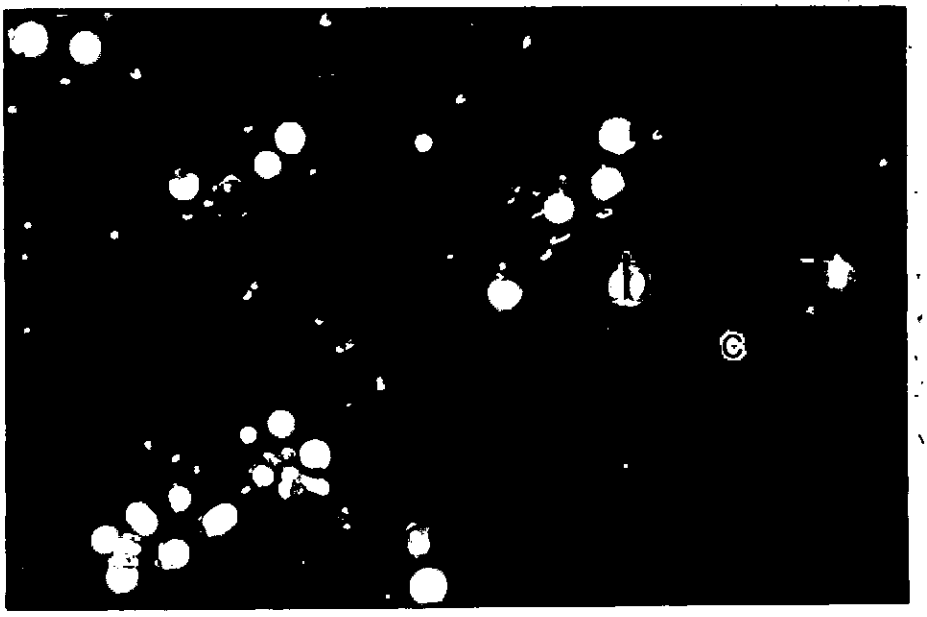
Plate 1 : Isolates from rotted onion tissue grown on onion extract agar (x 1)

Plate 2 : Isolates from rotted onion tissue grown on onion extract agar (x 1)

Plate 3 : A piece of rotted onion tissue removed from area of advancing lesion inoculated onto potato dextrose agar (x 5)

Areas marked a, b, c, d show isolate B_1, B_2, B_3 and B_4 respectively.

1



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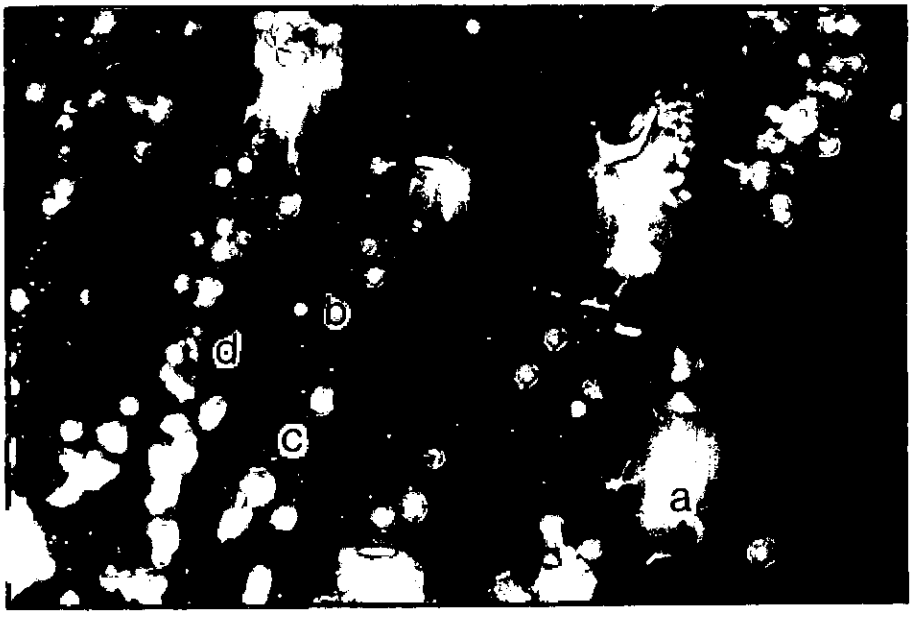

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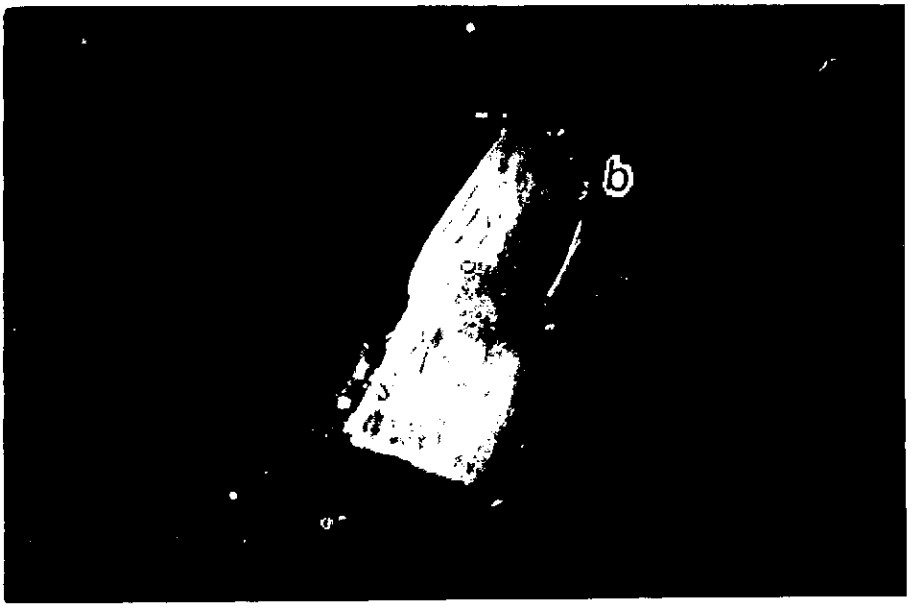


Table 1: General characteristics of the isolates:

Test	B_1	B_2	B_3	B_4
1. Cultural characteristics	White colonies, muccid in nature. About 5mm diameter after 48h at room temperature	Round, cream coloured colonies. About 3mm in diameter after 48h at room temperature on onion extract agar.	White colonies on onion extract agar. Colonies are round, about 3mm diameter after 48h at room temperature. After seven days colonies appear yellow.	Small white colonies. About 2mm diameter after 48h at room temperature on onion extract agar.
2. Gram stain	+ve cocci	-ve	-ve short rods	-ve rods
3. Stain with methylene blue	Round cells	Big ellipsoidal cells	Rod-shaped cells	Rod-shaped cells
4. Hugh and Leifson test	Ferm.	Ferm.	Oxi.	Oxi.

Test	B_1	B_2	B_3	B_4
5. Baird-Parker modification of H/L test	Ferm	Ferm	Oxi.	Oxi.
6. Catalase test	+	+	+	+
7. Oxidase test	-	-	+	+
8. V.P.test	+	Not done	-	-
9. Methyl Red test	-	Not done	+	+
10. Growth at 4°C on nutrient agar	-	+	+	-
11. Growth at 4°C on Potato dextrose agar	-	+	+	-
12. Growth at 44°C on nutrient agar	-	-	-	-

Test	B_1	B_2	B_3	B_4
13. Growth at 44°C on Potato dextrose agar	-	-	-	-
14. Indole production	-	-	-	-
15. Nitrate reduction	+	+	+	-
16. Motility test	-	Not done	+	+
17. Heamolysis on blood agar	-	Not done	-	-
18. Gelatin liquefaction	-	Not done	+	-
19. Coagulase test	-	Not done	-	-
20. Growth on Mac Conkey agar	+	Not done	+	+
21. Growth on Simmon Citrate agar	+	Not done	+	-

Test	B_1	B_2	B_3	B_4
22. Hydrogen Sulphide production	-	Not done	-	-
23. Ammonia gas production	-	Not done	+	+
24. Fermentation reactions on:				
Glucose	Acid + gas	Acid	-	-
Maltose	Acid + gas	-	-	-
Sucrose	Acid + gas	Acid	-	-
Lactose	Acid + gas	-	-	-
Galactose	-	-	-	-
25. Oxi reactions on:				
Glucose	-	Acid+gas	Acid+gas	Acid
Maltose	-	Acid+gas	-	Acid
Sucrose	-	Acid+gas	Gas	Acid
Lactose	-	-	Gas	-
Galactose	-	-	-	-
Mannitol	-	Acid+gas	-	-

Key: Ferm = Fermentative

Oxi = Oxidative

\+ = Positive reaction

\- = Negative reaction

Summary of biochemical characteristics of the isolates

Isolate B_1 is a Gram +ve non-motile coccus, fermentative on Hugh and Leifson and Baird-Parker modification of Hugh and Leifson media. It is catalase positive and can reduce nitrates to nitrites. It can grow on MacConkey agar, utilise citrate and is able to ferment glucose, maltose, sucrose and lactose giving acid and gas (Table 1).

Isolate B_2 is a yeast with big ellipsoidal cells when stained with methylene blue. It is fermentative on Hugh and Leifson and Baird-Parker modification of Hugh and Leifson media. It is catalase positive and can grow on nutrient agar and potato dextrose agar at 4°C. It is also capable of reducing nitrates to nitrites and can ferment and oxidise some sugars producing acid and gas (Table1).

Isolate B_3 is a Gram -ve motile short rod. It is oxidative on Hugh and Leifson and Baird-Parker modification of Hugh and Leifson media. It is catalase positive and oxidase positive. It is capable of growing on nutrient agar and potato dextrose agar at 4°C. It can reduce nitrates to nitrites and can liquefy gelatin. It can also grow on MacConkey agar and utilise citrate.

Isolate B_4 is a Gram -ve motile rod, capable of oxidising Hugh and Leifson and Baird - Parker modification of Hugh and Leifson media. It is catalase positive and oxidase positive. It can

grow on MacConkey agar and produce ammonia gas from nutrient broth. It can also oxidise glucose, maltose and sucrose producing acid in the medium (Table 1).

2. Establishment of pathogenicity:

Three of the isolates (i.e. B_2, B_3 & B_4) were observed to be capable of producing rot when inoculated into fresh onion bulbs (Plates 4, 5, 6 & 7). Isolate B_1 did not cause rot and was therefore not included in further studies.

In each case with B_2, B_3, and B_4 rot commenced from the point of inoculation about 48h on incubation at room temperature. Rot was preceded by the discolouration of the purple tissue at the point of inoculation. This was followed by gradual softening of the affected tissue. The colour eventually changed to yellow after the bulbs were inoculated with the bacterial isolates. After the onion bulbs were inoculated with the yeast, the tissues were soft and cream in colour resembling the colour of the yeast colony on onion extract agar.

Pathogenicity test carried out on the white onion variety and the shallots variety (an onion variety from Ghana) showed that the isolates could cause rot on these two varieties. However, garlic, the third onion variety employed in this study was not affected by the isolates.

Plate 4 : Onion bulb inoculated with *Candida utilis* (x1½)

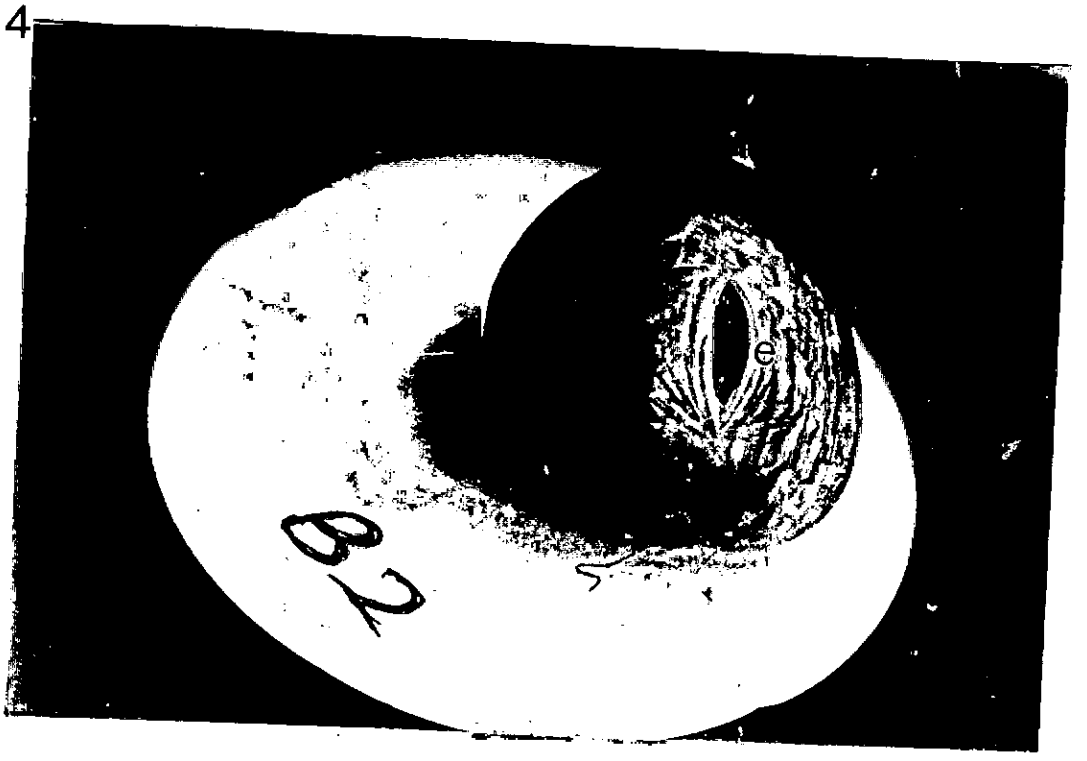
Plate 5 : Onion bulb inoculated with *Pseudomonas fluorescens* (x1½)

Plate 6 : Onion bulb inoculated with *Pseudomonas cepacia* (x1½)

Plate 7 : Onion bulb inoculated with sterile distilled water (x1½)

Areas marked e indicate rotted tissue by the isolates except in the control where there was no rot formation.

4



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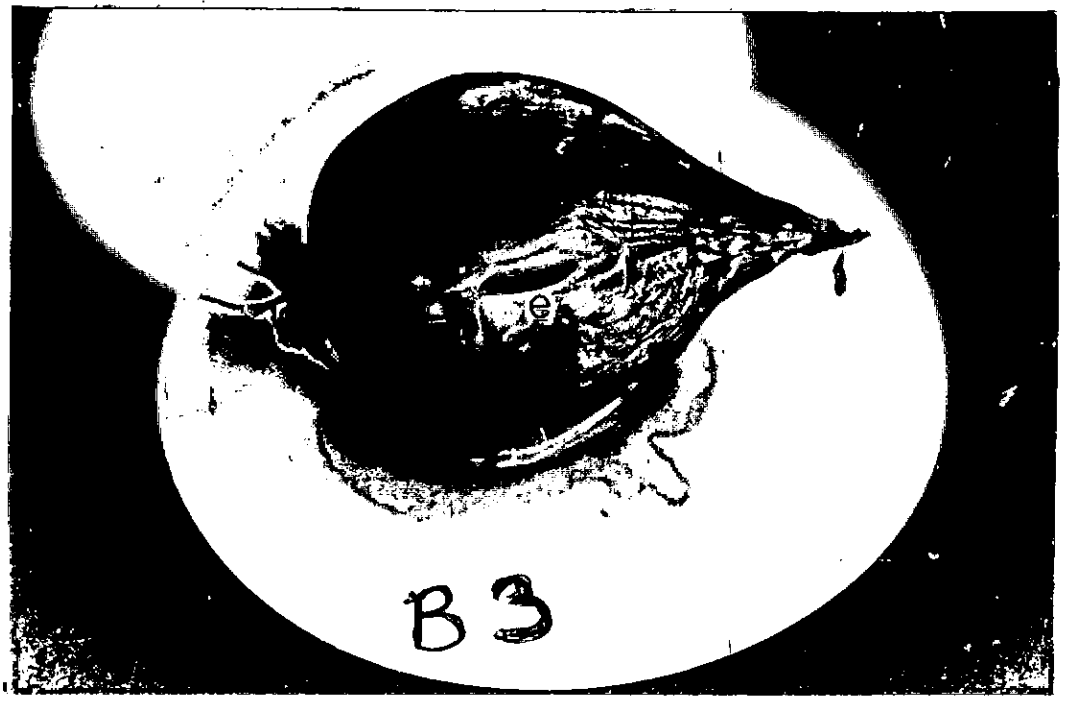

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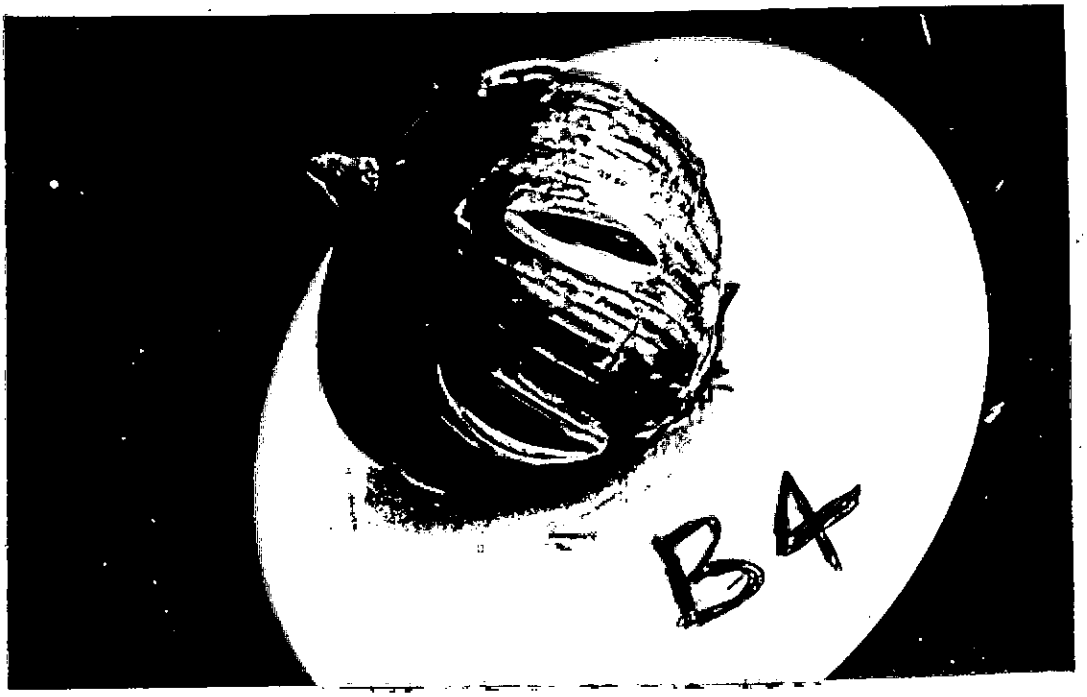

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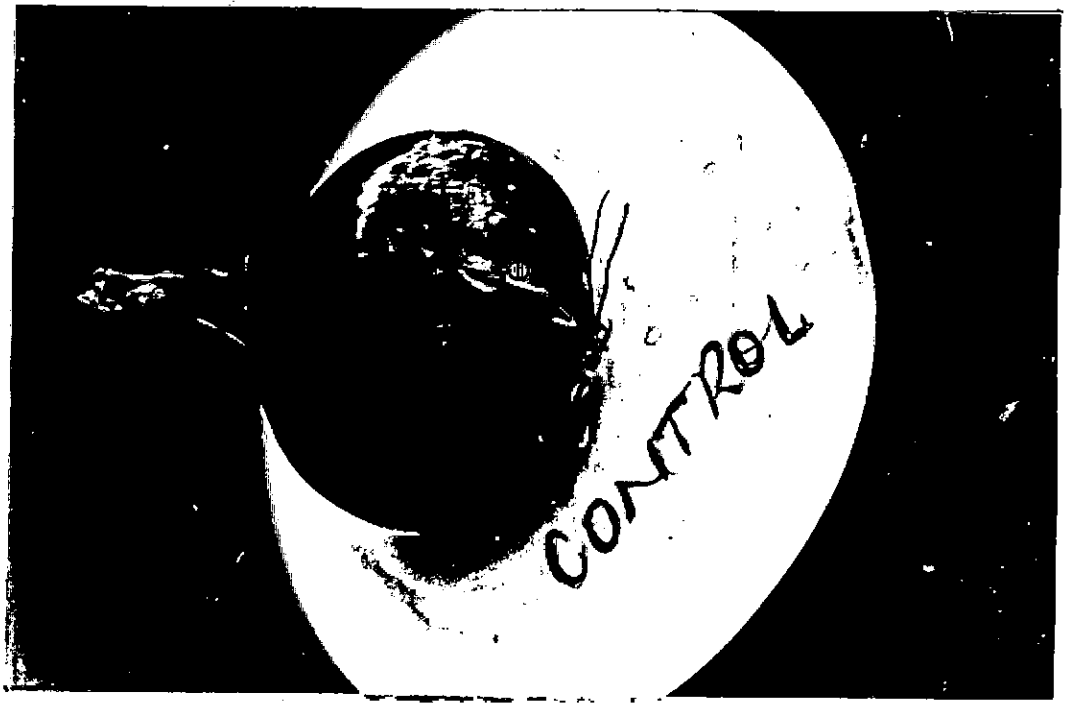


Pseudomonas fluorescens was found to be the most pathogenic of the three pathogenic isolates (diameter of rot after 6days was 2.53cm and 100% rot after 4days). Candida utilis was moderately pathogenic (diameter of rot after 6days was 1.98cm and 86% rot after 4days). Pseudomonas cepacia was the least pathogenic (diameter of rot after 6days was 1.48cm and 70% rot after 4days). (Fig 1a & b). The bulbs were inoculated with 10^7 cells of each organism.

The pathogens were reisolated from artificially inoculated onion bulbs. The organisms were examined and found to possess the characteristics of the original isolates inoculated into the bulbs. It was therefore concluded that isolates B_2, B_3 and B_4 were responsible for the rot of the onion bulbs.

Mode of entry into host tissue:

Experiments set up to determine the path of entry of the pathogens into the host showed that rot occurred on bulbs that were artificially wounded. There was no rot development when bulbs were surface inoculated (rubbing microbial suspensions all over the bulb). It was thus established that mechanical damage provided the portal for entry of pathogens. It was also observed that infection was restricted to the inoculated leaf bases whereas the inner leaf bases remained uninfected. (Plate 8).

Figure 1a: The development of rot on onion bulb inoculated with Pseudomonas fluorescens ●—●, Pseudomonas cepacia ○—○ and Candida utilis □—□

Healthy onion bulbs were inoculated with 10^7 cells of the pathogens. The diameter of rot was measured at 24h intervals for the three pathogens.

Fig. 1a

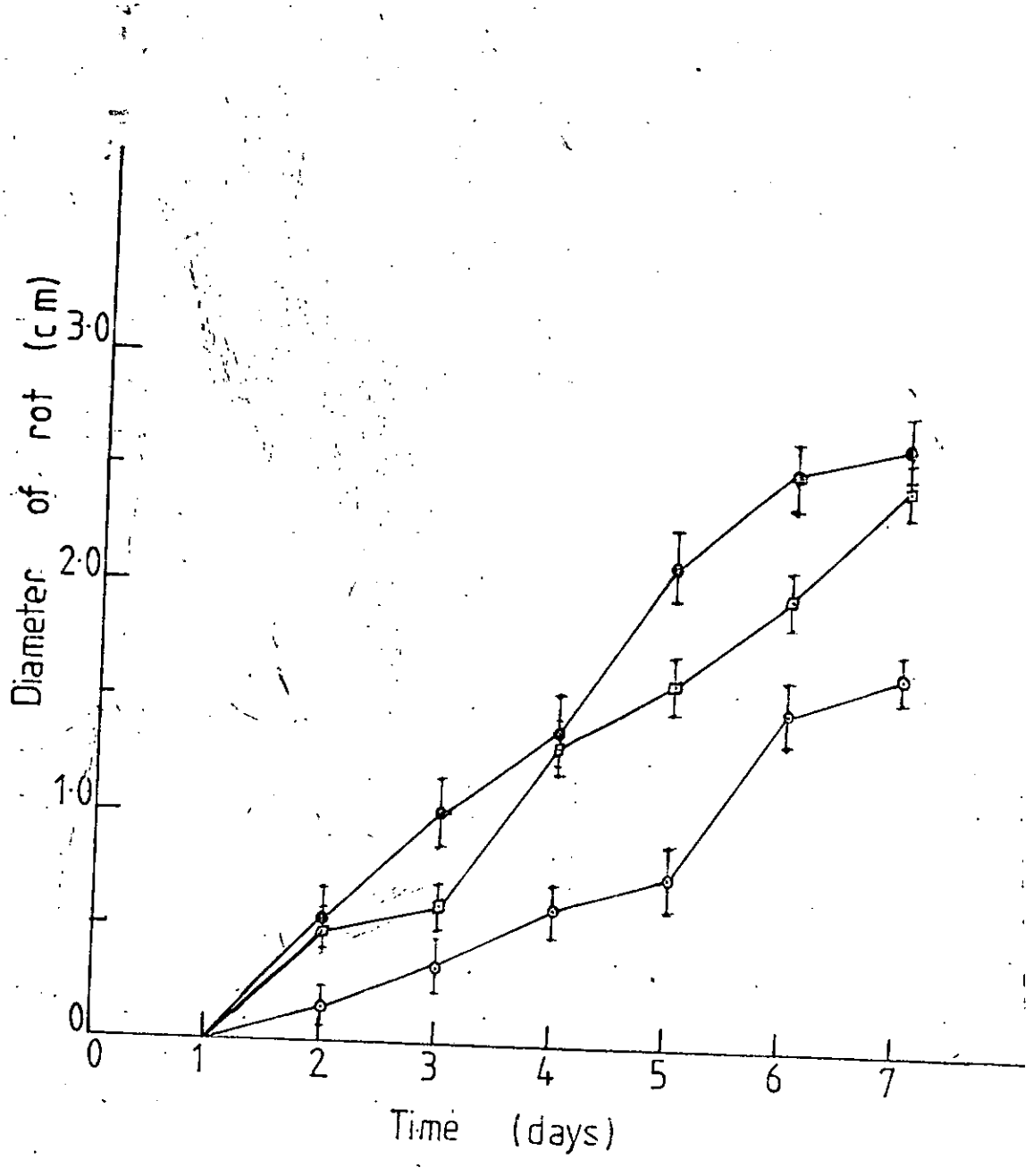


Figure 1b: Percentage rot on onion bulbs inoculated with *Pseudomonas fluorescens* ●—●, *Pseudomonas cepacia* O—O and *Candida utilis* □—□

Healthy onion bulbs were inoculated with 10^7 cells of the pathogens. At 24h intervals the number of inoculation sites showing rot formation (0.6cm diameter of rotted tissue) were calculated as percentage of the total number of sites inoculated

