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EARLY DETECTION AND DIFFERENTIATION OF MICROBIAL SPOILAGE OF BREAD USING ELECTRONIC NOSE TECHNOLOGY

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Supervisor:
Professor Naresh Magan

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This thesis is submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

DECLARATION

I hereby declare that the work presented in this thesis is the result of my own investigations, unless stated, and has not been accepted in a previous application for a degree.

Rachel Needham

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"Smells can be like fingerprints"

Grissom, C.S.I. Crime Scene Investigation, episode 2.22.

ABSTRACT

This study investigated the potential for use of electronic noses (e-noses) for early rapid detection and differentiation of bread spoilage before visible signs of growth occur. After 24 h incubation at 25°C it was possible to distinguish *Penicillium verrucosum*, *Aspergillus ochraceus,* and *Pichia anomala* from 3 different species of filamentous fungi before visible growth was observed on unmodified wheat agar using a conducting polymer based e-nose (BH114). Discrimination of controls was possible after 48 h. The BH114 e-nose was able to differentiate between *Pseudomonas fragi*, *Saccharomyces cerevisiae* and *P. verrucosum* growing on 0.97 a_w modified flour-based media after only 24 h. The BH114 e-nose was able to discriminate between *P. fragi* and *S. cerevisiae* growing in broth cultures and between different a_w controls in exponential growth (13.5 h). Discrimination of *Staphylococcus aureus* growing in different a_w broths and from uninoculated controls was achieved after 4.5 h.

The BH114 e-nose was also able to detect and differentiate microbial spoilage *in situ* using bread analogues. Discrimination was improved using an incubation temperature of 25°C when compared to 15°C. Discrimination of microbial and physiological (enzymic) spoilage of bread analogues was possible using e-nose technology, cfu counts and gas chromatograph-mass spectrometry (GC-MS) using an initial population of 10^6 spores/cells ml^{-1}. After 48 h differentiation of the spoilage types and between some of the microbial spoilage organisms was possible using the e-nose. A significant increase in populations was noted between 24 and 48 h. There were significant differences between microbial populations detected after 48 and 72 h. Analysis of volatile compounds produced, using GC-MS, showed that after 24 h *P. anomala* was the only treatment to produce 2-propanol, ethyl acetate, and pentanol. *P. anomala* also produced greater amounts of 3-methylbutanol when compared to *P. verrucosum*, *B. subtilis*, lipoxygenase spoilage and controls.

Differentiation between toxigenic and non-toxigenic strains of *Aspergillus parasiticus in vitro* was not achieved.   However, *in vitro* on unmodified 2% wheat agar it was possible to differentiate a non-toxigenic *P. verrucosum* strain from 4 citrinin producing strains and controls using the BH114 e-nose. On bread analogues it was possible to

discriminate two ochratoxin A (OTA) producing *P. verrucosum* strains after 24 h using an initial population of 10^6 spore ml^{-1}. Increased incubation resulted in only controls being discriminated. Using a lower initial population of 10^3 spores ml^{-1} only controls were discriminated after 24 h. However, after 48 h an OTA producing strain could also be differentiated.

The potential for use of e-noses as a tool for screening novel antioxidants was also investigated. It was possible to differentiate between broth samples with and without the antioxidants propyl paraben and butylated hydroxyanisole using both the conductance based e-nose (BH114) and a metal oxide and metal ion based e-nose (NST3220 lab emission analyser). When samples without antioxidant were removed it was possible to differentiate treatments containing antioxidant that had been inoculated with micro-organism and those that had not. The e-noses were also able to discriminate between sample times. Microbial populations and carbon dioxide levels increased with incubation time. *P. verrucosum* and *P. anomala* populations were greater in treatments without antioxidant whereas *B. subtilis* populations were greater in 0.97 a_w treatments containing antioxidant. CO_2 production was greater in inoculated treatments without an antioxidant except at 0.95 a_w *P. verrucosum* produced greater volumes in the presence of the antioxidant.

Using natural bread cross validation studies of 4 unknown contaminants (*P. anomala*, *P. verrucosum* and *B. subtilis* and controls) was performed. This showed that using initial populations of 10^3 spores/cells ml^{-1} the BH114 e-nose was able to differentiate between all the unknown treatments after 48 h and the NST3220 lab emission analyser after 72 h. CO_2 production could be used to detect controls but it was not possible to differentiate between the micro-organisms.

CONTENTS

LIST OF FIGURES AND PLATES

LIST OF TABLES

ABBREVIATIONS

ATP   - Adenosine triphosphate

a_w - water activity

BHA   - Butylated hydroxyanisole

BHT   - Butylated hydroxytoluene

CA   - Cluster Analysis

cfu   - colony forming units

DEFT   - Direct epifluorescent filter technique

DFA   - Discriminate Function Analysis

DNA   - Deoxyribonucleic acid

ELISA   - Enzyme linked immunosorbent assay

e-nose   - electronic nose

FIA   - flow injection analysis

GC-MS   - Gas chromatograph-mass spectrometry

HACCP   - Hazard analysis critical control points

HPLC   - High performance liquid chromatography

LAT   - Latex agglutination test

MEA   - Malt extract agar

MOSFET   - metal oxide semiconductor field effect transistor sensors

NYDA   - Nutrient yeast dextrose agar

NYDB   - Nutrient yeast dextrose broth

OTA   - Ochratoxin A

PARC   - Pattern recognition

PC   - Principle component

PCA   - Principle Components Analysis

PCR   - Polymerase chain reaction

PDA   - Potato dextrose agar

PP   - Propyl paraben

SHA   - Static headspace analysis

TBHQ - Butylated hydroxyquinone

Units of measurement

°C   - degrees Celsius

d   - day

h   - hour

sec   - seconds

min   - minutes

g   - gram

l   - litre (1000 ml)

ml   - millilitre (10^{-3} l)

μl   - microlitre (10^{-6} l)

w/v   - weight per volume

nm   - nanometer

CHAPTER 1

INTRODUCTION

1.1   INTRODUCTION

Food can be contaminated with spoilage or pathogenic micro-organisms. Spoilage organisms spoil the product so that it is not fit to eat or changes the taste so that the product is not desirable. They are generally not harmful if ingested. Pathogenic organisms, however, can cause food poisoning. Food poisoning can arise either through the ingestion of food containing micro-organisms, or by the ingestion of food containing toxins, which have been pre-formed by the micro-organisms. The cost of food poisoning to the food industry can be huge. It is not uncommon for the company producing a product implicated in a food poisoning outbreak to go bankrupt as a result. Companies producing similar products not directly involved in the outbreak can also suffer with a general decline in demand due to public concern that the same problem could occur. The cost of food spoilage is also great with loss of money due to wastage of product or raw materials. There is therefore a need for rapid methods to prevent contaminated foods reaching, and being consumed by the consumer.

In the past, control of the safety of foods has been mainly carried out by product testing of both raw materials and end products. The main problem with performing end-product testing is the high number of samples to be examined before one can decide on the safety of the product batch, especially when pathogens are expected to be heterogeneously distributed in the batch (de Boer & Beumer, 1999). Also end product testing detects only failures and does not identify causes. The Hazard Analysis Critical Control Points (HACCP) system is now generally considered the method of choice for ensuring the safety of foods (Jay, 1996; de Boer & Beumer, 1999). HACCP involves identifying places in the production process where hazards could occur (critical control

points, CCPs) and putting monitoring procedures in place to prevent these hazards occurring.

Even with a HACCP system in place samples still need to be tested for the presence of micro-organisms. Traditional cultural detection of most micro-organisms requires growth of the organism on selective media, which can take a number of days from isolation to identification. These methods are sensitive, inexpensive and give qualitative information on the number and the nature of the micro-organisms present in a food sample. However, conventional methods require several days to produce results because they rely on the ability of micro-organisms to multiply to visible colonies (de Boer & Beumer, 1999). Moreover, culture medium preparation, inoculation of plates, colony counting and biochemical characterisation make these methods labour intensive (de Boer & Beumer, 1999). Traditional methods are of limited value especially for the analysis of perishable foods since the foods are sold and eaten before the results of the tests are known. Rapid methods can reduce the time taken to achieve results from days to a few hours or even minutes.

Rapid methods commercially available include DNA probes, the Polymerase Chain Reaction (PCR), latex agglutination tests, direct epifluorescent filter techniques, Enzyme Linked Immunosorbant Assay (ELISA), conductance, impedance, bioluminescence, immunomagnetic beads and biochemical assays such as API 20E and Micro ID. Methods currently available for measuring mould contamination in food include: microscopic examination, culture on agar, electrical measurements of conductance and other changes in the electrical properties of the contaminated food

substratum and detection of fungal metabolites such as chitin, ergosterol or ATP (Girardin, 1997). These methods are briefly summarised in the following sections.

1.1.1 Colony forming units

Counting of colony forming units (cfus) has been the traditional method used to quantify fungal and bacterial populations. The simplicity of this method is however outweighed by its drawbacks. The method requires long incubation times on selective media, is labour intensive and measures only culturable organisms. CFUs are also not related to actual activity. Despite these drawbacks cfus are used as the standard method in the food industry.

1.1.2 Conductance and Impedance Methods

Conductance is the ability of a system to conduct an electrical charge. Microbial growth and metabolism produce a change in conductivity by metabolising substrates of low conductivity into smaller, mobile products of higher conductivity. An example of this, described by Silley & Forsythe (1996), is the conversion of glucose to two molecules of lactic acid with an increase in conductivity. Further metabolism converts the lactic acid and three oxygen molecules to carbonic acid, which is an even more effective electrical conductor. The smaller ions decrease the impedance, resistance to flow and therefore the conductance increases (Harrigan & Park, 1991). Problems are encountered with some selective media, e.g., those with a high salt concentration (Silley & Forsythe, 1996). This can be overcome by using the indirect method, which is used for yeasts, which produces a negative change in conductance. The indirect method detects production of carbon dioxide by detecting changes in a potassium

hydroxide solution, as carbon dioxide dissolves in it. Deak & Beuchat (1993) examined fruit juice concentrates containing an average microbial population of 35 cfu ml^{-1} by traditional plating techniques and direct and indirect impedance. The indirect method produced results 10 – 20 h before the direct method with detection after approximately 50 h. The detection time was shortened to an average of 14 h if the samples were incubated for 24 h at 25 °C.

1.1.3   DNA probes

DNA probes were the first nucleic acid based assays for the detection of food-borne pathogens and were introduced for food analysis in the early 1980's (Feng, 1997). Commercially available kits target ribosomal RNA (rRNA). The technique exploits the species specific signatures encoded by nucleic acid sequences using nucleic acid segments called probes. The method can be as quick as 45 minutes but requires overnight cultures, which can present some problems with the use of selective broths. DNA probes have been found to be highly specific (Patel & Williams, 1994). Samadpour *et al.* (1990) used DNA probes to detect shiga-like-toxin-producing *Escherichia coli* in a range of food samples. They were able to detect 1.3 cfu g^{-1} of sample with results obtained within 48 h even with an enrichment step incorporated into the method. DNA probes can be combined with other detection methods, which are described in the following sections.

1.1.4   Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is used more for identification of organisms than enumeration. Short segments of DNA, or primers, are used in a thermocycler to

amplify enzymatically a specific segment of the bacterial genome (Feng, 1997). The usefulness applied directly to foods is limited by the complex composition of food components, which inhibit PCR amplification (Swaminathan & Feng, 1994; Feng 1997). Only a few PCR assays have been developed into commercial kits. Fach & Popoff (1997) found that a duplex PCR method was rapid, sensitive and reliable for the detection and identification of strains of *Clostridium perfringens* in food samples. Duplex PCR utilises 2 sets of primers, which amplify 2 different fragments simultaneously in the same reaction. Laberge *et al.* (1996) achieved a detection limit of 1 to 10 *Cryptosporidium parvum* oocysts in 20 ml of artificially contaminated milk using PCR. Ingianni *et al.* (2001) combined DNA probes with PCR for the detection of *Listeria monocytogenes* in a range of foods. PCR combined with DNA probes produced more positive results and was more sensitive than PCR alone or traditional cultural methods. Results were also obtainable within a working day after a enrichment step of 68 h, which improved results.

1.1.5   Direct Epifluorescent Filter Technique

The direct epifluorescent filter technique (DEFT) is an extremely rapid microbiological analytical method, which has been used for many years for the direct quantification of microbial load in a variety of applications including predicting the shelf-life of foods. The method has gained acceptance for use with milk and milk-based products. The basic principles are that micro-organisms are trapped on a membrane filter, which is stained with a fluorescent dye, e.g., acridine orange, and then examined using epifluorescent microscopy. The total microbial population can then be rapidly enumerated using image analysis (Tortorello & Stewart, 1994). DEFT only takes

between 2 −30 mins to perform and provides the ability to distinguish between viable and non-viable bacteria. This is due to the use of acridine orange, which fluoresces different colours in cells during different phases of growth. A disadvantage of DEFT is that it only allows presumptive identification on morphology. A completely automated DEFT system has been developed. The method incorporates the use of 3 computers to attend to the sample preparation, staining, filtration, drying and image analysis stages (Sharpe, 1994).

1.1.6   Latex agglutination tests

Latex agglutination tests (LAT) incorporate the use of antibody coated coloured beads to agglutinate specific antigens, the bacterial cells. LAT are also available for analysis of bacterial toxins, which are sometimes known as reverse passive latex agglutination (RPLA) because the antibodies are attached (reverse) to latex beads (passive matrix) and used to detect soluble antigens (toxins) (Feng, 1997). LAT are simple to perform, very specific and yield results quickly. LATs are available for a range of bacteria. Hazeleger *et al*. (1992) found the *Campylobacter* test fast but produced false negatives and Manafi & Sommer (1992) found the *Salmonella* test had sensitivity and specificity of 96%.

Mould latex agglutination tests (MLA) depend on recognition of mould antigens. Two MLA tests specific for *Aspergillus* and *Penicillium* exopolysaccharide antigens have been developed (Girardin, 1997). These have however been removed from the market due to poor sales.

1.1.7 Adenosine triphoshate (ATP) bioluminescence

ATP bioluminescence is based on the reaction that naturally occurs in the North American firefly, *Photinus pyralis* (Kyriakides & Patel, 1994). The reaction, catalysed by the enzyme luciferase, uses the chemical energy contained within ATP (adenosine triphosphate) molecules to drive the oxidative decarboxylation of the co-factor luciferin. The resultant production of yellow-green light is detected using a luminometer. A linear relationship exists between the concentration of ATP and light output, because one photon of light is emitted for every ATP molecule consumed (Kyriakides & Patel, 1994). ATP is however the source of energy for all living organisms and therefore separation of bacterial or fungal ATP is the main problem encountered. ATP is predominately used for hygiene testing in the food industry. Samkutty *et al.* (2001) used ATP bioluminescence for the rapid assessment of bacteriological quality of raw milk. They found that ATP could be useful in predicting standard plate counts in raw milk.

1.1.8 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-Linked Immunosorbent Assays (ELISA) involve absorbing specific antibodies onto wells of microtitre plates and the addition of complementary antigens. The enzyme-linked antibody specific for a test antigen then binds to the antigen forming a double antibody sandwich. A visible colour change is produced by the addition of the substrate, which is measured spectrophotometrically.

Several sandwich ELISAs have been developed for detecting the most frequently isolated fungal food contaminants: *Aspergillus* and *Penicillium*, *Fusarium*, *Mucor* and

Rhizopus, *Cladosporium*, *Geotrichium* and *Botrytis* (Girardin, 1997). Using the ELISA protocol, a number of food samples can be tested simultaneously and automatically.

1.1.9 Chitin Assay

Chitin, a polymer of *N*-acetyl-D-glucosamine, is a major constituent of the walls of fungal spores and mycelium. It is not present in bacteria or foods. The assay as described by Pitt & Hocking (1994) involves alkaline hydrolysis of the sample causing partial depolymerisation of chitin to produce chitosan. Subsequent treatment with nitrous acid then causes partial solubilisation and deamination of glucosamine residues to produce 2,5-anhydromannose, which is estimated colourimetrically using 3-methyl-2-benzothiazolone hydrazone hydrochloride as the principle reagent. Improved assay sensitivity has been achieved by derivisation of glucosamine and other products with *o*-phtalaldehyde, separated by high performance liquid chromatography (HPLC) and detection of fluorescent compounds. The disadvantages of the assay are that it is rather complex requiring sophisticated and expensive equipment, the process is slow taking about 5 h, and it lacks sensitivity. Also chitin does not increase proportionally with fungal growth and the assay lacks sensitivity so the results can be misleading (Magan, 1993). Discrimination between viable and non-viable cells is also not possible.

1.1.10 Ergosterol Assay

Ergosterol is the major steroid produced by fungi. It occurs as a lipid of fungal cell membranes, so is inherently likely to be correlated with hyphal growth and biomass. For estimation, samples are blended with methanol, saponified with strong alkali,

extracted with petroleum ether, and fractionated by HPLC (Pitt & Hocking, 1994). Ergosterol is detected by ultraviolet absorption, optimally at 282nm, a wavelength at which other sterols exhibit little or no absorbance (Pitt & Hocking, 1994). Determination of the ergosterol content is a reliable measure of fungal growth and is a more sensitive measure of biomass than chitin content (Börjesson *et al*., 1990). Tothill *et al.* (1992) demonstrated a significant correlation between ergosterol content and total CFUs for naturally contaminated wheat at 0.95 a_w. The disadvantage of the assay is that it is time consuming and includes both viable and non-viable cells.

A comparison of time needed to perform a test, to obtain a result and sensitivity of some of the rapid methods mentioned are shown in Figure 1.1 and Table 1.1. New rapid methods are always being researched and developed for application for detection of micro-organisms. A new method, as described by Grow *et al.* (2003) called μSERS is a new biochip technology being developed for label-free detection of pathogens and their toxins. Another area being studied is the use of various biosensors (Deisingh, 2003; Leonard *et al*., 2003).

1.2   PRODUCTION OF VOLATILES/ODOURS

Micro-organisms produce a wide range of alcohols, ketones, aldehydes, esters, carboxylic acids, lactones, terpenes, sulphur and nitrogen compounds (Pasanen *et al*., 1996). Among the substances produced by fungi aliphatic alcohols, carbonyls, esters, lactones, aromatics and terpenoids dominate (Jeleń & Wąsowicz, 1998). These compounds represent both primary and secondary metabolites. The main metabolic pathways for secondary metabolite production are presented in Figure 1.2.
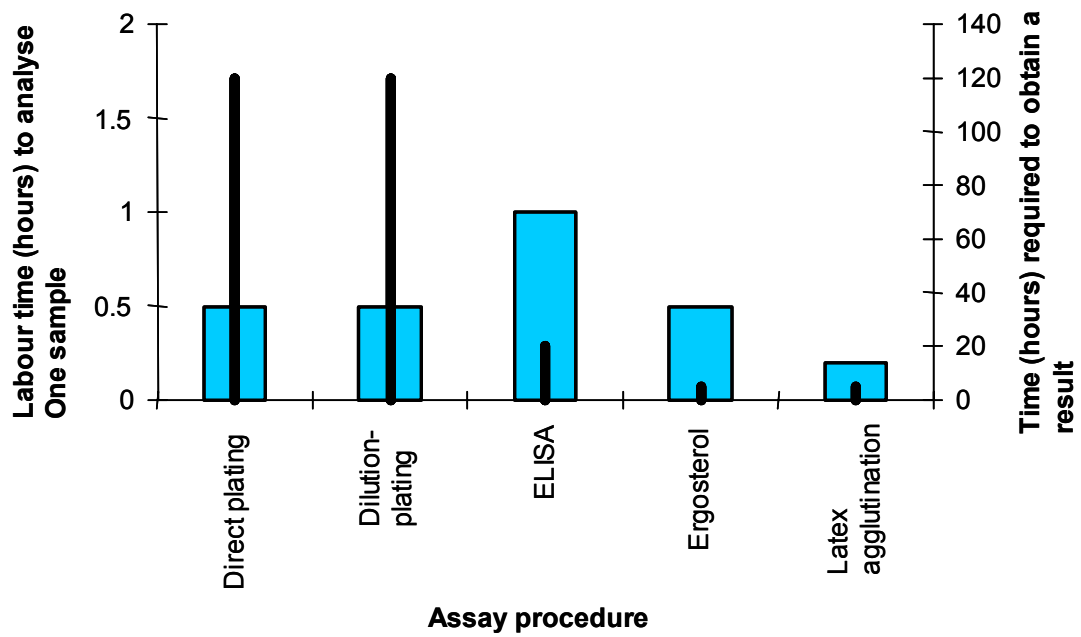
Figure 1.1 Comparison of the time required to perform (▮) and to obtain results (**I**)

for the different assays (adapted from Twiddy & Phillips, 1995).

Table 1.1 Detection limits of some rapid detection methods (adapted from Twiddy, 1994; Twiddy & Phillips, 1995; de Boer & Beumer, 1999).

Method	Detection limit CFU ml^{-1} or g^{-1}
Direct-plating	1
Dilution-plating	10-100
Impedimetry	1
Bioluminescence	10^4
ELISA	10^3-10^5
DEFT	10^3-10^4
Latex agglutination	10^2-10^3
Nucleic acid based assay	10^3
Ergosterol	10^4-10^5

Glucose → Aromatic secondary metabolites

Ethanol

Nitrogen compounds

Pyruvate

Malonate

Aromatic amino acids (Phenylalanine)

Amino acids (e.g. Leucine, Valine

Alcohols

TCA-cycle: Oxalo-acetate

Acetyl (Acetyl-CoA) → Polyketides → Ochratoxins

Mevalonate

Fatty acids → Methyl ketones

Methionine

Threonine ← Aspartate

Lactones

Esters

Aldehydes

Alcohols

Secondary alcohols

Geranyl pyrophosphate

Sulphur compounds

Farnesyl pyrophosphate

Monoterpenes 2-methylisoborneol

Sesquiterpenes Geosmin

Trichothecenes

Figure 1.2 Pathways involved in the production of different secondary metabolites (Pasanen *et al*., 1996; Magan & Evans, 2000).

Off-odours and taints arise in foods due to decomposition caused by endogenous enzymes, contamination or chemical oxidation (Hodgins & Simmonds, 1995). There are many examples of these including microbial spoilage which can lead to putrid, ammoniacal, musty/mouldy odours in a range of products due to sulphur volatiles, amines and chloroanisoles (Hodgins & Simmonds, 1995). Production of a volatile may be a way of removing inhibitory intermediates from the metabolism under unfavourable conditions (Magan & Evans, 2000). Many factors, such as substrate, temperature, oxygen concentration, age of the culture and microbial species have been observed to affect the composition of volatiles.

1.2.1 Bacterial volatiles

The volatiles from bacteria generally come from the breakdown of protein as well as carbohydrates, and consist of the products of decarboxylation and deamination of amino acids, which have been formed by the proteolytic degradation of proteins (Cowell & Ratcliffe, 1997). These vapours are generally methylamine, dimethylamine, trimethylamine and ammonia. They can also be sulphur based, e.g., hydrogen sulphide, methyl mercaptan, dimethyl disulphide, derived from sulphur containing amino acids such as cysteine and methionine (Cowell & Ratcliffe, 1997). Amines such as histamine are released from bacterial metabolism of the amino acid histidine: this is particularly a problem in fish such as mackerel and tuna (Cowell & Ratcliffe, 1997). The bacterial breakdown of proteins does not necessarily produce obnoxious smelling compounds. A variety of ketones and aldehydes are produced such as acetone, butanone, diacetyl, methyl butanal and acetaldehyde. Esters such as

methyl acetate and low molecular weight fatty acids are often also produced (Cowell & Ratcliffe, 1997).

Spoilage of milk products by *Pseudomonas fragi* is characterised by the production of a strawberry-like odour. Ethyl esters of butyric, hexanoic, and 3-methylbutanoic acid were shown to be the major contributors to the odour on the basis of their relative concentration in the headspace (Cormier *et al.,* 1991).

Off-odours can be used to indicate the degree of spoilage. Viehweg *et al.* (1989a; 1989b) showed that different stages of chicken carcass spoilage were linked with certain volatiles produced by the microflora. Mal-odours were attributable to the spoilage flora producing hydrogen sulphide, methanethiol and esters. In contrast, primary aliphatic alcohols were an index of freshness.

1.2.2   Fungal volatiles

Moulds can produce volatiles such as dimethyldisulphide, geosmin and 2-methylisoborneol, which can affect the quality of foods and beverages even when present in very small amounts (Filtenborg *et al.,* 1996).   These compounds are produced in large quantities in species specific combinations of different genera such as *Penicillium*, *Aspergillus* and *Fusarium* (Larsen & Frisvad, 1995a).

The fungi tend to attack foodstuffs with a high carbohydrate content producing alcohols and esters, such as ethanol and ethyl acetate, which are considered to be pleasant smelling by humans (Cowell & Ratcliffe, 1997).   Some secondary

metabolites, i.e., compounds not essential for mainstream metabolic processes, are responsible for the mouldy smell associated with fungi e.g. 3-octanol (Cowell & Ratcliffe, 1997).

Volatile fungal metabolites may be used to indicate the growth of moulds in stored cereals (Börjesson *et al.,* 1990; Börjesson *et al.,* 1992). Marked differences in the production of volatile metabolites have been reported between closely related species and even between strains of the same fungus (Börjesson *et al.,* 1992; Börjesson *et al.,* 1993). The stage and duration of fungal growth can influence volatile production.

Studies of Canadian barley (*Hordeum vulgare* L. cv. Bonanza) stored in granaries have revealed that the highest production of volatile fungal metabolites, precedes toxin production and maximal propagule counts (Abramson *et al.*, 1983). A number of compounds have been reported as fungal metabolites in grain with the most common being 3-octanone, 1-octan-3-ol, 2-methyl-1-propanol, and 3-methyl-1-butanol (Abramson *et al.*, 1983; Börjesson *et al.,* 1989; Börjesson *et al.,* 1992; Börjesson *et al.,* 1993).

1.3   PRINCIPLES OF OLFACTION

1.3.1   Human olfaction

The sensation of smell is dependent upon interaction of odourant molecules with a group of specialised nerve cells called olfactory receptors, which are located in the olfactory epithelium, a specialised area of tissue high up in the nose (Craven *et al.,* 1996; Bartlett *et al.,* 1997a, 1997b; Gibson *et al.*, 1997). Typically, odorants are

organic molecules with a relative molecular mass between about 30 and 300 Da containing one or two functional groups (Bartlett *et al.,* 1997b). They are also small, polar and often hydrophobic (Craven *et al.,* 1996). The hydrophobicity of a molecule is important, as the first step in olfactory recognition is the dissolution of the molecule in an aqueous mucous layer covering the olfactory receptor cells (Bartlett *et al.,* 1997a). The surface area of each olfactory cell is increased by a number of cilia, which lie in a thin aqueous, mucous layer covering the epithelium. G- receptor binding proteins located at the surface of the cilia interact with odorant molecules and cause excitation in the neurone. The number of different binding proteins is not known but has been estimated to be between 100 and 1000 (Craven *et al.*, 1996). There are about 100 million olfactory cells (50 million per nostril), which are believed both to amplify the signal and generate secondary messengers (Gardner & Bartlett, 1994). Many olfactory neurones appear to express only one of the many possible olfactory binding proteins and, since the number of olfactory neurones is large, there is therefore a large population of olfactory neurones containing any given olfactory binding protein (Gardner & Bartlett, 1994). The messengers control ion channels and thus generate signals that travel down axons from the olfactory nerves to about 5000 glomeruli nodes in the olfactory bulb. These signals are then further processed by about 100 000 mitral cells and then finally sent via a granular cell layer to the brain (Gardner & Bartlett, 1994). At the brain signals are decoded by using a kind of pattern recognition (Aishima, 1991). The different olfactory binding proteins have partially overlapping sensitivities to odorants (Gardner & Bartlett, 1994; Gibson *et al.*, 1997). For example, a particular olfactory neurone or set of neurones will respond to many different odorant molecules (Craven *et al.,* 1996).

Naturally occurring odours and flavours are usually made up of a large number of molecular components so that the perceived odour or flavour is the result of the integrated response of the olfactory system to the complex mixture (Craven *et al.,* 1996; Bartlett *et al.,* 1997b). The human olfactory system can discriminate aromas without separating mixtures into individual compounds (Aishima, 1991). For example, in coffee odour, more than 500 components have so far been identified and in beer flavours 400 distinct compounds are recognised as having an impact on the perceived flavour of the product (Bartlett *et al.,* 1997b). The high sensitivity of the human sensors enables very low levels of some compounds, down to parts per trillion in some cases, to be detected in a complex odour (Hodgins, 1995). Thresholds of odorant molecules in water that can be detected by a normal, healthy person are shown in Table 1.2.

1.3.2 Machine olfaction

The electronic nose parallels the human olfactory system in the following manner as described by Bartlett *et al*. (1997a). Olfactory receptors are represented by a group of chemical sensors, which produce a time-dependant electrical signal in response to an odour. Signal processing techniques can be used to reduce any noise and sensor drift. Pattern recognition software is the equivalent of the cerebral cortex of the brain, which is the final stage in the human olfactory process, which classifies and memorises odours (Bartlett *et al.,* 1997a). Electronic noses mimic the mammalian olfactory system by combining arrays of non-specific gas or odour sensors with pattern recognition software to create a system, which can be used to characterise or

Table 1.2 Human thresholds for various common odours (adapted from Craven *et al.,*

1996).

Odour type	Threshold (in water)
Green leaves	0.32 ppm
Rose	0.29 ppm
Thyme	86 ppb
Lemon	10 ppb
Off-flavour in white fish	0.01 ppb
Green pepper	0.001 ppb
Grapefruit	0.00002 ppb

discriminate complex mixtures without prior separation of the components of the mixture (Bartlett *et al.,* 1997b).

The basic components of the human olfactory system compared to the corresponding components in the electronic nose are illustrated in Figure 1.3. This shows the basic components of the human olfactory system and compares it with the construction of an electronic nose (Craven *et al.,* 1996). The human olfactory system consists of three essential elements: an array of olfactory receptor cells situated in the roof of the nasal cavity, the olfactory bulb which is situated just above the nasal cavity, and the brain. The electronic nose also has three roughly equivalent elements: the odour sensor array, data pre-processor and pattern recognition (PARC) engine.

The sensor array of an electronic nose has a very large information potential and will give a unique overall pattern of the volatiles (Haugen & Kvaal, 1998). There are, however, two basic differences between the human and electronic nose that should be kept in mind. The electronic nose has both large differences in sensitivity and selectivity from the human nose (Haugen & Kvaal, 1998). The sensors of an electronic nose respond to both odorous and odourless volatile compounds (Haugen & Kvaal, 1998).

Artificial olfaction
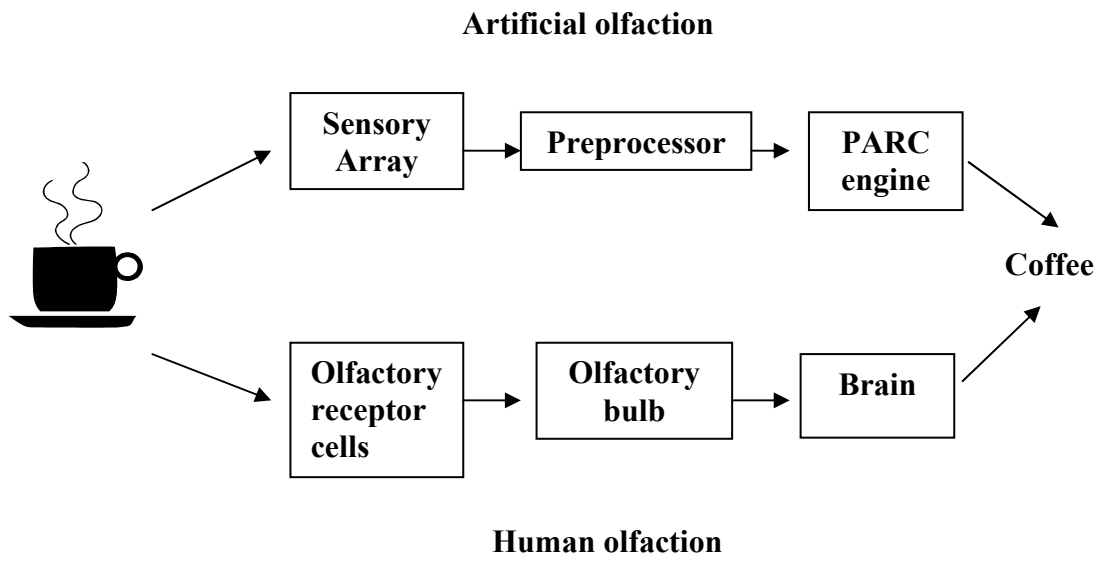
Human olfaction

Figure 1.3 Comparison of the basic elements of an artificial olfactory system with the human olfactory system (adapted from Craven *et al*., 1996; Bartlett *et al*., 1997a).

1.4   ELECTRONIC NOSES

Electronic nose is the colloquial name, first published in the literature by Julian Gardner at Warwick University in 1988, for an instrument made up of sensors combined with some sort of pattern recognition system, enabling discrimination between, or recognition of, simple or complex odours (Gibson *et al.,* 2000b; Schaller *et al.*, 1998). However, Persaud & Dodd (1982) were the first to demonstrate a sensor array for discrimination between odours. The earliest commercial electronic nose devices were launched in the early 1990s. Among the first were systems from Alpha MOS in 1993, Neotronics and Aromascan in 1994, and Bloodhound Sensors and HKR Sensorsyteme in 1995 (Gibson *et al.,* 2000b). A list of commercially available instruments is shown in Table 1.3. Since then electronic noses have been developed for qualitative classification of various kinds of environments.

1.4.1   Applications of Electronic noses

Electronic noses have been examined for use in a number of areas including food analysis (Schaller *et al*., 1998), monitoring environmental contamination such as insecticides (Baby *et al.,* 2000), product quality (Craven *et al.,* 1996), measuring breath alcohol concentration (Paulsson & Winquist, 1999; Paulsson *et al*., 2000), estimating the time of death of corpses (Snyder Sachs, 2003) and other medical uses (Gibson *et al*., 2000a). Their use in medicine could not only produce a more rapid treatment of the infection but also reduce the misuse of antibiotics (Gardner *et al*., 1998).

Table 1.3 Commercially available electronic nose instruments (adapted from Gibson *et al.,* 2000b; Vanneste & Geise, 2003; Zhang, 2003).

Company	*System*
Agilent Technologies	4440
Alpha MOS, France	Fox 2000, 3000, 4000, 5000
	alphaKronos, alphaPrometheus,
	alphaCentauri, alphaGemini
Bloodhound Sensors, UK	Bloodhound BH114,
	OEM-modules under development
Chemsensing Inc.	Chemsensing
Cyrano Sciences, US	Cyranose 320
Daimler Chyrsler Aerospace	SAM system
Electronic Sensor Technology, US	zNose
Element, Iceland	FreshSense
Environics Industry Oy, Finland	MGD-1
Forschungszentrum Karlsruhe	Sagas
HKR Sensorsysteme, Germany	QMB6/HS40XL, HS40/MS, MS-Sensor
	SensiTOF
Hewlett Packard, US	HP4440A
Lennartz Electronic, Germany	MOSES II
Marconi Applied Technologies	e-nose 5000
Microsensor Systems	ProSat
MoTech Sensoric, Germany	VOCmeter, VOCcheck, OEM-modules
Nordic Sensor Technologies/	NST 3210, NST 3220, NST 3220A,
Applied Sensor, Sweden	VOCseries, VOCcheck
OligoSense, Belgium	Under development
Osmetech, UK and US	OMA, MultiSampler-SP, CP sensors
Quartz technology	QTS-1
RST Rostock, Germany	Sam
Smart Nose, Switzerland	SMart Nose-300
WMA Airsense Analysentechnik,	PEN, i-PEN, PEN-EDU

Among the many applications, food analysis has been the topic considered more than any other (Di Natale *et al*., 1997). Electronic noses have been developed for the classification of a large variety of foods, such as coffees, meats, fishes, cheese, spirits, wines (Di Natale *et al*., 1997) and peppers (Korel *et al*., 2002). They have also been applied to looking at fruit ripeness (Brezmes *et al*., 2000; Brezmes *et al*., 2001) virgin olive oils (García-González & Aparicio, 2002; Oliveros *et al*., 2002), dairy products (Visser & Taylor, 1998; Ampuero & Bosset, 2003; Collier *et al*., 2003) and milk quality (Capone *et al*., 2001; Magan *et al.,* 2000). Checking of raw materials, for example quality taints and off-flavours (Craven *et al*., 1996), e.g. grain quality (Börjesson *et al.,* 1996; Jonsson *et al.,* 1997; Keshri *et al*., 1998; Olsson *et al.,* 2000; Olsson *et al*., 2002) and mite infestation of wheat (Ridgway *et al*.. 1999) have also been examined. Table 1.4 shows some of the food applications electronic noses have been applied to, and the sensor type used in the systems. Use of an electronic nose could allow the odour quality of a food to be continuously monitored from raw material intake through to production of the final food product. However few studies have examined discrimination between spoilage bacteria, yeasts and fungi growing on foodstuffs.

The human nose is the main tool used in many instances to evaluate the aroma of foods using highly trained sensory panels. However, this method is subjective, prone to error, difficult to correlate between different people, subject to fatigue (Balaban *et al*., 2000; Haugen, 2001) and expensive (Dainty, 1996). The food industry also uses techniques such as gas chromatography-mass spectrometry (GC-MS) to analyse

Table 1.4 Examples of applications of electronic noses to foodstuffs and beverages

(adapted from Bartlett *et al.,* 1997b)

Application	Sensor Type	Comments
Coffee	12 metal oxide	discrimination of Brazilian and Columbian coffee samples
	6 metal oxide	discrimination of *Coffee arabica* and *Coffea robusta* samples
	6 metal oxide	discrimination of roasting levels
Soft drinks	6 metal oxide	90% success in discriminating 6 brands of cola
Meat	5 metal oxide	84% success in detection of boar-taint
	10 MOSFETs and 4 metal oxide	determination of meat type and storage time
Liquor	6 metal oxide	problems caused by high alcohol levels
Perfume	8 bulk acoustic wave	5 different perfumes clearly discriminated
Wine	5 metal oxide	determination of vintages
	1 bulk acoustic wave	discrimination of wine types using transients
Beer	12 conducting polymers	discrimination of beers and lagers
	12 conducting polymers	detection of 0.5 ppm diacetyl taint
Food flavours	20 conducting polymers	discrimination of different food flavouring
Fish	6 metal oxide	determination of freshness
	8 amperometric	determination of freshness

odours, but these are time consuming, expensive and sometimes inadequate in identifying key odour compounds (Balaban *et al*., 2000; Deisingh, 2003).

Another advantage of an electronic nose compared to a human nose is the possibility of detecting toxic compounds. There is therefore room for an instrument that can mimic human olfaction and provide low-cost and rapid information.

1.4.2 Electronic nose sampling

There are two main odour sampling methods: static headspace analysis (SHA) and flow injection analysis (FIA). SHA is the more popular and low cost method. The sample to be examined is placed in a container and left so that the headspace becomes saturated with the odour (Craven *et al.,* 1996). This headspace is then transferred into the chamber containing the sensor array. The initial magnitude of response of the sensor array to the odour is large because the gas reaching the sensor array is saturated with the vapour, after a time the headspace is then removed from the chamber and replaced by clean air and the sensor responses return to their baseline values (Craven *et al.,* 1996). FIA is usually computer automated and employs a method where background gas (usually clean air) is constantly being pumped into the sensor chamber. Gas containing the odour is injected into the background gas before it reaches the sensor chamber. The ratio of the mixture of background gas to odour gas can be precisely controlled (Craven *et al.,* 1996).

Data pre-processing is an important step before classification can be performed (Dickinson *et al*., 1998). Signals from the sensor array are generally pre-processed in

some way in order to improve the quality of the information available and to optimise the array output before passing on to the pattern recognition system (Bartlett *et al.,* 1997b). Today the vast majority of systems developed on the electronic nose are based on steady-state measurements. Sensors are allowed to reach equilibrium and, after a predetermined time, the signal intensities from each sensor are measured (Dickinson *et al.,* 1998). The resulting response vectors are time independent and represent the absolute change in sensor signal with a measured odour. When classification is performed, these response vectors are often normalised to reduce the effect of concentration related fluctuations (Dickinson *et al.,* 1998). A variety of different pattern recognition techniques can be applied ranging from simple linear statistical methods to artificial neural networking approaches (Bartlett *et al.,* 1997b). The output from the sensor array may be interpreted via a variety of methods such as pattern recognition algorithms, principle component analysis, discriminant function analysis and artificial neural networks to discriminate between samples (Magan & Evans, 2000). A range of relatively non-specific sensors are able to detect the vapours which may, for instance, come from fungi, generally as alcohols, esters, aldehyde or ketones (Cowell & Ratcliffe, 1997).

1.4.3   Sensors

Sensors should be non-specific in their response to volatile organics and should possess good stability and sensitivity coupled with a fast response (Bartlett *et al.,* 1997b) as well as low sensitivity toward humidity and temperature (Schaller *et al.,* 1998). The most popular sensors used to develop electronic noses suitable for use within the food industry are:

Semiconductor metal oxide chemoresistive sensors: The oxide materials in the sensor contain chemisorbed oxygen species with which interaction of odour molecules alters the conductivity of the oxide (Bartlett *et al.,* 1997a). Sensor selectivity can be altered to different compounds by modification of the films by incorporating different amounts of noble metals or by changing operating temperatures (Börjesson *et al*., 1996; Dickinson *et al*., 1998). They are quite sensitive to combustible materials such as alcohols but are less efficient at detecting sulphur- or nitrogen-based odours (Bartlett *et al*., 1997a). The sensitivity of these types of sensors is quite good. They are relatively resistant to humidity and to ageing, and are made of particularly strong metals (Mielle, 1996). Metal oxides can also be used in a field-effect-transistor configuration, which are termed metal oxide semiconductor field effect transistor sensors (MOSFETs). MOSFET sensors are sensitive to a number of organic compounds.

Quartz-resonator sensors: Quartz resonator sensors consist of a piezoelectric quartz crystal oscillator coated with a sensing membrane such as acetyl cellulose or lecithin (Bartlett *et al.,* 1997a). Adsorption of odour molecules onto the membrane leads to changes in the resonant frequency the magnitude of which is related to the mass of the compound (Mielle, 1996) of the device due to a change in mass, provided that the viscoelastic effects are negligible (Bartlett *et al.,* 1997a). The selectivity of these sensors is dictated by the different µm-thick-coatings that are applied to the crystals surface (Dickinson *et al*., 1998). An alternative to these bulk acoustic wave devices is surface acoustic wave systems. These utilise waves emitted along the surface of a crystal by the electric field of surface deposited aluminium electrodes (Dickinson *et al*., 1998).

Conducting polymers: There are two main classes of conducting polymers, which are poly (pyrrole)s and poly(aniline)s (Bartlett *et al.,* 1997a). The materials are easy to process, so preparation of reproducible gas sensors is possible (Bartlett *et al.,* 1997a). The reversible adsorption of molecules to the films induces a temporary change in the electrical conductance of the film by altering the population of active charge carriers in the polymer structure (Dickinson *et al.*, 1998).

1.5   SPOILAGE OF BAKERY PRODUCTS

With bakery products water activity (a_w) is the most important factor affecting the type and rate of spoilage (Membré *et al.,* 1999). Commercially produced and properly handled bread generally lacks sufficient amounts of moisture to allow growth of any micro-organisms except moulds (Jay, 1996). As normal cooking temperatures destroy fungal spores, post-process contamination from airborne spores and contact with contaminated surfaces must be prevented (Sprenger, 1997). Filamentous fungi involved in spoilage of bread include *Rhizopus* and *Mucor* spp., *Penicillium* spp., *Eurotium* spp, *Aspergillus* spp. and *Monilia* (*Neurospora*) *sitophilia*. One of the most common is *Rhizopus stolonifer*, often referred to as the 'bread mould' (Jay, 1996). Storage of bread under conditions of low humidity retards mould growth. In addition to the economic losses associated with bakery products, another concern is the possibility of mycotoxin production. *Eurotium* species are usually the first fungi to colonize improperly dried, stored commodities, and when they grow, they increase the level of available water allowing other species, Aspergilli and Penicillia, which can produce toxins, to thrive (Abellana *et al.,* 2001). Losses of bakery products due to

mould spoilage vary between 1-5% depending on season, type of product and method of processing (Marín *et al.*, 2003).

Problems due to spoilage yeasts in bread usually result from post-baking contamination. Slicing machines, bread coolers, conveyor belts and racks have been identified as sources (Legan & Voysey, 1991; Scholte, 1996). Yeast spoilage is characterized by visible growth on the surface of products (white or cream patches) (Membré *et al.,* 1999). The most frequent and troublesome yeast is *Pichia butonii*, which is know as "chalk mould" (Seiler, 1981). This yeast can multiply rapidly on bread, with visible growth often apparent some time before mould occurs. Filamentous fungi are more common than yeasts on British breads. However, since filamentous fungi are more easily recognized than yeasts they generate the majority of complaints (Legan & Voysey, 1991).

Members of the *Bacillus* genus bring about bacterial spoilage of bread known as rope. This is of major economic concern to the baking industry (Bailey & von Holy, 1993). Ropiness, which is the most important spoilage of bread after mouldiness, occurs particularly in summer when the climatic conditions favour growth of bacteria (Rosenkvist & Hansen, 1995). It is mainly caused by *Bacillus subtilis* but *B. lichenformis*, *B. megaterium* and *B. cereus* have also been associated with ropy bread (Kirschner & von Holy, 1989; Bailey & von Holy, 1993; Thompson *et al.,* 1993; Rosenkvist & Hansen, 1995; Sorokulova *et al.*, 2003). The incidence of wheat bread spoilage caused by *Bacillus* has increased during the last few years presumably because more bread is produced without preservatives and often raw materials such as

bran and seeds are added (Rosenkvist & Hansen, 1995). Spoilage of bread by rope formation may constitute a health risk, high numbers of *B*. *subtilis* and *B. lichenformis* in foods may cause a mild form of food illness (Rosenkvist & Hansen, 1995). Consumption of ropy bread has been associated with foodborne illness in reports from Canada (Todd, 1982) and the United Kingdom (Sockett, 1991).

Rope spoilage can first become noticeable 12-24 h after the loaf has left the oven and is characterized by a distinctive sweet, fruit odour which has been likened to that of rotting pineapple (Kirschner & von Holy, 1989; Bailey & von Holy, 1993; Thompson *et al.,* 1993) or rotting melons (Rosenkvist & Hansen, 1995). A discoloured, sticky and soft breadcrumb subsequently follows this. Degradation of the breadcrumb is caused by the combined effects of microbial proteolytic and amylolytic enzymes breaking down starch (Bailey & von Holy, 1993; Thompson *et al.,* 1993; Rosenkvist & Hansen, 1995). Its sticky nature is due to the slime, extracellular polysaccharides, formed by certain rope-inducing strains (Bailey & von Holy, 1993; Thompson *et al.,* 1993; Rosenkvist & Hansen, 1995). At this stage web-like strands are visible when the crumb is broken (Bailey & von Holy, 1993).

The origins of the *Bacillus* species are reported to be raw materials, particularly flour, and from the bakery atmosphere, equipment surfaces and other raw materials (Bailey & von Holy, 1993; Rosenkvist & Hansen, 1995; Sorokulova *et al.*, 2003). Spores found in flour and other raw materials are resistant to heat and some of them can survive the baking process where temperatures in the centre of the crumb remains at a maximum of 97-101°C for only a few minutes (Kirschner & von Holy, 1989;

Rosenkvist & Hansen, 1995). Failure to reach this temperature in all parts of the bread greatly increases the proportion of surviving spores (Rosenkvist & Hansen, 1995). It has been found that all baking flours are contaminated with *Bacillus* spores as a result of cultivation and processing methods (Bailey & von Holy, 1993). As this type of spoilage only affects the central portions of the loaf in its initial stages it is seldom evident to consumers at time of purchase.

The problem of rope spoilage of bread has been minimised in the Western countries through the application of good manufacturing practices, advanced process control, high hygiene standards and the use of chemical preservatives according to specified legal limits (Kirschner & von Holy, 1989; Bailey & von Holy, 1993). However, the international trends towards the elimination of chemical preservatives, for example calcium propionate, from bread and other foodstuffs is expected to increase the risk of bread spoilage by rope inducing *Bacillus* strains (Bailey & von Holy, 1993).

There is little knowledge of the potential of using electronic nose technology to differentiate between bacteria, yeasts and filamentous fungi and between different strains of the same species. Very few studies have tried to discriminate between bacteria, yeasts and filamentous fungi in raw materials and bread-based matrices. Gibson *et al*. (1997) looked at detection and identification of 12 different pure cultures of bacteria using a Bloodhound BH-114 electronic nose. They correctly classified 93.4% using a neural network. They were also able to correctly classify 96.3% of 3 pathogenic yeasts. Keshri *et al*. (2003) were able to discriminate 5 out of 7 species of *Agaricus* growing on agar. Magan *et al*. (2000) showed that it was possible to

differentiate between unspoiled milk and the presence of yeasts and bacterial species. They were also able to differentiate between different concentrations of *Pseudomonas aureofaciens*. Keshri *et al*. (1998) looked at differentiation of spoilage fungi of grain growing on 2% milled wheat agar. They were able to discriminate and separate 3 out of 4 *Eurotium* species, a *Penicillium* species and *Wallemia sebi* after 48 h growth before the presence of visible growth. Subsequently, Keshri *et al*. (2002) were able to differentiate between 2 *Eurotium* species, *Penicillium chrysogenum* and uninoculated controls of 0.95 a_w bread analogues after 40-48 h.

1.6 PRESERVATIVES

Consumers require high quality, preservative-free, safe foods with an extended shelf-life (Brul & Coote, 1999). A reason why consumers are especially concerned about additives and contaminants is that these are not seen to be intrinsic to the food but are considered as added extras (Miller, 1991). Ever since the massive Anti-E Number lobby of the mid 1980's the bread industry has been working to reduce the number of additives and so called synthetic preservatives in a genuine effort to make bread as natural and as fresh as possible (Casdagli, 2000). Alternatives to preservatives, have been sought, that allow products to be presented in essentially the same form, but with modifications that make the product acceptable to a larger proportion of consumers (Smith, 1993). An example of this is the use of acetic acid (vinegar) as a preservative in place of propionic acid, vinegar being almost as effective as propionic acid as an antimycotic and more acceptable on the ingredient list (Smith, 1993). In the UK, when acetic acid is used as the bread preservative, it is added as vinegar (Voysey & Hammond, 1993). Both vinegar and calcium propionate (E282) are used as

preservatives in British bread (Voysey & Hammond, 1993). Preservation of bakery products commonly involves the use of propionates and sorbates, and sometimes benzoates and are added in concentrations that do not exceed 0.3% (Marín *et al*., 2003).

Antioxidants are substances, which prolong the shelf-life of foodstuffs by protecting them against deterioration caused by oxidation. Owing to differences in their molecular structure, the various antioxidants exhibit substantial differences in effectiveness when used with different types of foodstuffs and when used with different processing and handling conditions (Miková, 2001). Synthetic antioxidants such as butylated hydroxyanisole (BHA; E320), butylated hydroxytoluene (BHT; E321), butylated hydroxyquinone (TBHQ) and different esters of gallic acid were developed and shown to be very effective in increasing shelf-life of many foods. From the very many synthetic antioxidants which could potentially be used for the stabilization of foods, only a very few have gained any practical importance (Löliger, 1991). BHA is a mixture of 2-*tert*-butyl-4-hydroxyanisole (< 15%) and 3-*tert*-butyl-4-hydroxyanisole (> 85%) and is used in foods, cosmetics, food coating materials, waxes and vitamin A preparations (Verhagen *et al*., 1991).

Since food additives are subjected to the most stringent toxicological testing procedures, only a few synthetic antioxidants have been used in foods for human consumption for any length of time (Miková, 2001). Antioxidants are extensively tested for the absence of carcinogenicity and other toxic effects in themselves, in their oxidised form and in their reaction products with food constituents, for their

effectiveness at low concentration, and for the absence of the ability to impart an unpleasant flavour to the food in which they are used (Miková, 2001). Synthetic antioxidants have been very thoroughly tested for their toxicological behaviour, but some of them are becoming, after a very long period of use, under heavy pressure as new toxicological data impose more caution on their use (Löliger, 1991). Legislation has restricted the use and permitted levels of some currently accepted preservatives in different foods (Brul & Coote, 1999). Levels for the use of these antioxidants are generally limited to 100-200 ppm of BHA, BHT or TBHQ and often up to 200-500 ppm of the gallates (Löliger, 1991).

Reynoso *et al.,* (2002) and Torres *et al.,* (2003) studied the efficacy of single and mixtures of BHA and Propyl paraben (PP) on growth, production of the mycotoxin fumonisin and hydrolytic enzyme production by *Fusarium verticilliodes* and *F. proliferatum in vitro* on maize-based media. They found that a mixture of BHA and PP showed potential for control of growth and fumonisin production over a range of a_w levels.

Other natural solutions for use as preservatives have been researched including bateriocins, particularly from Lactic acid bacteria (Abee *et al.*, 1995; Corsetti *et al.*, 1998; Gänzle *et al.*, 1999; Cleveland *et al.*, 2001) and various essential oils (Nielsen and Rios, 2000; Guynot *et al.*, 2003; Suhr & Nielsen, 2003; Valero & Salmerón, 2003).

There is interest in rapid screening of novel antimicrobials. If antimicrobials, such as antioxidants, affect volatile production this could be used to screen new and novel

potential food preservatives. Therefore potential exists for use of electronic nose systems to screen preservatives by detecting the change in volatiles produced. Initial work would have to be performed to observe any effect background volatiles produced by the preservatives themselves would have on the sensor arrays used.

1.7   MYCOTOXINS

Mycotoxins are naturally occurring toxic secondary metabolites of fungi, which can be considered 'natural' contaminants, which may be present in food products. Several mycotoxins have been associated with, and implicated in, human and animal disease. Some are mutagenic and carcinogenic, some display specific organ toxicity, and some are toxic by other mechanisms (Jay, 1996). Mycotoxins encompass a wide spectrum of different chemicals and they may affect many target organs and systems, notably the liver, kidney, the nervous system, the endocrine system, and the immune system. Much of our concern is about chronic effects at low levels of exposure, and several mycotoxins have been classified by the International Agency for Research in Cancer as human carcinogens or possible human carcinogens (Kuiper-Goodman, 1995). At least 14 mycotoxins are carcinogens, with the aflatoxins being the most potent (Jay, 1996).

Concern about the potential hazards posed by aflatoxins started in the 1960s after some 100,000 turkey poults in England died as a result of aflatoxin exposure from their feed (Kuiper-Goodman, 1995; Jay 1996). Aflatoxins are highly substituted coumarins produced by the genus *Aspergillus*, and at least 18 closely related strains are known (Jay, 1996).

The ochratoxins are a group of at least 7 structurally related secondary metabolites of which ochratoxin A (OTA) is the most toxic and the best known (Jay, 1996). OTA is a mycotoxin produced by a variety of moulds in the genus *Aspergillus*, mainly *Aspergillus ochraceus* and also by *Penicillium verrucosum* (Creppy *et al.*, 1995). The latter is, however, able to produce the toxin at lower temperatures and are therefore more likely to be implicated as toxigenic species in countries with temperate climates (Osborne, 1980). The most threatening effects of OTA are its nephrotoxicity and carcinogenicity, since this mycotoxin is nephrotoxic to all animal species studied so far (Creppy *et al.*, 1995). In humans, a kidney disease, Balkan endemic nephropathy, has been correlated with the presence of ochratoxin (El-Shayeb, 1993). The consumption of OTA contaminated foods is a public health concern. The kidney is its main target tissue but OTA is distributed in all organs and produces in these tissues many chronic toxic effects, the more significant in terms of danger to health being nephrotoxicity, genotoxicity and carcinogenicity (Creppy *et al.*, 1995).

