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The Rapid Analysis of Fungal Growth in the Presence of Inhibitory Effects

Cranfield Health
Applied Microbiology

Masters by Research
Academic Year: 2010 - 2011

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September 2011

Abstract

For fungal contamination of foodstuffs, there are no fast, reliable, automated techniques to examine growth, nor have any predictive models been developed to describe the growth in the same way as for bacteria. Traditional plating methods can take 3 to 7 days to get adequate results depending on the fungal species utilised and well over a month for challenge testing, an unacceptable delay especially for the food industry. In this study two rapid analysis techniques were investigated, conductimetry (direct and indirect) and turbidimetry (Bioscreen), with the sole objective being to analyse their capability to detect fungal growth in optimum conditions and in the presence of inhibitory agents, in this case sorbic acid and vanillin. Three fungal (*Aspergillus niger, Fusarium oxysporum* and *Pencillium verrucosum* and one yeast species (*Saccharomyces cerevisiae*) were used, though only *A. niger* growth was analysed using both of the rapid analysis techniques. Two bacterial species (*Escherichia coli* and *Salmonella enterica* serovar typhimurium) were also tested using the conductimetry technique for comparison. It was found that both the impedance and turbidimetry methods provided a sensitive and rapid means of detecting, and, under standardised conditions, measuring the activity of micro-organisms. The rate of response showed close correlation with the concentration of both bacteria and spores in the initial inoculum for each strain tested so correlation curves could be constructed to estimate the number of viable cells and spores in a suspension. Moreover, both methods can be used for the accurate screening of potential antimicrobial substances. In comparison with the turbidimetry method though, the impedance method did show a greater deal of variability and there is the possibility it is unsuitable for the analysis of certain fungal species. In addition the direct impedance technique was found to be completely unusable for the analysis of fungal growth. Despite these disadvantages both are promising rapid alternatives to the standard plating technique.

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1 Aims and Objectives

The growth of fungi in foodstuffs contributes to the estimated 40% of the spoilage of all food grown for consumption. Mould (yeasts and filamentous fungi) are commonly found growing on surfaces – e.g. bread, cheese or on the surface of liquids or semi-solids, e.g. low pH drinks and yoghurts.

For bacterial contamination of foodstuffs – pathogen and to a lesser degree spoilage bacteria, predictive food microbiology has been used to describe the conditions required to inhibit the growth of these microbes. The traditional use of the growth curve has, however, often meant that such studies are lengthy and expensive to carry out. Indeed, the fact that many such studies are equally long as a challenge test has often been used to question the usefulness of such an approach. Advances have been in the use of automated techniques coupled to the development of data analysis to speed up the analyses of inhibition. Fungi, however, present a different problem. There are no fast, reliable, automated techniques to examine growth, nor have predictive models been developed to describe the growth in the same way as for bacteria.

Both the conductometric and turbidimetric techniques have shown great promise in the analysis of bacterial growth, however there is very little research into their possible use in the detection of fungal growth. In this study both will be utilised to analyse the growth of various bacterial, fungal and yeast species in the presence of inhibitory agents, with results compared against the traditional plating method, to determine whether they do have any applicability in the future, in particular within the food industry.

2 Introduction

Food spoilage is defined as a significant food deterioration, which produces a noticeable change in the taste, odour, or appearance of a product. Although exact figures of the total economic loss due to food spoilage are unknown it is clear that it constitutes an enormous financial burden. Post-harvest losses alone are reported to be as high as 25%; in tropical and developing countries losses could be as high as 50% (Prusky and Kolattukudy, 2007) and with the widespread use of monocultures becoming more common practice, vulnerability of crops to certain diseases is increasing. Yeasts and moulds such as *Zygosaccharomyces* spp, *Penicillum digitatum* and *Byssochlamys nivea* cause a significant portion of this food spoilage with plant diseases alone estimated to have cost over US$3 billion annually in North America (Barron, 2006). These high losses are attributed to mainly poor storage allowing and sustaining microbial colonisation. It is nearly impossible to completely sterilise food without the conditioning being detrimental to the taste of the product (e.g. milk is pasteurised not sterilised); this makes food preservation techniques necessary to prolong the shelf life of food.

2.1 PRESERVATION TECHNIQUES AND WEAK ACIDS

Food preservation can be defined as the process of treating and handling food in such a way as to stop or greatly slow down spoilage and prevent food-borne illness while maintaining nutritional value, texture and flavour. There are many ways to process and treat food to inhibit microbial growth and increase shelf life, some of which are reviewed in Table 2.1. One of the most common methods of food preservation is the addition of a chemical such as a weak acid. The documented use of weak acid preservatives to inhibit the growth of micro-organisms in foods and beverages extends back many centuries. John Evelyn in 1670 described the use of sulphur dioxide from burning sulphur in the preservation of cider (Rose and Pilkington, 1989). Sulphur dioxide and sulphites continue to be the method of choice for the preservation of wine. Other weak acid preservatives include acetic acid in pickles, propionic acid in bread and more recently sorbic and benzoic acids in soft drinks (Chichester and Tanner, 1972). Despite their various chemical structures, weak acid preservatives appear to share a common mode of action. All show increased inhibition at low pH, microbes are inhibited rather than killed, lag phases are prolonged, yields reduced and active transport prevented (Freese *et al.*, 1973)

Method	Effect on microbial growth
Refrigeration	Low temperature to retard growth
Freezing	Low temperature and reduction of water activity to prevent microbial growth, slowing of oxidation reactions
Drying, curing and conserving	Reduction in water activity sufficient to delay or prevent microbial growth
Vacuum and oxygen free modified atmosphere packaging	Low oxygen tension inhibits strict aerobes and delays growth of facultative anaerobes
Lactic fermentation	Reduction of pH value *in situ* by microbial action and sometimes additional inhibition by the lactic and acetic acids formed and by other microbial products. (e.g. ethanol, bacteriocins)
Sugar preservation	Cooking in high sucrose concentration creating too high osmotic pressure for most microbial survival
Ethanol preservation	Steeping or cooking in ethanol produces toxic inhibition of microbes. Can be combined with sugar preservation
Emulsification	Compartmentalisation and nutrient limitation within the aqueous droplets in water-in-oil emulsion foods
Pasteurization and appertization	Delivery of heat sufficient to inactivate target micro-organisms to the desired extent
Food irradiation	Delivery of ionising radiation to disrupt cellular RNA
Application of high hydrostatic pressure (Pascalization)	Pressure-inactivation of vegetative bacteria, yeasts and moulds
Pulsed electric field processing (PEF treatment)	Short bursts of electricity for microbial inactivation

Table 2.1 Various preservation techniques and their effect on microbial growth

2.1.1 Weak acid's proposed mechanism of action

In solution, weak acid preservatives exist in a pH-dependent equilibrium between the undissociated and dissociated state. Preservatives have an optimal inhibitory activity at low pH because this favours the uncharged, undissociated state of the molecule. It has long been held that with weak acid preservatives the undissociated acid is the inhibitor. After reviewing the effect of sorbic acid on bacterial cells Eklund (1983) suggested the undissociated acid was some 10-600 times more effective as an inhibitor than the dissociated state. The currently accepted theory of

preservative action suggests inhibition occurs mainly via the depression of internal pH (pH_i). Undissociated acid molecules are lipophilic and uncharged so pass readily through the plasma membrane by diffusion. Due to the more neutral pH of the cytoplasm acid molecules dissociate into charged anions and protons (Figure. 2.1), which cannot subsequently diffuse back across the plasma membrane. Intracellular acidification of the cell cytoplasm resulting from this accumulation of protons leads to inhibition of key metabolic activities involved in glycolysis and active transport and hence ATP yields (Freese *et al.,* 1973; Krebs *et al.*, 1983). A reduction in intracellular pH and thus in the proton motive force may also lead to reduced cellular uptake of amino acids and reduced growth (Freese *et al.*, 1973)
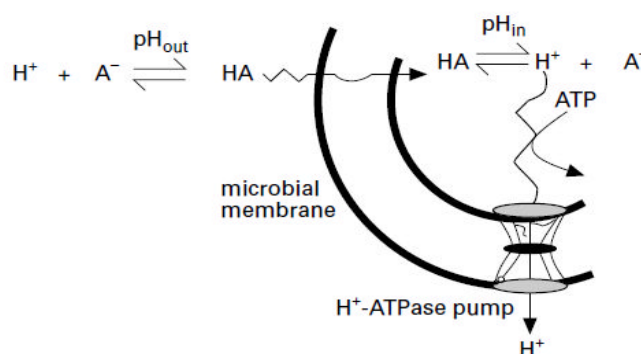
Figure 2.1 *Predicted medium and cytoplasmic weak-acid/anion equilibria. Only uncharged weak-acid molecules (HA) can diffuse freely across the plasma membrane. Charged anions (A^-) and protons (H^+) are retained within the cell; cytoplasmic protons are expelled by the membrane-bound H^+-ATPase (Lambert and Stratford, 1999).*

A number of other mechanisms of inhibition have also been proposed for weak acid preservatives including action on nutrient uptake, interference in signal transduction, stress on intracellular pH homeostasis and inhibition of various enzymatic reactions (Bracey *et al.*, 1998; Salmond *et al.*, 1984; Thevelein, 1994; York and Vaughan, 1964). Accumulation of toxic anions has also been suggested as a cause of inhibition (Eklund, 1985). In yeasts, it has been hypothesised that the actual inhibitory action of weak acid preservatives could be due to the induction of an energetically expensive stress response that attempts to restore homeostasis and results in the reduction of available energy pools for growth and other metabolic functions (Bracey *et al.*, 1998; Holyoak *et al.*, 1996). Sorbic acid in particular is thought to have an alternative mechanism of action. Acetic acid which has the same pK_a value as sorbic acid (4.76), and therefore should in theory release the same concentration of protons upon dissociation in the cell cytoplasm, actually has a minimum

inhibitory concentration 20 times higher than that of sorbic acid (Plumridge *et al*., 2004). In addition it has been calculated sorbic acid does not release sufficient protons to act as a classical weak acid preservative (Stratford and Anslow, 1998), experimental data confirms that sorbic acid has slight effect on internal pH (Stratford and Ueckert, 2000). This suggests the inhibitory action of sorbic acid is not due solely to intracellular acidification. It is thought, based on the relationship of inhibition with partition coefficient, that sorbic acid could have an effect on the plasma membrane in a manner similar to that of ethanol, affecting membrane fluidity and possibly causing leakage (Stratford and Anslow, 1998).

2.2 WEAK ACID RESISTANCE MECHANISMS

Weak acid preservatives are usually quite effective inhibitors against fungal growth and as such are commercially often used in such products as bread and carbonated beverages. However due to the toxicity of some weak acid preservatives and to prevent any negative consequences relating to their use legislation has been put in place to limit the maximum concentration of these compounds permitted to be added to food. In the European Union the use of sorbic and benzoic acids, as an example, is limited to as little as 300ppm and 150ppm respectively in certain products (Anon, 1995). However these levels of preservative can be ineffective; *Zygosaccharomyces bailii* for example has been shown to be capable of growth in the presence of 600mg/l of benzoic or sorbic acids or 2% acetic acid and at pH levels less than the pKa of the preservatives (Pitt, 1974). Furthermore the addition of sub-lethal concentrations of weak acid has been shown to promote tolerance in particular in yeast species. Cultures of *Saccharomyces cerevisiae* have been shown to exit the cell cycle and enter a long period of stasis in the presence of weak acid. Eventually resuming growth after several hours, now adapted, in that they will not display transient growth arrest if re-inoculated into fresh medium containing the same levels of weak acid (Piper *et al.*, 1997). Little is known about the actual mechanisms of resistance and adaptation of yeast and moulds to weak acid preservatives although there are several suggestions such as the bioconversion or inactivation of the preservative and utilisation of various pumps.