Fig. 1b

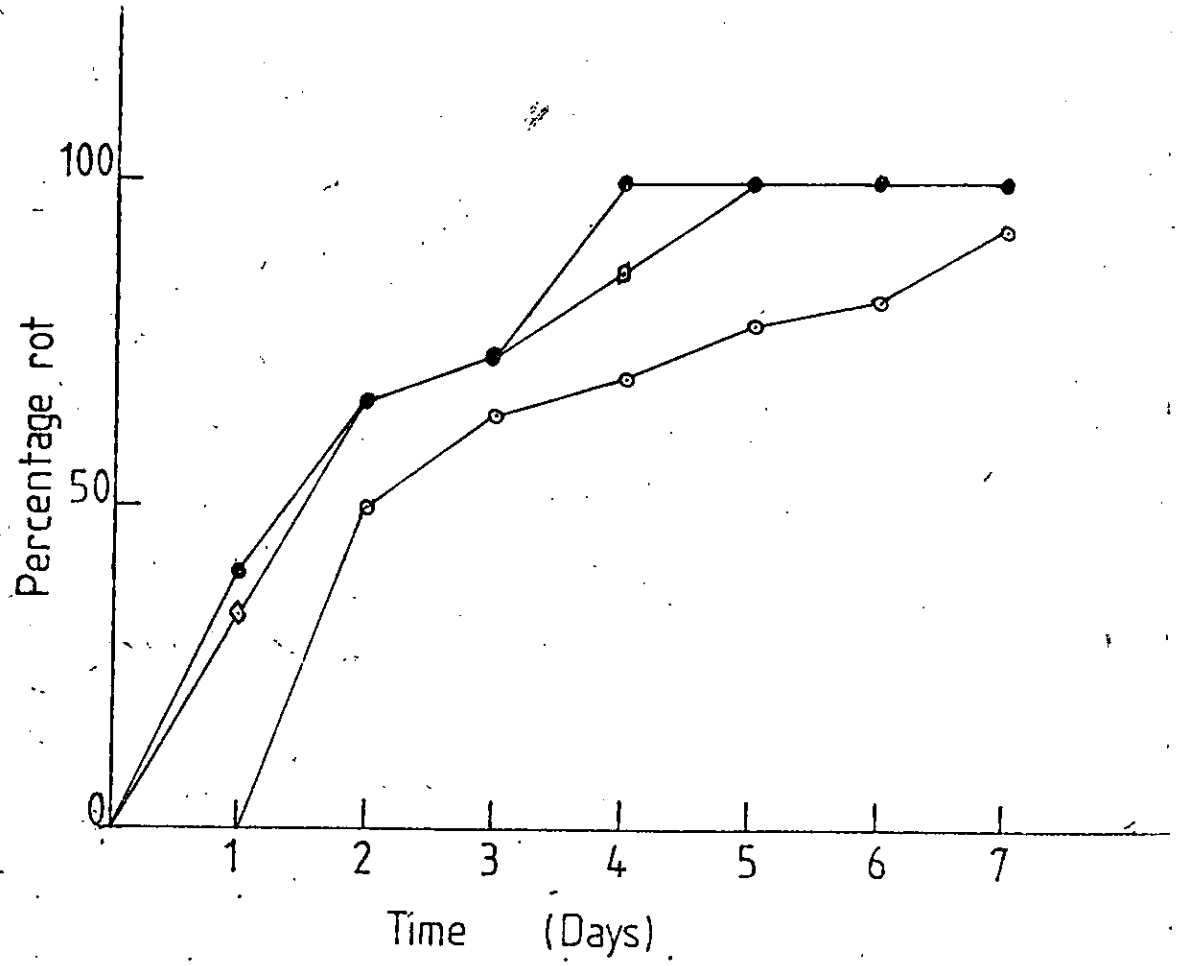
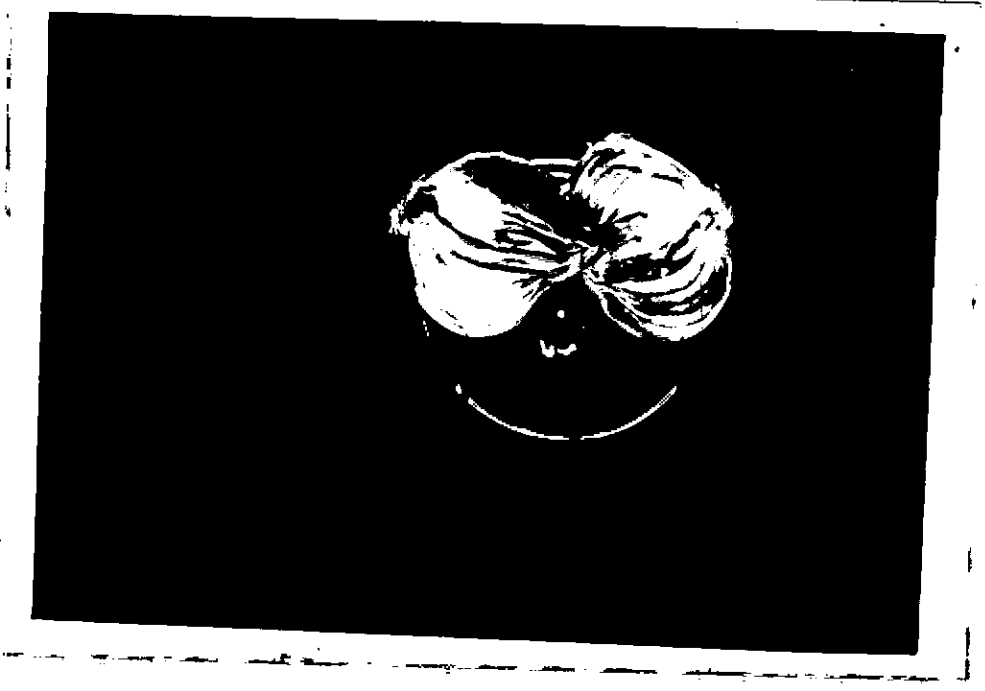


Plate 8: A longitudinal section of an onion bulb inoculated with the pathogens showing that infection is restricted to the leaf base wounded (X¼).

Arrows indicate infection on the outermost leaf base.

8



Histological studies

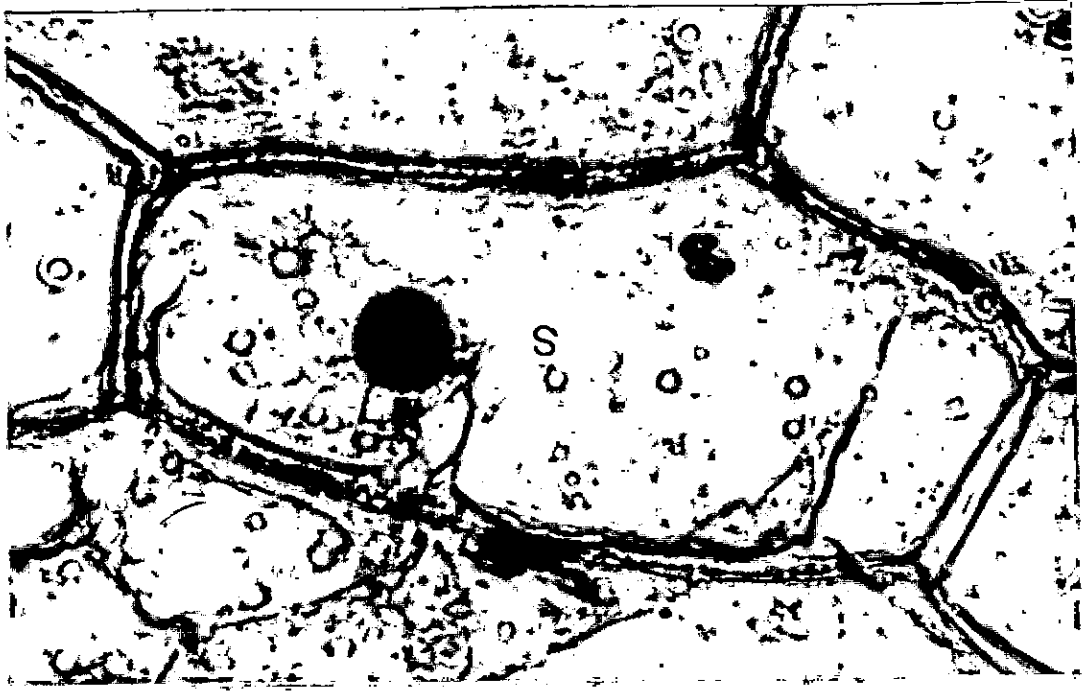
Microtome sections of bulbs inoculated with P.fluorescens were made from the advancing edge of the rot. Observations by means of a photomicroscope showed that the organisms penetrated the tissue of the host both intercellularly and intracellularly. The cellwalls of the host tissue were broken down and, consequently, total collapse of the cells occurred (Plates 9, 10, and 11).

Plate 9: Photomicrograph showing an uninfected intact onion cell (X 625) i.e. S

Plate10: Photomicrograph showing an infected tissue with collapsed cellwalls as a result of infection (X 625) i.e. R

Plate11: Photomicrograph showing an infected tissue with both collapsed cells (n) and intact cells (w) (X125)

9



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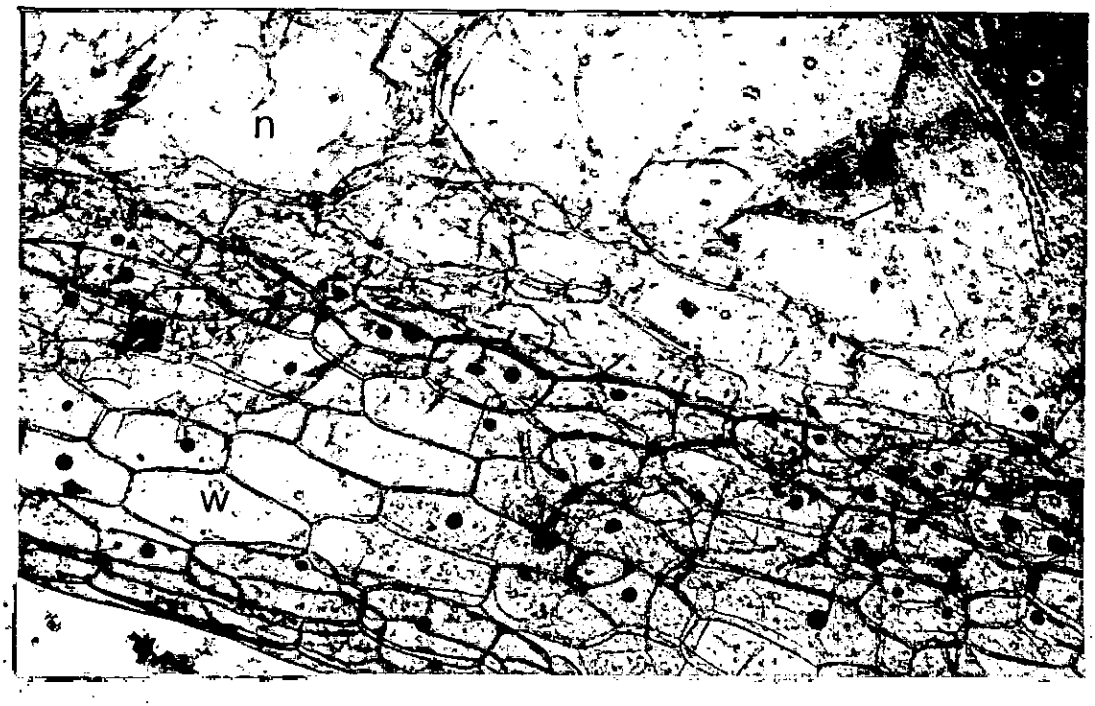


Effect of infection on the food contents of onions

Qualitative food tests carried out on both healthy and infected onion tissues showed the presence of sugars, amino acids and lipids. Bulbs inoculated with pathogens and incubated for five days were analysed for their food contents.

Paper chromatographic studies showed the presence of sucrose, fructose and glucose in the healthy bulbs. Infection however removed some of these sugars (Table 3). Total sugar content in the healthy bulb was 6.6%/g fresh weight and it was 0.255%, 0.36% and 0.12% for P.fluorescens, P.cepacia and C.utilis infections respectively (Table 2).

The amino acids found in the healthy tissue were glycine, alanine and tyrosine; but infection removed some of them (Table 3). Total amino acid in the healthy bulb was 0.019%/g fresh weight and it was 0.008%, 0.013% and 0.027% for P.fluorescens, P.cepacia and C.utilis infections respectively (Table 2).

There was no appreciable change in lipid content of the fresh bulb and bulbs infected with the bacterial pathogens, 0.53%/g fresh weight in the healthy bulb and 0.55% and 0.57% for P.flucrescens and P.cepacia infections respectively. However, bulb infected with C.utilis showed a decrease in lipid content (0.19%) (Table 2).

The water content of the bulbs showed little increase, 94%, 93% and 96% as compared to 91% in healthy bulb.

Table 2 : Concentration of food contents in the uninfected and infected leaf base of onions

Sample	Fresh weight (%/g)			
	Sugars	Amino Acid	Lipid	Water
1. Fresh onion	6.600	0.019	0.530	91
2. Onion infected with *P.fluorescens*	0.255	0.008	0.550	94
3. Onion infected with *P.cepacia*	0.360	0.013	0.570	93
4. Onion infected with *C.utilis*	0.120	0.027	0.190	96

Infected bulbs were incubated for five days at room temperature prior to analysis.

Table 3 : Summary of sugars and amino acids present in infected and healthy onions.

Sample	Sugars	Amino acids
1. Fresh onion	Glucose, fructose, sucrose	Glycine, alanine and tyrosine
2. Onion bulb infected with *P.fluorescens*	Glucose	Alanine and tyrosine
3. Onion bulb infected with *P.cepacia*	Glucose	Alanine and tyrosine
4. Onion bulb infected with *C.utilis*	Sucrose	Alanine and glycine

Effect of food substances on growth of isolates:

All the pathogens grew well on most of the food substances when incorporated into either nutrient medium or basal medium. They did not, however, show clearing on cellulose agar (Table 4) even though some growth occurred on the medium containing nutrients.

Table 4:

Medium	Candida utilis	P. fluorescens	P. cepacia
1.Lipid + Nutrient agar	+/H	+	+/H
Lipid + Agar	+	+	+
Lipid + Basal medium	+/H	+	+/H
2.Starch + Nutrient agar	+	+	+
Starch + Agar	+	+	+
Starch + Basal medium	+	+	+
3.Cellulose + Nutrient agar	+	+	+
Cellulose + Agar	-	-	-
Cellulose + Basal medium	+	+	+
4.Pectin + Nutrient agar	+	+	+
Pectin + Agar	+	-	-
Pectin + Basal medium	+	+	+

Medium	Candida utilis	P fluorescens	P cepacia
5.Protein +			
Nutrient agar	+	+	+
Protein + Agar	+	+	+
Protein + Basal			
medium	+	+	+

Key:- + = Growth

+/H = Growth and hydrolysis

\- = No growth

From the pathogenicity tests, P.fluorescens was the most virulent of the three isolates. It was therefore selected for subsequent detailed studies.

Growth studies of P. fluorescens

1. Growth on different solid media at different temperatures:

Pseudomonas fluorescens grew best at room temperature irrespective of the media used. There was no growth at 44°C while little growth occurred on some media at 4°C and 37°C (Table 5).

Nutrient agar and malt extract agar were the best media for growth (Table 5).

Table 5: Number of colonies of P.fluorescens developing on solid media at different temperatures when inoculated with 9.2×10^7 cells/ml.

Media	Number of colonies/ml of inoculum			
	4°C	29± 2°C	37°C	44°
1. MacConkey agar	$7.4 \times 10^{3\pm}$ .61	$3.4 \times 10^{7\pm}$ .47	-	-
2. Yeast extract agar	-	$2.8 \times 10^{7\pm}$ .74	-	-
3. Deoxycholate citrate agar	$7.12 \times 10^{3\pm}$ .74	$3.0 \times 10^{7\pm}$ .65	$3.2 \times 10^{3\pm}$ .78	-
4. Endo agar	-	$2.3 \times 10^{7\pm}$ .54	-	-
5. Onion extract agar	-	$8.7 \times 10^{7\pm}$ .72	$2.8 \times 10^{3\pm}$ .51	-
6. Tryptic Soy agar	-	$2.3 \times 10^{7\pm}$ .92	$1.8 \times 10^{3\pm}$ .45	-
7. Czapek solution agar	-	$6.5 \times 10^{5\pm}$ .85	-	-
8. Potato dextrose agar	$1.5 \times 10^{3\pm}$ .53	$4.5 \times 10^{\pm}$ .92	$5.8 \times 10^{5\pm}$ .35	-
9. Levine EMB agar	-	$5.3 \times 10^{5\pm}$ .84	$2.9 \times 10^{5\pm}$ .36	-
10. Malt extract agar	$3.1 \times 10^{3\pm}$ .21	$6.5 \times 10^{7\pm}$ .47	$1.5 \times 10^{7\pm}$ .40	-
11. Violet red bile agar	-	$4.5 \times 10^{7\pm}$ .62	$1.9 \times 10^{5\pm}$ .47	-
12. Nutrient agar	$1.9 \times 10^{3\pm}$ .47	$5.3 \times 10^{7\pm}$ .83	$3.3 \times 10^{7\pm}$ .88	-
13. Corn meal agar	-	$2.0 \times 10^{7\pm}$ .54	$3.8 \times 10^{5\pm}$ .17	-
14. Simmon citrate agar	-	$5.2 \times 10^{5\pm}$ .96	$2.0 \times 10^{5\pm}$ .47	-
15. Brilliant green agar	-	$1.7 \times 10^{7\pm}$ .47	$3.0 \times 10^{5\pm}$ .88	-

2. Growth in liquid media:

Nutrient broth was found to support more growth of P.fluorescens than onion broth. The bacterium grew in nutrient broth at 4°C, 29± 2°C and at 37°C; whereas growth in onion broth was observed only at 29± 2°C (Fig. 2a & b).

3. Growth on different carbon sources:

Pseudomonas fluorescens could utilise all the sugars used as sole carbon sources. Glucose was the best carbon

source while galactose supported the poorest growth (Fig 3a).

Growth was best at 1 - 2% ($^{W}/_{v}$) glucose concentration. Increasing glucose concentration above 2% ($^{W}/_{v}$) did not enhance growth; rather, growth was markedly retarded in 3 - 4% ($^{W}/_{v}$) glucose concentration (Fig 3b).

4. Growth on different nitrogen sources:

The nitrogen sources used in this study were sodium nitrate, tyrosine, alanine, glycine, ammonium sulphate and asparagin. Growth was best when the nitrogen source was glycine and poorest when alanine and tyrosine were used (Fig 4a).

A glycine concentration range of 0.8 - 1.06% ($^{W}/_{v}$) was observed to be required for the best growth. Growth in solutions containing 0.26 - 0.52% ($^{W}/_{v}$) glycine was very little while 1.33 - 1.6% ($^{W}/_{v}$) glycine resulted in very poor growth. Glycine used at 1.6% ($^{W}/_{v}$) in the medium appeared to have inhibitory effect on growth after 10h. (Fig 4b).

5. Growth at different hydrogen ion concentrations:

Growth of P.fluorescens did not occur in very acidic medium; for example pH = 2, instead there was a decrease in cell number as compared with the inoculum probably implying cell death. Also very alkaline medium of pH = 10 - 12 was inhibitory to growth and it appeared that the organisms disintegrated rather quickly. Growth was best at about neutral medium pH 6 - 8 (Fig 5).

Fig 2a : Growth of P.fluorescens in nutrient broth at different temperatures.

Growth of P.fluorescens was estimated on nutrient broth over a period of 30h. at different temperatures using the turbidimetric method. The temperatures used were 4°C ●——●, 29± 2°C O——O and 37°C □——□

Fig 2b : Growth of P. fluorescens in onion broth at different temperatures

Growth of P.fluorescens was estimated in onion broth over a period of 30h at different temperatures using the turbidimetric method. The temperatures used were 4°C ●——O, 29± 2°C O——O and 37°C □——□

Fig 2a

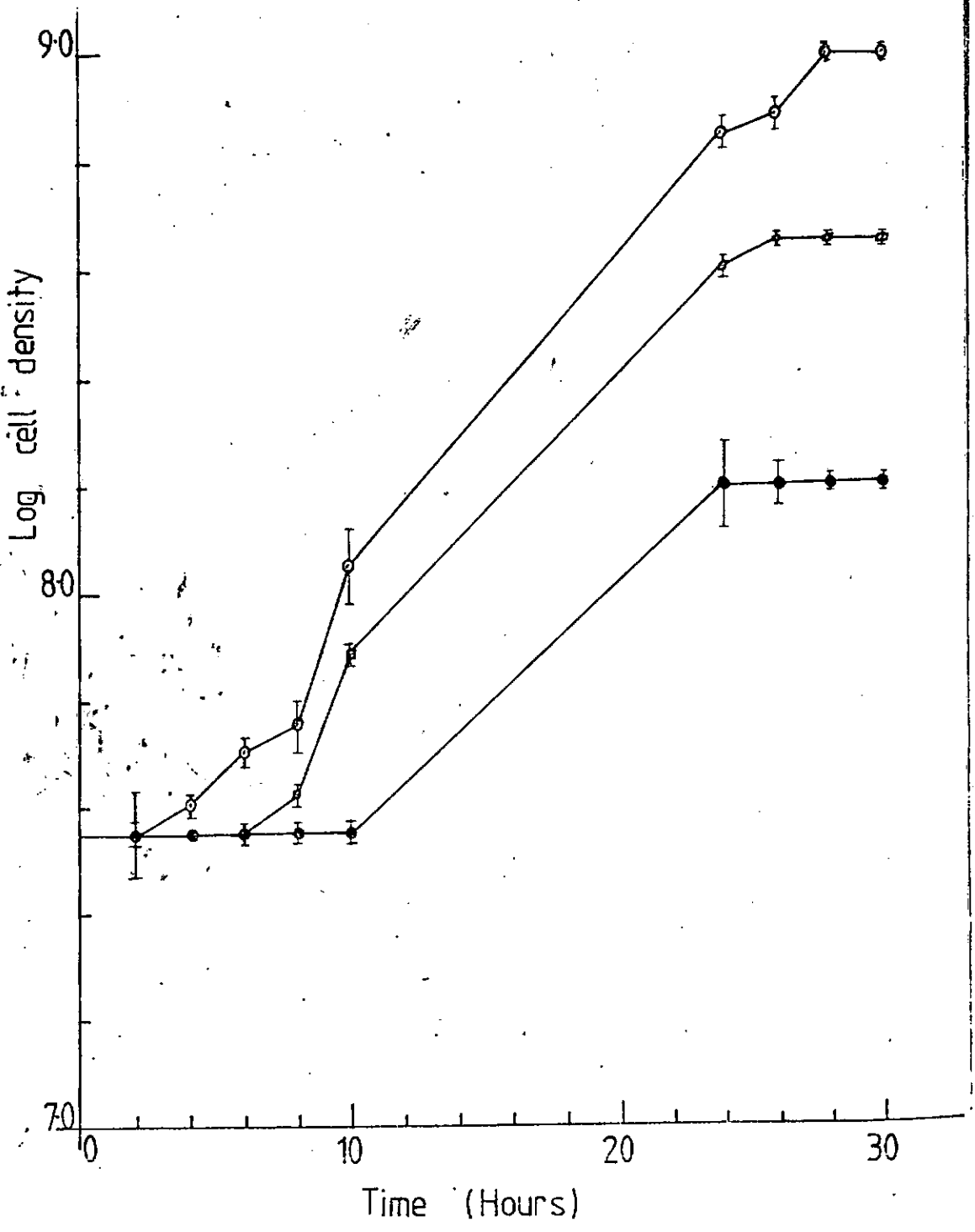


Fig. 2b

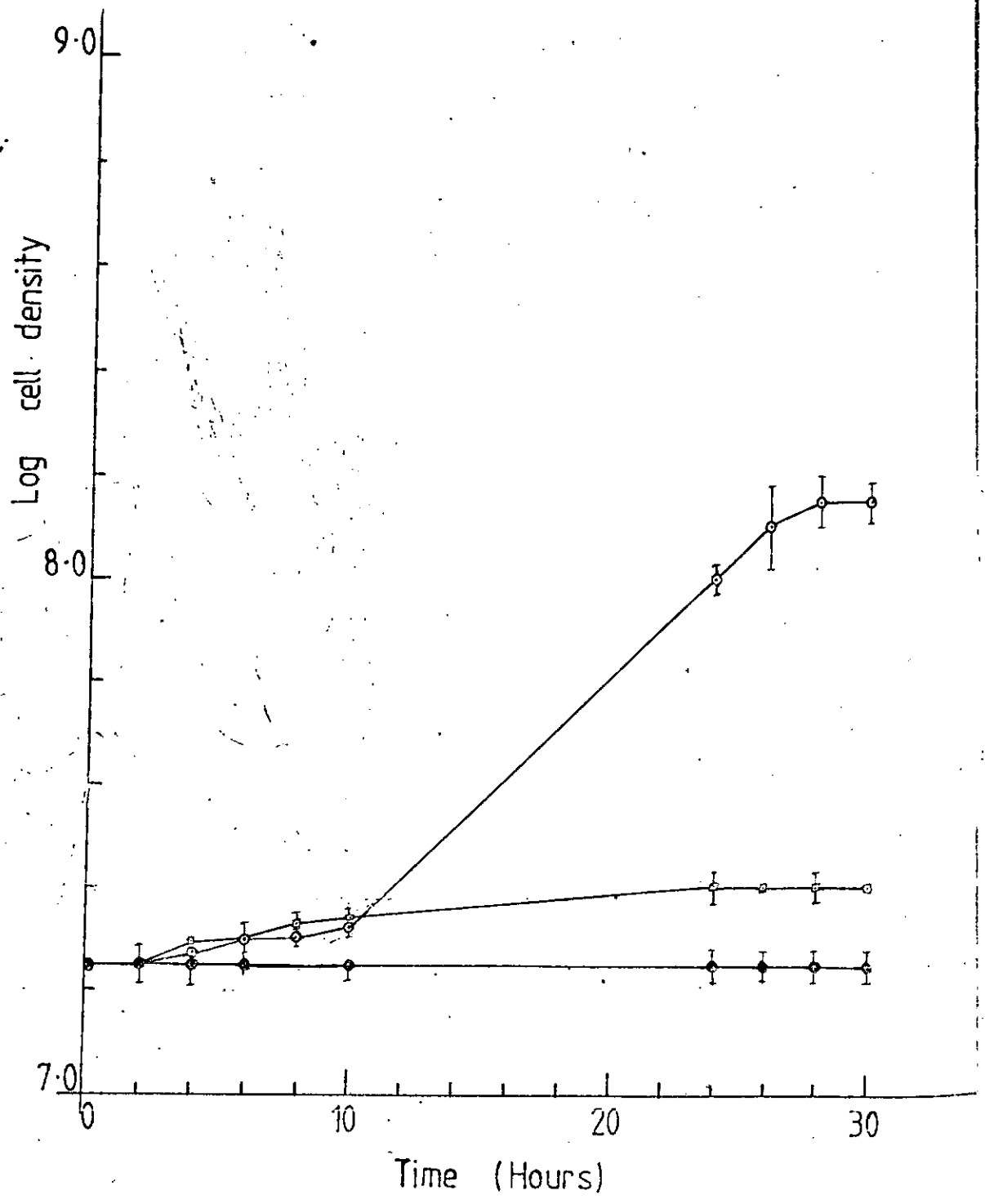


Fig 3a : Effect of different carbon sources on growth of P.fluorescens

Growth of P.fluorescens was estimated over a period of 30h on different carbon sources using the turbidimetric method. The carbon sources used were glucose x——x, fructose ⊠——⊠, sucrose O——O, maltose ●——● , saccharose △——△, galactose ▲——▲ , lactose ◧——◧ and mannitol ▩——▩

Fig 3b : Effect of different glucose concentrations on growth of P.fluorescens.