Some of the factors suggested to affect mycotoxin formation are; moisture, temperature, time, mechanical damage, O_2 and CO_2 levels, nature of substrate, fungal infestation, fungal strain differences, spore load, microbiological interactions, and invertebrate vectors (Abramson *et al.*, 1983).

Early detection potential exists for use of an electronic nose to differentiate toxin producing strains of filamentous fungi due to differences in volatile production profiles. Pasanen *et al.* (1996) noted a relationship between synthesis of mycotoxins and the relative proportions of different microbial volatile organic compounds.

Volatile terpenes seemed linked to formation of trichothecenes in *Fusarium*. A *Penicillium verrucosum* strain capable of synthesizing ochratoxin showed an accelerated production of volatile ketones compared to ketone production of a non-toxigenic strain. Zeringue *et al*. (1993) found $C_{15}H_{24}$ volatile compounds produced by aflatoxigenic strains of *Aspergillus flavus*, which were not produced by non-toxigenic strains. A correlation between the release of $C_{15}H_{24}$ volatile compounds and the initiation of aflatoxin biosynthesis was noted. A decline of aflatoxin synthesis and disappearance of the $C_{15}H_{24}$ compounds unique to aflatoxigenic *A. flavus* also occurred. Keshri & Magan (2000) were able to discriminate uninoculated controls, a non-toxigenic strain and 3 toxigenic strains of *Fusarium moniliforme* and *Fusarium proliferatum* after 48 h growth on 2% wheat meal agar. Olsson *et al*. (2002) used an electronic nose to detect OTA in barley grains and to predict if the level of toxin present was above or below 5 μg kg^{-1}. They were also able to predict levels of deoxynivalenol (DON). Very few detailed studies have been performed to determine the potential of electronic noses to differentiate between non-toxic and toxic strains of the same species producing one or more mycotoxin, e.g. OTA and citrinin.

1.8   OBJECTIVES OF THE PROJECT

The objectives of the project were to determine the potential of electronic nose technology for early rapid detection and differentiation of major microbial contaminants in bread, antioxidant screening and the potential for differentiation between toxigenic and non-toxigenic species. To achieve these aims the following questions were addressed.

1. Early detection and differentiation of spoilage bacteria, yeasts and filamentous fungi *in vitro*, before signs of visible growth.

2. *In situ* studies to differentiate contamination types on bread analogues of different a_w levels and incubation temperatures.

3. Differentiation of mycotoxigenic and non-mycotoxigenic strains of filamentous fungi both *in vitro* and *in situ* using a electronic nose.

4. The effect of initial concentration of inoculum on discriminatory potential of toxigenic and non-toxigenic strains of *Penicillium verrucosum*.

5. Rapid screening of novel antioxidants *in vitro* using electronic nose systems.

6. Differentiation of unknown samples on natural bread.

7. Comparison of electronic nose results with results from gas chromatography-mass spectrometry (GC-MS), CFUs and CO_2 measurements.

8. Comparison of 2 electronic nose systems using conducting polymer and metal oxide sensor arrays.

The flow diagram in Figure 1.4 shows the different phases of work performed to achieve these objectives.

Phase I: *In vitro* studies

Early detection and differentiation of spoilage bacteria, yeasts and fungi *in vitro* using an electronic nose:
- Different water activities of 2% wheat agar and flour agar
- Liquid media, NYDB, unmodified and 0.98 a_w including growth curves
- Different incubation times of sample bags
- Time to visible growth
- Validation

Phase II: Bread analogues

Early detection and differentiation of spoilage bacteria, yeasts and fungi *in situ* on bread analogues using an electronic nose:
- Different water activities and temperatures
- Comparison with GC-MS data
- CFUs
- Time to visible growth
- Validation

Phase III: Toxigenic and non-toxigenic fungal spoilage

Early detection and differentiation of mycotoxigenic and non-mycotoxigenic *Penicillium verrucosum* strains using an electronic nose:
- *In vitro* and *in situ* on bread analogues
- Time to visible growth
- Different initial microbial loads
- Validation

Phase IV: Antioxidant screening

- *In vitro* studies with a range of preservative levels using an electronic nose as a method of efficacy testing
- Comparison of 2 electronic nose systems
- CO_2 measurements
- Time to visible growth
- Validation
- CFUs
- Comparison with GC-MS data

Phase V: Inter-laboratory studies with natural bread

- Identification of unknown samples with 2 electronic nose systems
- CO_2 measurements
- CFUs
- Time to visible growth

Figure 1.4 Flow diagram of work performed to achieve the objectives of the project.

CHAPTER 2

MATERIALS AND METHODS

2.1   MICRO-ORGANISMS

Fungal, bacterial and yeast strains used are listed in Table 2.1. All were maintained on malt extract agar.

2.2   SUBSTRATES

2.2.1   Media for *in vitro* studies

a) Malt extract agar (MEA) was prepared by the addition of 48 g l^{-1} MEA (Merck, UK) to deionised water.

b) Wheat agar was prepared by the addition 2% (w/v) ground wheat grain and 2% (w/v) Technical agar No. 3 (Oxoid, Basingstoke, Hants) to deionised water.

c) Milled maize agar was prepared by the addition of 2% (w/v) milled maize (Argentina) and 2% (w/v) Technical agar No. 3 (Oxoid, Basingstoke, Hants) to deionised water.

d) Nutrient yeast dextrose agar (NYDA) was prepared by the addition of: 8 g l^{-1} Nutrient broth (Oxoid, Basingstoke, Hants), 5 g l^{-1} yeast extract powder (Lab M), 10 g l^{-1} D-(+)- glucose; corn sugar (Sigma, UK) and 2% (w/v) Technical agar No. 3 (Oxoid, Basingstoke, Hants) to deionised water.

e) Nutrient yeast dextrose broth (NYDB) was prepared by the addition of: 8 g l^{-1} Nutrient broth (Oxoid, Basingstoke, Hants), 5 g l^{-1} yeast extract powder (Lab M, UK) and 10 g l^{-1} D-(+)- glucose; corn sugar (Sigma, UK) to deionised water.

Table 2.1 List of microbial strains used in experiments.

Fungi

Alternaria tenuissima   IBT8320 (D.T.U., Denmark)

Aspergillus ochraceus   IBT21991 (CSL, York, England)

Aspergillus parasiticus   3, D64, D69, D106, T20, T47, T48 (Argentina)

Cladosporium herbarum IBT7961 (D.T.U., Denmark)

Penicillium verrucosum IBT22626, IBT21491, IBT5252, IBT22116,

                 IBT22025, IBT22123 (D.T.U., Denmark)

                 Vmmope 20-07 (CCFRA, Chipping Campden, England)

Bacteria

Bacillus cereus ATCC10876 (CCFRA, Chipping Campden, England)

Bacillus subtilis CRA14160 (CCFRA, Chipping Campden, England)

Pseudomonas fragi NCTC10689, CRA7221 (CCFRA, Chipping Campden, England)

Staphylococcus aureus ATCC25923 (CCFRA, Chipping Campden, England)

Yeasts

Debaromyces hansenii CBSnr941 (CCFRA, Chipping Campden, England)

Pichia anomala J121 (Sweden),

          NCPF3357 (CCFRA, Chipping Campden, England)

Saccharomyces cerevisiae NCTC505 (CCFRA, Chipping Campden, England)

            CRA3236 (CCFRA, Chipping Campden, England)

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f) Flour agar was prepared by the addition of 2% (w/v) white flour (Campden and Chorleywood Food Research Association (CCFRA), Chipping Campden, UK) and 2% (w/v) Technical agar No. 3 (Oxoid, Basingstoke, Hants) to deionised water.

g) Flour broth was prepared by the addition of 2% (w/v) white flour (CCFRA) to deionised water.

2.2.2 Modification of media water availability

Media were modified to the required water activity (a_w) by the addition of glycerol (Fisher Scientific, UK) to deionised water. The weight of glycerol added per 100 ml of deionised water to obtain the desired water activity is shown in Table 2.2. The solution was then measured to the desired volume before addition of ingredients. A_w of the media was checked using an Aqualab Series 3 quick start (Lab Cell, Basingstoke, Hants).

Table 2.2 Weight of glycerol added to deionised water to modify media water activity.

a_w	g of glycerol 100 ml^{-1}
0.955	23
0.964	18.4
0.971	14.72
0.982	9.2

2.2.3   Preparation of bread analogues

Bread analogues (pH 5.7 - 5.8) were prepared using a modified method of Patterson & Damoglou (1986). Dough was prepared by mixing 100 g white flour (CCFRA), 5 g margarine (Stork), 0.5 g salt (Tesco free running table salt), 0.5 g baking powder (Super Cook, gluten free), 0.5 g yeast extract (Allinson traditional dried active yeast), and a total volume of 70 ml of water and glycerol. To achieve analogues with 0.95 a_w, 10 g of glycerol was added per 100 g of dough with 60 ml of deionised water. For 0.97 a_w analogues 5 g of glycerol per 100g flour was added with 65 ml of deionised water. Dough was rolled out to a thickness of approximately 3 mm and cut into discs using a 75 mm diameter metal pastry cutter. Discs of dough were placed between two 15 cm^2 squares of aluminium foil and autoclaved at 121°C for 15 minutes. After autoclaving, whilst still warm, analogues were sprayed with 60% isopropanol before placing in Petri dishes using sterile tweezers. Bread analogues were subsequently incubated at 25°C in sealed containers containing water/glycerol solutions to maintain the relative humidity. After 24 h aliquots of spore suspension were pipetted randomly onto the surface of analogues (Fustier *et al*., 1998; Keshri *et al*., 2002), returned to the sealed containers and incubated at the desired temperature. Spore suspensions were prepared by transferring cells, spores or mycelium, with a sterile loop, to Universal bottles containing sterile deionised water, 0.01% of Tween 80 (polyoxyethylene-sorbitan monooleate, Sigma, UK) and Technical agar No. 3 (Oxoid, UK). Plate 2.1 shows bacteria, yeasts and filamentous fungi growing on bread analogues.
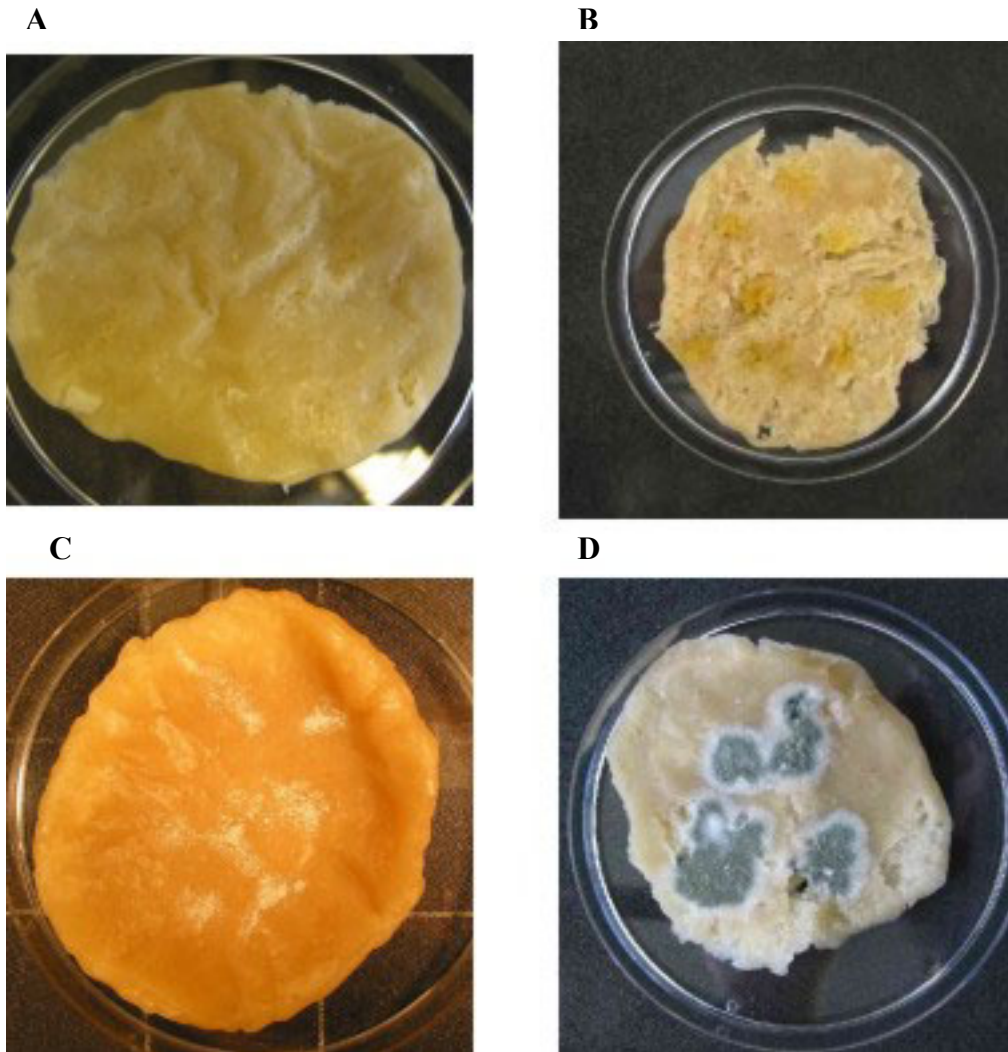
Plate 2.1 Spoilage micro-organisms grown on 0.95 a$_w$ bread analogues incubated at 25°C for 7 days: (A) Control uninoculated analogue (B) *Bacillus subtilis* (C) *Saccharomyces cerevisiae* and (D) *Penicillium verrucosum*.

2.3   ELECTRONIC NOSE SAMPLING

2.3.1   Preparation of solid samples for Bloodhound BH114 e-nose

Individual bread analogues (without Petri dishes) or Petri plates of agar, with the lid carefully removed, were placed in 500 ml capacity sampling bags (BDH, UK). Sample bags were inflated with a fixed volume of clean, filtered air and sealed. The bags were kept at 25°C for 1 hour before sampling to allow equilibration.

2.3.2   Preparation of liquid samples for Bloodhound BH114 e-nose

Liquid samples were analysed using 50 ml centrifuge tubes with modified lids containing a tube for flow of air and a tube for sampling (Figure 2.1). The ends of the tubes were sealed with parafilm until sampling.

2.3.3   Bloodhound BH114 electronic nose sampling

***Instrumentation*:** The Bloodhound BH114 electronic nose (Bloodhound Sensors Ltd., Leeds, UK) (Plate 2.2) uses an array of 14 conducting polymer sensors. Two calibration reference points are used by the electronic nose, a baseline, and a control sample derived reference point. Air is filtered through a charcoal filter attached to the rear of the machine before being passed over the sensors to set the baseline.

The sampling profile settings used were:

Time delay (secs)	5
Injection (secs)	9
Desorption (secs)	5
Baseline length (secs)	5
Hold time	0
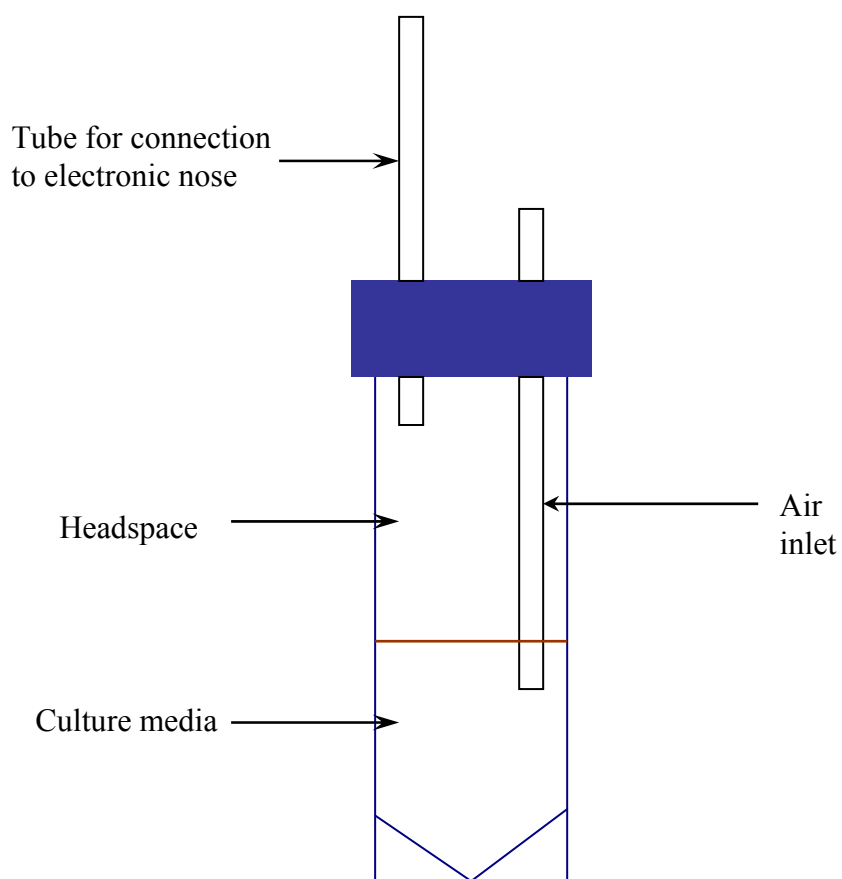
Figure 2.1 Schematic diagram of the sample tube used for liquid culture experiments with the Bloodhound BH114 electronic nose.

Plate 2.2 Bloodhound BH114 electronic nose.

Sampling: The headspace was sampled via a needle attached to a filter. The hepa-vent filter (size 0.3 μm) was used during sampling to prevent spores reaching and damaging the sensors. Samples were taken at random with one minute allowed between samples to enable the sensor array to return to baseline and prevent carry over of effects between samples.

2.3.4   NST3220 Lab Emission Analyser sampling

Nordic Sensor Technologies AB NST3220 Lab Emission Analyser (Sweden) (Plate 2.3) was used for comparison of 2 electronic nose systems and for CO_2 measurements.

Instrumentation: The NST3220 Lab Emission Analyser incorporates 2 sets of sensors, an array of 10 MOSFET and 12 MOS sensors. CO_2 measurements were obtained using the Ir-absorption CO_2 detector integrated in the sensor array.

Sampling: NST sample vials (30 ml), as shown in Plate 2.4, which have PTFE/silicon septa (Supelco, UK) in the lid, were placed in the auto sampler carousel. The temperature of the carousel was regulated at 20°C and samples analysed using the sample profile below.

Air baseline	30 secs
Sample	30 secs
Recovery	200 secs
Flush	100 secs (included in recovery)
Total sample time	4 min 20 sec

Plate 2.3 NST3220 Lab Emission Analyser with auto sampler carousel.

Plate 2.4 NST3220 sample vials containing natural bread.

2.4 EFFECT OF SAMPLE BAG INCUBATION TIME ON SENSOR RESPONSE

Wheat agar plates modified to 0.98 a_w were spread plated with 200 µl of a 1×10^6 spore ml^{-1} spore suspension of *A. parasiticus* 3 or *P. anomala* J121 or left uninoculated as a blank and incubated at 25°C. After 48 hours incubation plates were placed in sample bags and inflated. Inflated bags were left for 1, 2 or 4 hours before sampling with the electronic nose. Four replicates of each treatment were used per sample time. The experiment was performed using a new unused BH114 and a previously used BH114 e-nose. The new BH114 e-nose was used for all sampling thereafter.

2.5 DIFFERENTIATION OF MICROBIAL SPOILAGE *IN VITRO*

Unmodified a_w wheat agar plates were spread plated with 100 µl spore suspension of four fungi; *A. parasiticus* strain 3, *A. ochraceus*, *C. herbarum* and *P. verrucosum* IBT22626 and a yeast *P. anomala* J121. Inoculated and blank plates were incubated at 25°C. After 24, 48 and 72 h four replicates of each treatment were sampled with the Bloodhound BH114 electronic nose. The presence of visible growth was recorded. The experiment was repeated with the addition of *A. tenuissima* and an initial population of 1×10^6 cells or spores ml^{-1}, which was measured using a haemocytometer. The experiment was repeated using 0.98 a_w wheat agar with 5 replicates per treatment. The presence of any visible growth was recorded.

Flour agar modified to 0.97 a_w was spread plated with 100 µl of a 1×10^6 spore ml^{-1} spore suspension of *P. fragi* NCTC10689, *S. cerevisiae* NCTC505 and *P. verrucosum* Vmmope 20-07. Plates were incubated at 25°C and 4 replicates per treatment

sampled after 24, 48 and 72 h with the BH114 e-nose as described previously. Samples containing 2% v/v butanol, were also sampled. The experiment was repeated using 0.95 a_w flour agar.

2.6   LIQUID CULTURE STUDIES

2.6.1   Construction of growth calibration curves for bacteria and yeasts

Spore suspensions of *S. aureus* ATCC25923, *B. cereus* ATCC10876, *P. fragi* NCTC10689 and *S. cerevisiae* NCTC505 were diluted in sterile deionised water, Tween 80 and Technical agar no. 3 to give dilutions of 10, 25, 50, 75 and 100%. A 1 ml sample was transferred to Acryl-cuvettes (Sarstedt) and absorbance readings at 700 nm taken against a sterile deionised water, Tween 80 and technical agar no. 3 blank using a UVPC-210IPC Shimadzu spectrophotometer (Japan). Ten fold serial dilutions of the stock (100%) suspension were made and spread plated on NYDA. Graphs of cfu ml^{-1} versus absorbance were constructed. Micro-organisms were maintained on NYDA at 25ºC.

Subsequently, spore suspensions of *S. aureus*, *B. cereus*, *P. fragi* and *S. cerevisiae* were prepared and 1 ml taken to obtain the absorbance at 700 nm. Sterile 50 ml centrifuge tubes were filled with 18 ml of unmodified NYDB or 0.98 a_w NYDB. To this 2 ml of spore suspension was added and the tubes incubated at 25°C. After 4, 6.5, 23, 28.5, 30 and 47.5 h 1 ml was transferred to cuvettes and the absorbance at 700 nm recorded. Figures of time versus cfu ml^{-1} were constructed for each organism growing in each broth.

2.6.2   Electronic nose discrimination of liquid cultures

Spore suspensions of *S. aureus* ATCC25923, *B. cereus* ATCC10876, *P. fragi* NCTC10689 and *S. cerevisiae* NCTC505 were prepared in NYDB and the absorbance at 700 nm recorded. Sterile 50 ml centrifuge tubes containing 18 ml of unmodified or 0.98 a_w NYDB were inoculated with 2 ml of the spore suspension and incubated at 25°C. After 24 and 48 hours tubes were taken for sampling. One hour before sampling lids with tubes for sampling sealed with parafilm were transferred to the centrifuge tubes. Blank broth and 2% butanol samples were also tested. After sampling with the e-nose sample tubes were placed at 4°C until all had been sampled. Subsequently 1 ml of sample was transferred to cuvettes and the absorbance at 700 nm recorded.   Different tubes were used for 24 and 48 hours with 3 replicates of each organism per sample time.

The experiment was repeated sealing tubes with autoclaved sampling lids immediately after inoculation. *B. cereus, P. fragi* and *S. cerevisiae* were sampled after 13.5 and 39 h and *S. aureus* after 4.5 and 23.5 h. The initial population was also modified so all treatments contained 10^3 spores/cells ml^{-1}.

2.7   DIFFERENTIATION OF MICROBIAL SPOILAGE *IN SITU*

Bread analogues at 0.95 and 0.97 a_w were randomly inoculated with 100 µl of a 1×10^6 spores/cell ml^{-1} suspension of *S. cerevisiae* CRA3236, *P. verrucosum* IBT22626, *B. subtilis* CRA14160 or a mixture of the 3 and some left uninoculated to act as control samples. Analogues from both a_w, which were chosen to stimulate actual a_w of bread, were incubated at 15 and 25°C and 6 replicates per treatment analysed after

24, 48 and 72 h with the BH114 e-nose. Presence of any visible growth was also recorded.

2.8 COMPARISON OF BH114 E-NOSE AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY FOR DISCRIMINATION OF SPOILAGE OF BREAD ANALOGUES

In collaboration with Campden and Chorleywood Food Research Association (CCFRA, Chipping Campden, England) electronic nose data, GC-MS and CFU data were obtained for bread analogue samples. GC-MS and CFU were performed by CCFRA. Bread analogues at 0.95 a_w containing 2000 ppm calcium propionate (1.06 g per 300 g flour) were inoculated with 200 µl $1x10^6$ spores/cells ml^{-1} suspensions of *P. verrucosum* Vmmope 20-07, *B. subtilis* CRA14160 and *P. anomala* NCPF3357. Detection and differentiation of physiological spoilage was also tested by the addition of 150 µl of 50mM lipoxygenase Type 1-B from soybean (Sigma, UK). Six replicates per treatment were sampled after 0, 24, 48, 72 and 96 h. All e-nose data for 0 h and lipoxygenase data for 24 h was lost due to irreversible damage to the database.

2.9 DIFFERENTIATION OF MYCOTOXIGENIC AND NON-TOXIN PRODUCING STRAINS OF SPOILAGE MOULDS

2.9.1 Discrimination of toxin and non-toxin producing strains of *Aspergillus parasiticus in vitro*

Toxigenic (T20, D106, D69) and non-toxigenic (T47, T48, D64) strains of *A. parasiticus* were spread plated on 0.95 a_w milled maize agar using 200 µl of a $1x10^6$ spore ml^{-1} spore suspension per plate. Samples were incubated at 30°C. After 48 and

72 hours samples were taken and analysed as described previously. Presence of visible growth was also recorded.

2.9.2 Differentiation of toxin and non-toxin producing strains of *Penicillium verrucosum*

Initially 100 μl of 1×10^6 spores ml^{-1} was spread plated on 0.95 a$_w$ wheat agar and incubated at 25°C. After 24, 48 and 72 h five replicates per treatment were analysed with the BH114 e-nose.

Bread analogues at 0.95 a$_w$ were inoculated with 100 μl of 1×10^6 spores ml^{-1} suspensions of toxin and non-toxin producing strains of *P. verrucosum*. Strains used are shown in Table 2.3, which shows the mycotoxins produced by each strain on YES agar and bread analogues. Analogues were incubated at 25°C in sealed containers and four replicates per treatment sampled with the BH114 e-nose after 24, 48 and 72 h. The experiment was repeated using an initial spore concentration of 1×10^3 ml^{-1} and five replicates per treatment per sample time.

Table 2.3 Toxins produced by strains of *Penicillium verrucosum* on YES agar and bread analogue (supplied by EELA, Finland). Key: OTA- Ochratoxin A CIT- Citrinin

Strain	Believed	YES agar	Bread analogue
IBT5252	OTA + CIT	CIT	OTA + CIT
IBT22116	No toxin	CIT	CIT
IBT21491	CIT	CIT	CIT
IBT22025	OTA	No toxin	OTA
IBT22123	No toxin	CIT	OTA + CIT

2.10 ANTIOXIDANT SCREEN USING E-NOSE TECHNOLOGY

Initial investigations to observe levels of inhibition by two antioxidants, Butylated hydroxyanisole (BHA) (Sigma, UK) and propyl paraben (PP) (n-propyl-p-hydroxybenzoate, Sigma, UK), were performed on wheat agar at 0.95 and 0.97 a_w. Antioxidants were added at concentrations of 10, 20, 50 and 100 ppm dissolved in methanol. Wheat agar containing methanol was also used as a control to check that the antioxidant inhibited growth and not methanol. Suspensions containing 1×10^6 cells/spores ml^{-1} of *P. anomala* NCPF3357, *D. hansenii*, *S. cerevisiae* CRA3236, *P. verrucosum* IBT22626 and *P. verrucosum* Vmmope 20-07 were point inoculated at the centre of the agar and suspensions of *B. cereus* ATCC10876, *B. subtilis* CRA14160 and *P. fragi* CRA7221 were streaked across the middle of the plate. After 2, 3, 5, 7, 11 and 28 days incubation at 25°C colony diameter of the point inoculated plates was measured. Streak plates were assigned a score (1-10) depending on the level of growth, with 10 being very good growth.

An antioxidant screen was performed using both the BH114 e-nose and NST3220 lab analyser in collaboration with CCFRA who carried out GC-MS measurements and CFU measurements. Centrifuge tubes (50ml) with 30 ml 2% flour broth were prepared as shown in Table 2.4. After 24, 48 and 72 h incubation at 25°C, 5 ml of broth was transferred to NST sample vials and 4 replicates per treatment analysed with the BH114 e-nose and NST3220 lab analyser.

Table 2.4 Flour broth conditions used for antioxidant screen.

Inoculated with 100 μl of $1x10^6$ spore/cells ml^{-1} of *B. subtilis* CRA14160, *P. verrucosum* IBT22626 and *P. anomala* NCPF3357.

0.97 a_w 50ppm PP

0.95 a_w 50ppm PP

0.97 a_w 50ppm BHA

0.95 a_w 50ppm BHA

0.97 a_w no antioxidant

0.95 a_w no antioxidant

Uninoculated

0.97 a_w 50ppm PP

0.95 a_w 50ppm PP

0.97 a_w 50ppm BHA

0.95 a_w 50ppm BHA

0.97 a_w no antioxidant

0.95 a_w no antioxidant

2.11 DISCRIMINATION OF UNKNOWN SAMPLES ON NATURAL BREAD

Discs of bread, cut from sliced loaves of white bread (supplied by CCFRA) using a 75 mm metal pastry cutter, were placed in Petri dishes. Bread discs were then incubated in sealed containers measuring 295 x 295 x 170 mm containing two 250 ml beakers of water/glycerol solution with an a_w of 0.97 for 24 h before being inoculated. Vials containing unknown freeze dried samples labelled A, B, C and D were resuspended with 4 ml of diluent, consisting of sterile deionised water, Tween 80 and technical agar No. 3, to give suspensions containing 1×10^3 spores/cells ml^{-1}. Bread discs were randomly inoculated with 100 µl of spore suspension, placed in the sealed containers and incubated at 25°C. Five replicates of each treatment were removed for sampling after 24, 48 and 72 h. Samples were first analysed using the BH114 e-nose and then half a disc was placed in NST sample vials and analysed using the NST3220 lab analyser. CFU measurements were performed by CCFRA. Presence of any visible growth was recorded. Samples were *P. anomala* NCPF3357, *P. verrucosum* IBT22626, *B. subtilis* CRA14160 or blank vials.

2.12 ANALYSIS OF DATA

BH114 Electronic nose data: BH114 e-noses produce 5 values for each of the 14 sensors based on the sample response curve. Figure 2.2 illustrates a typical sensor response curve produced and the readings taken.
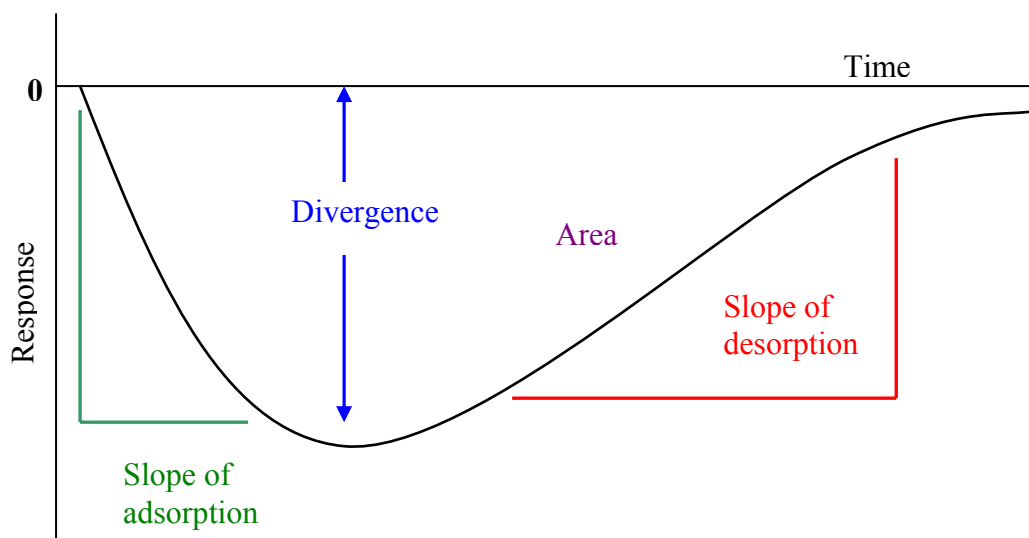
Figure 2.2 A typical BH114 electronic nose sensor response curve showing the measured parameters.

The parameters are:

Divergence (div) – the maximum deflection from the baseline.

Absorption (abs) – rate of increase in response on injection of the sample headspace.

Desorption (des) – rate of decrease in response.

Ratio – ratio of absorbance to desorption.

Area – area under the response curve.

Before applying statistical analysis, histograms of replicate reproducibility were produced using Microsoft Excel and the divergence measurements. Any obvious outliers were removed. Principle Components Analysis (PCA), Discriminant Function Analysis (DFA) and Cluster Analysis (CA) were applied to normalised divergence and area measurements using the statistical program xlSTAT (Microsoft Excel add-in, version 3.4).

PCA was initially applied to measurements from all sensors, using Spearman correlation. By flagging significant correlations, when performing the initial PCA, sensors not showing significant correlations could be removed. Sensor number 13 was always removed from analysis unless stated. DFA and CA were only applied to measurements from the significant sensors.

Principle Component Analysis is a statistical technique used to summarise the pattern of correlation among observed variables. It also reduces a large number of observed variables to a smaller number of factors. DFA involves finding one or more linear combinations of the original variables that maximise the between-group differences relative to within group differences. Cluster analysis was performed on the Malahanobis's squared distance between groups, produced by DFA, to construct dendrograms. Cross validation of samples, at 95% significance level, was performed using DFA.

NST33220 lab analyser data: Analysis of NST3220 data was performed using NST Senstool (Nordic Sensor Technologies, Sweden). PCA and Partial Least Squared (PLS) were applied to normalised response measurements.

CFU and GC-MS data: CFU and GC-MS measurements were analysed using Microsoft Excel to produce figures of means with standard error bars.

CHAPTER 3

RESULTS

3.1 EFFECT OF SAMPLE BAG INCUBATION TIME ON CLUSTERING

The degree of discrimination achieved using a range of static incubation periods of the sample bag was investigated. Leaving the sample bag for 1 h before sampling with the new BH114 electronic nose system produced only a small amount of overlap of control and *A. parasiticus* clusters as shown in Figure 3.1 accounting for 84% of the total data. However, if *A. parasiticus* replicate A4 was considered as an outlier and omitted from the cluster there would be no overlap. After 4 h there was no overlap of different treatments (Figure 3.2a). It was, however, decided for all subsequent experiments to use a 1 h static incubation of samples before sampling of the head space for volatiles. Results using an older, previously used, BH114 e-nose produced larger clusters with more overlap as shown in Figure 3.2b. This shows the PCA results after 4 h incubation with the new and older BH114 e-noses. DFA performed on data from both the older and new BH114 e-noses together showed a difference between data from the two e-noses. Figure 3.3 shows the DFA discrimination of the older e-nose and the new system containing 98% of the total data. The new BH114 e-nose was used for all subsequent experiments.

3.2 DIFFERENTIATION OF MICROBIAL SPOILAGE *IN VITRO*

Initial experiments, on agar, showed the potential to distinguish between species of filamentous fungi that are involved in spoilage of bread products using an e-nose system. Discrimination of the spoilage yeast, *P. anomala* from the filamentous fungal species was possible after only 24 h growth on 2% wheat agar at 25°C. Figure 3.4 shows the individual clusters achieved using DFA with some overlap of *C. herbarum* and *A. parasiticus*.
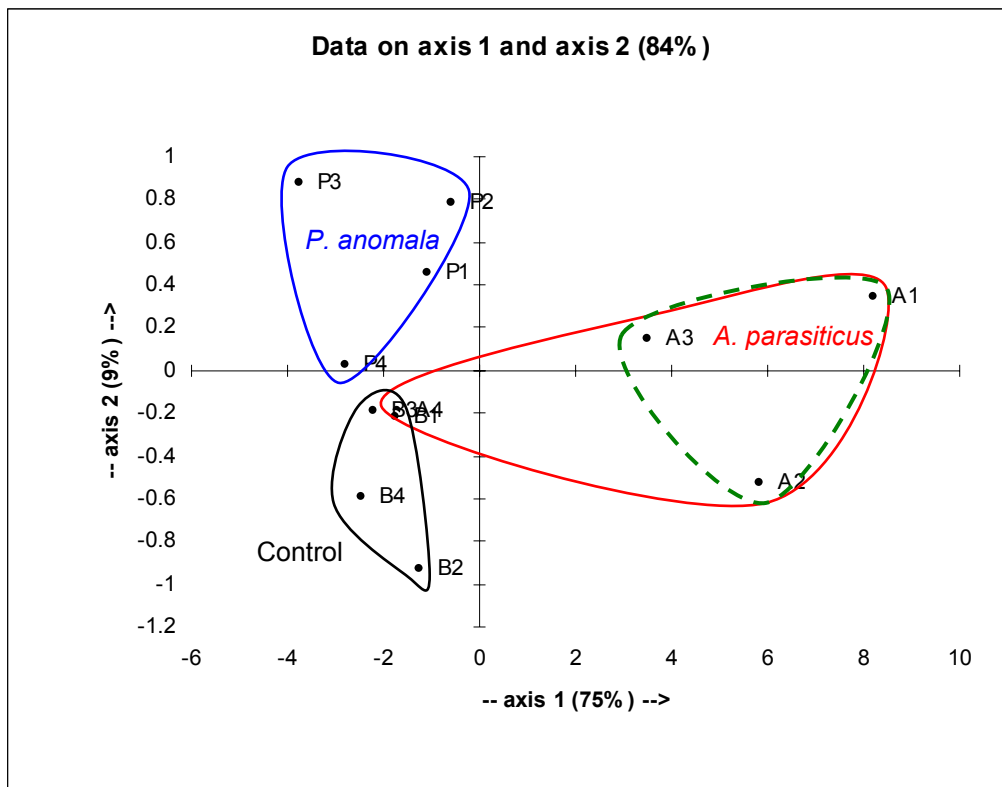
Figure 3.1 PCA of *Aspergillus parasiticus*, *Pichia anomala* and uninoculated control 0.98 a_w 2% wheat agar based on their volatile profiles after 48 h at 25°C and 1 h static incubation of sample bags. The dashed line shows the cluster if replicate A4 is considered an outlier.
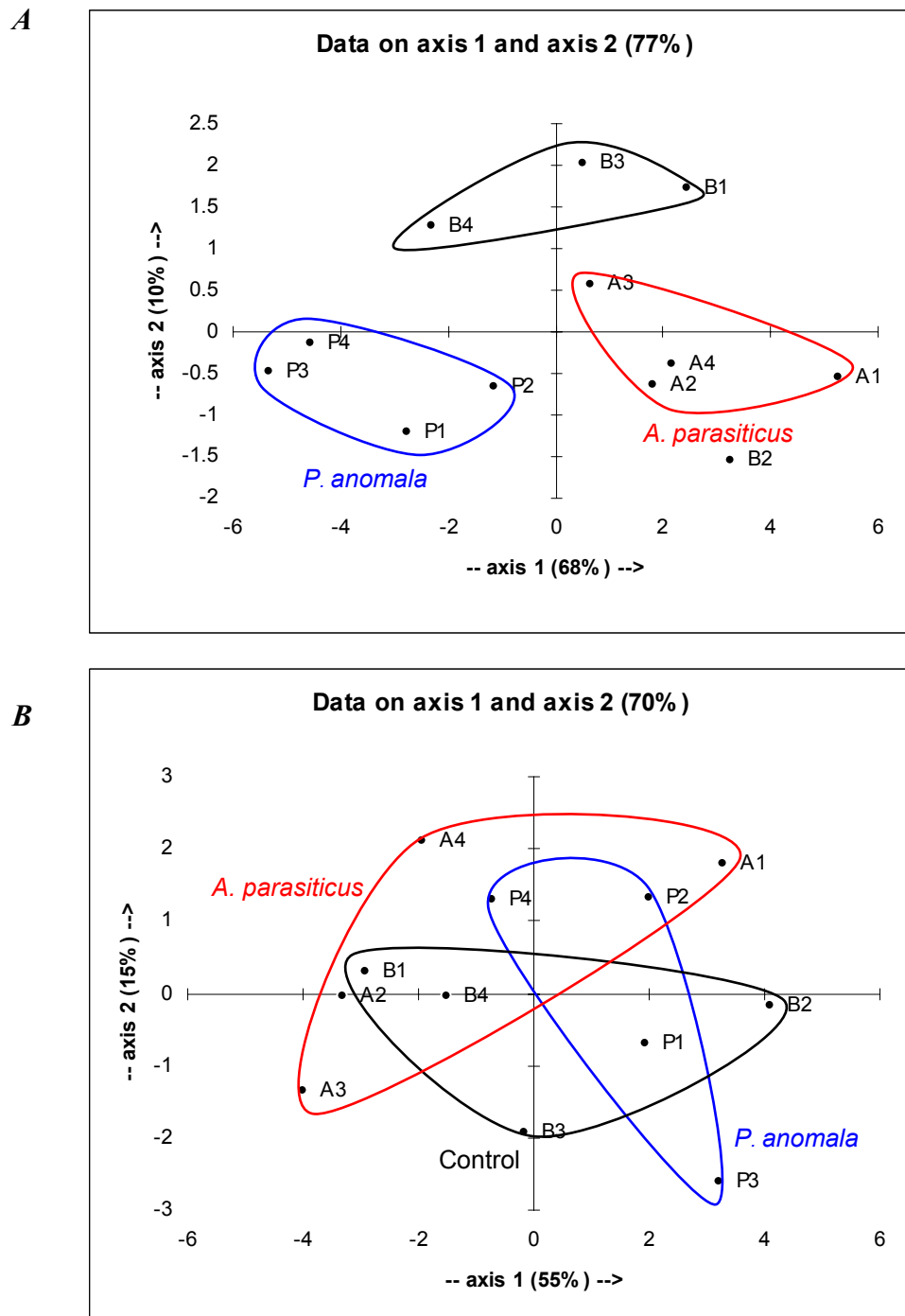
Figure 3.2 PCA of *Aspergillus parasiticus*, *Pichia anomala* and uninoculated control 0.98 a_w 2% wheat agar based on their volatile profiles after 48 h growth at 25°C and 4 h static incubation of sample bags. *A* shows results using a new BH114 e-nose and *B* using a three year old BH114 e-nose.
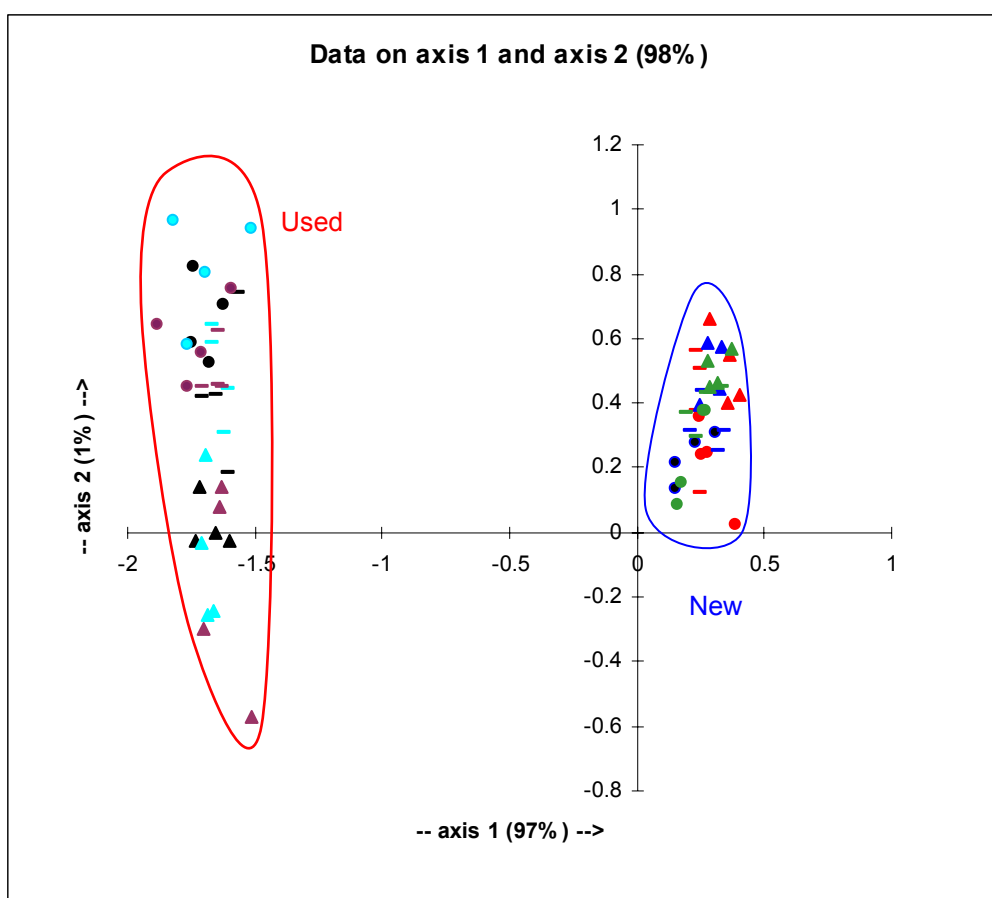
Figure 3.3 DFA of normalized divergence responses to volatiles produced by *Aspergillus parasiticus*, *Pichia anomala* and uninoculated control 0.98 a_w 2% wheat agar after 48 h at 25°C and static incubation of sample bags for 1, 2 and 4 h using a used and a new BH114 e-nose.

Key to treatments:

		1 h	2 h	4 h
New e-nose	Control	B ●	C —	D ▲
	A. parasiticus	A ●	H —	I ▲
	P. anomala	P ●	Q —	R ▲
Older e-nose	Control	E ●	F —	G ▲
	A. parasiticus	J ●	K —	L ▲
	P. anomala	S ●	T —	U ▲
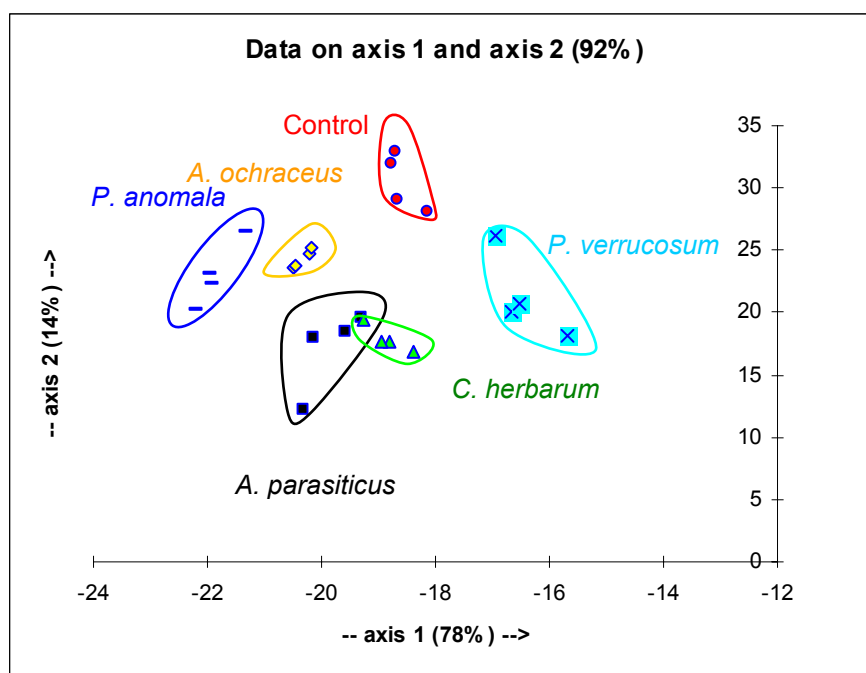
Figure 3.4 DFA of normalized BH114 e-nose divergence responses to volatiles produced by four filamentous fungi and a spoilage yeast after 24 h growth on unmodified 2% wheat agar at 25°C.

Separate clusters indicate that the treatments can be discriminated from one another. The CA dendrogram, in Figure 3.5, shows that it was possible to differentiate *P. verrucosum* and *P. anomala* but not between the other filamentous fungi and the controls after 24 h. This is due to the distance between these and the other treatments being greater than the distance indicated by the dashed red line. This discrimination was possible before any visible signs of growth occurred on any plates. After 48 h controls could be distinguished from inoculated plates as shown in Figure 3.6. However it was not possible to discrimination between the different micro-organism as indicated by CA (Figure 3.7). After 72 h *A. parasiticus* was also distinguishable. Only *P. anomala*, *A. ochraceus* and *A. parasiticus* showed visible signs of growth after 48 h but all treatments showed signs of growth after 72 h.

When the experiment was repeated with the addition of another filamentous fungus, *A. tenuissima*, 72 h were required for discrimination of controls and a filamentous fungus (Figure 3.8). Cross validation gave approximately 65% correct classification of treatments with a significance level of 95%. Table 3.1 shows examples of treatments correctly classified. The percentage successful validation for these samples is highlighted.

Sensor reproducibility for replicates of each treatment was checked by comparison of replicate profiles for all sensors. It was noted that sometimes the first replicate was not always as good as the other replicates (Figure 3.9).
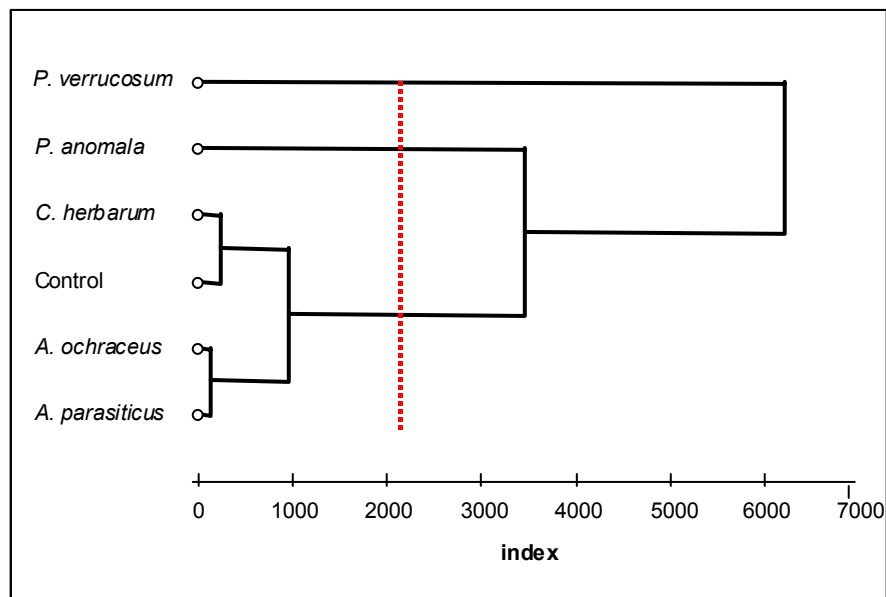
Figure 3.5 CA dendrogram of four filamentous fungi and a spoilage yeast based on their volatile profiles after 24 h at 25°C on unmodified 2% wheat agar.
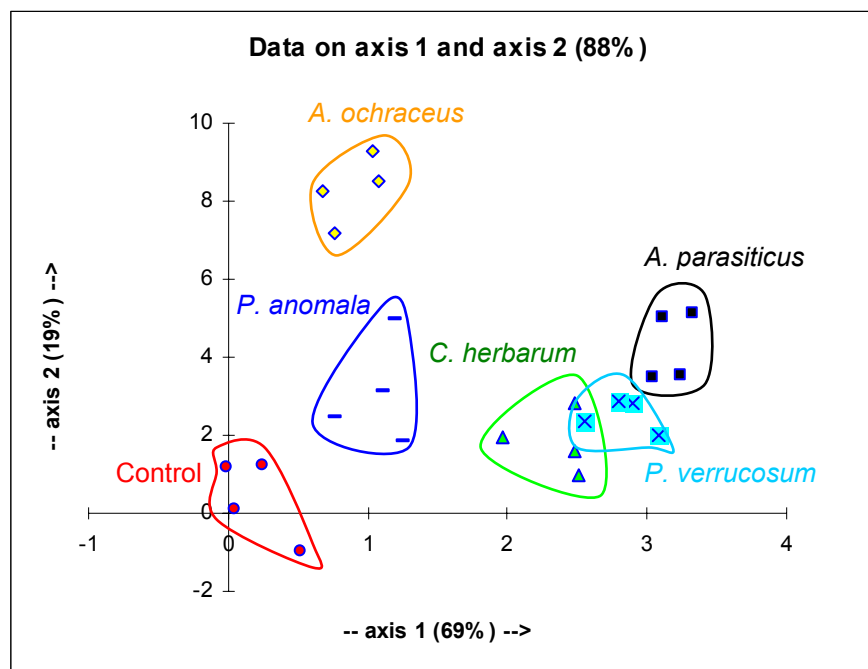
Figure 3.6 DFA of normalized BH114 e-nose divergence responses to volatiles produced by four filamentous fungi and a spoilage yeast after 48 h at 25°C on unmodified 2% wheat agar.
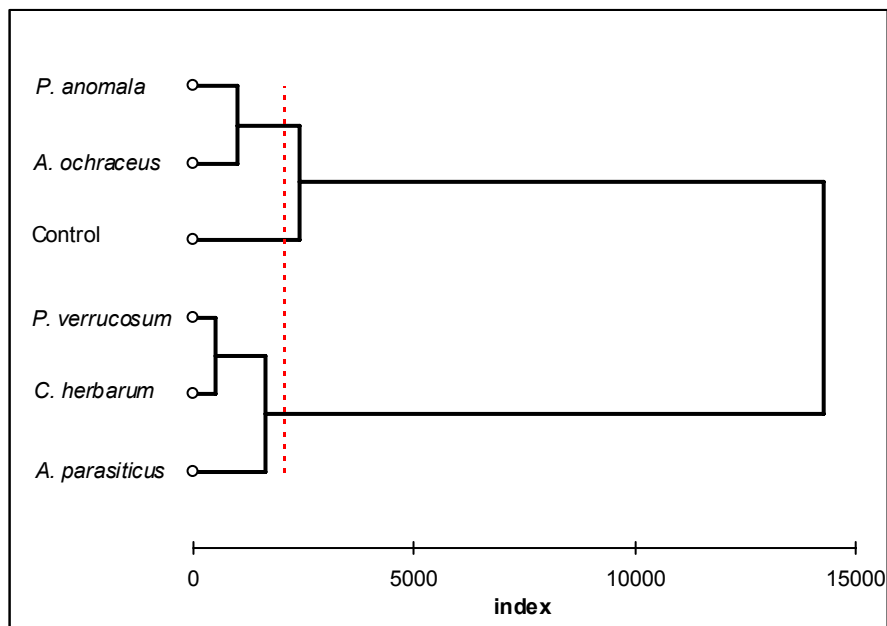
Figure 3.7 CA dendrogram of four filamentous fungi and a spoilage yeast based on their volatile profiles after 48 h at 25°C on unmodified 2% wheat agar.
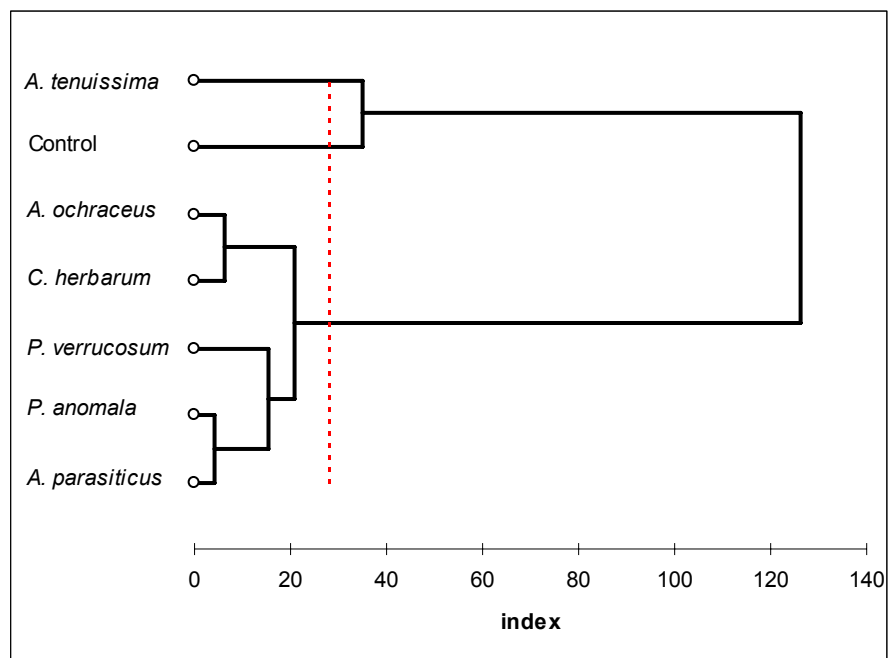
Figure 3.8 CA dendrogram of five filamentous fungi and a spoilage yeast based on their volatile profiles after 72 h at 25°C on unmodified 2% wheat agar.