2.2.1 Bioconversion of weak acids

The ability of filamentous fungi to metabolise weak acid preservatives is well established, with early reports by Melnick *et al* (1954) suggesting that degradation of sorbic acid occurs as a result of β-oxidation with CO_2 and H_2O as end products. It is thought similar acids could undergo a similar process of inactivation. Several authors have reported that some fungi can metabolise sorbic acid and thereby detoxify it, for example Lukas (1964) and Marth *et al.* (1966) reported that *Aspergillus niger* and moulds in the genus *Penicillium* could degrade sorbic acid to the volatile compound 1, 3-pentadiene via a decarboxylation reaction, which eliminated the inhibitory sorbate from the medium and allowed growth. 1, 3- pentadiene has also been observed in sorbate treated noncarbonated beverages (Goetz *et al.*, 1978) and feta cheese (Horwood *et al.*, 1981) confirming microbial involvement in its production. A number of other bio-transformations of sorbic acid have also been seen. Kurogochi reported that sorbic acid was metabolised by *Mucor* species to give 4-hexenol (Kurogochi *et al.,* 1975) and by *Geotrichium* species to 4-hexonic acid and ethyl sorbate (Kurogochi *et al*, 1974). A number of volatile derivatives of sorbic acid have been found in wine, which has undergone spoilage (Crowell and Guymon, 1975). Although the spoilage was attributed to the growth and metabolism of lactic acid bacteria, no evidence is presented for the microbiology, it is possible that this spoilage could be attributed to moulds.

Although the exact metabolic pathways and implicated enzymes that convert sorbic acid into its metabolite products are still elusive, it is thought the suppression of the carboxyl group of sorbic acid is the likely cause of its inactivation. Table 2.2 shows the various volatiles isolated and identified from sorbate treated wine; in each the carboxyl group of sorbic acid has undergone a reaction including esterification, reduction, decarboxylation and ether formation. The evidence presented in the table suggests that the free carboxyl group is necessary for microbial activity. In order to grow, the moulds must chemically suppress the charged carboxyl group and so these bio-transformations could represent a detoxification (Kinderlerer and Hatton, 1990). Similar chemical modification of aliphatic acids was observed with a white wine spoilage mould, *Chrysosporium* (Kinderlerer *et al.*, 1988). Lipoxygenase enzymes have also been linked, mainly because they are widely distributed and are known to react preferentially with unsaturated fatty acids with *cis,cis*-1,4-pentadiene systems like in sorbic acid (Galliard and Chan, 1980). There have been numerous

reports of resistant yeasts, but none have indicated that degradation of weak acid preservatives occurs, suggesting an alternative mechanism of resistance. It has been hypothesised that yeasts can adapt the composition or structure of their membranes to reduce the diffusion coefficient of preservatives across the plasma membrane (Brul and Coote, 1999). Alternatively Warth (1977) found that resistance to preservatives by *Z. bailii* resulted primarily from an inducible, energy requiring system, which transports preservatives from the cell.

Class	Compound observed
Alcohol	4-hexenol
	2,4-hexadien-1-ol
	3,5-hexadien-2-ol
Esters	Ethyl sorbate
Ethers	1-ethoxyhexa-2,4-diene
	2-ethoxyhexa-3,5-diene
Hydrocarbon	1, 3 pentadiene

Table 2.2 *Volatile compounds isolated from sorbate-treated wine. (Crowell and Guymon, 1975)*

2.2.2 Role of the H^+-ATPase pump

The resistance of spoilage yeasts has been extensively studied and is thought to depend to some degree on the H^+-pumping P-type membrane ATPase. In normal cell function, it mainly contributes to the generation of the electrochemical potential across the membrane, a potential that drives nutrient uptake and that regulates ion and pH balance (Serrano, 1991). In the presence of weak acids it has been shown via measurements of H^+-ATPase activity in purified plasma membranes (Piper *et al.*, 1997; Viegas and Sa-Correia, 1991) and proton extrusion by intact cells (Holyoak *et al.*, 1996) that H^+-ATPase is strongly activated. Furthermore by lowering the expression of the gene encoding H^+-ATPase (Holyoak *et al.*, 1996) or inhibiting H^+-ATPase activity (Kubo and Lee, 1998) the sensitivity of cells to weak acids is increased. Despite this evidence of its apparent importance in the resistance of microbes to weak acid inhibition at first sight, raising the pH_i

through the use of the H^+-ATPase appears to be a futile, ATP-wasting activity because a higher pH_i will cause a further influx of preservative and consequent lowering of pH_i.

Lambert and Stratford (1999) however showed that proton pumping is not a futile cycle and H^+-ATPase will raise pH_i, albeit slowly and with great expense in terms of ATP. This can be explained by the fact that for every proton pumped from the cytoplasm by H^+-ATPase activity one anion remains accumulated inside the cell. Since for any given internal or external pH there is a set ratio of acid: base concentrated in the cell (Figure 2.2), the presence of these extra accumulated anions means more acid is within the cell than permitted, so therefore to restore chemical equilibrium the acid has to flow out, allowing pH_i to rise a little. Figure 2.3 models this recovery and shows the process is time dependent on preservative concentration. However because the recovery to a pH_i that can sustain exponential growth is slow the lag times in stressed cells are increased. Assuming the usage of ATP in the proton pumping activity is diverted from cell growth, this explains the loss of cell yield.

It was once hypothesised that continuous proton pumping and ATP usage throughout growth was necessary to sustain weak acid resistance leading to metabolic exhaustion of the cells (Warth, 1988). Lambert and Stratford (1999) demonstrated, however, that once a pH_i that permitted exponential growth was reached, no further proton pumping was required. This seemed to be further confirmed when it was found an integral membrane protein, Hsp30; down regulated the increased activity of membrane ATPase. It was deduced that Hsp30 acts as a molecular 'switch' down regulating the activity of the membrane ATPase to conserve energy pools, which would otherwise be consumed by the enzyme trying to restore homeostasis (Braley and Piper, 1997). Indeed Piper *et al* (1997) showed *hsp30* mutant cells when stressed showed unusually low ATP levels, which could have been a reflection of their excessive H^+-ATPase activity.
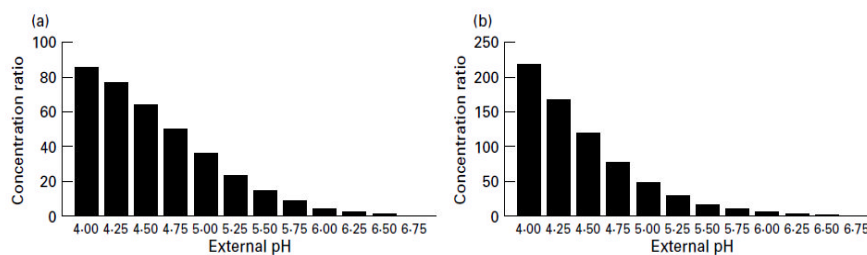
Figure 2.2 Predicted concentration ratios of preservatives from medium to cytoplasm, based on known proportions of undissociated acid/anion at each pH value. Concentrations are calculated assuming pHi to be 6·75, due either to infinite buffering or to proton removal. (a) Sorbic acid/sorbate; (b) benzoic acid/benzoate (Lambert and Stratford, 1999).
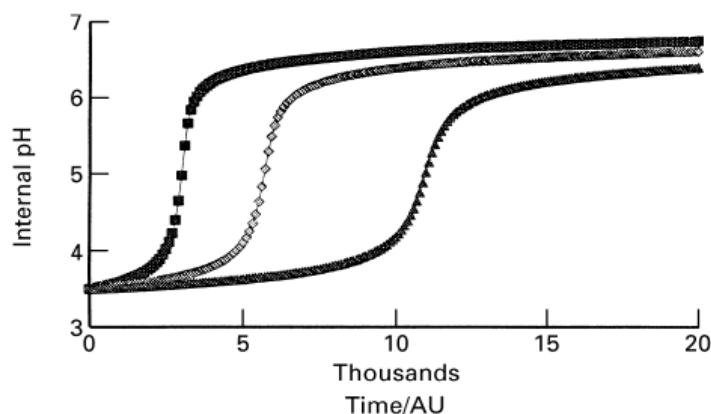
Figure 2.3 The rise of pH$_i$ from pH 3·5 by proton pumping, despite further weak-acid influx. Sorbic acid concentrations used were 0·5 mmol l^{-1} (■), 1 mmol l^{-1} (◇) and 2 mmol l^{-1} (▲). Time is in arbitrary units. Increased time is required (lag phase) to raise pH$_i$ with increased preservative concentration (Lambert and Stratford, 1999).

2.2.3 Possible role of Pdr12 in resistance

Studies by Henriques *et al* (1997) have however shown that *Saccharomyces cerevisiae* actively extrudes [C-14] labelled benzoic acid, suggesting there is an alternative efflux system to H$^+$-ATPase, which actively removes accumulated acid anions from the cell. Supporting this, data gathered by Piper *et al* (1998) demonstrated the existence of an ABC transporter, Pdr12, which actively extrudes preservative anions. Its role in weak acid resistance was seemingly confirmed when cells lacking the transporter proved to be hypersensitive to water-soluble mono-carboxylic acids of relatively short aliphatic carbon chain length (Piper *et al.*, 1998). However the main problem with this theory of proton and anion extrusion from yeast cells is that it would create a

futile and energetically expensive cycle. In response Piper *et al* 2001 suggested that yeast cells limit the diffusional entry of the undissociated acid, using cell wall mannoproteins, which have shown to limit porosity in yeasts (De Nobel and Barnett, 1991). Furthermore acid adapted *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* can maintain an intracellular versus extracellular distribution of benzoate that is not in equilibrium (Herniques *et al.*, 1997; Piper *et al.*, 1998; Warth, 1977; Warth, 1988). However the hypothesis suggested by Lambert and Stratford (1999) of slow recovery from weak acid stress via just H^+-ATPase activity seems the more likely and favourable mechanism.

2.3 ESSENTIAL OILS AN ALTERNATIVE TO WEAK ACID PRESERVATION

With the increasing level of weak acid tolerance, rising concern about the potentially harmful side effects of synthetic additives and consumer demand for fresh or minimally processed products containing no artificial preservatives, the interest in the use of natural antimicrobials is growing. Several herbs, spices, plants and their extracts, normally added to foods for their flavouring properties are of particular interest due to their documented antimicrobial activity (Hitokoto *et al.*, 1980; Mishra and Dubey, 1994; Sacchetti *et al.*, 2005; Velluti *et al.*, 2003). Among these, vanillin (4-hydroxy-3-methylbenzaldehyde), a major constituent of vanilla beans and principle flavouring compound used in numerous foods such as ice cream, chocolate and confectionary products has shown to be an able inhibitor of yeasts and moulds. Cerrutti and Alzamora (1994) found that the addition of 2000ppm vanillin had an important inhibitory effect during 40 days of storage at 27°C on the growth response of *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii* and *Debaromyces hansenii* in laboratory media and in apple puree at pH 3.5 and a water activity (a_w) of 0.99 and 0.95. In another study by Lopez-Malo *et al* (1995), the incorporation of vanillin (~450ppm-1050ppm) into fruit-based agars inhibited the growth of *Aspergillus flavus*, *Aspergillus niger, Aspergillus ochraceus* and *Aspergillus parasiticus* for 2 months. It has also been reported that the compound can act as an antioxidant in complex foods containing polyunsaturated fatty acids (Burri *et al.*, 1989). From these results and observations it has been suggested that vanillin could have the potential to be a total or partial substitute for common preservatives to inhibit the growth of yeast and moulds. Other essential oils and their constituents such as basil, tea tree oil, mint, thymol, cinnamic aldehyde, eugenol, carvacrol,

anethole and linalool among others have also shown similar antifungal effects (Cox *et al.*, 2000; Hitokoto *et al.*, 1980; Lachowicz *et al.*, 1998; Vazquez *et al.*, 2001).