Growth of P.fluorescens was estimated over a period of 30h at different glucose concentrations using the turbidimetric method. The concentrations used were in g/100ml distilled water and they were 0.25% x——x , 0.5% ⊠——⊠ 1.0% O——O , 1.5% ◉——◉ , 2.0% △——△ 3.0% ▲——▲ and 4.0% □——□

Fig. 3a

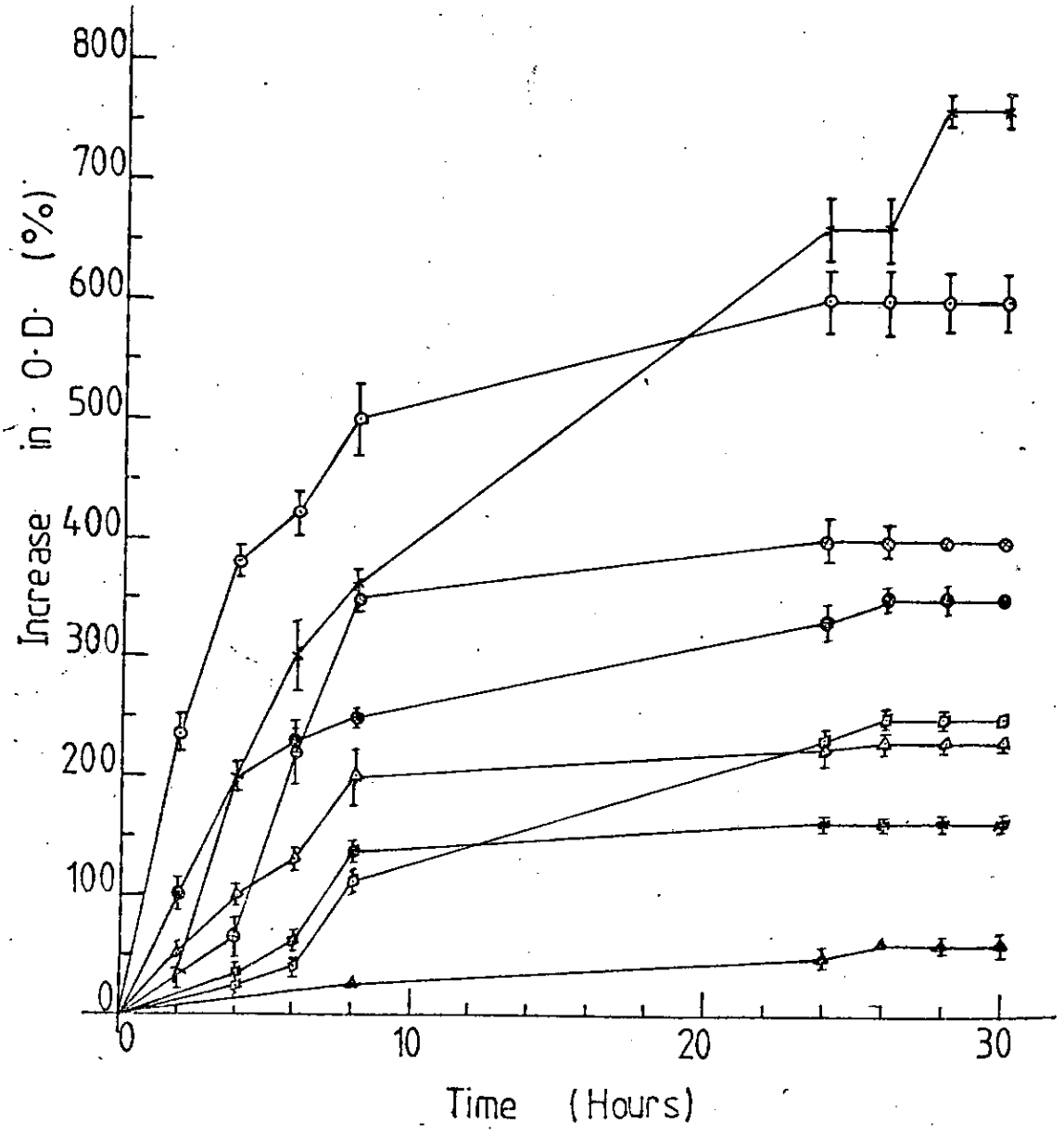

800
700
600
500
400
300
200
100
0
Increase in O·D· (%)
0
10
20
30
Time (Hours)

Fig. 3b

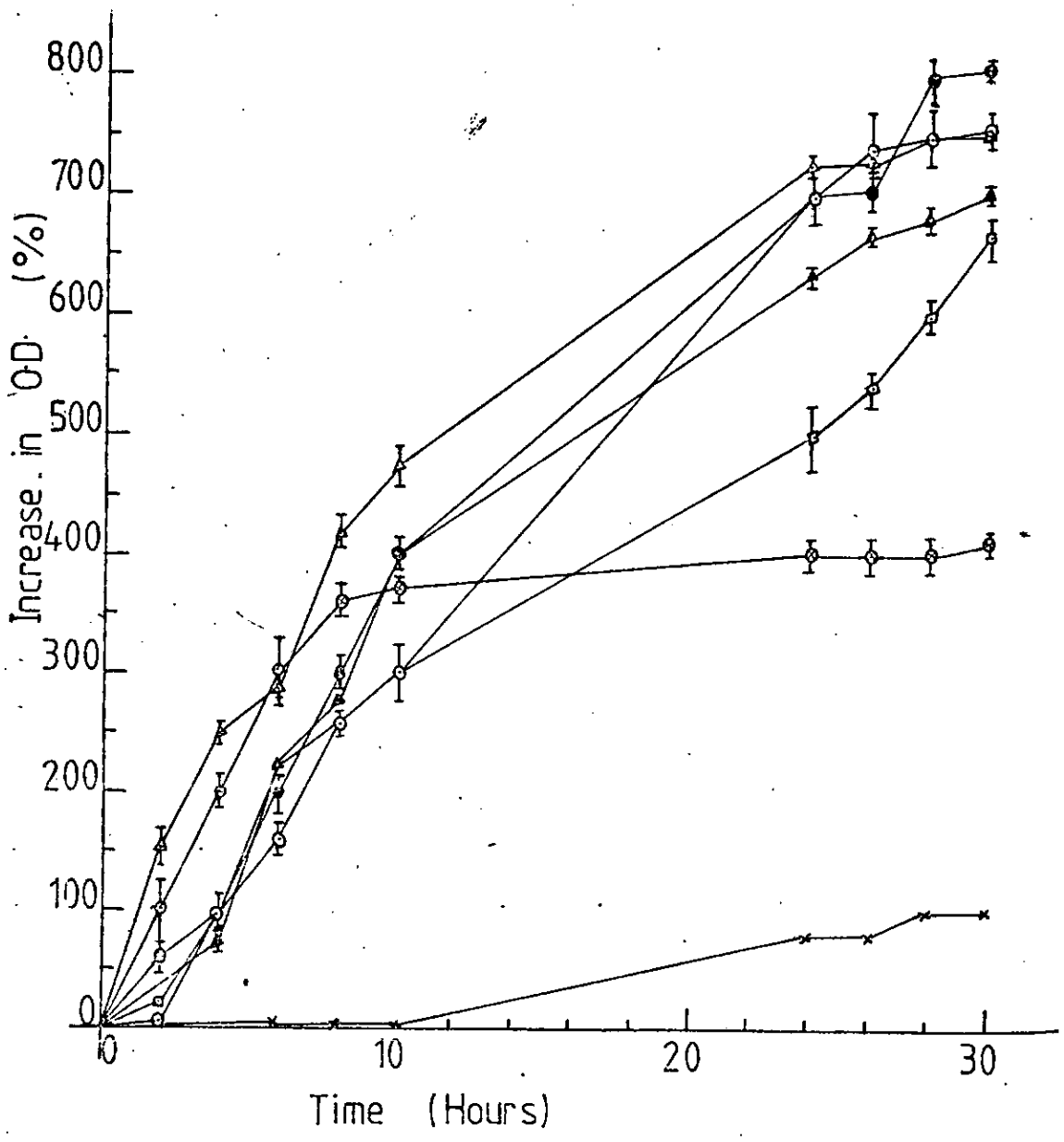

800
700
600
500
400
300
200
100
0
Increase in OD (%)
0
10
20
30
Time (Hours)

Fig 4a : Effect of different nitrogen sources on growth of P.fluorescens

Growth of P.fluorescens was estimated over a period of 30h on different nitrogen sources using the turbidimetric method. The nitrogen sources were sodium nitrate x——x, tyrosine ⊠——⊠ , glycine O——O , alanine ⊙——⊙, ammonium sulphate △——△ and asparagin ▲——▲

Fig 4b :: Effect of different glycine concentrations on growth of P.fluorescens

Growth of P.fluorescens was estimated over a period of 30h at different glycine concentrations using the turbidimetric method. The concentrations were in g/100ml distilled water and they were 0.26% x——x , 0.52% ⊠——⊠ , 0.8% O——O , 1.06% ⊙——⊙ , 1.33% △——△ and 1.6% ▲——▲

Fig. 4a

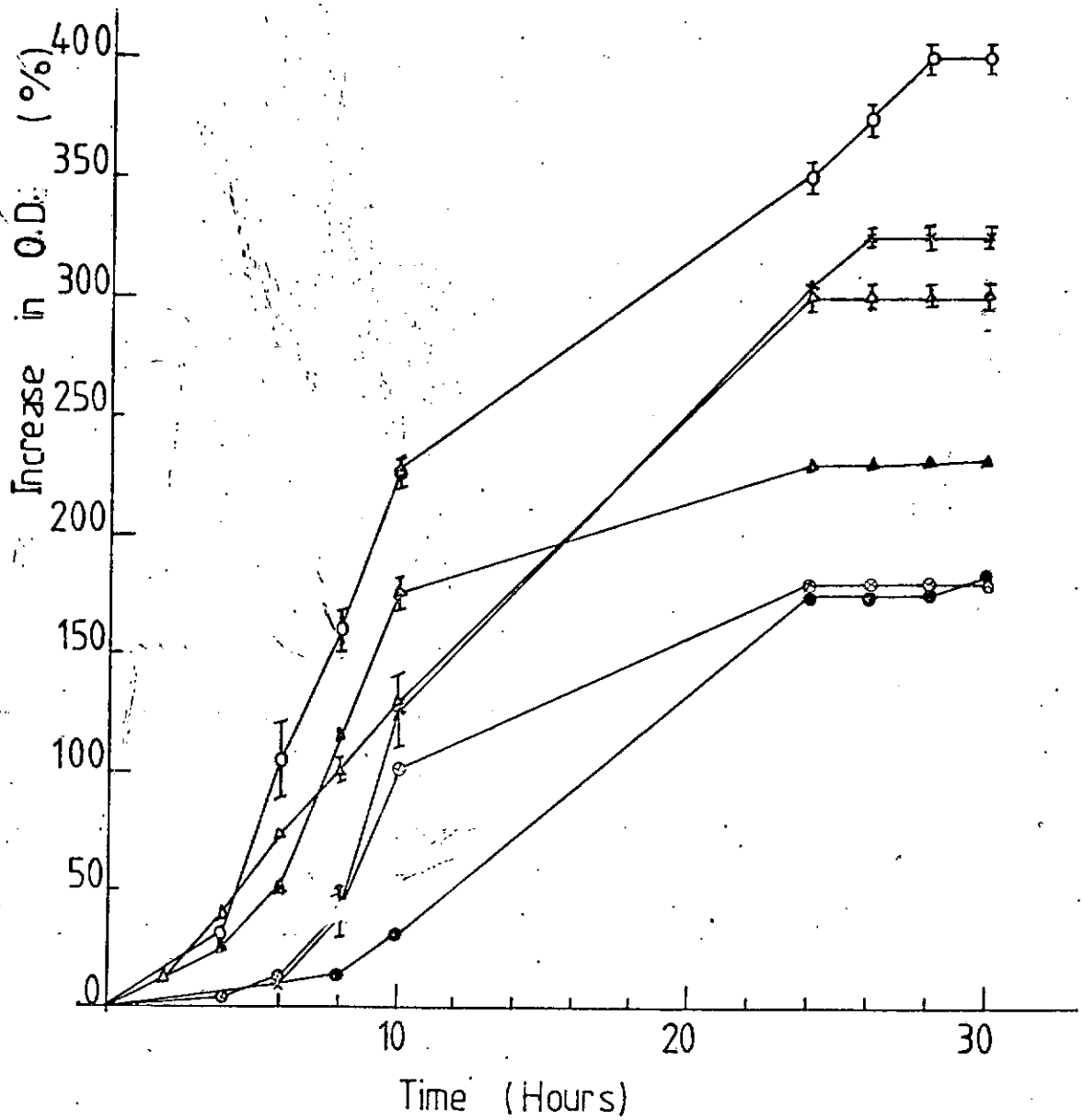

Increase in O.D. (%)
400
350
300
250
200
150
100
50
0
10
20
30
Time (Hours)

Fig. 4b

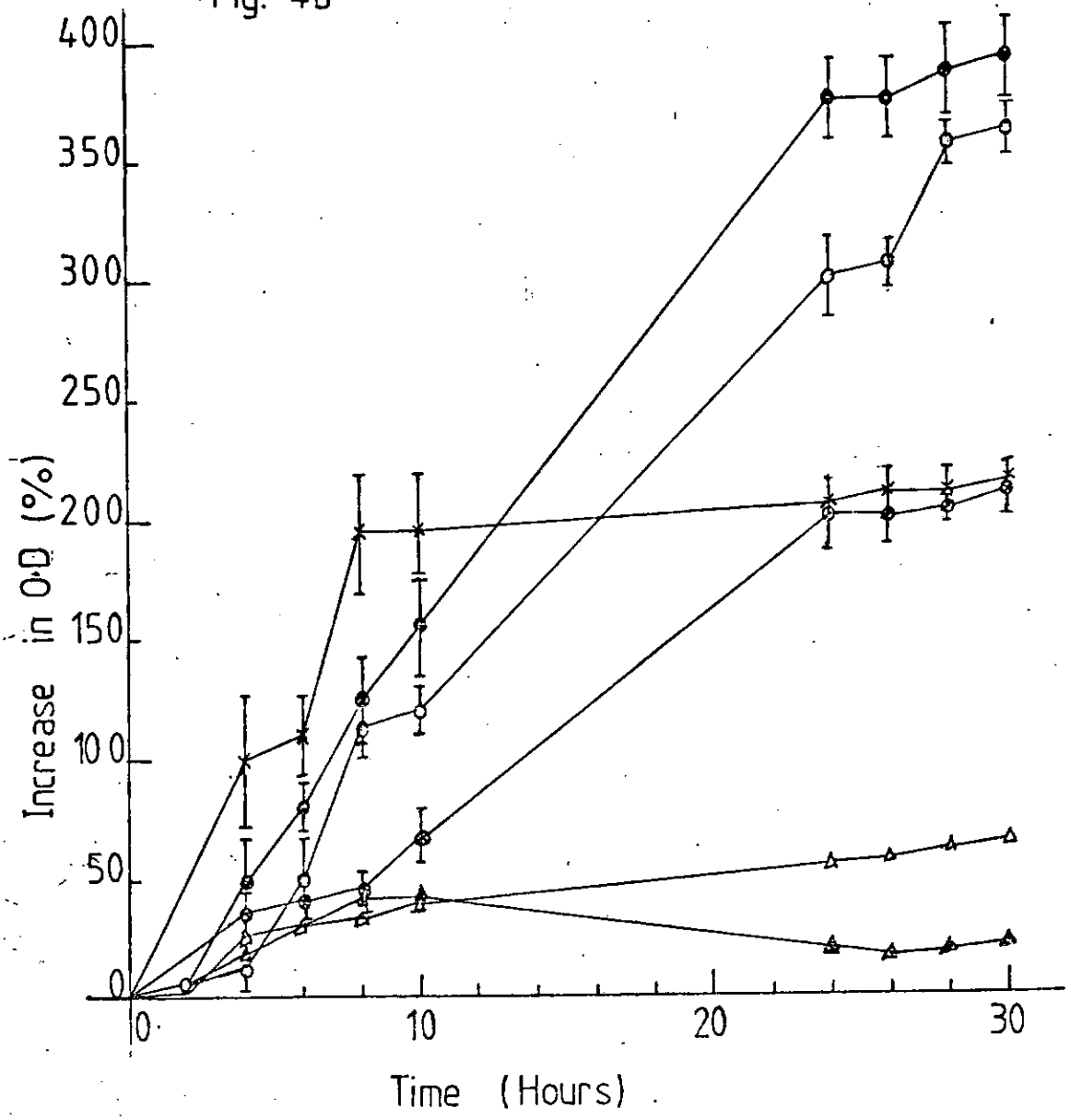
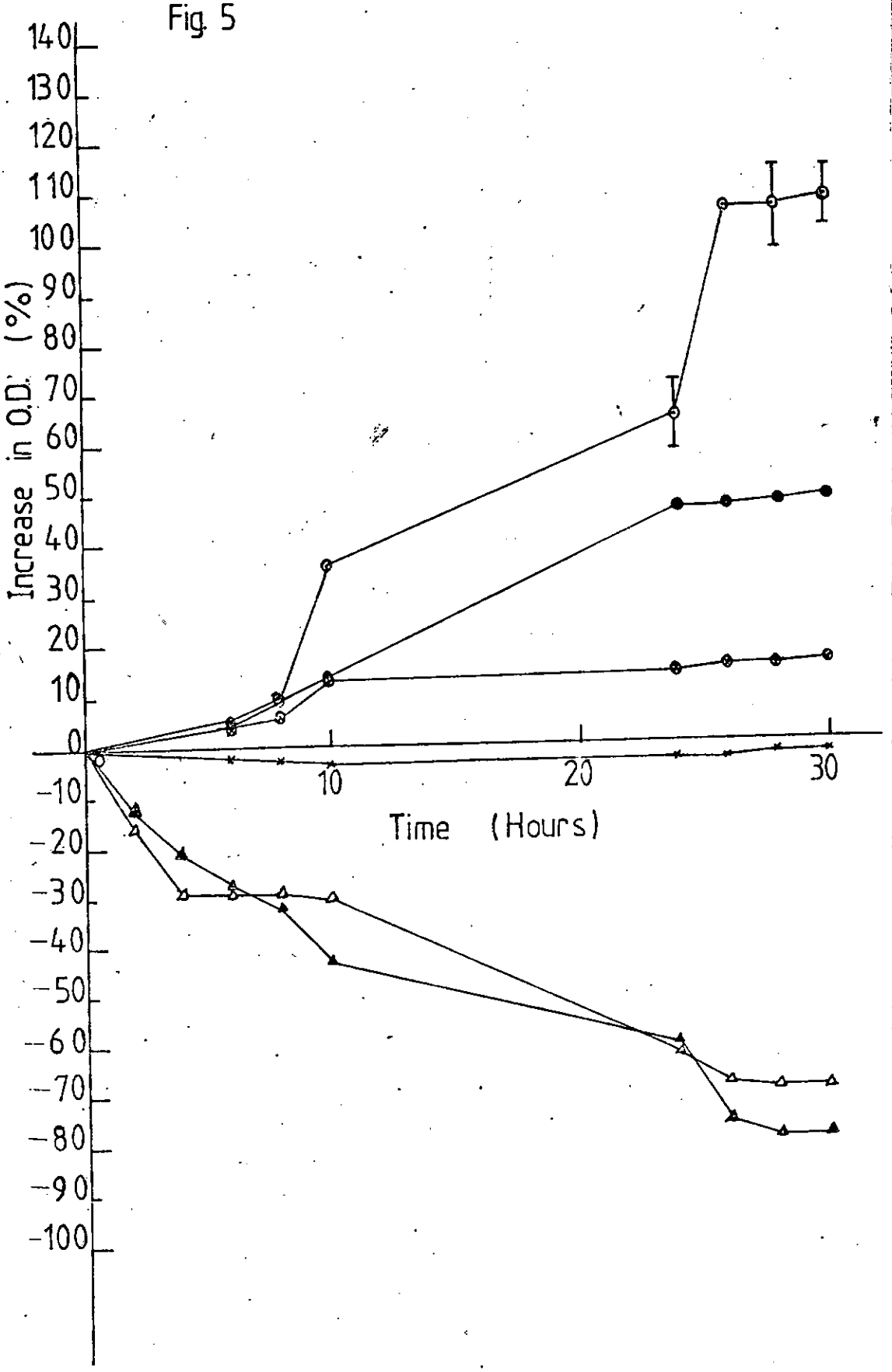

400
350
300
250
200
150
100
50
0
Increase in O.D (%)
0
10
20
30
Time (Hours)

Fig 5 : Effect of pH on growth of P.fluorescens

Growth of P.fluorescens was estimated over a period of 30h at different hydrogen ion concentrations of pH 2 x——x , pH 4 ⊠——⊠ , pH 6 o——o , pH 8 ⊙——⊙ pH 10 △——△ and pH 12 ▲——▲ using the turbidimetric method.

Fig. 5


140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
Increase in O.D. (%)
10
20
30
Time (Hours)

6. Growth on the onion bulb.

Experiment carried out to show the number of cells of P.fluorescens present in rotted tissue as days of incubation progressed showed that there was an increase till the 5th day of incubation after which there was a slight decrease till the 7th day (Fig 7)

Rot development also increased steadily up to the 7th day even though increase in cell number did not proceed further than the 5th day (Fig 6).

Fig 6 : Extent of rot development on onion bulb inoculated with P.fluorescens

Rot development was estimated by measuring the diameter of rotted tissue every day after inoculation for 7 days.

Fig. 6

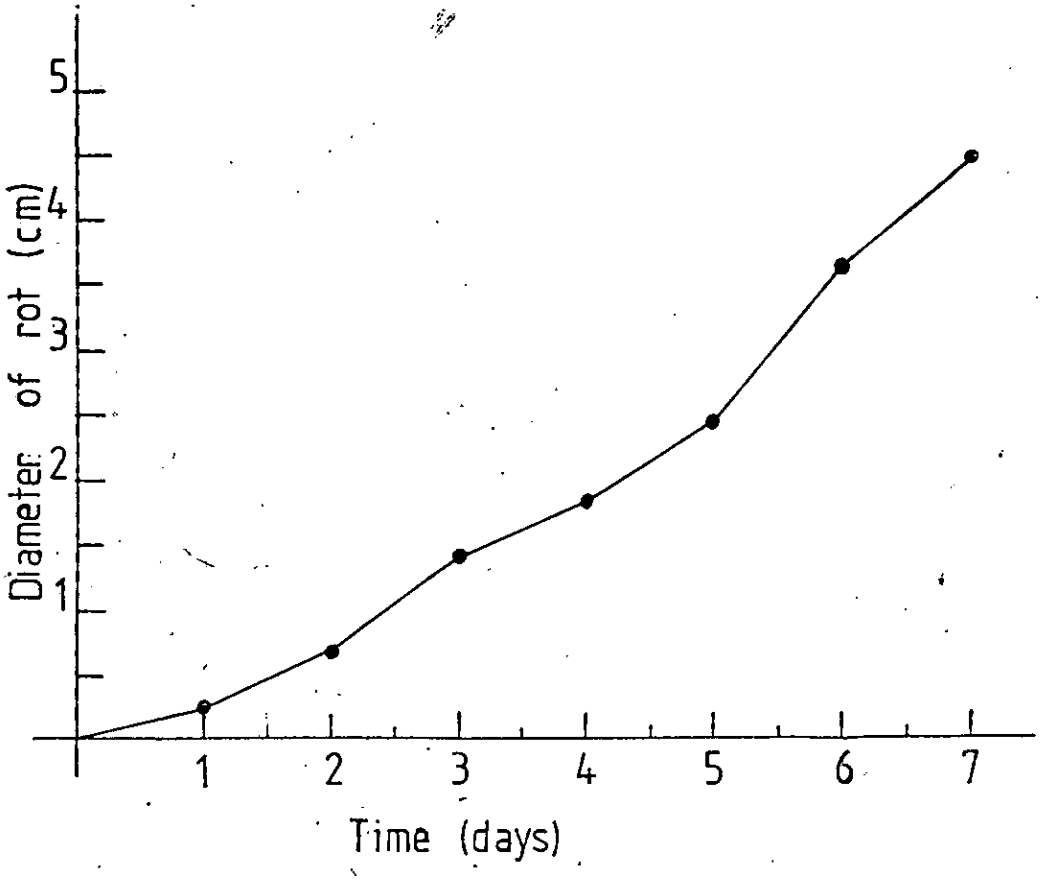


Fig 7 : Growth of P.fluorescens when inoculated into onion bulbs

Onion bulbs were inoculated with 3.2×10^9 cells and incubated at room temperature. At 24h intervals rotted tissue was removed with the aid of a 1cm diameter cork borer. The tissue was crushed in 10ml sterile distilled water and the suspension estimated for total cells present using the dilution plate method.

