Chitin assays in the estimation of fungi

This item was submitted to Loughborough University's Institutional Repository by the/an author.

Additional Information:

- A Master's Thesis. Submitted in partial fulfilment of the requirements for the award of Master of Philosophy at Loughborough University.

Metadata Record: https://dspace.lboro.ac.uk/2134/33187

Publisher: © Rana Makki Taki

Rights: This work is made available according to the conditions of the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) licence. Full details of this licence are available at: https://creativecommons.org/licenses/by-nc-nd/4.0/

Please cite the published version.
CHITIN ASSAYS IN THE ESTIMATION OF FUNGI

by

RANA MAKKI TAKI

A Master’s Thesis

Submitted in partial fulfilment of the requirements

for the award of

Master of Philosophy of Loughborough University

12th June 1997

© by RANA MAKKI TAKI, 1997
ACKNOWLEDGEMENTS

I wish to express my gratitude to my husband for his financial and moral support and for his patience throughout this period of study.

I would also like to thank my supervisor Dr.R.K.Dart. for his help, advice and guidance throughout this research and in the preparation of this thesis.
SUMMARY

Spoilage by fungi is a very considerable problem throughout the world. A variety of different methods are available to detect and enumerate fungi. In this study enumeration of the fungus *Aspergillus niger* was carried out using a traditional microbiological “dilution plating” method involving total and viable counts of fungal spores. This assay gave inaccurate results and inconsistent data but it is not fundamentally flawed. The failure of this assay at counting fungal spores is due to the characteristics of spores. However, it can be used for the detection of fungi in variety of commodities.

Another more rapid method of estimation of fungal biomass and the extent of contamination can be carried out by the assay of chitin. This is a biochemical method based on the spectrophotometric determination of chitin measured as equivalents of glucosamine, formed after acid or alkaline hydrolysis of chitin. In this study, alkaline hydrolysis of chitin in fungi was carried out in the presence and absence of bacteria to investigate the interference of bacteria with the assay. The bacteria used were typical Gram positive and Gram negative bacteria commonly found in spoiled food. They included *Bacillus cereus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The results obtained show that the Gram negative bacteria inhibit the assay, and the behaviour of Gram positive bacteria varies from species to species. The presence of Gram positive bacteria with the fungus *Aspergillus niger* cause underestimation of the fungal biomass.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CERTIFICATE OF ORIGINALITY</td>
<td>i</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES AND TABLES</td>
<td></td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 What Are Fungi?</td>
<td></td>
</tr>
<tr>
<td>1.2 Role of Fungi in Nature</td>
<td></td>
</tr>
<tr>
<td>1.2.1 Beneficial Role</td>
<td></td>
</tr>
<tr>
<td>1.2.2 Harmful and Destructive Role</td>
<td></td>
</tr>
<tr>
<td>1.3 Conditions Leading to Mycotoxin Contamination of food and feed</td>
<td></td>
</tr>
<tr>
<td>1.4 Mycotoxins in Commodities</td>
<td></td>
</tr>
<tr>
<td>1.4.1 Ochratoxins</td>
<td></td>
</tr>
<tr>
<td>1.4.2 Citrinin</td>
<td></td>
</tr>
<tr>
<td>1.4.3 Patulin</td>
<td></td>
</tr>
<tr>
<td>1.4.4 Penicillic Acid</td>
<td></td>
</tr>
<tr>
<td>1.4.5 Trichothecenes</td>
<td></td>
</tr>
<tr>
<td>1.4.6 Zearalenone</td>
<td></td>
</tr>
</tbody>
</table>
1.4.7 Aflatoxins

1.5 Estimation of Fungal Biomass

1.5.1 Chitin Assay

1.5.2 Ergosterol Assay

1.5.3 Impedimetry

1.5.4 Estimation of ATP

1.5.5 Pectinesterase Activity

1.6 Objectives  

2 EXPERIMENTAL WORK

2.1 Counting Assay

2.1.1 Preparation and Harvesting of Fungal Spores

2.1.2 Total Counts

2.1.3 Viable Counts

2.2 Chitin Assay

2.2.1 Definition

2.2.2 Explanation

2.3 Testing of Chitin Assay

2.4 Chitin Assay on Fungi

2.4.1 Growth and Preparation of Fungal Suspension

2.4.2 Chitin Analysis

2.5 Chitin Assay on Bacteria

2.5.1 Growth and Preparation of Bacterial Suspensions

2.5.2 Chitin Analysis
2.6 Chitin Assay on Mixed Fungi and Bacteria

2.7 Counting of Bacteria

3 RESULTS AND DISCUSSIONS

3.1 Counting Assay

3.2 Chitin Assay on Glucosamine, Chitosan and Chitin

3.3 Chitin Assay on Aspergillus niger

3.4 Chitin Assay on Bacteria

3.4.1 Gram Negative Rod: Pseudomonas aeruginosa

3.4.2 Gram Positive Bacteria: a) Bacillus cereus

b) Staphylococcus aureus

3.5 Assay of Aspergillus niger in the Presence of Bacillus cereus

3.6 Assay of Aspergillus niger in the presence of Staphylococcus aureus

3.7 Counting of Bacteria

4 CONCLUSIONS

APPENDIX 1

REFERENCES
LIST OF FIGURES AND TABLES

Table 1  Total and Viable Count of *Aspergillus niger* Spores  31

Fig. 1  Structure of The Aflatoxins  7

Fig. 2  Chitin Hydrolysis  11

Fig. 3  Glucosamine Weight Against Optical Density  32

Fig. 4  Chitosan Weight Against Optical Density  33

Fig. 5  Chitin Weight Against Optical Density  34

Fig. 6  Optical Density Versus *Aspergillus niger* (mycelium) Dry Weight  35

Fig. 7  Optical Density Versus *Aspergillus niger* (mycelium + spores) Dry Weight  36

Fig. 8  Optical Density Versus *Bacillus cereus* Dry Weight  37

Fig. 9  Graph of Fixed Amount of *Aspergillus niger* Mycelium With Varying  38
    Amounts of *Bacillus cereus* (1)

Fig. 10  Graph of Fixed Amount of *Aspergillus niger* Mycelium With Varying  39
    Amounts of *Bacillus cereus* (2)

Fig. 11  Graph of Fixed Amount of *Aspergillus niger* Mycelium With Varying  40
    Amounts of *Bacillus cereus*

Fig. 12  *Bacillus cereus* colony Number Versus Dry Weight  41

Fig. 13  Optical Density Versus *Staphylococcus aureus* Dry Weight  42
    (linear correlation)

Fig. 14  Optical Density Versus *Staphylococcus aureus* Dry Weight  43
    (polynomial correlation)

Fig. 15  Graph of Fixed Amount of *Aspergillus niger* Mycelium With Varying  44
    Amounts of *Staphylococcus aureus* (1)
Fig. 16 Graph of Fixed Amount of *Aspergillus niger* Mycelium With Varying Amounts of *Staphylococcus aureus* (2) 45

Fig. 17 Graph of Fixed Amount of *Aspergillus niger* Mycelium With Varying Amounts of *Staphylococcus aureus* (3) 46

Fig. 18 *Staphylococcus aureus* Colony Number Versus Dry Weight 47
INTRODUCTION

1.1 What are Fungi?

Fungi are eukaryotic organisms which have sufficient distinctive features to set them apart from both animals and plants. They lead a parasitic, symbiotic or saprophytic existence and take sustenance by absorption of decaying organic matter, or from the living cells of other plants or animals.