2.3.1 Proposed mechanisms of action for essential oils

Little information is available on these natural antimicrobials and their action on fungal cells, even with bacteria it is still largely unknown, but has been suggested to depend on their chemical structure. Eugenol (the main component of clove and cinnamon oils) and carvacrol (from oregano oil) as an example are phenols, which is a class of chemical compounds consisting of a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group. It is this reactive phenolic OH group that is thought to provide the antimicrobial activity via forming hydrogen bonds with the active sites of target enzymes (Farag *et al.*, 1989). Indeed, Nychas (1995) indicated phenolics could denature the enzymes responsible for spore germination, or interfere with amino acids involved in germination. Prindle and Wright (1977) showed however, that the mode of action of phenolic compounds was actually concentration dependent. At low concentrations, phenolics did indeed affect enzymatic activity related to energy production, but at high concentrations they cause protein precipitation. Alternatively cell wall perturbation has been suggested as a possible mechanism of action. With bacteria thymol and carvacrol have been found to disintegrate the outer membrane of *Escherichia coli* and *Salmonella* typhimurium (Helander *et al.*, 1998). Furthermore Ultee *et al*. (1999) found that in the presence of 1mM carvacrol depletion of intracellular ATP pools and dissipation of proton motive force components of *Bacillus cereus* was seen. This detrimental effect on the proton motive force and dissipation of pH regardless of glucose availability described by Lambert *et al* (2001) suggests leakage of specific ions. Indeed, acting on the permeability barrier of the cytoplasmic membrane various preservatives including essential oils and phenols cause leakage of various substances, such as ions, ATP, nucleic acids and amino acids (Cox *et al.*, 1998; Helander *et al.*, 1998; Tassou *et al.*, 2000; Tranter *et al.*, 1993; Ultee *et al.*, 1999). Though these studies were performed on bacteria Conner *et al* (1984) demonstrated that most essential oils (at approx. 100mg l^{-1}) impair the respiratory activity of *Saccharomyces cerevisiae*, therefore it is possible that essential oils and phenolic compounds could have similar effects on fungal cells.

2.3.2 Limitations of essential oils as food preservatives

Although they seem like perfect replacements for synthetic additives, essential oils and their constituents do have limitations, which could possibly hinder their use in foods as preservatives. Due to their strong flavour and fragrance chemistry taste considerations are the first hurdle, since in many cases effective antimicrobial doses exceed organoleptically acceptable levels. When used in practice the compounds are only implemented at concentrations that just have growth inhibitory effects rather than fungicidal or are used in combined methods with other preservatives like potassium sorbate rather than as a sole preservative. Furthermore essential oils have proven to be difficult to incorporate into complex food systems, mainly because of their poor solubility, often requiring solvents such as alcohol to properly incorporate them into solution. Lopez-Malo *et al* (1995) also found that vanillin had a lower inhibitory effect in mango and banana containing systems compared with other fruits. Cerrutti and Alzamora (1994) had similar findings with 3000ppm vanillin in banana puree (pH 4) needed to inhibit growth of three yeast strains, but only 2000ppm was required in apple puree. It is thought this difference could be attributed to the higher fat and/or protein content in mango (0.2% fat and 0.7% protein) and banana (0.3% fat and 1.2% protein), which are known to bind and/or solubilise phenolic compounds, reducing their availability for antimicrobial activity (Cerrutti and Alzamora, 1994; McCance and Widdowson, 1993; McNeil and Schmidt, 1993). In addition Therefore essential oils could be unsuitable for use as preservatives in foods with higher fat or protein content.

Another potential limitation is the possibility of biotransformation, which could influence the antimicrobial effectiveness of the compounds. Studies have also shown that certain yeasts are able to bioconvert vanillin to its respective alcohol and acid derivatives (Fitzgerald *et al*., 2003). *Zygosaccharomyces bailii* in particular was shown to be able to completely remove a concentration of 750ppm vanillin from culture media after 24 hours, and 1500ppm after 48 hours (Fitzgerald *et al*, 2003). As Figure 2.4 indicates the bioconversion of vanillin is in fact a constitutive process and not inducible as previously thought (De Wulf and Thonart, 1989). This means that the enzymes responsible for the conversion are produced under all physiological conditions and not only expressed in certain conditions, meaning the bioconversion of vanillin is not a specific stress response to the presence of vanillin. The yeasts used in these studies were fermentative in nature and it appears most likely that non-specific aryl alcohol dehydrogenase enzymes are involved, this

means structurally similar compounds like eugenol could also be inactivated in such ways, though it is uncertain. It is also unclear if non-fermentative spoilage organisms would be able to perform such bioconversions, but the possibility still poses a problem for the future use of essential oils and their derivatives in foods. Taking all these limitations into consideration until further analysis of the mode of action of essential oils, interactions with food systems and minimum inhibitory concentrations are reviewed it is unlikely these natural antimicrobials will be utilised as sole preservatives replacing weak acids completely in the near future.
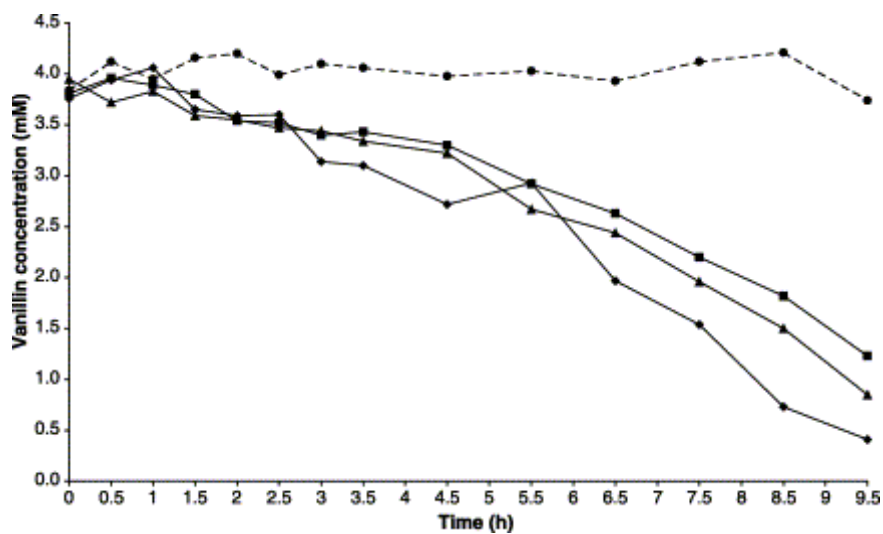
Figure 2.4 *Vanillin concentration in YEPD (pH 4.0) with an initial level of 5 mM and inoculated with S. cerevisiae (NCYC 956) previously grown in the absence (◆) or presence of 1 mM (■) or 5 mM (▲) vanillin. In the control flask yeast cells were omitted (●) (Fitzgerald et al., 2003)*

2.3.3 Hurdle technology

A way to reduce the impact of some of these limitations is to use the so-called combined methods technique. This method advocates the deliberate combination of existing and novel preservation techniques. The hypothetical basis of this is that a combination of preservative factors (hurdles) gives a multi target disturbance of homeostasis (Leistner, 1995). Due to their concerted or suggested synergistic effect (Leistner, 1978), combinations are considered, therefore, to achieve better results than or at least equivalent to those of a single inhibitory action. It also means the individual 'hurdles' may be set at lower intensities than would be required if only a single inhibitor was used. This is advantageous in the case of essential oils because if they can be used at lower

intensities their strong flavour and fragrance chemistry is less likely to compromise the taste or quality of the product, but still ensure food safety due to the presence of other preservative factors. Traditional approaches for the preservation of products based on this hurdle approach include generally a reduction of pH (2.5-4.1) and water activity (a_w, 0.92-0.98), in combination with slight heat treatment and addition of a preservative, although other hurdles like redox potential (Eh) and competitive microorganisms are used (Alzamora *et al.*, 1993; Lopez-Malo *et al.*, 1994; Leistner, 1999). Fruit products undergoing this sort of treatment have been reported to be microbiologically stable after storage at 25°C for at least 4 months (Alzamora *et al.*, 1993; Lopezmalo *et al.*, 1994). Essential oils have shown promise as a possible hurdle with synergistic effects observed when vanillin and potassium sorbate were used in combination. Matamoros-Leon *et al* (1999) established that with a slight reduction in pH and water activity, ~3mM vanillin in combination with ~2mM potassium sorbate could inhibit the growth of *Penicillium digitatum*, *Penicillium glabrum* and *Penicillium italicum* for 1 month. So the possible future of essential oils in food preservation could be within the combined method approach rather than as a sole preservative.

2.4 SCREENING

To assess the efficacy of such antimicrobials in foods and to determine the mycological quality of products screening is implemented. However the analysis of fungal growth in the presence of inhibitors, especially in a mixture, can take months. These sorts of time delays are unacceptable in a highly competitive industry, making a rapid analysis method highly advantageous; consequently several non-traditional procedures have been developed including radiometry, bioluminescence and impedimetry.

2.4.1 Impedance microbiology

Impedance microbiology is a rapid method that enables qualitative and quantitative monitoring of microorganism's growth by measuring changes in electrical conductivity (Wawerla *et al.*, 1999). It is a growth-based analysis allowing the differentiation between viable and dead cells (Figure 2.5), and making enumeration more precise than with conventional methods such as counting chambers.
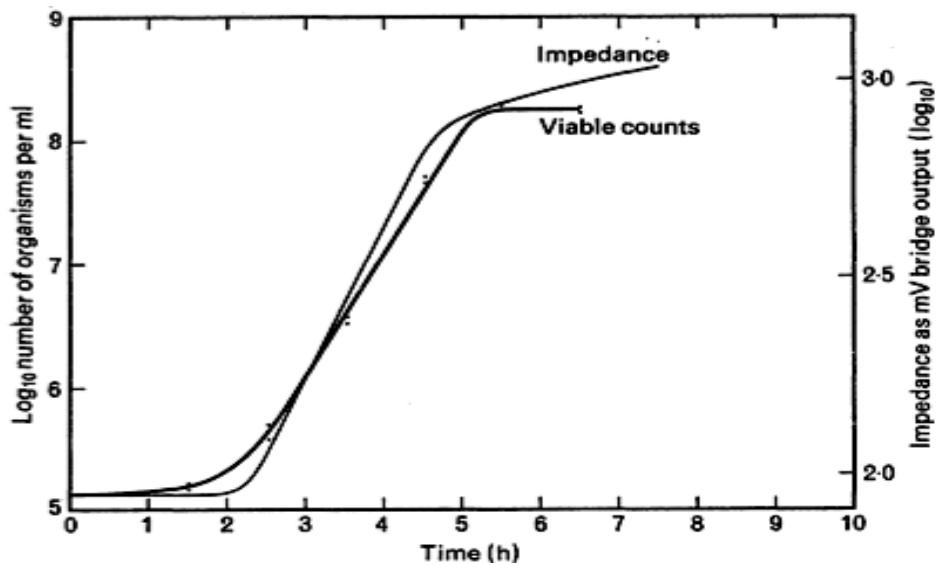
Figure 2.5 *Curves of changes in comparative impedance and of number of viable organisms in a culture of Escherichia coli growing in PPLO broth (Ur and Brown, 1975).*