Fig. 7

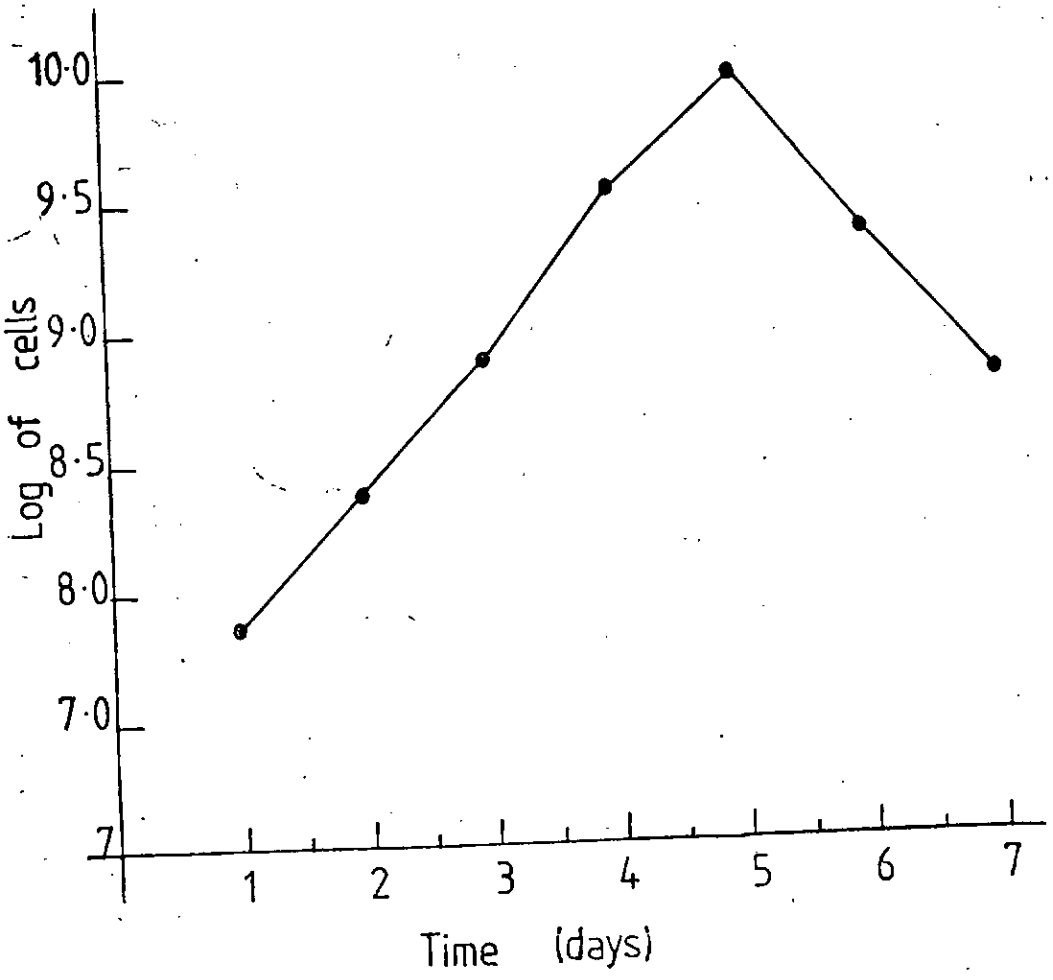


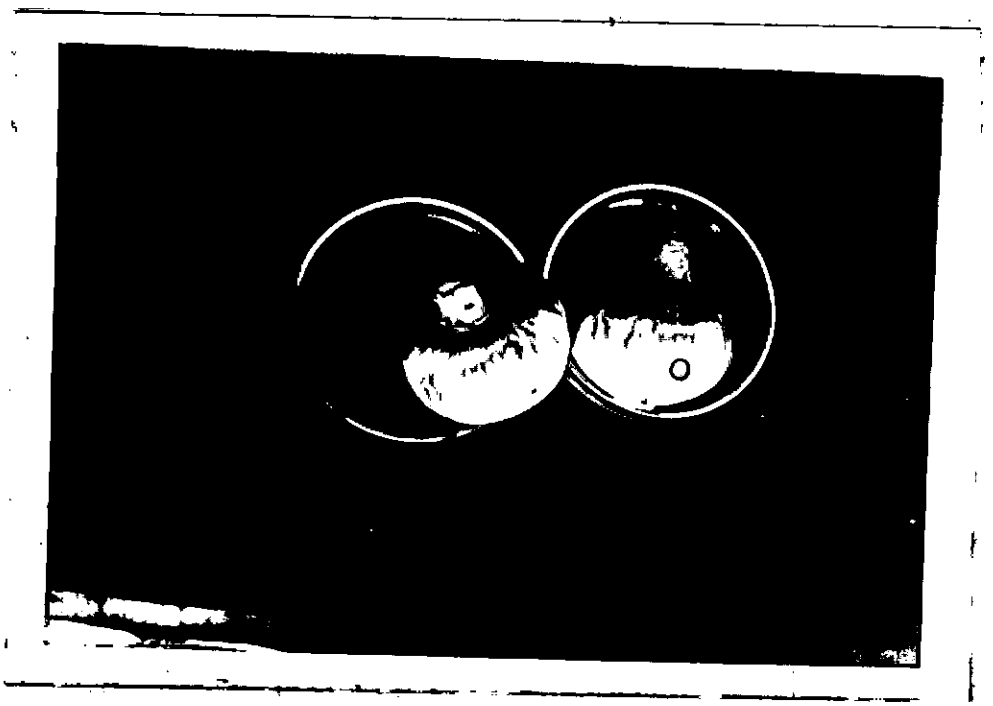
Plate 12A : Onion bulbs inoculated with P.fluorescens and incubated at room temperature for 3 days (x½)

Plate 12B : Onion bulbs inoculated with sterile distilled water and incubated at room temperature for 3 days (x½)

Inoculated areas are marked O

12A

12B



Pathogenicity tests on other vegetables

Tests on pathogenicity of *P.fluorescens* on cucumber, carrots, Irish potatoes, garlic and the white variety of onions showed that the pathogen was only effective on the white variety of onions and not on the others (Plates 13A, B, C, D, E, F, G, H, I and J).

Plate 13: (A) Cucumber inoculated with *P.fluorescens* (x½) - s

(B) Cucumber inoculated with sterile distilled water (x½) - r

(C) Potatoes inoculated with *P.fluorescens* (x½) - m

(D) Potatoes inoculated with sterile distilled water - n (x½)

(E) Carrots inoculated with *P.fluorescens* (x½) - t

(F) Carrots inoculated with sterile distilled water - u (x½)

(G) Garlic inoculated with *P.fluorescens* (x⅓) - w

(H) Garlic inoculated with sterile distilled water - z (x⅓)

(I) White variety onions inoculated with *P.fluorescens* - h (x¼)

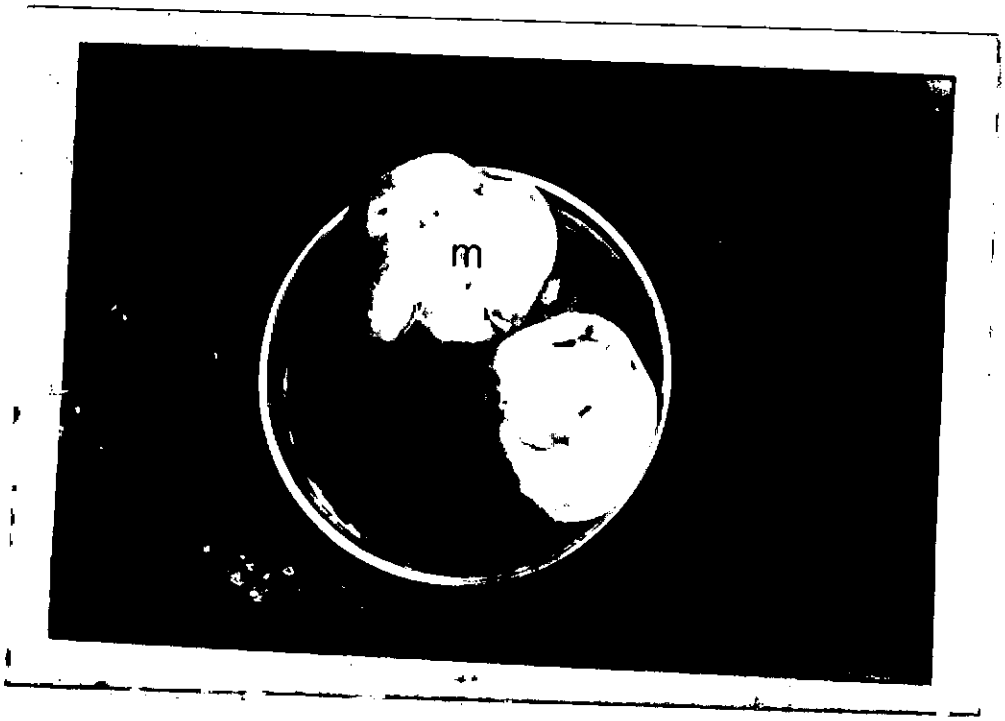
(J) White variety onions inoculated with sterile distilled water - g (x¼)

All incubations were done at room temperature for 7 days.

13A

B

C



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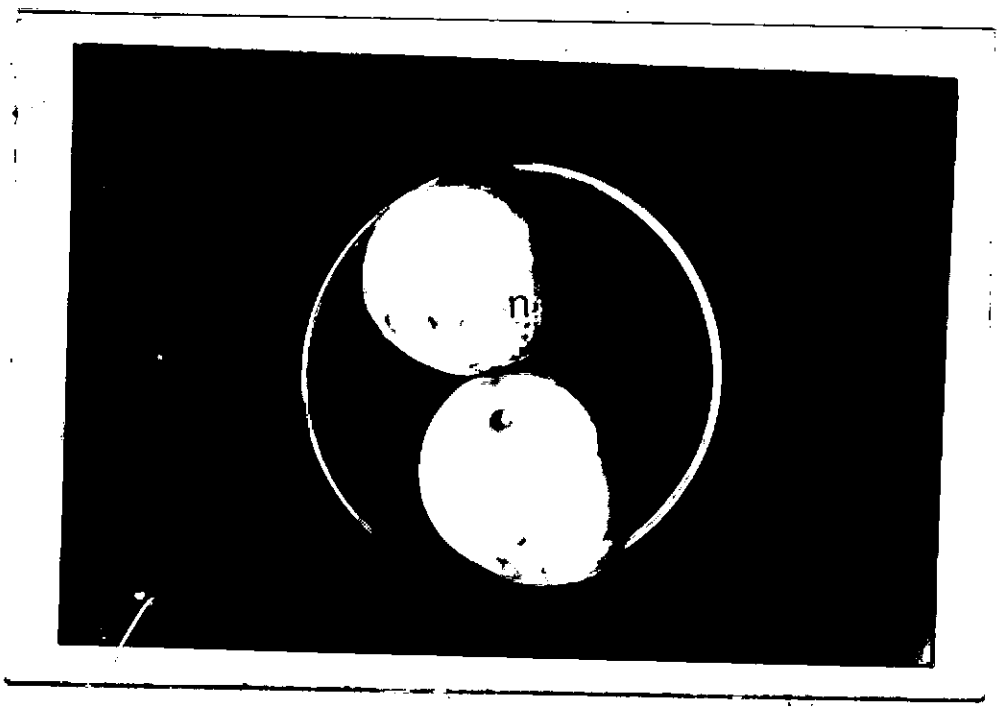
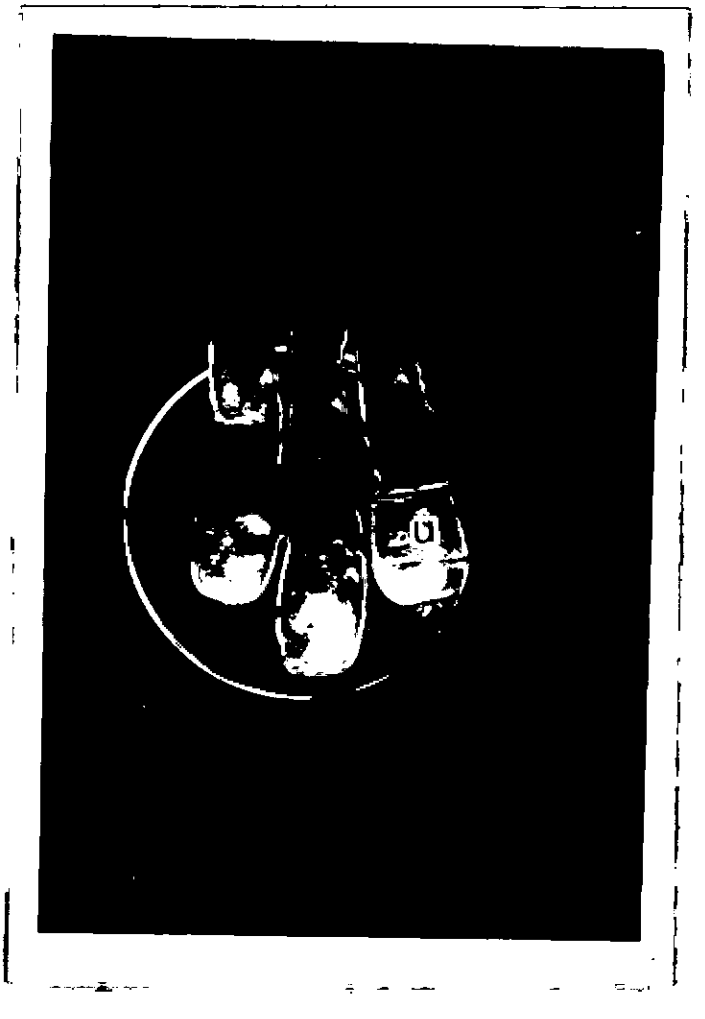
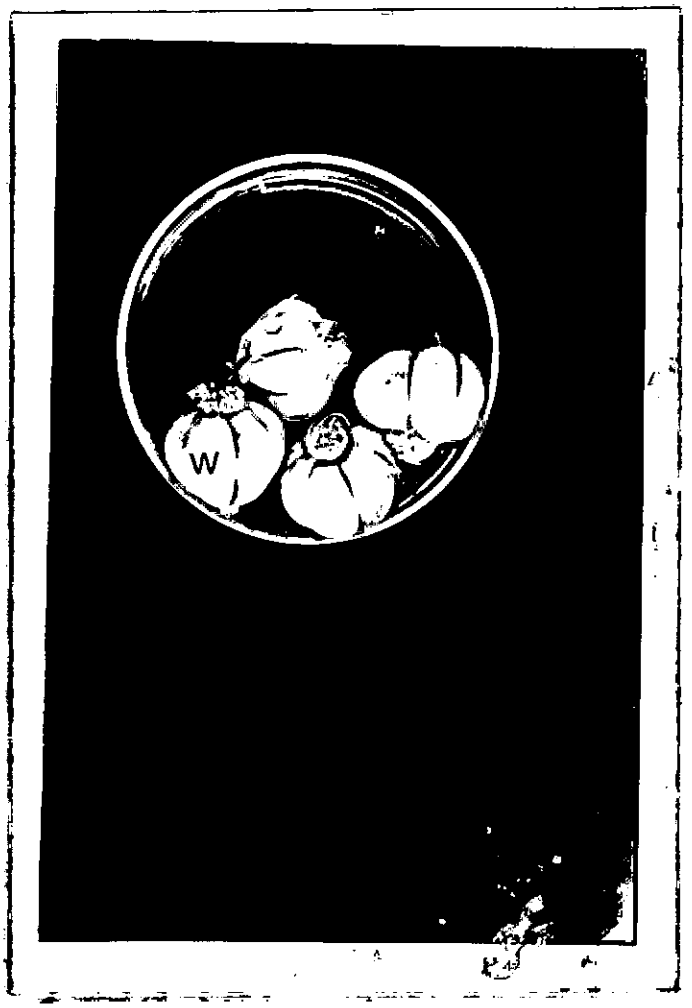


E

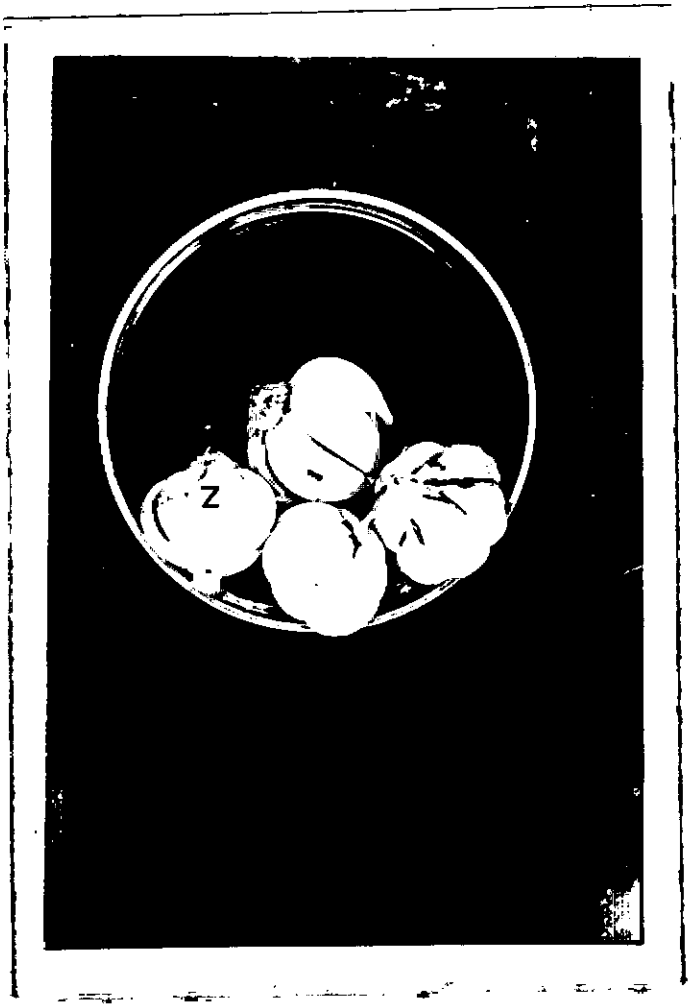
F



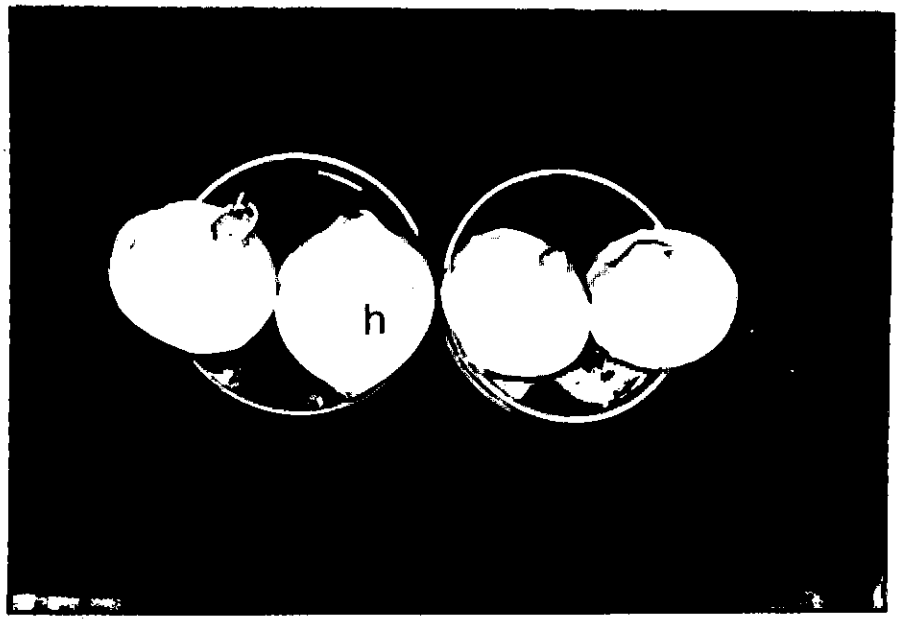
G



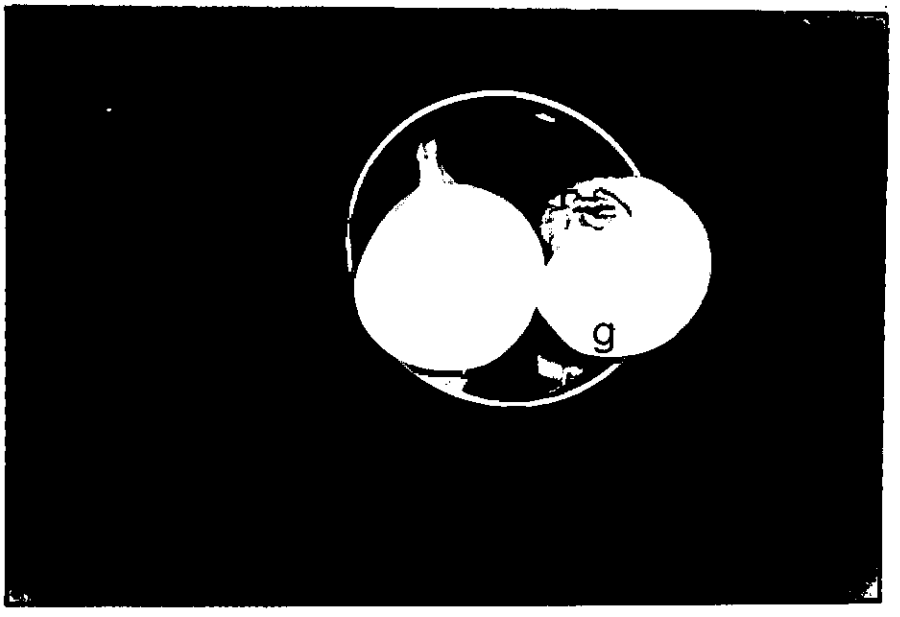
H



I



J



Influence of environmental conditions on development of onion rot caused by Pseudomonas fluorescens.

1. Effect of temperature:

Room temperature (29± 2°C) was the best temperature for development of rot. There was no rot formation at -20°C and at 4°C while at 37°C very little rot developed (Table 6).

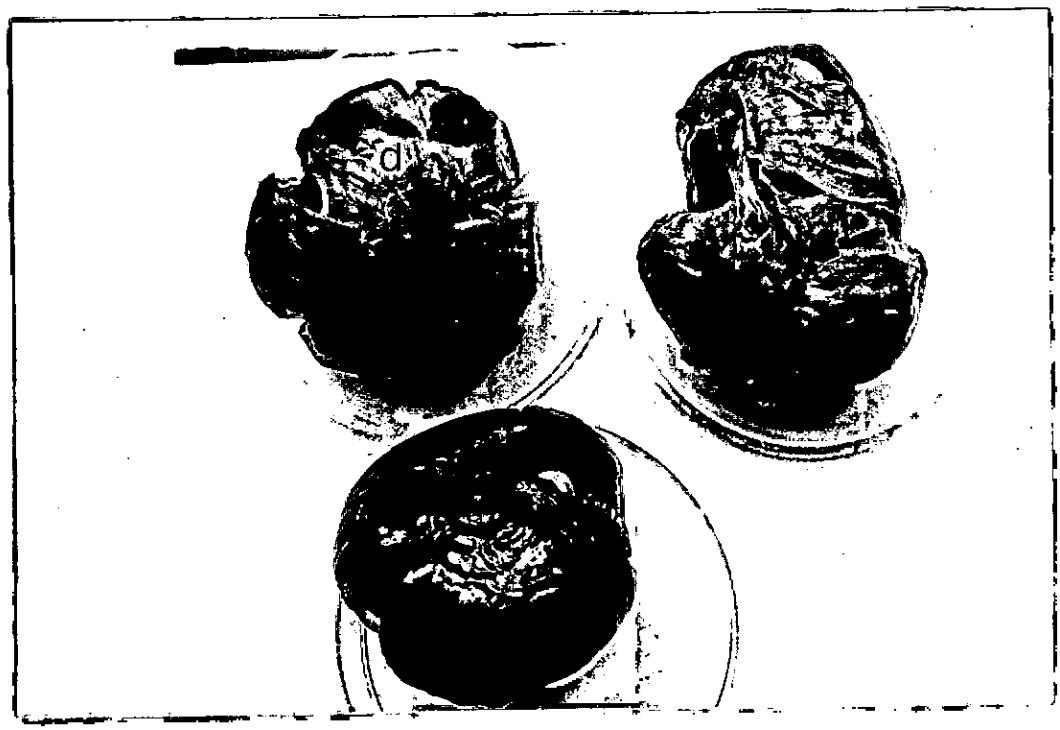
There was total collapse of the inoculated and control samples incubated at 44°C (Plate 14).

Table 6: The effect of temperature on onion rot development by P.fluorescens after 7 days incubation

Temperature (°C)	Diameter of rot (cm)
Control	0
4	0
44	0
-20	0
29± 2	4.36 ± 0.17
37	0.21 ± 0.14

Plate 14 : Onion bulbs inoculated with
P.fluorescens and distilled water - d
They were incubated at 44°C for
7 days (x½).

14



2. Effect of relative humidity

High relative humidity favoured development of rot. Rot development was not observed at 50% R.H. Fifty percent rot was observed at 85 - 90% R.H. (Table 7).

Table 7: Effect of relative humidity on onion rot development after incubation for 7 days.

R.H. (%)	Diameter of rot (cm)	% maximum rot
Control	0	0
50	0 ± 0.019	0
55	0.06 ± 0.04	22.3
60	0.15 ± 0.09	22.8
65	0.27 ± 0.10	23.4
70	0.42 ± 0.13	25.7
75	0.68 ± 0.21	22.9
80	1.38 ± 0.22	31.7
85	2.38 ± 0.21	46.5
90	2.99 ± 0.15	64.9
95	3.40 ± 0.16	88.4
100	4.53 ± 0.20	100

The greatest amount of rot was assigned 100% and lesser amounts of rot expressed as percentage of it.

3. Effect of anaerobic condition on rot development

Incubation of both inoculated and uninoculated bulbs in an atmosphere containing only nitrogen gas led to total collapse of bulbs within 7 days like the bulbs incubated at 44°C (Plate 14).