Fungi can reproduce both sexually and asexually. The fundamental processes of sexual systems are by meiosis followed by the fusion of two nuclei to give a diploid cell. The usual method of vegetative multiplication is by the production of spores. In general, asexual reproduction is more important for the propagation of the species and the major means of increasing the numbers of fungi. The asexual cycle is usually repeated several times during a season, whereas the sexual stage of many fungi occurs only once a year.

Fungi produce primary and secondary metabolites. Primary metabolites are substances essential for the maintenance and growth of the living cell and its structure. They include sugars, lipids and nucleic acids. Secondary metabolites (e.g. antibiotics, toxins, alkaloids, isoprenoids) are derived in various ways from the primary metabolites and are not essential for growth.
1.2 Role of Fungi in Nature

1.2.1 Beneficial Role

In nature, fungi are of prime importance as decomposers of organic material and are responsible with the bacteria for the recycling of carbon, nitrogen and other vital elements. They also increase the fertility of the soil by inducing various changes which eventually result in the release of plant nutrients in a form available to green plants.

Fungi are used in a number of industrial processes involving fermentation, such as the making of bread, wines, beers, fermentation of the cacao bean, ripening of many cheeses and ageing of meats.

They are also employed in the commercial production of many organic acids and of some vitamin preparations and are responsible for the manufacture of a number of antibiotics. One familiar example is the production of penicillin by strains of *Penicillium chrysogenum*.

1.2.2 Harmful and Destructive Role

Fungi are responsible for much of the disintegration of organic matter and as such they affect us directly by destroying food, fabrics, leather and other consumer goods. They cause the majority of known plant diseases and a number of diseases of animals and of man.

Fungi also synthesise mycotoxins in stored products. The association of microbial toxins in food or feed products with human and animal disease follows consumption of the product.
1.3 Conditions Leading to Mycotoxin Contamination of Food and Feed

Numerous fungi produce mycotoxins in the living plant, on decaying plant material and in stored produce. Toxigenic mould invasion takes place during plant growth, at harvest time and after harvest. Invasion by fungi follows stresses on the plant, insect damage, damage by other fungi, damage by animals or birds and mechanical damage during harvest. After harvest, mould growth depends on numerous factors including the moisture level of the produce, temperature and humidity. This can be influenced by the rapidity of drying, aeration, the microbiological ecosystem, insects, mixing of grain, accidental wetting of the grain by condensation or leakage and the development of hot spots.

1.4 Mycotoxins in Commodities

The biologically active fungal metabolites that are of significant occurrence in food and feed are: Ochratoxin A, Citrinin, Patulin, Penicillic acid, Trichothecenes, Zearalenone and the Aflatoxins (Stoloff, 1976).

The greatest attention has been given to the aflatoxins, but other mycotoxins potentially capable of causing damage on ingestion have been considered.

1.4.1 Ochratoxins

Ochratoxins are toxic metabolites isolated from strains of *Aspergillus ochraceus* as well as *Penicillium viridicatum*. These toxins are widely found in soils and on food commodities such as nuts, dried fish, grains, beans, peas, coffee, spices, alfalfa and meats (Scott *et al.*, 1970, 1972; Saito *et al.*, 1974).
Ochratoxin A, is the major toxic component and has been reported as teratogenic to mice and rats (More et al., 1974; Hayes et al., 1974). A dose of 200μg ochratoxin A per kg body weight has been reported to produce nephrosis in pigs (Krogh et al., 1973).

1.4.2 Citrinin

Citrinin is a toxic metabolite originally isolated from Penicillium citrinum. Citrinin producing isolates of Penicillium citrinum have been obtained from yellow-coloured rice associated with toxic symptoms in Japan (Saito et al., 1971). However the occurrence of citrinin as a contaminant of foodstuffs has been associated with Penicillium viridicatum and causes kidney damage in experimental animals (Ambrose et al., 1946). It always occurs as a co-contaminant with ochratoxin.

1.4.3 Patulin

Patulin is a toxic carcinogenic metabolite elaborated by species of the genera Aspergillus and Penicillium. Patulin has been found on a wide range of agricultural commodities including grain, malt, flour, sausages, bread and fruits. Rotten apples contaminated by Penicillium expansum often contain patulin. The primary interest in Patulin derives from the fact that it is a proven carcinogen in laboratory animals (Ayres et al., 1980).
1.4.4 Penicilllic Acid

It has been isolated from at least ten species of Penicillium and Aspergillus. Penicilllic acid is considered as a potentially dangerous mycotoxin implicated in carcinogenesis (Thorpe et al., 1974). It has been found in mouldy tobacco from commercial storage, in mouldy corn and beans.

1.4.5 Trichothecenes

Trichothecenes are a group of chemically related and biologically active secondary metabolites produced by Fusarium species. One common producer, Fusarium tricinctum has been isolated from field corn, sorghum, peas and rice. These toxins are considered as natural contaminants of corn, and have been implicated in economically important mycotoxicoses such as mouldy corn toxicosis of cattle and poultry (Hsu et al., 1972) and stachybotryotoxicosis, a disease mainly of horses (Eppley & Bailey, 1973).

1.4.6 Zearalenone

Zearalenone is a secondary metabolite produced by various species of Fusarium such as Fusarium tricinctum and Fusarium moniliforme, but particularly by Fusarium roseum. It is usually produced on maize and barley in storage, and was first isolated from maize infected with the fungus Gibberella zeae, the perithecial stage of Fusarium graminearum (Stob et al., 1962). It is notable because of its eostrogenic and anabolic activity in animals. It causes hyper-eostrogenism in swine after the ingestion of infected maize or barley.
Although many of these toxins have been shown to be toxic at various levels, it would appear that the majority of the mycotoxin survey work has been confined to the aflatoxins.

1.4.7 Aflatoxins

Aflatoxins are a group of toxic secondary metabolites which were discovered as contaminants of certain animal feeds. The generic name "Aflatoxins" was applied to the group of toxic compounds produced by some strains of *Aspergillus flavus*.

The term aflatoxin normally refers to the group of bisfuranocoumarin metabolites. The major members are designated as B1, B2, G1, G2. Delineation of the four substances was made on the basis of their fluorescent colour (blue=B, green=G). Determination of the molecular formulas of the toxins demonstrated that aflatoxins B2, and G2 are dihydro derivatives of the parent aflatoxins, B1 and G1.

Aflatoxin B1, the most common of the group in natural contamination of foodstuffs is the most potent liver carcinogen known for experimental animals. The first study that associated aflatoxins in food supply with incidence of human liver cancer was done in Uganda by Alpert *et al.*, (1971). Of the 480 food samples collected from various areas of Uganda during 1966-1967, almost 4% contained more than 1ppm total aflatoxins, which is regarded as a heavy contamination. More importantly, there was a geographical relationship between the contamination and the distribution of liver cancer in Uganda. Keen and Martin (1971) measured the extent of aflatoxin contamination in peanuts collected from various areas in Swaziland and at the same time estimated the geographical distribution of the liver cancer cases based on cancer
Fig. 1 Structure of the Aflatoxins

B₁

G₁

B₂

G₂
registry data for 1964 to 1968. In areas where contamination of peanuts was high, the estimated incidence of liver cancer was high. In addition, inhabitants of the high liver cancer areas tended to eat more peanuts than those in other areas.