Owens (1985) stated from an electrical point of view when two electrodes are placed in an electrolyte-containing solution and a potential difference is applied across them a drift of ions is produced in which ions move towards the electrode of opposing charge. The steady flow of charge (current) is maintained by ions taking electrons from or handing electrons to the electrode. The ability of a solution to conduct current is evaluated from measurement of the resistance of the solution (impedance), which is dependent on its ionic composition; it is this that microorganism growth affects. The impedance between two electrodes can be modelled by a series circuit, as shown in Figure 2.6. It includes the medium conductance G_m, the interface conductance G_i and capacitance C_i of each electrode, and also G as the total conductance. Roughly, C_i represents the double-layer capacity of the electrode-electrolyte interface (Felice, 1995). Impedance theory is more extensively reviewed at Eden and Eden (1984), Owens (1985) and by Kell and Davey (1990).
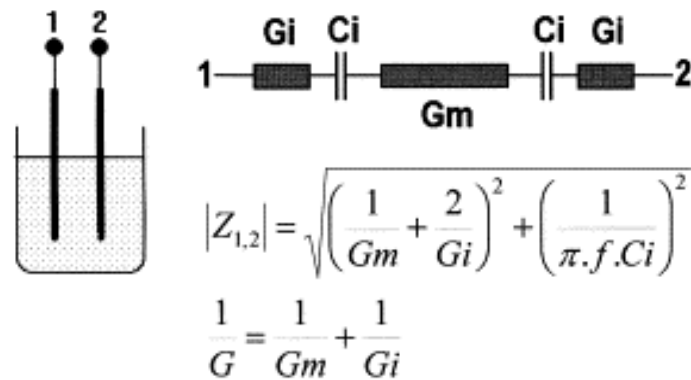
Figure 2.6 *Electrical circuit equivalence between two electrodes. $Z_{1,2}$, impedance modulus; G, total conductance; G_i, interface conductance; C_i, interface capacitance; G_m, medium conductance (Felice et al., 1999).*

2.4.2 Direct impedance

Impedimetric measurements can be made one of two ways, directly or indirectly. In the direct technique, a pair of electrodes is immersed in growth medium that is inoculated with the test culture, a low frequency voltage is then applied, and when impedance values increase or decrease beyond a threshold value they are recorded. In general the change in impedance is caused by metabolic processes and the release of ionic metabolites from live cells into the growth medium (Ur and Brown, 1975; Yang and Bashir, 2008). In bacteria the main origin of ion release into the medium is via energy metabolism (catabolism). Bacteria consume oxygen and sugars and produce carbon dioxide and organic acids, basically changing the generally uncharged or weakly charged substrates of growth media into highly charged smaller end products. This increases the conductivity and capacitive potential of growth medium causing a reduction in impedance. For example, after the conversion of glucose to two molecules of lactic acid, further metabolism will take the lactic acid and three oxygen molecules to carbonic acid. The smaller more mobile carbonate ion is a more effective ionic conductor than the lactate ion (Silley and Forsythe, 1996). There are other possible sources such as ion exchange, which do make small contributions to fluctuations in the ionic composition of media and therefore consequent changes in conductivity, yet undoubtedly catabolism is the major source. However in a study by Owens *et al*. (1992) for yeast cultures the metabolic activity primarily responsible for impedance changes was found to be the uptake of charged ammonium ions as the nitrogen source and the reaction of protons with pH buffer compounds. Though this study was primarily aimed at yeast cultures Watson-Craik *et al*

(1989) found no difference in the behaviour between yeasts and moulds in their studies and, therefore, it seems likely mechanisms of conductance change could be similar.

2.4.3 Problems with direct impedance

For yeasts and moulds and other organisms, due to their different metabolic processes growth does not result in large changes in impedance when measured directly. It is speculated the production of non-ionised end products such as ethanol instead of strongly ionised metabolites is the cause. However yeasts have also been shown to absorb ions from solution, which would result in a net decrease in medium conductivity (Suomalainen, and Oura, 1971). Direct impediometry does have another disadvantage; it is highly dependent on the media composition. It is expected that different growth mediums will yield impedance curves with varying size and amplitude as seen in Figure 2.7, this is attributed to differences in the buffering capacity and chemical composition of different media impacting on ion movement and organism growth rates. However this sensitivity can be a problem when using growth media with a high salt content or low pH, which is a common feature of many selective media, as their already high ionic composition, would create artificially high readings making detectable growth indistinguishable from media interference. Furthermore it has been shown that conductance changes recorded do not always coincide with the growth that is present due to media composition influencing the results.

Watson-Craik *et al* (1989) showed that addition of product samples, such as fruit juices, to yeast extract/soy peptone culture media could greatly influence and even reverse the direction of conductance changes. Following up on this Owens *et al* (1992) measured the impedance changes during growth of *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii* and *Rhodotorula rubra* (Figure 2.8) in culture medium containing glucose, tartrate pH buffer and ammonium ions as sole nitrogen source in comparison with a medium containing L-asparagine as the sole nitrogen source. Decreases in impedance were observed in glucose-ammonium cultures of all three yeasts while little change occurred in cultures with L-asparagine. This shows the choice of nitrogen source has large implications on the results attained and is largely attributed to the fact L-asparagine is uncharged and so uptake leads to no excretion of protons which would cause the consequent changes in conductance of pH buffers. Taking all these issues into account to make the direct technique an effective procedure the correct growth medium must be used, ideally one with low

conductivity and when metabolised allows the production of highly ionised metabolites. These problems among others can be overcome by using the indirect impedance method.
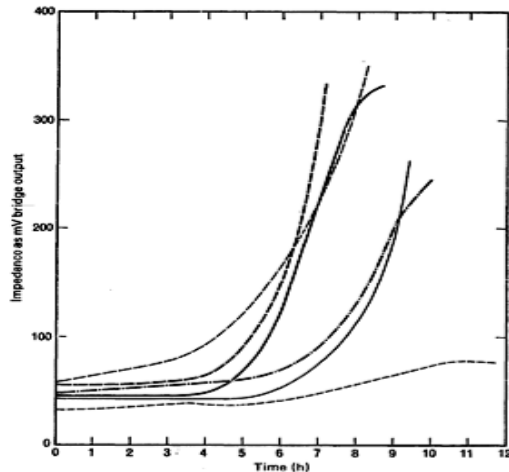
Figure 2.7 *Comparative impedance curves of the Oxford Staphylococcus grown in different media: Comparative PPLO broth, supplemented PPLO broth, nutrient broth, peptone water, tryptose-phosphate broth and glucose broth (Ur and Brown, 1975)*
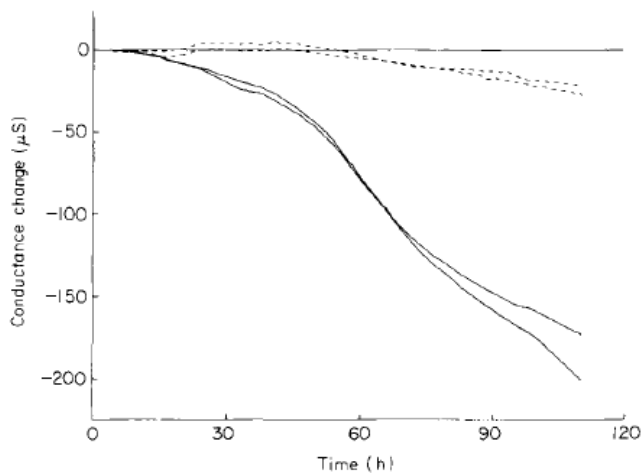
Figure 2.8 *Effect of nitrogen source on conductance change due to growth of Rhodotorula rubra in base YCBT. Conductance was monitored in 2ml medium in Malthus 2ml electrode systems incubated at 25°C ——————— , $(NH_4)_2SO_4$; -----------, L-asparagine (Owens et al., 1992).*

2.4.4 Indirect impedance

The indirect impedance technique monitors microorganism metabolism via the production of carbon dioxide (CO_2) (Owens *et al*., 1989). There are multiple setups for this technique as shown in Figure 2.9. With the Sy-lab BacTrac and Don Whitley RABIT system potassium hydroxide is added to the impedance tube across the electrodes. The inoculated culture medium is in a separate chamber and not in contact with the electrodes or the potassium hydroxide (KOH). The unit is then tightly sealed so that any CO_2 produced is a result of normal microorganism metabolism and not atmospheric.
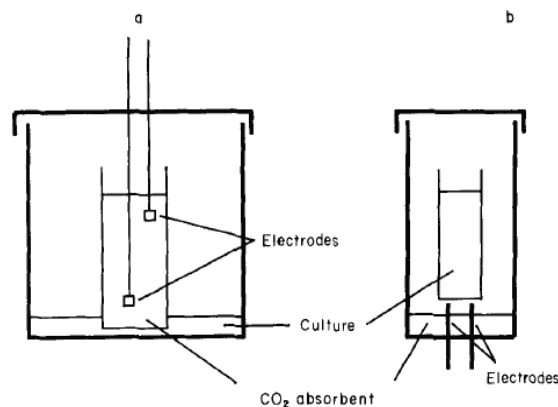
Figure 2.9 *The different measuring cell set ups for indirect impedance analysis are displayed. (a) Malthus system (b) BacTrac (Owens et al., 1989)*

This technique largely relies on the potassium hydroxide absorbing the CO_2 and the chemical reaction shown in equation 2.1 taking place. As the CO_2 is absorbed by the alkaline KOH, at pH values above 11, it is converted to carbonate which leads to a net change in molar conductivity. The net change in molar conductivity is a consequence of the high molar conductivity of the hydroxide ion. Using this net change in molar conductivity a microorganism's growth rate can be deduced.

$$CO_2 + 2OH^- \leftrightarrows CO_3^{2-} + H_2O$$

Equation 2.1

Due to the fact this technique is still a growth based analysis like the direct technique it is still affected to some degree by media composition, however unlike the direct method because the

media is not in contact with the electrodes unmodified selective and non-selective medium can be used regardless of supplementation or high ionic composition due to pH or salinity. Furthermore because CO_2 production is the focus of this analysis it is not dependent on a microorganism's ability to produce highly ionising metabolites, which as stated before can be highly variable, and dependent on media composition. In comparison CO_2 is a major metabolic end product produced by all microorganisms, which undergo aerobic respiration, and is a useful indicator to monitor the growth of most bacteria, yeasts and moulds.