Table 3.1 Cross validation of filamentous fungi and a spoilage yeast on 2% wheat agar at 25°C. Key; **A**- *A. parasiticus*; **B**- Control; **C**- *C. herbarum*; **E**- *A. ochraceus*; **P**- *P. anomala*; **T**- *A. tenuissima*; **V**- *P. verrucosum*; – indicates not included in cross validation. Highlighted values show the correct classification percentages.

	actual	x-val	A	B	C	E	P	T	V
P. verrucosum 24 h	V	V	0	0.041	0.038	0	0	-	0.921
P. anomala 24 h	P	P	0.041	0	0	0.112	0.847	-	0
Control 48 h	B	B	0	0.868	0.026	0.012	0.094	-	0
Control 72 h	B	B	0	0.931	0	0	0.019	-	0.051
A. tenuissima 72 h	T	T	0	0.096	0.052	0	0	0.812	0.04
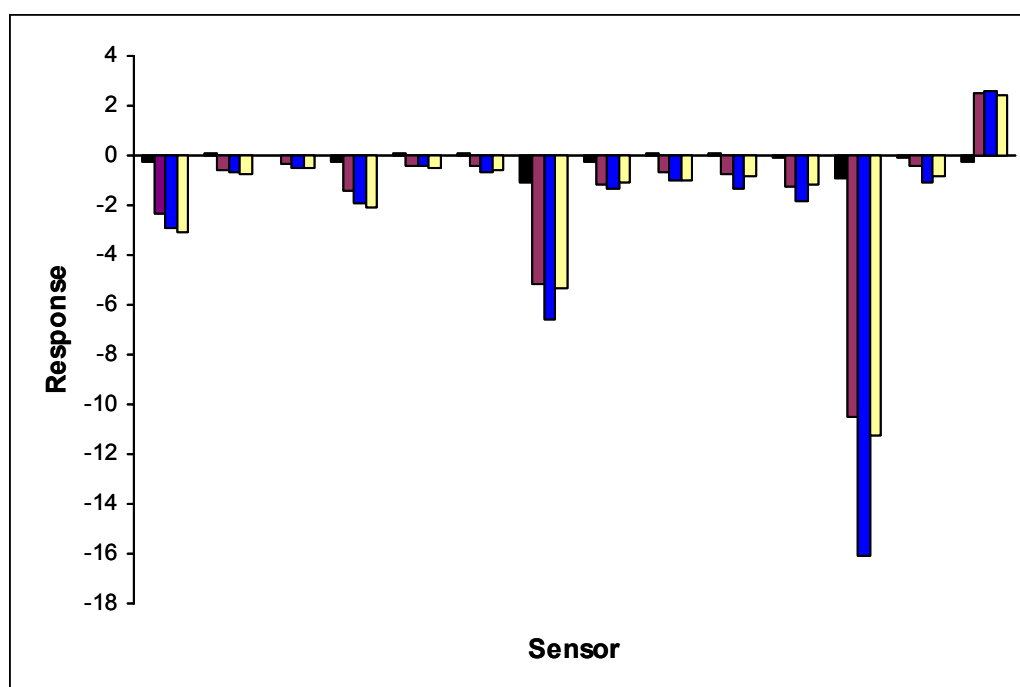
Figure 3.9 Histogram of BH114 e-nose sensor reproducibility for control replicates after 24 h at 25°C.

The first replicate has a smaller response compared to the other 3 replicates. When the first replicate was removed and the data re-analysed DFA produced better clustering. Removal of the first replicate also altered CA dendrograms so that after 48 h the controls, *P. anomala* and *A. parasiticus* could be discriminated (Figure 3.10) but after 72 hours only *C. herbarum* could be differentiated (Figure 3.11).

Modifying the water availability of the 2% wheat agar to 0.98 a_w improved differentiation. CA differentiated between micro-organisms and controls after 48 h. All micro-organisms could be differentiated, except the filamentous fungi *A. tenuissima* and *A. parasiticus* (Figure 3.12). After 72 h, when some visible growth was observed, only controls could be discriminated (Figure 3.13).

The effect of the addition of the flour to be used in bread analogues to agar was examined at different water availabilities. After 24 h on 0.97 a_w flour agar separate clusters were visible with both PCA and DFA (Figure 3.14a), which accounts for 95% of the data. CA, however only differentiated butanol and *S. cerevisiae* (Figure 3.14b). With an increased incubation time of 48 h treatments became closer with some overlap (Figure 3.15a). Butanol was discriminated from all other treatments at all sample times, however 48 h were required for controls to be discriminated (Figure 3.15b). Results obtained at 0.95 a_w, are not shown as after 1 week only *P. verrucosum* showed any visible signs of growth.
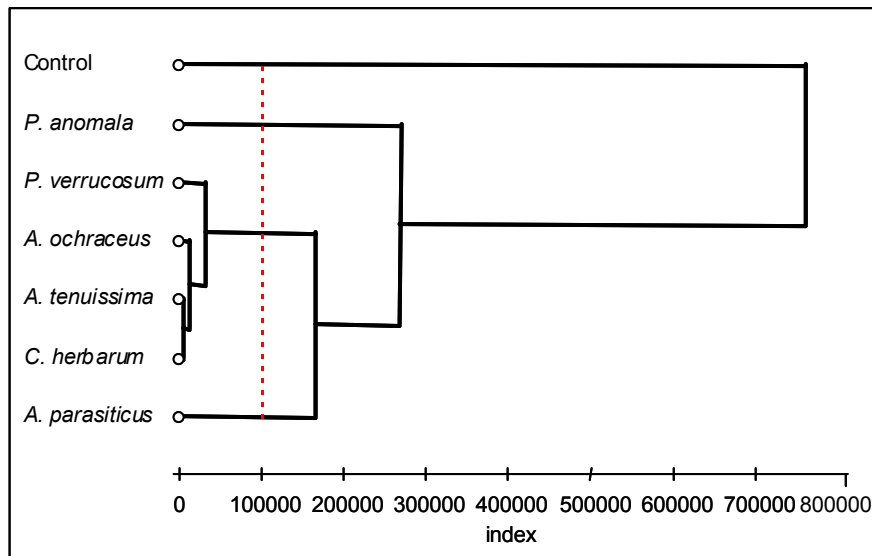
Figure 3.10 CA dendrogram of five filamentous fungi and a spoilage yeast based on their volatile profiles after 48 h at 25°C on unmodified 2% wheat agar with the first replicates removed.
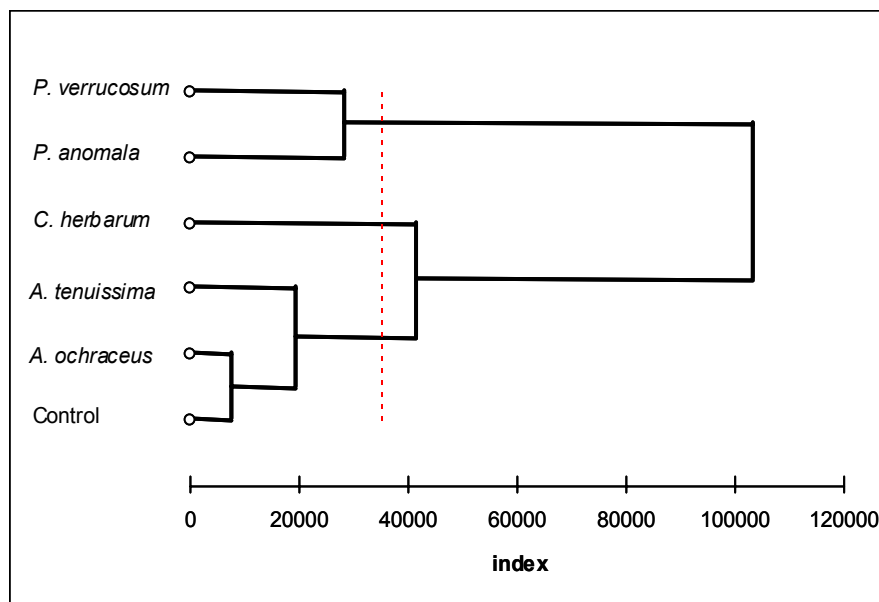
Figure 3.11 CA dendrogram of five filamentous fungi and a spoilage yeast based on their volatile profiles after 72 h at 25°C on unmodified 2% wheat agar with the first replicates removed.
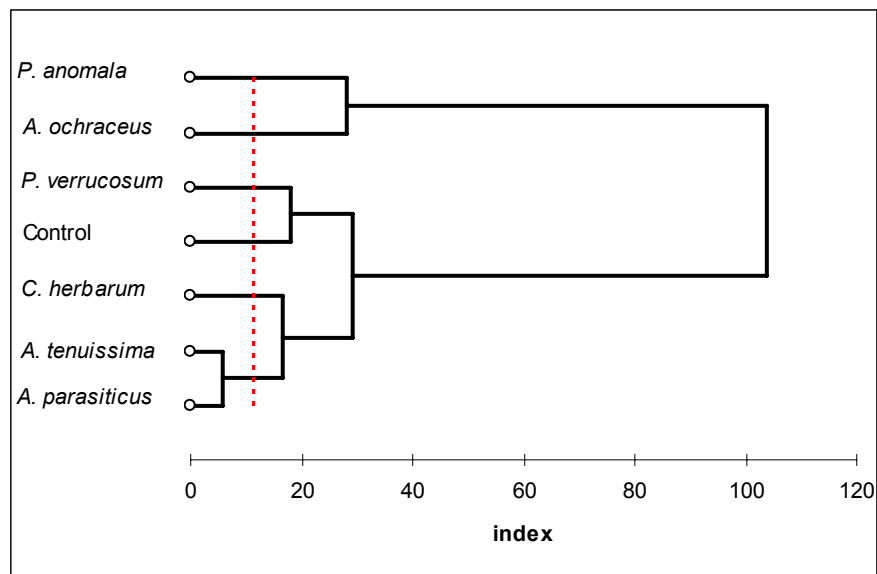
Figure 3.12 CA dendrogram of five filamentous fungi and a spoilage yeast based on their volatile profiles after 48 h at 25°C on 0.98 a_w 2% wheat agar.
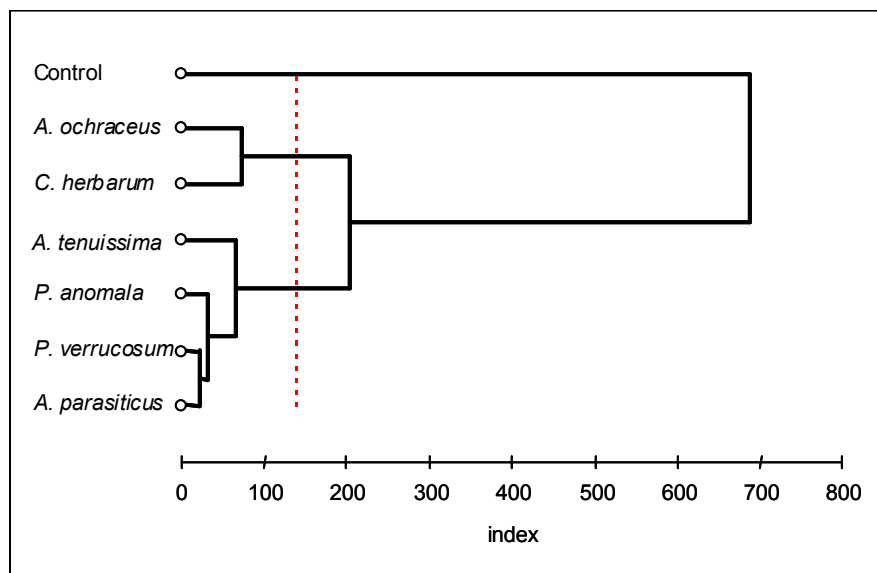
Figure 3.13 CA dendrogram of five filamentous fungi and a spoilage yeast based on their volatile profiles after 72 h at 25°C on 0.98 a_w 2% wheat agar.
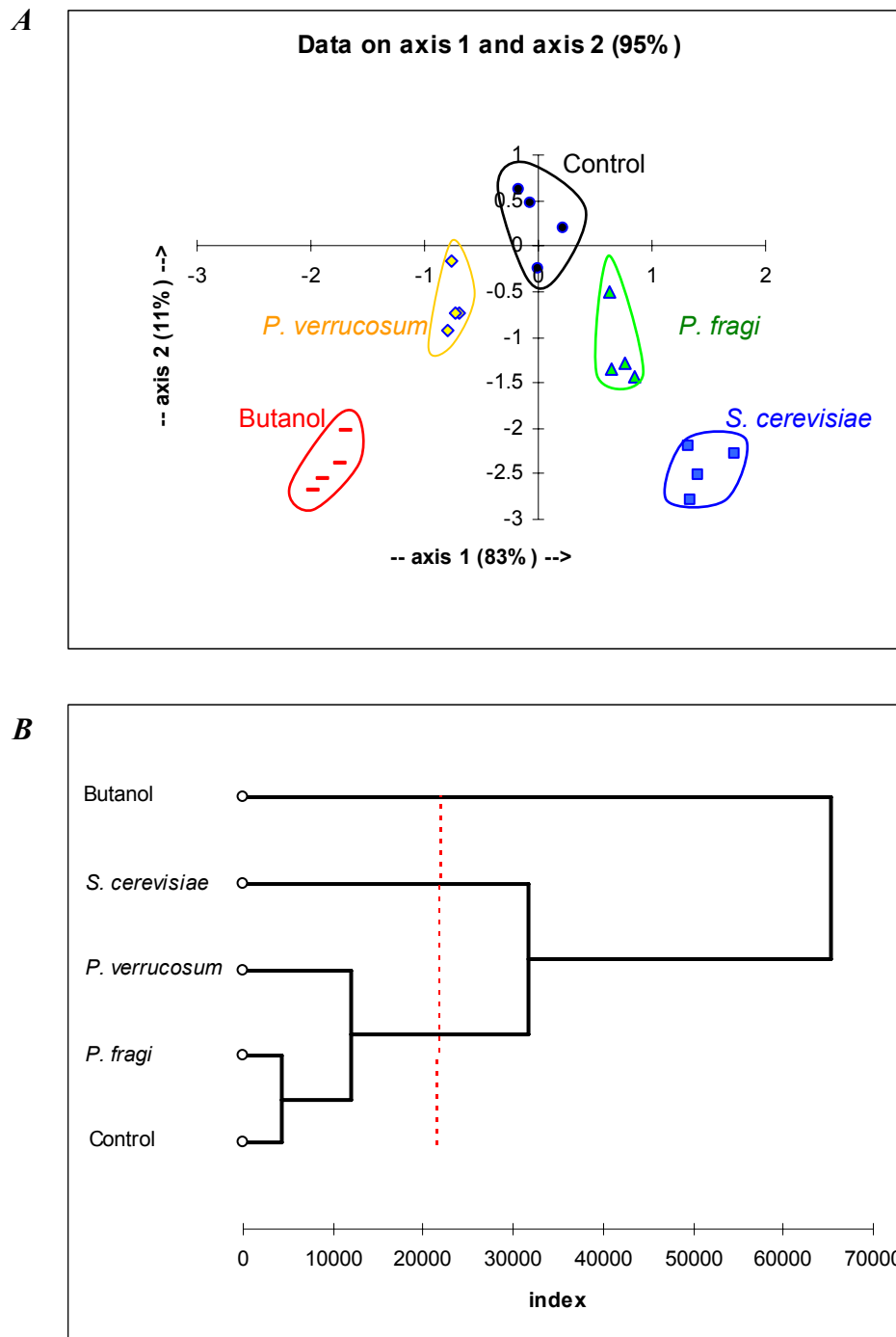
Figure 3.14 Differentiation of *Penicillium verrucosum*, *Saccharomyces cerevisiae*, *Pseudomonas fragi* and controls based on their volatile profiles after 24 h at 25°C on 2% flour agar modified to 0.97 a_w using a BH114 e-nose. *A* DFA and *B* CA dendrogram.

A

B
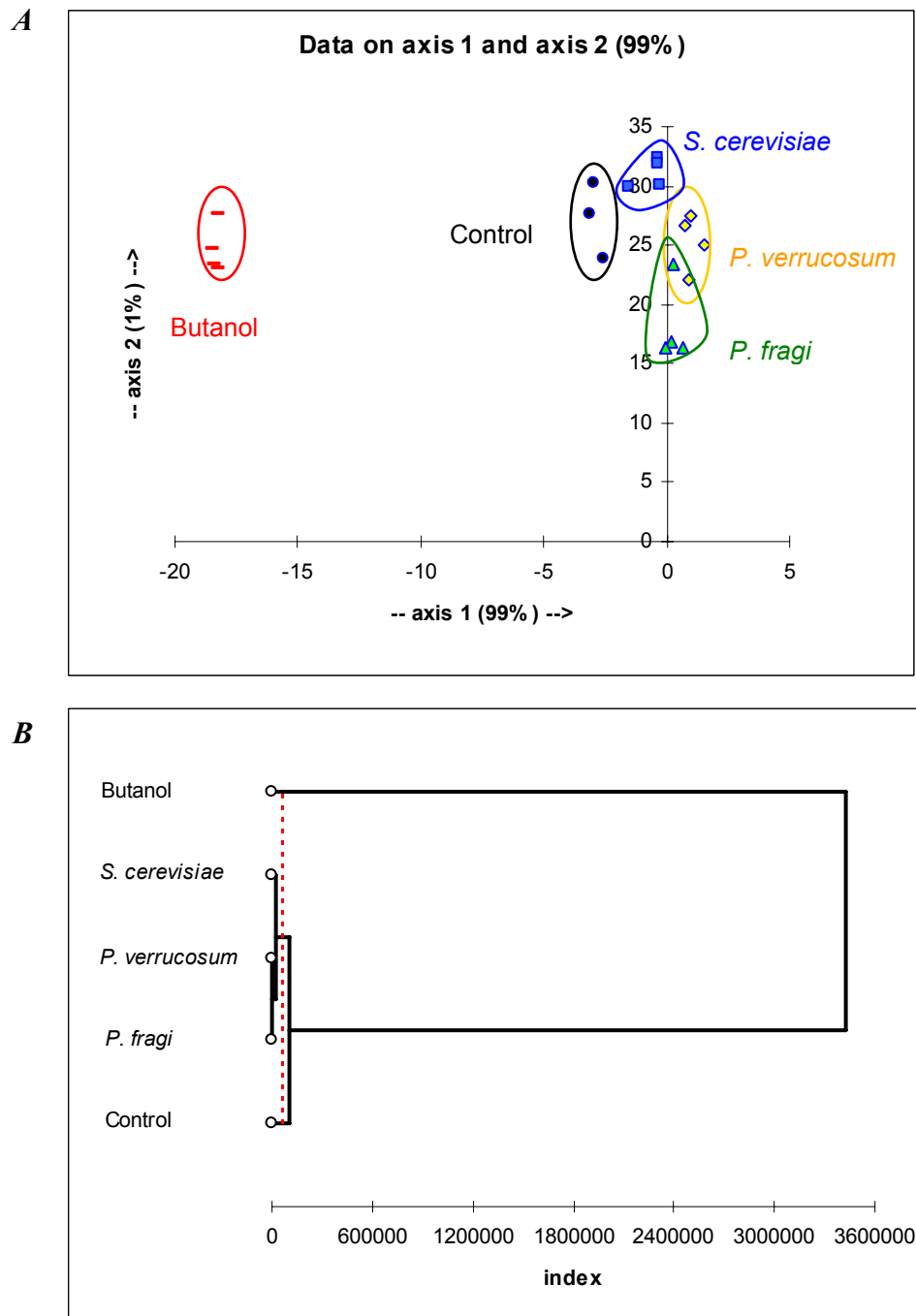
Figure 3.15 Differentiation of *Penicillium verrucosum*, *Saccharomyces cerevisiae*, *Pseudomonas fragi* and controls based on their volatile profiles after 48 h at 25°C on 2% flour agar modified to 0.97 a_w using a BH114 e-nose. *A* DFA and *B* CA dendrogram.

Analysing data without the butanol samples gave discrimination of controls and *P. verrucosum* after 24 h (Figure 3.16). After 72 h visible signs of *P. verrucosum* growth was present. Cross validation gave approximately 60% correct classification of treatments with a significance level of 95% after 24 h. The percentage decreased with increasing sample time. Table 3.2 shows the treatments correctly classified with the percentages highlighted.

Table 3.2 Cross validation of microbial spoilage of 0.97 a_w 2% flour agar at 25°C. Key: **BL**- control; **BU**- butanol; **PF**- *P. fragi*; **PV**- *P. verrucosum*; **SC**- *S. cerevisiae* **-** indicates not included. Highlighted values show the correct classification percentages.

	actual	x- val	BL	BU	PF	PV	SC
S. cerevisiae 24 h	SC	SC	0.021	0	0.134	0.01	0.835
Control 48 h	BL	BL	0.769	0	0.063	0.067	0.101
Butanol 48 h	BU	BU	0.021	0.989	0	0	0
P. verrucosum 24 h	PV	PV	0.047	-	0.009	0.941	0.003
Control 24 h	BL	BL	0.876	-	0.06	0	0.064

3.3 LIQUID CULTURE STUDIES

The potential for detection and differentiation in liquid culture at different water availabilities was examined. Calibration curves of cfu ml^{-1} against absorbance at 700 nm were produced for each micro-organism. These are shown in Figures 3.17 - 3.20. The equations produced were used to calculate cfu ml^{-1} in subsequent liquid culture studies. Initially samples were tested after 24 and 48 h. CA of samples incubated for 24 h showed that *S. cerevisiae*, *S. aureus* and *B. cereus* growing in the different a_w broths could be discriminated from one another but there was no discrimination between the different micro-organisms (Figure 3.21). After 48 h only *S. aureus* and *P. fragi* could be discriminated in the different a_w broths (Figure 3.22).
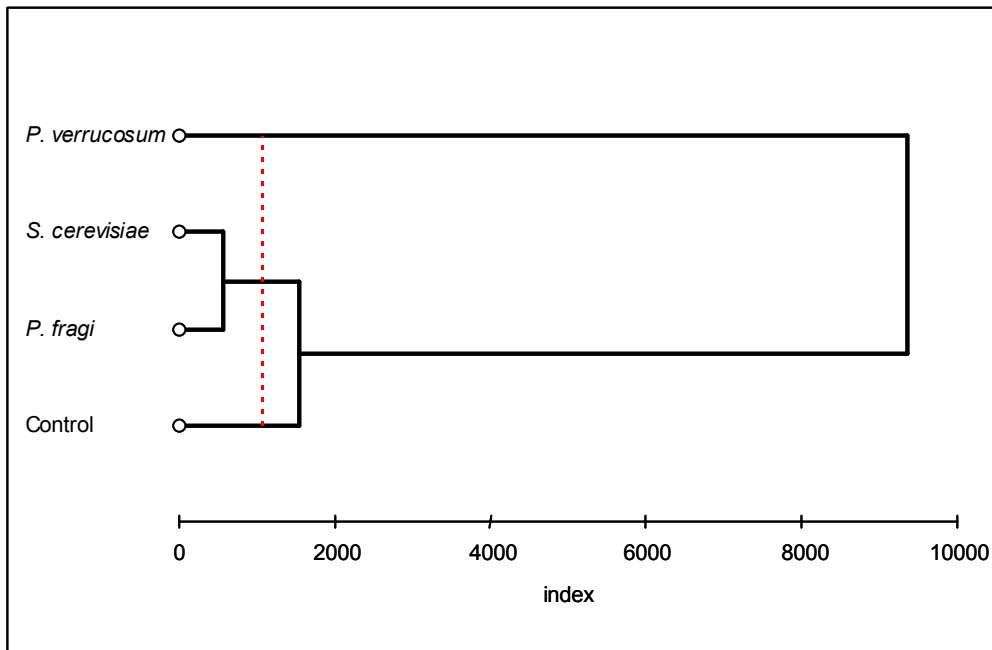
Figure 3.16 CA dendrogram of *Penicillium verrucosum*, *Saccharomyces cerevisiae*, *Pseudomonas fragi* and controls based on their volatile profiles after 24 h incubation at 25°C on 0.97 a_w 2% flour agar.
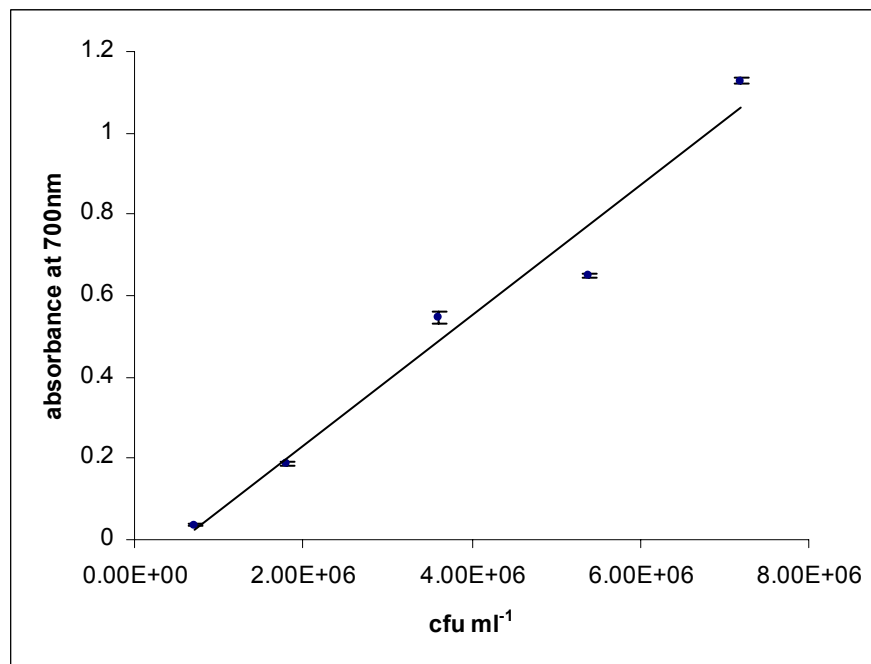
Figure 3.17 Calibration curve of cfu ml^{-1} against absorbance at 700nm for *Bacillus cereus* in NYDB. $y = 2x10^{-7}x – 0.0905$ $R^2 = 0.9678$
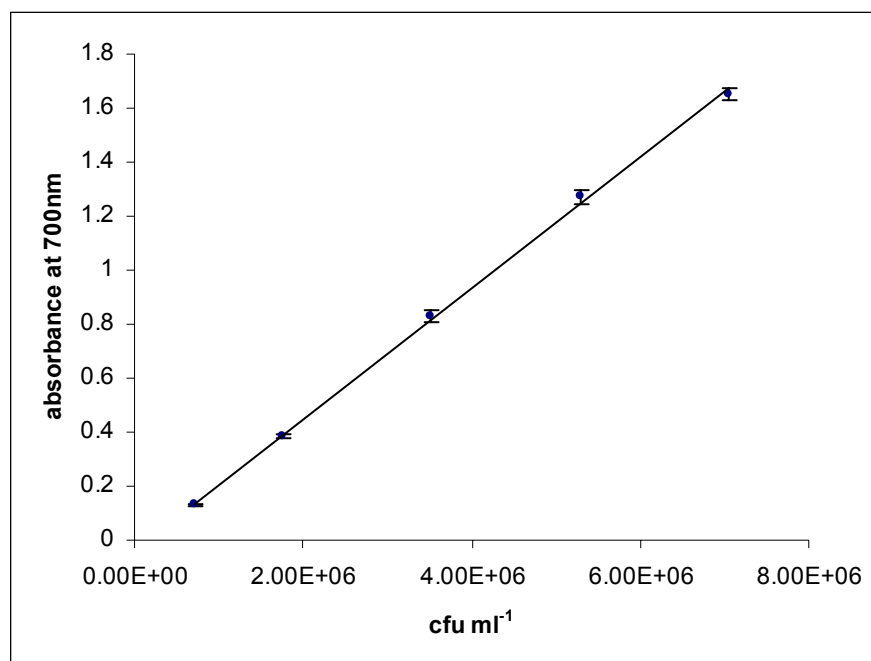
Figure 3.18 Calibration curve of cfu ml^{-1} against absorbance at 700nm for *Staphylococcus aureus* in NYDB. $y = 2x10^{-7}x – 0.0335$ $R^2 = 0.9991$
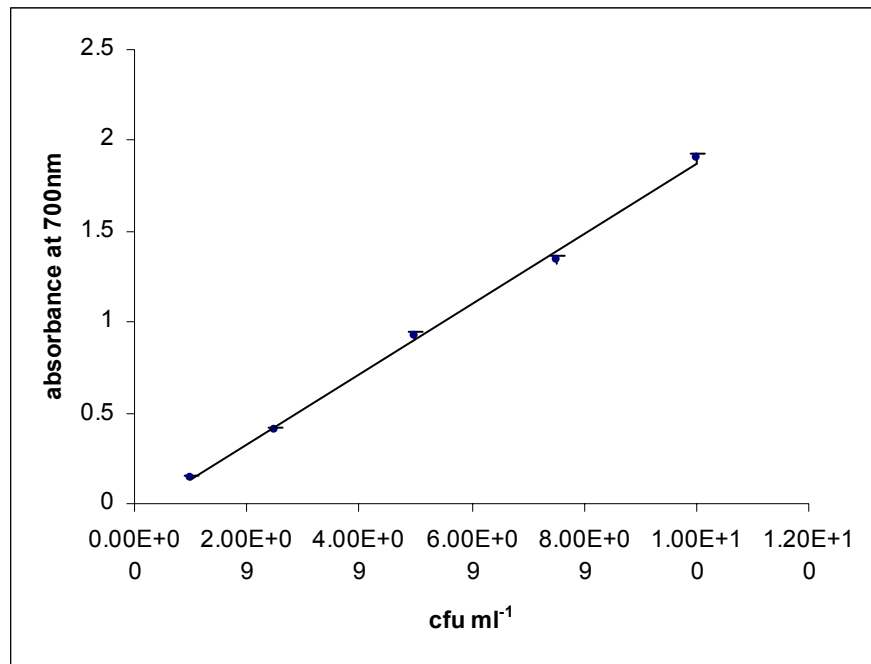
Figure 3.19 Calibration curve of cfu ml^{-1} against absorbance at 700nm for *Pseudomonas fragi* in NYDB. $y = 2x10^{-10}x - 0.0596$ $R^2 = 0.9981$
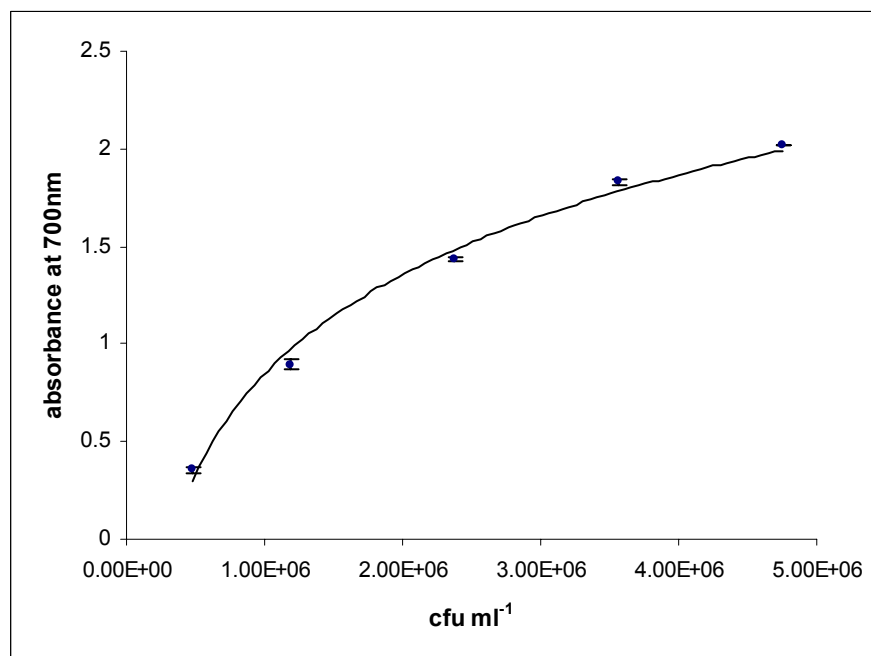
Figure 3.20 Calibration curve of cfu ml^{-1} against absorbance at 700nm for *Saccharomyces cerevisiae* in NYDB. $y = 0.7353Ln(x) - 9.3123$ $R^2 = 0.9917$
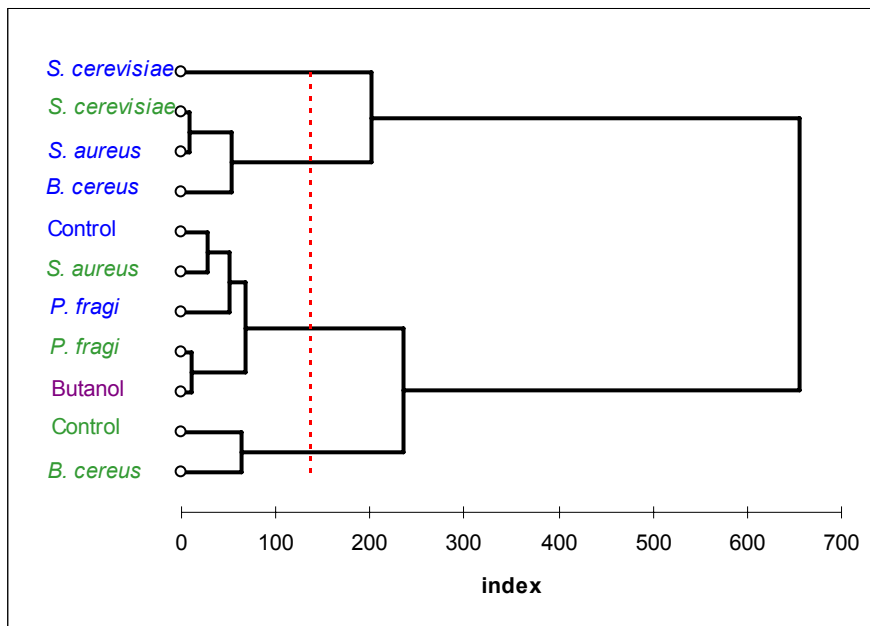
Figure 3.21 CA dendrogram of three spoilage bacteria and a spoilage yeast in unmodified and 0.98 a_w NYDB based on their volatile profiles after 24 h at 25°. Key to treatments: unmodified NYDB; 0.98 a_w NYDB.
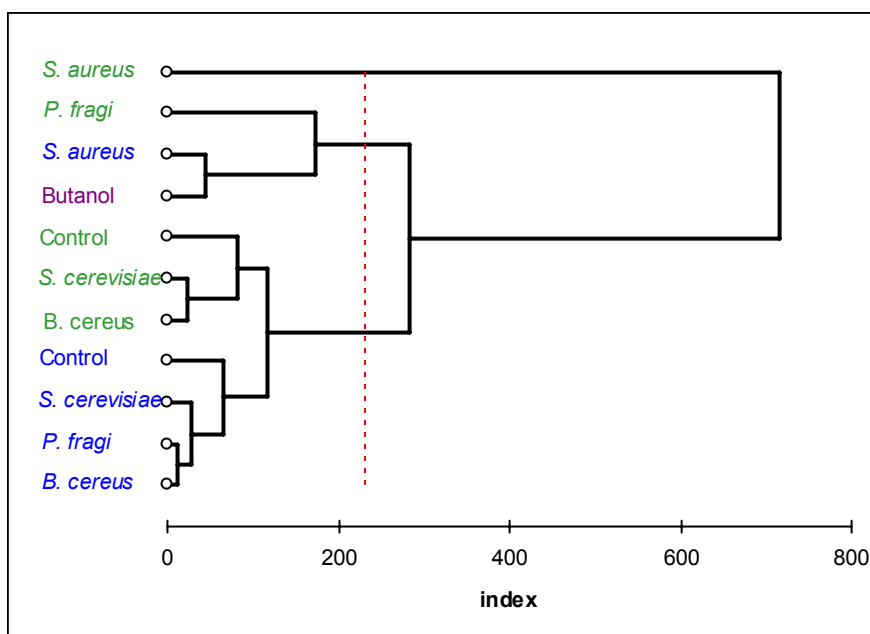
Figure 3.22 CA dendrogram of three spoilage bacteria and a spoilage yeast in unmodified and 0.98 a_w NYDB based on their volatile profiles after 48 h at 25°. Key to treatments: unmodified NYDB; 0.98 a_w NYDB.

Curves of cfu ml^{-1} against time were plotted for four micro-organisms (*B. cereus, S. aureus, P. fragi and S. cerevisiae*) grown in unmodified and 0.98 a$_w$ modified NYDB (Figures 3.23 –3.26). These were used to obtain sample times in exponential and stationary growth phases. For *B. cereus*, *P. fragi* and *S. cerevisiae* 13.5 and 39 h were chosen and for *S. aureus* 4.5 and 23.5 h. The experiment was repeated using these sampling times and initial populations of ~1x10^3 cfu ml^{-1}. Electronic nose sampling after 13.5 h produced clusters with some overlap using DFA, which included 81% of the data (Figure 3.27). Figure 3.28 shows the cluster analysis discrimination between *P. fragi* and *S. cerevisiae* growing in the different a$_w$ broths and between the different control broths. It was also possible to distinguish between all the micro-organisms and controls in unmodified NYDB. After 39 h growth PCA produced separation of samples in unmodified and 0.98 a$_w$ NYDB (Figure 3.29) with 87% of separation being accounted for by PC1. CA after 39 h showed *S. cerevisiae* and *B. cereus* could not be differentiated from one another as shown in Figure 3.30. It was noted from calculations of growth rates, in Table 3.3, that *P. fragi* grew fastest in both broths and that all micro-organisms grew faster in unmodified NYDB. Sampling of *S. aureus* after 4.5 h, as shown in Figure 3.31a, gave discrimination of the micro-organism in the different a$_w$ broths and from the controls and butanol. It was possible after 23.5 h to distinguish between NYDB with and without *S. aureus* but not between the different water availabilities (Figure 3.31b).
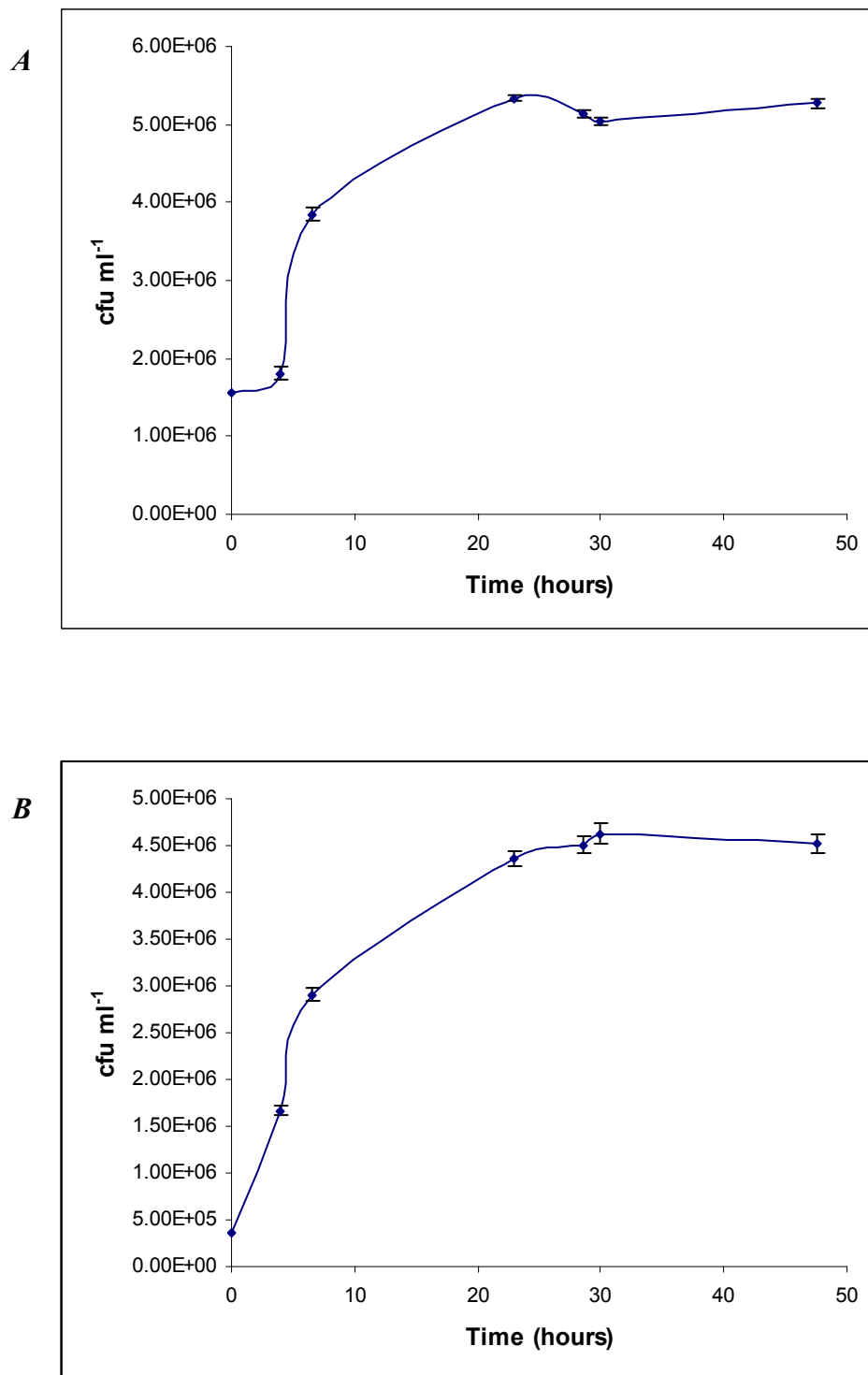
Figure 3.23 Growth curves for *Bacillus cereus* in *A* unmodified NYDB and *B* 0.98 a_w NYDB incubated at 25°C.
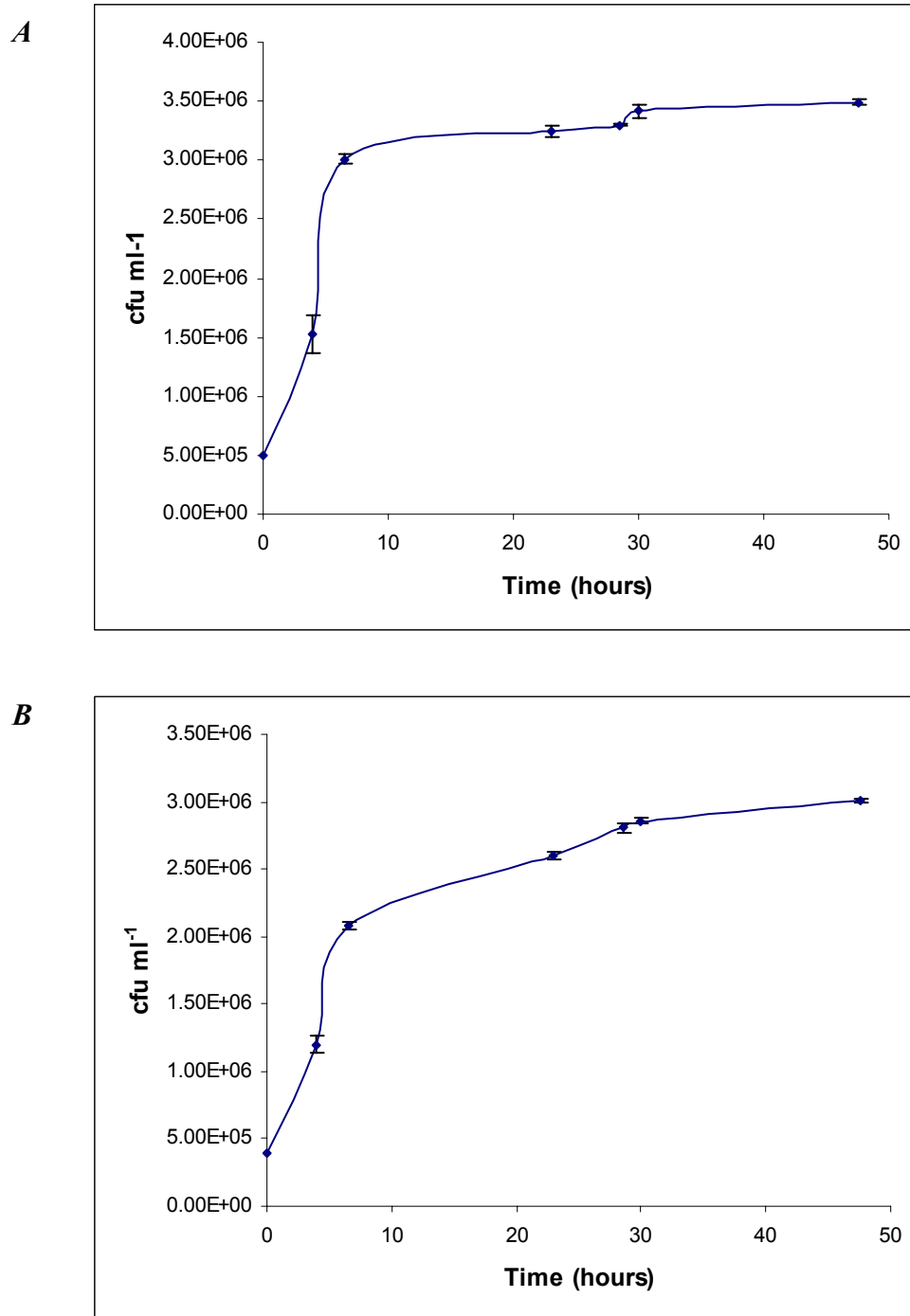
Figure 3.24 Growth curves for *Staphylococcus aureus* in *A* unmodified NYDB and *B* 0.98 a_w NYDB incubated at 25°C.
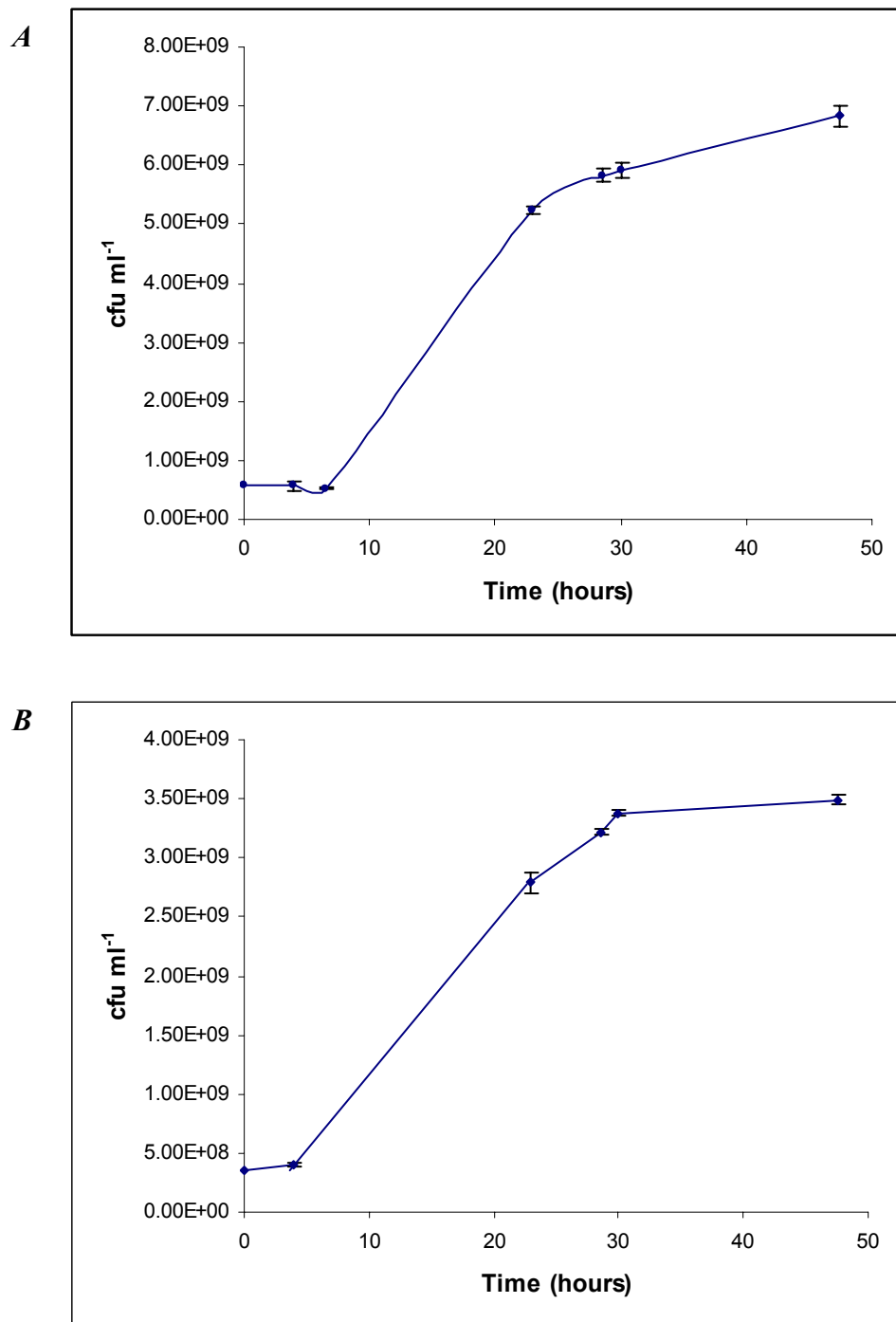
Figure 3.25 Growth curves for *Pseudomonas fragi* in *A* unmodified NYDB and *B* 0.98 a_w NYDB incubated at 25°C.
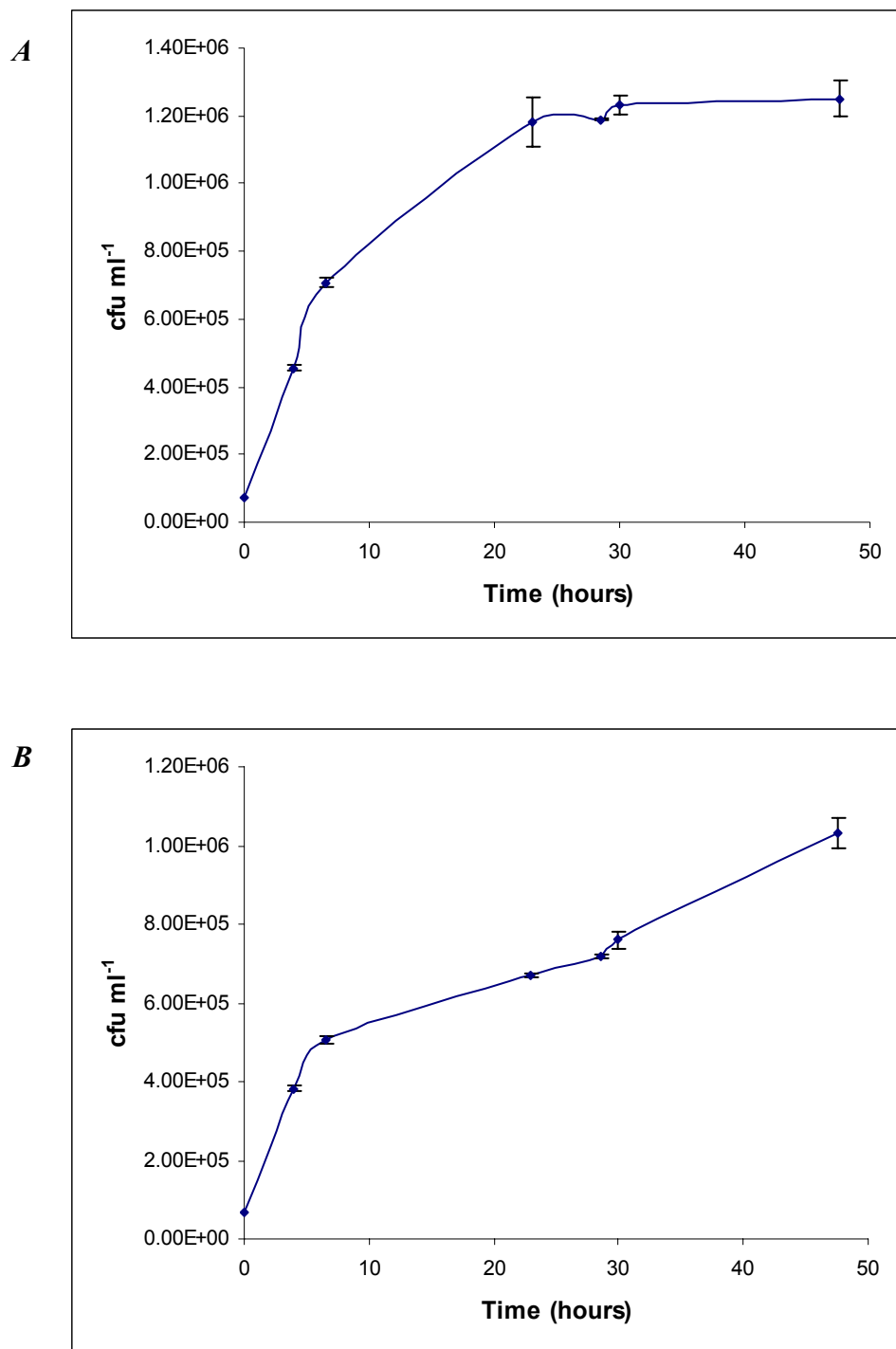
Figure 3.26 Growth curves for *Saccharomyces cerevisiae* in *A* unmodified NYDB and

B 0.98 a$_w$ NYDB incubated at 25°C.
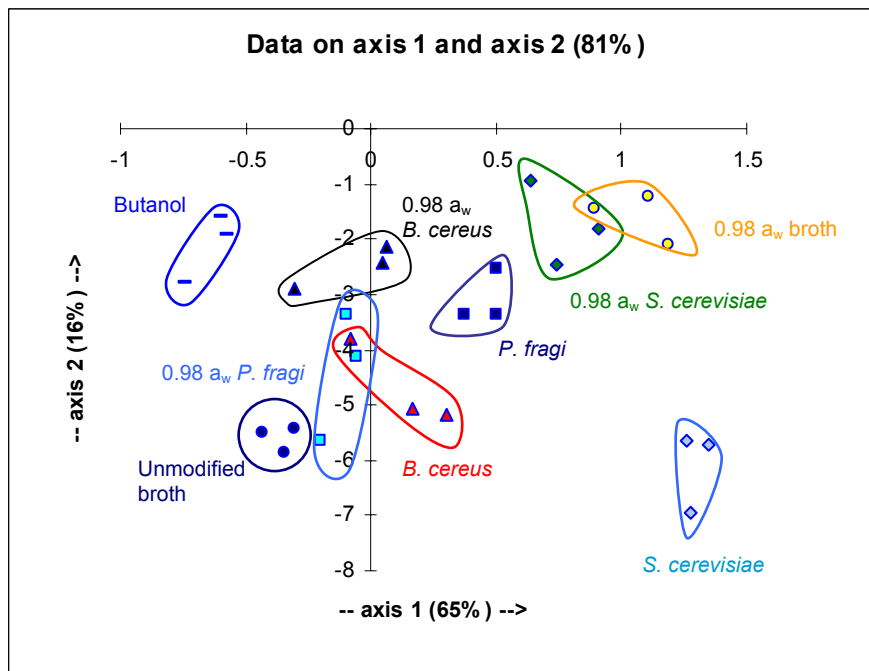
Figure 3.27 DFA of BH114 e-nose normalized divergence responses to volatiles produced by *Bacillus cereus, Pseudomonas fragi* and *Saccharomyces cerevisiae* after 13.5 h incubation at 25°C in unmodified and 0.98 a_w NYDB.
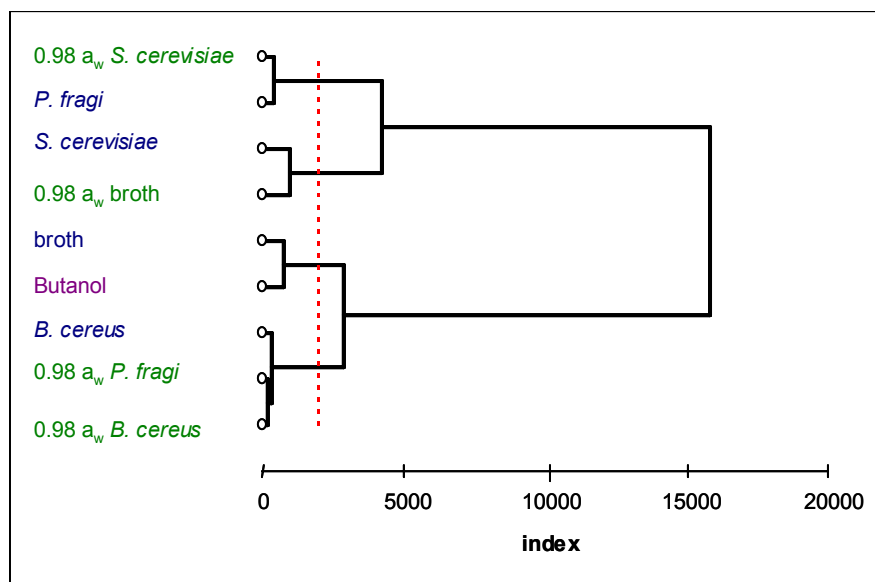
Figure 3.28 CA dendrogram of *Bacillus cereus, Pseudomonas fragi* and *Saccharomyces cerevisiae* in unmodified and 0.98 a_w NYDB based on their volatile profiles after 13.5 h at 25°C. Key to treatments: unmodified NYDB; 0.98 a_w NYDB.
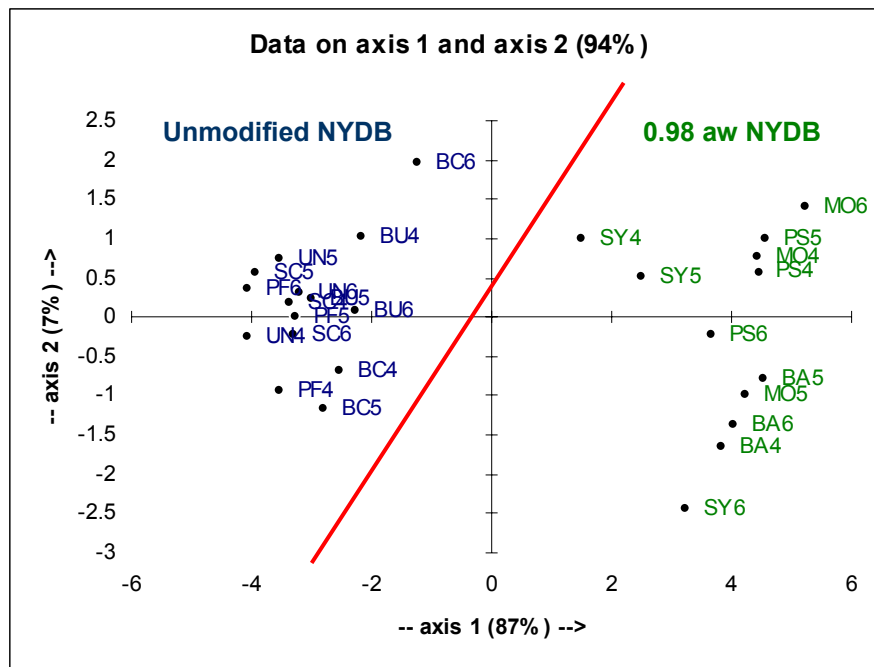
Figure 3.29 PCA of *Bacillus cereus, Pseudomonas fragi* and *Saccharomyces cerevisiae* based on their volatile profiles after 39 h growth in unmodified and 0.98 a_w NYDB at 25°C.