Aflatoxin has also an acute toxicity. In 1967, Ling et al., reported on an intoxication of 26 persons with three deaths in two Taiwanese farming villages. The victims suffered oedema of the lower extremities, abdominal pain and vomiting but not fever. The most incriminating evidence comes from Thailand (Bourgeois et al., 1971). Reye's syndrome which occurs in epidemic proportions in North Eastern Thailand is a children's diseases characterised by vomiting, hypoglycaemia, convulsions, hyperammoniemia, coma, and usually death. The conclusion that can be drawn from these studies in Africa and South East Asia, is that there is strong epidemiological evidence incriminating aflatoxin B1 as having an active part in hepatocarcinogenesis in man.

After feeding a diet containing 20% of toxic peanut meal for six months, nine out of eleven rats developed multiple liver tumours, and two of these displayed lung metastases (Lancaster et al., 1961).

Marth (1967) associated aflatoxin with mouldy peanut meal which caused the death of a large number of poultry. The source of toxic material was found to be peanut meal used in the diet and imported from Brazil. The fungus Aspergillus flavus was recovered from peanut meal and found to produce the toxin. In addition, Purchase et al., (1968) found that rats and cattle were able to modify and excrete some of the toxin in milk, (aflatoxin M1 and M2). The amount of aflatoxin M1 excreted is directly related to the level of aflatoxin B1 in the feed.
Toxin production is only one effect produced by storage fungi. Others are a decrease in the germination percentage of malting barley and seeds to be used for planting and a discoloration and/or damage in seeds. There is also the production of off-flavours. Heating also occurs which is usually accompanied by a drastic reduction in quality or complete spoilage (Christensen et al., 1969).

The genus Aspergillus consists of a number of species, the most important of which in terms of spoilage are *Aspergillus flavus*, *Aspergillus glaucus* and *Aspergillus candidus*. Special attention has been given to *Aspergillus flavus* because of its ability to produce aflatoxins which are carcinogenic and potentially hazardous to human and animal health.

### 1.5 Estimation of Fungal Biomass

A number of chemical and biochemical techniques have been used to estimate the extent of fungal growth in a commodity. These systems mostly rely on some unique component of the fungus that is not found in other micro-organisms, or in foods. Many of these methods are still in the developmental phase.

#### 1.5.1 Chitin Assay

Chitin is a polysaccharide, a major constituent of the wall of fungal spores and mycelium of most fungi (Bartnicki-Garcia, 1968). It also occurs in the invertebrate exoskeleton (Parson, 1981) but not in food. In higher plants, cellulose plays the role of chitin where it forms the microfibrillar component which provides the skeletal frame work for the cell wall (Muhlethaer, 1967).
The chitin molecule consists of a long chain polymer of N-acetylglucosamine (GlcNac) units linked by 1:4\ β glucosidic bonds. The detection of chitin in commodities and plants indicates the presence of mycelial mass. Chitin assay is based on the estimation of glucosamine and chitosan formed by acid or alkaline hydrolysis of chitin. A number of studies have indicated that chitin assay is a valuable technique for estimating the extent of fungal invasion in foods and plants. Troppan et al., (1976) have reported a method for the determination of the mass of chitin containing fungal pathogens in plants. It is based upon the release of glucosamine on hydrolysis by hydrochloric acid. Holan et al., (1980) analysed chitin in biological materials. The method used was based on gas liquid chromatography measurements of acetic acid liberated by acid or alkaline hydrolysis of chitin: Hubbard et al., (1979), Line (1980) and Vignon et al., (1986) have worked with measurements of fungal contamination by means of chitin determination.

Chitin assay is most effectively assayed by the method of Ride & Drysdale (1972). The method used was based on the alkaline deacetylation of chitin at 30°C to chitosan. The results showed that the level of aldehyde expressed as glucosamine was related to fungal dry weight. The glucosamine content of the fungi indicated by the alkaline degradation method gave lower results than acid hydrolysis due to partial depolymerisation of chitin by the alkaline hydrolysis.
Fig. 2 Chitin Hydrolysis

![Diagram of chitin hydrolysis process](image)

-Chitin

-Chitosan

-Glucosamine

-2,5-Anhydro-mannose
Bethlenfalvay et al., (1981), measured the extent of invasion by fungi in soybeans by the means of alkaline hydrolysis. Their results showed that a glucosamine standard would severely underestimate the amount of chitin present. The average percentage of chitin which reacted in the colourimetric assay was 37.2%. A GLcN standard would be applicable to direct calculation of fungal chitin only if complete, but non-destructive conversion of chitin to GLcN were achieved.

Frey et al., (1994) determined the extent of invasion of fungi in dry roots by the means of alkaline hydrolysis (Ride & Drysdale, 1972). Chitin was determined as glucosamine multiplied by 2.67; this factor being determined by the incomplete chitin digestion of 36-37% (Bethlenfatvay et al., 1981).

The chitin assay has some shortcomings and has been severely criticised by some authors (e.g. Sharma et al., 1977). The relationship between dry weight and chitin content varies at least two-fold for different food spoilage fungi (Cousin et al., 1984). Some foods contain naturally occurring amino sugars such as glucosamine and galactosamine which should be removed by acetone extraction prior to hydrolysis (Whipps & Lewis, 1980). Products from rot-free tomatoes gave positive glucosamine assay even after acetone extraction (Cousin et al., 1984).

The chitin assay has also been criticised as a measure of fungal biomass on the grounds of background interference (Johnson & McGrill, 1990). Not only does the chitin assay not distinguish between living and dead mycelia, but also it does not distinguish between fungal and faunal biomass. In samples collected from the field, the exoskeletons of micro-arthropods (Sharma et al., 1977), and the non living organic matter in soil (Grant & West, 1986) could interfere with the hydrolysis and contribute to the results. Inside the roots, the presence of plant pathogenic fungi
could contribute to the chitin analysis as could insect eggs. Bethlenfalvay & Ames (1987) did not obtain a good correlation between soil chitin and hyphal length.

1.5.2 Ergosterol Assay

The study of the chemical constitution of fungal wall has showed that typically the walls are composed of polysaccharides, protein, lipid and melanin. Steroids belong to the lipid fraction and are a group of substances extractable with organic solvents but which undergo no saponification - the unsaponifiables. The role of sterols may be that of stabilising the semi-permeable cytoplasmic membrane and they may be involved in the selective permeability of such membranes. It has been established that the synthesis of sterols is oxygen dependant.

Ergosterol is the major steroid produced by fungi, but it is not produced in quantity by other systems (Weete, 1974) and therefore, ergosterol can be used as a measure of fungal invasion in foods and raw materials.