2.4.5 Turbidimetry method

The Bioscreen C Automated Microbiological Analyser (Labsystems Corp., Helsinki, Finland (Figure 2.10)) is an automated multi-well spectrophotometer, which directly measures microorganism growth via changes in turbidity (optical density). The changes in turbidity or the formation of colour in the culture medium due to the growth of microorganisms is measured using the optical arrangement shown in Figure 2.11. In this optical arrangement the halogen lamp emits a light beam, which is carried through the chopper and a UV-mirror to the filter wheel. The interference filters of the wheel are selected using the PC software (usually the 600nm filter is used to measure turbidity). The filtered beam of light enters an optical fibre, which leads the light upward to the lens of the measuring device below the honeycomb plates. After passing through a honeycomb plate well the light enters the detector. The measuring device is moved along the Y-axis while the incubator tray with the plate is moved along the X-axis. This enables rapid measurement of all wells in the plate. Using this device and by measuring the turbidity of the medium over time, an optical density (O.D.) curve can be generated. The curve reflects the growth (multiplication) of the organism of interest.
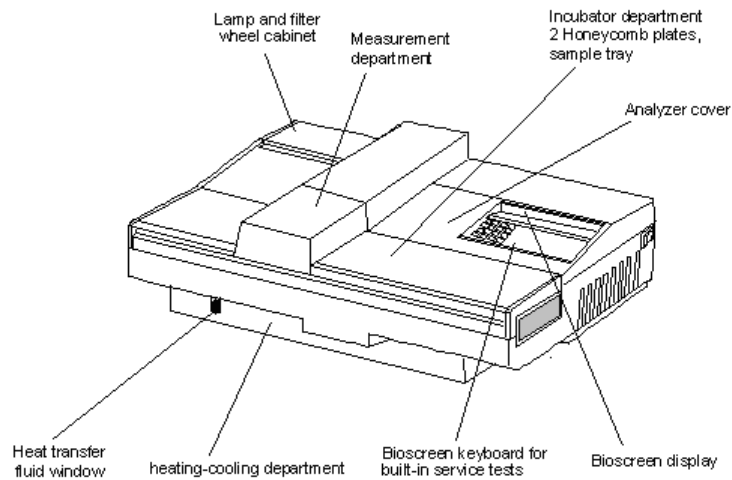
Figure 2.10 *The components of the Bioscreen C automated microbiological analyser.*
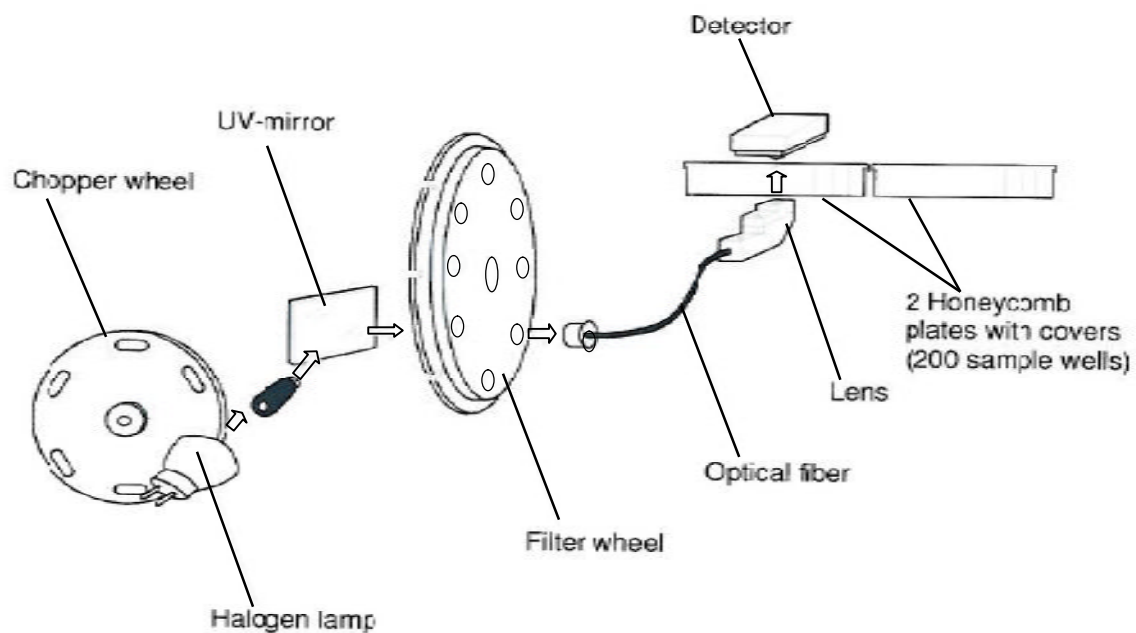
Figure 2.11 *The optical arrangement of the Bioscreen C automated microbiological analyser.*

The Bioscreen has proven to be an efficient, reproducible and rapid screening method for bacteria (Dalgaard *et al*., 1994; Johnston,1998) and has seen applications in monitoring the effect of biocides and preservatives on bacterial growth (Korkeala *et al*., 1992; Lambert *et al*., 1998; Olasupo *et al*., 2003). However optical density and the Bioscreen are rarely used for the analysis of

Page 31

fungal growth. This could be in part because filamentous fungal growth in liquid medium often does not in most cases produce the easily detectable change in broth turbidity characteristic of microbial growth (Figure 2.12). This is largely due to the fact filamentous fungi grow as hyphae, which are multi-cellular cylindrical, thread-like structures specifically adapted for growth on solid surfaces, therefore when grown in liquid medium filamentous fungi readily adhere to the walls of any container they are inoculated in (Figure 2.13). For Bioscreen analysis this pattern of growth is undesirable and can lead to inconsistent results (Figure 2.14), it also could be argued that results obtained do not give a true estimate of the amount of growth actually occurring, as the majority of the biomass of the fungus is attached to the container and not evenly distributed within the medium. Despite this optical density can be used to assess fungal growth effectively (Tauk-Tornisielo *et al.*, 2007).

Figure 2.12 *The colour change induced by bacterial growth in sterile growth medium.*

Figure 2.13 *The growth of Penicillium verrucosum in liquid growth medium.*
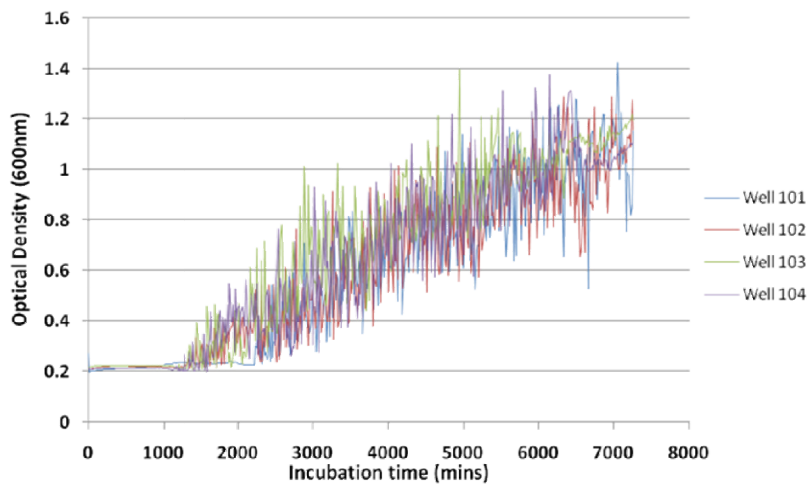
Figure 2.14 *Analysis of growth of Penicillium glabrum in liquid medium using the Bioscreen C.*

3 Methods

3.1 STRAINS, CULTURE MEDIA AND GROWTH CONDITIONS

Saccharomyces cerevisiae (ATCC 9763), *Penicillium verrucosum*, *Fusarium oxysporum*, *Aspergillus niger, Escherichia coli* (ATCC 11229), *Salmonella enterica* serovar typhimurium (ATCC 53648) were employed. All mould isolates were industry specimens. *E.coli* and *S.*typhimurium strains were grown and maintained on tryptone soya agar (TSA; Oxoid CM131) slopes. *S.cerevisiae, A. niger*, *F. oxysporum* and *P.verrucosum* strains were grown and maintained on malt extract agar (MEA; Oxoid CM59). The mould cultures were sub-cultured every 14 days. All agar was prepared following the manufacturer's instructions and autoclaved at 121°C for 20 minutes prior to use. Stock cultures of all isolates were stored in a cold room at 4°C.

3.2 PREPARATION OF INOCULA

E.coli and *S.* typhimurium strains were grown overnight in flasks containing 100ml of tryptone soya broth (TSB; Oxoid CM129) and incubated in a 150 rpm-shaking incubator at 37°C. The cells were harvested by centrifugation at 3000 rpm for 10 minutes. The supernatant was then removed and the cells subsequently re-suspended in 5ml 0.85% sterile saline (Oxoid) to provide a bacterial concentration between 10^8-10^9 CFU ml^{-1}. Fresh cultures were prepared for each experiment. To create different inoculums sizes the original bacterial suspension was then 10 fold serially diluted in 0.85% sterile saline. Bacteria were enumerated using plate counts; 0.1ml of dilutions 10^{-4}, 10^{-5} and 10^{-6} were plated onto TSA in triplicate. After incubation at 37°C for 24 hours, colonies were counted.

The protocol for *E.coli*, S. typhimurium and *S.cerevisiae* was largely the same except with *S. cerevisiae* malt extract broth (MEB; Oxoid CM57) and MEA was used and the cultures were incubated at 30°C for about 3 days.

P. verrucosum, *F. oxysporum* and *A. niger* cultures were grown on MEA for 5 days at 25°C. Spores were harvested by adding 10ml sterile 0.85% sterile saline to the plates and scraping the agar surface to dislodge the spores. Mycelial debris was removed by filtration through a glass wool plug

into a sterile universal bottle. Total spore concentrations per ml were determined using a Neubaeur haemocytometer. The original spore suspension was then half fold serially diluted in 10 ml of 0.85% sterile saline. For Bioscreen analysis prior to the serial dilution step the original spore suspension was diluted to an optical density (OD) of 0.5 at 600nm. Fresh cultures were prepared for each experiment

3.3 SORBIC ACID AND VANILLIN SOLUTIONS

A stock solution of sorbic acid was prepared by dissolving potassium sorbate in distilled water to give a 2% (wt/vol) solution. The stock solution was subsequently filter sterilised using a 0.2μm filter.

A stock solution of vanillin was prepared by dissolving vanillin in 10ml pure ethanol and then adding 90ml distilled water once all the compound was dissolved to give a 2% (wt/vol) solution. The stock solution was subsequently filter sterilised using a 0.2μm filter.

3.4 STANDARD GROWTH ANALYSIS TECHNIQUE

MEA was sterilised for 20 minutes at 121°C, cooled, aseptically divided and the necessary amount of potassium sorbate added under sterile conditions. The concentrations of potassium sorbate used were 50, 100, 200, 300, 400, 500, 600, 700 and 800ppm. The agar was then acidified using 1M hydrochloric acid to pH 4.6 for one set of plates and pH 2.5 for the other. Triplicate plates for each concentration of sorbic acid were centrally inoculated with 2μl of spore suspension to give a circular inoculum 2mm in diameter. Growth controls without antimicrobials were prepared and inoculated as above. The inoculated plates and controls were incubated at 25°C. The radial diameter of colonies was recorded every day over 30 days. Increase in diameter of each plate was plotted as a function of incubation time, and radial growth was obtained from the slope by linear regression of the linear phase of growth (Horner and Anagnostopoulos, 1973; Lopez-Malo *et al.*, 1995). This was performed for all the mould isolates; however analysis of growth in the presence of antimicrobials was only performed on *A. niger*.

3.5 IMPEDANCE ANALYSIS

Impedance measurements were performed on a BacTrac[TM] 4300 (SY-LAB Gerate GmbH, Neupurkerdorf, Austria) microbiological analyser, a fully automated impedance instrument used to

monitor microbial growth in liquid samples. This instrument enables electrical changes in the growth medium resulting from microbial metabolic processes (M-value; media impedance) and changes in the ionic layers on the electrode surfaces to be measured (E-value; electrode impedance).

The following detection parameters were used: For direct analysis the E-threshold was set at +10%, for indirect analysis the M-threshold was set at -25%, the time to detection was defined as the time taken to surpass these thresholds. A warm up time of 1 hour was set for all experiments. These settings were suggested as standard by the BacTrac^{TM} operational manual.

TSB was used for indirect (only with *E.* coli) and direct impedance analysis, even though it does show conductive properties and poor readings when measuring changes in media impedance, when using electrode impedance viable results were still recorded. MEB however is too conductive for use as a medium for direct analysis so could only be used for indirect analysis of fungal species.

3.5.1 Direct analysis

For direct analysis the impedance cells consisted of disposable polythene cells (20ml) containing four long electrodes. The cells were prepared by adding 9ml of TSB and 1ml of inoculum prepared earlier. This process was repeated for each dilution and done in duplicate. The cells were then loosely sealed and incubated in the BacTrac^{TM} at 30°C and 37°C for up to 24 hours (25°C and 30°C for up to 72 hours for *S. cerevisiae*) with measurements taken every 10 minutes. Two negative controls were also tested alongside the inoculated cells which contained just 10ml of sterile TSB, this was done to determine the effect of the conductivity of the media on the results and to check for any contaminants. Direct impedance analysis was performed on *S.cerevisiae*, *E. coli* and *S.* tyhpimurium strains only. Results were analysed using the Microsoft Excel spreadsheet.