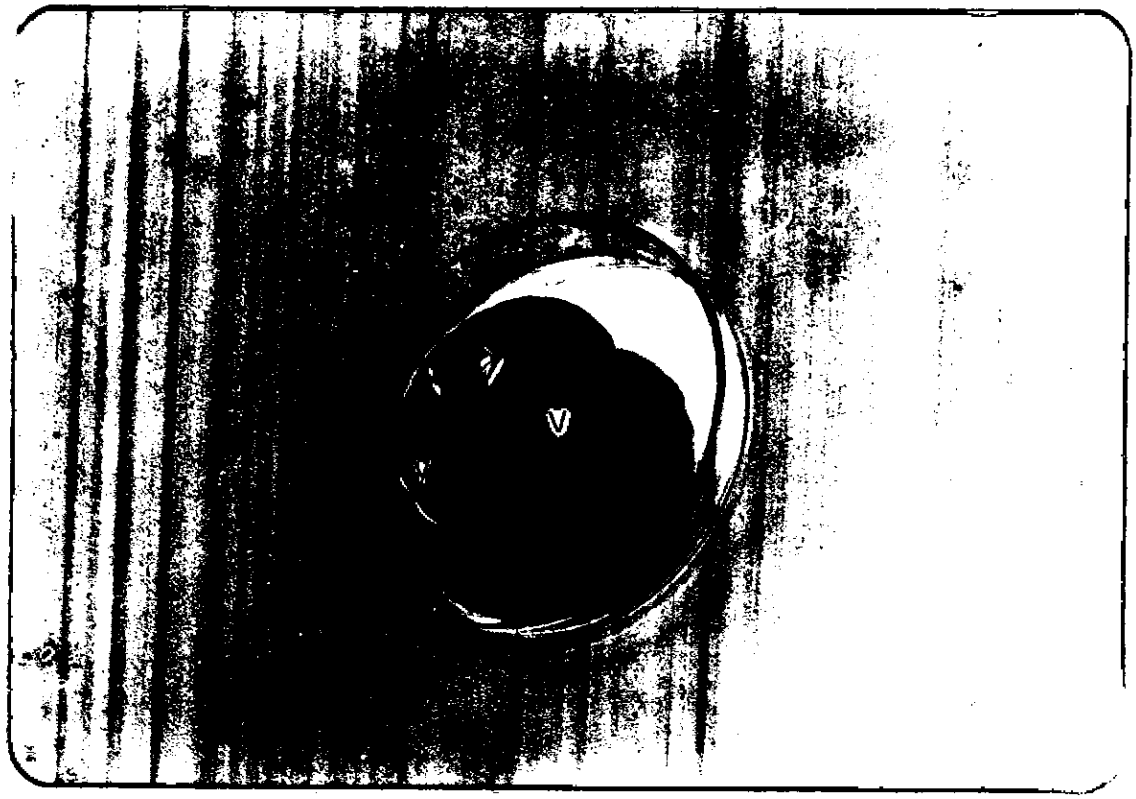
Plate 15A : Onion bulb inoculated with P.fluorescens and incubated at room temperature of 29° ± 2°C and relative humidity of 100% for 7 days (x¼)

Plate 15B : Onion bulb inoculated with P.fluorescens and incubated at room temperature of 29° ± 2°C and relative humidity of 80% for 7 days (x¼).

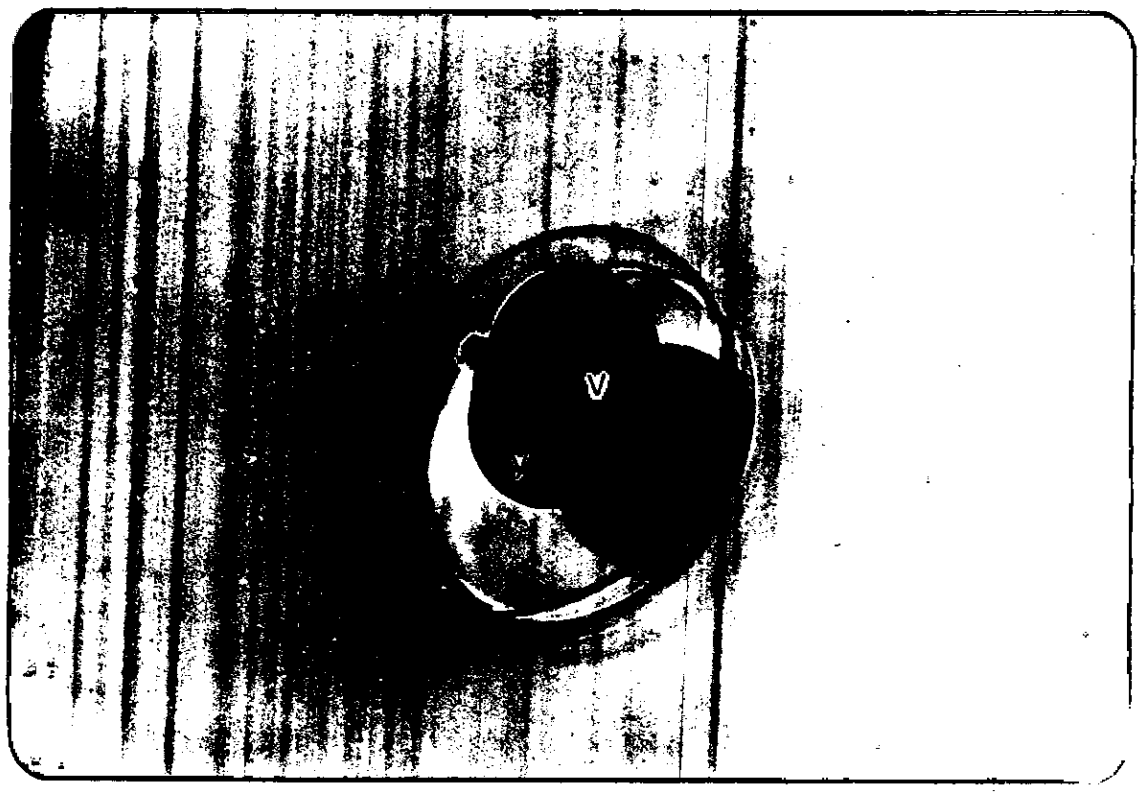
v shows areas of rot

y shows healthy tissues

15A



B



Enzyme Studies

1. Conditions for P.fluorescens enzyme production in culture.

Growth studies of P.fluorescens on different media containing pectin as carbon source showed that it was possible for the organism to produce pectic enzyme in culture media. The following preliminary experiments were aimed at establishing the best cultural conditions for pectic enzyme secretion by the organism.

(a) Effect of various carbon sources:

The carbon sources used were glucose, galacturonic acid, pectin, sodium polypectate, potato pectin and potato sodium polypectate. Results showed that pectic enzymes production by P.fluorescens was inductive (Table 8). No pectic enzyme was induced when carbon sources were glucose and galacturonic acid. Pectin, potato pectin and potato sodium polypectate all induced pectic enzyme production and the enzyme reduced the viscosity of the reaction mixture at pH 5 (Table 8).

Pectin was the best carbon source when present alone in the culture media and it was capable of producing an enzyme activity of 28.5 ± 0.42. Pectin was therefore used for production of pectic enzyme for all other preliminary experiments.

The effect of the concentration of pectin on enzyme activity was also investigated. High concentrations of 3 - 4% ($^{w}/v$) pectin was not favourable for enzyme activity. The best concentration was 2% ($^{w}/v$) (Table 9)

Table 8: Viscosity reducing activity of culture filtrate of P.fluorescens grown on media in different carbon sources.

Pectic substrate	Enzyme activity/ml
Glucose	0
Galacturonic acid	0
Sodium polypectate	0
Pectin	28.5 ± 0.42
Potato pectin	119.5 ± 0.51
Potato sodium polypectate	22.5 ± 0.34

Enzyme activity expressed in relative viscometric units was calculated from $\frac{1000}{t}$ where t = time (minutes) for 50% loss in viscosity of reaction mixture.

Table 9: Effect of different concentrations of pectin on enzyme activity.

Pectin (% $^{w}/_{v}$)	Enzyme activity /ml
1	79.9 ± 0.63
2	119.5 ± 0.59
3	0
4	0

(b) Effect of age of culture on enzyme activity:

Age of culture was found to affect enzyme activity and production. Results showed that viscosity reduction of the reaction mixture increased gradually up to the 6th day, and reached its peak, decreased till the 8th day and then increased again till the 10th day when the experiment was terminated (Fig 8).

(c) p^H effect on enzyme activity:

Enzyme activity was highest at pH 5 and was zero at pH 9. The inclusion of Ca^{++} in the reaction mixture slightly reduced the activity at pH 5 and there was no activity with Ca^{++} at pH 9 (Fig 9).

Fig 8 : Pectic enzyme activity of P. fluorescens on different days of incubation during a 10-day incubation period at 27°C

Pseudomonas fluorescens was grown in a basal medium containing pectin (2% W/v) as the only carbon source. The culture filtrates were obtained for assay using millipore filtration every day for 10 days. Enzyme activity was estimated viscometrically.

Fig. 8

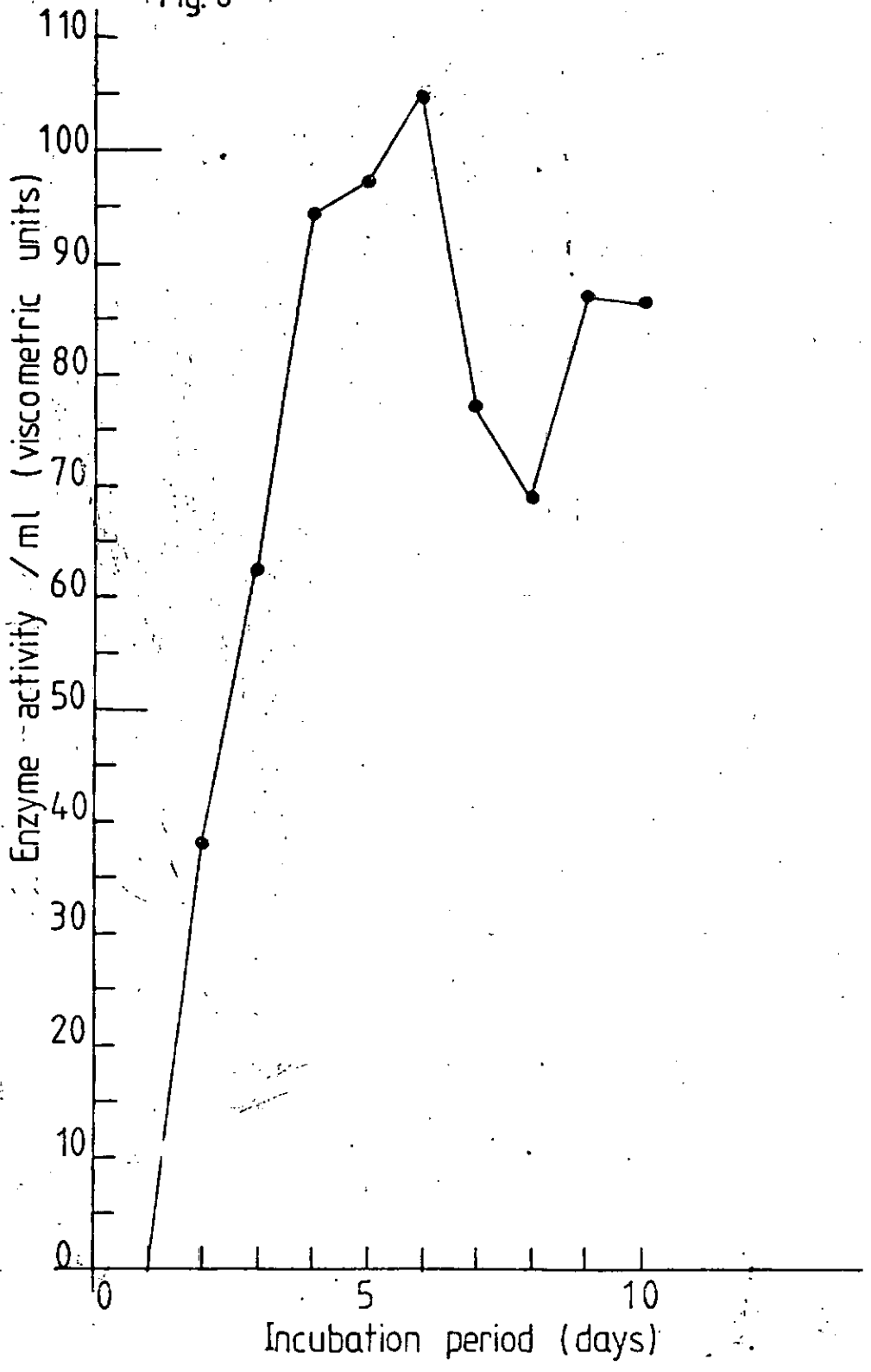
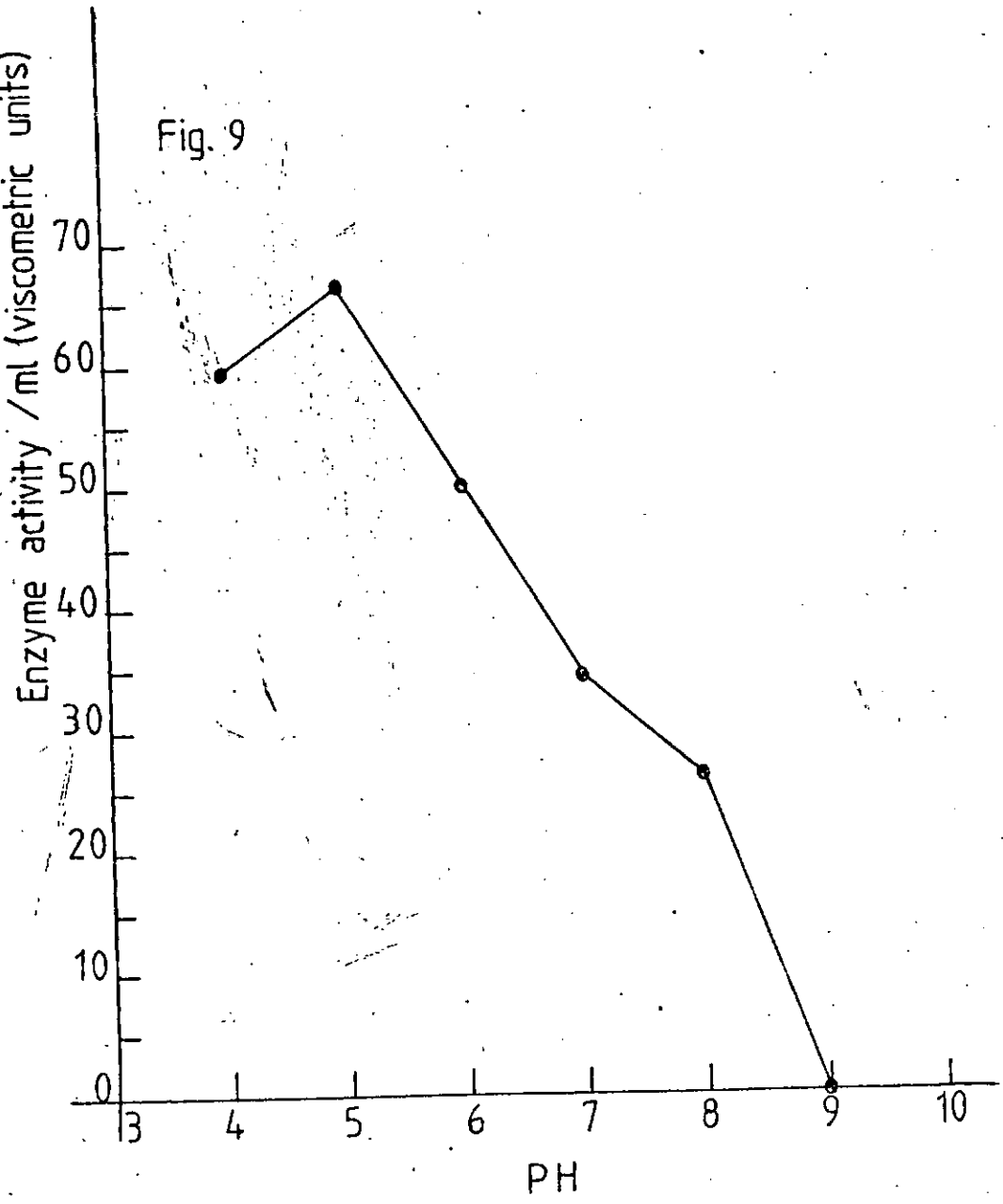

110
100
90
80
70
60
50
40
30
20
10
0
Enzyme - activity / ml (viscometric units)
0
5
10
Incubation period (days)

Fig 9: Effect of pH on activity of pectic enzyme from P.fluorescens at 27°C.

Pseudomonas fluorescens was inoculated into a basal medium containing pectin 2% (W/v) as the only carbon source. The culture filtrate was obtained using millipore filtration after 7 days incubation. Activity was estimated viscometrically using buffer solutions of pH 4 - 9 in the reaction mixtures.

Fig. 9


Enzyme activity /ml (viscometric units)
70
60
50
40
30
20
10
0
3
4
5
6
7
8
9
10
PH

Thiobarbituric acid reaction:

The thiobarbituric acid reaction carried out on the culture filtrate and rot extract showed maximum absorbance at 515nm. This implied the presence of hydrolytic polygalacturonase (Fig 10a & 10b)

Fig 10a : Thiobarbituric acid reaction on culture filtrate.

Pseudomonas fluorescens was inoculated in a medium containing only pectin as the carbon source and incubated for 7 days at room temperature. Thiobarbituric acid reaction was carried out on the culture filtrate.

Fig 10b : Thiobarbituric acid reaction on rot extract

Pseudomonas fluorescens was inoculated on fresh onion bulbs and incubated for 7 days at room temperature. The rotted tissues were removed and the filtrate extracted using millipore filtration. Thiobarbituric acid reaction was carried out on the rot extract.

Fig. 10a

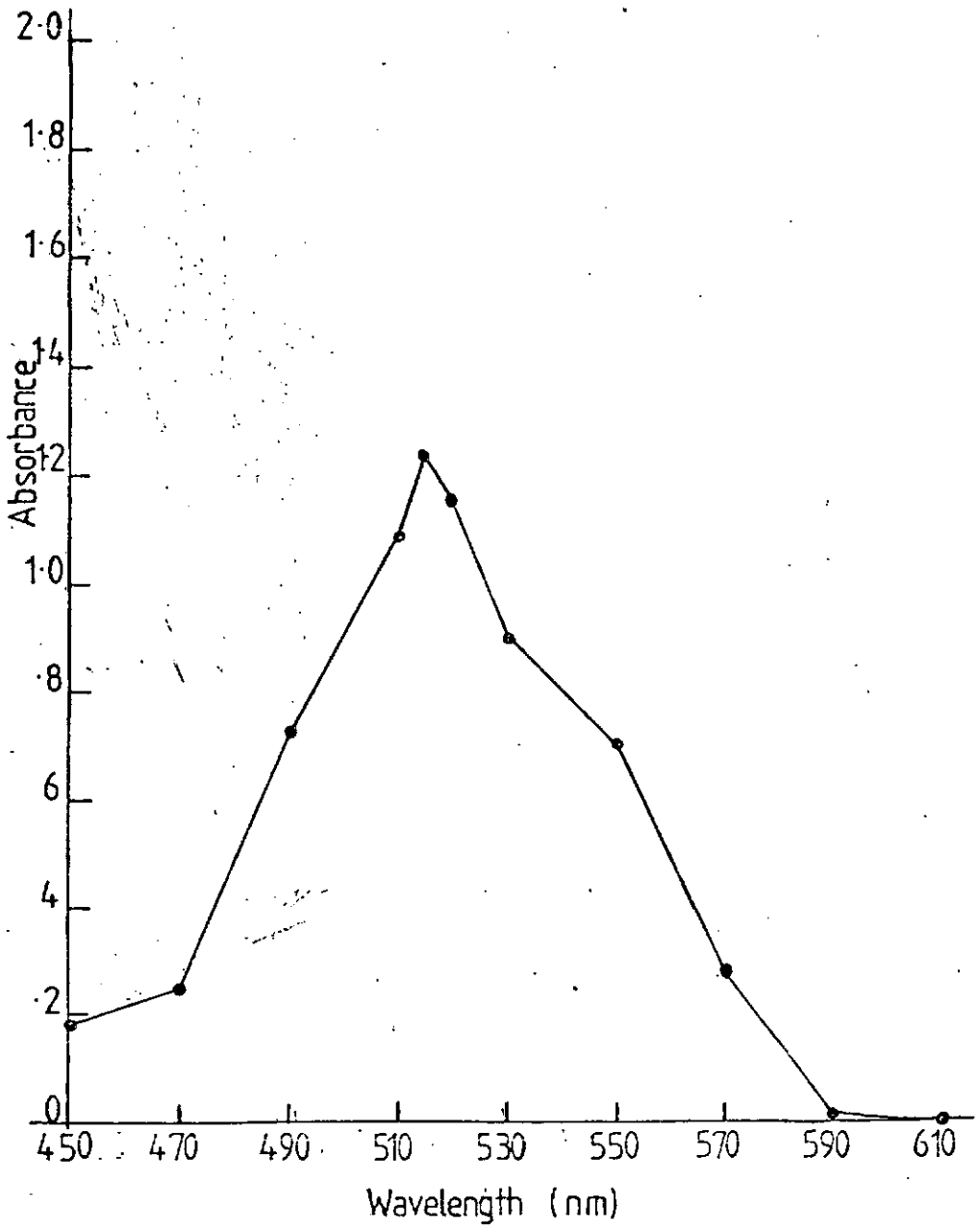
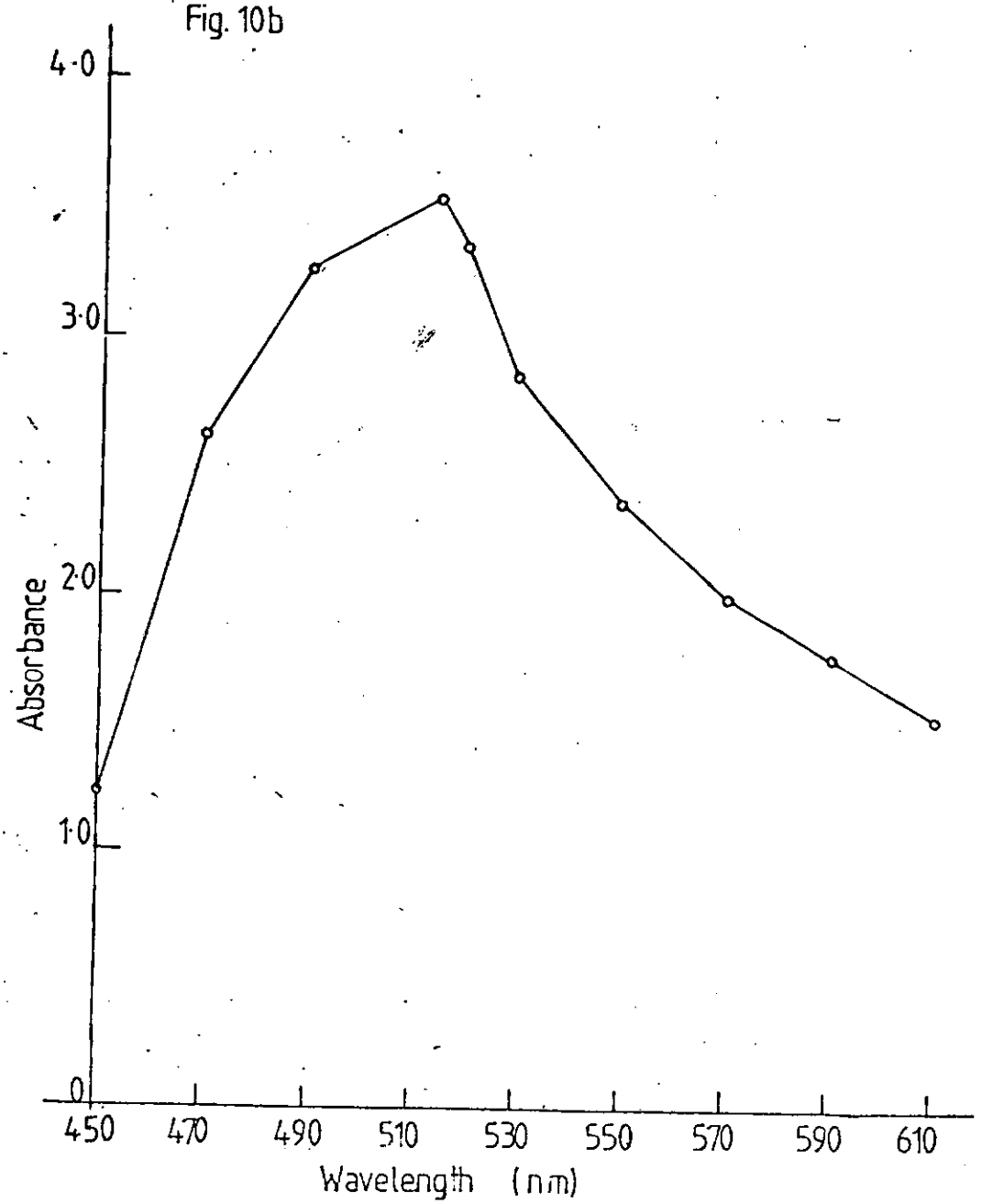

2·0
1·8
1·6
1·4
1·2
1·0
·8
6
4
·2
0
Absorbance
450
470
490
510
530
550
570
590
610
Wavelength (nm)

Fig. 10b


4·0
3·0
2·0
1·0
0
Absorbance
450
470
490
510
530
550
570
590
610
Wavelength (nm)

Assay of crude rot extract

(a) Effect of extractants on activity of crude rot extract

Enzyme activity was present when extraction was carried out with sodium chloride and was increased almost 100% when dithiothreitol was added. Activity was inhibited when ascorbic acid was added to the sodium chloride solution.

Activity of the rot extract was highest at pH 4 and inhibited at pH 9 with or without the presence of calcium ions (Table 10).

Table 10 : Enzyme activity of rot extract in viscometric units /ml using different extractants at different pH values.

Rot extract with different extractants	pH values							
	4	5	5+Ca^{++}	6	7	8	9	9+ Ca^{++}
1. Rot extract + NaCl	79	57.4	50.5	0	0	0	0	0
2. Rot extract + NaCl + dithiothreitol	138.8	111.1	104.1	0	0	0	0	0
3. Rot extract + NaCl + ascorbic acid	0	0	0	0	0	0	0	0
4. Fresh onion extract + NaCl + dithiothreitol	0	0	0	0	0	0	0	0

(b) Effect of inoculum concentration on enzyme activity of rot extract

Experiments carried out to find the effect of inoculum concentration on enzyme activity showed that there is an increase in activity of the enzyme as the inoculum concentration increased (Fig 11).

(c) Effects of days of incubation on enzyme activity of rot extract.

Enzyme activity of the rot extract increased till the 3rd day. There was a drop in activity on the 5th day and an increase again till the 9th day of incubation when the experiment was terminated (Fig 12).

Fig 11 : Effect of inoculum concentration on enzyme activity.

Varying concentrations (10^1 - 10^{13}) of cells /ml of P.fluorescens were inoculated into healthy onion bulbs and incubated at room temperature for 5 days. At the end of incubation, enzyme activity of the rot extracts produced at these inoculum concentrations were estimated viscometrically.