Key to treatments: BA- *B. cereus* in 0.98 a_w NYDB; BC- *B. cereus* in unmodified NYDB; BU- Butanol; PF- *P. fragi* in unmodified NYDB; PS- *P. fragi* in 0.98 a_w NYDB; SC- *S. cerevisiae* in unmodified NYDB; SY- *S. cerevisiae* in 0.98 a_w NYDB; MO- 0.98 a_w NYDB controls; UN- unmodified NYDB controls.
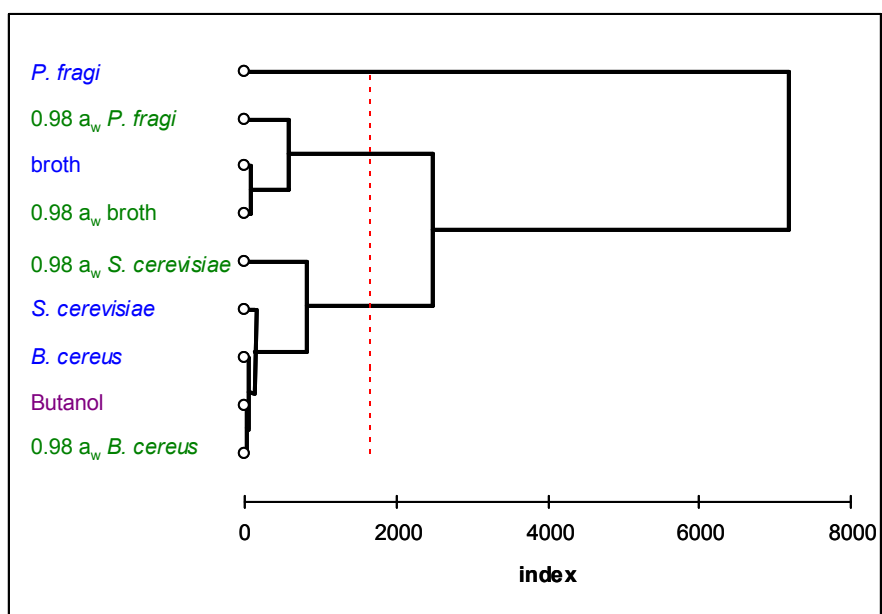
Figure 3.30 CA dendrogram of *Bacillus cereus, Pseudomonas fragi* and *Saccharomyces cerevisiae* in unmodified and 0.98 a_w NYDB based on their volatile profiles after 39 h at 25°C. Key to treatments: unmodified NYDB; 0.98 a_w NYDB.

Table 3.3 Growth rates of *Bacillus cerevisiae, Pseudomonas fragi* and *Saccharomyces cerevisiae* in unmodified and 0.98 a_w NYDB at 25°C. * Indicates that the cfu could not be calculated.

	Initial population	Population after 13.5h	Approx rate cfu h^{-1}	Population after 39 h	Approx rate cfu h^{-1}	Approx rate 13.5 – 39 h
Unmodified B. cereus	4.63 a 10^3	4.66×10^6	3.45×10^5	7.41×10^6	1.90×10^5	1.08×10^5
Unmodified P. fragi	9.90×10^3	3.35×10^9	2.48×10^8	5.93×10^9	1.38×10^8	1.01×10^8
Unmodified S. cerevisiae	8.55×10^3	6.70×10^5	4.90×10^4	1.71×10^6	4.36×10^4	4.08×10^4
0.98 a_w B. cereus	2.04 a 10^3	3.29×10^6	2.44×10^5	5.71×10^6	1.46×10^5	9.49×10^4
0.98 a_w P. fragi	9.20×10^3	9.54×10^8	7.07×10^7	4.50×10^9	1.16×10^8	1.39×10^8
0.98 a_w S. cerevisiae	7.79×10^3	*	*	8.51×10^5	2.16×10^4	*

Figure 3.31 CA dendrograms of *Staphylococcus aureus* in unmodified and 0.98 a_w NYDB based on their volatile profiles after *A* 4.5 h and *B* 23.5 h at 25°C.
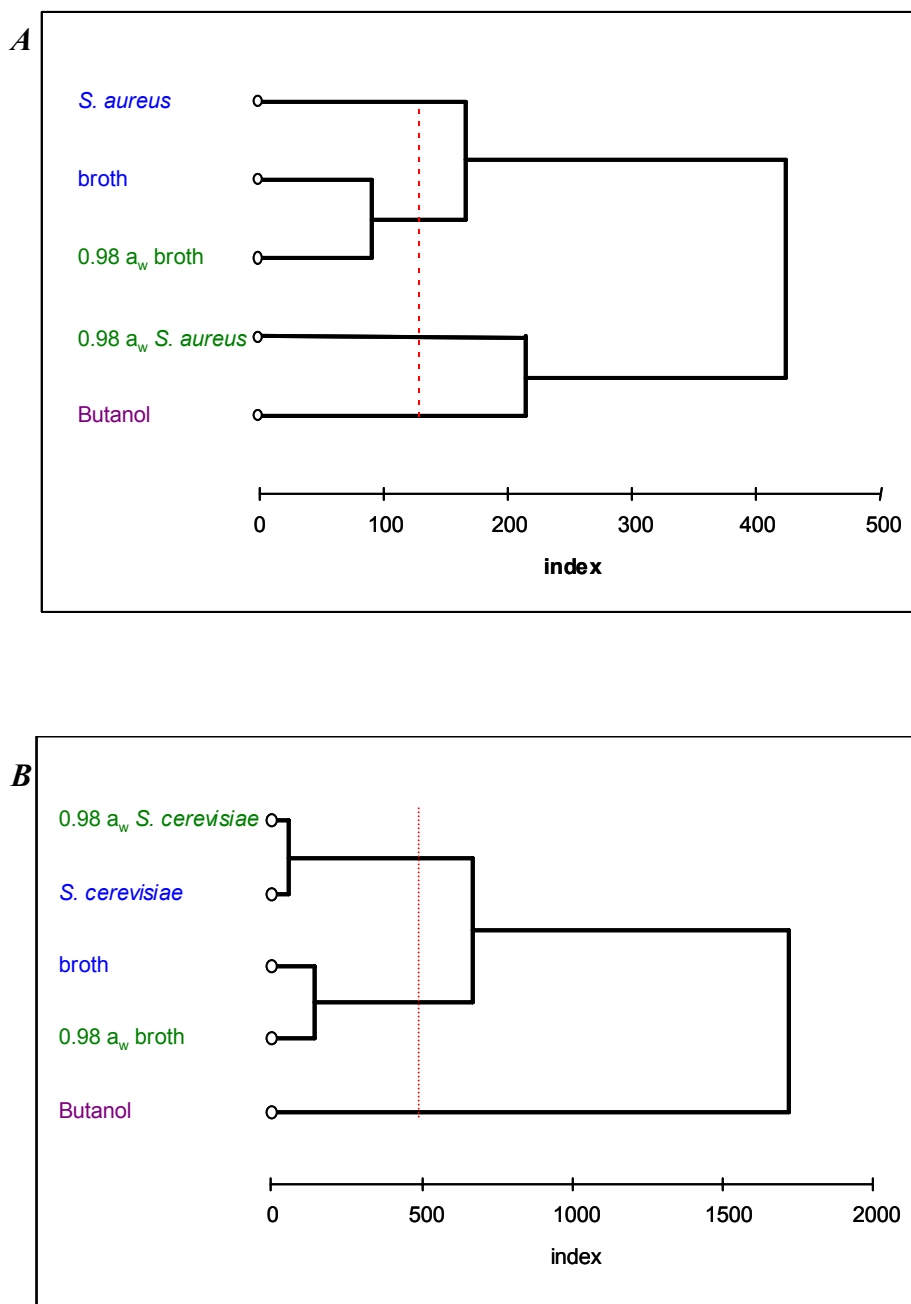
3.4 DIFFERENTIATION OF MICROBIAL SPOILAGE *IN SITU*

The effect of different water availabilities and temperatures on detection and differentiation of microbial spoilage species inoculated on bread analogues was examined using the conducting polymer e-nose system.

3.4.1 Bread analogues at 25°C and 0.95 a_w

After 24 h incubation at 25°C on 0.95 a_w bread analogues, clusters were obtained with DFA (Figure 3.32). It was possible to differentiate the controls, *S. cerevisiae* and *B. subtilis* but not between *P. verrucosum* and the treatment containing a mixture of the 3 micro-organisms. Similar results were obtained after 48 h as shown in the PCA plot shown in Figure 3.33, which accounts for 97% of the data. Microscopic growth of *S. cerevisiae* and *P. verrucosum* was noted after 48 h. Although the PCA accounted for 99% of the data after 72 h, overlapping of the treatments occurred. Cross validation gave approximately 50% correct classification of treatments with a significance level of 95%. Table 3.4 shows examples of treatments correctly classified with the percentages highlighted.

Table 3.4 Cross validation of microbial spoilage of 0.95 a_w bread analogues at 25°C. Key: **CO** - control; **BS** - *B. subtilis*; **MX** – Mix; **PV** – *P. verrucosum*; **SC** – *S. cerevisiae*. Highlighted values show the correct classification percentages.

	actual	x-val	BS	CO	MX	PV	SC
24 h	CO	CO	0	0.893	0.047	0.060	0
24 h	BS	BS	0.877	0	0.003	0.011	0.109
48 h	CO	CO	0.095	0.754	0.041	0.033	0.077
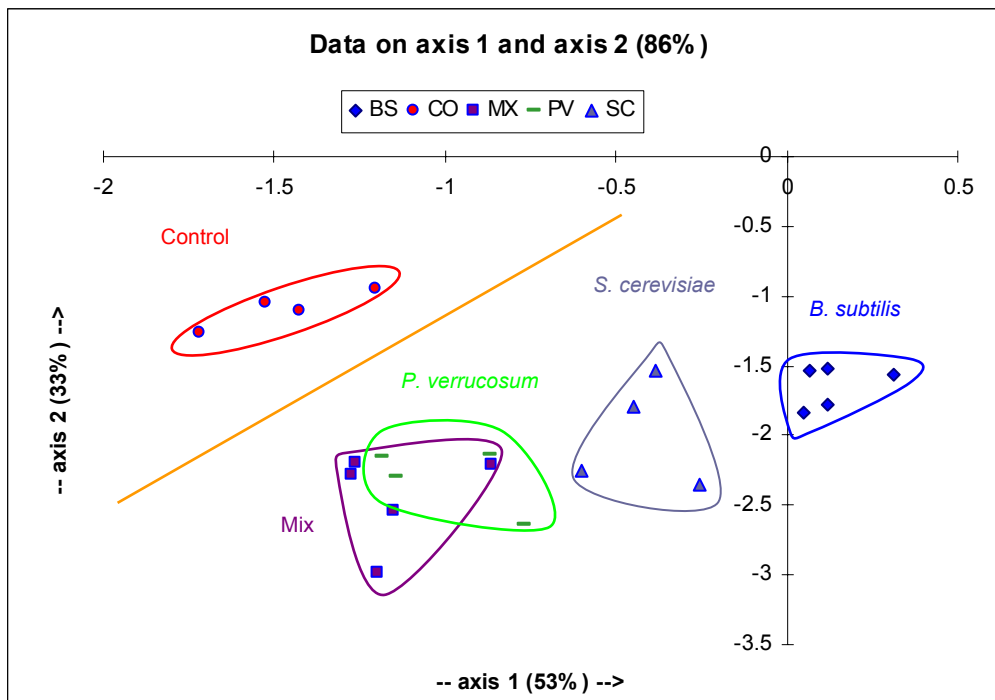
Figure 3.32 DFA of normalized BH114 e-nose divergence responses to volatiles produced by microbial spoilage organisms after 24 h growth on 0.95 a$_w$ bread analogues at 25°C.
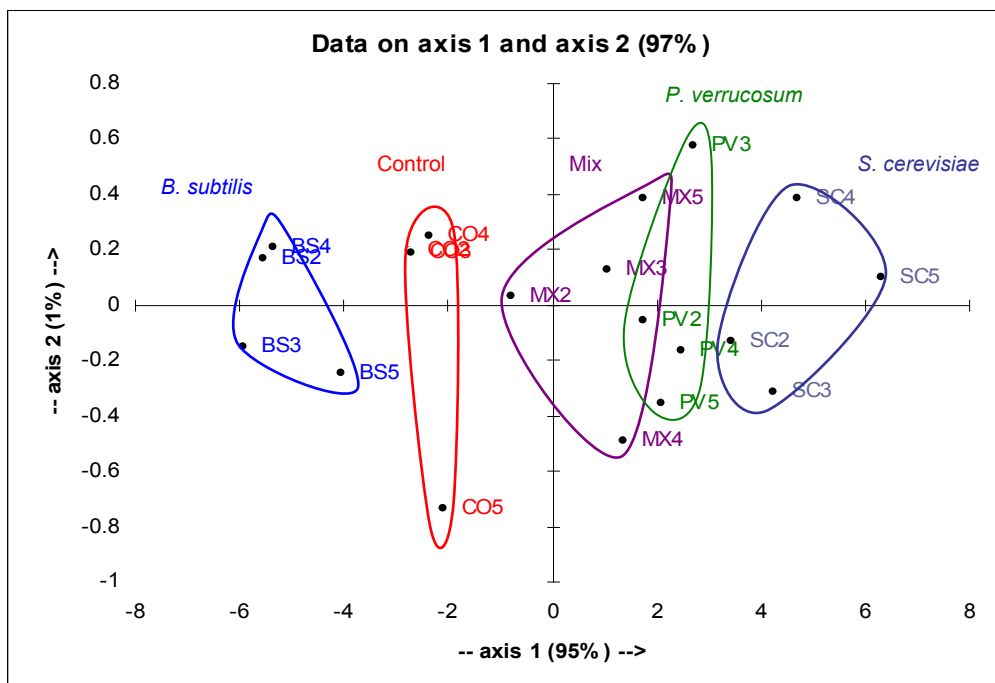
Figure 3.33 PCA of normalized BH114 e-nose divergence responses to volatiles produced by microbial spoilage organisms after 48 h growth on 0.95 a$_w$ bread analogues at 25°C.

3.4.2   Bread analogues at 25°C and 0.97 a_w

Differentiation of treatments was achieved after 24 h growth on 0.97 a_w bread analogues at 25°C as shown in the PCA plot in Figure 3.34. However overlap of controls with *B. subtilis* and mixed treatments occurred. After 48 h is was possible to only discriminate *P. verrucosum* from the other treatments (Figure 3.35). It was still not possible to differentiate controls after 72 h (Figure 3.36) although *P. verrucosum*, *S. cerevisiae* and the mixed treatment could be discriminated. Visible growth of *P. verrucosum* and *S. cerevisiae* occurred after 48 h and on the mixed treatment after 72 h. Cross validation gave approximately 50% correct classification of treatments with a significance level of 95%. Table 3.5 shows examples of treatments correctly classified with the percentage of them being that treatment highlighted.

Table 3.5 Cross validation of microbial spoilage of 0.97 a_w bread analogues at 25°C. Key: **CO** - control; **BS** - *B. subtilis*; **MX** – Mix; **PV** – *P. verrucosum*; **SC** – *S. cerevisiae*. Highlighted values show the correct classification percentages.

	actual	x-val	BS	CO	MX	PV	SC
24 h	SC	SC	0.14	0.0736	0.0134	0.008	0.765
48 h	PV	PV	0	0.056	0.041	0.891	0.012
72 h	MX	MX	0.094	0.004	0.861	0.041	0

3.4.3   Bread analogues at 15°C and 0.97 a_w

After 24 h incubation at 15°C only *S. cerevisiae* could be distinguished on 0.97 a_w bread analogues (Figure 3.37) while the mix and *P. verrucosum* clustered together. After 48 and 72 h there was no differentiation of any treatment.
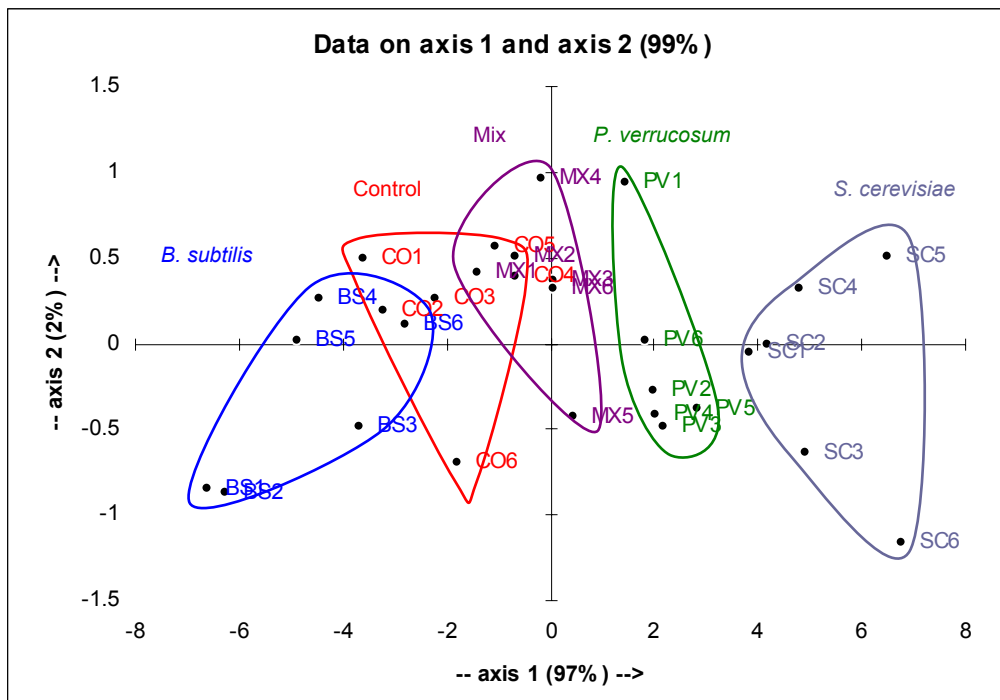
Figure 3.34 PCA of normalized BH114 e-nose divergence responses to volatiles produced by microbial spoilage organisms after 24 h growth on 0.97 a_w bread analogues at 25°C.
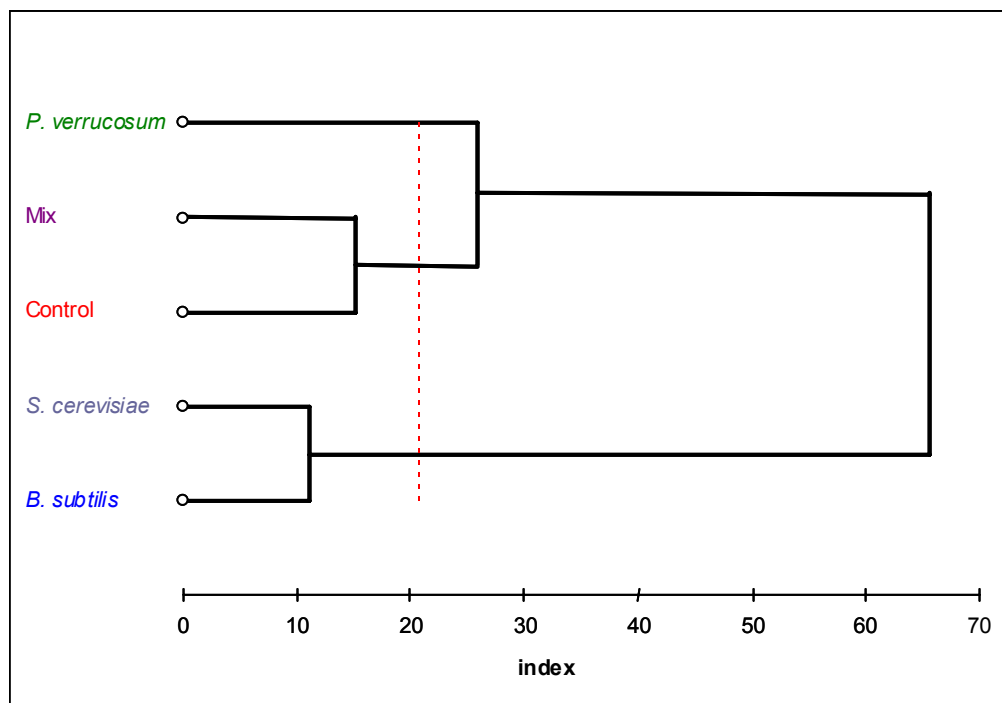
Figure 3.35 CA dendrogram of normalized BH114 e-nose divergence responses to volatiles produced by microbial spoilage organisms after 48 h growth on 0.97 a_w bread analogues at 25°C.
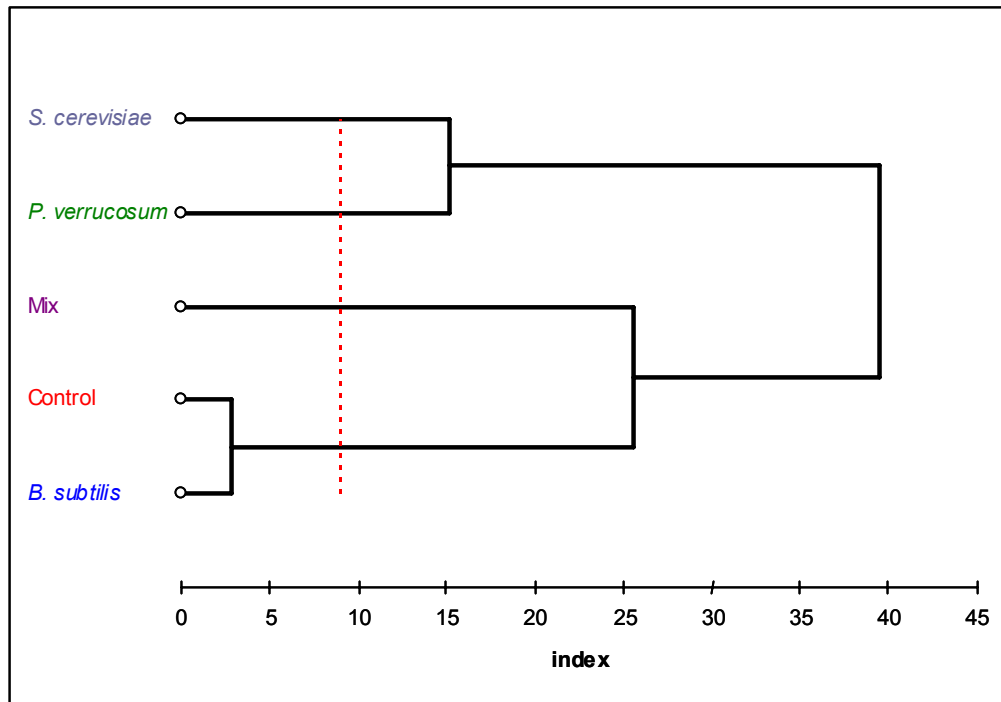
Figure 3.36 CA dendrogram of normalized BH114 e-nose divergence responses to volatiles produced by microbial spoilage organisms after 72 h growth on 0.97 a_w bread analogues at 25°C.
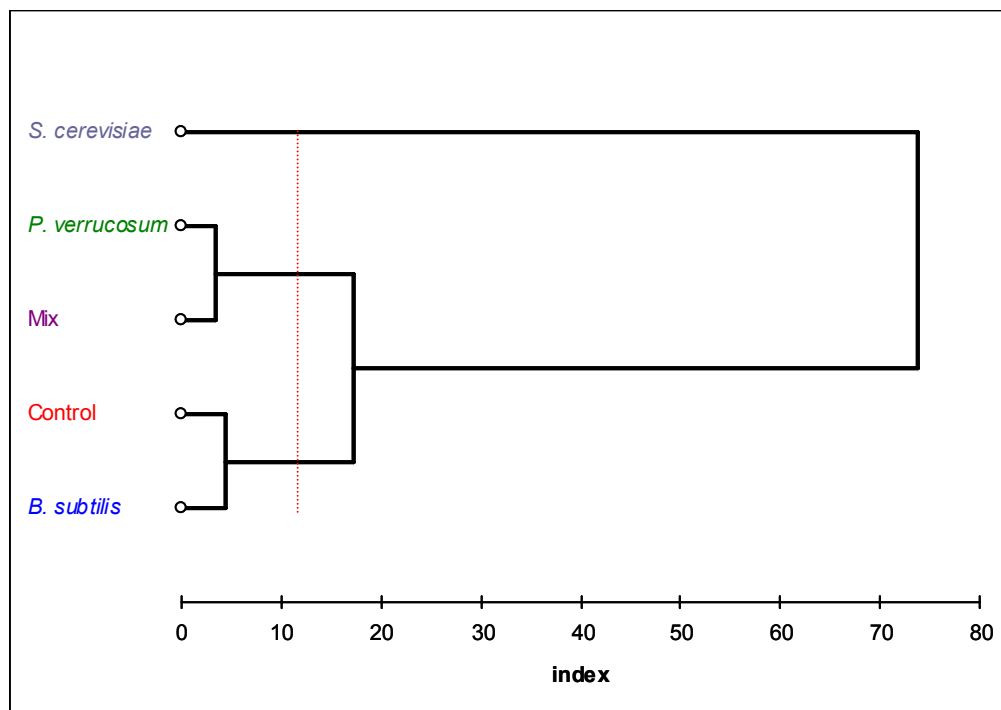
Figure 3.37 CA dendrogram of normalized BH114 e-nose divergence responses to volatiles produced by microbial spoilage organisms after 24 h growth on 0.97 a_w bread analogues at 15°C.

3.4.4 Bread analogues at 15°C and 0.95 a_w

After 24 h (Figure 3.38) and 48 h incubation at 15°C on 0.95 a_w bread analogues clusters of treatments were obtained, but significant overlap occurred. Controls, *S. cerevisiae* and *B. subtilis* could be differentiated after 72 h (Figure 3.39). There was similarity between volatiles produced by *P. verrucosum* and the mixed microbial treatment. Cross validation only correctly classified 45% of treatments at 95% significance level. Table 3.6 shows these results.

Table 3.6 Cross validation of microbial spoilage of 0.95 a_w bread analogues at 15°C. Key: **CO** - control; **BS** - *B. subtilis*; **MX** − Mix; **PV** − *P. verrucosum*; **SC** − *S. cerevisiae*. Highlighted values show the correct classification percentages.

	actual	x-val	BS	CO	MX	PV	SC
72 h	SC	SC	0.12	0.075	0	0	0.805
72 h	CO	CO	0.137	0.854	0.08	0.001	0
72 h	BS	BS	0.809	0.079	0.03	0.03	0.052

Table 3.7 shows a summary of the time taken to achieve visible growth on the different bread analogues at both temperatures. This shows that visible growth of *P. verrucosum* and *S. cerevisiae* occurred after 48 h at 25°C on bread analogues at both water availabilities. Four days were required for and visible signs of growth at 15°C.

Using CA it was possible to differentiate treatments growing on the different a_w bread analogues at both 25°C (Figure 3.40) and 15°C (Figure 3.41) with all sample times. It was also possible to differentiate between treatments growing at the different temperatures at 0.95 (Figure 3.42) and 0.97 a_w (Figure 3.43) at all sample times.
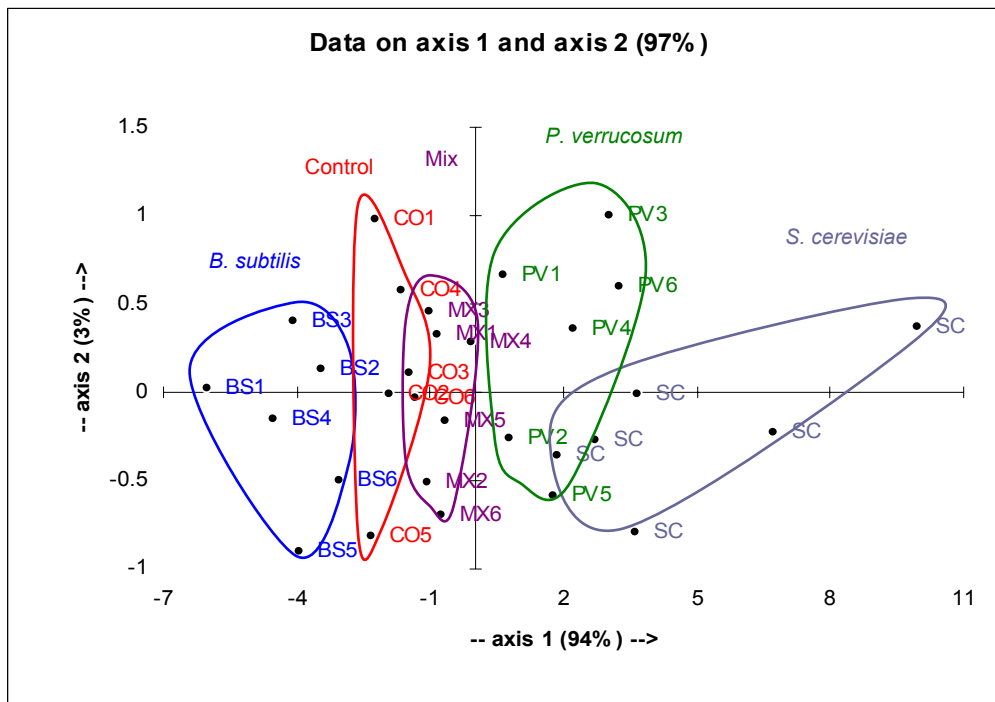
Figure 3.38 PCA of normalized BH114 e-nose divergence responses to volatiles produced by microbial spoilage organisms after 24 h growth on 0.95 a$_w$ bread analogues at 15°C.
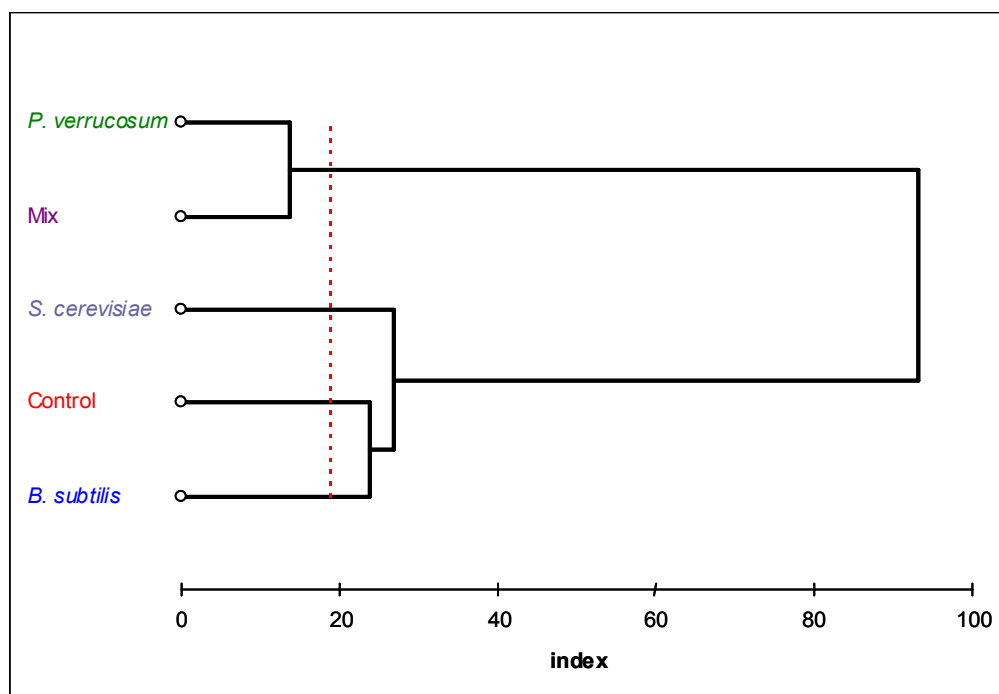
Figure 3.39 CA dendrogam of normalized BH114 e-nose divergence responses to volatiles produced by microbial spoilage organisms after 72 h growth on 0.95 a$_w$ bread analogues at 15°C.

Table 3.7 Time taken to achieve visible growth on bread analogue at different water availabilities and temperatures.

		15°C				25°C			
		24 h	48 h	72 h	actual	24 h	48 h	72 h	actual
0.95 a_w	**Control**	-	-	-	-	-	-	-	-
	P. verrucosum	-	-	-	4 days	-	+	+	48 h
	B. subtilis	-	-	-	>9 days	-	-	-	5/6 days
	S. cerevisiae	-	-	-	>9 days	-	+	+	48 h
	Mix	-	-	-	8 days	-	-	+	72 h
0.97 a_w	**Control**	-	-	-	-	-	-	-	-
	P. verrucosum	-	-	-	5 days	-	+	+	48 h
	B. subtilis	-	-	-	>10 days	-	-	-	75 h
	S. cerevisiae	-	-	-	4 days	-	+	+	48 h
	Mix	-	-	-	5 days	-	-	+	72 h
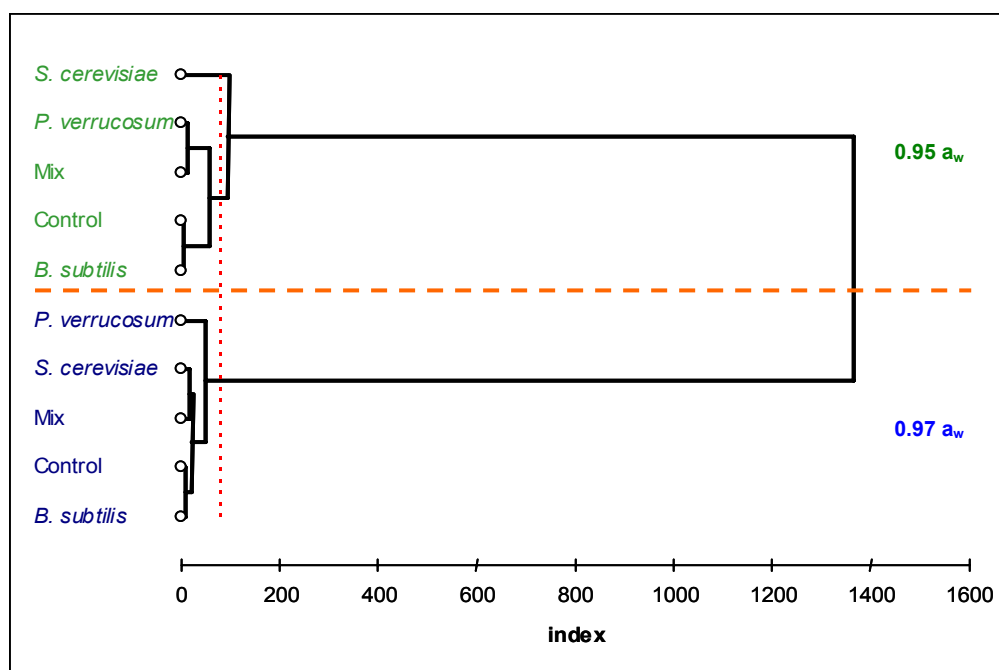
Figure 3.40 CA dendrogram comparing volatile production profiles of microbial spoilage organisms grown for 24 h on 0.95 and 0.97 a_w bread analogues at 25°C.
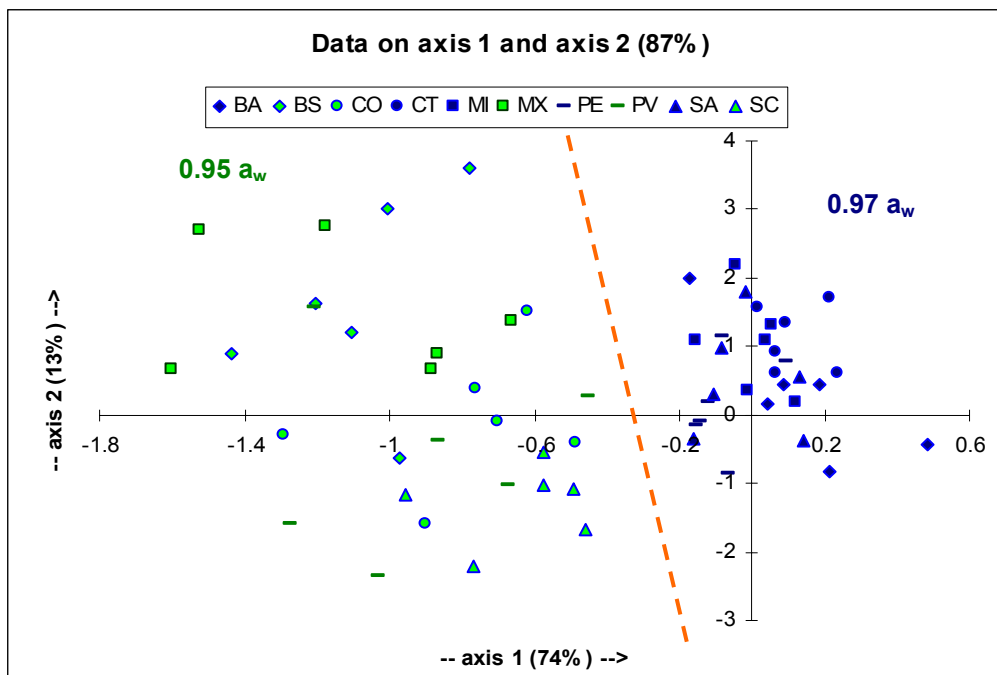
Figure 3.41 DFA of normalized BH114 e-nose divergence responses comparing volatile production profiles of microbial spoilage organisms grown for 24 h on 0.95 and 0.97 a_w bread analogues at 15°C. **Key to treatments**: BA- *B. subtilis* at 0.97 a_w; BS- *B. subtilis* at 0.95 a_w; CO- 0.95 a_w controls; CT- 0.98 a_w controls; MI- 0.98 a_w mixture; MX- 0.95 a_w mixture; PE- *P. verrucosum* at 0.97 a_w; PV- *P. verrucosum* at 0.97 a_w; SA- *S. cerevisiae* at 0.97 a_w; SC- *S. cerevisiae* at 0.97 a_w
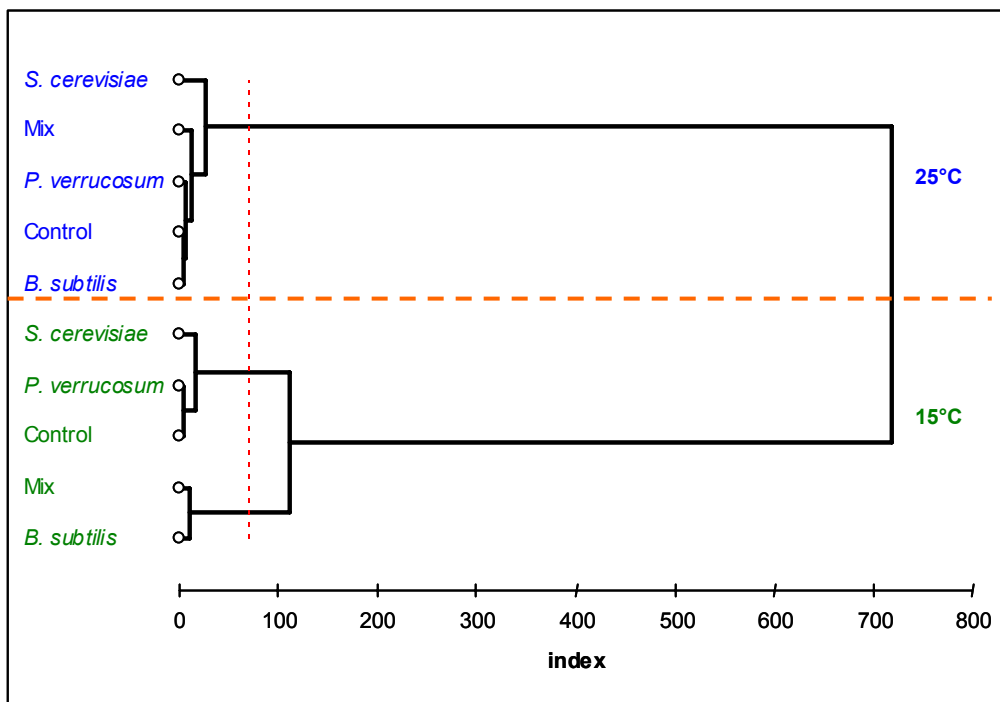
Figure 3.42 CA dendrogram comparing volatile production profiles of microbial spoilage organisms grown for 24 h on 0.95 a_w bread analogues at 15°C and 25°C.
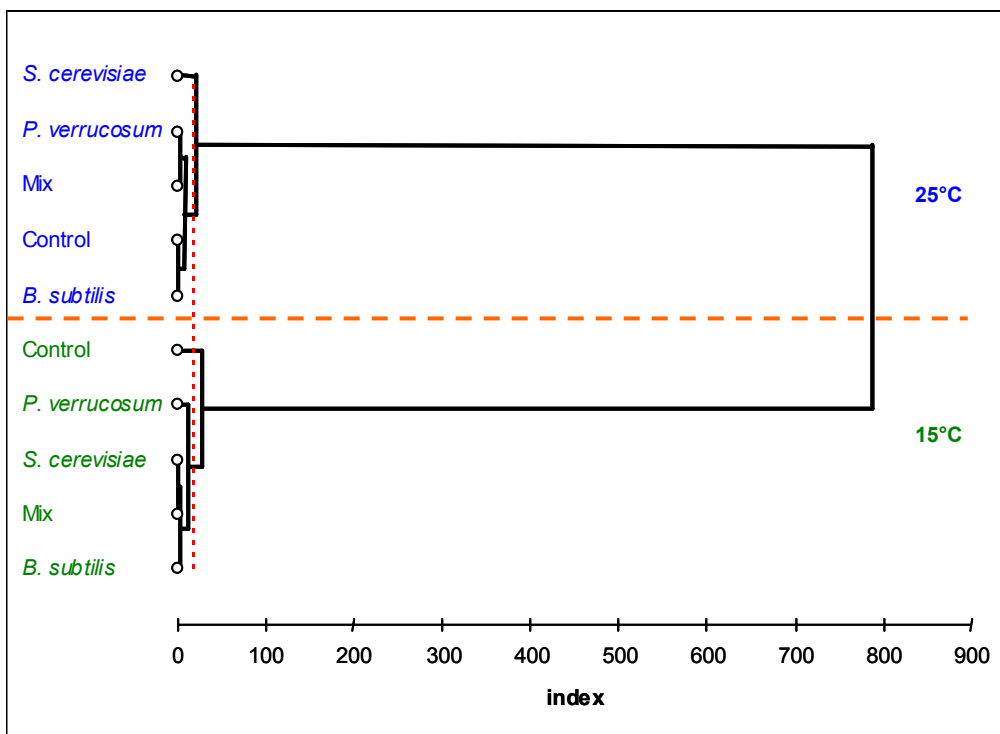
Figure 3.43 CA dendrogram comparing volatile production profiles of microbial spoilage organisms grown for 72 h on 0.97 a_w bread analogues at 15°C and 25°C.

3.5 COMPARISON OF A BH114 E-NOSE AND GC-MS FOR DISCRIMINATION OF MICROBIAL SPOILAGE ORGANISMS ON BREAD ANALOGUES

Comparison of a BH114 e-nose system and GC-MS for differentiation of microbial and physiological spoilage of bread analogues containing 2000ppm calcium propionate was examined.

Using the BH114 e-nose after 24 h it was possible to discriminate control and *P. verrucosum* inoculated analogues using CA as shown in Figure 3.44. After 48 h physiological spoilage by lipoxygenase, could also be differentiated (Figure 3.45). Removal of the lipoxygenase treatment from the PCA allowed differentiation between microbial species (Figure 3.46). However with increased sample time there was no differentiation of spoilage.

The microbial populations changed with time as shown in Figure 3.47. Populations of *P. verrucosum* did not increase as significantly as *B. subtilis* and *P. anomala*. There were no visible signs of growth or spoilage after 96 h incubation in this study.

From the 59 compounds detected by the GC-MS, 11 found to be important in microbial spoilage previously by Börjesson *et al*. (1992) and Magan & Evans (2000) were compared. The 11 compounds were 2-propanol, 2-butanone, ethyl acetate, 3-methylbutanal, 1-butanol, pentanol, methylbenzene, hexanal, 1-hexanol, styrene and 2-pentylfuran.
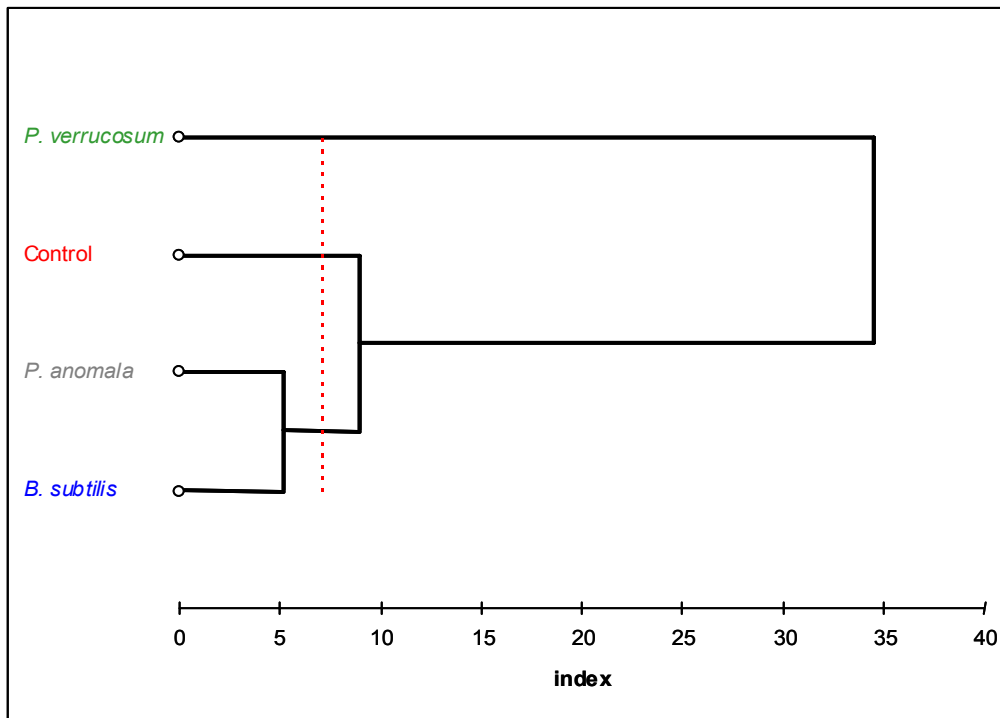
Figure 3.44 CA dendrogram of *Penicillium verrucosum*, *Pichia anomala* and *Bacillus subtilis* based on their volatile profiles after 24 h at 25°C on 0.95 a_w bread analogue containing 2000ppm calcium propionate.
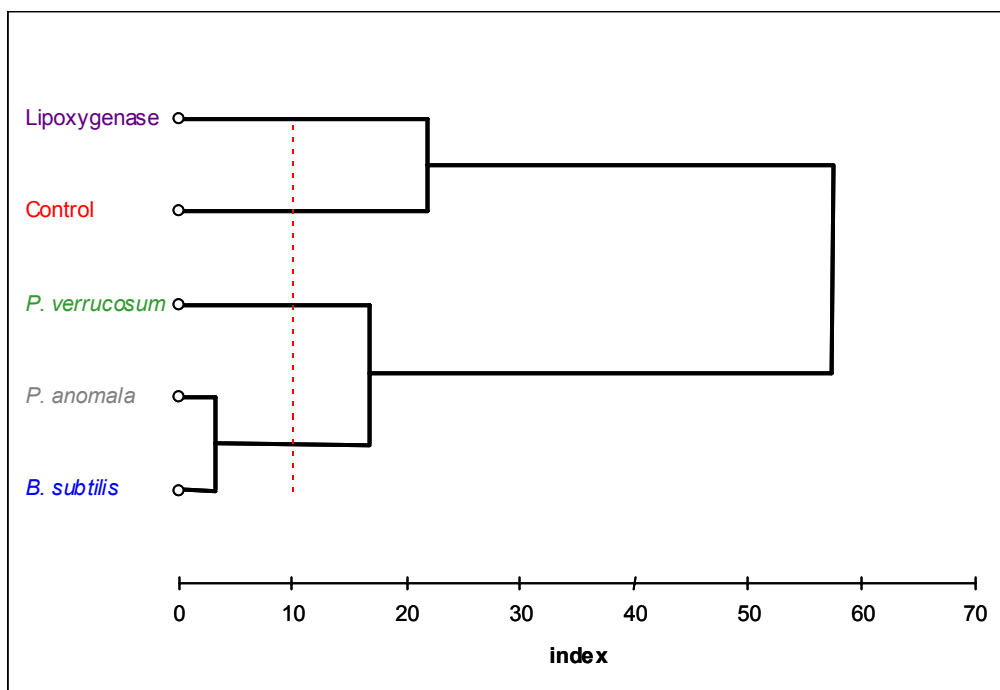
Figure 3.45 CA dendrogram of microbial and physiological spoilage based on their volatile production profiles after 48 h at 25°C on 0.95 a_w bread analogue containing 2000ppm calcium propionate.
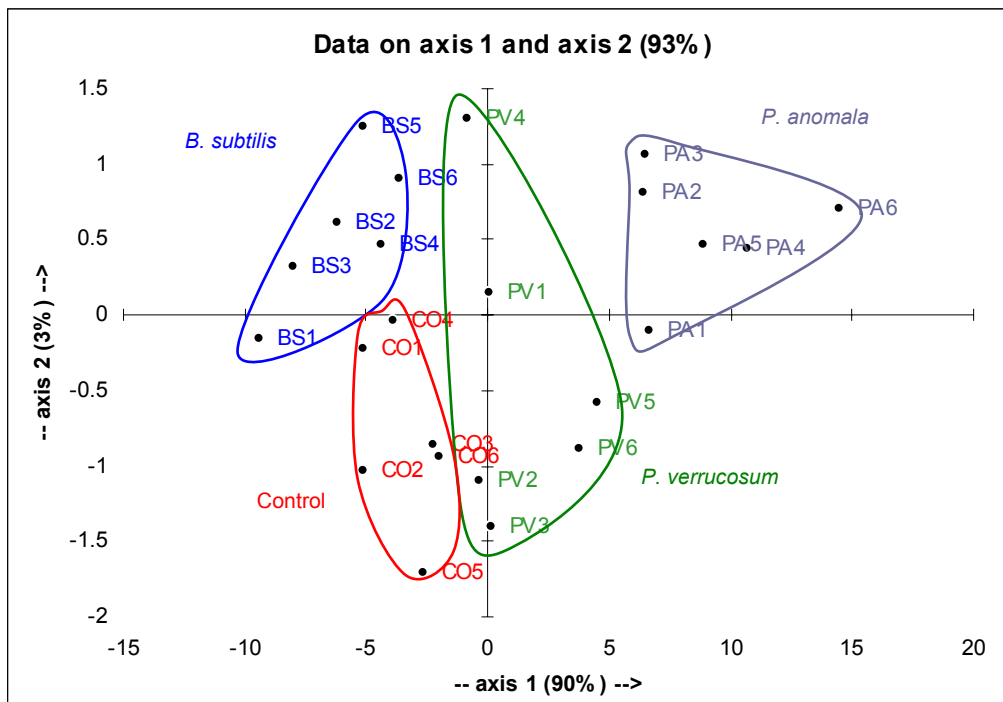
Figure 3.46 PCA of *Penicillium verrucosum*, *Pichia anomala* and *Bacillus subtilis* based on their volatile profiles after 48 h at 25°C on 0.95 a_w bread analogue containing 2000ppm calcium propionate.
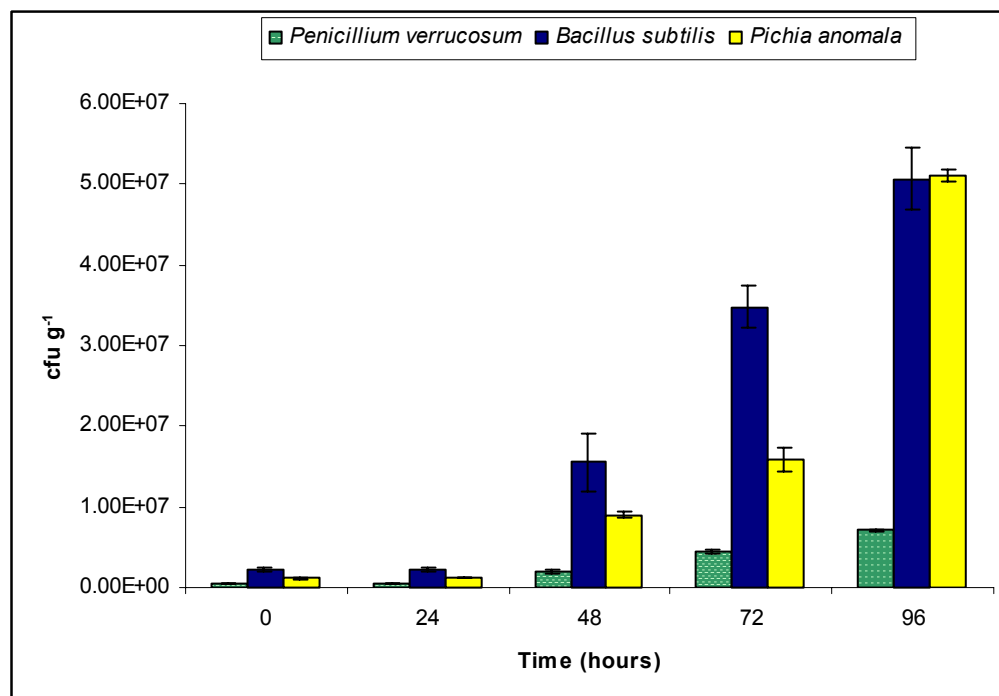
Figure 3.47 Microbial cfu g^{-1} against time for *Penicillium verrucosum*, *Pichia anomala* and *Bacillus subtilis* grown on 0.95 a_w bread analogue containing 2000ppm calcium propionate at 25°C.