3.5.2 Indirect analysis

For indirect analysis the impedance cells consisted of disposable polythene cells (20 ml) containing two short electrodes and an inner polythene reaction cell (7 ml). The outer cells were prepared by adding 2 ml of 0.2% potassium hydroxide, which filled the cells to just above the electrodes. The inner reaction cells were prepared by adding 4.5ml MEB and 0.5ml of inoculum prepared earlier

(1ml inoculum to 4ml broth was used for *P.verrucosum*, *F. oxysporum* and *A. niger* strains, also for *E. coli* TSB was used). This process was repeated for each dilution and done in duplicate. Immediately after inoculation the inner reaction cells were aseptically placed into each of the outer cells and the cells tightly sealed. They were then incubated in the BacTrac™ at 25°C and 30°C for up to 5 days (30°C and 37°C for up to 24 hours for *E.coli*) with measurements taken every 10 minutes. Two negative controls were also tested alongside the inoculated cells, which contained just 5 ml of sterile TSB or MEB, to check for any contaminants or if any atmospheric CO_2 was affecting the experiments. Indirect impedance analysis was performed on *S.cerevisiae*, *E. coli A. niger*, *F. oxysporum* and *P.verrucosum* strains only. Results were analysed using the Microsoft Excel spreadsheet

3.5.3 Impedance analysis in the presence of sorbic acid

The indirect analysis method was used exclusively to analyse the effects of sorbic acid on the growth of *A. niger*. *P. verrucosum* was not analysed because of its slow growing nature and time constraints on the study. MEB was sterilised for 20 minutes at 121°C, cooled, aseptically divided and the necessary amount of potassium sorbate added under sterile conditions. The concentrations of potassium sorbate used were 100, 200, 300 and 400ppm. The broth was then acidified using 1M hydrochloric acid to pH 4.6 for one set of plates and pH 2.5 for the other. After media preparation the method for addition of inoculum and preparation of impedance reaction cells is the same as stated in section 3.4.2.

3.6 TURBIDIMETRY ANALYSIS

Semi solid MEA was prepared using the following formulation: MEB, 8g; purified agar, 0.5g; distilled water, 400ml and autoclaved at 121°C for 20 minutes. Once cooled to room temperature 5ml of semi solid MEA was transferred to a sterile universal and 1 ml of inoculum added. The universal was gently shaken to ensure the inoculum and media were evenly mixed and no bubbles were formed, this process was repeated for each of the inoculums. A Bioscreen 10×10 micro-array plate was then prepared in which each of the columns (except the wells of the last row) had 300 µl of inoculated semi solid MEA added. The last row of 10 wells for each dilution acted as negative controls and contained wells just 300µl of semi solid MEA. The plate was then incubated for 10 days at 25 °C. The Bioscreens were set to take to take an optical density (OD) reading every 15 minutes at 600nm with no shaking. The time to detection (TTD) was defined as the time to produce

an OD=1. Bioscreen analysis was only performed on *A. niger*. Results were analysed using the Microsoft Excel spreadsheet

3.6.1 Turbidimetry analysis in the presence of sorbic acid and vanillin

Turbidimetry analysis was only used to monitor the effects of sorbic acid on the growth of *A. niger*. Semi solid agar was prepared in the same way as stated in section 3.5 however after cooling the media was aseptically divided and the necessary amount of potassium sorbate added under sterile conditions. The concentrations of potassium sorbate used were 400, 600, 1000, 1400, 1800, 2200, 2600, 3000, 3400 and 3800ppm. 5ml of media for each concentration of sorbic acid was then transferred to individual sterile universals and 1 ml of inoculum added (a single standard inoculum containing ~1.2 x10^6 spores/ml was used). Media without sorbic acid was also prepared and inoculated as above. The universals were then gently shaken to ensure the inoculum and media were evenly mixed and no bubbles formed. A Bioscreen 10×10 micro-array plate was then prepared in that all wells, except those of column one, received 200µl inoculated standard medium. To the wells of column one 400µl of the ten dilutions of sorbic acid was added. Using a multiwall pipette, 200µl of the sorbic acid dilutions was then removed from column one and added to the wells of column two and mixed. The process was repeated across the plate (setting up a half fold dilution series within the plate), discarding 200µl from the wells of column ten. Well ninety contained only 200µl of inoculated standard growth medium and acted as a control. Each plate was done in duplicate and incubated for 10 days at 25 °C. The Bioscreens were set to take to take an optical density (OD) reading every 20 minutes at 600nm with no shaking. The time to detection (TTD) was defined as the time to produce an OD=0.6. Bioscreen analysis was only performed on *A. niger*. This process was repeated for sorbic acid at pH 2.5 and vanillin at pH 4.6. Results were analysed using the Microsoft Excel spreadsheet

3.6.2 Turbidimetry analysis of the combined effects of sorbic acid and vanillin

The combined effect of sorbic acid and vanillin on growth was only tested on *A. niger*. Semi solid agar was prepared in the same way as stated in section 3.5 however after cooling the media was aseptically divided and the necessary amount of potassium sorbate and vanillin added under sterile conditions. The concentrations of potassium sorbate and vanillin used were 100, 200, 300, 400, 500, 600, 700, 800 and 900ppm. 5ml of media for each concentration of sorbic acid and vanillin was then transferred to individual sterile universals and 1 ml of inoculum added (a single standard inoculum containing ~1.2 x10^6 spores/ml was used). Media without sorbic acid and

vanillin was also prepared and inoculated as above. The universals were then gently shaken to ensure the inoculum and media were evenly mixed and no bubbles formed. A Bioscreen 10×10 micro-array plate was then prepared in that each well in column one (except well one) received 200µl of sorbic acid infused inoculated media (with the lowest concentration in well two and the highest in well ten). Each well of row one (except well one) received 200µl of vanillin infused inoculated media (with the lowest concentration in well eleven and the highest in well ninety one). Every subsequent well (except well one) was filled in a similar fashion (sorbic acid concentration low to high vertically and vanillin concentration low to high horizontally) but with 100µl of sorbic acid and 100µl of vanillin infused inoculated media. Each well where there was a combination of inhibitor was mixed thoroughly using a pipette. Well one contained only 200µl of inoculated standard growth medium and acted as a control. Each plate was done in duplicate and incubated for 10 days at 25 °C. The Bioscreens were set to take to take an optical density (OD) reading every 20 minutes at 600nm with no shaking. The time to detection (TTD) was defined as the time to produce an OD=0.6. Results were analysed using the Microsoft Excel spreadsheet.

3.7 STATISTICAL ANALYSIS

Regression lines for log microbial count (*Y*) vs time to detection (*X*) were compared for the different incubation temperatures, analysis type and organisms. Generation time estimation was performed by dividing the regression line gradient by 3.322. The time taken for one organism to reach the threshold reference value was determined using the *y* intercept of the regression line. The microbial population required to achieve a time to detection of 0 was calculated by dividing the gradient of the regression line by its intercept.

For non-linear regression, time to detection data was transformed to the reciprocal. Such transformations stabilise the data variance, allowing a less-biased analysis. Samples that showed no growth during the period of the experiment were not used in the model fitting but were retained for comparison. A simple mathematical model, the Lambert-Pearson model (Lambert and Pearson, 2000 (equation 3.1)) was used to fit the observed dose response profile. This is because a normal minimum inhibitory concentration (MIC) test gives only one piece of information - the least concentration in a serial dilution which prevented visible growth. Turbidimetry and conductimetry methods however obtain information on the level of inhibition at sub-MIC concentrations, as well as providing the MIC itself. Further the data obtained can be modelled by the Lambert-Pearson

model, which provides a measure of the dose response of the antimicrobial. It can be shown that the standard model for looking at the effect of pharmaceutics - the Hill Equation (Hill, 1910)- is an approximation to the Lambert-Pearson model. Hence the method and the analyses are superior in several ways: 1. gives the MIC, 2. gives value to the data at sub-MIC concentrations, 3. provides a dose response, and 4. is compatible with the standard lab methods and the standard method of analysis (i.e. the Hill model).

$$RTD = P_0 EXP\left[-1\left(\frac{x}{P_1}\right)^{P_2}\right]$$

Equation 3.1

Where RTD is the rate to detection (1/time), *x* is the inhibitor concentration (ppm), P_0 is the reference time to detection, P_1 is the concentration at maximum slope and P_2 is a slope parameter.

The MIC for a single inhibitor was defined as the intercept of the concentration axis to the tangent at the maximum gradient of the RTD/log concentration curve. From the Lambert-Pearson model this can be formulated as

$$MIC = P_1 EXP\left(\frac{1}{P_2}\right)$$

Equation 3.2

The non-inhibitory concentration (NIC) is the concentration below which normal uninhibited growth was observed, this was defined as the intercept of the tangent at the maximum gradient of the RTD/log concentration curve. From the Lambert-Pearson model this can be formulated as

$$NIC = P_1 EXP\left(\frac{1-e}{P_2}\right)$$

Equation 3.3

where *e* is the value of the exponential of 1 (approximately 2.718). All data was fitted using the Microsoft Excel spreadsheet package.

For the analysis of the combined effect of sorbic acid and vanillin, the model of Lambert and Lambert (2003) was used

$$RTD = P_0 EXP\left(-\left(\left(\frac{x}{P_1}\right)^{C_1} + \left(\frac{y}{P_3}\right)^{c_2}\right)^{c_q}\right)$$

Equation 3.4

If either of the concentrations (*x* or *y*) is zero then the equation is equivalent to that of Lambert and Pearson: i.e. P_1 and P_3 are the concentrations at maximum slope for each inhibitor in the absence of any other inhibitor. If the concentration of *y* is zero these expressions ensure that the product of C_1 and C_q equals P_2 and equivalently for inhibitor *y* when the concentration of *x* is zero. If the value of P_2 (from the Lambert Pearson model) is approximately 1 for each inhibitor, then in combination if there is a purely additive effect i.e. no synergy or antagonism present, then C_q will be approximately 1, and the modelled values of P_1 and P_3 will be equivalent to the values found individually (Lambert and Bidlas, 2007).

3.8 VOLATILE ORGANIC COMPOUND ANALYSIS

3.8.1 ATD GC-MS

The standard stainless-steel automated thermal desorption (ATD) sorbent tubes containing dual packing, comprising of 50% Tenax® TA and 50% Carbotrap (Markes International Limited, Llantrisant, UK), were used following conditioning. Conditioning was carried out by purging with helium carrier gas for 2 minutes at ambient temperature (approximately 25°C) followed by 30 minutes at 335°C. Conditioned cartridges were sealed with brass locking caps and stored at 4°C until required for use.

3.8.2 Collection

MEA was sterilised for 20 minutes at 121°C, cooled, aseptically divided and the necessary amount of potassium sorbate added under sterile conditions. The concentrations of potassium sorbate used were 100, 500, and 800ppm. The agar was then acidified using 1M hydrochloric acid to pH 4.6. Triplicate plates for each concentration of sorbic acid were centrally inoculated with 2µl of an *A. niger* spore suspension. To collect the volatiles produced by growth these inoculated plates were placed in suitably sized Nalophan® bags (Kalle Ltd. Witham, UK). The bags were then secured using a plastic tie wrap on one end and a custom made fitting made from a polypropylene

tube fitted with a Swagelok fitting on the other end. The bags were then filled with zero grade air. After 7 days incubation this fitting was connected to a TSI SP730 pump to pre-concentrate VOCs into TD tubes at a flow rate of 100ml/min for 5 minutes.

3.8.3 Internal standard

To quantify the VOCs in the samples an internal standard, d8-toluene in methanol, was used. This was supplied as 1ml vial of 1000µg ml^{-1} d8-toluene in methanol which was subsequently diluted to a final concentration of 100ng µl^{-1}. 5ml aliquots were then dispensed into screw top vials and stored at -80°C.

Standard was applied using a calibration solution loading rig (CSLR) (Markes International Limited, Llantrisant, UK) and a Hamilton gas syringe to deposit 0.5µl (50ng d8-toluene) on each tube. To quantify compounds indentified by GC-MS the peaks of each compound was compared to the height of the standard peak and scaled up to a litre.