Fig.11

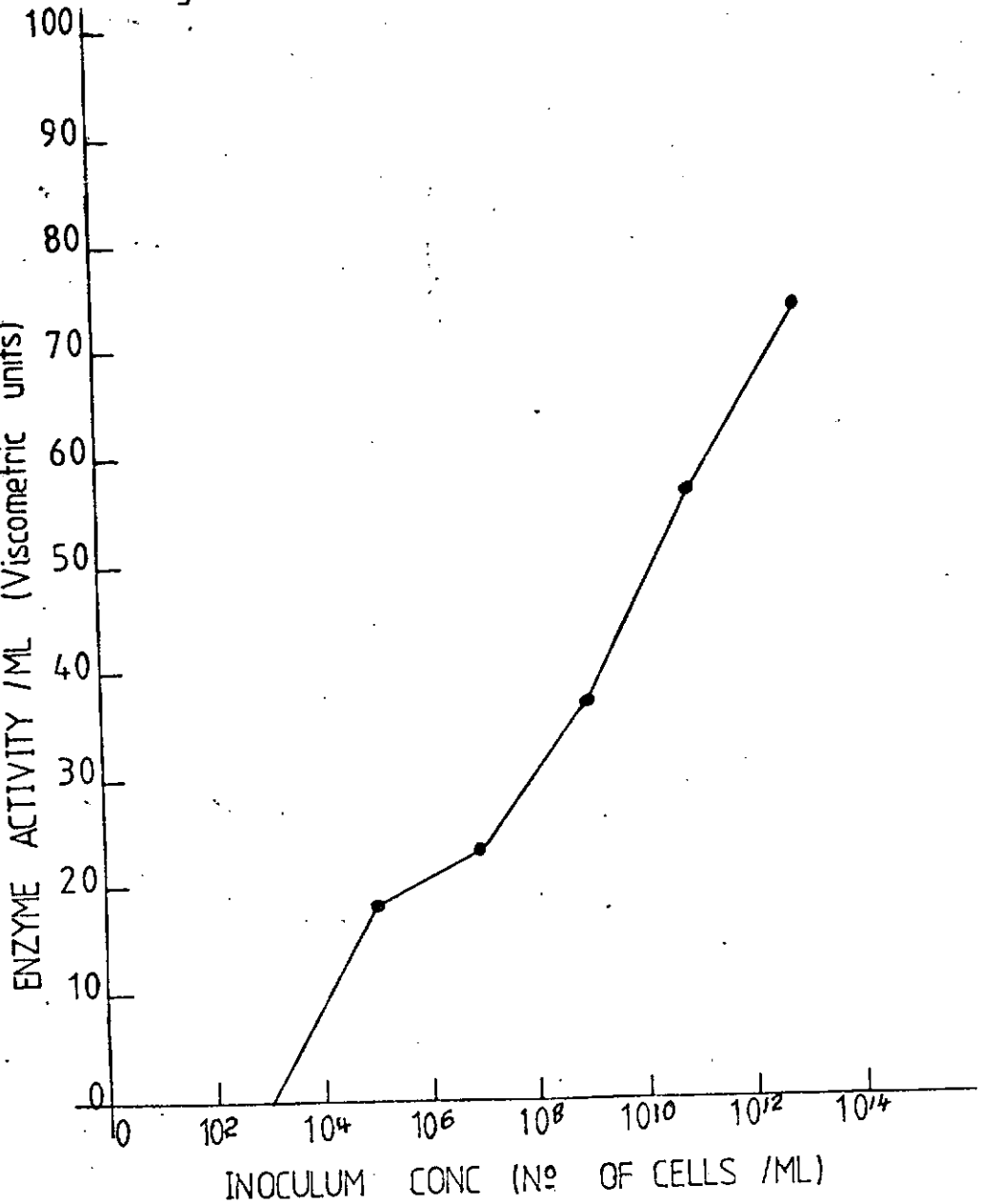
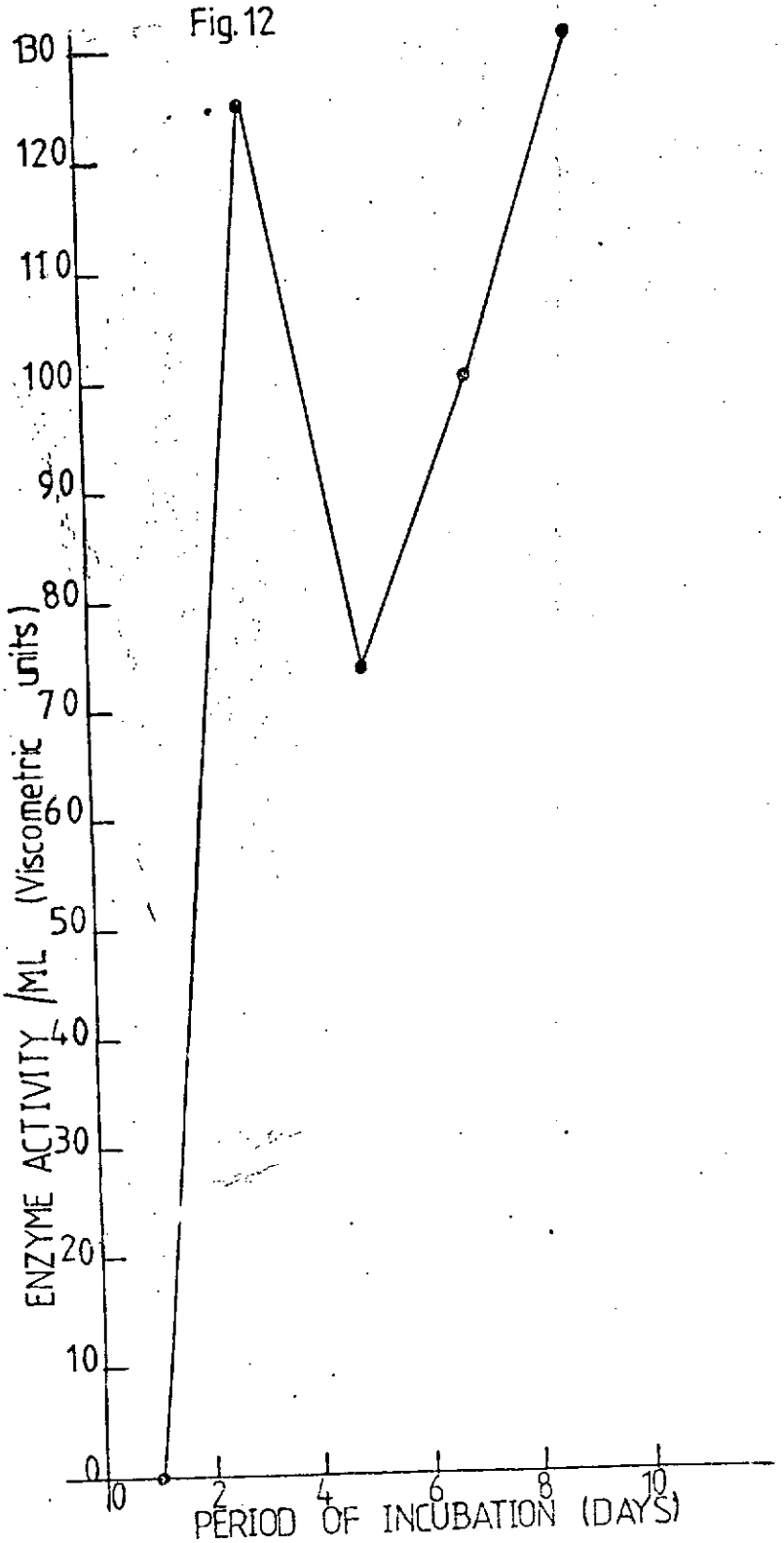

100
90
80
70
60
50
40
30
20
10
0
ENZYME ACTIVITY /ML (Viscometric units)
0
10²
10⁴
10⁶
10⁸
10¹⁰
10¹²
10¹⁴
INOCULUM CONC (Nº OF CELLS /ML)

Fig 12: Effect of age of rot on enzyme activity. Pseudomonas fluorescens was inoculated into healthy onion bulbs and incubated at room temperature. At 24h intervals rotted tissue was removed and extract estimated for enzyme activity viscometrically.

Fig. 12


130
120
110
100
90
80
70
60
50
40
30
20
10
0
ENZYME ACTIVITY /ML (Viscometric units)
0
2
4
6
8
10
PERIOD OF INCUBATION (DAYS)

Release of reducing sugar by pectic enzyme

Viscometric assay showed that both the crude culture filtrate and rot extract were capable of reducing the viscosity of pectin after 30 minutes. The thiobarbituric acid (TBA) reaction on the two filtrates showed the presence of hydrolytic polygalacturonase. This enzyme is known to break down pectic substrates (pectin) to galacturonic acid (Fig 13a & 13b). It was therefore necessary to investigate the affinity of substrate for the enzyme both *in vivo* and *in vitro*, and also the enzymatic release of free galacturonic acid in these two systems.

The estimation of free reducing sugar using Nelson - Somogyi method (1944) at different times showed a peak value at 20 minutes (Table 11). Activity at high substrate concentration -like 2% ($^{W}/v$) - and above was low (Table 11). The low enzyme activity was thought to be due to (1) impurity in pectic substrate used in the assay, and (2) presence of reducing sugar in the filtrates.

Attempts were therefore made to reduce the free reducing sugar in the filtrate by using some standard purification procedures. Known volume of the filtrate was dialysed against distilled water at 4°C for 24h. To the dialysate (50ml) was added ammonium sulphate to achieve a saturation level of 80% (Arinze, 1978; Aribisala, 1978). The mixture was allowed to precipitate in the cold for 3 - 4h. The precipitate was centrifuged at 10,000g for 30 minutes in a Beckman refrigerated centrifuge, model J - 21 C. The precipitate was

dissolved in 10ml cold distilled water and stored at -20°C. Protein was estimated by using the method of Lowry et al (1951).

At each of all the substrate concentrations used (0.5%, 0.2%and 0.15% ($^{W}/v$)), both in vivo and in vitro systems, there was an increase in enzyme activity up to 15 minutes. Beyond this time the pattern varied from one concentration to the other (Fig 13a and 13b).

Enzyme activity was also investigated at other substrate concentrations as shown in Table 12. Rate of enzyme activity was calculated by using the linear part of the graph.

A plot of velocity against substrate concentration is shown in Fig 14a and 14b. In vitro studies showed that increase in enzyme activity could be observed up to substrate concentration of 0.2% ($^{W}/v$) after which there was a decrease. However, in vivo studies showed continuous increase in activity up to 0.5% ($^{W}/v$).

Table 11: Effect of two substrate concentrations on release of reducing groups during a 40 minute period of incubation.

Incubation period (mins)	Free galacturonic acid (μg) released at two different pectin concentrations	
	0.5% ($^{w}/_{v}$)	2% ($^{w}/_{v}$)
0	10.4	48.6
10	11.0	50.2
20	15.2	50.8
30	12.1	49.6
40	9.0	36.5

Table 12: Effect of pectin concentrations on the rate of release of free galacturonic acid by pectic enzyme in the in vivo and in vitro systems.

Sample	Pectin concentration [S] ($^{w}/_{v}$)	$I/[S]$	Velocity (μg/min)	I/V
Culture filtrate (in vitro system)	0.02	50	0.44	2.27
	0.05	20	0.87	1.14
	0.10	10	1.15	0.86
	0.15	6.6	1.41	0.70
	0.20	5.0	1.73	0.57
Rot extract filtrate (in vivo system)	0.10	10	1.17	0.85
	0.15	6.6	1.29	0.77
	0.25	4.0	1.68	0.59
	0.35	2.8	1.89	0.52
	0.50	2.0	2.21	0.45

Fig 13a: The amount of free galacturonic acid released in the *in vitro* system at different time intervals for two substrate concentrations

The enzyme reaction was carried out by using two substrate concentrations of 0.2% ($^{W}/v$) ●——● and 0.15% ($^{W}/v$) O——O pectin. The amount of galacturonic acid released at intervals of 5 minutes was estimated by using the Nelson-Somogyi method.

Fig 13b: The amount of free galacturonic acid released in the *in vivo* system at different time intervals for two substrate concentrations.

The enzyme reaction was carried out by using two substrate concentrations of 0.15% ($^{W}/v$) O——O and 0.5% ($^{W}/v$) ●——● The amount of galacturonic acid released at intervals of 5 minutes was estimated by using the Nelson-Somogyi method.

Fig. 13a

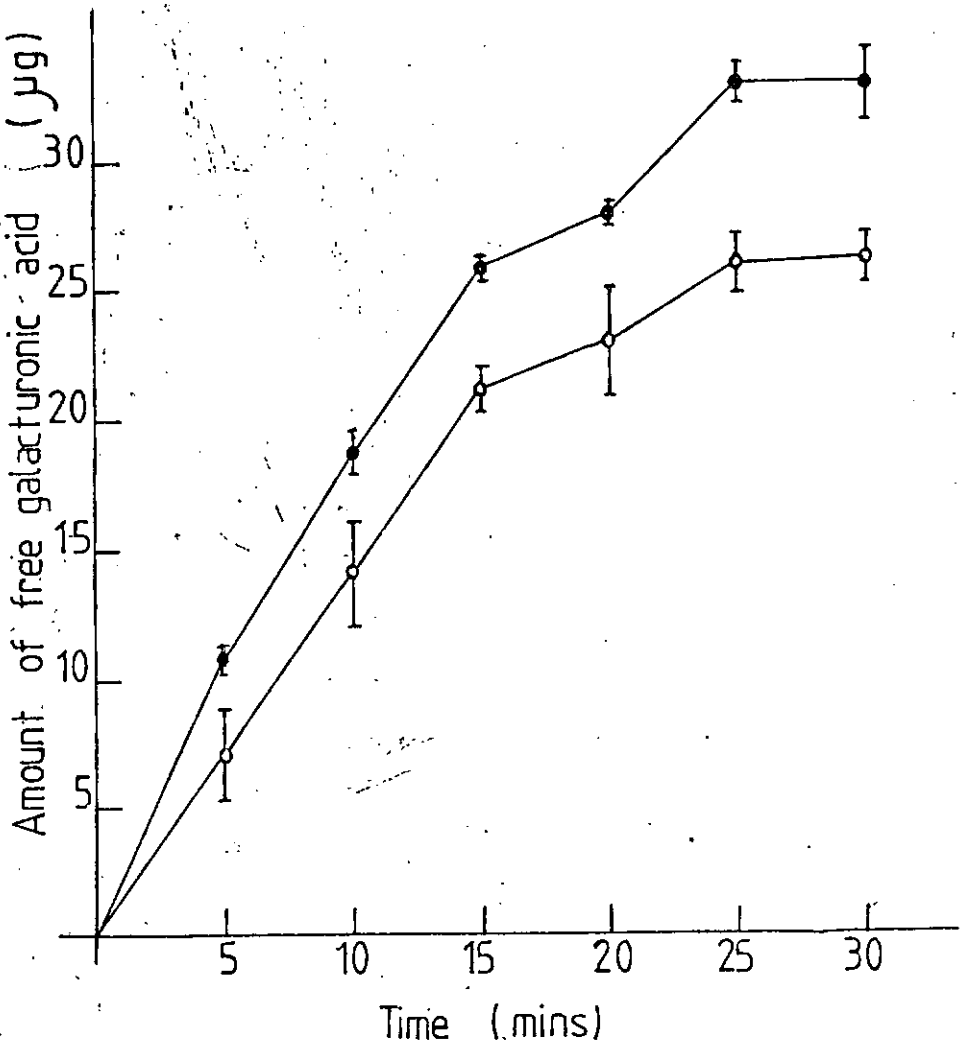

Amount of free galacturonic acid (µg)
30
25
20
15
10
5
5
10
15
20
25
30
Time (mins)

Fig. 13b

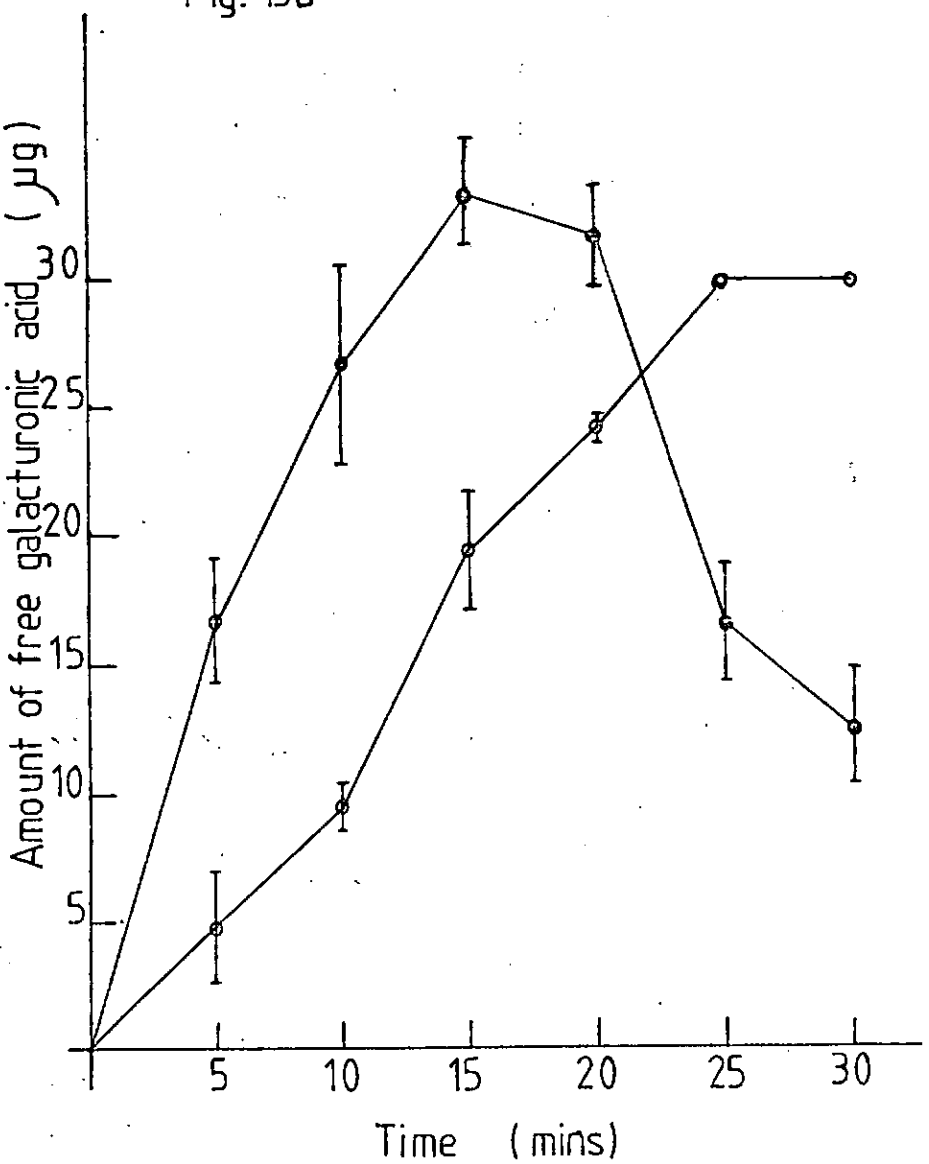

Amount of free galacturonic acid (µg)
30
25
20
15
10
5
5
10
15
20
25
30
Time (mins)

Fig 14a : Rate of release of free galacturonic acid in the in vitro system when different pectin concentrations were used.

Five days old culture filtrate was assayed for amount of free galacturonic acid at different pectin concentrations using the Nelson-Somogyi method. Velocities were calculated at 15 minutes.

Fig 14b :: Rate of release of free galacturonic acid in the in vivo system when different pectin concentrations were used.

Rot extract filtrate from a 5-day old rot was assayed for the amount of free galacturonic acid at different pectin concentrations using the Nelson-Somogyi method. Velocities were calculated at 15 minutes.

A plot of $^1/V$ against $^1/S$ for the *in vivo* system showed a straight line with a slope of 0.051. Best line was plotted by linear regression of y and x (Fig 15) using the regression equation:-

$$y = m\,x + c$$

where m is the slope, c is the intercept on y axis, x and y are $\frac{1}{[S]}$ and $\frac{1}{V}$ respectively.

$$\text{Slope} = \frac{km}{Vm} = \frac{\text{Michealis constant}}{\text{Maximum velocity}}$$

$$Vm = \frac{1}{\text{Intercept}}$$

Intercept = 0.377

Vm = 2.65

$$\text{Slope} = \frac{km}{2.65}$$

$\therefore$ km = 2.65 x 0.051

= .0135g/litre = 22.5 μM

For the *in vitro* system, the slope is 0.037 (Fig 16)

Intercept = 0.43

Vm = 2.32

$\therefore$ km = 2.32 x 0.037

= 0.0085g/litre = 14.1 μM.

Fig 15 : The Lineweaver-Burk reciprocal plot ($^1/V$ against $^1/[S]$) for the *in vitro* system. The Km (Michealis constant) was obtained from this reciprocal plot. V is the rate of release of free galacturonic acid.

Fig. 14b

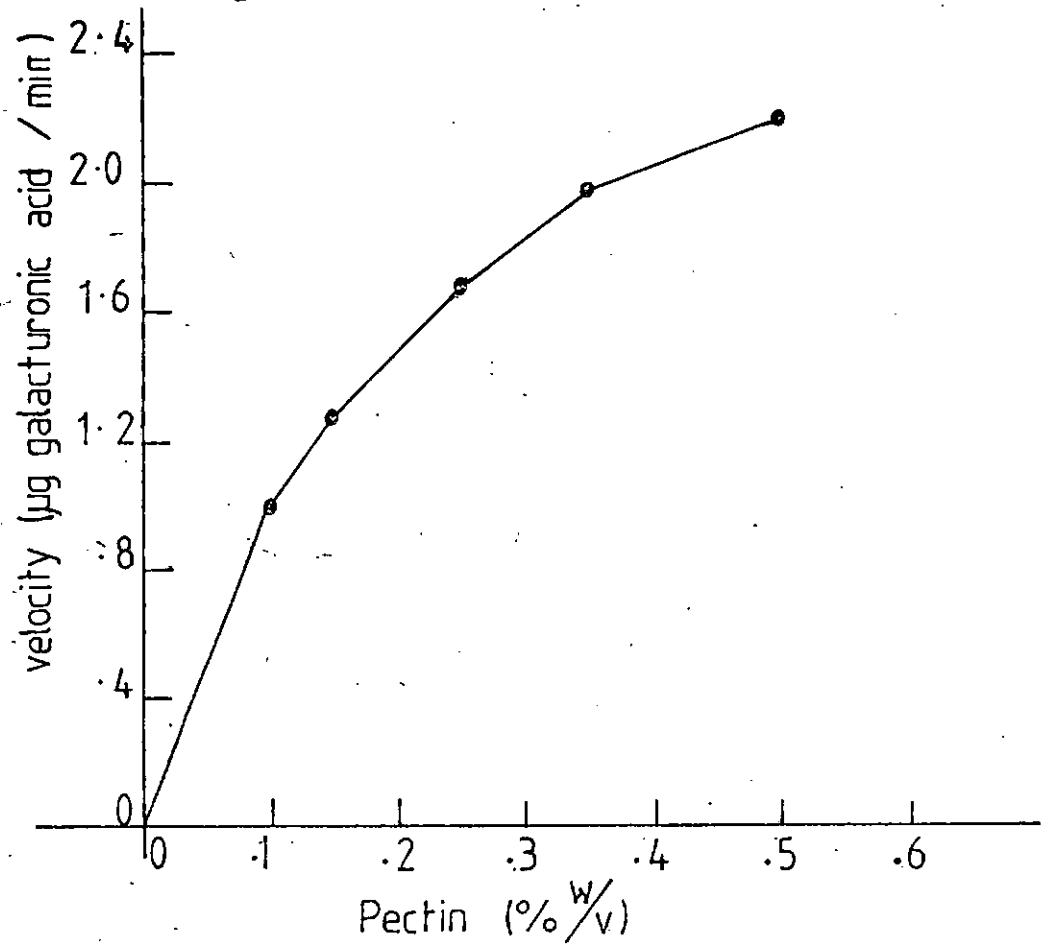

velocity (µg galacturonic acid / min)
2·4
2·0
1·6
1·2
·8
·4
0
0
·1
·2
·3
·4
·5
·6
Pectin (% w/v)

Fig. 14a

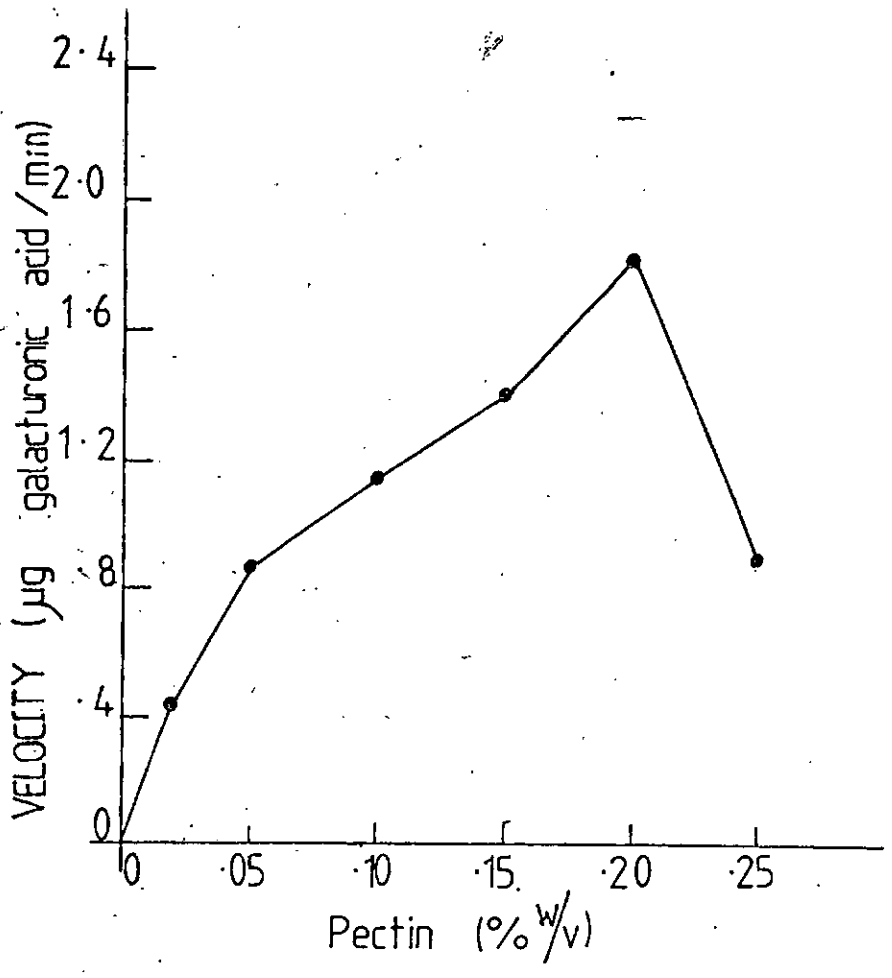

2·4
2·0
1·6
1·2
·8
·4
0
VELOCITY (µg galacturonic acid / min)
0
·05
·10
·15
·20
·25
Pectin (% w/v)