The methodology for estimating ergosterol in cereals has been described by Seitz et al. (1977,1979). Samples are extracted by homogenising in methanol followed by saponification with strong alkali, extraction with petroleum ether and then fractionation by high-pressure liquid chromatography. Ergosterol is detected by ultraviolet absorption; optimally at 282nm, a wavelength at which other sterols exhibit little or no absorbency.

The ergosterol assay is reported to have a high sensitivity and, in contrast to the chitin assay, requires only one hour for completion. It is also more sensitive to early fungal growth and invasion of foods, and holds promise as a routine technique for quality control purposes. Lee et al (1977,1980) extracted leaves and stems by
refluxing in toluene-methanol and then they assayed ergosterol as its acetate derivative by capillary gas chromatography. Griffiths et al. (1985) extracted and saponified leaf samples in one step with ethanol-KOH, and assayed by hplc. Newell et al. (1988) compared various periods of methanol refluxing (with and without KOH), and various lengths of methanol homogenisation for four types of salt marsh plant materials followed by hplc assay. They found that homogenisation (two times for 2 minutes) and refluxing (2h) in methanol were equally effective in extracting ergosterol.

Amezeder et al., (1989) proposed a method for the rapid and accurate determination of ergosterol in microbial cells for routine analysis. This method involves a mild acid hydrolysis to labilize the sterol and a subsequent alkaline saponification. The method allows the rapid separation of sterols within ten minutes by liquid chromatography on reversed-phase columns using an optimised mobile phase composed of methanol, ethanol, water and isopropanol.

Many studies have therefore been carried out to determine the relationship between fungal growth and ergosterol production in vitro in liquid media, and in agricultural production (Matcham et al., 1985; Cahagnier et al., 1983; Nout et al., 1987). Fungal biomass and ergosterol yield were found to correlate well in liquid culture but ergosterol content was found to be influenced by substrate composition, extent of aeration and growth phase of the mycelium (Marfleet et al., 1991; Harris & Magan 1992).
1.5.3 Impedimetry

Impedance is the resistance to the flow of an alternating current. It has other definitions but the basic principle behind its use in food mycology is that the impedance of the growth medium can be altered by changes in the chemical composition of the medium brought about by metabolising cells. The technology is applied by allowing cells to replicate to a concentration where measurable impedance changes occur. The time taken to reach this level, (detection time-DT) is a function of the initial concentration of cells, the growth conditions (medium and temperature), and the metabolic activity of the organism. Several investigators have applied this principle to evaluate microbial levels in milk, (Cady et al, 1978), in vegetables, (Hardy et al. 1977), in cereal grain products, (Sorells, 1981) and meat, (Martin & Seby, 1980). Weihe (1984), developed a methodology for classifying frozen concentrated orange juice samples as acceptable, (<10^4 cfu/ml), or unacceptable, (>10^4 cfu/ml), using a rapid, automated impedance screen. The study showed that most metabolising organisms altered the growth medium by creating ion pairs and/or converting large uncharged molecules into small, charged molecules. These conversions lead to a decrease in impedance, (shown for bacteria). Yeasts, however, alter the medium in other ways as the changes to the medium lead to an increase in the impedance. One possible explanation was that yeast actually remove ions from the solution.

Several papers have been published on impedance measurements in both bacteria and yeast but a not a great deal of research has been carried out on impedance changes caused by fungi. It was however shown by Jarvis in 1984, that changes in the electrical properties of the medium for at least 30 species of storage fungi could be
related to the number of conidia inoculated. It was also noted that in the examination of food samples it was necessary to inhibit bacterial and yeast growth when studying fungal contamination. Antibiotics can be incorporated into the growth medium to achieve this. Adak et al., (1987) used the impedimetric method to detect trichothecene mycotoxins. Yeast strains (195), 74 strains of moulds and 20 strains of bacteria were screened for sensitivity to 1µg per ml of T-2 toxin. Growth inhibition was assessed by measuring changes in impedance using a Bactometer 32 impedimeter. Twelve (60%) of the bacteria, 20 (27%) of the moulds and 38 (19%) of the yeasts were found to be sensitive to T-2 toxin. Eight bacterial strains, eight moulds and 16 yeast strains were tested against four other trichothecene mycotoxins also at 1µg per ml. These were diacetoxycirpenol (DAS); deoxynivalenol (DON); roridin A and verrucarin A. Sensitivity to these varied. Little response was obtained to DON. The macrocyclic trichothecenes (roridin A and verrucarin A) were markedly more toxic than the others to the fungi, but not to the bacteria. This area definitely requires further research but it must be remembered that changes in impedance do not always correlate directly with fungal growth rate, but rather to metabolic activity.

1.5.4 Estimation of ATP

Adenosine triphosphate, (ATP), is common to all living organisms and is considered the universal transfer agent of chemical energy between energy-yielding and energy-requiring reactions. It has also been suggested as a measure of microbial biomass, and bioluminescence techniques provide a very sensitive assay (Jarvis et al., 1983). Suberkropp et al., (1983) extracted the ATP by homogenisation of the sample in
sulphuric-oxalic acids and quantified it by using a Turner luminometer to measure luciferin-luciferase light emission.

The method has been applied to bacterial contamination of foods (Standard, 1983) and a system has been developed to measure yeast ATP, (Patel and Williams, 1985). Unfortunately fungal ATP has not been well studied, but there are several inherent problems with fungal ATP measurements which have made the technique difficult to carry out. One such problem is that separating both mycelium and conidia from food material is often very difficult. Another problem is that the fungi must be separated from other micro-organisms, (bacteria), before ATP estimation. Extraction of molecules from fungal cells is also notoriously difficult. It must be remembered that fungal ATP must be clearly distinguished from 'total ATP' in the food and the technique becomes impractical in living plant cells as they contain high levels of ATP. Suberkropp et al., (1993) compared ATP and ergosterol as indicators of fungal biomass associated with leaves decomposing in laboratory microcosms and streams. In all studies, the sporulation rates of the fungi colonising leaves were also determined to compare patterns of fungal reproductive activity with patterns of mycelial growth. They found that during leaf degradation, ATP concentrations exhibited significant, positive correlation with ergosterol concentrations in the laboratory and when leaves had been air dried prior to being submerged in a stream. However, when freshly shed leaves were submerged in a stream, the concentration of ATP and ergosterol were negatively correlated during degradation. This appeared to be due to the persistence of leaf-derived ATP in freshly shed leaves during the first 1-2 weeks in the stream. Estimates of fungal biomass from ergosterol concentrations of leaf litter were up to 3 times those calculated from ATP concentrations. Ergosterol
concentrations provide a more accurate indication of fungal biomass in situations in which other organisms make significant contributions to ATP pools.

1.5.5 Pectinesterase Activity

A number of authors worked in this field by measuring the pectinase activity as a rapid means for detecting the presence of spoilage fungi (Sreekantiah & Johns, 1963, Sreekantiah et al., 1973, Abdel-Fattah & Mabrouk, 1976; Dart & Offem, 1980).

Kratka & Vesely (1979) analysed the activity of pectinase, amylase and saccharase in Phythium spp. and studied the effect of pH and time of incubation (24h - 15 days on the cultivation of fungi. The results showed the same pattern in all fungal species examined.