3.8.4 GC-MS method

Pre-concentrated volatile species were subsequently analysed using an Autosystem XL gas chromatograph equipped with an ATD 400 thermal desorption system and TurboMass MS (Perkin Elmer, Wellesley, MA). The carrier gas used throughout was CP grade helium (BOC gases, Guilford, Surrey, UK) and further purified using a Mat-Sen inline filter (Sigma). Tubes were desorbed by purging for 2 minutes at ambient temperature then for 5 minutes at 300°C. Volatiles purged from the tubes were captured on a cold trap which was initially maintained at 30°C. Once desorption of the tube was complete, the trap was heated to 320°C using the fastest possible heating rate and maintained at that temperature for 5 minutes whilst the effluent was transferred to the GC via a heated transfer line at 180°C coupled directly to the GC column.

A Zebron ZB624 chromatographic column was used (Phenomexex, Torrance, CA, USA). This is a wall coated open tubular column (30m x 0.4mm x 0.25mm), the liquid phase compromising a 0.25µm layer of 6% cyanpropylphenyl and 94% methylpolysiloxane. The gas chromatograph oven was maintained at 50°C for 4 minutes.

4 Results

4.1 IMPEDANCE ANALYSIS OF BACTERIAL GROWTH

4.1.1 *Escherichia coli*

Figure 4.1 and Figure 4.2 (see page 45 and page 46 respectively) present the direct and indirect impedance analysis of the effect of incubation temperature and inoculum size on *Escherichia coli* growth. Detection times were defined as the time at which the conductivity of the KOH solution decreased by 25% (indirect analysis) or the growth medium increased by 10% (direct analysis). A higher threshold was used for indirect analysis because it is known to produce a larger conductance response than the direct method when measuring capacitance as in this experiment (Deak and Beuchat,1993). It has to be noted due to this being impedance analysis, the results do not necessarily represent an increase in bacterial cell number as with optical density, but instead an increase in metabolic activity, which is usually associated with cell growth.

As shown in Table 4.1 and Table 4.2 (see page 45 and page 46 respectively) a good linear relationship between *E. coli* concentration and detection time was obtained for both the indirect and direct analysis (correlation coefficient: $R^2 \geq 0.93$), with the rate of response found to be significantly ($p \leq 0.05$) dependent on inoculum size for both techniques (length of the impedance lag period inversely proportional to the size of the inoculum). There was also little variability in the detection times of replicate inocula at each inoculum level for the direct technique (no replicates were performed for indirect analysis). However detection times for the same experimental conditions were on average higher for indirect analysis. This lag is caused by the fact the technique relies on potassium hydroxide (KOH), a strong alkali absorbing CO_2 produced by *E.coli* and the resulting reaction taking place (equation 2.1) as the reaction cell isn't in direct contact with the electrodes (Figure 2.9). Unfortunately the reaction is not as efficient as directly monitoring ionic changes in growth medium caused by microbial growth (direct analysis), resulting in the elevated detection times seen here.

A similar response to inoculum size was also recorded in other studies for direct impedance analysis of *E. coli* growth (Cady *et al.,* 1977; Ur and Brown, 1975). Ur and Brown (1975) showed at 37°C using direct impedance analysis a 10-fold increase in the inoculum size reduced the response time by approximately one hour, this was also seen in results obtained by Cady *et al.* (1978). In the same inoculum range as stated in Ur and Brown (1975) the response time in this experiment was reduced by an average of 50 minutes at 37°C. This similarity despite the difference in equipment and growth medium used does seem to suggest that the direct impedance method is a fairly reproducible technique for bacteria or *E. coli* in particular. Though it has to be noted that Ur and Brown (1975) do not state what they define as their threshold for detection, so the reproducibility between these two systems cannot be conclusively ascertained. For indirect analysis however there is little research into the actual inoculum size effect, though results from Sawai *et al* (2002) when using an inoculum of approximately 10^3 CFU ml^{-1} produced a similar result to that shown in this experiment, in that a characteristic drop in impedance was seen and a detection time of roughly 10 hours was recorded. Timms *et al*. (1996) with an unidentified inoculum size saw a 25% decrease in impedance at 44°C within 5 hours. Though the results from Sawai *et al*. (2002) are double the detection time recorded in this experiment, it should not reflect on the reproducibility of this technique but could be instead due to the choice of alkali in the case of Sawai *et al* (2002) it was sodium hydroxide (NaOH) which could be less efficient absorbent of CO_2 in comparison with KOH or media influence could have taken an effect.

As expected the incubation temperature was found to have a significant impact ($p \leq 0.05$) on the growth rate of *E. coli*. The specific generation time (time for metabolic activity to double) calculated from Table 4.1 was 0.41 hours for 30°C and 0.25 hours for 37°C. The slope of the regression lines were then used to calculate the *y* intercept and therefore the detection time of a single *E. coli* cell; this was found to be 11.79 hours at 30°C and 6.98 hours at 37°C. For Table 4.2 the generation time was calculated at 0.45 hours for 30°C and 0.25 hours for 37°C. Once again the slope of the regression lines was used to calculate the *y* axis intercept which were found to be; 13.46 hours for 30°C and 7.94 hours for 37°C. Both direct and indirect impedance analysis conclude that the optimum temperature for the growth of *E. coli* to be 37°C. This is an unsurprising result considering *E. coli* is a common human pathogen and commensal organism commonly found in the gastrointestinal tract. Interestingly plate counts performed by Cady *et al*. (1978) on *E. coli* at 35°C found the generation time to be 20 minutes, a figure quite close to those calculated in these

experiments, meaning the assumption that the metabolic activity mirrors the physical growth is most likely correct

.

Figure 4.1 *Linear regression analysis of the results from the monitoring of E. coli growth in TSB at 37℃ and 30℃ using the direct impedance method (table 8.1 and table 8.2).*

a	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	6.98	0.17	6.61	7.35	0.98	16
log bacteria count	-0.82	0.03	-0.88	-0.75		

b	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	11.79	0.18	11.42	12.17	0.99	16
log bacteria count	-1.36	0.03	-1.43	-1.29		

Table 4.1 *Statistical output for the regression analysis of the results from the monitoring of E. coli growth in TSB at (a) 37℃ and (b) 30℃ using the direct impedance method*

Figure 4.2 *Linear regression analysis of the results from the monitoring of E. coli growth in TSB at 37℃ and 30℃ using the indirect impedance method (table 8.3 and table 8.4).*

a	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	7.94	0.59	6.43	9.44	0.93	7
log bacteria count	-0.82	0.10	-1.07	-0.56		

b	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	13.46	0.29	12.76	14.16	0.99	8
log bacteria count	-1.50	0.05	-1.63	-1.38		

Table 4.2 *Statistical output for the regression analysis of the results from the monitoring of E. coli growth in TSB at (a) 37℃ and (b) 30℃ using the indirect impedance method*

4.1.2 *Salmonella enterica* serovar typhimurium

Figure 4.3 (see page 48) presents the direct impedance analysis of the effect of incubation temperature and inoculum size on *Salmonella enterica* serovar typhimurium growth. Detection times were defined as the time at which the conductivity of the growth medium increased by 10%.

As shown in Table 4.3 a good linear relationship between *S.* typhimurium concentration and detection time was obtained (correlation coefficient: $R^2 \geq 0.97$), with the rate of response found to be significantly ($p \leq 0.05$) dependent on inoculum size (length of the impedance lag period inversely proportional to the size of the inoculum). There was also little variability in the detection times of replicate inocula at each inoculum level. Similarly Gibson (1988) also found a high correlation between log_{10} counts of three Salmonella species and detection times during direct impedance analysis at 35°C. Though the indirect technique was not performed on *S.* typhimurium in this experiment, results obtained by Bolton (1990) studying Salmonella growth using the indirect technique also found a similar inoculum dependent response and drop in impedance as seen with the direct technique and *E. coli* (Figure 4.2). These results and similarities does seem suggest that the direct and indirect impedance analysis of bacterial growth to be a fairly reproducible and reliable method.

As expected the incubation temperature was found to have a significant impact ($p \leq 0.05$) on the growth rate of *S.* typhimurium. The specific generation time calculated from Table 4.3 was 0.37 hours for 30°C and 0.24 hours for 37°C. The slope of the regression lines was then used to calculate the *y* intercept and therefore the detection time of a single *S.* typhimurium cell; this was found to be 13.24 hours at 30°C and 8.01 hours at 37°C. From these results it can be concluded that the optimum temperature for the growth of *S.* typhimurium to be 37°C. This is a similar result to those seen in the Figure 2.1, which again is unsurprising since like *E. coli, S.* typhimurium is a common human pathogen. However in comparison with *E. coli* from the generation times calculated it does seem that *S.* typhimurium does have a marginally faster metabolic activity and therefore possibly faster growth rate especially at 30°C. Though the detection times calculated for a single bacterium of each organism (*y* axis intercepts) is lower at both temperatures for *E. coli.* This suggests that *E. coli* produces more ionising metabolites than *S.* typhimurium as a part of its normal metabolic process causing greater conductance changes in the medium. This would explain the higher generation times but lower detection times recorded for *E. coli*, as even though *S.* typhimurium has a faster metabolic process creating a shallower slope on the regression line, the metabolites produced are not as ionising in comparison with *E. coli* leading to the higher detection times and *x* axis intercept for both temperatures seen in Figure 4.3. Yet in plate counts performed by Cady *et al* (1978) it was calculated the generation time of *Salmonella enteridis* (a structurally similar bacterium to *S.* typhimurium) to be 20 minutes at 35°C, the same as *E. coli*. This

a possible indication how much more precise a measurement impedance analysis is in comparison to the standard plating technique, however the difference in generation times at 37°C was only marginal so realistically a visual difference in growth populations probably would not be seen.

Figure 4.3 *Linear regression analysis of the results from the monitoring of S. typhimurium growth in TSB at 30℃ and 37℃ using the direct impedance method (table 8.5 and table 8.6).*

a	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	8.01	0.19	7.61	8.42	0.97	16
log bacteria count	-0.80	0.03	-0.87	-0.72		

b	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	13.24	0.28	12.64	13.85	0.98	15
Log bacteria count	-1.23	0.05	-1.34	-1.12		

Table 4.3 *Statistical output for the regression analysis of the results from the monitoring of S. typhimurium growth in TSB at (a) 37℃ and (b) 30℃ using the direct impedance method*

4.2 IMPEDANCE ANALYSIS OF FUNGAL GROWTH

4.2.1 *Saccharomyces cerevisiae*

Figure 4.4 and Figure 4.5 (see page 51 and page 52 respectively) present the direct and indirect impedance analysis of the effect of incubation temperature and inoculum size on *Saccharomyces cerevisiae* growth. Detection times were defined as the time at which the conductivity of the KOH solution decreased by 25% (indirect analysis) or the growth medium increased by 10% (direct analysis). Tryptone soya broth (TSB) though not the ideal media for analysing yeast growth had to be used for the direct impedance analysis, due to the high conductivity of malt extract broth (MEB), which was used for the indirect analysis portion of this experiment. TSB in control studies (results not shown) produced an increase in capacitive impedance (E-value) of less than +2% at most after 72 hours, with the threshold at +10% this was deemed acceptable.

As shown in Table 4.4 and Table 5.5 (see page 51 and page 52 respectively) a good linear relationship between *S. cerevisiae* concentration and detection time was obtained for both the indirect and direct analysis (correlation coefficient: $R^2 \geq 0.96$), with the rate of response found to be significantly ($p \leq 0.05$) dependent on inoculum size for both techniques (length of the impedance lag period inversely proportional to the size of the inoculum). There was also little variability in the detection times of replicate inocula at each inoculum level for the direct technique (no replicates were performed for indirect analysis). Furthermore the detection times were noticeably elevated across both techniques in comparison with the results from *E. coli* and *S.* typhimurium, this is because *S. cerevisiae* has a slower growth rate. Though as with the *E.* coli results detection times for the same experimental conditions were on average higher for indirect analysis regardless.