Fig. 15

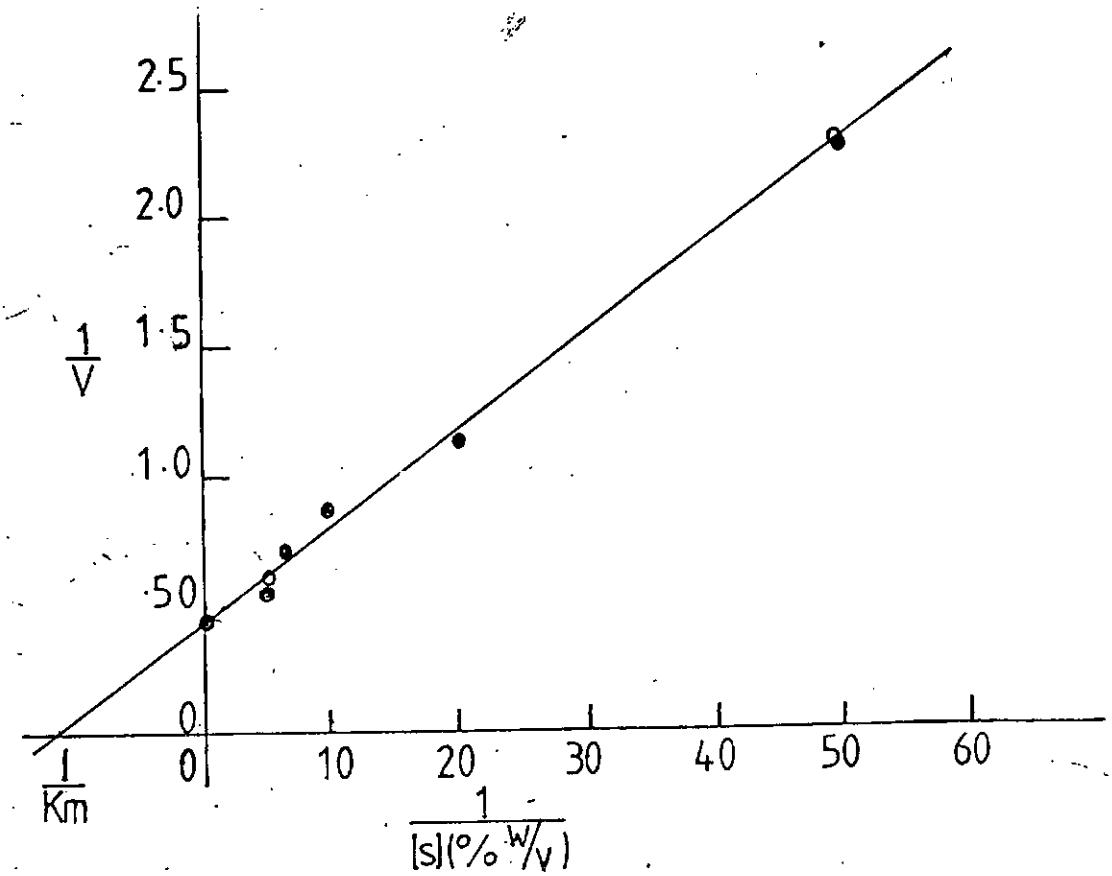
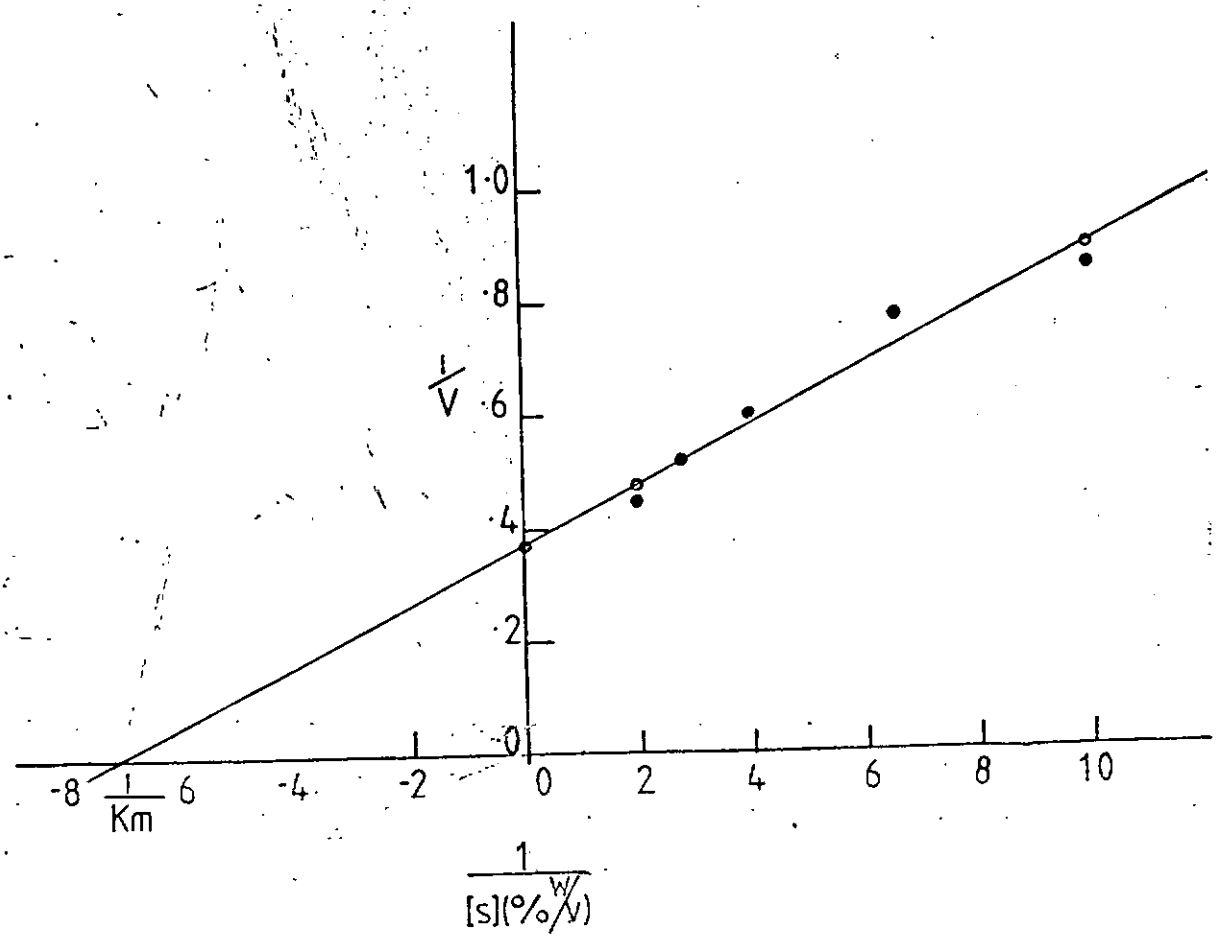


Fig 16 : The Lineweaver-Burk reciprocal plot ($^{1}/V$ against $^{1}/[S]$) for the *in vivo* system. Km (Michealis constant) was obtained from this reciprocal plot. V is the rate of release of free galacturonic acid.

Fig. 16



Macerating activity:

Potato discs and onion tissue were macerated considerably by the end of three hours in the crude culture filtrate and rot extract. Partially purified filtrates showed some degree of maceration by one hour.

Cup - plate assay:

Partially purified culture filtrate and rot extract showed some little degree of activity on the agar plates by forming white precipitates around the wells.

Polyphenol content of onions

Pathogens are known to be able to break down the natural defences of plant tissue to cause infection (Wood, 1967). Some compounds are responsible for the host defensive mechanism; some of which are the polyphenols.

Determination of polyphenol content of the different leaf bases of a bulb, that is from the outermost to the innermost leaf bases, showed a gradual decrease from the outermost to the innermost. Also the first two leaf bases of healthy onion bulb of about the same size and weight were selected and investigated. This was to give an indication of the polyphenol content of the outermost leaf base which is exposed to attack as compared to the inner leaf bases. Results showed that the outermost leaf base contained more polyphenols (Table 13a and 13b).

It was therefore necessary to investigate the effect of infection on the polyphenol content. When the outermost leaf base was inoculated and incubated for 9 days there was an appreciable increase. From 59.28 µg/g. tissue to 112.20 µg/g tissue. Also high inoculum concentration 4.5×10^{13} cells of P.fluorescens increased polyphenol content from 49.28 µg/g tissue to 112.57 µg/g tissue.

Table 13a : Polyphenol content of different leaf bases in a single onion bulb.

Leaf base	Phenolic content (µg tannic acid /g tissue)
1	59.04 ± 3.04
2	65.06 ± 1.52
3	46.80 ± 1.27
4	39.00 ± 2.54
5	45.50 ± 1.02
6	34.70 ± 3.13
7	28.00 ± 0.23
8	29.70 ± 4.04

Table 13b : Polyphenol content of the first two leaf bases on onion bulbs of about the same weight.

Leaf base	Weight of bulb (g)	Phenolic content (µg tannic acid/ g tissue
Bulb A: 1	48.9 ± 5.61	54.2 ± 2.82
2		35.2 ± 1.50
Bulb B: 1	52.1 ± 4.84	52.0 ± 3.21
2		25.9 ± 2.28
Bulb C: 1	53.5 ± 4.90	48.9 ± 3.14
2		31.3 ± 1.02
Bulb D: 1	46.3 ± 3.18	34.8 ± 1.72
2		27.7 ± 1.07

Fig 17 : Effect of inoculum concentration on polyphenol content of rotted tissue kept at room temperature for 7 days.

Different concentrations (10^1 - 10^{13}) cells of P.fluorescens were inoculated into the first leaf base of healthy onion bulbs and incubated at room temperature for 7 days. The rotted tissue at these different inoculum concentrations were estimated for total polyphenol content by using the Folin-Denis reagent.

Fig. 17

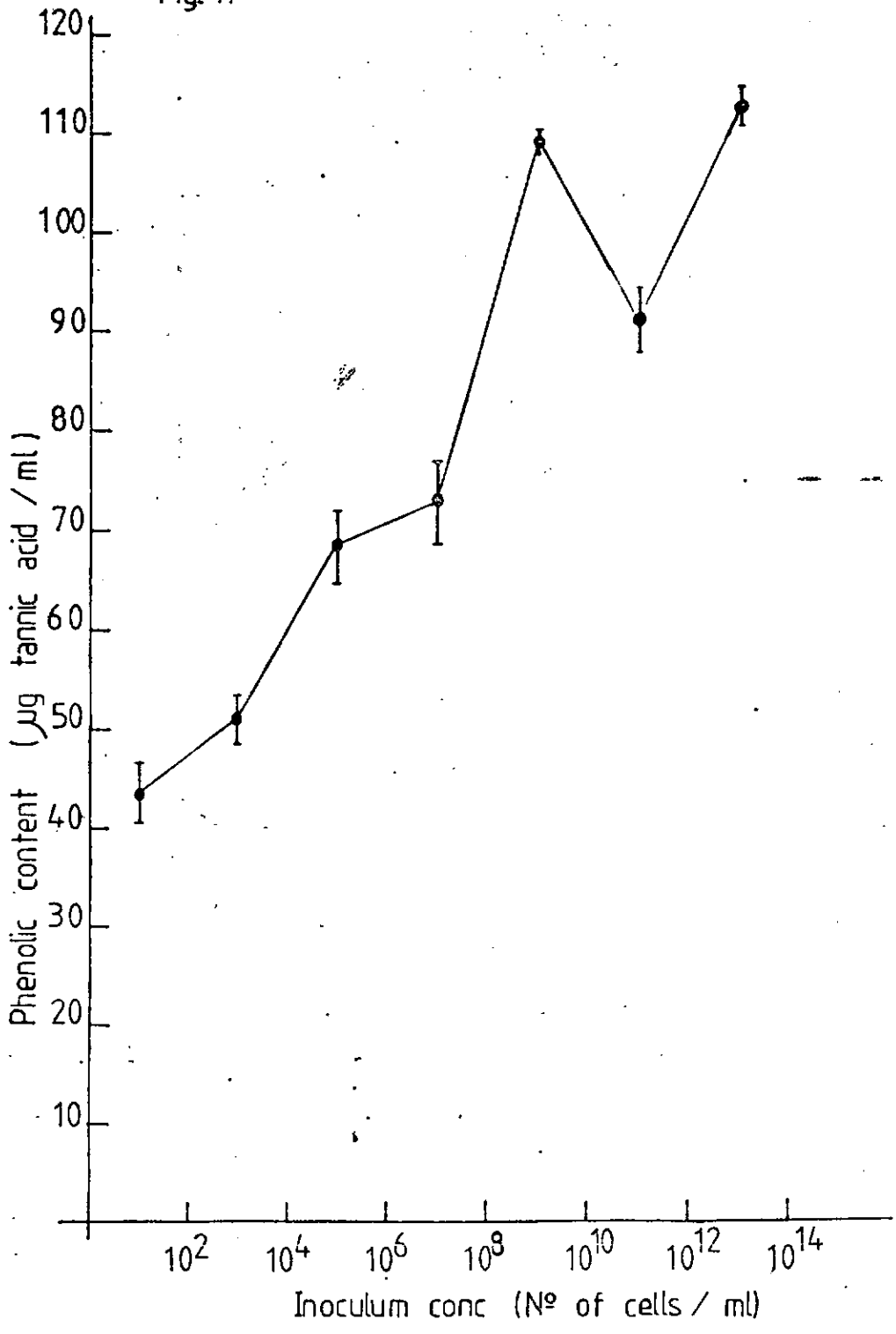
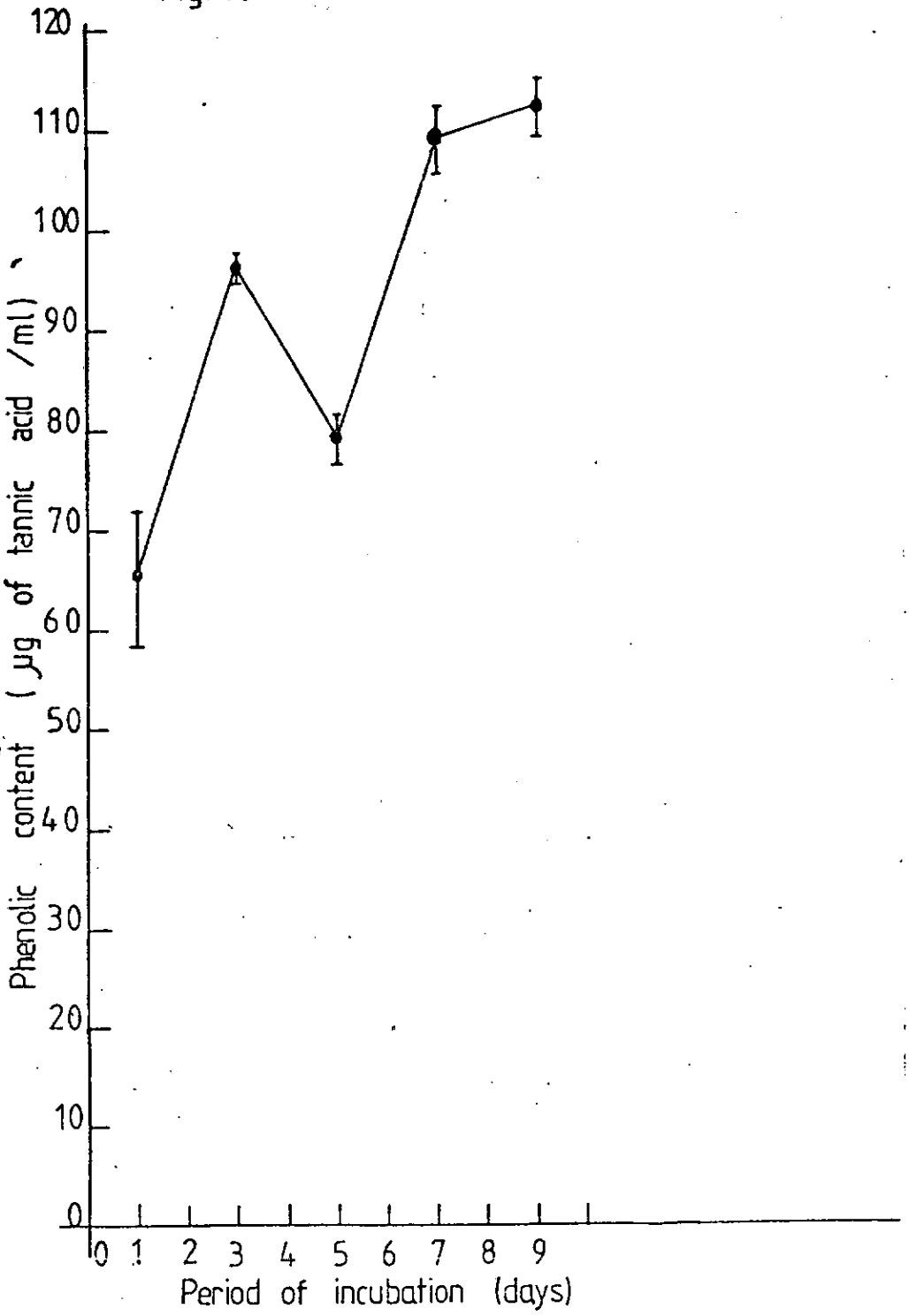

120
110
100
90
80
70
60
50
40
30
20
10
Phenolic content (µg tannic acid / ml)
10^2
10^4
10^6
10^8
10^{10}
10^{12}
10^{14}
Inoculum conc (№ of cells / ml)

Fig 18 : Effect of incubation period on the total polyphenol content in the infected tissue incubated at room temperature.

Pseudomonas fluorescens was inoculated into healthy onion bulbs and incubated at room temperature for 9 days. At 24h intervals rotted tissue was removed and estimated for total polyphenol content by using Folin - Denis reagent.

Fig. 18


120
110
100
90
80
70
60
50
40
30
20
10
0
Phenolic content (µg of tannic acid /ml)
0 1 2 3 4 5 6 7 8 9
Period of incubation (days)

Discussion

Post-harvest spoilage of the purple variety of onions (*Allium cepa*.L) in Nigeria is thought to be due to the deteriorative activities of micro-organisms. In the present study some micro-organisms were isolated from rotted onion bulbs using onion extract agar which provided a good medium for their growth. Kado and Heskett (1970) claimed that standard procedures for isolating plant pathogenic bacteria from diseased material and soil are facilitated by use of selective and diagnostic media.

Three of the four isolates from rotted onion bulbs were pathogenic. The pathogenic organisms were isolated from the advancing edge of the rot. Cother, Derbyshire and Brewer (1976) also got an onion pathogen when they isolated from the advancing edge of the lesion.

The isolates were identified as *Pseudomonas fluorescens*, *Pseudomonas cepacia*, *Candida utilis* and *Staphylococcus* sp which was not pathogenic. Buckholder (1942) associated *P.allicola* with onion rot and in 1950 showed that *P.cepacia* caused the sour skin disease of onions. Work done in India by Hingorani and Malta (cited by Rangaswani, 1962) described a soft rot of onion caused by *P.marginalis* and Volcani (cited by Rangaswani, 1962) reported a bacterial disease of onions caused by *P.syringae*. Goto (1972) in Japan reported a bacterial leaf spot on onions caused by a pathogenic strain of *P.syringae*

Van Hall. Taylor (1974) associated three pathogens, P.allicola, Lactobacillus sp and Erwinia-like organism with bacterial rot of stored onions. Also Cother, Derbyshire and Brewer (1976) found an onion pathogen that had a nutritional spectra similar to that of P.aeruginosa and P. fluorescens.

Some fungal pathogens have also been associated with onion spoilage. Some species of Alternaria and Macrosporium have been associated with onion spoilage (Skiles, 1953; Nolla, 1927) while Clark and Lorbeer (1973) reported a disease of onion caused by Botrytis cinerea. Also Maude and Presley (1977) reported a neck rot disease on onion bulbs caused by Botrytis allii. In the study reported here, Candida utilis is being reported to be an onion pathogen. Buckholder (1950) reported that some yeasts were secondary invaders in the sour skin disease of onions. It is also known that some species of Candida can form films and spoil foods high in acid and salt (Frazier, 1978), though onion extract has pH of 6.3 and so it is not likely to belong to this class of foods.

Pseudomonas fluorescens was the most pathogenic of the isolates, followed by Candida utilis, and P.cepacia was the least pathogenic (Fig.1a & b). P.fluorescens is usually regarded as a saprophyte in soils (Klement, 1963); its ability to cause spoilage therefore might be due to the fact that it can multiply in plant cells under favourable conditions. Green et al (1974) showed that P.aeruginosa was fairly

common in agricultural soils and could multiply and persist within plant tissue. Also Irwin (1972) found that P.cepacia could be isolated from onion field soil and so was implicated as inoculum source of the pathogen when they cause increasing losses of bulbs in both field and storage. However, P.fluorescens has also been implicated as the causative agent in the pink eye disease and storage rot of potato tubers (Huether and McIntyre, 1969).

The infection caused by P.fluorescens when reproduced under laboratory conditions usually started as discolouration of the inoculated area, followed by gradual softening of the tissue. The softening of tissue resulted from collapse of affected tissue and the conversion of tissue into a mass of soft, slimy, semi-solid material as shown by the histological study (Plates 9, 10 & 11). This is similar to the findings of Buckholder (1950) that in the sour skin disease of onion caused by P.cepacia, the infected scales became yellow and slimy.

The inability of P.cepacia to be pathogenic in this study is very surprising as it is the well known onion pathogen (Buckholder, 1950; Kawamoto and Lorbeer, 1974). It is likely that it needed environmental conditions different from those provided in the present study. Cother, Derbyshire and Brewer (1976) had reckoned that the failure of P.aeruginosa to infect leaves and topped onion bulbs under laboratory conditions might be due to inability of the bacterium to infect cells except under extremely specific conditions.

The three pathogens P.cepacia, P.fluorescens and Candida utilis caused rot on the white variety of onions and shallots (Allium ascalonicum) but were not effective on garlic. It is possible that garlic contains some inhibitory chemicals. However, Goto (1972) working in Japan found an onion pathogen highly pathogenic on Welsh onion (Allium fistalosum) and bulb onion (Allium cepa), moderately pathogenic on shallots (Allium ascalonicum), slightly pathogenic on rakkyo (Allium chinese) and peach (Prunus persica). Pseudomonas fluorescens, normally very pathogenic on onion (Allium cepa), did not cause rot on Irish potatoes, carrots and cucumber. It is possible that the plant organs have some structural or physiological (or both) barriers against infection by the bacterium. Similarly, P.cepacia isolated from rotted onion bulbs did not rot potato tubers, narcissus bulbs and calla lilly rhizome (Buckholder,1950).

Studies on the mode of entry of the pathogen into the host showed that it needed a wound to be able to infect healthy tissue. It must be that it could not degrade the thin membraneous skin on the leaf bases or that it needed to establish itself first in injured cells which provided exposed nutrients before it could develop the capability to infect healthy tissue. Some workers have found that most plant pathogens needed a wound to infect their host. Buckholder (1950); Kawamoto and Lorbeer (1974) found that onion decay caused by P.cepacia could be reproduced in the laboratory only

when the bulbs were wounded and artificially inoculated with the bacterium. From the results in the present study, it could be recommended that care should be taken at every stage from the field to the consumer : harvesting, transporting, storing and displaying in the market, so as not to create a portal of entry for rot-causing pathogen. It was also found that infection was usually restricted to the infected leaf base (Plate 8). It is possible that the pathogen could not break down the constituents of the cuticle as found by Cother, Derbyshire and Brewer (1976) when microscopic observations of the leaves inoculated with P.aeruginosa were made.

Biochemical analysis of healthy onion bulb showed that it contained glucose, fructose, and sucrose. These were also the only soluble sugars found in onion bulbs by Cother, Derbyshire and Brewer (1976). They also observed that the onion pathogen could not utilise sucrose for growth but results in the present author's study showed that some onion pathogens could utilise sucrose for growth (Table 3).

Biochemical studies of the uninfected and infected tissues showed that the sugar content was reduced by the bacterial pathogens. The pathogens probably utilised the sugars for growth. Arinze, (1974) found that the carbohydrate content, especially the sugars, were used up during infection of sweet potato by L.threobromae. There was no appreciable change in the amino acid and lipid contents (Table 2)

though these bacterial species were found to be proteolytic and lipolytic. It is possible that these substances were not in the forms readily available for use by the organisms. However, infection caused by Candida utilis caused a decrease in the sugar and lipid contents but an increase in the amino acid content (Table 2). This increase might be due to its ability to convert some other materials in the medium to amino acids through intermediary metabolism.

Growth studies of P.fluorescens on different solid media showed that it grew best on nutrient media and malt extract agar at temperature ranges of 4° - 37°C (Table 5, Fig 2). It also grew on onion extract agar at 29° - 37°C though not at 4°C (Table 5). This may explain why infection was not caused when the inoculated bulbs were incubated at 4°C. It is likely that growth was not favoured at this temperature even when present in the susceptible host. It is not surprising that media containing onion extract supported growth because Starr(1959) had found that the gross nutritive requirements for the growth of practically any bacterial phytopathogen could be met by organic substances present in the suitable hosts.

When the effect of carbon and nitrogen sources on growth were investigated, it was found that P.fluorescens was able to utilise all the carbon and nitrogen sources (Fig 3a and 4a) used in the study.

Glycine was the best nitrogen source. This is not surprising as it was found in healthy onion tissue and it was used up during infection (Table 3) though Buckholder (1948) claimed that plant pathogens could not use organic nitrogen for growth. Glucose was the best carbon source and this is similar to the findings of Chi and Hanson (1964) that glucose was the best carbon source for the growth of Fusarium solani and F.roseum. Though these substances supported growth, high concentrations of glycine (1.6% w/v) in the medium became toxic to the organism (Fig 4b). Olutiola (1978) also found that though growth and sporulation of Fusarium oxysporum increased with increase in concentration of carbon and nitrogen sources in the medium, there was a decline in the rate of these processes when nitrogen concentration exceeded 50mM.

Work done on effect of environmental conditions on growth of P.fluorescens showed that it grew best at 29± 2°C, a temperature most favourable for development of rot in the laboratory and in nature. However, infection was restricted at 4°C. Buckholder (1948) had stated that the approximate optimum temperature for growth of most bacterial pathogens is 27°C. Lauritzen (1926) found that the optimum temperature for growth of Alternaria radicine (an organism causing black rot of carrot) and the temperature most favourable for infection of carrots to be about 28°C, while Misra and Haque (1962) reported the maximum growth of Sclerotium rolfsii causing

storage rot of potatoes at 30°C.

Growth of the pathogen was inhibited at 44°C and the infected as well as the uninfected onion bulbs all collapsed completely at this temperature (Plate 14). This is likely due to denaturation of the essential enzymes at high temperature thereby causing death of the tissue of onions and cells of the bacterium.