Dart & Offem (1980) studied methods for detection and estimation of low levels of spoilage fungi. One of them was based on the estimation of pectinesterase produced by the fungus when grown in a medium supplemented with pectin. The enzyme hydrolyses the ester linkage of the pectin to produce methanol and polygalacturonic acid. The methanol was measured using gas liquid chromatography. The results showed a straight line correlation between inoculum size (number of spores) and methanol production. Preliminary studies were reported on pure and mixed spore suspensions of Aspergillus and Penicillium species. Practical applications for this technique have yet to be developed.
1.6 Objectives

The purpose of this research work was to investigate experimentally the enumeration of fungi by the counting assay and estimation of fungal biomass by the chitin assay. This was carried out in both the presence and absence of bacteria to assess bacterial interference with the chitin assay. It was decided to use Aspergillus niger in these assays, since these are thermotolerant fungi, able to grow between 8°C and 45°C (Panasenko, 1967) and they are among the most destructive moulds known.

The advantage of alkaline hydrolysis lies in the reduction of time required, which is significantly less than acid hydrolysis which takes more than 16 hours (Swift, 1973, Braid & Line, 1980; Vignon et al., 1985). In addition, the vigorous alkaline treatment reduces the possibility of interference from plant material.
EXPERIMENTAL WORK

2.1 Counting Assay
Fungal spores in commodities were counted by traditional plate counts. This counting assay involves (a) preparation and harvesting of fungal spores (b) determination of total counts and (c) the determination of viable counts.

2.1.1 Preparation and Harvesting of Fungal Spores
The sporulation medium used in this assay was 'Czapek Dox agar'. This medium was prepared in Roux bottles and inoculated with a pure culture of *Aspergillus niger* maintained as slopes in universal bottles. Fungi (mycelium and spores) were suspended in nutrient broth before inoculation. The Roux bottles were incubated at 30°C until a thick mycelial mat covered with spores had grown over the surface of the medium; this process required three days. The spores were harvested into Roux bottles by adding 1/4 strength Ringers solution or distilled water. The contents were then filtered through a cotton wool plug into a conical flask to separate the mycelium. The filtrate was centrifuged for 10 minutes at 5000 r.p.m. The supernatant was decanted and the spores were resuspended in Ringers solution. This spore suspension was stored at 4°C until required.

2.1.2 Total Counts
The spore suspension was serially diluted to give a dilution of 10^-6. The number of spores were determined by means of a counting chamber (Improved Neubauer Chamber). The average number of spores per square and per ml were then calculated for each dilution.
2.1.3 Viable Counts
Plates containing fungal sporulation medium (Czapek Dox agar) were inoculated with 1ml of the appropriate spore dilution ($10^{-3}$...$10^{-6}$) and incubated at 30°C for 48 hours. The fungal colonies for each dilution were counted using an electronic colony counter and the number of viable spores per ml of the standard suspension was calculated.

2.2 Chitin Assay

2.2.1 Definition
The chitin assay was carried out as spectrophotometric determination of chitin in fungi. This measured chitin as equivalents of glucosamine formed after acid or alkaline hydrolysis of chitin. In this study, chitin was subjected to alkaline hydrolysis following the method of Ride & Drysdal (1972). As the hydrolysis of chitin in a concentrated alkali leads to a partially de-acetylated mixture of chitosaccharides (Forester & Webber, 1960; Ride & Drysdale, 1972), chitin can be determined as equivalents of glucosamine and the figure obtained must be multiplied by 2.67, a factor which makes allowance for the incomplete chitin digestion. This has been estimated at 36-37% (Bethlenfalvay et al 1981).

The experimental details are shown in appendix 1.

2.2.2 Explanation
The treatment of chitin with concentrated alkali at high temperatures causes partial depolymerisation and extensive deacetylation to produce a group of compounds collectively known as chitosan (Foster & Webber, 1960; Horton & Lineback, 1965). This remains insoluble if the alkali is removed with progressive dilutions of ethanol in water (Roelofsen & Hoette, 1951). The removal of alkali with aqueous ethanol produces much higher yields of chitosan than when water alone was used as the
washing solvent (Ride & Drysdale, 1972). Celite suspension (as a filter aid) aided centrifugation and removal of the supernatant. The deamination of chitosan results in both production of aldehyde and cleavage of the chitosan rendering it soluble. Potassium bisulphate breaks down the aldehydes. Chitosan usually contains residual acetyl groups (Foster & Webber, 1960) which render portions of the molecule resistant to deamination. Thus only some of the glucosamine residues in the original chitin are converted to aldehyde and can be measured by the method described. An additional centrifugation was introduced into the colourimetric assay to remove the residue resistant to nitrous acid. The 2:5 anhydromannose is the final residue of chitin hydrolysis. This molecule which possesses a free aldehyde group on carbon atom one, reacts with MBTH, a specific reaction of aldehyde groups, and gives a blue colour in the presence of FeCl₃ (Tsuji et al, 1969 a,b). The incubation period with MBTH, which takes 1hr at room temperature is reduced to 3 minutes at 100°C (Ride & Drysdal, 1972).

2.3 Testing of Chitin Assay

Glucosamine, chitosan and chitin were assayed as described in the previous section starting at the appropriate points. For glucosamine and chitosan, the assay started from the colourimetric step, while for chitin the assay started from the hydrolysis step. The assay was carried out on chitosan and chitin which had been soaked in water for 24 hours. At the end of the assay the optical density for each amount was read spectrophotometrically at 650nm.

A blank sample was prepared as described in the section on experimental details using 1.5ml of water as a blank.
2.4 Chitin Assay on Fungi: \textit{Aspergillus niger}

2.4.1 Growth and Preparation of Fungal Suspension

Fungi in nature can be found in two forms: (a) mycelium in the early stages of growth (b) mycelium and spores in the late stage of growth; thus two fungal suspensions were prepared: a mycelial suspension, and a mycelial and spores suspension. Chitin assay was carried out on both suspensions separately to compare them.

To prepare a mycelial suspension, a litre conical flask containing 400ml Czapek Dox liquid medium was inoculated with a fresh spore suspension and incubated in a rotary shaker at 120 r.p.m. at 30°C for 2-3 days. Mycelia were filtered by centrifugation three times (500 r.p.m.) using sterile distilled water as a washing solvent. The final residue was homogenised in sterile distilled water using a blender for two minutes. The mycelial suspension was kept at 4°C until required for the assay.

To prepare mycelial and spore suspensions, Roux bottles containing Czapek Dox liquid medium were inoculated with fresh fungi maintained on slopes and incubated at 30°C for three days, or until a mycelial mat was covered with spores. Mycelia and spores were filtered with the same procedure as used for mycelia, then kept at 4°C until required for the assay.

2.4.2 Chitin Analysis

Different amounts of mycelial suspension and mycelial and spores suspension were centrifuged, mixed with 3ml of KOH and hydrolysed at 130°C for 1 hour. The following procedure of chitin assay was carried out as mentioned before (experimental details), then the optical density for each amount was read spectrophotometrically at 650 nm.
2.5 Chitin Assay on Bacteria

After chitin estimation was carried out on fungi, it was also carried out on different species of bacteria to investigate whether bacteria would interfere with the assay.