Figure 3.48A shows ethyl acetate, 2-propanol and 3-methylbutanal were produced in large quantities by *P. anomala*, after 24 h. When these 3 compounds were removed it was possible to observe differences between the compounds produced by all treatments as shown in Figure 3.48B. This shows that pentanol was only produced by *P. anomala*. Styrene levels were similar for all treatments and controls and *P. verrucosum* produced lower amounts of 2-butanone. PCA of GC-MS data didn't allow differentiation of any treatments. CA using 2-propanol, 2-butanone, 3-methylbutanal, pentanol, 1-hexanol, and 2-pentylfuran discriminated *P. anomala* and lipoxygenase spoilage from the other treatments (Figure 3.49). The production of compounds altered with sample time as shown in Figure 3.50. There was an increase in production of 3-methylbutanol and a decrease in hexanal by *P. verrucosum* with increasing sample time.

3.6 DISCRIMINATION OF MYCOTOXIGENIC AND NON-TOXIGENIC STRAINS OF FILAMENTOUS FUNGI USING VOLATILE PRODUCTION PROFILES

Use of the conducting polymer based BH114 e-nose system to differentiate between toxin and non-toxin producing strains of *Aspergillus parasiticus* and *P. verrucosum* was examined *in vitro* and *in situ*.
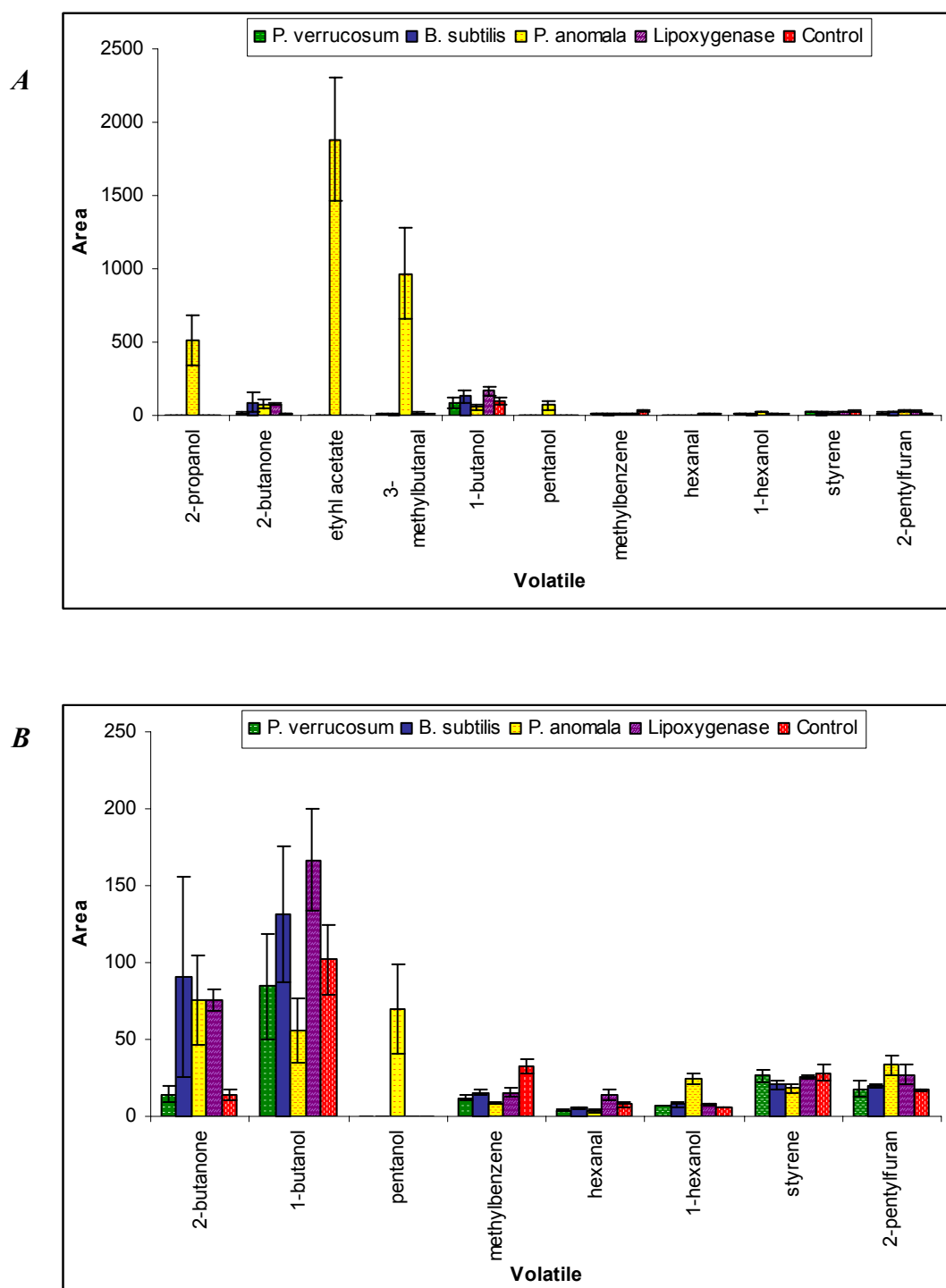
Figure 3.48 Levels of compounds produced by microbial and physiological spoilage detected using gas chromatography-mass spectrometry after 24 h growth on 0.95 a_w bread analogues containing 2000ppm calcium propionate. *A* all 11 compounds and *B* with 3 removed.
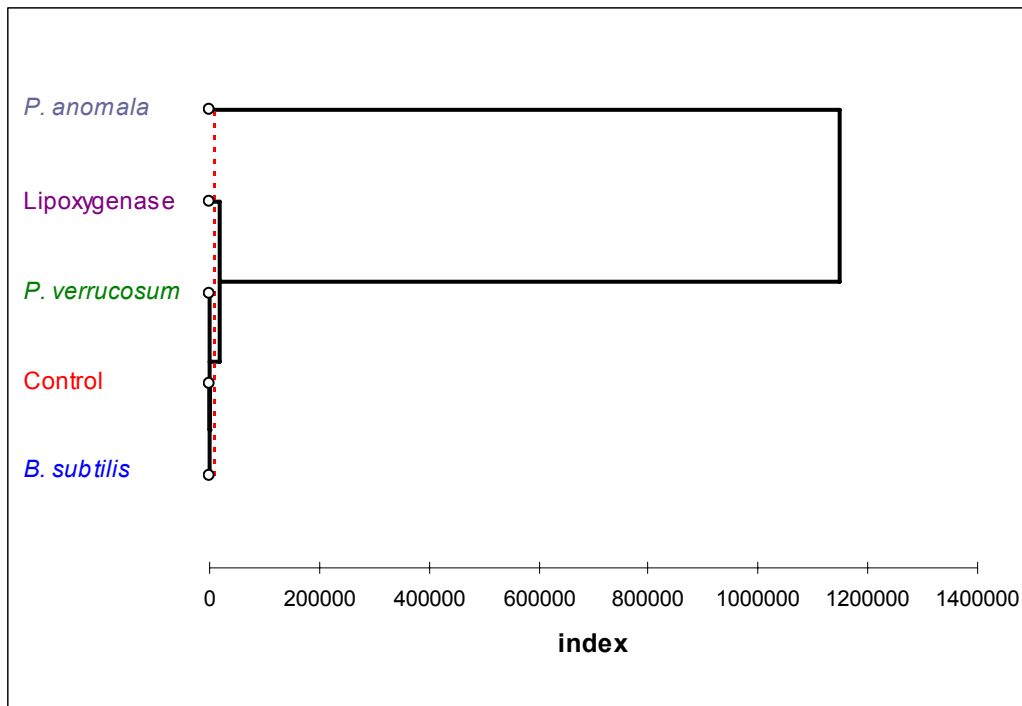
Figure 3.49 CA dendrogram of microbial and physiological spoilage of 0.95 a_w bread analogues incubated at 25°C for 24 h using production levels of 2-propanol, 2-butanone, 3-methylbutanal, pentanol, 1-hexanol, and 2-pentylfuran determined by gas chromatograph-mass spectrometry.
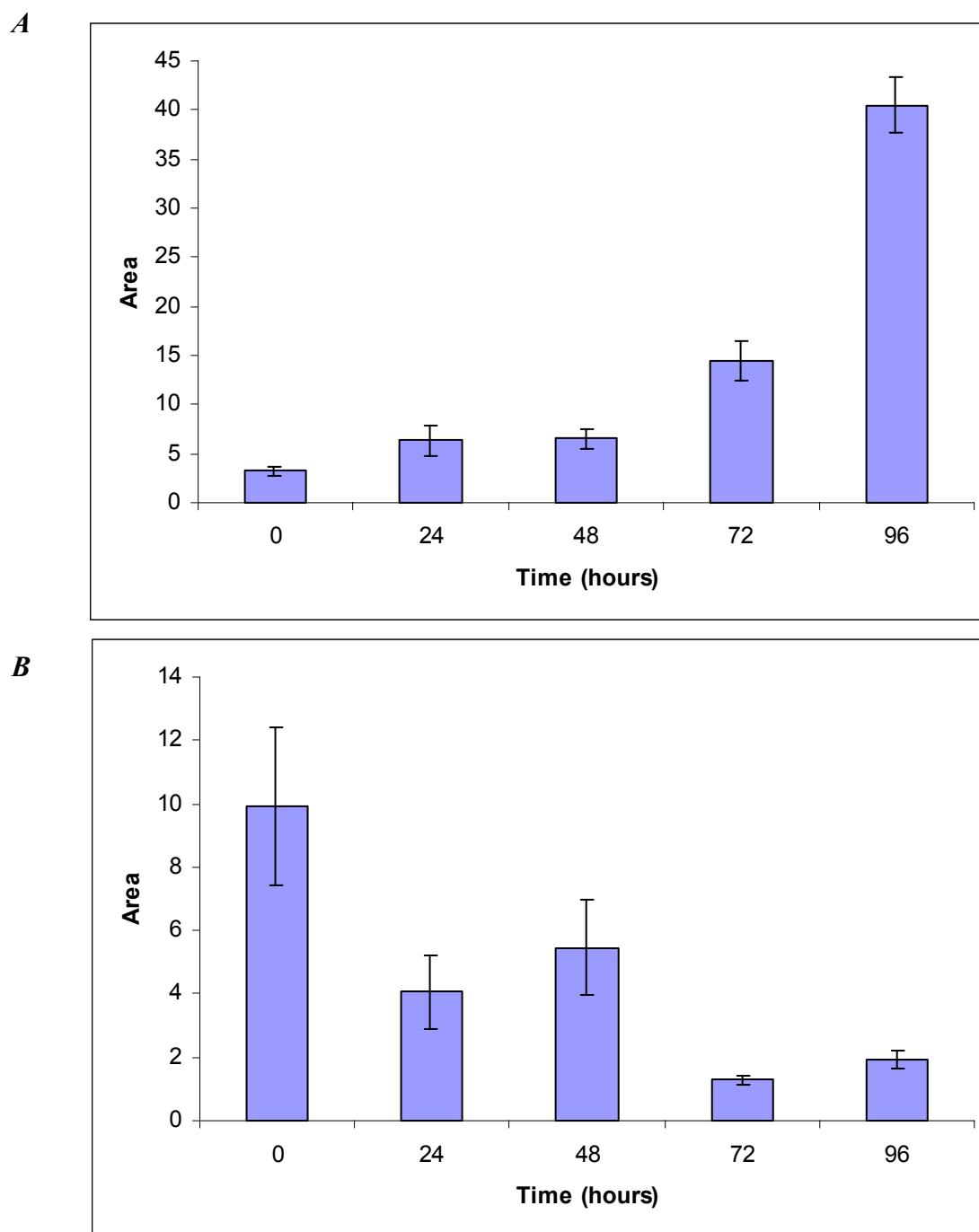
Figure 3.50 Levels of 3-methylbutanal (**A**) and hexanal (**B**) produced by *Penicillium verrucosum* on 0.95 a_w bread analogues containing 2000ppm calcium propionate detected using gas chromatography-mass spectrometry.

3.6.1   Discrimination of mycotoxigenic and non-toxigenic *Aspergillus parasiticus* strains

PCA (Figure 3.51) allowed differentiation of controls from *A. parasiticus* inoculated maize based agar media after 48 h. There was some overlap of the non-mycotoxigenic strain D64 with the mycotoxin producing strains. Cluster analysis showed only one toxigenic strain (D106) could be discriminated from the others (Figure 3.52). Figure 3.53 shows that after 72 h DFA produced differentiation of controls, one mycotoxin producing strain (D106) and one non-toxigenic strain (D64). A different toxigenic strain, T20, could be discriminated from the other *A. parasiticus* strains using CA (Figure 3.54).   Microscopic growth was observed after 48 h.

3.6.2   Differentiation of mycotoxigenic and non-toxigenic *Penicillium verrucosum* strains

Figure 3.55 shows the PCA plot for *P. verrucosum* strains *in vitro* on 2% unmodified wheat agar after 24 h growth at 25°C. It was possible to differentiate controls and a non-toxigenic strain IBT22025 from 4 strains, which produce citrinin. After 48 h the non-toxigenic strain IBT22025 could be distinguished but controls could not be differentiated from one of the citrinin producing strains (IBT5252) as shown in Figure 3.56.

Using agar with a water activity of 0.95 produced less differentiation with overlap of strains after 24 h (Figure 3.57). Differentiation improved slightly after 48 h with one of the citrinin producing strains (IBT5252) being discriminated as shown in the PCA plot in Figure 3.58, which accounts for 96% of the data.
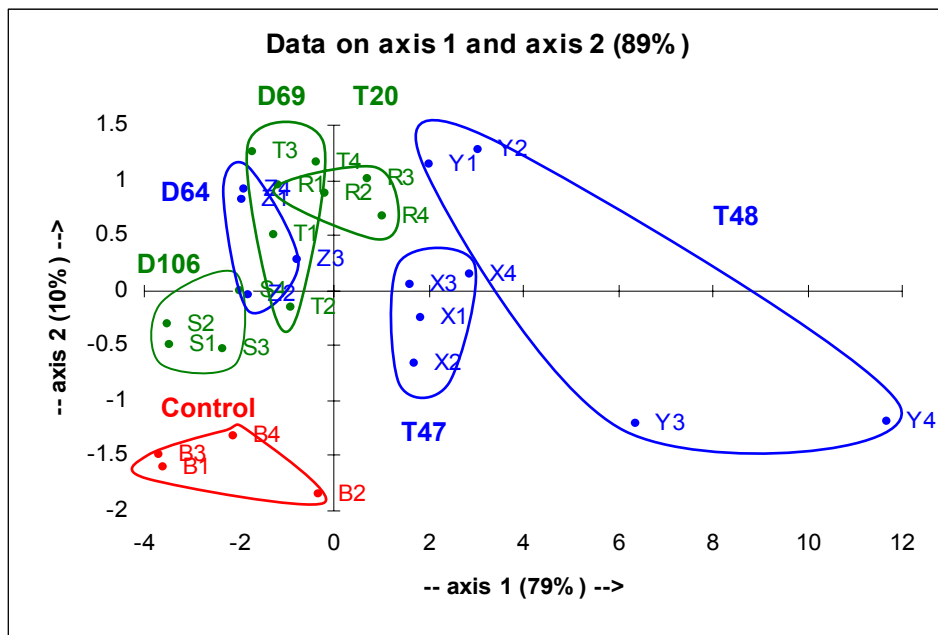
Figure 3.51 PCA of normalized BH114 e-nose divergence responses to volatiles produced by mycotoxigenic and non-mycotoxigenic strains of *Aspergillus parasiticus* after 48 h incubation at 25°C on 0.95 a$_w$ 2% milled maize agar.   Key: mycotoxigenic strains - T20, D106, D69; non-mycotoxigenic strains – T47, T48, D64.
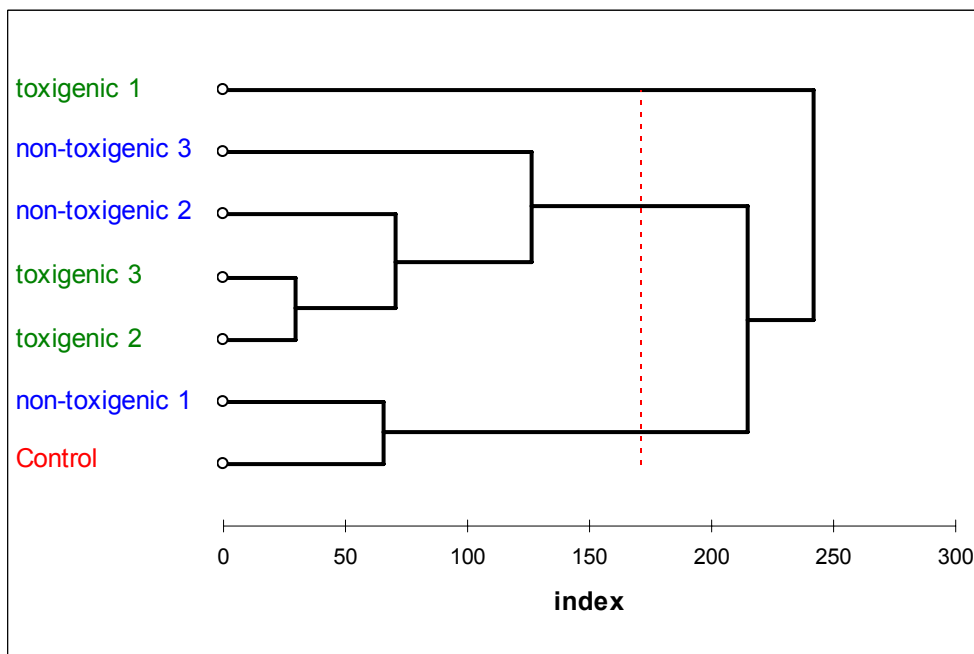
Figure 3.52 CA dendrogram of mycotoxigenic and non-mycotoxigenic *Aspergillus parasiticus* strains based on their volatile profiles after 48 h incubation at 25°C on 0.95 a$_w$ 2% milled maize agar. Key: toxigenic 1 - T20; toxigenic 2 - D106; toxigenic 3 - D69; non-toxigenic 1 – T47; non-toxigenic 2 - T48; non-toxigenic 3 - D64.
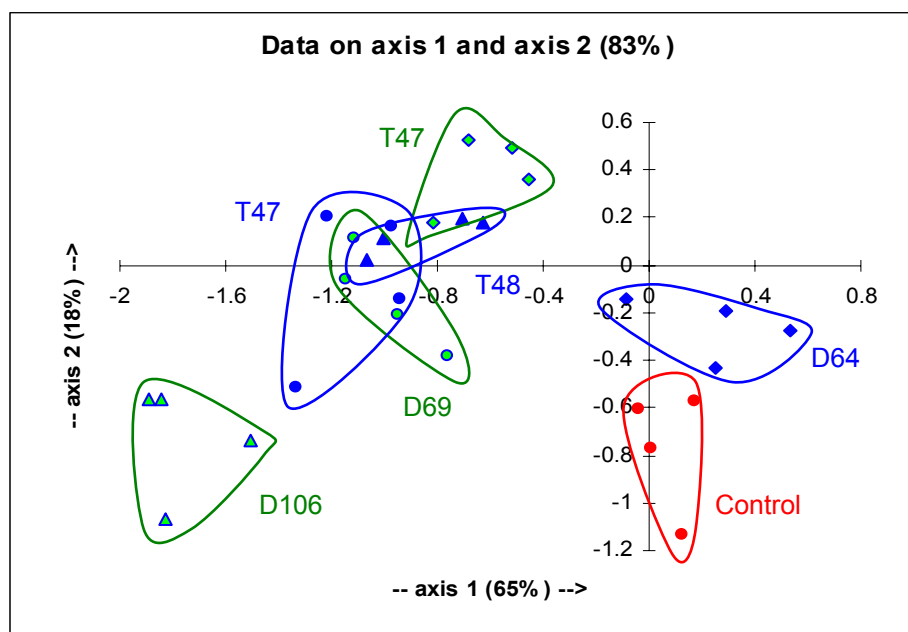
Figure 3.53 DFA of normalized BH114 e-nose divergence responses to volatiles produced by mycotoxigenic and non-mycotoxigenic strains of *Aspergillus parasiticus* after 72 h incubation at 25° on 0.95 a_w 2% milled maize agar.

Key : mycotoxigenic - T20, D106, D69   non-mycotoxigenic – X, Y, D64
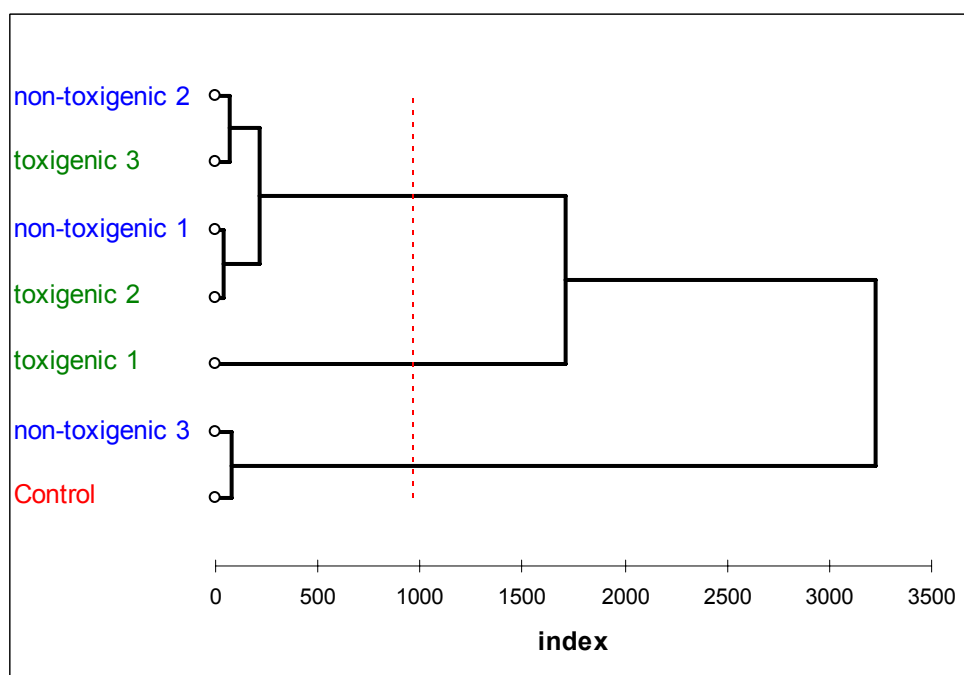
Figure 3.54 CA dendrogram of mycotoxigenic and non-mycotoxigenic *Aspergillus parasiticus* strains based on their volatile profiles after 72 h incubation at 25°C on 0.95 a_w 2% milled maize agar. Key: toxigenic 1 - T20; toxigenic 2 - D106; toxigenic 3 - D69; non-toxigenic 1 – T47; non-toxigenic 2 - T48; non-toxigenic 3 - D64.
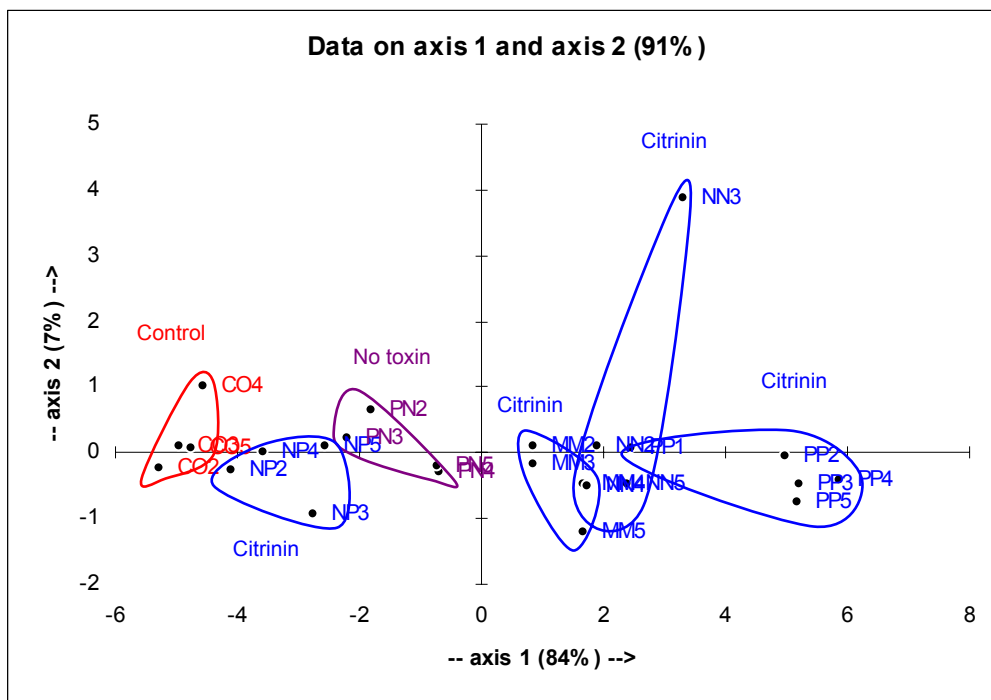
Figure 3.55 PCA of mycotoxigenic and non-mycotoxigenic *Penicillium verrucosum* strains based on their volatile profiles after 24 h incubation at 25°C on unmodified 2% wheat agar. Key: non-toxin producer IBT22025 (PN), citrinin producers - IBT5252 (PP), IBT22116 (NN), IBT21491 (NP), IBT22123 (MM).
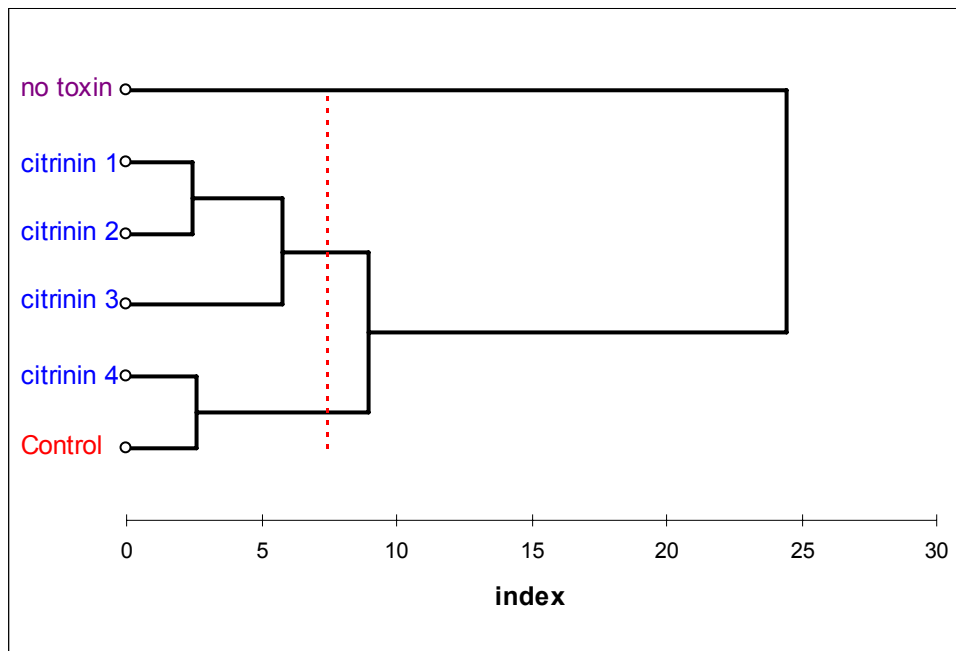
Figure 3.56 CA dendrogram of mycotoxigenic and non-mycotoxigenic *Penicillium verrucosum* strains based on their volatile profiles after 48 h incubation at 25°C on unmodified wheat agar. Key: non-toxin producer IBT22025, citrinin 1 - IBT5252, citrinin 2 - IBT22116, citrinin 3 - IBT21491 , citrinin 4 - IBT22123 .
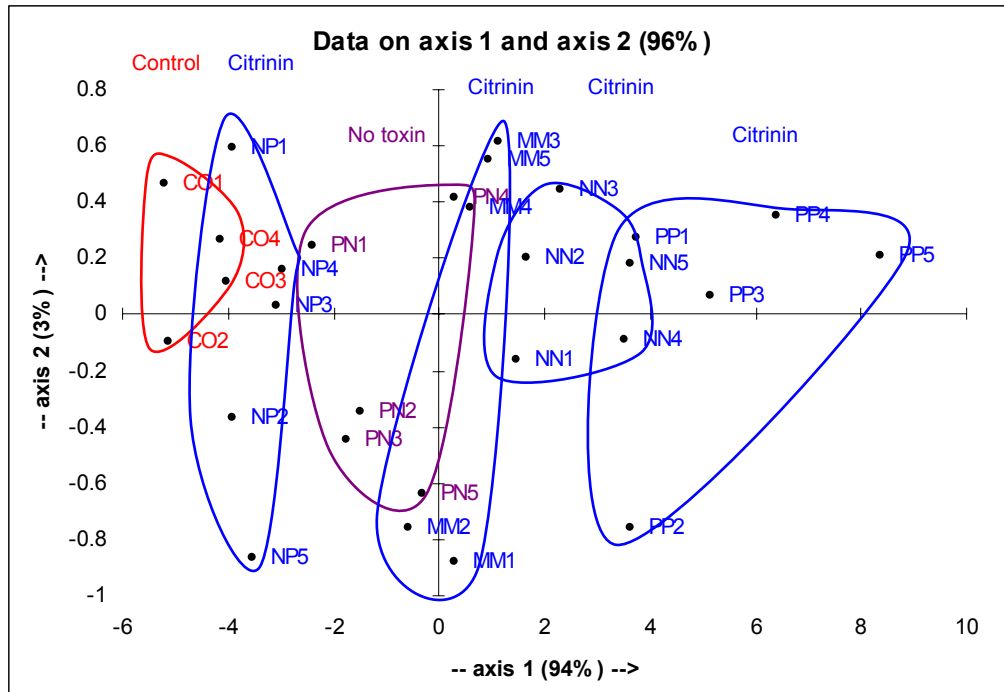
Figure 3.57 PCA of mycotoxigenic and non-mycotoxigenic *Penicillium verrucosum* strains based on their volatile profiles after 24 h incubation at 25°C on 0.95 a_w 2% wheat agar. Key: non-toxin producer IBT22025 (PN), citrinin producers- IBT5252 (PP), IBT22116 (NN), IBT21491 (NP), IBT22123 (MM).
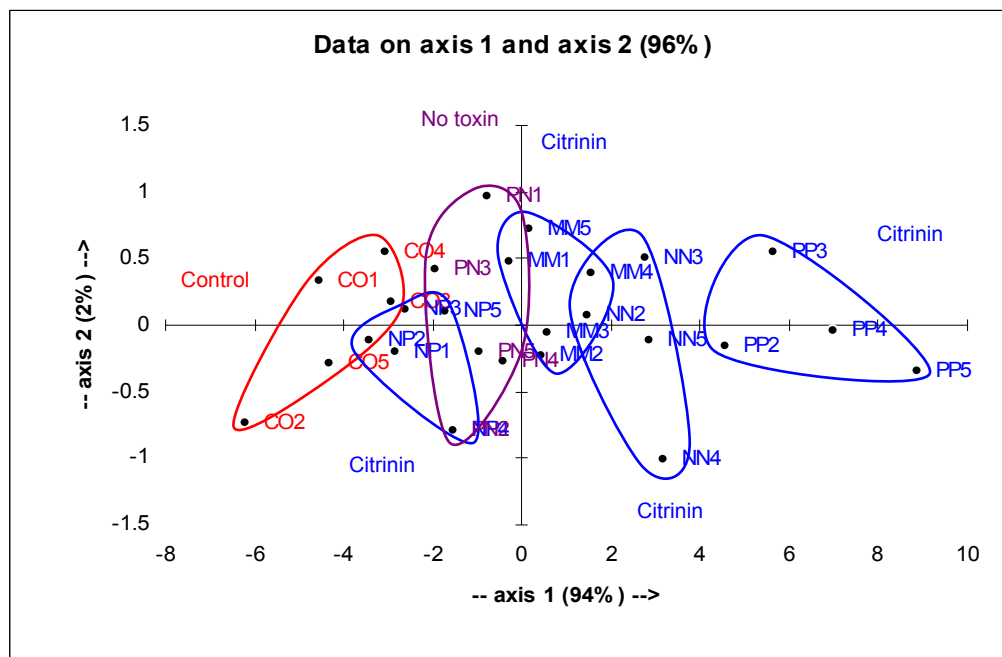
Figure 3.58 PCA of mycotoxigenic and non-mycotoxigenic *Penicillium verrucosum* strains based on their volatile profiles after 48 h incubation at 25°C on 0.95 a_w 2% wheat agar. Key: Non-toxin producer IBT22025 (PN), citrinin producers- IBT5252 (PP), IBT22116 (NN), IBT21491 (NP), IBT22123 (MM).

Seventy-four hours were required for discrimination of controls although there was no differentiation of toxin producing strains as shown in Figure 3.59.

In situ studies on 0.95 a_w bread analogues showed discrimination of OTA producing strains after 24 h (Figure 3.60). With increased incubation times the controls were discriminated but there was no differentiation of the different toxin producing strains as shown in the CA dendrogram (Figure 3.61). Using a low initial inoculum of 1×10^3 spores ml^{-1} discrimination of controls after only 24 h was possible (Figure 3.62). However, no differentiation of the different toxin producing strains was possible. After 48 h the controls could still be differentiated together with an OTA producing strain (IBT5252) as shown in Figure 3.63. Cross validation correctly classified approximately 65% of treatments correctly (p = 0.05). Table 3.8 shows results with the correctly classified treatments highlighted.

Table 3.8 Cross validation of *Penicillium verrucosum* strains on 0.95 a_w wheat agar. Key: **CO** - control; **MM** – IBT22123; **NN**- IBT22116; **NP** – IBT21491; **PN** – IBT22025; **PP** – IBT5252. Highlighted values show the correct classification percentages.

	actual	x-val	CO	MM	NN	NP	PN	PP
24 h 10^3	PP	PP	0.0009	0.0122	0.0297	0.0164	0	0.9407
48 h 10^6	PP	PP	0.0012	0.0009	0.3222	0.0289	0	0.6468
48 h 10^6	CO	CO	0.9343	0.0023	0	0.0011	0.0619	0.0005
48 h 10^6	PP	PP	0.1761	0.078	0.0001	0	0.0007	0.7452
48 h 10^6	PN	PN	0.0730	0.0845	0.0202	0.0028	0.7388	0.0806

It was possible to differentiate between samples grown on bread and agar as shown by the CA dendrogram in Figure 3.64. Different initial inoculum levels could also be discriminated (Figure 3.65).
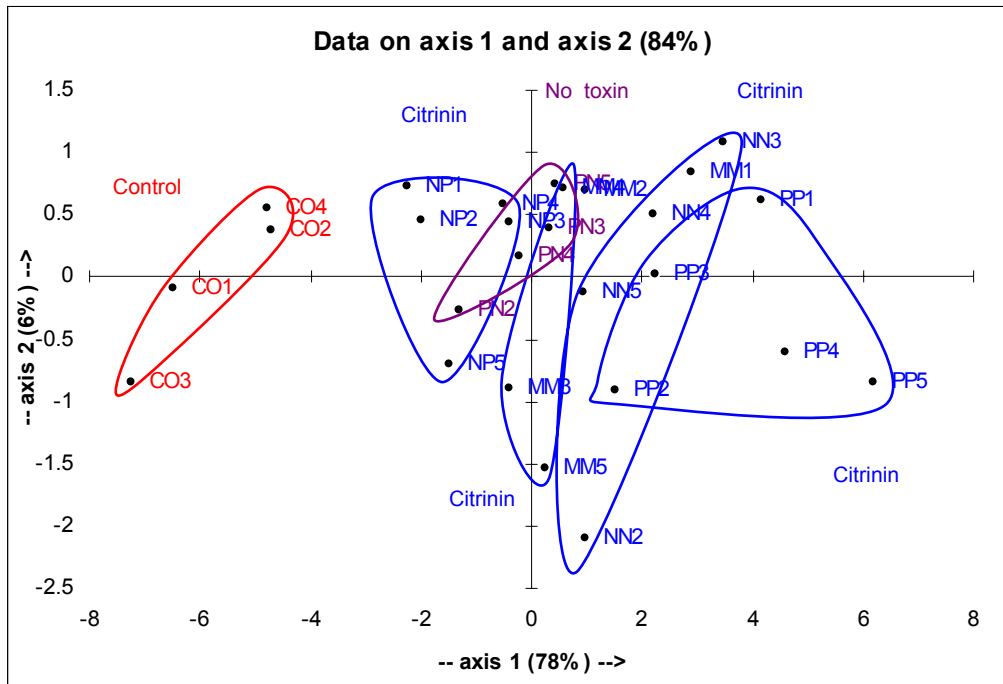
Figure 3.59 PCA of mycotoxigenic and non-mycotoxigenic *Penicillium verrucosum* strains based on their volatile profiles after 72 h incubation at 25°C on 0.95 a_w 2% wheat agar. Key: non-toxin producer IBT22025 (PN), citrinin producers- IBT5252 (PP), IBT22116 (NN), IBT21491 (NP), IBT22123 (MM).
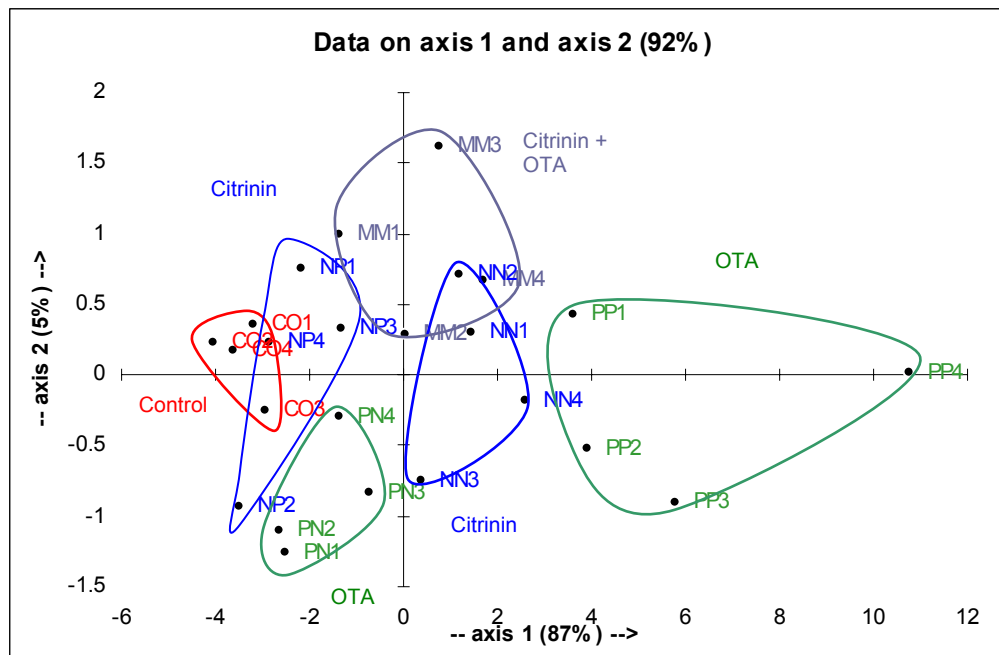
Figure 3.60 PCA of mycotoxigenic and non-mycotoxigenic *Penicillium verrucosum* strains based on their volatile profiles after 24 h incubation at 25°C on 0.95 a_w bread analogue with an initial population of $1x10^6$ spores ml^{-1}. Key: citrinin + OTA producer- IBT22123 (MM) OTA producers- IBT5252 (PP), IBT22025 (PN), citrinin producers- IBT22116 (NN), IBT21491 (NP).
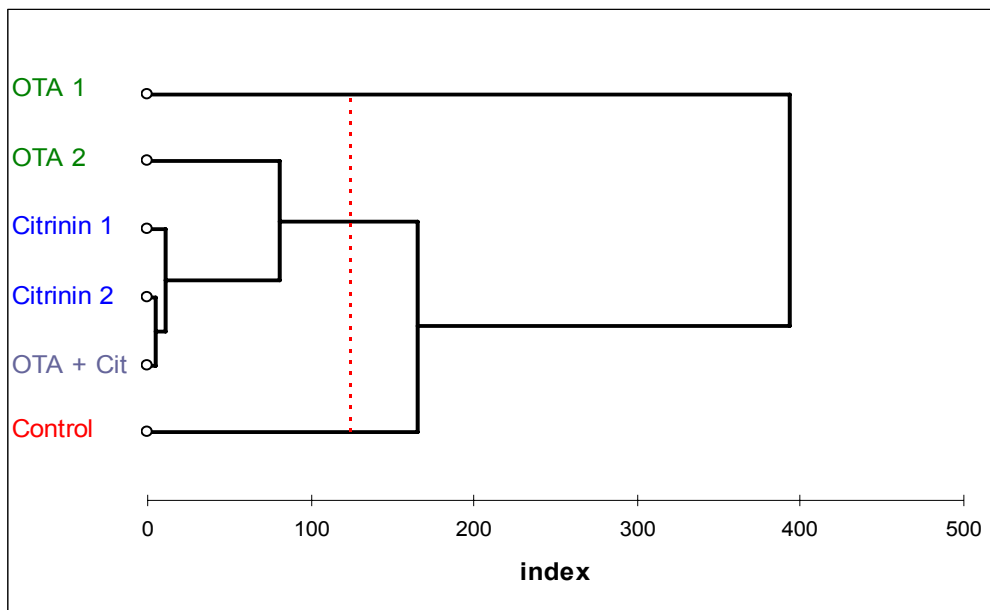
Figure 3.61 CA dendrogram of mycotoxigenic and non-mycotoxigenic *Penicillium verrucosum* strains based on their volatile profiles after 48 h incubation at 25° on 0.95 a_w bread analogue with an initial population of $1x10^6$ spores ml^{-1}. Key: citrinin (Cit) + OTA producer- IBT22123, OTA 1- IBT22025, OTA 2- IBT5252, citrinin 1- IBT22116, citrinin 2- IBT21491, OTA- ochratoxin A producer.
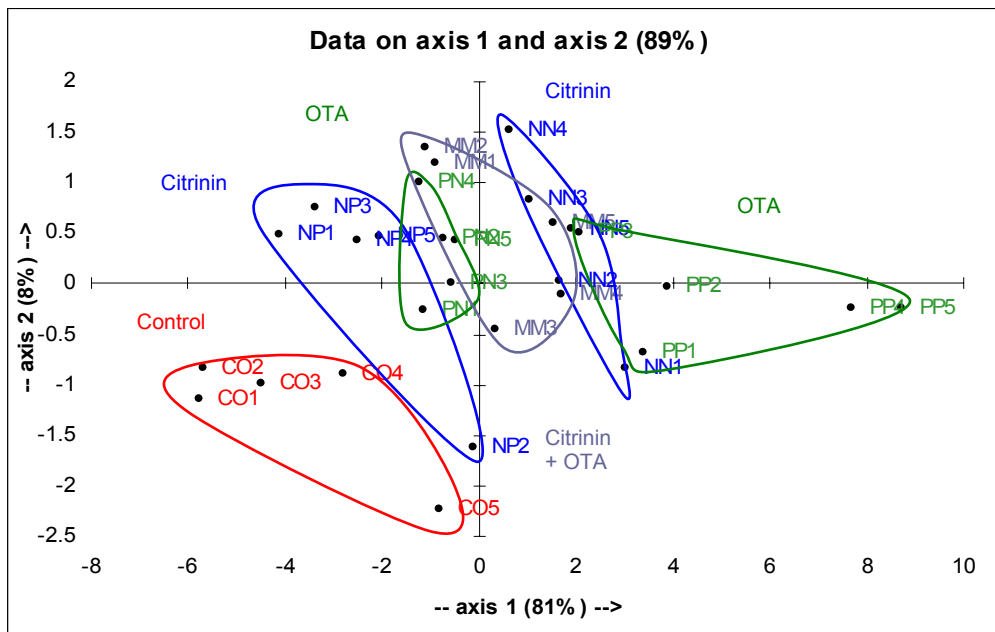
Figure 3.62 PCA of mycotoxigenic and non-mycotoxigenic *Penicillium verrucosum* strains based on their volatile profiles after 24 h incubation at 25°C on 0.95 a$_w$ bread analogue with an initial population of 1×10^3 spores ml^{-1}. Key: citrinin + OTA producer- IBT22123 (MM), OTA producers- IBT5252 (PP), IBT22025 (PN), citrinin producers- IBT22116 (NN), IBT21491 (NP).
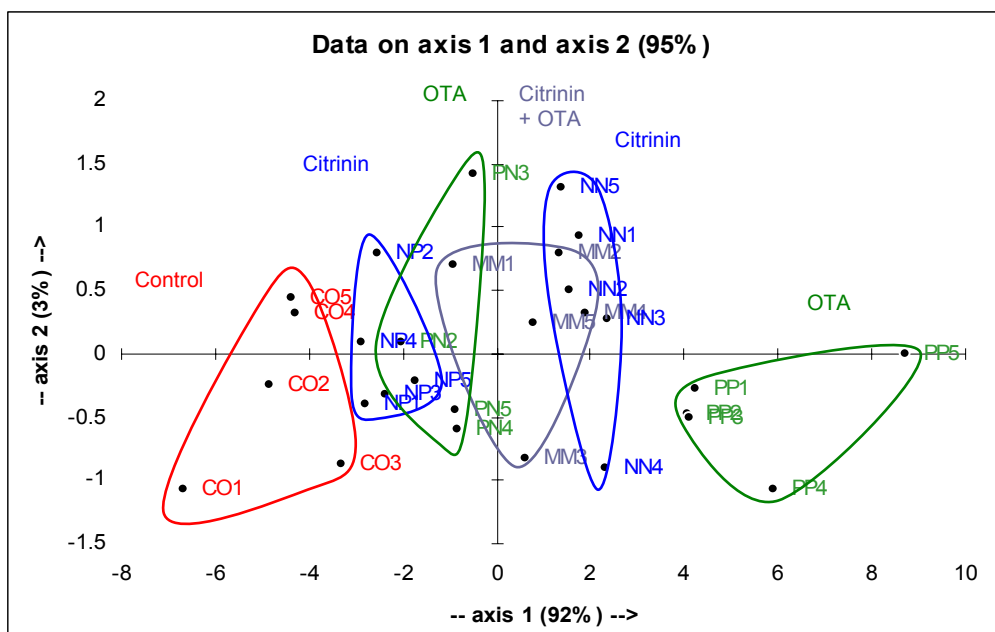
Figure 3.63 PCA of mycotoxigenic and non-mycotoxigenic *Penicillium verrucosum* strains based on their volatile profiles after 48 h incubation on $0.95a_w$ bread analogues with an initial population of $1x10^3$ spores ml^{-1}. Key: citrinin + OTA producer-IBT22123 (MM), OTA producers- IBT5252 (PP), IBT22025 (PN), citrinin producers-IBT22116 (NN), IBT21491 (NP).
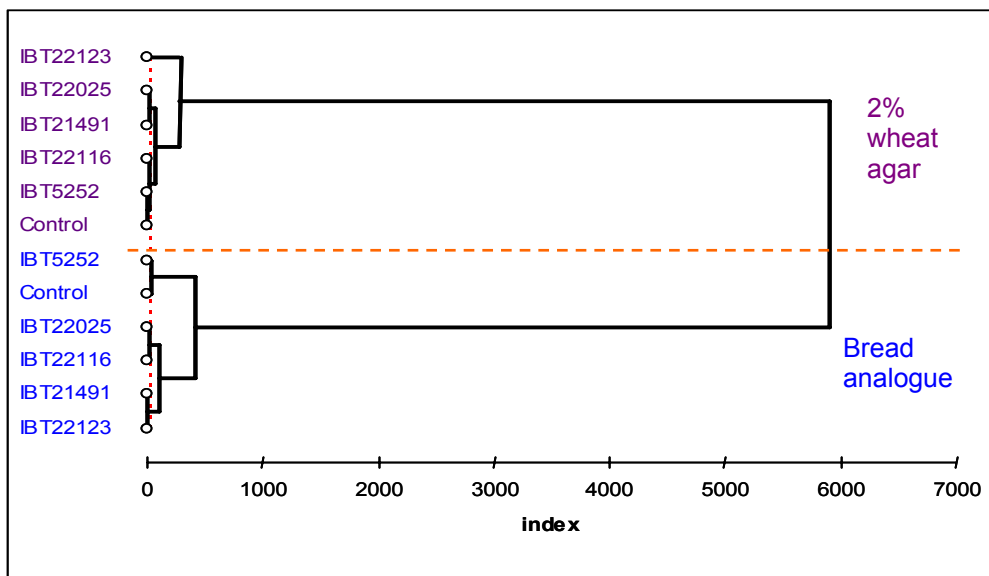
Figure 3.64 CA dendrogram comparing mycotoxigenic and non-mycotoxigenic *Penicillium verrucosum* strains grown on 0.95 a_w bread analogues and 0.95 a_w 2% wheat agar based on their volatile profiles after 72 h at 25°C.
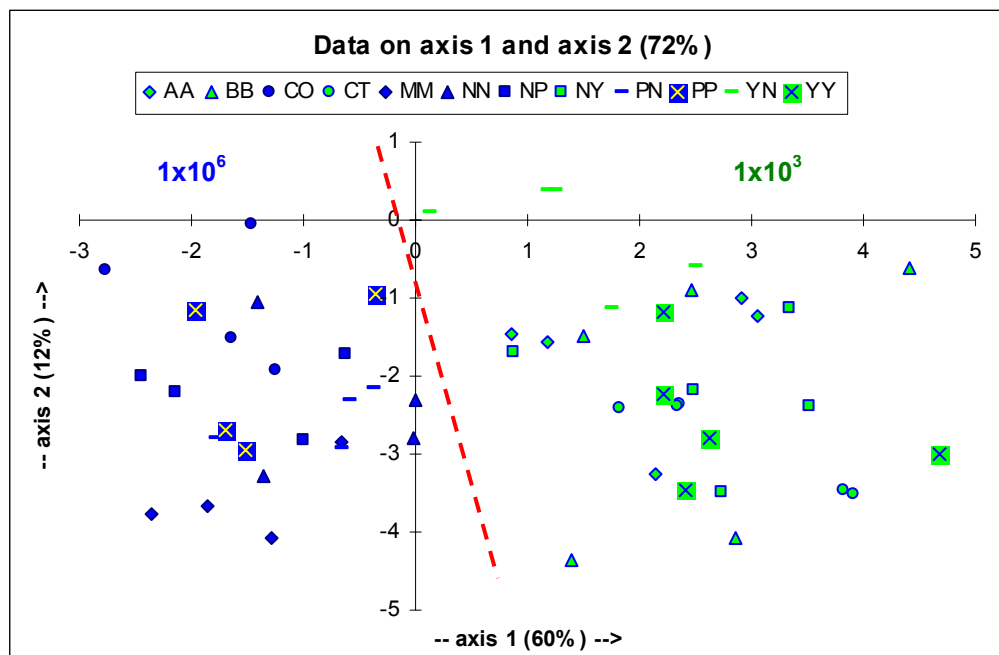
Figure 3.65 DFA of normalized BH114 e-nose divergence response comparing *Penicillium verrucosum* strains grown for 24 h at 25°C on 0.95 a_w bread analogues with initial populations of 1×10^3 and 1×10^6 spores ml^{-1}.

3.7 SCREENING OF ANTIOXIDANTS USING E-NOSE TECHNOLOGY

The potential for use of e-nose technology as a rapid method for screening of natural preservatives was investigated.