Relative humidity was found to play a major role in rot development in this study. Increase in relative humidity of the atmosphere favoured increase in rot. It is likely that the organism grew best under high moisture content. Kawamoto and Lorbeer (1974) found that P. cepacia was capable of infecting and multiplying within wounded onion leaves under high moisture condition. Similarly Cother, Derbyshire and Brewer (1976) found that moist environment around the onion bulbs after a heavy dew would be conducive to the maintenance and increase of bacterial population.

Onion bulbs incubated in an atmosphere of nitrogen gas disintegrated by seven days even when they were not inoculated with the pathogen. This happened probably because tissues of onion bulbs are alive and must require oxygen for respiration.

Vegetables, especially onions because of their susceptibility to microbial rot in storage, should be kept under controlled environment. Tomkins (1951) found that a high relative humidity in store

is necessary for commodities susceptible to serious evaporation losses but in the present study high relative humidity favoured microbial rotting of the produce. Robinson, Browne and Burton (1975) suggested that although reduction in temperature is probably the single most important factor in successful storage of most commodities, it is closely followed by the need to minimize evaporative losses. They claimed that crops vary considerably in their water loss depending upon the exposed area and the nature of the surface. For example, both potatoes and onions have a minimal exposed surface per volume ratio, and, in both, the surfaces are protected from excessive evaporation. In the onion bulb the protective layers are the dried scale leaf bases. It might be preferable to store onions at a low relative humidity to reduce microbial hazard.

It had been suggested that before onion bulbs can be stored, it is necessary to dry the foliage (especially in the neck region) as this shrivels on drying to form a seal which prevents ingress of pathogens (Jones and Mann, 1963). Similarly, Stow (1976) found that pre-harvest defoliation of onion bulbs did not increase rotting in store but did increase sprouting which is a major problem in storage.

It is the view of the present author that onion can be stored in an environment of low relative humidity and low temperature. It must be noted that

the bulbs should be used immediately after removal from this environment as they would become more susceptible to attack by pathogens because of the high ambient relative humidities in Nigeria especially during the rainy season in the southern states.

It is known that for an organism to cause infection, it must have the ability to break down the natural defence mechanism of the host plant, one of which is the cellwall (Bateman and Millar, 1966; Wood, 1973). The plant cellwall is a complex, ordered structure and constitutes a barrier to microbial invasion of cells. Micro-organisms therefore require specific enzymes for the degradation of the wall to penetrate it. Pathogens have evolved a means to recognise the chemical structure in plant cellwalls, and elaborate the appropriate enzymes to dismantle the various cellwall constituents (Bateman and Basham, 1976).

Pectic substances are the primary constituents of the middle lamella and are structural elements in the primary wall (McClendon, 1964; Talmadge et al, 1973). Many phytopathogenic fungi and bacteria are known to produce enzymes that degrade pectic substances (Wood, 1967). Those enzymes that split the α - 1,4 glycosidic bonds in pectin or pectic acids have been associated with the phenomenon of tissue maceration and have been considered to function in aiding pathogens to spread within their host (Bateman, 1963; Duel and Stutz, 1958).

Pectic enzymes can also be found in higher plants because Besford and Hobson (1972) associated pectic enzymes with softening and ripening of tomato fruits. Also McCready, McComb and Jansen, (1955) found high concentrations of endo-polygalacturonase during ripening of certain fruits like tomato and avocado.

In this study, P.fluorescens was able to grow on pectin as sole carbon source (Table 8). During growth the organism released proteins into the medium as shown by ammonium sulphate precipitation and degraded pectin to release reducing sugars (Fig 14) (Nelson, 1944). The culture filtrate exhibited pectinolytic activity. Extracts of soft rot tissues from onions also showed pectolytic activity as shown by viscometric assay. It can be concluded therefore that the presence of pectic enzyme and the ability to produce soft rot which appeared similar to the storage breakdown phase of the disease suggested their importance in the disease syndrome.

Thaysen and Bunker (1927) found that many aerobic pectin decomposers are pathogenic bacteria which resemble P.fluorescens group or Bacterium coli forms generally found in grass and hay and in their biochemical reactions. The fluorescent pectolytic bacteria isolated from plant soft rot tissues are currently grouped as P.fluorescens sometimes as biotype II of that species or as P.marginalis (Folsom and Friedman, 1959; Elliot, Billing and Hayward, 1966; Huether and McIntyre, 1969; Duodoroff and Palleroni, 1974).

Some other pectolytic bacteria were commonly implicated in soft rot diseases of plants (Sands and Hankin, 1975). Pseudomonas fluorescens produced pectic enzymes when involved in the pink eye disease and storage soft rot of potato tubers. (Huether and McIntyre, 1969). Also. P.cepacia was reported to elaborate a pectic enzyme in diseased onions (Ulrich, 1975).

The pectic enzyme from P.fluorescens was produced inductively. This confirms the observation by previous workers that some pathogens produce chain-splitting enzymes in response to pectic substances (Gäumann and Böhni, 1947; Singh and Wood, 1956; Fergus and Wharton, 1957). Some, however, are produced constitutively (Moran and Starr, 1968; Hsu and Vaughan, 1969).

Pseudomonas fluorescens has the ability to produce enzymes capable of degrading pectic substances hydrolytically and not by trans-elimination, as shown by the thiobarbituric acid reaction (Albersheim, Neukom and Deuel, 1960). From the present study, the activity of this enzyme was demonstrated in culture filtrate of P.fluorescens as well as in extracts of infected onion tissue. This finding agrees with earlier reports that enzymes that hydrolytically degrade pectic constituents are produced by a variety of phytopathogenic micro-organisms (Cole and Bateman, 1969; Van Etten and Bateman, 1969; Mullen, 1974). It is also possible

that some microbes can produce the trans-eliminative type of pectic enzyme as demonstrated by P.fluorescens infecting potatoes (Huether and McIntyre, 1969).

The optimum pH for the activity of pectic hydrolases is generally acidic (Albersheim, Neukom and Deuel, 1960; Bateman, 1966; Hall and Wood, 1970; Rombouts and Pilnik, 1972). The hydrolase produced by P.fluorescens in this study has an optimum pH between 4 - 5 (Fig 9, Table10). Ulrich (1975) found an endo-polygalacturonase produced by P.cepacia in diseased onions to have an optimum pH range of 4 - 4.6.

The activity of the pectic enzyme studied in this investigation increased in the culture filtrate and rot extract for a period and a decrease in the activity set in. The decrease may be occasioned by modification in conditions within the culture medium. For example, change in pH arising from accumulation of certain substrates and the accumulation of certain products inhibitory to enzyme activity will cause a decrease in enzyme activity. Wood (1967) found that when pectinic acids were added to media, they reduced the pH to lower values and if pectin esterase was secreted, acid was produced which reduced the pH to values unfavourable to growth of pathogens.

Growth of the pathogen within the bulb was not so pronounced in the first seven days though enzyme activity increased till the third day and again from the fifth day till the ninth day (Fig 12).

Wood (1967) found that enzyme activity continued to increase after active growth had stopped and enzyme secretion might still be conspicuous when conditions did not permit rapid growth of the pathogen.

Pectic enzymes have been implicated as being responsible for pathogenicity in most plants. It is therefore possible that any substance that can inhibit the activity of these enzymes should offer resistance to plants. Extracts of a number of plant species are known to inhibit pectic enzymes *in vitro* (Bell et al, 1962; Mahadevan, Kuc and Williams, 1965). Also the occurrence of such substances *in vivo* has been associated with resistance to pathogenic fungi (Byrde, 1959; Mahadewan, Kuc and Williams, 1965). In many cases the inhibitors were phenols or their oxidation products. Their free radicals (metabolic intermediates) reacted readily with biochemical constituents capable of electron interaction, such as $-NH_2$ and OH groups (Patil and Dimond, 1967). This makes it possible for the free radicals to prevent or limit some infections.

Despite the presence of these inhibitors in plants, some micro-organisms are still able to break down the defence mechanisms of plants and cause infection. *Pseudomonas fluorescens* must be one of such organisms as it is able to cause infection on onion bulbs though the volatile compounds present in onions have some anti-bacterial properties (Lewis et al, 1977).

It is known that apple fruits infected with Sclerotinia frutigena contained oxidised phenols that inactivated polygalacturonase in vitro (Cole, 1958). Byrde et al, (1973) found the active form of the enzyme in the lesion as well as in tissue outside the zone of accumulated phenols.

The quantity of the polyphenols present in healthy onion bulbs were investigated. There was slight variation in total polyphenol content from one bulb to the other and the outermost leaf bases had more polyphenols than the inner leaf bases (Table 13). This can be due to the fact that the outermost leaf bases are more exposed to infection since they are usually outermost.

It was found, however, that polyphenol content increased with infection (Fig 17 and 18). Huang and Agrios (1979) found that in the skin disease of apples, higher amounts of phenols were present in the infected than in the healthy tissue. It is also believed that development of necrotic symptoms in plants in response to infection results from accumulation of phenolic compounds (Solymosy, Farkas and Király, 1959; Parish, Zaitlin and Siegel, 1965; Rahe et al, 1969).

Besides the fact that polyphenols offer resistance to plants during infection, their presence in diseased tissue might account for the change in colour that accompanies injury or infection. Clark and Lorbeer (1975) found that catechol

present in onions might function as the substrate for the brown staining reaction when onions were infected with Botrytis cinerea. Also the browning of apples in response to injury was due to accumulation of phenolic compounds which were oxidised and polymerised to form the brown substances observed after injury or infection (Walker, 1962). It is therefore possible that the yellow colouration of the purple variety of onions during infection by P.fluorescens may be due to the presence of some phenolic compounds. Buckholder (1950) however attributed the yellow colouration in the sour skin of onions to formation of aggregate of minute yellow masses under the epidermis of the scales.

In conclusion, although the findings in the present studies and reports of earlier investigators point to the presence of large amounts of phenolic compounds in onion leaves and other plants after infection thereby suggesting that those substances might be able to confer resistance to microbial attack, the rapid rate at which P.fluorescens, P.cepacia and C.utilis rot onion bulbs demonstrates that they have been able to overcome the defence mechanisms of the onion host. Since these micro-organisms grew very weakly and were not able to cause any appreciable rot at low temperatures and low relative humidities, it is recommended that onion bulbs be stored at such conditions after harvest until they are required for distribution or consumption.

Summary

The micro-organisms responsible for the spoilage of purple variety of onions (*Allium cepa*. L) in Lagos area were isolated on onion extract agar and potato dextrose agar. They were identified as *Pseudomonas fluorescens* Migula, *Pseudomonas cepacia* Buckholder and *Candida utilis* (Hennberg) Lodder and Kreger-Van Rig. They produced soft rots and were also pathogenic on the white variety of onions and shallots though they could not cause rot on garlic. *Pseudomonas fluorescens* was the most pathogenic of the three isolates. The diameter of rot increased with days of incubation and the severity of rot decreased correspondingly with a decrease in inoculum potential.

Pathogens penetrated the host tissue through wounds on the surface of the bulbs. Small cuts on the surface of the bulbs were sufficient to permit entry by the pathogens.

When food substances such as starch, cellulose, protein (meat), fat and pectin were incorporated into the basal medium, they all supported growth, though they did not produce any clearing on cellulose agar, and *P.fluorescens* could not hydrolyse starch.

Biochemical analysis of the food substances present in infected and non-infected onion bulbs showed that the bacterial pathogens used up some sugars and there was no appreciable change in total amino acid and lipid content. *Candida utilis*

however utilised most of the sugars and lipids but caused an increase in the amino acid content. There was a slight increase in the water content of the infected tissue since soft rot was formed by the pathogens.

Growth studies of P.fluorescens showed that it grew on a variety of solid and liquid media at 29 ± 2°C. The pathogen utilised glucose, lactose, galactose, sucrose, maltose, fructose, saccharose and mannitol though growth was best on glucose. Similarly the nitrogen sources, sodium nitrate, ammonium sulphate, asparagin, glycine, tyrosine and alanine were used for growth. Glycine was the best nitrogen source but high concentration of it (1.6% W/v) inhibited growth.

The pH optima for growth of P.fluorescens was 6 which is similar to the pH of onion extract of 6.3.

The storage environment was found to play an important role in rot development. Maximum rot of onions occurred at 29 ± 2°C and at a high relative humidity of 100%.

Histological analysis of infected and non-infected onion tissues showed that P.fluorescens penetrated the tissue intercellularly and intracellularly. This resulted in breaking down of the cellwalls giving rise to a soft, mushy water-soaked appearance of yellow colouration.

Bacterial cell population in the infected tissue increased till the fifth day before a decrease occurred.

The pathogen produced a pectinase enzyme in culture media and in the rotted tissue. The enzyme was produced inductively when pectic substrates were present in the media. The optimum pH for crude culture filtrate's enzyme activity was pH 5 and for rot extract pH 4.

Enzyme activity of the crude culture filtrate reached a peak on the sixth day, decreased slightly by the eighth day and then increased again till the tenth day. Also for the crude rot extract, enzyme activity increased initially till the third day, decreased by the fifth day and then increased till the ninth day. Activity increased with increase in diameter of rot and with increase in inoculum potential.

Activity of the enzyme increased when the filtrates were precipitated with ammonium sulphate (80% saturation.) There was inhibition of enzyme activity by high substrate concentration.

Polyphenols were present in the healthy onion tissue but their concentration increased when the bulbs were infected.

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Appendix

Table A1

Extent of infection on onion bulbs inoculated with P.fluorescens and incubated at room temperature for 7 days.

Inoculum concentration showing No. of cells	Extent of rot estimated as diameter of rot (cm)						
	1day	2 days	3 days	4 days	5 days	6 days	7days
Control	0	0	0	0	0	0	0
3.2×10^3	0	0	$0.10 \pm .05$	$0.10 \pm .05$	$0.25 \pm .10$	$0.20 \pm .11$	$0.30 \pm .12$
3.2×10^4	0	0	$0.10 \pm .10$	$0.15 \pm .09$	$0.20 \pm .10$	$0.25 \pm .12$	$0.50 \pm .11$
3.2×10^5	0	$0.25 \pm .11$	$0.35 \pm .11$	$0.60 \pm .08$	$0.75 \pm .17$	$1.03 \pm .13$	$1.25 \pm .12$
3.2×10^6	0	$0.40 \pm .13$	$0.81 \pm .13$	$1.15 \pm .12$	$1.75 \pm .15$	$1.81 \pm .15$	$1.93 \pm .16$
3.2×10^7	0	$0.55 \pm .13$	$1.02 \pm .15$	$1.39 \pm .15$	$2.11 \pm .14$	$2.53 \pm .14$	$2.65 \pm .14$
3.2×10^8	0	$0.65 \pm .10$	$1.30 \pm .13$	$1.64 \pm .16$	$2.31 \pm .17$	$3.50 \pm .15$	$3.71 \pm .18$
3.2×10^9	$0.23 \pm .10$	$0.70 \pm .11$	$1.42 \pm .11$	$1.83 \pm .10$	$2.47 \pm .14$	$3.62 \pm .12$	$4.47 \pm .12$

Table A2

Percentage rot as shown by P.fluorescens when inoculated into healthy onion bulbs and incubated at room temperature for 7 days.

Inoculum concentration	Percentage rot from 1 - 7 days						
	1	2	3	4	5	6	7
Control	0	0	0	0	0	0	0
3.2×10^3	0	0	40	40	60	60	73.3
3.2×10^4	0	0	40	40	60	60	80
3.2×10^5	0	33.3	46.6	60.3	73.3	93.3	100
3.2×10^6	0	40	46.6	73.3	100	100	100
3.2×10^7	0	40	66.6	73.3	100	100	100
3.2×10^8	0	46.6	66.6	73.3	93.3	100	100
3.2×10^9	33.3	66.6	100	100	100	100	100

Table B1

Extent of infection on onion bulbs inoculated with *P.cepacia* and incubated at room temperature for 7 days.

Inoculum concentration showing No. of cells	Extent of rot estimated as diameter of rot (cm)						
	1 day	2days	3days	4days	5days	6days	7days
Control	0	0	0	0	0	0	0
4.2×10^1	0	0	0	0	0	0	0
4.2×10^2	0	0	0	0	0	0	$0.10 \pm .05$
4.2×10^3	0	0	$0.07 \pm .05$	$0.08 \pm .05$	$0.08 \pm .05$	$0.10 \pm .06$	$0.23 \pm .09$
4.2×10^4	0	0	$0.15 \pm .10$	$0.15 \pm .08$	$0.22 \pm .12$	$0.40 \pm .13$	$0.45 \pm .15$
4.2×10^5	0	0	$0.23 \pm .11$	$0.25 \pm .07$	$0.30 \pm .10$	$0.60 \pm .10$	$0.95 \pm .13$
4.2×10^6	0	0.10	$0.30 \pm .12$	$0.45 \pm .13$	$0.70 \pm .14$	$1.15 \pm .13$	$1.37 \pm .11$
4.2×10^7	0	$0.15 \pm .10$	$0.35 \pm .12$	$0.60 \pm .12$	$0.75 \pm .15$	$1.48 \pm .15$	$1.65 \pm .10$

Table B2

Percentage rot as shown by P.cepacia when inoculated into healthy onion bulbs and incubated at room temperature for 7 days.

Inoculum concentration	Percentage rot after 1 - 7 days						
	1 day	2 days	3 days	4 days	5 days	6 days	7 days
Control	0	0	0	0	0	0	0
4.2×10^1	0	0	7.1	21.4	21.4	21.4	21.4
4.2×10^2	0	0	7.1	21.4	35.7	50	57.1
4.2×10^3	0	0	21.4	35.7	50	50	78.5
4.2×10^4	0	0	21.4	35.7	42.8	64.2	78.5
4.2×10^5	0	7.1	50	42.8	100	100	100
4.2×10^6	0	50	64.2	70	78.5	81.8	92.8
4.2×10^7	0	50	64.2	70	78.5	81.8	92.8

Table B2

Percentage rot as shown by P.cepacia when inoculated into healthy onion bulbs and incubated at room temperature for 7 days.

Inoculum concentration	Percentage rot after 1 - 7 days						
	1 day	2 days	3 days	4 days	5 days	6 days	7 days
Control	0	0	0	0	0	0	0
4.2×10^1	0	0	7.1	21.4	21.4	21.4	21.4
4.2×10^2	0	0	7.1	21.4	35.7	50	57.1
4.2×10^3	0	0	21.4	35.7	50	50	78.5
4.2×10^4	0	0	21.4	35.7	42.8	64.2	78.5
4.2×10^5	0	7.1	50	42.8	100	100	100
4.2×10^6	0	50	64.2	70	78.5	81.8	92.8
4.2×10^7	0	50	64.2	70	78.5	81.8	92.8

Table C1

Extent of infection on onion bulbs inoculated with Candida utilis and incubated at room temperature for 7 days.

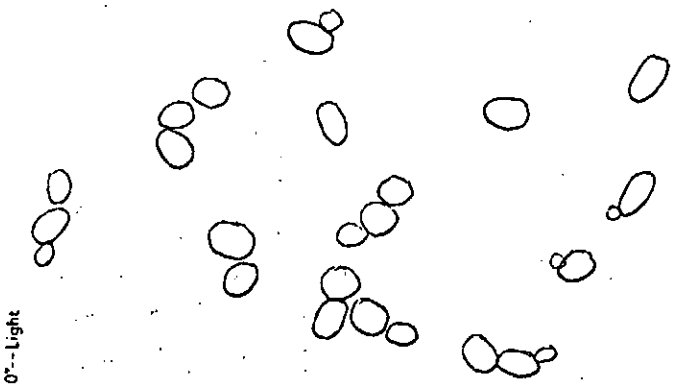
Inoculum concentration showing No. of cells	Extent of rot estimated as diameter of rot (cm).						
	1day	2 days	3 days	4 days	5 days	6 days	7 days
Control	0	0	0	0	0	0	0
4.5×10^2	0	0	0	0	0	$0.10^{\pm}.07$	$0.10^{\pm}.08$
4.5×10^3	0	0	0	0	$0.10^{\pm}.07$	$0.25^{\pm}.10$	$0.31^{\pm}.11$
4.5×10^4	0	0	0	$0.05^{\pm}.05$	$0.28^{\pm}.09$	$0.30^{\pm}.09$	$0.48^{\pm}.13$
4.5×10^5	0	$0.10^{\pm}.05$	$0.20^{\pm}.07$	$0.35^{\pm}.09$	$0.65^{\pm}.12$	$0.78^{\pm}.10$	$1.0^{\pm}.13$
4.5×10^6	0	$0.30^{\pm}.09$	$0.45^{\pm}.13$	$1.14^{\pm}.15$	$1.38^{\pm}.13$	$1.60^{\pm}.11$	$1.77^{\pm}.12$
4.5×10^7	0	$0.48^{\pm}.10$	$0.60^{\pm}.16$	$1.33^{\pm}.15$	$1.59^{\pm}.13$	$1.98^{\pm}.12$	$2.47^{\pm}.12$
4.5×10^8	0	$0.65^{\pm}.11$	$1.12^{\pm}.12$	$1.55^{\pm}.10$	$1.98^{\pm}.11$	$2.95^{\pm}.13$	$3.15^{\pm}.15$

Table C2

Percentage rot as shown by *Candida utilis* when inoculated into healthy onion bulbs and incubated at room temperature for 7 days.

Inoculum concentration	Percentage rot after 1 - 7 days						
	1 day	2 days	3 days	4 days	5 days	6 days	7 days
Control	0	0	0	0	0	0	0
4.5×10^2	0	0	0	33.3	33.3	33.3	53.3
4.5×10^3	0	6.6	53.3	53.3	66.6	66.6	80
4.5×10^4	0	33.3	53.3	60	73.3	73.3	86.6
4.5×10^5	0	33.3	53.3	60	60	73.3	73.3
4.5×10^6	6.6	53.3	66.6	66.6	86.6	86.6	86.6
4.5×10^7	33.3	66.6	73.3	86.6	100	100	100
4.5×10^8	33.3	66.6	100	100	100	100	100

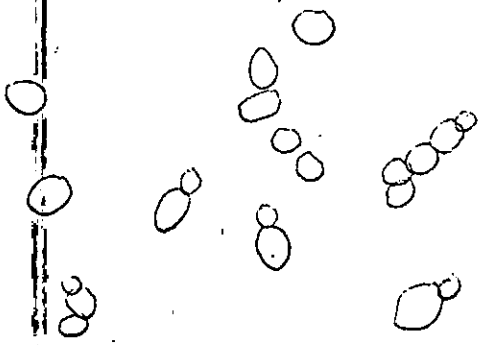
YM broth 24 hrs.



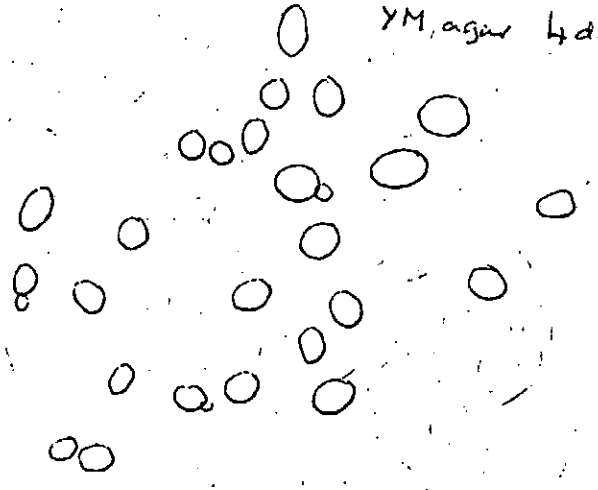
× 1600

YM broth 4 days.

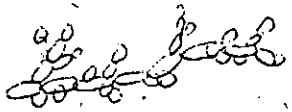
× 4



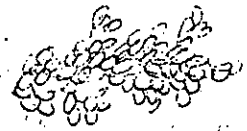
YM, agar 4 days.



CMA 21 days.



Anamorph



Teleomorph

(Not to scale)

NATIONAL COLLECTION OF YEAST CULTURES

Cat. No. X4 Species Candida utilis Strain 02 Alt. Cat. No.

Deposited by Dr. Ibe, University of Nigeria Date April 1979

Isolated by

SOURCE Isolated from soft rot of ~~[illegible]~~ onions

INFORMATION

REFERENCES

MORPHOLOGY
Liquid Medium Difco YM broth (0711-01)

24 hours	Cells oval; occurring singly, in pairs or short chains. $(3-4)\times(3-6)\mu$.
~~72 hours~~ 4 days.	Cells round to oval; occurring as above. $(2-4)\times(3.5-5)\mu$.
21 ~~14~~ days	Culture forms a non-flocculent deposit and a slight ring.
Solid Medium	
~~72 hours~~ 4 days	Cells oval; occurring mostly singly. $(1-4)\times(1-6)\mu$.
~~1 month~~ 21 days	Streak culture is cream, shiny, smooth.

SLIDE CULTURES

P. D. A. 21 days.	Very good pseudomycelial development. Long, irregularly branched chains of oval cells. Oval blastospores along long but pseudomycelium.
C. M. A. "	As for PDA, but pseudomycelial cells longer and blastospores mostly at chains at ~~V~~ertex.
~~G. Y. E.~~	No arthrospores or bathrospores.

ASCOSPORE FORMATION

~~Potato~~ WA —	~~V8 Carrot~~ —	~~Sodium~~ Potassium Acetate —	Mackelvey's agar
Gorodkowa's agar —			
Ascus			
Ascopores			

Moore's Modern Methods, Ltd., London, E.C.4
To repeat order state Plain White Ledger, Size 8" x 10" — Light

No. 14 | SPECIES Candida utilis.

SUGAR FERMENTATION

	Micro-method	Time	Durham-tube method	Time
Dextrose			+	4 days
Fructose			+	21 days
Galactose			+	"
Maltose			+	4 days
Sucrose			+	21 days
Lactose			−	"
Melibiose			+	4 days
Raffinose			Starch −	21 days

ASSIMILATION OF CARBON COMPOUNDS

Glucose	+	Trehalose	−	L-arabinose	+	Adonitol	−
Galactose	−	Melibiose	−	D-arabinose	−	Dulcitol	−
Sucrose	+	Raffinose	+	D-ribose	+	D-mannitol	+
Maltose	+	Melezitose	+	L-rhamnose	+ −	D-sorbitol	+
Lactose	−	Soluble starch	−	Ethanol	+	α-methyl glucoside	+
L-sorbose	−	Inulin	+	Glycerol	+	Salicin	+
Cellobiose	+	D-xylose	+	Erythritol	−	Citric acid	+
		Lactic acid	+	Succinic acid	+	Inositol	−
				Ammonium sulphate	+	Ethylamine hcl	+

Nitrate Assimilation very weak +

Hydrolysis of Fat − | Splitting of Arbutin + | Splitting of Aesculin

Starch production − | Urease activity −

Growth with ethanol as sole carbon source

Growth in vitamin-free medium: −ve
Growth on 60% sugar medium: −ve
" " 50% " " : −ve
Growth with 100 ppm. actidione: +ve
Growth at 37°C: +ve
Acid formation on chalk agar: −ve.