2.5.1 Growth and Preparation of Bacterial Suspension

Three different species of bacteria were grown to be used with the assay: *Pseudomonas aeruginosa* (Gram-ve rod), *Bacillus cereus* (Gram+ve rod) and *Staphylococcus aureus* (Gram+ve coccus).

Conical flasks (1l litre) containing 400ml nutrient broth were inoculated separately with the bacteria mentioned above, incubated overnight in a rotary shaker at 37°C for *Ps. aeruginosa* and 30°C for *B.cereus* and *St aureus*. After incubation the bacteria were washed by centrifugation three times at 5000 r.p.m using sterile distilled water as a washing solvent. Each bacterial preparation was diluted with sterile distilled water and kept at 4°C. Bacterial preparations were checked for contamination before any assay was carried out.

2.5.2 Chitin Analysis

Different amounts were taken from each of the bacterial suspensions prepared and centrifuged at (5000 r.p.m superspeed) for 20 minutes. The bacteria harvested were mixed with 3ml of KOH strength and heated at 130°C for 1hour. The chitin assay was carried out as described in the experimental details. The optical density for each amount was read at 650nm.

2.6 Chitin Assay on Mixed Fungi and Bacteria

After chitin estimation was carried out on the bacteria, it was also carried out on mixed fungal and bacterial preparations with the aim of investigating the effect of bacteria on the estimation of fungi.
Pseudomonas aeruginosa did not show a clear pattern of behaviour with chitin assay and further experiments with this organism were discontinued.

The other bacteria, Bacillus cereus and Staphylococcus aureus were mixed with Aspergillus niger and assayed for chitin as described previously. A variety of ratios of bacteria to fungi were measured.

2.7 Counting of Bacteria

The number of colonies of Bacillus cereus and Staphylococcus aureus were determined using serial dilutions and traditional plate counts.

Plates containing nutrient agar medium were inoculated with 1ml of the appropriate dilution and incubated for 24 hours at 30°C. The bacterial colonies for each dilution were counted using an electronic colony counter and the number of viable bacteria per ml in the standard suspension was calculated.
RESULTS AND DISCUSSION

3.1 Counting Assay
Table 1 shows the results obtained after the counting assay was carried out on *Aspergillus niger*. Eight serial dilution were prepared from the same standard suspension. The results shown in table 1 indicates that no correlation between dilution and count could be obtained using a counting chamber. There might be number of reasons to explain such an outcome. Firstly, the spores might clump, causing the standard spore suspension to loose its uniformity. The serial dilution would thus be affected causing the lack of correlation between dilution and count. Secondly, the size and density of fungal spores are greater than those of bacteria, so they sediment much more rapidly in dilution tubes. Sedimentation in the pipette is also surprisingly rapid. Both of these could introduce an error if the samples are not subjected to complete agitation. Thirdly an error which might be introduced is due to operator fatigue during the counting of spores microscopically. Table 1 also shows the number of colonies per ml for each dilution counted using a viable counting method. These results were also inaccurate and unreliable due to the reasons mentioned above.

3.2 Chitin Assay on Glucosamine, Chitosan and Chitin
Chitin assay was carried out on different amounts of glucosamine solutions and different amounts of chitosan and chitin suspensions separately in the aim of comparison and testing the assay. A relationship was developed between optical density and a) Glucosamine b) Chitosan and c) Chitin weight. These relationships were presented separately in Figures 3, 4, 5 respectively. Figure 3 shows a strong linear correlation between optical density and glucosamine, it also shows that the line
passes through the origin as expected. Although the chitosan and chitin curves also show linear correlation (Figures 4 and 5), they do not pass through the origin and the standard deviation for each point is higher than those of glucosamine. This may be due to the fact that chitosan and chitin are natural compounds taken from different natural sources with different chemical compositions and different contaminants.

Another important fact is that the time required for a complete dissolution of chitosan and chitin was not determined. It was noticed that when prepared suspensions of chitosan and chitin with different concentrations were not left for the same period of time to dissolve before the assays were carried out, the standard deviations were higher, and less accurate results were obtained.

It is therefore preferable to use glucosamine as a calibration material unless using purified chitosan and chitin taken from one source.

3.3 Chitin Assay on Aspergillus niger

Alkaline hydrolysis of the chitin in Aspergillus niger was carried out on different amounts of mycelium, and mycelium and spores with the aim of comparing assays of the early stage and the late stages of growth of fungi. The linear relationship between the optical density and the dry weight of mycelium (Fig6) and mycelium and spores (Fig7) is shown. Figure 6 gives more accurate results than Figure 7, because it passes through the origin as expected. Furthermore, the standard deviation for each point in Figure 6 is less than that in Figure 7. This inaccuracy in Figure 7 can again be explained by the characteristic of fungal spores sticking together. The above results confirm the failure of the counting assays with fungal spores.

It is therefore preferable to work with mycelium rather than with mycelium and spores for two reasons: Firstly, the results with mycelium are more accurate, and secondly, the method then assesses the early stages of growth of fungi as sporulation is a secondary feature.
3.4 Chitin Assay on Bacteria

Chitin assays were carried out on different amounts of the bacteria, the species *Pseudomonas aeruginosa, Bacillus cereus* and *Staphylococcus aureus* being used to determine whether they interfere with the assay.

3.4.1 Gram Negative Rod: *Pseudomonas aeruginosa*

From the results obtained it was noticed that the behaviour of *Pseudomonas aeruginosa* with chitin assay is not clear and the results obtained were not linear. It seemed that this bacterium inhibited the assay and the inhibition increased as the bacterial amount increased. The results were non repeatable in spite of the assay being attempted on a number of occasions. Work with *Pseudomonas aeruginosa* was therefore discontinued. It can be concluded that using chitin assays for the estimation of fungal biomass in the presence of *Pseudomonas aeruginosa* cannot be a reliable method to follow.

3.4.2 Gram Positive Bacteria

a) *Bacillus cereus*

Figure 8 shows a linear correlation between optical density and the *Bacillus cereus* dry weight. This linearity continues to reach the maximum linear allowed value of the machine of 1. Figure 8 also shows that the optical density obtained for levels of *Bacillus cereus* below $4 \times 10^7$ organisms is negligible, and that the lower limit of detection of *Bacillus cereus* using this method is $5 \times 10^7$.

b) *Staphylococcus aureus*

Figure 13 shows an initial linear correlation between optical density and *Staphylococcus aureus* dry weight. This linearity continues until reaches a point corresponding to $1.7 \times 10^{13}$ after which the relationship becomes polynomial as shown
in Figure 14. The limit of detection of *Staphylococcus aureus* using this method is about $3.36 \times 10^{12}$ colonies and below this number the optical density is negligible.