3.7.1 Initial screen

An antioxidant concentration of 50ppm was chosen to use in e-nose experiments as at this concentration between 50-75% inhibition was achieved. At 100ppm complete inhibition was achieved for some species. Concentrations of 10 and 20ppm did not significantly inhibit growth, particularly of the bacteria. Tables of the level of growth inhibition for each micro-organism are shown in Appendix 1. Plate 3.1 shows inhibition of *P. verrucosum* growth by 50 and 100 ppm BHA. Plate 3.2 shows inhibition of *B. subtilis* and *B. cereus* growth by PP. The ranking system of assigning scores out of 10 is also shown.

3.7.2 Antioxidant screen using a BH114 e-nose, NST3220 lab emission analyser, GC-MS and CFUs

The Bloodhound BH114 e-nose and NST3220 lab emission analyser were able to differentiate samples without antioxidant from those containing BHA or PP (Figures 3.66 and 3.67). It was possible to discriminate between inoculated and uninoculated treatments without antioxidant at all sample times. To observe if the e-noses could detect the difference between samples containing micro-organisms and those without, in the presence of antioxidant, data was reanalysed using treatments only containing either PP or BHA.
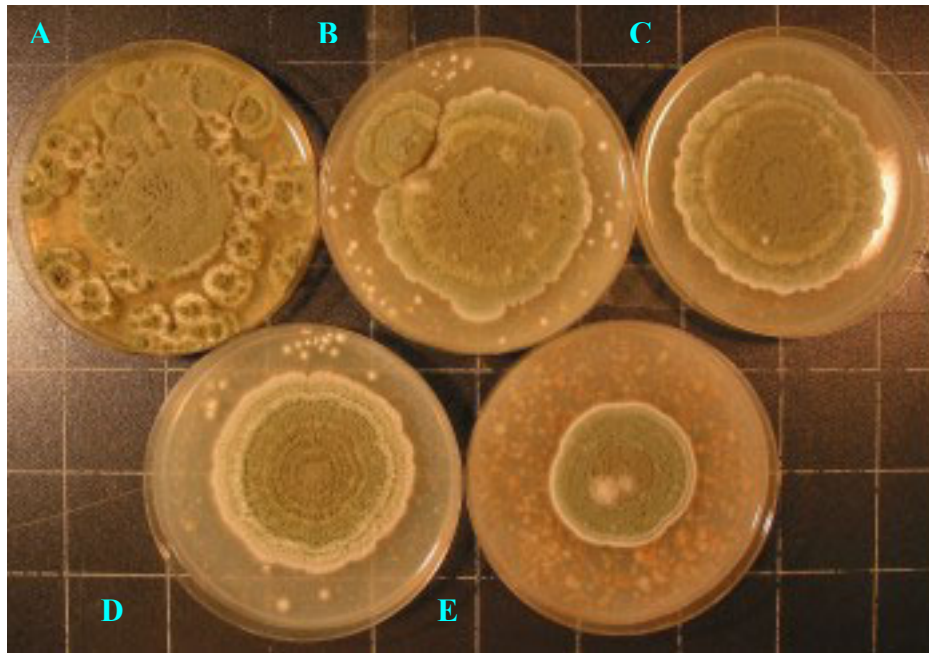
Plate 3.1 *Penicillium verrucosum* growing on 0.95 a$_w$ 2% wheat agar at 25°C showing the effect of different concentrations of the antioxidant butylated hydroxyanisole. Key to treatments: A- no antioxidant; B- 10ppm; C- 20ppm; D- 50ppm; E- 100ppm.
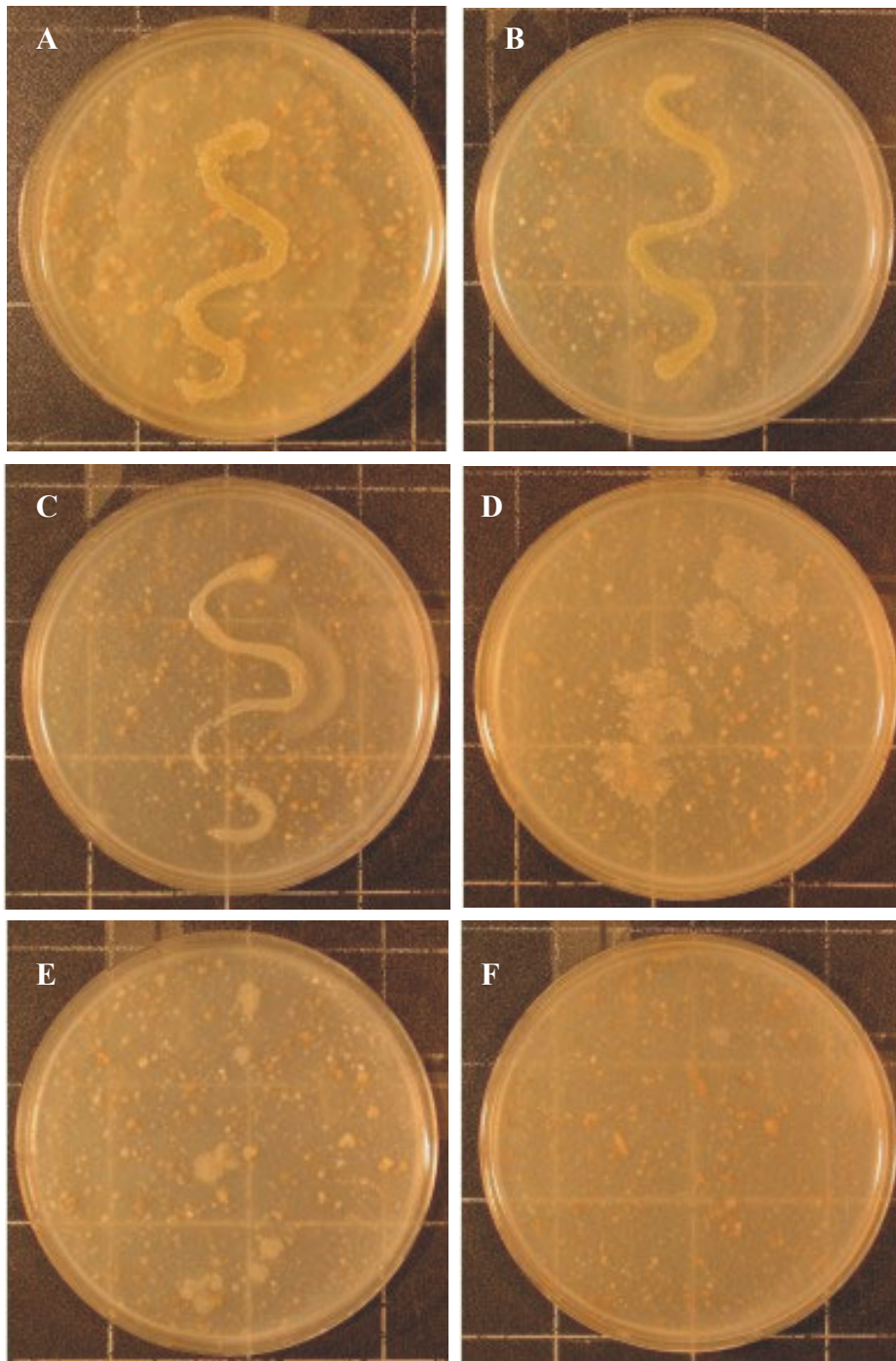
Plate 3.2 Inhibition of growth of *Bacillus subtilis* and *Bacillus cereus* on 2% wheat agar containing the antioxidant propyl paraben incubated at 25°C. Key: score out of 10; A-10; B-9; C- 7; D- 6; E- 3; F- 1.
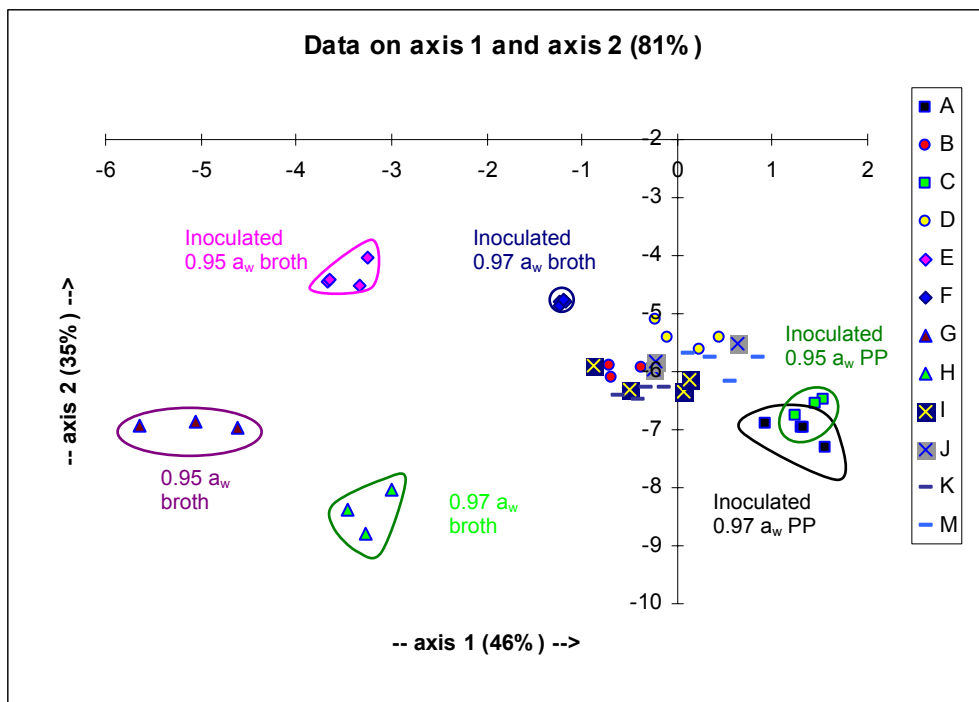
Figure 3.66 DFA of normalized divergence responses to volatiles produced by *Penicillium verrucosum* after 24 h growth in 2% flour broth at 25°C with and without the antioxidants propyl paraben and butylated hydroxyanisole measured with a BH114 electronic nose.

Key to treatments:

Inoculated   A = 0.97 a_w flour broth with PP

B = 0.97 a_w flour broth with BHA

C = 0.95 a_w flour broth with PP

D = 0.95 a_w flour broth with BHA

E = 0.95 a_w flour broth no antioxidant

F = 0.97 a_w flour broth no antioxidant

Controls   G = 0.95 a_w flour broth no antioxidant

(uninoculated) H = 0.97 a_w flour broth no antioxidant

I = 0.95 a_w flour broth with PP

J = 0.97 a_w flour broth with PP

K = 0.95 a_w flour broth with BHA

M = 0.97 a_w flour broth with BHA
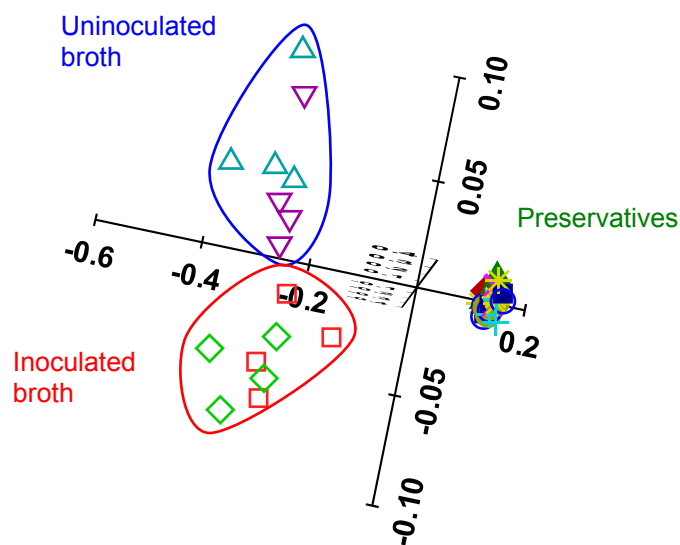
Figure 3.67 PCA of normalized response data to volatiles produced by *Bacillus subtilis* after 24 h growth in 2% flour broth at 25°C with and without the antioxidants propyl paraben and butylated hydroxyanisole measured with a NST3220 lab emission analyser. Key to treatments:

Inoculated	BA = 0.97 a_w flour broth with PP
	BB = 0.97 a_w flour broth with BHA
	BC = 0.95 a_w flour broth with PP
	BD = 0.95 a_w flour broth with BHA
	BE = 0.95 a_w flour broth no antioxidant
	BF = 0.97 a_w flour broth no antioxidant
Controls	GG = 0.95 a_w flour broth no antioxidant
(uninoculated)	HH = 0.97 a_w flour broth no antioxidant
	II = 0.95 a_w flour broth with PP
	JJ = 0.97 a_w flour broth with PP
	KK = 0.95 a_w flour broth with BHA
	MM = 0.97 a_w flour broth with BHA

The conducting polymer BH114 e-nose system was not able to differentiate treatments with and without *B. subtilis* after 24 h in the presence of either PP or BHA (Figure 3.68). However, this was possible in the presence of both antioxidants after 48 h. The PCA, which accounted for 93% of the data showed discrimination between uninoculated and inoculated flour broths but not between the different water activity treatments (Figure 3.69). The BH114 e-nose was able to discriminate broth containing *P. verrucosum* after 24 h (Figure 3.70a) and 48 h. However, after 72 h the 0.95 a_w uninoculated flour broth overlapped with the inoculated treatments (Figure 3.70b). It was possible to differentiate the 0.97 a_w flour broth containing *P. anomala* from the uninoculated broths after 24 h. However, the 0.95 a_w inoculated broth treatments overlapped with the uninoculated broths (Figure 3.71). As shown in the PCA in Figure 3.72 the conducting polymer sensor array was able to discriminate between uninoculated and inoculated treatments and between the different water activities of the inoculated broths after 48 h.

Using the metal oxide and iron sensor arrays (NST3220) it was possible to differentiate *B. subtilis* inoculated broth after 24 h (Figure 3.73) using 3D PCA including the 3rd PC. Differentiation was improved after 48 h (Figure 3.74). After 24 h the NST3220 was also able to differentiate *P. verrucosum* inoculated broths from uninoculated flour broths as shown in the PCA in Figure 3.75 with the addition of the fourth PC. *Penicillium verrucosum* inoculated flour broth could also be discriminated from uninoculated flour broths after 48 and 72 h using PCA with the 3rd PC. *P. anomala* could be differentiated from uninoculated broths after 24 h with the NST3220 using 2 PCs (Figure 3.76). Differentiation was also achieved after 48h (Figures 3.77) with the addition of the 4th PC and 72 h with the addition of the 3rd PC.
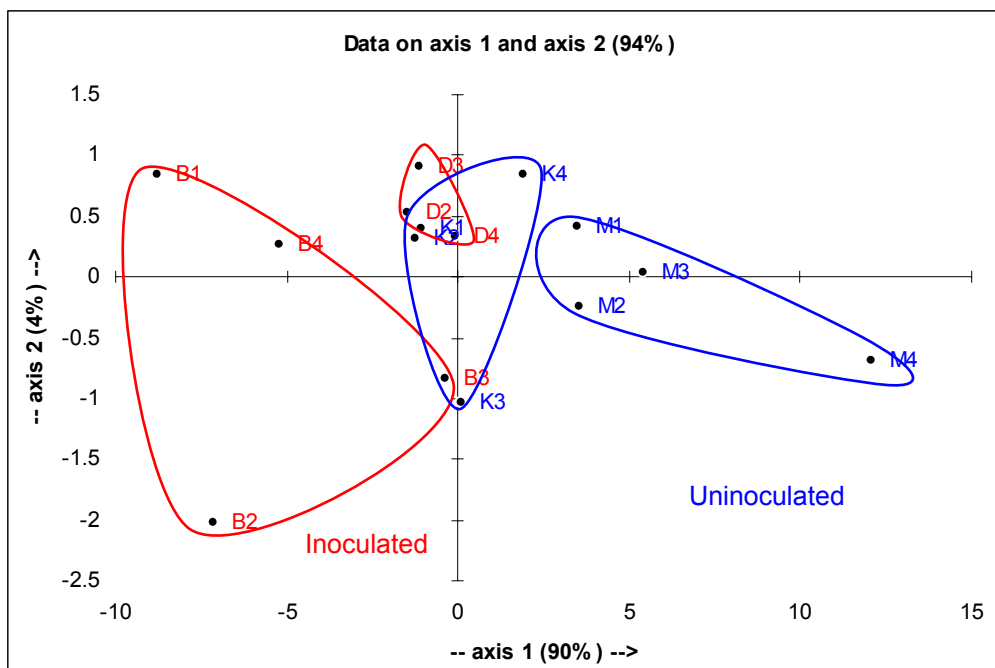
Figure 3.68 PCA of normalized BH114 e-nose divergence responses to volatiles produced by *Bacillus subtilis* after 24 h at 25°C in 2% flour broth containing butylated hydroxyanisole. Key to treatments: B- inoculated 0.97 a_w broth, D- inoculated 0.95 a_w broth, K- uninoculated 0.95 a_w broth, M- uninoculated 0.97 a_w broth.
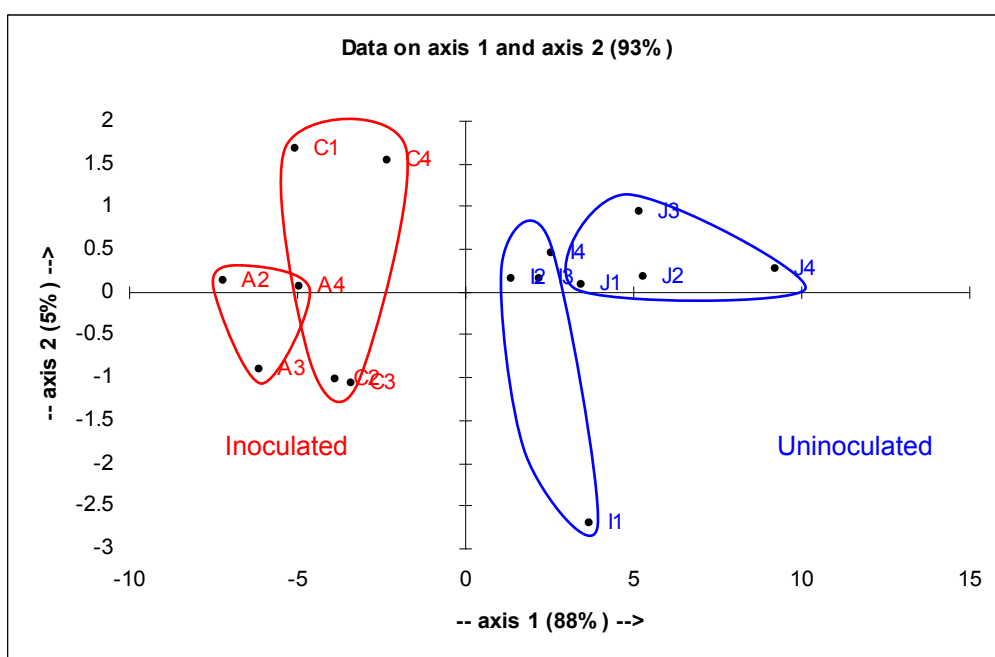
Figure 3.69 PCA of normalized BH114 e-nose divergence responses to volatiles produced by *Bacillus subtilis* after 48 h at 25°C in 2% flour broth containing propyl paraben. Key to treatments: A- inoculated 0.97 a_w broth, C- inoculated 0.95 a_w broth, I- uninoculated 0.95 a_w broth, J- uninoculated 0.97 a_w broth.

A

B
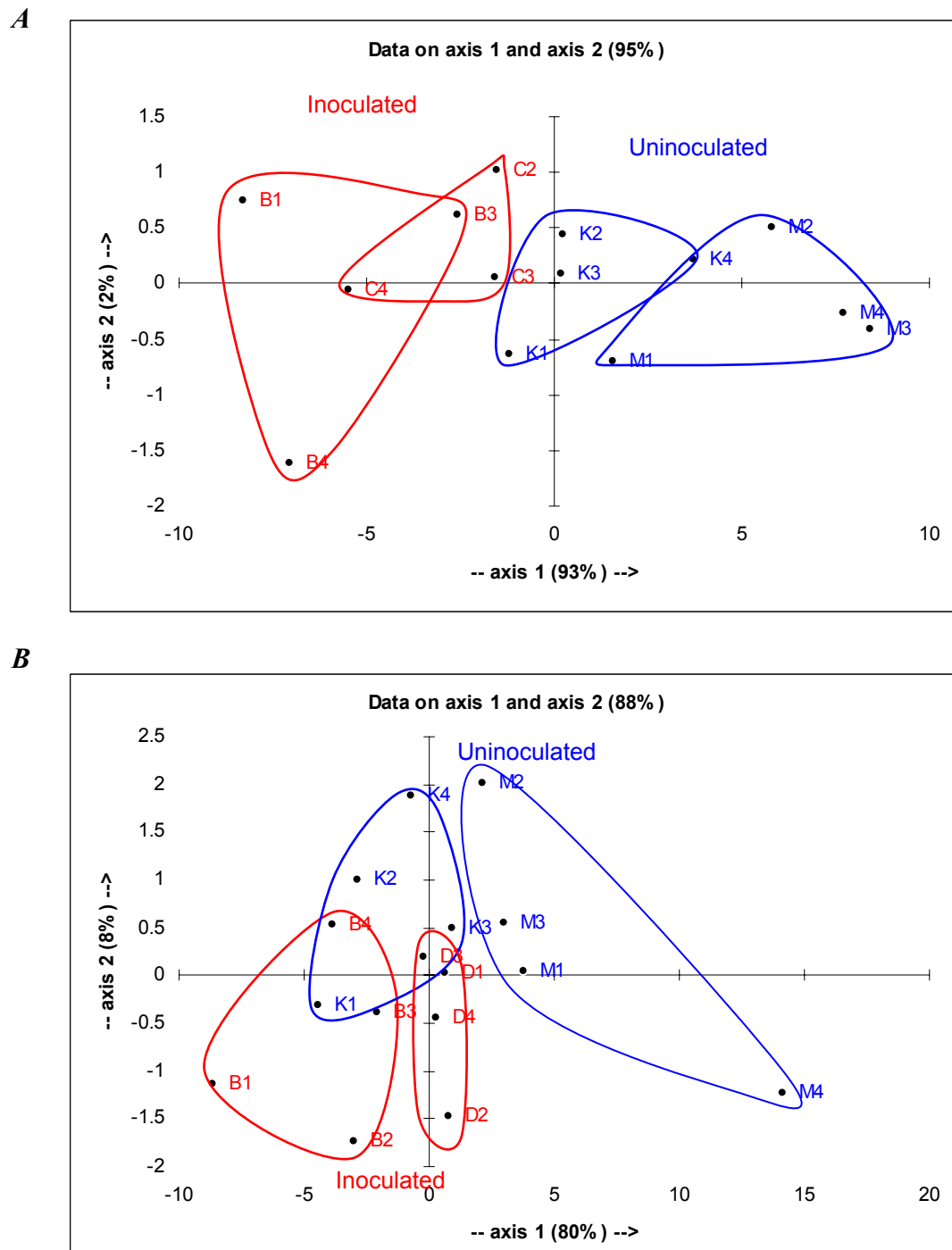
Figure 3.70 PCA of normalized BH114 e-nose divergence responses to volatiles produced by *Penicillium verrucosum* after *A* 24 h and *B* 72 h at 25°C in 2% flour broth containing butylated hydroxyanisole. Key to treatments: B- inoculated 0.97 a_w broth, D- inoculated 0.95 a_w broth, K- uninoculated 0.95 a_w broth, M- uninoculated 0.97 a_w broth.
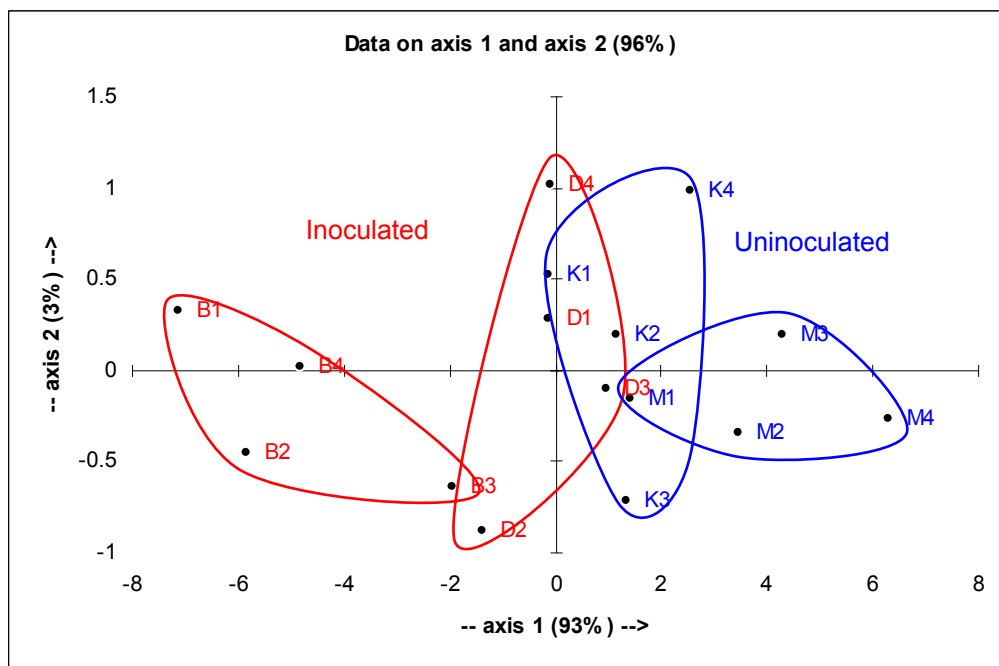
Figure 3.71 PCA of normalized BH114 e-nose divergence responses to volatiles produced by *Pichia anomala* after 24 h at 25°C in 2% flour broth containing butylated hydroxyanisole. Key to treatments: B- inoculated 0.97 a_w broth, D- inoculated 0.95 a_w broth, K- uninoculated 0.95 a_w broth, M- uninoculated 0.97 a_w broth.
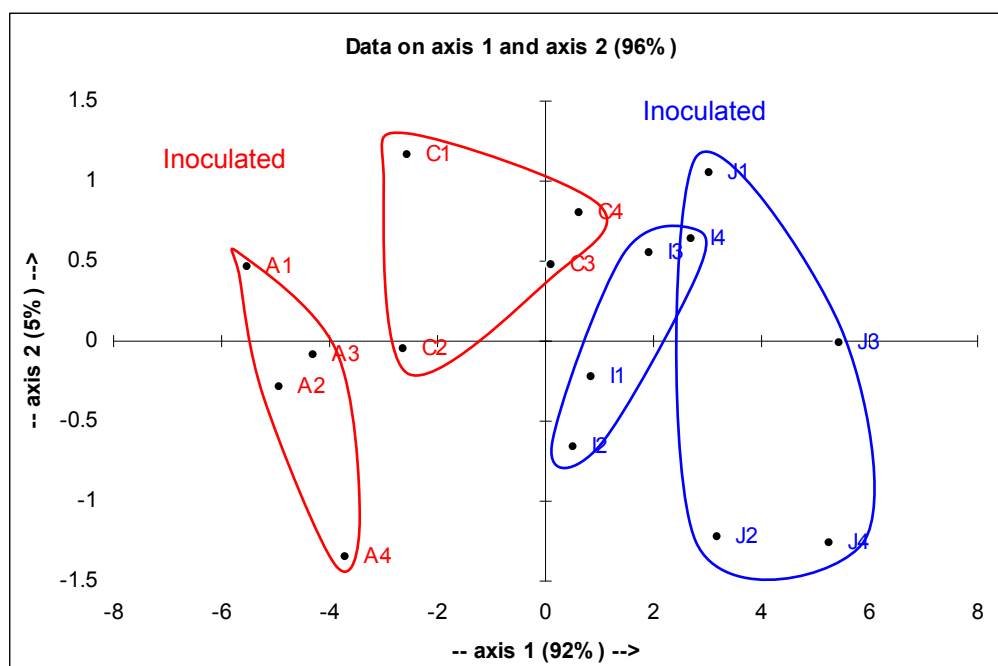
Figure 3.72 PCA of normalized BH114 e-nose divergence responses to volatiles produced by *Pichia anomala* after 48 h at 25°C in 2% flour broth containing propyl paraben. Key to treatments: A- inoculated 0.97 a_w broth, C- inoculated 0.95 a_w broth, I- uninoculated 0.95 a_w broth, J- uninoculated 0.97 a_w broth.
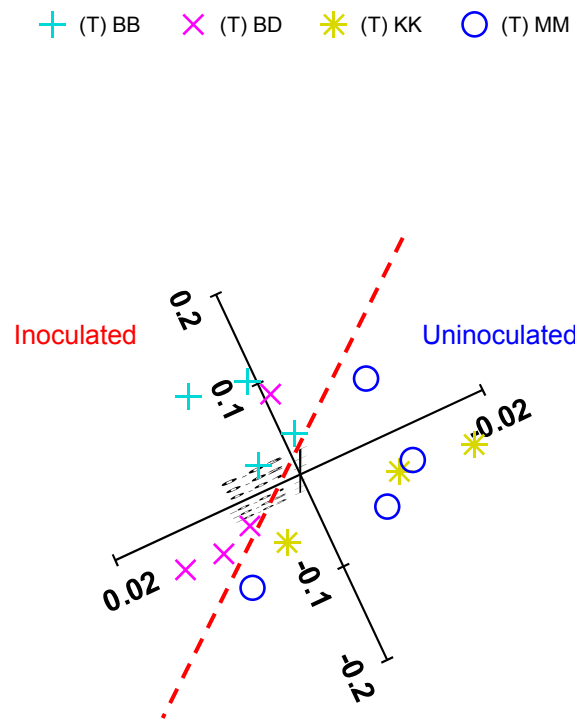
Figure 3.73 PCA of normalized response data to volatiles produced by *Bacillus subtilis* after 24 h at 25°C in 2% flour broth containing butylated hydroxyanisole measured with a NST3220 lab emission analyser. Key to treatments: BB- inoculated 0.97 a_w broth, BD- inoculated 0.95 a_w broth, KK- uninoculated 0.95 a_w broth, MM- uninoculated 0.97 a_w broth.
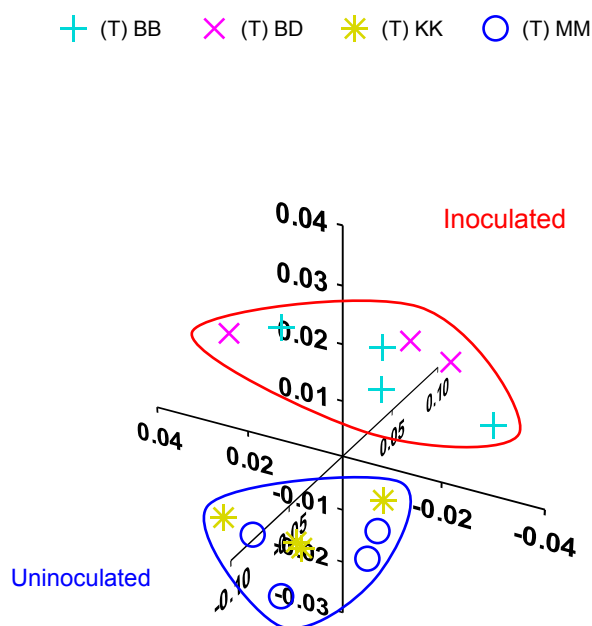
Figure 3.74 PCA of normalized response data to volatiles produced by *Bacillus subtilis* after 48 h at 25°C in 2% flour broth containing butylated hydroxyanisole measured with a NST3220 lab emission analyser. Key to treatments: BB- inoculated 0.97 a_w broth, BD- inoculated 0.95 a_w broth, KK- uninoculated 0.95 a_w broth, MM- uninoculated 0.97 a_w broth.
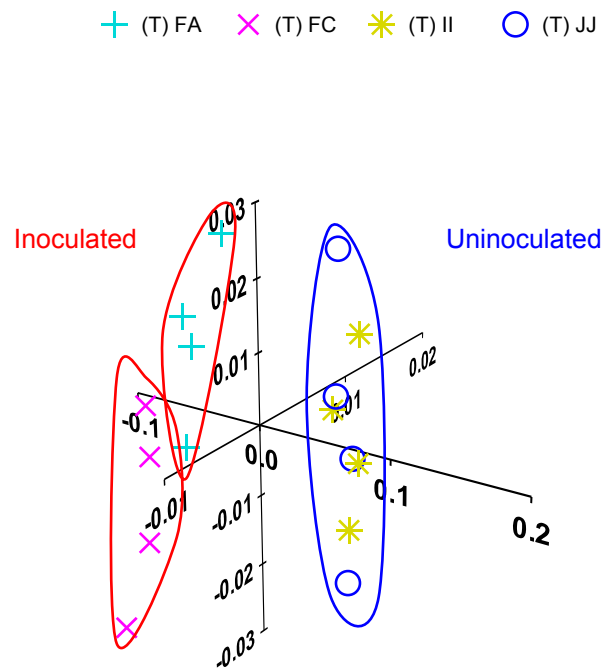
Figure 3.75 PCA of normalized response data, with the 4ᵗʰ PC, to volatiles produced by *Penicillium verrucosum* after 24 h at 25°C in 2% flour broth containing propyl paraben measured with a NST3220 lab emission analyser. Key to treatments: FA-inoculated 0.97 a_w broth, FC- inoculated 0.95 a_w broth, II- uninoculated 0.95 a_w broth, JJ- uninoculated 0.97 a_w broth.
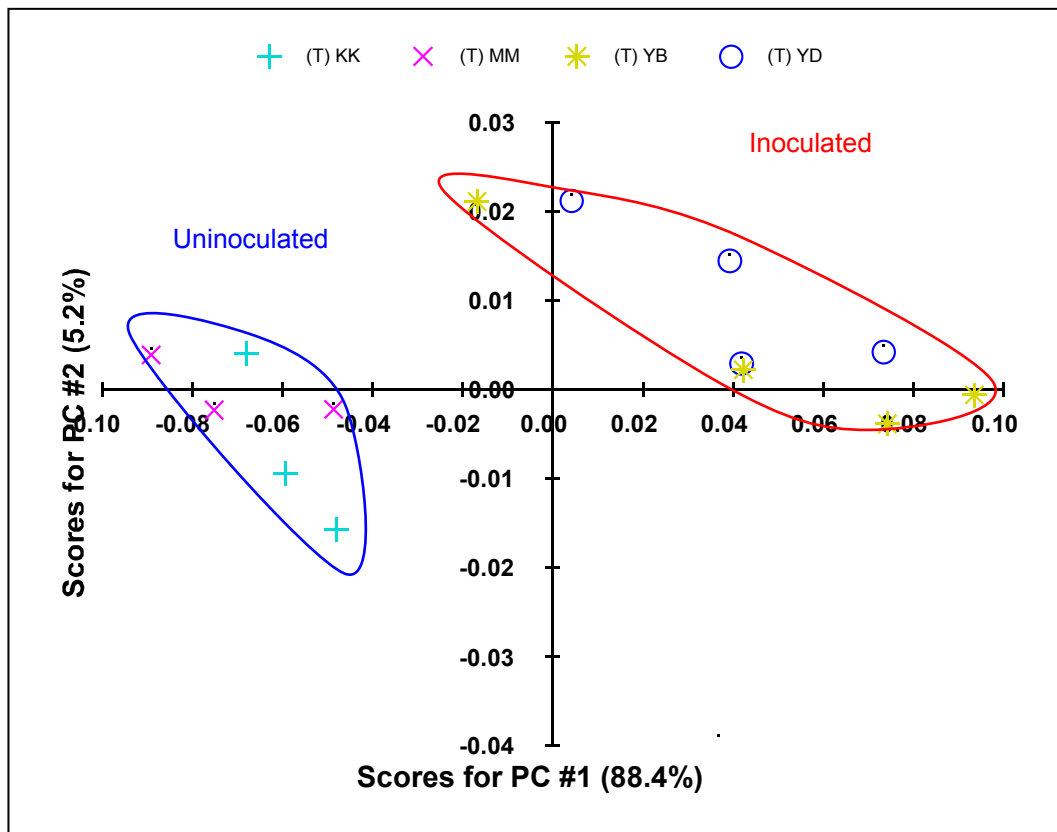
Figure 3.76 PCA of normalized response data to volatiles produced by *Pichia anomala* after 24 h at 25°C in 2% flour broth containing butylated hydroxyanisole measured with a NST3220 lab emission analyser. Key to treatments: YB- inoculated 0.97 a_w broth, YD- inoculated 0.95 a_w broth, KK- uninoculated 0.95 a_w broth, MM-uninoculated 0.97 a_w broth.
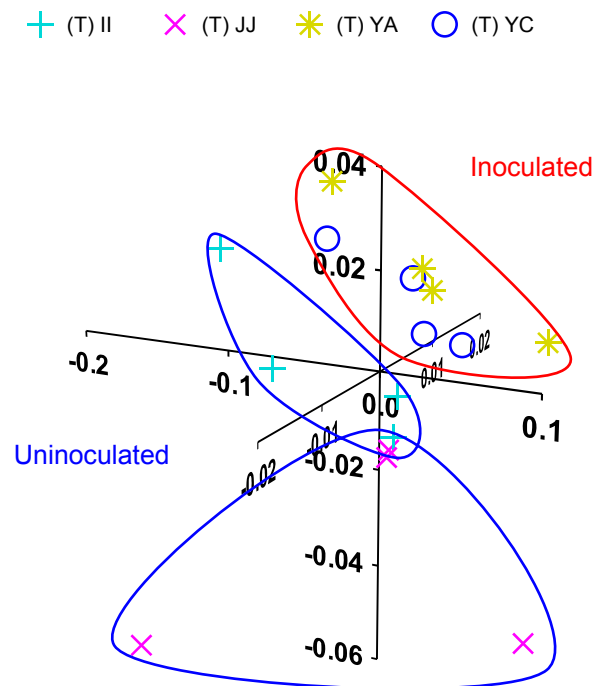
Figure 3.77 PCA of normalized response data to volatiles produced by *Pichia anomala* after 48 h at 25°C in 2% flour broth containing propyl paraben measured with a NST3220 lab emission analyser. Key to treatments: YA- inoculated 0.97 a_w broth, YC- inoculated 0.95 a_w broth, II- uninoculated 0.95 a_w broth, JJ- uninoculated 0.97 a_w broth.

When data from all sample times was analysed together as shown in Figure 3.78 it was possible to discriminate the different sample times with a shift from left to right with *B. subtilis*, *P. anomala* and *P. verrucosum*.

Carbon dioxide levels, measured by the NST3220, increased with sample time and were higher in inoculated treatments particularly after 48 and 72 h (Figures 3.79-3.81). CO_2 production by *P. verrucosum* and *P. anomala* was greater in 0.97 a_w flour broth without antioxidants. At 0.95 a_w, *P. verrucosum* produced greater amounts of CO_2 in the presence of the antioxidants. The populations isolated also increased with time. Figures 3.82 and 3.83, show that the populations of *P. verrucosum* and *P. anomala* increased significantly between 24 and 48 h. Both micro-organisms produced higher cfu counts in the control flour broth without an antioxidant. *B. subtilis* cfu g^{-1} increased significantly between 24 and 48 h. It was however, not possible to see the cfus for 0 and 24 h when all times were plotted together (Figure 3.84a). When 0 and 24 h were compared alone it was found that there was a significant increase between 0 and 24 h (Figure 3.84b*). B. subtilis* grew better at 0.97 aw and in the presence of the antioxidants.

From the 60 compounds identified by GC-MS only 5 of the 11 compounds found to be important in spoilage odours (methylbenzene, hexanal, hexanol, styrene and 2-pentylfuran) were detected and analysed for differences between samples. Figures 3.85 – 3.89 show the change with time in the levels of the 5 compounds in microbial inoculated broth samples. Methylbenzene, hexanol and 2-pentylfuran levels produced by *P. anomala* were less than those produced by *B. subtilis* and *P. verrucosum*. *P. anomala* also produced smaller volumes of hexanal at time 0.
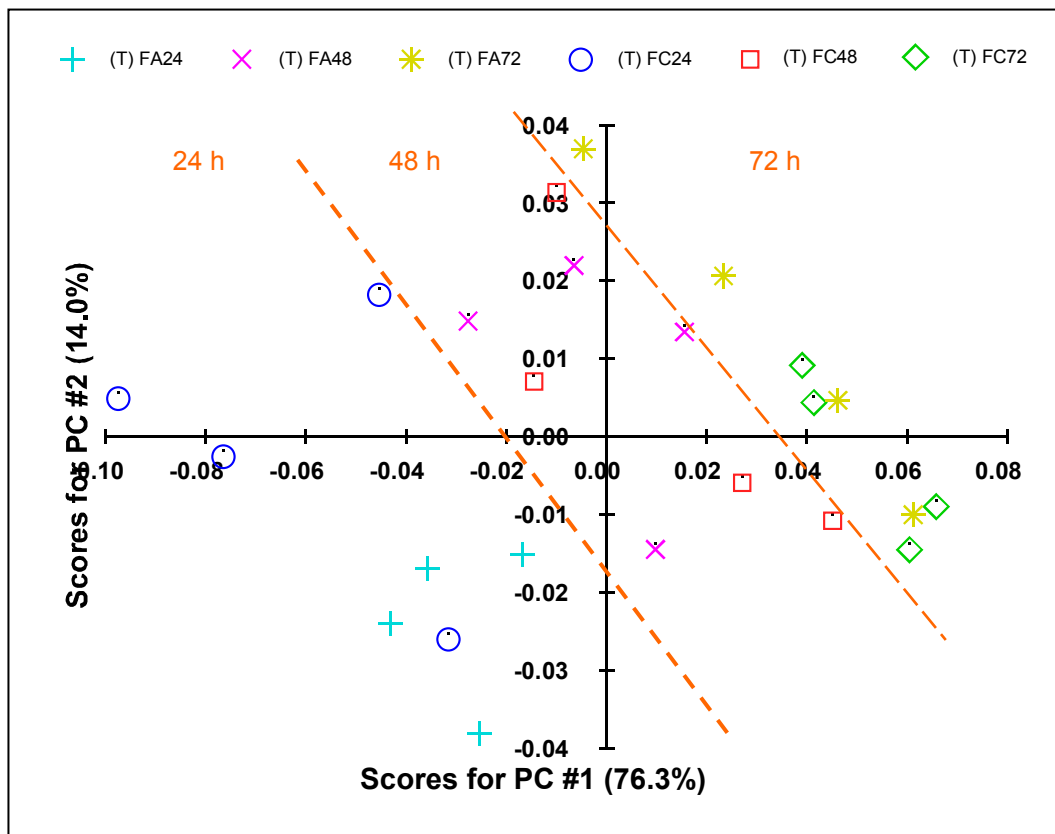
Figure 3.78 PCA of normalized response data to volatiles produced by *Penicillium verrucosum* after 24, 48 and 72 h at 25°C in 2% flour broth containing propyl paraben measured with a NST3220 lab emission analyser.

Key to treatments: FA- 0.97 a_w flour broth

FC- 0.95 a_w flour broth

24, 48 + 72 – sample time
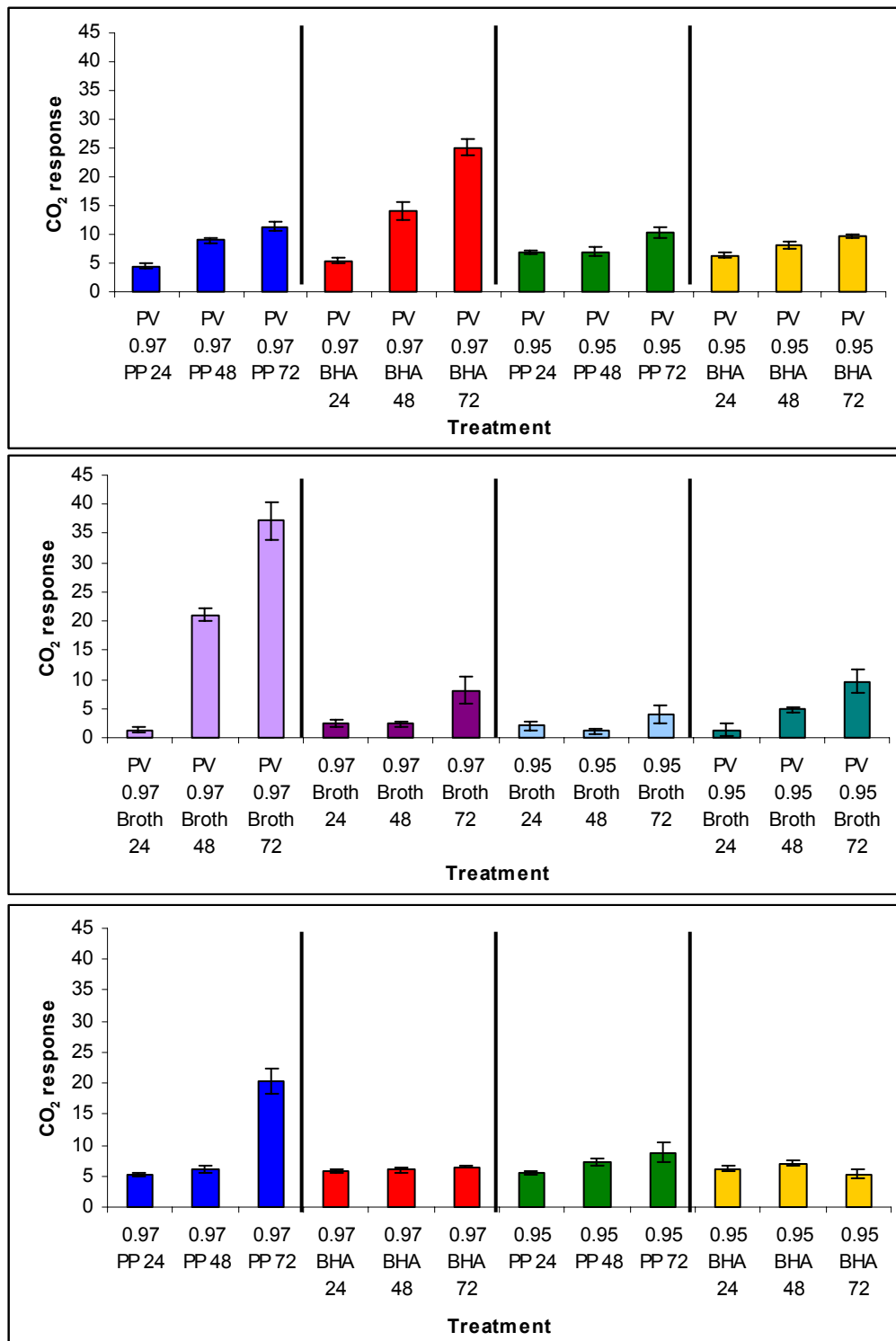
Figure 3.79 Carbon dioxide levels measured by NST3220 for *Penicillium verrucosum* in flour broth with and without antioxidant at 25°C. Key to treatments: PV- inoculated with *P. verrucosum*; 0.95 and 0.97- water activity of flour broth; PP- presence of propyl paraban; BHA- presence of butylated hydroxyanisole; broth – broth without antioxidant; 24, 48 and 72- sample time.
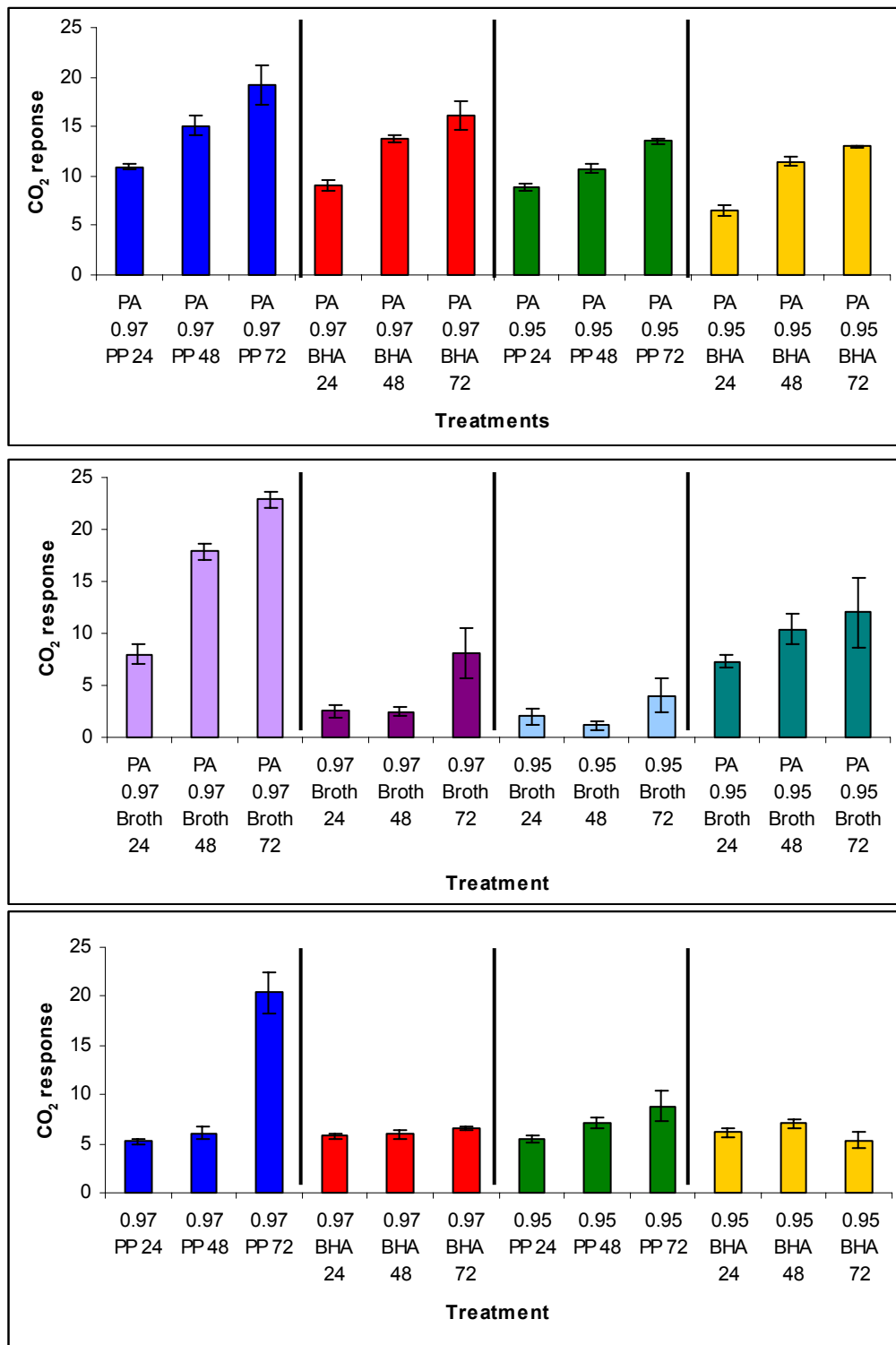
Figure 3.80 Carbon dioxide levels measured by NST3220 for *Pichia anomala* in flour broth with and without antioxidant at 25°C. Key to treatments: PA- inoculated with *P. anomala*; 0.95 and 0.97- water activity of flour broth; PP- presence of propyl paraban; BHA- presence of butylated hydroxyanisole; broth – broth without antioxidant; 24, 48 and 72- sample time.
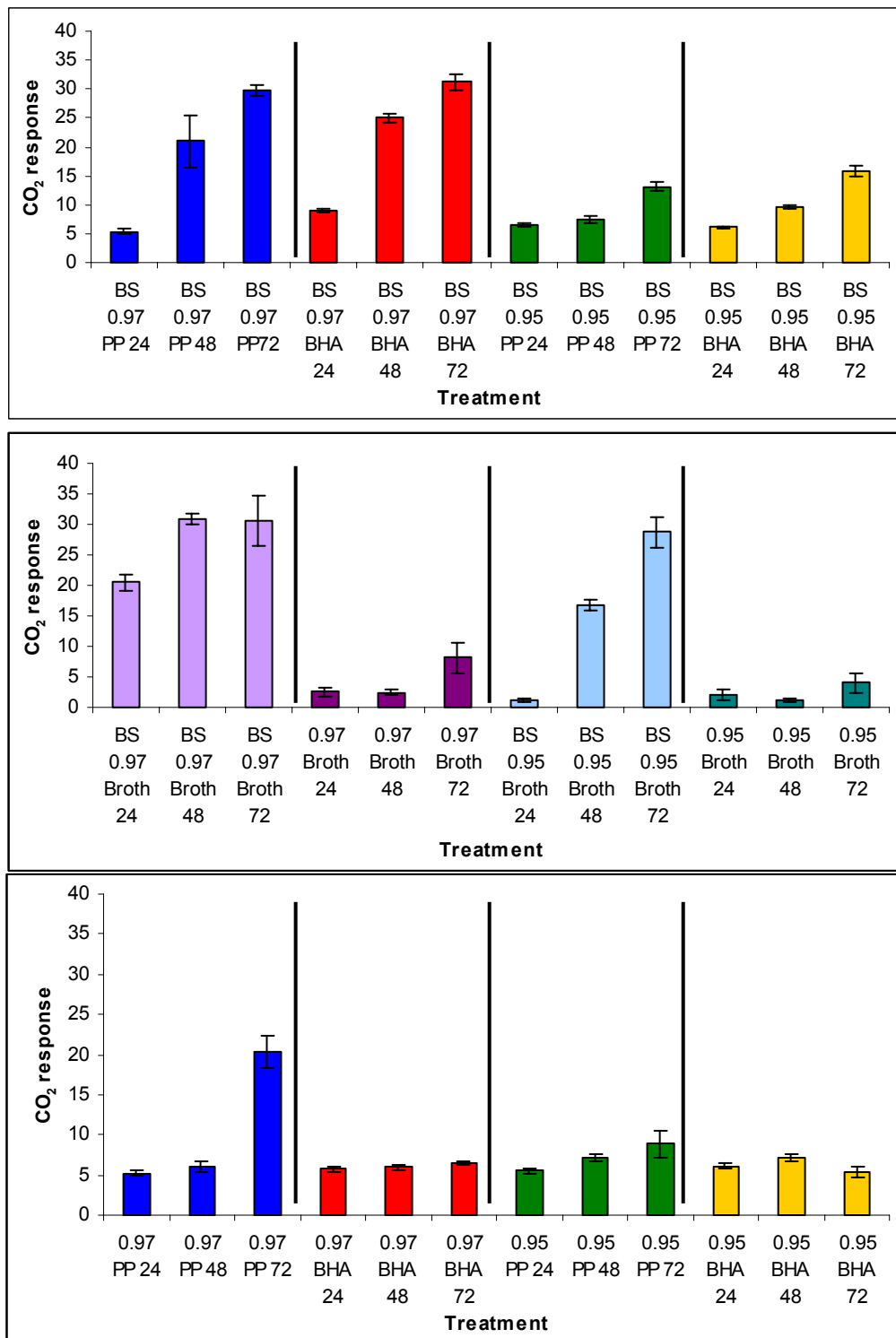
Figure 3.81 Carbon dioxide levels measured by NST3220 for *Bacillus subtilis* in flour broth with and without antioxidant at 25°C. Key to treatments: BS- inoculated with *B. subtilis*; 0.95 and 0.97- water activity of flour broth; PP- presence of propyl paraban; BHA- presence of butylated hydroxyanisole; broth – broth without antioxidant; 24, 48 and 72- sample time.
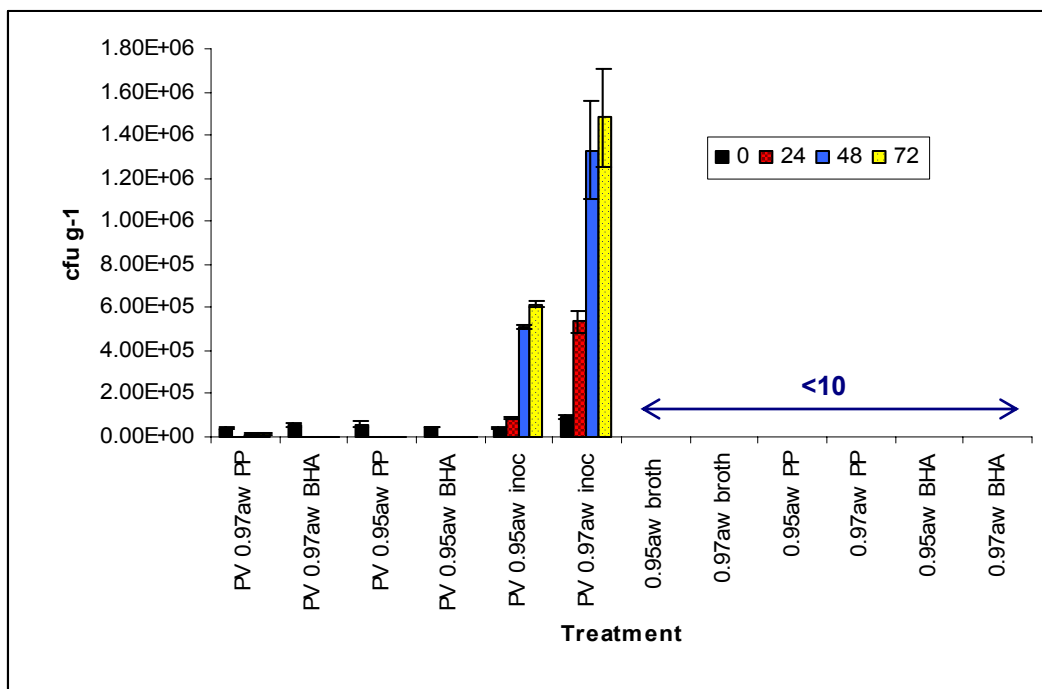
Figure 3.82 Comparison of cfu g^{-1} over time for *Penicillium verrucosum* grown at 25°C in 2% flour broth at 0.95 and 0.97 a$_w$ with and without antioxidants propyl paraben and butylated hydroxyanisole. Key to treatments: PV - inoculated with *P. verrucosum*; 0.95aw and 0.97aw – flour broth water activity; PP- presence of propyl paraban; BHA presence of butylated hydroxyanisole; broth and inoc – broth without antioxidant.
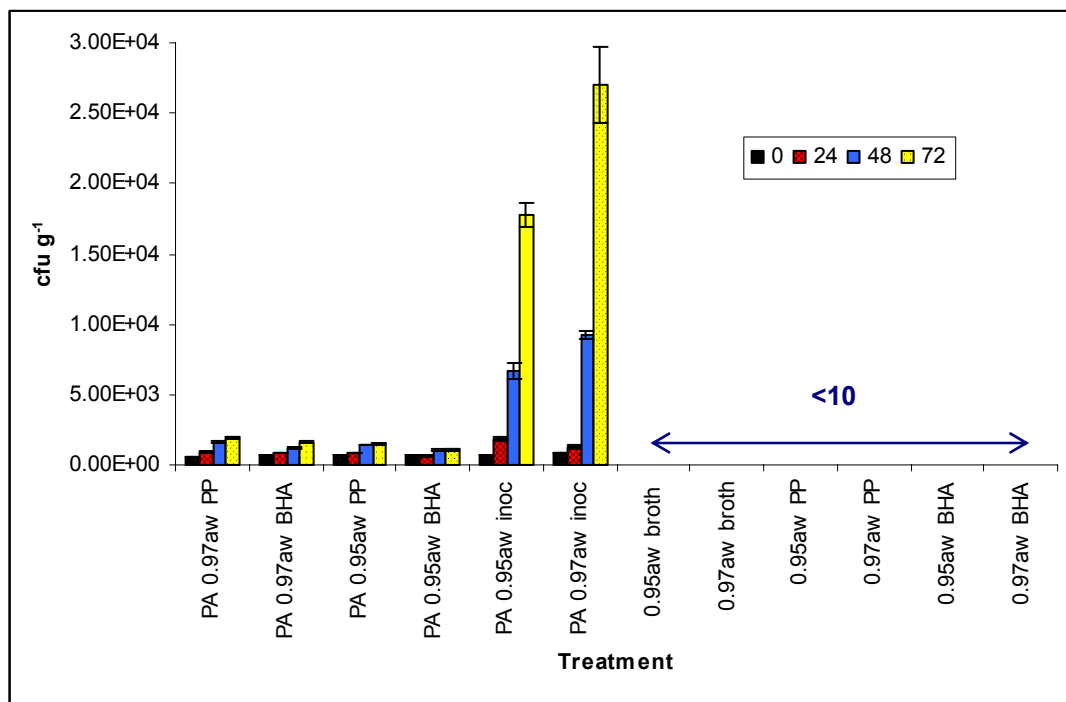
Figure 3.83 Comparison of cfu g^{-1} over time for *Pichia anomala* grown at 25°C in 2% flour broth at 0.95 and 0.97 a$_w$ with and without antioxidants propyl paraben and butylated hydroxyanisole. Key to treatments: PA - inoculated with *P. anomala*; 0.95aw and 0.97aw – flour broth water activity; PP- presence of propyl paraban; BHA presence of butylated hydroxyanisole; broth and inoc – broth without antioxidant.
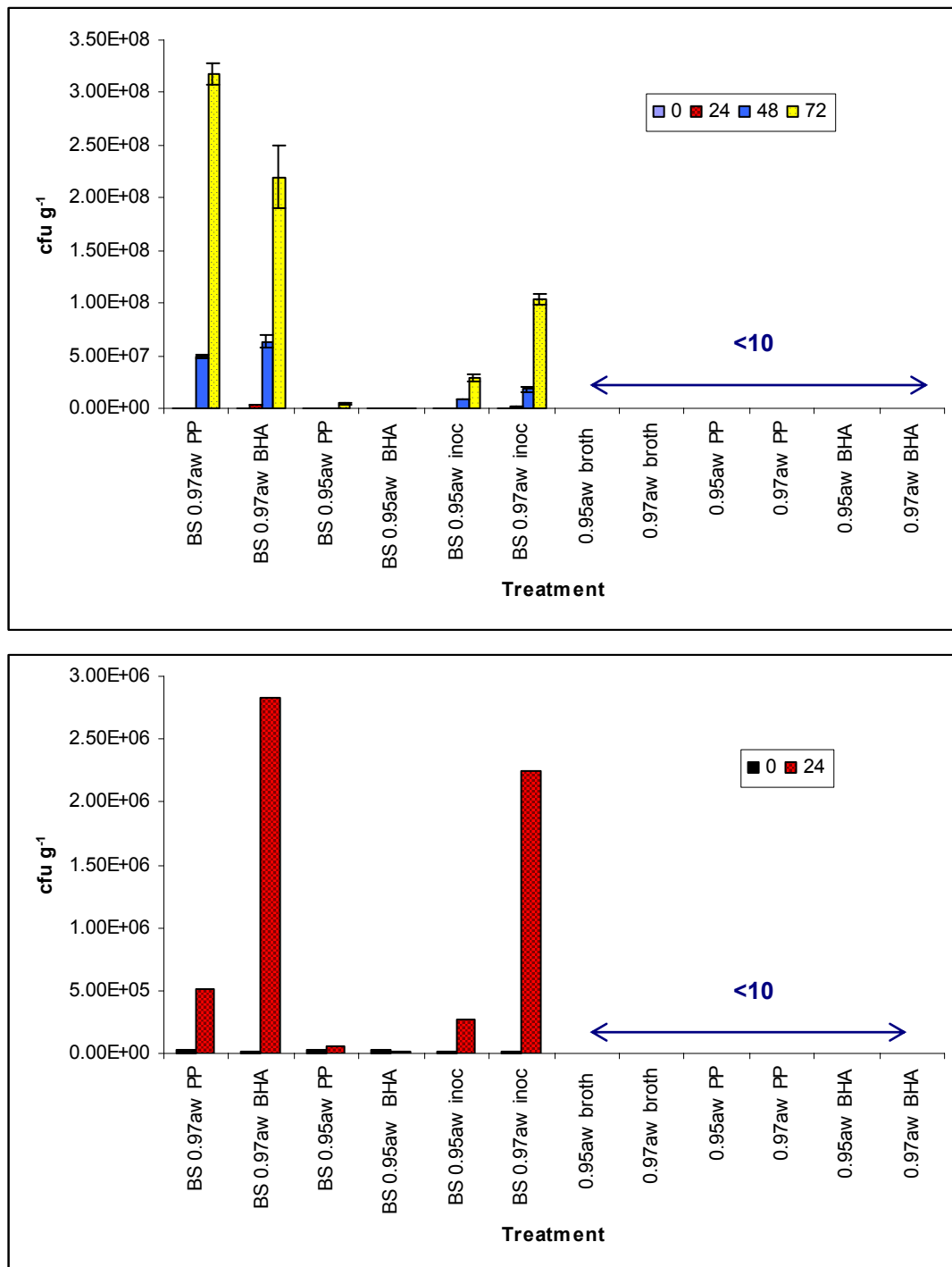
Figure 3.84 Comparison of cfu g^{-1} over time for *Bacillus subtilis* grown at 25°C in 2% flour broth at 0.95 and 0.97 a$_w$ with and without antioxidants propyl paraben and butylated hydroxyanisole, ***A*** with all times and ***B*** at time 0 and 24 h. Key to treatments: BS - inoculated with *B. subtilis*; 0.95aw and 0.97aw – flour broth water activity; PP- presence of propyl paraban; BHA presence of butylated hydroxyanisole; broth and inoc – broth without antioxidant.
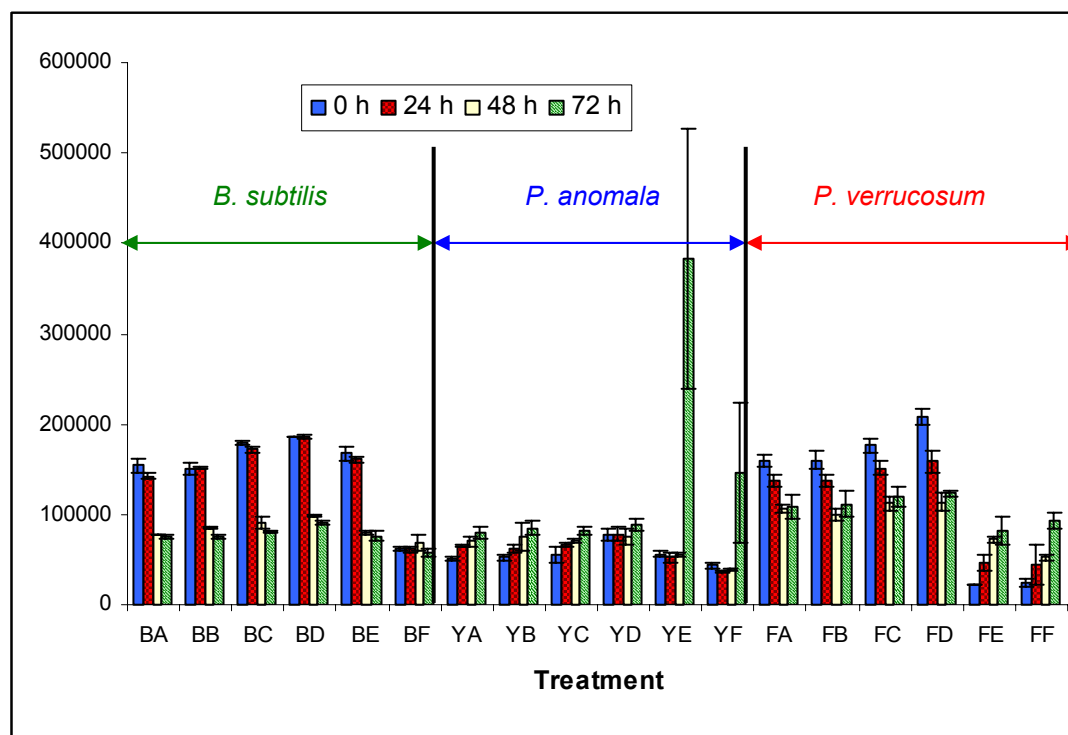
Figure 3.85 Methylbenzene levels produced by *Bacillus subtilis*, *Pichia anomala* and *Penicillium verrucosum* in 2% flour broth with and without antioxidants propyl paraben and butylated hydroxyanisole at 25°C measured by GC-MS.