### 3.5 Assay of *Aspergillus niger* in the Presence of *Bacillus cereus*.

Chitin assay was carried out on a mixed culture of *Aspergillus niger* and *Bacillus cereus* with the intention of investigating the effect of bacteria on fungal biomass estimation. Figures 9 to 11 show the variation of optical density with *Bacillus cereus* dry weight for different values of *Aspergillus niger* dry weight. They also show the expected curves based on the sum of the optical densities that were calculated from the curve fit equation obtained for the fungus and bacterium separately. It is obvious that the expected figures deviated from the experimental ones and the deviation is beyond the standard deviation for each point. Furthermore, this difference between the experimental and the expected results increases as the levels of *Bacillus cereus* increases, especially for high levels of *Aspergillus niger* (Figure 11). It can be concluded from these figures that the *Aspergillus niger* biomass is underestimated in the presence of *Bacillus cereus* especially at higher values of the fungus. For small amounts of the fungus the underestimation varies from 4.5 to 9 percent as shown in Figures 9 and 10. However, for a high mycelial mass the underestimation increases and varies from 10 to 24 percent as the *Bacillus cereus* mass increases.

### 3.6 Assay of *Aspergillus niger* in the Presence of *Staphylococcus aureus*

Chitin assay was carried out on a mixed culture of *Aspergillus niger* and *Staphylococcus aureus*. Figures 15 to 17 show the variation between optical density with *Staphylococcus aureus* dry weight for the different values of *Aspergillus niger*. It is obvious that the experimental curves are below those expected which are based also on the sum of the optical densities that were calculated from the curve fit obtained for the fungus and the bacterium separately. The difference between the
experimental and the expected results is beyond the standard deviation of each experimental point. It can be concluded that the presence of *Staphylococcus aureus* causes an underestimation of about 8 percent of *Aspergillus niger* biomass using chitin assay.

### 3.7 Counting of Bacteria

The number of colonies for *Bacillus cereus* and *Staphylococcus aureus* were counted using the plate count method. Figures 12 and 18 show a linear relationship between the dry weight for each of *Bacillus cereus* and *Staphylococcus aureus* with the number of colonies.

*Bacillus cereus* is a bacterium which gives spores at a certain stage of growth, thus the counting method was carried out with a fresh bacterial preparation to avoid the contribution of spores to the dry weight.
Table 1

Total and Viable Count of *Aspergillus niger* Spores

The standard spore suspension of the fungus *Aspergillus niger* was serially diluted. The total count of the spores was carried microscopically and the viable count was carried out by the method of plate counts. This work was repeated eight times with eight serial dilutions prepared from the same standard suspension.
<table>
<thead>
<tr>
<th>Dilution</th>
<th>Av no of sp/sq</th>
<th>No of sp/ml</th>
<th>No of colonies/ml</th>
<th>No of viable sp/ml in std suspension</th>
<th>Mean ± std dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-1}$</td>
<td>uncountable</td>
<td>7.68x10^6</td>
<td>uncountable</td>
<td>51</td>
<td>5.1x10^6 ± 1.05x10^6</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>1.92</td>
<td>1.32x10^6</td>
<td>uncountable</td>
<td>18</td>
<td>1.8x10^5 ± 8.4x10^4</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>0.33</td>
<td>10^6</td>
<td>uncountable</td>
<td>over 300</td>
<td>1.75x10^6 ± 1.25x10^6</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>0.25</td>
<td>6.4x10^5</td>
<td>uncountable</td>
<td>over 200</td>
<td>4.7x10^6 ± 3.5x10^6</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>0.16</td>
<td>3.2x10^5</td>
<td>5</td>
<td>5x10^5</td>
<td>6x10^6 ± 3x10^6</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>0.08</td>
<td>3.2x10^5</td>
<td>6</td>
<td>6x10^6</td>
<td>6x10^6 ± 3x10^6</td>
</tr>
</tbody>
</table>

### Additional Notes
- The table represents the average numbers of spores per square (sp/sq), total spores per milliliter (sp/ml), number of colonies per milliliter, and viable spores per milliliter in standardized suspension for different dilutions.
- The mean values are given along with their standard deviation (± std dev).
Fig. 3

Glucosamine Weight Against Optical Density

The standard solution used was glucosamine-hydrochloride. A graph was plotted between optical density and weight of glucosamine (mg). The calculation was based on the weight of glucosamine, the weight of HCL was not taken into account.

\[ y = 16.09x \quad r = 0.99 \]

Each point is the mean of three assays, two determinations per assay.
The optical density was determined for different amounts of chitosan. A graph was plotted between optical density and weight of chitosan (mg).

\[ y = -0.04 + 0.032 \quad r = 0.9 \]

Each point is the mean of three assays, two determinations per assay.
The optical density was determined for different amounts of chitin. A graph was plotted between weight of chitin against optical density (mg).

\[ y = 0.05 + 0.006x \quad r = 0.97 \]

Each point is the mean of three assays, two determinations per assay.
The optical density was determined for different amounts of mycelial suspension. A graph was plotted between optical density and mycelial dry weight (mg).

\[ y = 0.494 \times \quad r = 0.99 \]

Each point is the mean of three assays, two determinations per assay.
Optical density

versus

Aspergillus niger (mycelium + spores) dry weight

The optical density was determined for different amounts of mycelial and spores suspension. A graph was plotted between optical density and mycelium plus spores dry weight(mg).

\[ y = 0.065 + 0.285 \times \quad r = 0.95 \]

Each point is the mean of three assays, two determinations per assay.
The optical density for different amounts of *Bacillus cereus* suspension was determined. A graph was plotted between optical density and *Bacillus cereus* dry weight (mg)

\[ y = 0.068 \times \quad r = 0.99 \]

Each point is the mean of three assays, two determinations per assay.
Bacillus cereus dry weight (mg)

O.D. 1

0

0 5 10 15 20

Bacillus cereus dry weight (mg)
A suspension containing a fixed amount of *Aspergillus niger* mycelium equivalent to 0.2 mg dry weight was assayed with varying amounts of *Bacillus cereus* suspension.

**Experimental results**

\[ y = 0.056 x + 0.056 \]

\[ r = 0.99 \]

The expected results based on the addition of the optical densities calculated from the curves fit obtained for *Aspergillus niger* alone and *Bacillus cereus* alone were plotted.

**Expected results**

\[ y = 0.099 + 0.068 x \]

\[ r = 1 \]

Each point is the mean of three assays, two determinations per assay.
Graph of Fixed Amount of *Aspergillus niger* Mycelium

With

Varying Amount of *Bacillus cereus*

A suspension containing a fixed amount of *Aspergillus niger* mycelium equivalent to 0.4mg dry weight was assayed with varying amounts of *Bacillus cereus*.

Experimental results ■ \[y = 0.063x + 0.159\]

\[r = 0.995\]

The expected results based on the addition of the optical densities calculated from the curves fit for *Aspergillus niger* alone and *Bacillus cereus* alone.

Expected results ● \[y = 0.068x + 0.198\]

\[r = 1\]

Each point is the mean of two assays, two determinations per assay.
Bacillus cereus dry weight (mg)

O.D.

2 4 6 8 10

Bacillus cereus dry weight (mg)
A suspension containing a fixed amount of *Aspergillus niger* mycelium equivalent to 1.21 mg dry weight was assayed with varying amounts of *Bacillus cereus*.

**Experimental results**  
\[ y = 0.047x + 0.542 \]  
\[ r = 0.99 \]

The expected results based on the addition of the optical densities calculated from the curves fit obtained for *Aspergillus niger* alone and *Bacillus cereus* alone.

**Expected results**  
\[ y = 0.068x + 0.599 \]  
\[ r = 1 \]

Each point is the mean of three assays, two determinations per assay.
The number of colonies for different amounts of *Bacillus cereus* suspension were determined by the counting assay, for each of these amounts the appropriate dry weight was determined. A curve was plotted between colony number and dry weight.

\[ y = 7.14 \times 10^7 x + 5 \times 10^5 \]

\[ r = 0.98 \]

Each point is the mean of two determinations.
colonies no. x10^8

Bacillus cereus dry weight (mg)
The optical density for different amounts of *Staphylococcus aureus* suspension was determined. For each of these amounts the appropriate dry weight was determined. A curve was plotted between optical density and *Staphylococcus aureus* dry weight (mg).

\[ y = 0.03 \times \quad r = 0.99 \]

Each point is the mean of three assays, two determinations per assay.
Staphylococcus aureus dry weight (mg)
The optical density for different amounts of *Staphylococcus aureus* suspension was determined, for each of these amounts the appropriate dry weight was determined. A curve was plotted between optical density and *Staphylococcus aureus* dry weight(mg).

\[ y = -0.0016x^2 + 0.042x - 0.0117 \quad r = 0.99 \]

Each point is the mean of three assays, two determinations per assay.
Staphylococcus aureus dry weight (mg)
A suspension containing a fixed amount of *Aspergillus niger* mycelium equivalent to 0.2mg dry weight was assayed with varying amounts of *Staphylococcus aureus*.

Experimental results ■ \[ y = 0.07 + 0.026 \times \]
\[ r = 0.099 \]

The expected results based on the addition of the optical densities calculated from the curves fit obtained for *Aspergillus niger* alone and *Staphylococcus aureus* alone.

Expected results ● \[ y = 0.098 + 0.031 \times \]
\[ r = 1 \]

Each point is the mean of three assays, two determinations per assay.
A suspension containing a fixed amount of *Aspergillus niger* mycelium equivalent to 0.4mg dry weight was assayed with varying amounts of *Staphylococcus aureus*.

**Experimental results**

\[ y = 0.166 + 0.028x \]

\[ r = 0.099 \]

The expected results are based on the addition of the optical densities calculated from the curves fit obtained for *Aspergillus niger* alone and *Staphylococcus aureus* alone.

**Expected results**

\[ y = 0.199 + 0.031x \]

\[ r = 1 \]

Each point is the mean of three assays, two determinations per assay.
A suspension containing a fixed amount of *Aspergillus niger* mycelium equivalent to 1.21mg dry weight was assayed with varying amounts of *Staphylococcus aureus*.

Experimental results
\[ y = 0.551 + 0.0282x \]
\[ r = 0.994 \]

The expected results based on the addition of the optical densities calculated from the curves fit obtained from *Aspergillus niger* alone and *Staphylococcus aureus* alone were plotted.

Expected results
\[ y = 0.603 + 0.031x \]
\[ r = 0.99 \]

Each point is the mean of three assays, two determinations per assay.
The number of colonies for different amounts of *Staphylococcus aureus* suspension were determined by the counting assay, for each of these amounts the appropriate dry weight was determined. A curve was plotted between colony number and dry weight.

\[ y = 2 \times 10^{12} x + 4 \times 10^{10} \quad r = 0.992 \]

Each point is the mean of two determinations.
Staphylococcus aureus dry weight (mg)

Colonies no. $\times 10^{12}$

![Graph showing the relationship between Staphylococcus aureus dry weight and colonies number.]
CONCLUSION

The assessment of fungi in foodstuffs and others commodities is of considerable importance due to the damage that they cause. There are a variety of methods available for the determination of fungi and fungal metabolites in many products. None of these methods however takes into account the influence of bacteria on the method of measurement. Bacteria of various types are however frequently present in considerable numbers in produce damaged by fungi.

This research has studied the experimental estimation of fungi in the presence of varying amounts of different bacteria. The results indicate that the influence of bacteria on the chitin assay varies from bacterial species to species and is not always predictable. The results obtained with *Pseudomonas aeruginosa* were not repeatable. Those obtained with *Bacillus cereus* were linear whilst those obtained with *Staphylococcus aureus* were only linear to a certain level. In all cases the presence of bacteria interfered with the assay of chitin and therefore this assay cannot be regarded as a reliable method for the estimation of fungi in the presence of quantities of bacteria.

The results also indicate that the chitin method is not suitable even when no bacteria are present. The results obtained using mycelia only (Fig6) and mycelia which have commenced sporing (Fig7) show that there is a significant difference. The graph obtained for mycelia and spores also shows a much greater standard deviation then that for mycelia only. Therefore, before using the chitin assay for fungi in produce,
it would be necessary to know the level of sporulation which the fungus had undergone. This would be extremely difficult to determine.

It can therefore be concluded that the assay of chitin is not a reliable method for the estimation of spoilage fungi.
APPENDIX 1

Experimental Details

The assay of chitin involves two steps: These are the conversion of chitin to chitosan and the colourimetric assay (Ride & Drysdale, 1972), as described below

1) Conversion of Chitin to Chitosan

   Reagents:
   a) 120g % (w/v) potassium hydroxide KOH
   b) 75% (v/v) aqueous ethanol
   c) 40% aqueous ethanol
   d) celite suspension (as a filter aid)-the supernatant obtained by allowing a suspension of 1g of celite in 20ml of 75% ethanol to stand for 2 minutes.

   Procedure:
   • Preparation of fungal suspensions.
   • Centrifugation of different amounts of fungal suspension (1500g, 10minutes, 2°C) and removing of the supernatant.
   • Mixing of the residue with 3ml of KOH solution and heating at 130°C for 1 hour.
   • After cooling, mixing of the alkaline solution with 8ml of 75% ethanol and allowing to stand in ice-water for 15minutes.
   • Layering of the celite suspension (0.9 ml) on top and recentrifugation of the tubes at (1500g, 10minutes, 2°C).
   • Removing of the supernatant, washing of the residue with 40% ethanol and recentrifugation at (1500g, 10minutes, 2°C).
   • Repeating the procedure twice with cold distilled water as the washing solvent.
   • Diluting of the final residue to 1.5ml (corresponding to the volume of water in which the chitosan was suspended).
2) Colourimetric Assay

**Reagents:**

a) 5% (w/v) sodium nitrite NaNO₂

b) 5% potassium hydrogen sulphate KHSO₄

c) 12.5% ammonium sulfamate NH₄SO₃NH₂

d) 0.5% 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) prepared fresh daily.

e) 0.5% ferric chloride FeCl₃ stored at 4°C and discarded after 3 days.

**Procedure:**

- Transferring of 1.5ml level; equal volumes of chitosan suspension, NaNO₂ and KHSO₄ to a centrifuge tube, shaking for 15 minutes and centrifugation at (1500g, 2°C) before removing of two samples (1.5ml) of the supernatant.

- Addition to each of these samples 0.5ml NH₄SO₃NH₂ and after shaking for 5 minutes, addition of 0.5ml of 0.5% MBTH.

- Heating of the mixture in a boiling water bath for 3 minutes, and after cooling addition of 0.5ml of 0.5% FeCl₃.

- Standing for 30minutes, and reading the optical density at 650 nm in 1cm cells.
REFERENCES