Key to treatments:

B. subtilis inoculated treatments; BA = 0.97 a_w flour broth with PP, BB = 0.97 a_w flour broth with BHA, BC = 0.95 a_w flour broth with PP, BD = 0.95 a_w flour broth with BHA, BE = 0.95 a_w flour broth no antioxidant, BF = 0.97 a_w flour broth no antioxidant.

P. anomala inoculated treatments; YA = 0.97 a_w flour broth with PP, YB = 0.97 a_w flour broth with BHA, YC = 0.95 a_w flour broth with PP, YD = 0.95 a_w flour broth with BHA, YE = 0.95 a_w flour broth no antioxidant, YF = 0.97 a_w flour broth no antioxidant.

P. verrucosum inoculated treatments; FA = 0.97 a_w flour broth with PP, FB = 0.97 a_w flour broth with BHA, FC = 0.95 a_w flour broth with PP, FD = 0.95 a_w flour broth with BHA, FE = 0.95 a_w flour broth no antioxidant, FF = 0.97 a_w flour broth no antioxidant.

Figure 3.86 Hexanal levels produced by *Bacillus subtilis*, *Pichia anomala* and *Penicillium verrucosum* in 2% flour broth with and without antioxidants propyl paraben and butylated hydroxyanisole at 25°C measured by GC-MS.

Key to treatments:

B. subtilis inoculated treatments; BA = 0.97 a_w flour broth with PP, BB = 0.97 a_w flour broth with BHA, BC = 0.95 a_w flour broth with PP, BD = 0.95 a_w flour broth with BHA, BE = 0.95 a_w flour broth no antioxidant, BF = 0.97 a_w flour broth no antioxidant.

P. anomala inoculated treatments; YA = 0.97 a_w flour broth with PP, YB = 0.97 a_w flour broth with BHA, YC = 0.95 a_w flour broth with PP, YD = 0.95 a_w flour broth with BHA, YE = 0.95 a_w flour broth no antioxidant, YF = 0.97 a_w flour broth no antioxidant.

P. verrucosum inoculated treatments; FA = 0.97 a_w flour broth with PP, FB = 0.97 a_w flour broth with BHA, FC = 0.95 a_w flour broth with PP, FD = 0.95 a_w flour broth with BHA, FE = 0.95 a_w flour broth no antioxidant, FF = 0.97 a_w flour broth no antioxidant.

Figure 3.87 Hexanol levels produced by *Bacillus subtilis*, *Pichia anomala* and *Penicillium verrucosum* in 2% flour broth with and without antioxidants propyl paraben and butylated hydroxyanisole at 25°C measured by GC-MS.

Key to treatments:

B. subtilis inoculated treatments; BA = 0.97 a_w flour broth with PP, BB = 0.97 a_w flour broth with BHA, BC = 0.95 a_w flour broth with PP, BD = 0.95 a_w flour broth with BHA, BE = 0.95 a_w flour broth no antioxidant, BF = 0.97 a_w flour broth no antioxidant.

P.anomala inoculated treatments; YA = 0.97 a_w flour broth with PP, YB = 0.97 a_w flour broth with BHA, YC = 0.95 a_w flour broth with PP, YD = 0.95 a_w flour broth with BHA, YE = 0.95 a_w flour broth no antioxidant, YF = 0.97 a_w flour broth no antioxidant.

P. verrucosum inoculated treatments; FA = 0.97 a_w flour broth with PP, FB = 0.97 a_w flour broth with BHA, FC = 0.95 a_w flour broth with PP, FD = 0.95 a_w flour broth with BHA, FE = 0.95 a_w flour broth no antioxidant, FF = 0.97 a_w flour broth no antioxidant.

Figure 3.88 Styrene levels produced by *Bacillus subtilis*, *Pichia anomala* and *Penicillium verrucosum* in 2% flour broth with and without antioxidants propyl paraben and butylated hydroxyanisole at 25°C measured by GC-MS.

Key to treatments:

B. subtilis inoculated treatments; BA = 0.97 a_w flour broth with PP, BB = 0.97 a_w flour broth with BHA, BC = 0.95 a_w flour broth with PP, BD = 0.95 a_w flour broth with BHA, BE = 0.95 a_w flour broth no antioxidant, BF = 0.97 a_w flour broth no antioxidant.

P.anomala inoculated treatments; YA = 0.97 a_w flour broth with PP, YB = 0.97 a_w flour broth with BHA, YC = 0.95 a_w flour broth with PP, YD = 0.95 a_w flour broth with BHA, YE = 0.95 a_w flour broth no antioxidant, YF = 0.97 a_w flour broth no antioxidant.

P. verrucosum inoculated treatments; FA = 0.97 a_w flour broth with PP,FB = 0.97 a_w flour broth with BHA, FC = 0.95 a_w flour broth with PP, FD = 0.95 a_w flour broth with BHA, FE = 0.95 a_w flour broth no antioxidant, FF = 0.97 a_w flour broth no antioxidant.

Figure 3.89 2-pentylfuran levels produced by *Bacillus subtilis*, *Pichia anomala* and *Penicillium verrucosum* in 2% flour broth with and without antioxidants propyl paraben and butylated hydroxyanisole at 25°C measured by GC-MS.

Key to treatments:

B. subtilis inoculated treatments; BA = 0.97 a_w flour broth with PP, BB = 0.97 a_w flour broth with BHA, BC = 0.95 $a_w$ flour broth with PP, BD = 0.95 a_w flour broth with BHA, BE = 0.95 a_w flour broth no antioxidant, BF = 0.97 a_w flour broth no antioxidant.

P.anomala inoculated treatments; YA = 0.97 a_w flour broth with PP, YB = 0.97 a_w flour broth with BHA, YC = 0.95 a_w flour broth with PP, YD = 0.95 a_w flour broth with BHA, YE = 0.95 a_w flour broth no antioxidant, YF = 0.97 a_w flour broth no antioxidant.

P. verrucosum inoculated treatments; FA = 0.97 a_w flour broth with PP,FB = 0.97 a_w flour broth with BHA, FC = 0.95 a_w flour broth with PP, FD = 0.95 a_w flour broth with BHA, FE = 0.95 a_w flour broth no antioxidant, FF = 0.97 a_w flour broth no antioxidant.

Levels of hexanal produced by *B. subtilis* and *P. verrucosum* decrease with time whereas *P. anomala* produced consistent volumes. *P. verrucosum* produced significantly higher levels of styrene than *B. subtilis* and *P. anomala*.

Comparing the compounds at individual sample times with control treatments showed differences in production between samples with and without micro-organisms. Hexanol was not produced by *P. anomala* or in the BHA control broths at time 0 (Figure 3.90) but after 72 h levels were higher than treatments with *B. subtilis* and *P. verrucosum* (Figure 3.91). Figure 3.92 shows styrene levels were greater in broths containing *P. verrucosum*. After 72 h the level of methylbenzene produced was similar in all treatments except *P. verrucosum* in the 0.95 a_w flour broth control treatment (Figure 3.93).

3.8 DISCRIMINATION OF UNKNOWN SAMPLES ON NATURAL BREAD

Discrimination of unknown treatments A (*P. anomala*), B (*B. subtilis*), C (*P. verrucosum*) and D (controls) were investigated using initial population levels relevant to specification limits for natural white bread.

The BH114 e-nose could differentiate *P. anomala* and *B. subtilis* after 24 h as shown in the PCA in Figure 3.94, which accounts for 94% of the total data. There was slight overlap of *P. verrucosum* and controls. After 48 h (Figure 3.95) and 72 h (Figure 3.96) the BH114 could differentiate all 4 treatments. CA however, could only differentiated between *P. anomala* and *B. subtilis* after 72 h (Figure 3.97). Cross validation of samples could not be performed due to insufficient replication.
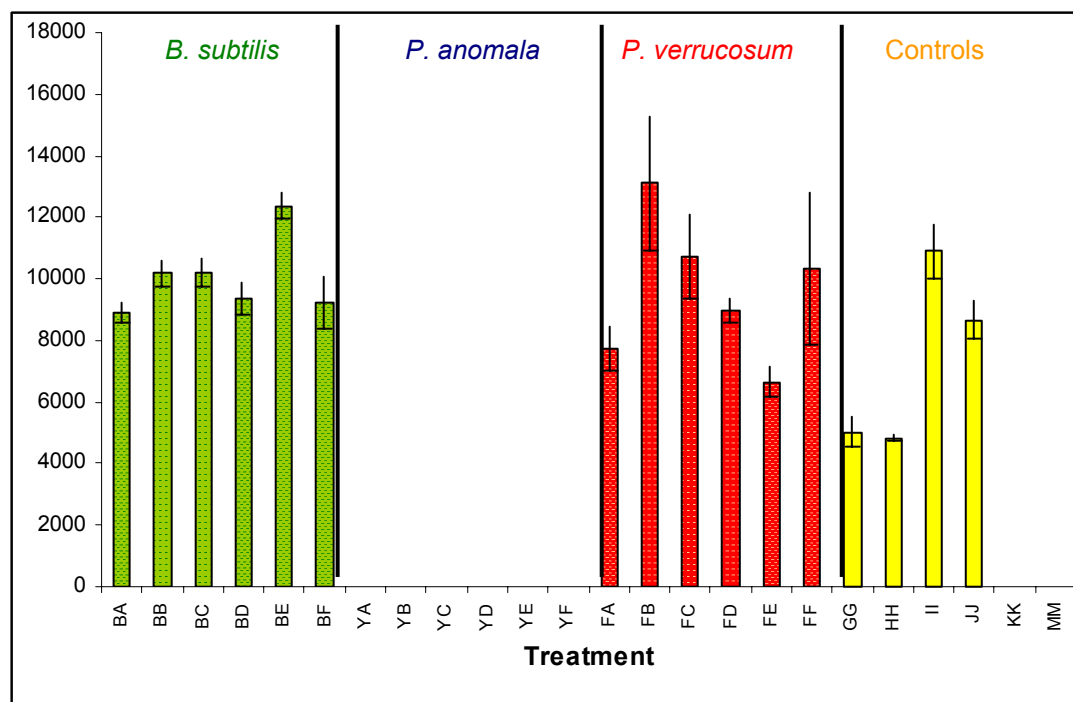
Figure 3.90 Hexanol levels produced by *Bacillus subtilis*, *Pichia anomala*, *Penicillium verrucosum* and control 2% flour broth with and without antioxidants propyl paraben and butylated hydroxyanisole at 25°C measured by GC-MS after 0 h.

Key to treatments:

B. subtilis inoculated treatments; BA = 0.97 a_w flour broth with PP, BB = 0.97 a_w flour broth with BHA, BC = 0.95 a_w flour broth with PP, BD = 0.95 a_w flour broth with BHA, BE = 0.95 a_w flour broth no antioxidant, BF = 0.97 a_w flour broth no antioxidant.

P.anomala inoculated treatments; YA = 0.97 a_w flour broth with PP, YB = 0.97 a_w flour broth with BHA, YC = 0.95 a_w flour broth with PP, YD = 0.95 a_w flour broth with BHA, YE = 0.95 a_w flour broth no antioxidant, YF = 0.97 a_w flour broth no antioxidant.

P. verrucosum inoculated treatments; FA = 0.97 a_w flour broth with PP,FB = 0.97 a_w flour broth with BHA, FC = 0.95 a_w flour broth with PP, FD = 0.95 a_w flour broth with BHA, FE = 0.95 a_w flour broth no antioxidant, FF = 0.97 a_w flour broth no antioxidant.

Control treatments; GG = 0.95 a_w flour broth no antioxidant, HH = 0.97 a_w flour broth no antioxidant, II = 0.95 a_w flour broth with PP, JJ = 0.97 a_w flour broth with PP, KK = 0.95 a_w flour broth with BHA, MM = 0.97 a_w flour broth with BHA.

Figure 3.91 Hexanol levels produced by *Bacillus subtilis*, *Pichia anomala*, *Penicillium verrucosum* and control 2% flour broth with and without antioxidants propyl paraben and butylated hydroxyanisole at 25°C measured by GC-MS after 72 h.

Key to treatments:

B. subtilis inoculated treatments; BA = 0.97 a_w flour broth with PP, BB = 0.97 a_w flour broth with BHA, BC = 0.95 a_w flour broth with PP, BD = 0.95 a_w flour broth with BHA, BE = 0.95 a_w flour broth no antioxidant, BF = 0.97 a_w flour broth no antioxidant.

P.anomala inoculated treatments; YA = 0.97 a_w flour broth with PP, YB = 0.97 a_w flour broth with BHA, YC = 0.95 a_w flour broth with PP, YD = 0.95 a_w flour broth with BHA, YE = 0.95 a_w flour broth no antioxidant, YF = 0.97 a_w flour broth no antioxidant.

P. verrucosum inoculated treatments; FA = 0.97 a_w flour broth with PP, FB = 0.97 a_w flour broth with BHA, FC = 0.95 a_w flour broth with PP, FD = 0.95 a_w flour broth with BHA, FE = 0.95 a_w flour broth no antioxidant, FF = 0.97 a_w flour broth no antioxidant.

Control treatments; GG = 0.95 a_w flour broth no antioxidant, HH = 0.97 a_w flour broth no antioxidant, II = 0.95 a_w flour broth with PP, JJ = 0.97 a_w flour broth with PP, KK = 0.95 a_w flour broth with BHA, MM = 0.97 a_w flour broth with BHA.

Figure 3.92 Styrene levels produced by *Bacillus subtilis, Pichia anomala, Penicillium verrucosum* and control 2% flour broth with and without antioxidants propyl paraben and butylated hydroxyanisole at 25°C measured by GC-MS after 24 h.

Key to treatments:

B. subtilis inoculated treatments; BA = 0.97 a_w flour broth with PP, BB = 0.97 a_w flour broth with BHA, BC = 0.95 a_w flour broth with PP, BD = 0.95 a_w flour broth with BHA, BE = 0.95 a_w flour broth no antioxidant, BF = 0.97 a_w flour broth no antioxidant.

P.anomala inoculated treatments; YA = 0.97 a_w flour broth with PP, YB = 0.97 a_w flour broth with BHA, YC = 0.95 a_w flour broth with PP, YD = 0.95 a_w flour broth with BHA, YE = 0.95 a_w flour broth no antioxidant, YF = 0.97 a_w flour broth no antioxidant.

P. verrucosum inoculated treatments; FA = 0.97 a_w flour broth with PP,FB = 0.97 a_w flour broth with BHA, FC = 0.95 a_w flour broth with PP, FD = 0.95 a_w flour broth with BHA, FE = 0.95 a_w flour broth no antioxidant, FF = 0.97 a_w flour broth no antioxidant.

Control treatments; GG = 0.95 a_w flour broth no antioxidant, HH = 0.97 a_w flour broth no antioxidant, II = 0.95 a_w flour broth with PP, JJ = 0.97 a_w flour broth with PP, KK = 0.95 a_w flour broth with BHA, MM = 0.97 a_w flour broth with BHA.

Figure 3.93 Methylbenzene levels produced by *Bacillus subtilis*, *Pichia anomala*, *Penicillium verrucosum* and control 2% flour broth with and without antioxidants propyl paraben and butylated hydroxyanisole at 25°C measured by GC-MS after 72 h. Key to treatments:

B. subtilis inoculated treatments; BA = 0.97 a_w flour broth with PP, BB = 0.97 a_w flour broth with BHA, BC = 0.95 a_w flour broth with PP, BD = 0.95 a_w flour broth with BHA, BE = 0.95 a_w flour broth no antioxidant, BF = 0.97 a_w flour broth no antioxidant.

P.anomala inoculated treatments; YA = 0.97 a_w flour broth with PP, YB = 0.97 a_w flour broth with BHA, YC = 0.95 a_w flour broth with PP, YD = 0.95 a_w flour broth with BHA, YE = 0.95 a_w flour broth no antioxidant, YF = 0.97 a_w flour broth no antioxidant.

P. verrucosum inoculated treatments; FA = 0.97 a_w flour broth with PP,FB = 0.97 a_w flour broth with BHA, FC = 0.95 a_w flour broth with PP, FD = 0.95 a_w flour broth with BHA, FE = 0.95 a_w flour broth no antioxidant, FF = 0.97 a_w flour broth no antioxidant.

Control treatments; GG = 0.95 a_w flour broth no antioxidant, HH = 0.97 a_w flour broth no antioxidant, II = 0.95 a_w flour broth with PP, JJ = 0.97 a_w flour broth with PP, KK = 0.95 a_w flour broth with BHA, MM = 0.97 a_w flour broth with BHA.

Figure 3.94 DFA of BH114 e-nose normalized divergence responses to volatiles produced by 4 unknown samples on natural white bread after 24 h at 25°C. Key to treatments: A- *P. anomala*, B- *B. subtilis*, C- *P. verrucosum* and D- controls.
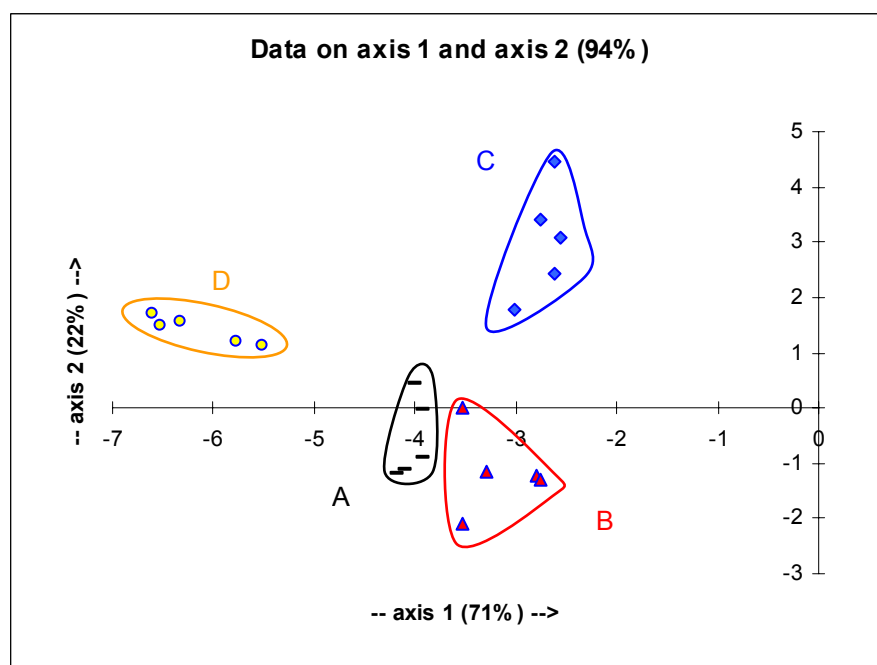
Figure 3.95 DFA of BH114 e-nose normalized divergence responses to volatiles produced by 4 unknown samples on natural white bread after 48 h at 25°C. Key to treatments: A- *P. anomala*, B- *B. subtilis*, C- *P. verrucosum* and D- controls.
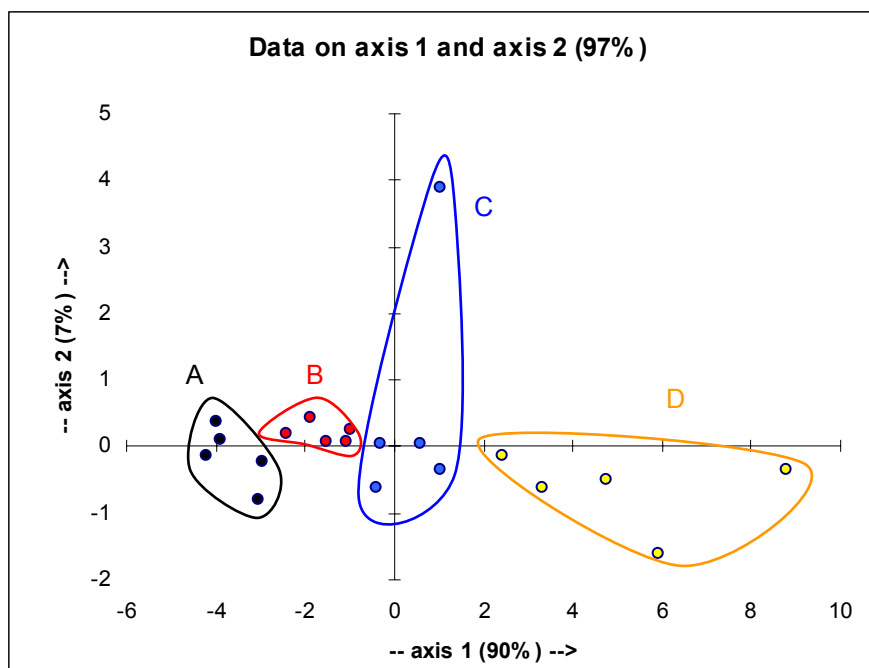
Figure 3.96 DFA of BH114 e-nose normalized divergence responses to volatiles produced by 4 unknown samples on natural white bread after 72 h at 25°C. Key to treatments: A- *P. anomala*, B- *B. subtilis*, C- *P. verrucosum* and D- controls.
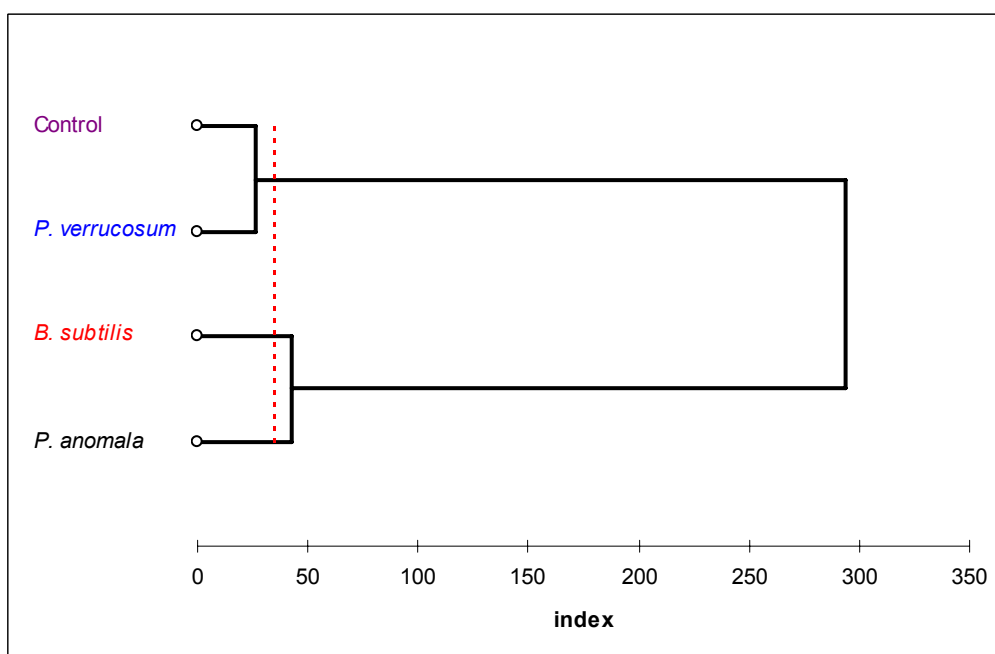
Figure 3.97 CA dendrogram of 4 unknown samples on natural white bread after 72 h at 25°C based on their volatiles profiles using a BH114 e-nose.

Visible signs of bacterial growth occurred in treatment B after 72 h and of *P. verrucosum* in treatment C after 72 h.

Using the NST3220 lab emission analyser only *P. anomala* could be discriminated after 24 h as seen in the PCA in Figure 3.98a. With an increased incubation time of 48 h it was possible to differentiate *P. anomala* and the control but not *B. subtilis* and *P. verrucosum* (Figure 3.98b). The NST3220 was able to discriminate between all treatments after 72 h using PCA with the addition of the 3rd PC as shown in Figure 3.99.

CO$_2$ levels were highest in treatments A (*P. anomala*), B (*B. subtilis*) and C (*P. verrucosum*) (Figure 3.100) compared to treatment D (controls) with a significant increase in levels from 24 to 48 h. *P. anomala* and *B. subtilis* produced similar levels of CO$_2$.

Population analysis showed that treatment A (*P. anomala*), (Figure 3.101), was the only treatment with yeasts present. Only bacteria was present on treatment B (*B. subtilis*) (Figure 3.102), which was found on all treatments but was present in higher levels in B. Treatment C (*P. verrucosum*) (Figure 3.103) was the only treatment with filamentous fungi present. Treatment D (controls) also only had bacteria present as shown in Figure 3.104.

In summary this study showed that controls could be discriminated from microbial treatments but that differentiation between bacterial, yeast and filamentous fungus species varied with time of incubation and microbial load.
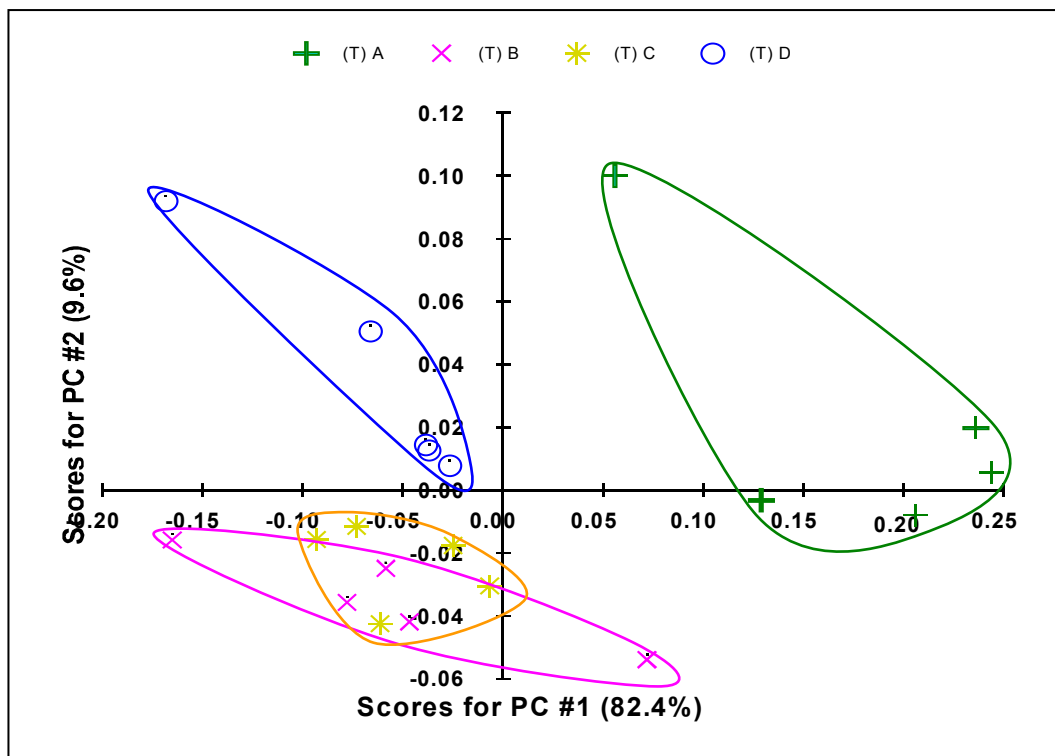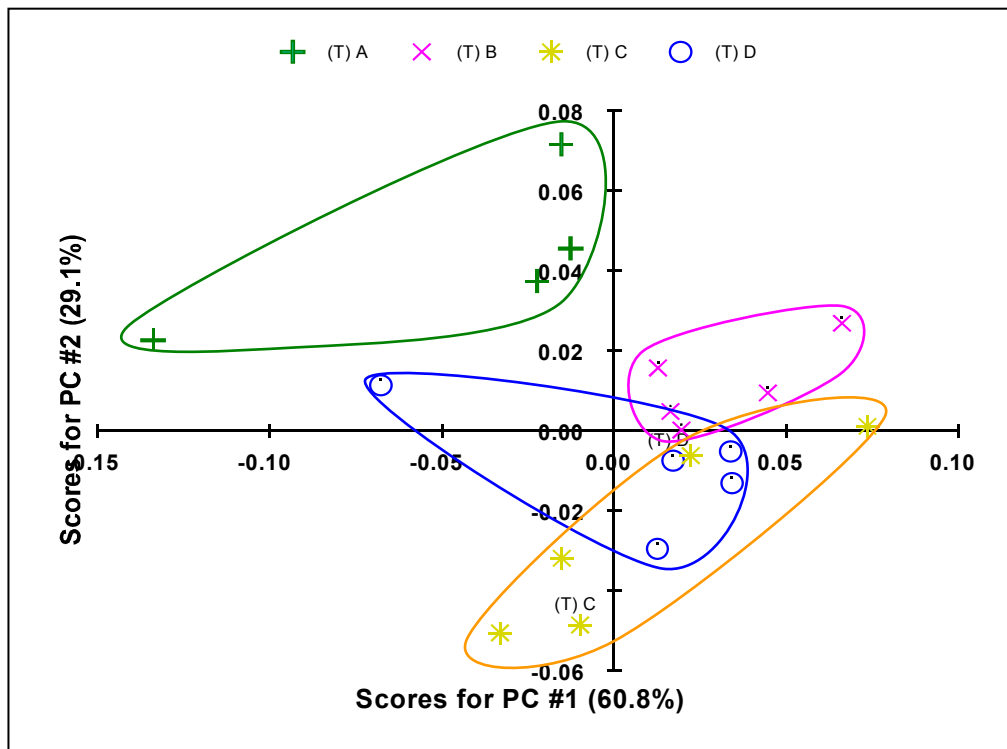
Figure 3.98 PCA of normalized response data from a NST3220 lab emission analyser for 4 unknown samples on natural white bread after 24 h (**A**) and 48 h (**B**) at 25°C. Key to treatments: A- *P. anomala*, B- *B. subtilis*, C- *P. verrucosum* and D- controls.
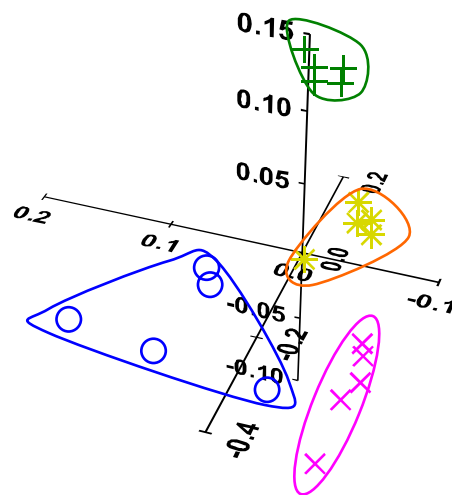
Figure 3.99 PCA of normalized response data from a NST3220 lab emission analyser for 4 unknown samples on natural white bread after 72 h with 3rd PC. Key to treatments: A- *P. anomala*, B- *B. subtilis*, C- *P. verrucosum* and D- controls.
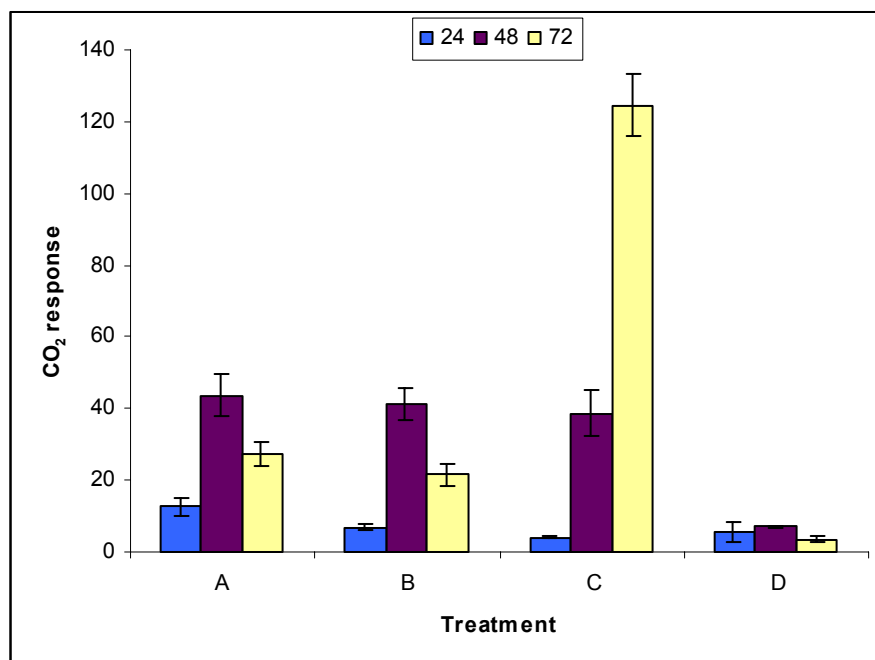
Figure 3.100 Carbon dioxide levels, measured by a NST3220 lab emission analyser, for 4 unknown treatments on natural white bread at 25°C. Key to treatments: A- *P. anomala*, B- *B. subtilis*, C- *P. verrucosum* and D- controls.
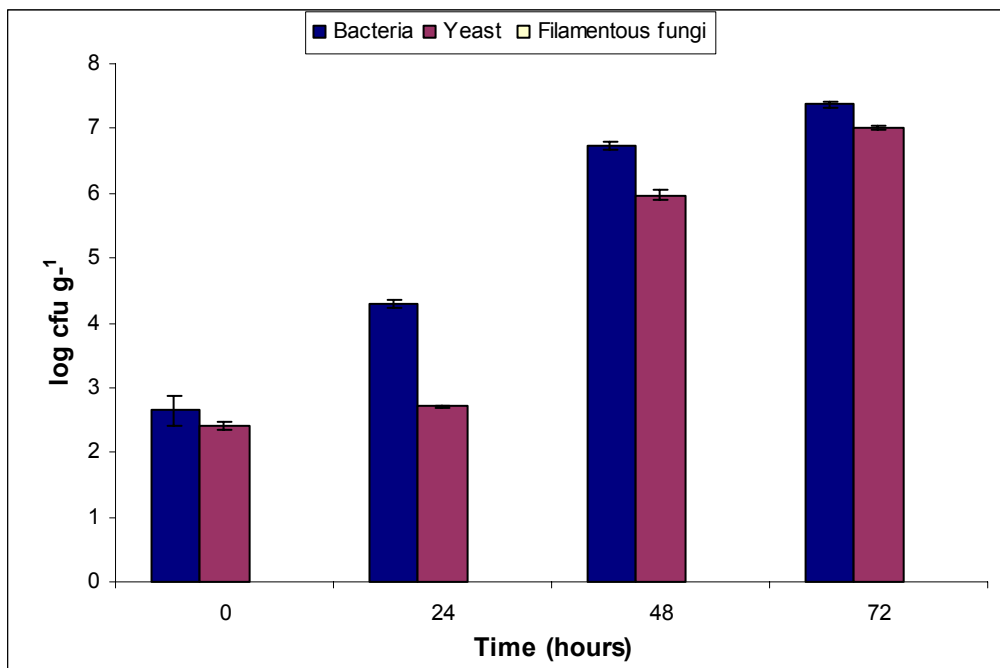
Figure 3.101 Histogram of cfu g^{-1} for unknown treatment A (*Pichia anomala*) on natural white bread at 25°C.
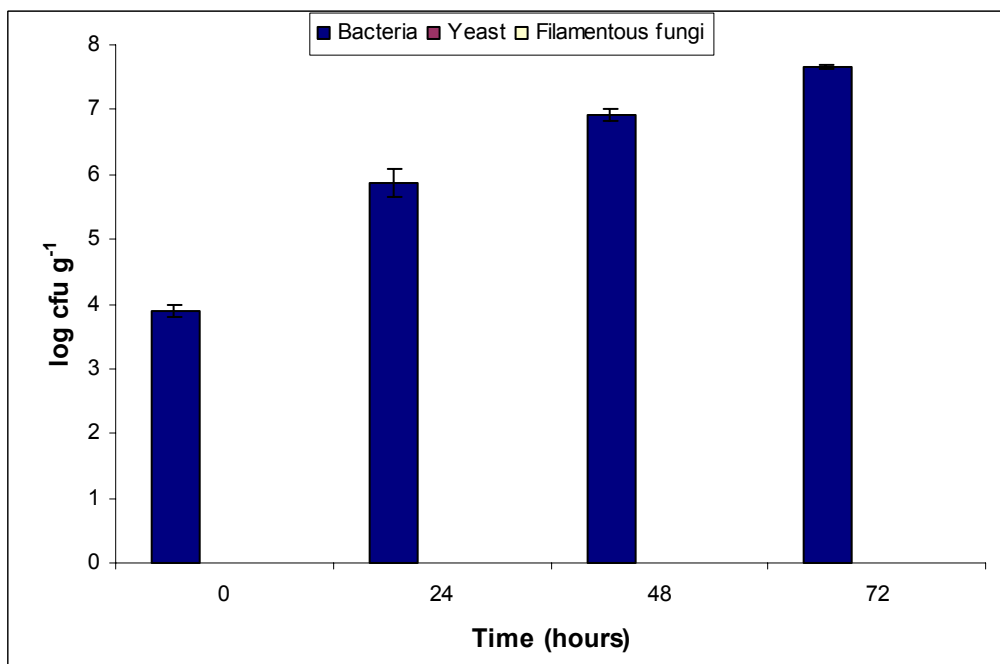
Figure 3.102 Histogram of cfu g^{-1} for unknown treatment B (*Bacillus subtilis*) on natural white bread at 25°C.
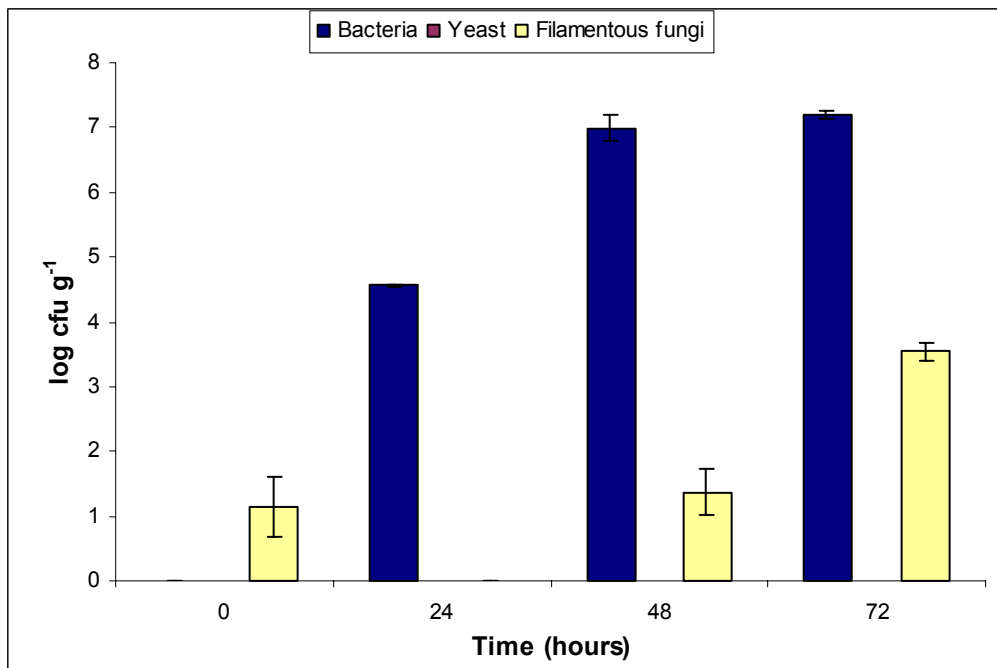
Figure 3.103 Histogram of cfu g^{-1} for unknown treatment C (*Penicillium verrucosum*) on natural white bread at 25°C.
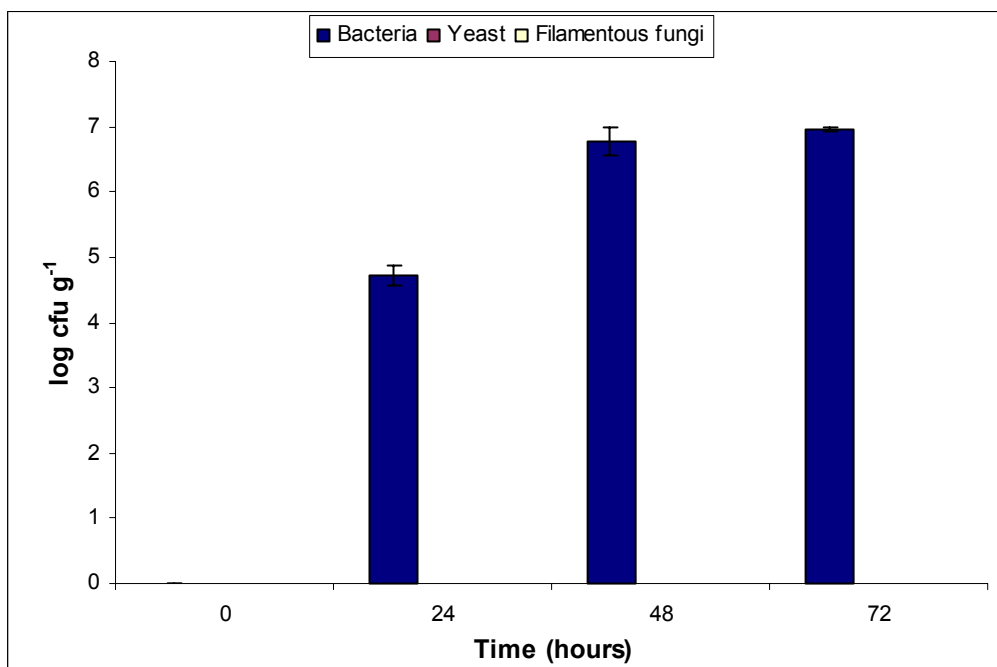
Figure 3.104 Histogram of CFU g^{-1} for unknown treatment D (controls) on natural white bread 25°C.

CHAPTER 4

DISCUSSION

4.1 EFFECT OF SAMPLE BAG INCUBATION TIME ON DISCRIMINATION OF MICRO-ORGANISMS

Only a slight improvement in separation of clusters was observed by increasing the static incubation period of sample bags. Therefore an incubation time of 1 hour was chosen for all subsequent experiments. This was also because in industry a shorter time period is desired to decrease the time taken to achieve results and to allow more samples to be examined. Replicate A4 in the PCA shown in Figure 3.1 (page 61), with an incubation time of 1 h, could be an outlier due to sensor drift or deflation of the sample bag reducing the concentration of volatiles. Keshri *et al*. (1998) also left sample bags for 1 h before using a BH114 e-nose to discriminate spoilage fungi on wheat-based agar media. Gibson *et al*. (1997) however only left samples for 2 minutes before sampling with a BH114 e-nose and obtained 100% correct classification of 7 bacteria. However, their studies were carried out on liquid cultures and not on solid agar-based media or food matrices.

4.2 DIFFERENTIATION OF MICROBIAL SPOILAGE *IN VITRO*

These studies showed the potential for use of an e-nose system to detect and differentiate microbial spoilage early before signs of visible growth occur. After 24 h it was possible to distinguish the filamentous mycotoxigenic fungi *P. verrucosum* and *A. ochraceus,* and a yeast *P. anomala* from 3 other species of filamentous fungi before visible growth was observed on unmodified wheat agar using PCA. For discrimination of controls, uninoculated agar, 48 h were required.

Modifying the a_w of the agar to 0.98 produced better discrimination between micro-organisms based on their volatile production profile. After 72 h, no differentiation between micro-organisms was possible, suggesting that once growth has commenced the volatile fingerprints detected by the conducting polymer array was similar. However, environmental conditions may affect volatile production and allow better discrimination of different micro-organisms. Changes in volatile production also occurred with time and could account for the lack of discrimination with longer incubation periods as different volatiles are produced during different stages of growth as previously reported by Börjesson *et al*., (1989). The sensitivity of the conducting polymer sensors may also vary with volatile types.

A possible explanation for the first few samples not being as reproducible as the other replicates of the same treatment is that the e-nose may need to perform a few samples to stabilise the sensors or to "warm up". Removal of these replicates often altered the degree of differentiation. For example with all replicates only *A. tenuissima* was distinguishable and this required 72 h. However, when the initial replicates were removed, *P. anomala* and *A. parasiticus* could be distinguished after 48 h. For all subsequent experiments three or four samples of air were taken before randomised sampling of the treatments. Sensor reproducibility was checked before analysis performed.

It was possible to discriminate between all micro-organisms when grown on 2% flour agar modified to 0.97 a_w after only 24 h using DFA. With increased sample time the extent of differentiation decreased as after 48 h only controls could be distinguished

using CA. Clusters were observed using PCA and DFA. However, clusters became closer with more overlap with time. The flour used in these studies was that subsequently used to prepare bread analogues.

Cross validation correctly classified approximately 65% of the treatments grown on wheat agar and 60% on flour agar. The percentages decreased with increased sample time due to decreased levels of discrimination. Differentiation may be improved using 3D PCA. Addition of a 3rd PC can separate treatments, which appear to overlap, on a different plane. Capone *et al*. (2001) produced 3D PCA graphs in their analysis of monitoring milk rancidity, which allowed them to observe separate clusters and a move of direction with increased rancidity of milk. PCA is an unsupervised technique because the classification of the individual data points is not used in the analysis. DFA, in comparison, is a supervised technique, which uses a priori classifications assigned by the user to obtain the separation. Goodner *et al*. (2001) noted that the smaller the group size the more erroneous classifications were made using multivariate analyses such as PCA and DFA and that larger sample sizes minimized the random noise allowing true differences to be shown. Overlap of treatments could also be due to sensor drift or due to the sensors not being sensitive to some of the important volatiles produced.

Marked differences in the production of volatile metabolites by fungi have been reported between closely related species and even between strains of the same species. Börjesson *et al*. (1989) studied the production of volatiles by various fungi during growth on moist wheat. They identified ten different volatiles produced by four fungi.

The most common volatiles were alcohols, alkanes and terpenes, with alcohols representing more than 80% of the volatiles found after 6 days. Some compounds were found to occur predominantly at the early stages of growth with 3-methyl-1-butanol being produced by *A. flavus* and *Penicillium cyclopium* during the first 2 days. This could account for why, with increased incubation, the degree of discrimination of micro-organisms decreased. They also showed that during fungal growth the total concentration of volatiles does not rise significantly, but that the concentration of individual compounds changes. Compounds such as straight and branched saturated alkenes have been shown to be derived from polystyrene Petri dishes alone (Larsen and Frisvad, 1994), which could also affect the volatile profiles detected.

Cormier *et al.* (1991) suggested that some major components made little or no contribution to the aroma, whereas other components present in low concentrations caused strong perceptions. Of the 90 compounds detected from *P. fragi*, growing in skimmed milk, only 26 were odour active. Ethyl acetate, which occurred in large quantities, was not significant to odour. They found ethyl butyrate; ethyl 3-methylbutanoate and ethyl hexanoate were major contributors to odours whereas other compounds contributed to complexity and richness. This may account for not being able to discriminate between micro-organisms even if there is a difference in smell. Differentiation would depend on whether the sensors detected the volatiles present in small concentrations.

Gervais (1990) showed the effect of a_w on volatile production by the filamentous fungus *Trichoderma viride* and the yeast *Sporidiobolus salmonicolor*. Their work found that 2-heptanone production by *T. viride* increased with decreasing medium a_w from 0.98 to 0.96 over a period of 7 days at 25°C. Furthermore, their study also demonstrated a drastic decrease in 2-heptanone production by *T. viride* when the relative humidity (r.h.) of the air was reduced from 99% to 80% for 10 h. The return to 99% r.h. resulted in an increase in the production of volatiles. Production of γ-decalactone by *S. salmonicolor* was high at 0.99-0.97 a_w and markedly decreased above and below this range. The production of different volatile profiles with different water activities could account for the better discrimination observed in media modified to 0.98 a_w.

Previous studies with e-nose technology have looked mainly at differentiation between bacteria only or spoilage fungi. Keshri *et al*. (1998) have noted the potential for early detection of spoilage fungi using a BH114 e-nose system. They achieved good replication of volatile patterns of the same species and were able to differentiate between agar blanks, three out of four *Eurotium* spp. tested, a *Penicillium* spp. and *Wallemia sebi* grown on 2% wheat agar modified to 0.95 a_w after 48 h using DFA. There was some overlap of the related species *E. amstelodami* and *E. herbariorum*.

Gibson *et al*. (1997) were able to correctly classify 93.4% of 12 different bacteria and a pathogenic yeast from static headspaces formed from freshly inoculated agar plates with a BH114 e-nose using neural networks. Analysis of a subset of seven bacteria

gave 100% correct classification. Three similar yeast cultures were also compared and correctly classified at a level of 96.3%.

Sampling with a BH114 e-nose Pavlou *et al*. (2002) were able to differentiated between agar blanks and individual bacterial species. Fourteen strains of *Clostridium* and 12 strains of *Bacteroides fragilis* were incubated on blood agar plates for 16 h at 37°C then left in sample bags for 30 mins before sampling the headspace. A total of eight unknowns were correctly discriminated into bacterial groups.

Many studies have used GC-MS to analyse volatile profiles for discrimination of filamentous fungi. Larsen and Frisvad (1994) were able to distinguish between *Penicillium clavigerum* and *Penicillium vulpinum* on the basis of their volatile profiles produced when grown on Czapek yeast autolysate agar. Collecting volatiles by diffusive sampling onto tubes containing carbon black or Tenax TA and analysing with GC-MS they noted that *P. clavigerum* produced geosmin whilst *P. vulpinum* produced a number of different unsaturated compounds, e.g. mono- and sesquiterpenes. Larsen and Frisvad (1995b) were able to differentiate 25 different species of *Penicillium* grown on yeast extract sucrose agar using Tenax TA and GC-MS. They concluded that volatile profiles could be used in detection and classification of fungi at the species level using multivariate statistical analysis. When analysing volatile profiles of 47 different *Penicillium* grown on 3 different agar media Larsen and Frisvad (1995c) noted that more than half the metabolites were detected from only one species and nearly all taxa produced a unique profile of volatile metabolites.

4.2.1   Production of volatiles in liquid culture

It was possible to distinguish between bacteria and yeast growing in two different a_w broths but not between micro-organisms after 24 h growth. Sampling in exponential and stationary phases of growth improved differentiation.   It was possible to discriminate between *P. fragi* and *S. cerevisiae* growing in the different a_w broths and between the different a_w control broths during exponential growth (13.5 h).   In stationary growth phase (39 h), separation of samples in the unmodified medium and at 0.98 a_w was possible. *S. aureus* was discriminated in the different a_w broths and from the uninoculated treatments during the exponential growth phase.   In the stationary growth phase there was discrimination of uninoculated and inoculated samples but not between the different a_w broths. All micro-organisms grew fastest in unmodified broth. The ability to distinguish between unmodified and 0.98 a_w broths could be due to the different growth rates observed between the broths.

Gardner *et al.* (1998) showed the potential for use of an e-nose to predict both the class of bacteria and the growth phase, which indicates that a different volatile fingerprint is produced during different growth phases. Using *Escherichia coli* and *S. aureus*, grown in broth over a 12 h period, they were able to correctly identify the bacterial species and growth phase with percentages of 96% and 81% respectfully.   They did not however examine the affect of environmental conditions such as a_w and temperature.

A summary of *in vitro* differentiation of micro-organisms based on their volatile fingerprints in this study compared with that in the literature is given in Table 4.1

Table 4.1 Summary comparison of differentiation achieved *in vitro* using microbial volatile production profiles.

	Method	Achieved	cfu levels
This study	e-nose	Differentiation of controls from inoculated plates after 24 h, with discrimination of yeast and between some fungal species.	10^6 spores/cells ml^{-1}
This study	e-nose	Differentiation of bacteria and yeast growing in two a_w broths during exponential growth phase.	10^6 cells ml^{-1}
Gardner *et al.* (1998)	e-nose	Predicted growth phase and type of bacteria in liquid media.	10^6 - 10^7 cells ml^{-1}
Gibson *et al.* (1997)	e-nose	Correct classification of seven bacteria on an agar medium. Correct classification of three yeast cultures on agar medium.	Not specified
Keshri *et al.* (1998)	e-nose	Differentiation of spoilage fungi after 48 h on 2% wheat agar.	1.5 x 10^6 spores ml^{-1}
Pavlou *et al.* (2002)	e-nose	Discrimination of *Clostridium* and *Bacteroides fragilis* strains on blood agar after 16 h incubation.	10^6 cells ml^{-1}
Larsen and Frisvad (1994)	GC-MS	Discriminated between 7 day old cultures of *Penicillium clavigerum* and *Penicillium vulpinum*.	>10^6 spores ml^{-1}
Larsen and Frisvad (1995b)	GC-MS	Differentiated between 25 different species of *Penicillium* after 11 days growth on agar	Not specified
Larsen and Frisvad (1995c)	GC-MS	Noted from 47 different *Penicillium* strains over half produced unique volatiles. Used diffusive sampling over 14 days.	Not specified

4.3   DIFFERENTIATION OF MICROBIAL SPOILAGE *IN SITU*

These studies showed the potential for use of an e-nose to detect and differentiate microbial spoilage *in situ* using bread analogues. Whereas synthetic media are the most reproducible in terms of their composition and ease of preparation, studies on the natural product or analogues are required since their composition differs and can alter the volatile production profiles. Fustier *et al*. (1998) demonstrated that even with identical exposures the inoculation levels varied greatly as a function of the exposed medium when studying growth of spoilage micro-organisms on potato dextrose agar (PDA) and yellow layer cakes. Growth was found to be faster on PDA.

In the present study it was possible to discriminate the controls, *B. subtilis* and *S. cerevisiae* but not *P. verrucosum* or a mixture of the 3 micro-organisms on bread analogues after 24 h at 25°C. A possible reason for the lack of discrimination between *P. verrucosum* and the mixed treatment is that when visible growth occurred in this treatment only fungal growth was apparent. Better discrimination was achieved using an incubation temperature of 25°C and 0.95 a_w. This could be due to the temperature favouring microbial growth as indicated in Table 3.7, which showed that visible growth occurred with all treatments at 25°C before visible growth was observed at 15°C. The percentage of correctly classified treatments was also slightly higher (50% compared to 45%) with an incubation temperature of 25°C.

Keshri *et al*. (2002) were able to differentiate between uninoculated control bread analogues modified to 0.95 a_w and analogues contaminated with *Penicillium chrysogenum* after 24 h at 25°C using a BH114 e-nose. After 40 h they were able to

differentiate between two *Eurotium* spp., *P. chrysogenum* and controls with initial spore populations of approximately 10^3 cfu g^{-1} substrate using PCA analysis. They also investigated activities of seven hydrolytic enzyme. Activities of three were found to be significantly different from the controls after 72 h; these were N-acetyl-β-D-glucosaminindase, α-D-galactosidase and β-D- glucosidase. Magan *et al*. (2003) were also able to detect and differentiate spoilage mould growth on bread analogues within 24-40 h of inoculation before signs of visible growth. Other measures of fungal activity including enzyme production and enumeration of fungal populations showed differences only after more than 48 h of incubation. Jain et al. (1991) and Magan (1993) have also suggested hydrolytic enzymes as good early indicators for fungal spoilage prior to visible growth. However, as shown, discrimination is achieved earlier using an e-nose.

Keshri (2001) investigated the detection limit of the conducting polymer based BH114 e-nose for spoilage fungi and was able to detect a limit of 1×10^2 spores ml^{-1} after 24 h on bread analogue. However, there was some overlap between 1×10^2 and 1×10^4 spores ml^{-1} for both *Eurotium chevalieri* and *P. chrysogenum*. The upper limit of 1×10^6 spores ml^{-1}, as used in these studies, was clearly differentiated.

4.4 COMPARISON OF A BH114 ELECTRONIC NOSE AND GAS CHROMATOGRAPHY - MASS SPECTROMETRY FOR DISCRIMINATION OF SPOILAGE OF BREAD ANALOGUES

This study showed for the first time the potential to discriminate between microbial and physiological spoilage of bread using both e-nose technology and GC-MS. After

48 h incubation differentiation of the types of spoilage and between some of the microbial spoilage organisms was possible with the BH114 e-nose using CA. Significant temporal increases in CFU occurred. After 48 and 72 h there were significant differences between the populations of each micro-organism with higher populations of *B. subtilis* followed by *P. anomala* then *P. verrucosum*. Analysis of volatile compounds produced with GC-MS showed that after 24 h *P. anomala* was the only treatment to produce 2-propanol, ethyl acetate, and pentanol and also produced greater amounts of 3-methylbutanol than the three other types of spoilage. These compounds could be used for identification of *P. anomala* spoilage. When comparing the other 8 compounds it was noted that there was more difference in some compounds such as 1-butanol but not in others such as styrene. To be able to differentiate all spoilage micro-organisms, physiological spoilage and controls more of the 59 volatiles detected would probably need to be analysed for volatiles specific to each spoilage type. It was also found that the levels of volatiles produced differed with time. Discrimination between microbial and physiological spoilage was greater using the e-nose and cfu when compared with GC-MS, which was only able to discriminate *P. anomala*.

Gas chromatography (GC) and Gas chromatography-mass spectrometry (GC-MS) have been utilised by many researchers to observe volatile production profiles of micro-organisms, particularly filamentous fungi associated with grain spoilage. Growing *Penicillium aurantiogriseum* on six different agars, including wheat meal agar, and analysing volatiles produced, Börjesson *et al*. (1990) discovered that the dominant compounds were alcohols of low molecular weight and sesquiterpenes. Börjesson *et*

al. (1993) identified five volatile compounds with prominent off-odours and noted that geosmin, 1-methoxy-3-methylbenzene and methylphenol were produced in large amounts by some off-odorous fungi, which contributed to their unpleasant odour. However, they also found that 3-methylfuran, 2-methyl-1-propanol and 3-methyl-1-butanol were much more commonly produced than the off-odorous compounds.

Larsen and Frisvad (1994) were able to distinguish between 2 different *Penicillium* species based on their volatile profiles by collecting volatiles. Larsen and Frisvad (1995b) noted that nearly all *Penicillium* spp. they tested produced a unique volatile fingerprint. Olsson *et al*. (2000) noted the main volatile compounds of grain with normal odour were 2-hexenal, benzaldehyde and nonanal, while 3-octanone, methylheptanone and trimetylbenzene were the main volatile compounds of grain with off-odours.

Börjesson *et al*. (1989) collected and identified volatile fungal metabolites during fungal growth on wheat. When production of volatiles during different fungal growth phases was investigated they found that some compounds were mainly produced during early growth stages. The production of terpenes varied greatly between the examined species, and they proposed that these compounds could possibly be used for the recognition of species. This could account for the different levels of discrimination achieved with different sample times.

4.5 DISCRIMINATION OF MYCOTOXIGENIC AND NON-TOXIGENIC STRAINS OF FILAMENTOUS FUNGI

Potential for use of an e-nose system for early detection and differentiation of toxigenic and non-toxigenic strains of fungal species on bread analogues was demonstrated for *P. verrucosum*.

Discrimination between mycotoxigenic and non-toxigenic strains of *A. parasiticus* was not achieved with only one toxigenic strain being distinguished after 48 h and a different toxigenic strain after 72 h using CA. Longer incubation times may be required, as toxins may not be produced within 72 h. However, an increase in sample time would result in visible growth of the fungi. Since the aim of this work was early detection of microbial spoilage before signs of visible growth on bakery products, longer incubation times were considered unrealistic.

The lack of differentiation may also be due to the substrate as Reiss (1982) noted that *A. parasiticus* and *A. flavus* grew better on cake than on bread and that malt extract agar was a less favourable substrate for aflatoxin synthesis than bakery products. Perhaps better differentiation may have also been achieved if the *A. parasiticus* strains were grown on bread analogues. Since a differentiation of aflatoxigenic and non-aflatoxigenic strains is important studies on both maize and groundnut are needed to evaluate the potential for this approach.

Subsequently more detailed studies with *P. verrucosum* were performed as this species is more important in bread contamination. These showed that there is potential for use

of an e-nose to differentiate between mycotoxigenic and non-toxigenic strains of *Penicillium verrucosum* before signs of visible growth. *In vitro* studies on 2% wheat agar showed that it was possible to differentiate a non-toxin producing *P. verrucosum* strain from 4 citrinin producing strains and controls. With a modified a_w of 0.95 a_w it was not possible to differentiate the non-toxigenic strain and 72 h were required for discrimination of controls. On bread analogues it was possible to discriminate two OTA producing strains after 24 h but with increased incubation only controls were differentiated. Starting with a lower initial spore concentration of $1x10^3$ cfu ml^{-1} reduced the number of strains, which could be discriminated. It was not possible to differentiate the non-toxin producers, which could be differentiated with the higher initial spore inoculum. This may indicate that the threshold for discrimination of mycotoxin producers is above $1x10^3$ cfu ml^{-1}. Cross validation of strains was better with the initial population of $1x10^6$ cfu ml^{-1} with 65% correctly classified.

Even in inoculated bread not all mycotoxigenic fungi are able to produce mycotoxins (Legan, 1993), which may account for the inability to discriminate between some of the strains. Again early analysis before the visible presence of growth was of interest, which probably occurred before significant mycotoxin synthesis. This may explain the level of discrimination achieved.

Previously, Keshri and Magan (2000) obtained similar results *in vitro* on 2% wheat agar at 0.95 a_w using strains of *Fusarium moniliforme* ($\equiv$ *F. verticillioides*) and *Fusarium proliferatum*. With a BH114 e-nose they achieved good reproducibility of volatile patterns between replicates of the same treatment. PCA indicated that

discrimination could be achieved between uninoculated controls, a non-mycotoxigenic strain and three mycotoxin producing strains for both *Fusarium* species after 48 h. Measurements were not taken after 24 h incubation.

Olsson *et al*. (2002) investigated the possibility of using fungal volatiles as indicators of mycotoxins in grain. Using an electronic nose and GC-MS, they noted the potential for predicting levels of the mycotoxins OTA and deoxynivalenol (DON) with either the e-nose or GC-MS. Zeringue *et al*. (1993) examined the headspace of four aflatoxigenic and four non-toxigenic strains of *A. flavus* in submerged culture using gas chromatograph over 10 days. They discovered that *A. flavus* produced several distinct compounds, which were only present in aflatoxin producing strains. Sesquiterpenes peaked in 3-day cultures and were not present in earlier or later cultures. There was a correlation noted between release of sesquiterpenes and initiation of aflatoxin biosynthesis, and a correlation between decline of aflatoxin synthesis and the disappearance of sesquiterpenes unique to aflatoxigenic *A. flavus*. Jeleń *et al*. (1995) also investigated sesquiterpene production of mycotoxigenic and non-toxigenic strains of *Fusarium sambucinum* infecting wheat grain incubated for 5 days at 25°C. They showed that the toxin producing strains produced greater amounts of diverse sesquiterpenes compared to non-toxigenic strains. They also found that the profiles of volatile sesquiterpenes were similar for all mycotoxigenic isolates examined, although the concentration varied in the different strains, whereas for non-toxigenic isolates the volatile profiles were different. Börjesson et al. (1989) also found that different strains of *Fusarium culmorum* gave rise to entirely different patterns of volatile metabolites.

Pasanen *et al*. (1996) showed differences between the volatile production patterns for toxigenic and non-toxigenic strains of *P. verrucosum,* on oat grains using GC. The main volatiles detected included oct-1-en-3-ol, 3-methylbutan-2-ol, octan-1-ol, octan-3-ol, octan-3-one, hexan-2-one, heptan-2-one, α-pinene, and limonene. A relationship between the synthesis of mycotoxins and the relative proportion of different volatile groups was also detected, which was proposed to probably result from similar metabolic pathways. The production of volatile terpenes appeared to be linked to the formation of trichothecenes in *Fusarium sporotrichiodes* cultures. A OTA producing strain of *P. verrucosum* showed accelerated production of volatile ketones compared to that by a non-toxigenic strain. However, in their study the spore concentrations used were much lower for the non-toxigenic than the toxigenic strain. Recently, Abramson *et al*. (2004) looked at mycotoxins, ergosterol, and odour volatiles produced in duram wheat during granary storage at 16% and 20% moisture content. Signals from nine metal-oxide sensors used to monitor odour volatiles showed good correlation with OTA formation at 20% moisture content.

When analysed together it was possible to differentiate between strains growing on bread and agar and between bread samples with different initial populations. As mentioned previously volatile production can alter with growth on different substrates.

Potential may also exist to use e-noses to differentiate toxin producing pathogenic bacteria such as *Staphylococcus aureus* on food, which can produce a wide array of toxins.

4.6 SCREENING OF ANTIOXIDANTS USING E-NOSE TECHNOLOGY

To control contamination and growth of spoilage micro-organisms on bread chemical preservatives such as potassium sorbate and calcium propionate are incorporated. Typically bakeries use 0.2% calcium propionate based on flour weight although the maximum permitted level is 0.3% in England and Wales (Legan, 1993). The need to reduce concentrations of chemical preservatives in foodstuffs as a response to consumer demand for artificial additive free, fresh and mild processed foods has increased significantly. Also recently calcium propionate has been linked with attention deficit disorder in children (Dengate and Ruben, 2002). This study has examined, for the first time, the potential for use of electronic noses as a tool for screening novel antioxidants and preservative.

The antioxidants butylated hydroxyanisole (BHA) and propyl paraben (PP) were chosen because they are not odorous and therefore should not greatly affect the sensor responses. Antioxidants were also chosen because BHA, although used in foods primarily to prevent auto-oxidation of lipids, has been shown to possess antimicrobial activity against a wide range of micro-organisms. PP, which is a derivative of benzoic acid, also has a proven antimicrobial activity.

Analysis of all treatments at both a_w levels only allowed differentiation of samples without an antioxidant. Due to the high number of samples it was difficult to see differences between treatments containing an antioxidant. However, when samples without antioxidant were removed from analysis it was possible to see differences between antioxidant treatments alone and those inoculated with the micro-organisms *P.*

verrucosum, *P. anomala* and *B. subtilis* for both antioxidants. The BH114 was able to differentiate *P. verrucosum* after 24 h and *B. subtilis* and *P. anomala* after 48 h whereas the NST3220 was able to discriminate all after 24 h. However a 3rd PC was needed for *P. verrucosum* and *B. subtilis* when analysing the NST3220 data, which was not possible with the BH114 analysis. It was also possible to discriminate between the sample times.

Carbon dioxide levels and cfus increased with incubation time and were greater in inoculated samples when compared to uninoculated controls. Larger populations of micro-organisms were detected in 0.97 a_w treatments when compared to 0.95 a_w. Populations of *P. verrucosum* and *P. anomala* were significantly greater in broths containing no antioxidant. In contrast *B. subtilis* populations were greater in broths containing the antioxidants after 48 and 72 h. When grown in 0.97 a_w broth *P. verrucosum* and *B. subtilis* produced more CO_2 in the presence of antioxidant after 24 h, however after 48 and 72 h more CO_2 was produced in broth without an antioxidant.

GC-MS showed differences in production of compounds between micro-organisms for example hexanol production by *P. anomala* was significantly less than that by *B. subtilis* and *P. verrucosum*. There was no significant difference between levels of volatiles produced by *P. anomala* in broths with and without antioxidant. However, production of 2-pentylfuran by *B. subtilis* was lower in broth without an antioxidant after 48 and 72 h. There were significant differences in production of methylbenzene, hexanal and 2-pentlyfuran by *P. verrucosum* growing in treatments with and without an antioxidant.

Stimulation of growth was observed with low concentrations of antioxidant, especially of *B. subtilis*, which has been noted previously by other authors. Marín *et al*. (2002) showed that sub-optimal concentrations, 0.003% and sometimes 0.03%, of the weak acid preservatives calcium propionate, potassium sorbate and sodium benzoate led to an enhancement of fungal growth on bakery products. Guynot et al. (2002) also noted that sorbate, benzoate and propionate at 0.03% acted as a growth promoter of *Eurotium* isolates on sponge cake analogues. Marín *et al*. (2003) when studying the efficacy of sorbic acid and potassium sorbate on growth of different *Eurotium* isolates when added to a bakery product analogue noted that 0.025% and 0.05% concentrations of preservative always enhanced growth. Arroyo (2002) observed that the use of very low concentrations of BHA and particularly PP (50 ppm) on wheat flour agar resulted in enhancement of mould growth. Other authors have suggested the possible adverse effect of BHA at very low doses (10 ppm) on fungal toxin production, finding almost twice the amount of toxin produced in the presence of 10 ppm BHA compared to the controls and with no effect on mycelial growth (Ray and Bullerman, 1982).

Measurement of growth rates has been used to assess the effect of novel preservatives on grain. Reynoso *et al*. (2002) noted an increase in lag phase of *Fusarium verticilliodes* and *Fusarium proliferatum* with a combination of PP and BHA incorporated in a maize based media. For both *Fusarium* species PP alone or in combination with BHA, at concentrations of 0.5 and 1 mM reduced growth rates by >85%. However, at low concentrations of antioxidant (0.5 mM) they observed some stimulation in fumonisin toxin production. Torres *et al*. (2003) also showed the potential for PP and BHA to be used for the treatment of maize grain for controlling

mycotoxigenic *Fusarium* species. They noted that both antioxidants had an effect on growth and also decreased production of the mycotoxin fumonisin. Arroyo (2002) showed that BHA had a stronger antifungal activity than PP especially at low concentrations (50-150 ppm) *in vitro*. Fungal growth was completely inhibited by both antioxidants at doses of 500ppm.

Thomson (1992) concluded that BHA was a more effective phenolic antioxidant than PP for inhibiting growth of several *Aspergillus*, *Penicillium* and *Fusarium* species on PDA. They also found higher concentrations of antioxidant were needed for *Aspergillus* than for *Penicillium* and *Fusarium* cultures. However, no interaction with the matrix or the effect of a_w or temperature was considered

The antioxidants may also have a different inhibitory effect when added to bread. Guynot *et al*. (2003) found that cinnamon leaf, clove bay, lemongrass and thyme essential oils totally inhibited 7 filamentous fungi when tested on a wheat flour based medium. However, when the essential oils were tested in sponge cake analogues the antifungal activity detected was much more limited.

4.7   DISCRIMINATION OF UNKNOWN SAMPLES ON NATURAL BREAD

White bread was inoculated with four unknown treatments (A- *P. anomala*, B- *B. subtilis*, C- *P. verrucosum* and D- control) at levels important in bread spoilage as stated in the UK bread specifications.

The BH114 e-nose was able to differentiate between all treatments after 48 h and the NST3220 after 72 h. A possible explanation for not being able to differentiate between *B. subtilis* and the other treatments is that serial dilutions showed presence of bacteria on all treatments although levels were higher on analogues inoculated with treatment B (*B. subtilis*) after 72 h. Plate counts could be used to identify the different treatments if the background levels of bacteria were considered.

CO_2 measurement could be used to detect the control treatment but there was no significant difference between the different micro-organisms except for a large increase of *P. verrucosum* after 72 h. Analysis of CO_2 also showed a significant increase in production between 24 and 48 h. Börjesson *et al*. (1989) also noted a rise in the concentration of CO_2 and volatiles two days after inoculation of fungi, before growth was visually detectable. Börjesson *et al*. (1992) noted that the production of volatiles was more strongly correlated with accumulated CO_2 production than with actual CO_2 production and more strongly correlated with ergosterol contents of grain than with numbers of CFU when analysing six fungal species on moistened and autoclaved wheat and oat grains using GC-MS.

Natural food studies have predominately been in relation to mould contamination of raw product, particularly wheat and barley grain, which have been reviewed by Schnürer *et al*. (1999) and Magan and Evans (2000). Some studies have recently examined the activity and relative colonisation patterns of cakes by spoilage moulds (Abellana *et al*., 1999a; 1999b) in relation to environmental regimes, but not for early detection or volatile production.

Using a BH114 e-nose Keshri (2001) was able to differentiate fungal species on natural bread from uninoculated controls after 24 h. Using GC-MS, Harris *et al*. (1986) examined several *actinomycetes* and fungi grown on agar and whole wheat bread. They noticed that for *Penicillium roqueforti*, *A. flavus* and *A. niger*, more alcohols and ketones such as 3-octanol and 1-octen-3-ol were produced in greater amounts on bread when compared to agar substrate. They proposed that the high yield of 8-carbon compounds on whole wheat bread compared to the agar medium was probably related to the lack of fatty acids in the latter medium.

Electronic noses have largely been applied to microbial quality classification of grain. Jonsson *et al*. (1997) able to predict whether samples of different grain types were good, mouldy, weakly or strongly musty. Evans *et al*. (2000) achieved a 92.3% classification of grain as good or bad with no bad samples misclassified as good. Börjesson *et al*. (1996) obtained correct classification of approximately 75% of grain samples using a four-class system of mouldy/musty, acid/sour, burnt or normal and 90% when using a two-class system of good or bad. Olsson *et al*. (2000) used an e-nose to classify maize samples with only 3 samples out of 40 being misclassified. They also noted a stronger correlation of e-nose measurements with predicted ergosterol level was obtained than with predicted cfu values.

Magan et al (2003) developed a real-time grain monitoring system containing 32 conducting polymer sensors, which was able to evaluate samples of grain in less than 10 minutes and give information on whether the sample was "good", "bad" or "intermediate" using neural networks. Thus for further development and application of

e-nose technology to the bakery production industry in quality assurance, appropriate

e-nose assays combined with real time analysis and investigation of neural networks is

required.

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1   CONCLUSIONS

➤ Static incubation of sample bags for 1 h is sufficient to achieve early detection and differentiation of microbial spoilage.

➤ Early detection of microbial spoilage, based on their volatile production profiles, was achieved *in vitro* on agar-based media.   Differentiation of *P. verrucosum*, *A. ochraceus* and *P. anomala* was possible, using initial populations of 10^6 spores/cells ml^{-1}, after only 24 h.

➤ The BH114 e-nose could discriminate *P. fragi*, *S. aureus* and *S. cerevisiae* grown in different water activity broths in exponential and stationary phases of growth.

➤ It was possible to differentiate between bread analogues with and without microbial spoilage using a BH114 e-nose before signs of visible growth occurred. Differentiation of *B. subtilis*, *S. cerevisiae* and controls was possible after 24 h.

➤ Discrimination of microbial spoilage on bread analogues was improved using an incubation temperature of 25°C when compared to 15°C.

➤ An E-nose and cfu counts produced better discrimination of microbial and physiological spoilage of bread analogues than GC-MS.

➤ A BH114 e-nose could differentiate between microbial and physiological spoilage (by lipoxygenase) of bread analogues after 48 h.

➤ Measurements of microbial populations showed significant differences between populations of each micro-organism after 48 and 72 h.

➤ Analysing volatiles produced using GC-MS only allowed differentiation of *P. anomala* from *B. subtilis*, *P. verrucosum*, lipoxygenase and controls after 24 h

as it was the only treatment to produce 2-propanol, ethyl acetate and pentanol. GC-MS did however show differences between volatile profiles of microbial and physiological spoilage over time.

➢ There is potential for use of electronic noses to differentiate between toxigenic and non-toxigenic strains of micro-organisms. It was possible to differentiate two OTA producing strains of *P. verrucosum in situ* on bread analogues after 24 h incubation.

➢ An initial population between 10^3 and 10^6 spores ml^{-1} is required for early differentiation of mycotoxigenic and non-mycotoxigenic strains of *P. verrucosum* as with an initial population of 10^3 discrimination required at least 72 h.

➢ There is potential for use of an e-nose as a rapid method for screening of novel antioxidants. A BH114 e-nose and NST3220 lab emission analyser were able to differentiate broth samples containing an antioxidant (either propyl paraben or butylated hydroxyanisole) that had been inoculated with micro-organisms and those that had not.

➢ The e-noses were better at discriminating samples than GC-MS.

➢ Differentiation of four unknown samples on natural bread was possible with a BH114 e-nose, after 48 h incubation, and a NST3220 lab emission analyser, after 72 h, using initial microbial populations of 10^3. CO_2 measurements, taken by the NST3220, could be used to discriminate *P. verrucosum*, *B. subtilis* and *P. anomala* from controls but not to differentiate between the microbial species.

➢ Addition of a 3rd or 4th PC can increase discrimination by separating treatments on another plane.

5.2   FUTURE WORK

1. Develop and apply artificial neural networks for identification of unknown samples.

2. Apply to commercially available bread. This could include different types of bread, for example, wholegrain and white. The range of products tested could also be extended to other bakery products.

3. Further studies of antioxidant or preservative screening. These could include incorporating the antioxidant in bread analogues for preservative screening and looking at other novel antioxidants or preservatives. Look at using different levels of antioxidant (below recommended limits) to observe if an electronic nose can differentiate between the different levels of inhibition and be used to decide on the ideal concentration.

4. Investigate the detection limits of the electronic nose by using different initial inoculum levels to observe the level needed for good differentiation of micro-organisms.

5. Comparison with other rapid methods for early detection and differentiation of bread spoilage along with commercial viability.

6. Direct comparisons of QA applications with existing techniques.

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APPENDIX I

ANTIOXIDANT SCREEN
RAW DATA

APPENDIX I ANTIOXIDANT SCREEN RAW DATA

Inhibition of *Bacillus cereus* growth on 0.95 a_w 2% wheat agar by different concentrations of antioxidant propyl paraben.

Key: PP = propyl paraben; meth = methanol; - = no growth

Time	Concentration of PP in 0.95 a_w 2% wheat agar					
	no PP no meth	no PP + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	3	3	2	2	1	-
	3	3	2	2	1	-
			2	2	1	-
3 d	5	5	2	2	1	-
	5	5	2	2	1	-
			2	2	1	-
5 d	8	8	2	2	1	-
	8	8	2	2	1	-
			3	2	1	-
7 d	10	10	4	4	4	-
	10	10	4	4	4	-
			4	4	4	-

Inhibition of *Bacillus cereus* growth on 0.97 a_w 2% wheat agar by different concentrations of antioxidant propyl paraben.

Key: PP = propyl paraben; meth = methanol

Time	Concentration of PP in 0.97 a_w 2% wheat agar					
	no PP no meth	no PP + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	3	3	2	2	2	1
	3	3	2	2	2	1
			2	2	2	1
3 d	5	5	5	5	4	3
	5	5	5	5	4	3
			5	5	4	3
5 d	8	8	6	6	6	4
	8	8	6	6	6	4
			6	6	6	4
7 d	10	10	8	8	6	5
	10	10	8	8	6	6
			8	8	6	5

Inhibition of *Bacillus cereus* growth on 0.95 a$_w$ 2% wheat agar by different concentrations of antioxidant butylated hydroxyanisole.

Key: BHA = butylated hydroxyanisole; meth = methanol; - = no growth

| Time | Concentration of BHA in 0.95 a$_w$ 2% wheat agar | | | | | |
	no BHA no meth	no BHA + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	3	3	2	2	2	-
	3	3	2	2	2	-
			2	2	2	-
3 d	5	5	2	2	4	-
	5	5	2	2	4	-
			2	2	4	-
5 d	8	8	2	2	4	-
	8	8	2	2	4	-
			2	2	4	-
7 d	10	10	4	4	6	-
	10	10	4	4	6	-
			4	4	6	-

Inhibition of *Bacillus cereus* growth on 0.97 a$_w$ 2% wheat agar by different concentrations of antioxidant butylated hydroxyanisole.

Key: BHA = butylated hydroxyanisole; meth = methanol; - = no growth

| Time | Concentration of BHA in 0.97 a$_w$ 2%wheat agar | | | | | |
	no BHA no meth	no BHA + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	3	3	1	1	*	-
	3	3	1	1	*	-
			1	1	*	-
3 d	5	5	3	2	*	-
	5	5	3	2	*	-
			3	2	*	-
5 d	8	8	3	3	*	½
	8	8	3	3	*	½
			3	3	*	½
7 d	10	10	4	4	*	1
	10	10	4	4	*	1
			4	4	*	1

Inhibition of *Bacillus subtilis* growth on 0.95 a_w 2% wheat agar by different concentrations of antioxidant propyl paraben.

Key: PP = propyl paraben; meth = methanol; - = no growth

Time	Concentration of PP in 0.95 a_w 2% wheat agar					
	no PP no meth	no PP + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
d	1	1	-	-	-	-
	1	1	-	-	-	-
			-	-	-	-
5 d	2	2	-	-	-	-
	2	2	-	-	-	-
			-	-	-	-
7 d	4	4	1	-	-	-
	4	4	1	-	-	-
			-	-	-	-
11 d	5	5	1	1	-	-
	5	5	1	1	-	-
			1	1	-	-

Inhibition of *Bacillus subtilis* growth on 0.97 a_w 2% wheat agar by different concentrations of antioxidant propyl paraben.

Key: PP = propyl paraben; meth = methanol; - = no growth

Time	Concentration of PP in 0.97 a_w 2% wheat agar					
	no PP no meth	no PP + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
3 d	½	-	-	-	-	-
	½	-	-	-	-	-
			-	-	-	-
5 d	1	½	-	-	-	-
	1	½	-	-	-	-
			-	-	-	-
7 d	2	2	1	1	1	-
	2	2	1	1	1	-
			1	1	1	-
11 d	2	2	2	2	1	1
	2	2	2	2	1	1
			2	2	1	1

Inhibition of *Bacillus subtilis* growth on 0.95 a_w 2% wheat agar by different concentrations of antioxidant butylated hydroxyanisole.

Key: BHA = butylated hydroxyanisole; meth = methanol; - = no growth

| Time | Concentration of BHA in 0.95 a$_w$ 2% wheat agar | | | | | |
	no BHA no meth	no BHA + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
3 d	1	1	-	-	-	-
	1	1	-	-	-	-
			-	-	-	-
5 d	2	2	-	-	-	-
	2	2	-	-	-	-
			-	-	-	-
7 d	4	4	1	1	3	-
	4	4	1	1	3	-
			1	1	3	-
11 d	5	5	2	2	3	-
	5	5	2	2	3	-
			2	2	3	-

Inhibition of *Bacillus subtilis* growth on 0.97 a_w 2% wheat agar by different concentrations of antioxidant butylated hydroxyanisole.

Key: BHA = butylated hydroxyanisole; meth = methanol; - = no growth

| Time | Concentration of BHA in 0.97 a$_w$ 2% wheat agar | | | | | |
	no BHA no meth	no BHA + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
3 d	½	-	-	-	-	-
	½	-	-	-	-	-
			-	-	-	-
5 d	1	½	-	-	-	-
	1	½	-	-	-	-
			-	-	-	-
7 d	2	2	1	1	2	½
	2	2	-	1	2	1
			-	1	2	1
11 d	2	2	2	2	2	1
	2	2	2	2	2	1
			2	2	2	1

Inhibition of *Debaromyces hansenii* growth on 0.95 a$_w$ 2% wheat agar by different concentrations of antioxidant propyl paraben.

Key: PP = propyl paraben; meth = methanol; - = no growth

| Time | Concentration of PP in 0.95 a$_w$ 2% wheat agar | | | | | |
	no PP no meth	no PP + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
3 d	9 x 8	9 x 9	7 x 8	7 x 6	7 x 7	-
	10 x 8	8 x 9	6 x 7	8 x 6	6 x 7	-
			7 x 8	7 x 7	6 x 7	-
5 d	10 x 9	9 x 9	7 x 8.5	7 x 7	7.5 x 7	3 .5 x 4
	9 x 9	9 x 9.5	7 x 7	8 x 7	6 .5 x 7	3 x 4
			7.5 x 8	7 x 7	7 x 7	2.5 x 4
7 d	10 x 10	11 x 9	11 x 9	12 x 9	10 x 9	6 x 7
	10 x 11	10 x 11	11 x 10	13 x 9	10 x 8	5 x 6
			12 x 9	11 x 9	10 x 9.5	6 x 7

Inhibition of *Debaromyces hansenii* growth on 0.97 a$_w$ 2% wheat agar by different concentrations of antioxidant propyl paraben.

Key: PP = propyl paraben; meth = methanol; - = no growth

| Time | Concentration of PP in 0.97 a$_w$ 2% wheat agar | | | | | |
	no PP no meth	no PP + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
3 d	10 x 8	10 x 8	9 x9	9 x 9	10 x 8	9 x 8
	10 x 8	11 x 7	8 x 9	8 x 9	9 x 9	8 x 9
			10 x 8	10 x 9	9 x 9	9 x 9
5 d	10 x 9	9 x 12	9 x 9	9 x 9.5	10 x 9	10 x 9
	9 x 11	11 x 8.5	8 x 9.5	9 x 9	9 x 10	9 x 9
			10 x 9	10 x 10	9.5 x 9	9 x 9
7 d	10 x 10	12 x 9	13 x 9	18 x 9	12 x 9	10 x 8
	10 x 11	9 x 13	12 x 10	17 x 9	14 x 8	11 x 9
			15 x 8	18 x 9	16 x 9	11 x 10

Inhibition of *Debaromyces hansenii* growth on 0.95 a$_w$ 2% wheat agar by different concentrations of antioxidant butylated hydroxyanisole.

Key: BHA = butylated hydroxyanisole; meth = methanol; - = no growth

Time	Concentration of BHA in 0.95 a$_w$ 2%wheat agar					
	no BHA no meth	no BHA + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
3 d	9 x 8	9 x 9	8 x 8	7 x 6	7 x 7	-
	10 x 8	8 x 9	6 x 7	7.5 x 6	6.5 x 7	-
			7 x 7	7 x 7	6 x 7	-
5 d	9 x 10	9 x 12	8 x 9	9 x 8	7 x 7	5 x 4
	10 x 8.5	11 x 8.5	7 x 8	9 x 8	7 x 7	3 x 4.5
			8.5 x 7	8 x 7	8 x 7	4 x 4.5
7 d	10 x 10	12 x 10	10 x 9	9 x 10	8 x 8	5 x 4
	10 x 11	10 x 12	10 x 9.5	10 x 9	9 x 8.5	6 x 5
			10 x 10	9 x 8	9 x 8	5.5 x 5

Inhibition of *Debaromyces hansenii* growth on 0.95 a$_w$ 2% wheat agar by different concentrations of antioxidant butylated hydroxyanisole.

Key: BHA = butylated hydroxyanisole; meth = methanol; - = no growth

Time	Concentration of BHA in 0.97 a$_w$ 2% wheat agar					
	no BHA no meth	no BHA + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
3 d	10 x 8	10 x 8	11 x 7	9 x 8	7 x 7	7.5 x 6
	10 x 8	11 x 7	11 x 7	9 x 8	7 x 7	8 x 6.5
			10 x 7	8 x 7	8 x 7	7 x 8.5
5 d	9 x 10	9 x 12	8 x 10	9 x 8	7 x 7	7.5 x 6
	11 x 8.5	11 x 8.5	11 x 8	9 x 8	7 x 7	8 x 6.5
			11 x 7	8 x 7	8 x 7	7 x 8.5
7 d	10 x 10	12 x 9	10 x 8	9 x 10	9 x 8	9 x 9
	10 x 11	9 x 13	11 x 9	10 x 9	9 x 9	8 x 8
			11 x 10	10 x 8	9 x 8	8 x 8

Inhibition of *Pichia anomala* growth on 0.95 a$_w$ 2% wheat agar by different concentrations of antioxidant propyl paraben.

Key: PP = propyl paraben; meth = methanol; - = no growth

Time	Concentration of PP in 0.95 a$_w$ 2% wheat agar					
	no PP no meth	no PP + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
3 d	7 x 8	7.5 x 8	7 x 5	4 x 6	6 x 5	-
	8 x 7.5	8 x 8	6 x 5	6 x 5	5 x 4	-
			6.5 x 7	6 x 7	6 x 4	-
5 d	8 x 9	9 x 9	7 x 7.5	7 x 7	7.5 x 7	3 .5 x 4
	9 x 9	9 x 9.5	7 x 7	8 x 7	6 .5 x 7	3 x 4
			7.5 x 8	7 x 7	7 x 7	2.5 x 4
7 d	10 x 10	10 x 9.5	10 x 9	10 x 9	10 x 9	6 x 6
	10 x 9	10 x 10.5	10 x 10	11 x 9	10 x 8	5 x 6
			11 x 9	10 x 10	9 x 9.5	6 x 6.5

Inhibition of *Pichia anomala* growth on 0.97 a$_w$ 2% wheat agar by different concentrations of antioxidant propyl paraben.

Key: PP = propyl paraben; meth = methanol; - = no growth

Time	Concentration of PP in 0.97 a$_w$ 2% wheat agar					
	no PP no meth	no PP + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
3 d	6 x 8	7 x 8	7 x 7	7 x 7	6.5 x 8	7 x 6
	7 x 8	7 x 7	8 x 6.5	8 x 7	8 x 9	6.5 x 6
			7 x 8	9 x 9	7 x 8	6.5 x 7
5 d	9 x 9	9 x 10	8 x 9	8 x 9.5	9 x 9	8 x 8.5
	9 x 10	10 x 9.5	8 x 8.5	9 x 8.5	9 x 8	7.5 x 8
			9 x 9	9 x 10	9.5 x 9	7 x 8
7 d	10 x 10	12 x 9	11 x 10	14 x 9	11 x 9.5	9 x 8
	10 x 11	10 x 12	11 x 10	15 x 10	12 x 9	10 x 9
			13 x 8	15 x 9	14 x 9	10 x 10

Inhibition of *Pichia anomala* growth on 0.95 a$_w$ 2% wheat agar by different concentrations of antioxidant butylated hydroxyanisole.

Key: BHA = butylated hydroxyanisole; meth = methanol; - = no growth

Time	Concentration of BHA in 0.95 a$_w$ 2% wheat agar					
	no BHA no meth	no BHA + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
3 d	9 x 8	8 x 9	7 x 6	7 x 6	6.5 x 6	-
	8 x 8	8 x 8.5	6 x 5	7.5 x 6	6.5 x 5	-
			6 x 7	7 x 7	6 x 5	-
5 d	9 x 9.5	9 x 10	8 x 8.5	8.5 x 8	6 x 7	4.5 x 4
	9 x 8.5	10 x 8.5	7 x 8	8.5 x 8	7 x 6.5	3 x 4
			8 x 7	7.5 x 7	7 x 7	4 x 3.5
7 d	10 x 10	11 x 10	10 x 9	9.5 x 8	8 x 8	5 x 4
	10 x 11	11 x 9.5	9 x 9.5	10 x 9	9 x 8.5	5 x 5
			10 x 10	9 x 8	8 x 8	4.5 x 5

Inhibition of *Pichia anomala* growth on 0.97 a$_w$ 2% wheat agar by different concentrations of antioxidant butylated hydroxyanisole.

Key: BHA = butylated hydroxyanisole; meth = methanol; - = no growth

Time	Concentration of BHA in 0.97 a$_w$ 2% wheat agar					
	no BHA no meth	no BHA + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
3 d	10 x 8	10 x 8	10 x 7	8.5 x 8	7 x 7	6.5 x 5
	10 x 8	11 x 7	9.5 x 7	9 x 8	7 x 7	6 x 6.5
			9 x 8	8 x 7.5	6.5 x 7	7 x 6.5
5 d	9 x 10	9 x 12	8 x 10	9 x 8	7.5 x 7	7.5 x 6
	11 x 8.5	11 x 8.5	10.5 x 8	9 x 8	7 x 7	8 x 6.5
			10 x 7	8 x 7	8 x 7	7 x 7.5
7 d	10 x 10	12 x 9	10 x 8	9 x 10	9 x 8	8 x 9
	10 x 11	10 x 12.5	11 x 9	10 x 9	9 x 9	7.5 x 8
			10 x 10	10 x 8	9 x 8	8 x 8

Inhibition of *Penicillium verrucosum* IBT22626 growth on 0.95 a_w 2% wheat agar by different concentrations of antioxidant propyl paraben.

Key: PP = propyl paraben; meth = methanol; - = no growth

Time	Concentration of PP in 0.95 a_w 2% wheat agar					
	no PP no meth	no PP + meth	10 ppm	20 ppm	50 ppm	100 ppm
2 d	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
3 d	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
5 d	10 x 11	8 x 7	5 x 5	-	-	-
	12 x 11	9 x 8	6 x 5	-	-	-
			5 x 6	-	-	-
7 d	15 x 16	13 x 12	13 x 12	12 x 11	10 x 8	-
	16 x 16	14 x 13	12 x 13	12 x 13	8 x 8	-
			13 x 13	11x 11	8 x 9	-
11 d	26 x 25	25 x 25	20 x 21	20 x 19	15 x 16	-
	25 x 26	23 x 25	20 x 20	19 x 18	15 x 14	-
			20 x 20	20 x 19	14 x 19	-
28 d	covers	covers	covers	60 x 60	62 x 50	25 x 23
	Petri dish	Petri dish	Petri	70 x 50	56 x 60	25 x 22
			dish	58 x 59	60 x 53	23 x 24

Inhibition of *Penicillium verrucosum* IBT22626 growth on 0.97 a_w 2% wheat agar by different concentrations of antioxidant propyl paraben.

Key: PP = propyl paraben; meth = methanol; - = no growth

Time	Concentration of PP in 0.97 a_w 2% wheat agar					
	no PP no meth	no PP + meth	10 ppm	20 ppm	50 ppm	100 ppm
48 h	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
72 h	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
5 d	13 x 14	9 x 8	9 x 9	7 x 8	7 x 7	-
	11 x 10	10 x 8	9 x 9	9 x 9	7 x 7	-
			10 x 9	8 x 9	7 x 7	-
7 d	16 x 15	14 x 13	13 x 13	12 x 12	10 x 10	6 x 5
	15 x 15	13 x 13	14 x 13	12 x 11	10 x 9	5 x 5
			12 x 13	11 x 11	10 x 9	4 x 5
11 d	24 x 25	21 x 22	20 x 20	20 x 20	15 x 17	10 x 10
	28 x 29	22 x 20	20 x 21	22 x 21	15 x 16	10 x 11
			20 x 21	21 x 20	17 x 16	9 x 9
28 d	covers	covers	55 x 56	54 x 55	47 x 47	25 x 25
	Petri dish	Petri dish	53 x 60	55 x 55	45 x 46	33 x 34
			50 x 60	54 x 56	44 x 43	32 x 29

Inhibition of *Penicillium verrucosum* IBT22626 growth on 0.95 a_w 2% wheat agar by different concentrations of antioxidant butylated hydroxyanisole.

Key: BHA = butylated hydroxyanisole; meth = methanol; - = no growth

Time	Concentration of BHA in 0.95 a_w 2% wheat agar					
	no BHA no meth	no BHA + meth	10 ppm	20 ppm	50 ppm	100 ppm
48 h	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
72 h	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
5 d	10 x 11	8 x 7	6 x 5	6 x 5	5 x 5	-
	12 x 11	9 x 8	7 x 6	7 x 6	5 x 5	-
			7 x 6	7 x 6	4 x 5	-
7 d	15 x 16	13 x 12	12 x 14	10 x 10	10 x 10	5 x 6
	16 x 16	14 x 13	14 x 13	12 x 10	10 x 9	6 x 5
			13 x 13	10 x 10	9 x 9	6 x 6
11 d	26 x 25	25 x 25	20 x 19	22 x 20	20 x 18	10 x 10
	25 x 26	23 x 25	20 x 19	20 x 21	18 x 18	11 x 12
			19 x 18	20 x 19	18 x 17	12 x 11
28 d	covers	covers	55 x 55	56 x 55	50 x 50	40 x 36
	Petri dish	Petri dish	covers	60 x 58	51 x 52	40 x 35
			cover	60 x 55	51 50	38 x 38

Inhibition of *Penicillium verrucosum* IBT22626 growth on 0.97 a_w 2% wheat agar by different concentrations of antioxidant butylated hydroxyanisole.

Key: BHA = butylated hydroxyanisole; meth = methanol; - = no growth

Time	Concentration of BHA in 0.97 a_w 2% wheat agar					
	no BHA no meth	no BHA + meth	10 ppm	20 ppm	50 ppm	100 ppm
48 h	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
72 h	-	-	-	-	-	-
	-	-	-	-	-	-
			-	-	-	-
5 d	13 x 14	9 x 10	9 x 10	10 x 10	6 x 5	-
	11 x 10	10 x 8	11 x 10	11 x 9	7 x 4	-
			10 x 10	10 x 10	5 x 6	-
7 d	16 x 15	14 x 13	13 x 13	12 x 13	10 x 11	8 x 9
	15 x 15	13 x 13	12 x 13	13 x 13	12 x 11	8 x 7
			12 x 12	13 x 13	12 x 1	8 x 8
11 d	24 x 25	21 x 22	24 x 23	21 x 20	18 x 17	14 x 13
	28 x 29	22 x 20	23 x 24	23 x 24	18 x 19	15 x 16
			23 x 20	23 x 20	17 x 18	12 x 14
28 d	covers	covers	56 x 58	55 x 57	43 x 45	38 x 36
	Petri dish	Petri dish	56 x 55	53 x 56	44 x 41	37 x 38
			57 x 55	56 x 60	42 x 44	38 x 36

APPENDIX II

PUBLICATIONS

APPENDIX 2 PUBLICATIONS

PAPERS

Needham, R & Magan, N. (2002). Detection and differentiation of microbial spoilage organisms of bakery products *in vitro* and *in situ*. In *Proceedings of the Ninth International Symposium on Olfaction and Electronic Nose, ISOEN'02, Rome, Sep 29-Oct 2, 2002,* pp.385-388. Edited by A. D'Amico & C. DiNatale. Rome: Aracne.

Magan, N, Keshri, G., Needham, R. & Sneath, R. (2003). Use of electronic nose technology for the early detection of spoilage moulds in cereal products. In *Advances in Stored Product Protection,* pp. 139-143. Edited by P. Credland,, M. Armitage, C. H. Bell & P. M. Colgan. CABI International.

Needham, R. & Magan, N. (2003). Detection and differentiation of toxigenic and non-toxigenic *Penicillium verrucosum* strains on bakery products using an electronic nose. *Aspects of Applied Biology*, **68**, 217-221.

Needham, R., Williams, J., Beales, N., Voysey, P. & Magan, N. ( ). Early detection and differentiation of spoilage of bakery products. Accepted by Sensors and Actuators B.

Several papers are currently in production

POSTERS

Needham, R & Magan, N. (2002). Use of Electronic Nose Technology to discriminate spoilage Micro-organisms Growing on Bakery Products. Silsoe Postgraduate Conference 2002 and Howard Eggins Meeting and Postgraduate Symposium. Liverpool July 10th-11th 2002.

Needham, R. & Magan, **N. (2002)** Detection and Differentiation of Microbial Spoilage Organisms of Bakery Products *in vitro* and *in situ*. 9th International Symposium on Olfaction and Electronic Nose ISOEN'02. Rome, Italy Sep. 29th – Oct. 2nd 2002.

Needham, R. & Magan. N. (2003) Detection and Differentiation of toxigenic and Non-Toxigenic *Penicillium verrucosum* Strains on Bread using Electronic Nose Technology. Mycotoxins in Food Production Systems. Bath, England June 25th-27th 2003.

Needham, R., Williams, J. & Magan, N. (2003) Early Detection and Differentiation of spoilage Micro-organisms of Bakery Products. The 10th International Symposium on Olfaction and Electronic Nose ISOEN'03. Riga, Latvia June 26th-28th 2003.