Similar responses to inoculum size and general *S. cerevisiae* growth were also recorded in other studies for direct and indirect impedance analysis of *S. cerevisiae* and unidentified yeast growth (Deak and Beucaht, 1993; Matsunaga *et al*. 1979; Owens *et al*. 1989; Owens *et al*. 1992). Results obtained from Deak and Beuchat (1993) like those from Owens *et al.* (1992) show a strong correlation between population size and detection time (or response time), however interestingly Deak and Beuchat (1993) found that in contrast to the findings from this experiment the indirect technique was actually 10-15 hours quicker than the direct technique at detecting similar yeast populations. Though it has to be noted that the results from Deak and Beuchat (1993) showed a

greater degree of variability (R^2 of regression lines equalling 0.69 for direct and 0.73 for indirect) and instead of a pure culture being analysed it was a mixed culture of unidentified yeasts. Furthermore it was not stated for the direct technique whether the capacitive (E- value) or media (M-value) impedance was monitored. This would have a great effect on the results as yeasts are known to produce only relatively small changes in media impedance unlike bacteria (Owens *et al.*1992) and hence would create elevated detection times. Though no other studies found directly compare direct and indirect impedance analysis, results from Owens *et al* (1992) seem to coincide with the findings seen in Figure 4.5, suggesting these are correct and with results from *E. coli* and S. typhimurium showing direct analysis to be the more rapid analysis technique, it would be expected the same to be true for *S. cerevisiae*.

As expected the incubation temperature was found to have a significant impact ($p \leq 0.05$) on the growth rate of *S. cerevisiae*..The specific generation time calculated from Table 4.4 was 1.15 hours for 25°C and 0.79 hours for 30°C. The slope of the regression lines was then used to calculate the *y* intercept and therefore the detection time of a single *S. cerevisiae* cell; this was found to be 30.28 hours at 25°C and 21.8 hours at 30°C. For Figure 4.5 the generation time was calculated at 1.19 hours at 30°C. Once again the slope of the regression lines was used to calculate the *y* axis intercept which were found to be; 31.4 hours at 30°C. The results from the direct analysis of *S. cerevisiae* growth concluded that the optimum incubation temperature for growth is 30°C. It would be fair to assume looking at previous results where indirect analysis mirrored the results of the direct analysis (Figure 4.1 and Figure 4.2) that a drop in metabolic activity and therefore increase in detection times would have also been seen if *S. cerevisiae* growth had been monitored at 25°C using the indirect technique. Plate counts by Cady *et al* (1978) calculated the generation time of *S. cerevisiae* at 32°C to be 1.5 hours, a much higher figure than the generation times calculated from both Table 4.4, Table 4.5 and growth at 25°C, suggesting unlike results for bacteria the metabolic activity of *S. cerevisiae* is not directly proportional to cellular growth.
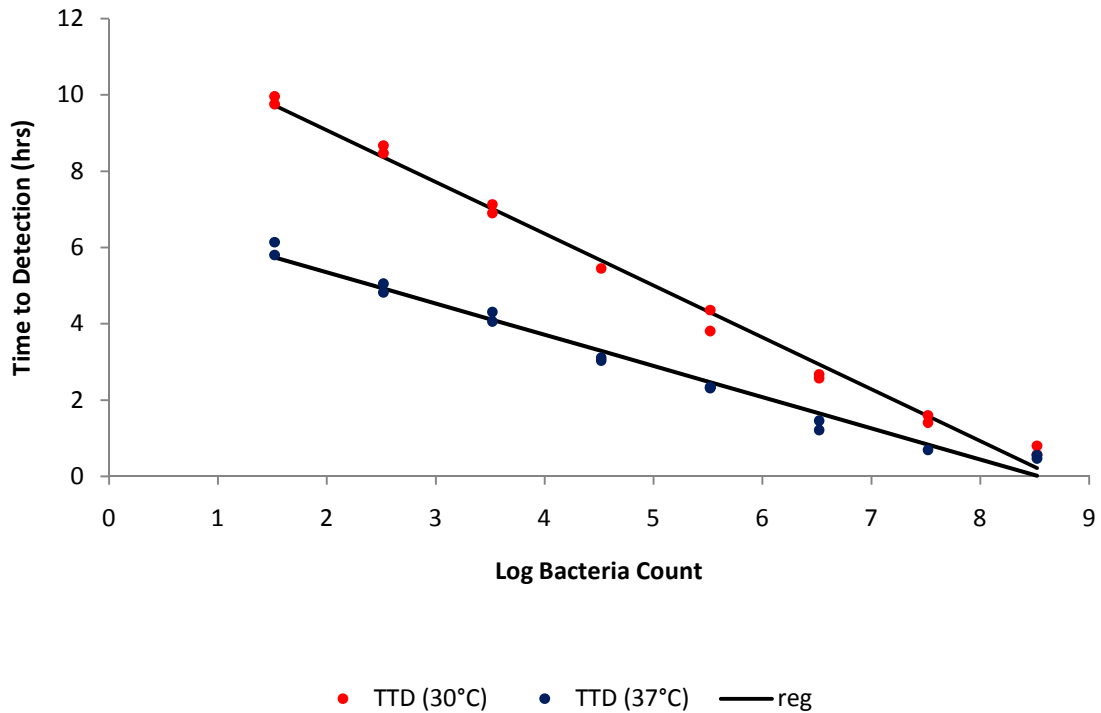
Figure 4.4 *Linear regression analysis of the results from the monitoring of S. cerevisiae growth in TSB at 30℃ and 25℃ using the direct impedance method (table 8.7 and table 8.8).*

a	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	21.83	0.61	20.49	23.17	0.96	14
log cell count	-2.62	0.15	-2.95	-2.30		

b	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	30.28	0.55	29.09	31.47	0.99	14
log cell count	-3.84	0.13	-4.13	-3.55		

Table 4.4 *Statistical output for the regression analysis of the results from the monitoring of S. cerevisiae growth in TSB at (a) 30℃ and (b) 25℃ using the direct impedance method*
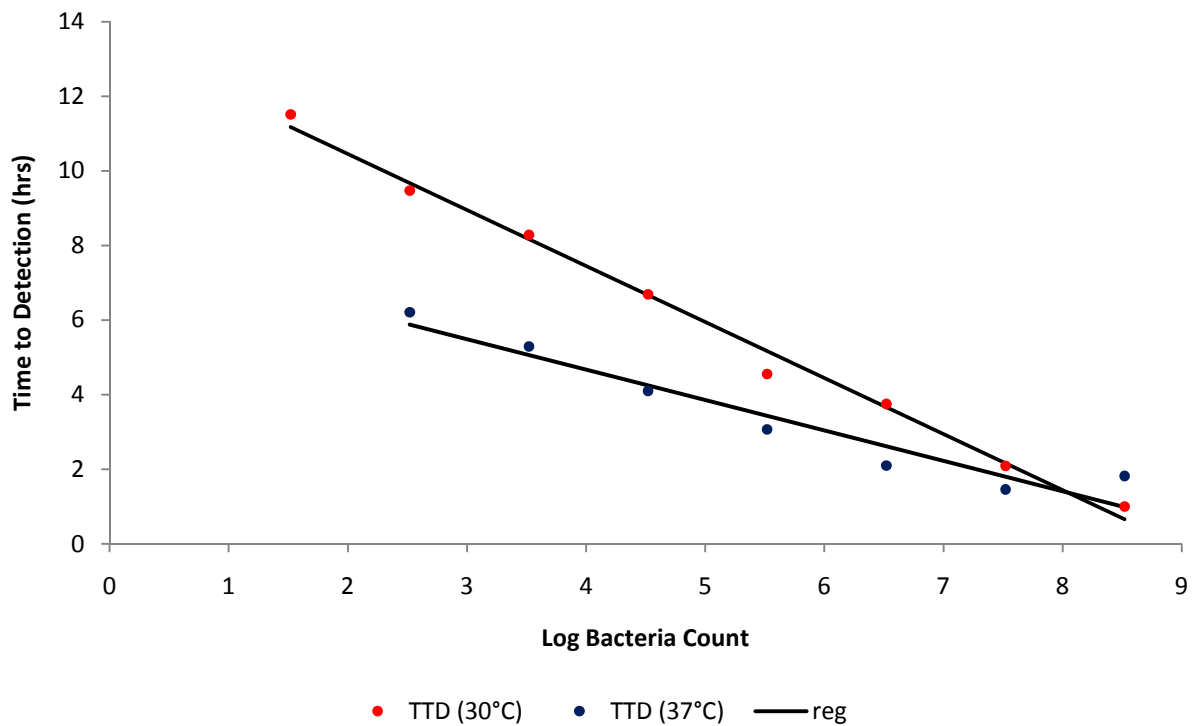
Figure 4.5 Linear regression analysis of the results from the monitoring of S. cerevisiae growth in TSB at 30$°C$ using the indirect impedance method (table 8.9).

	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	31.40	0.35	30.51	32.30	0.998	7
log cell count	-3.99	0.08	-4.19	-3.78		

Table 4.5 Statistical output for the regression analysis of the results from the monitoring of S. cerevisiae growth in TSB at 30$°C$ using the indirect impedance method

4.2.2 *Penicillium verrucosum*

Figure 4.6 (see page 54) presents the indirect impedance analysis of the effect of incubation temperature and inoculum size on *Penicillium verrucosum* growth. Detection times were defined as the time at which the conductivity of the KOH solution decreased by 25%. To confirm this detection time threshold was still adequate for the analysis of filamentous fungal growth, others were tested. As shown in Figure 4.7 (see page 55) it was found that though a threshold of -5% would provide a more rapid analysis the detection times showed a greater deal of variability (R^2 = 0.9) in comparison with a threshold of -25% (R^2 = 0.96). Furthermore a threshold of -50% was deemed unnecessary, as the results obtained were the same as with a -25% threshold (the gradient of the

regression lines are only marginally different so generation times calculated would be largely unaffected by the change in threshold). Therefore a threshold of -25% was deemed satisfactory for growth analysis. Radial growth measurements for *P. verrucosum* are also presented in Figure 4.8 (see page 56). Direct impedance analysis was not conducted on *P. verrucosum* mainly due to the fact as proved previously MEB which is the standard growth media for this organism is unsuitable for this type of analysis due to its high conductivity. Also it is known that filamentous fungi produce electrolytes by metabolism at a slow rate and also readily absorb electrolytes (Eden and Eden, 1984) causing no to very little changes in conductivity (Sawai *et al.,* 2003).

In a similar result to *S. cerevisiae*, *E. coli* and *S.* typhimurium, as shown in Table 4.6 a good linear relationship between *P. verrucosum* concentration and detection time was obtained (R^2 = 0.96), with the rate of response found to be significantly ($p \le 0.05$) dependent on inoculum size (length of the impedance lag period inversely proportional to the size of the inoculum). There was also little variability in the detection times of replicate inocula at each inoculum level. Unlike bacteria and yeasts there is very little research concerning the impedance analysis of filamentous fungal growth, making comparison difficult, though results obtained by Sawai *et al* (2003) for *Aspergillus niger* and *Rhizopus stolonifer* also showed the characteristic change in conductance and linear relationship between inoculum size and detection time seen in this study.

The specific radial growth rate of *P. verrucosum* was calculated from Table 4.8, it was deduced the growth rate of *P. verrucosum* was 0.036mm per hour from a starting inoculum of 1.6 x 10^6 spores/ml (log 6.2). At this rate visual growth was not identified until 72 hours into the experiment, while 1cm of growth took 261 hours (10.86 days). In comparison for impedance analysis the specific generation time calculated from Table 4.6 was 5.06 hours for 30°C. The slope of the regression lines was then used to calculate the *y* intercept and therefore the detection time of a single *P. verrucosum* spore; this was found to be 127.17 hours (5.3 days) at 30°C. Therefore it can be concluded that despite the standard plate technique being the more common method used, it is a lot slower, also because impedance analysis doesn't rely on the identification of visual growth but the metabolic activity its ability to detect smaller inoculum sizes is far superior. Results from Sawai *et al* (2003) support these findings.
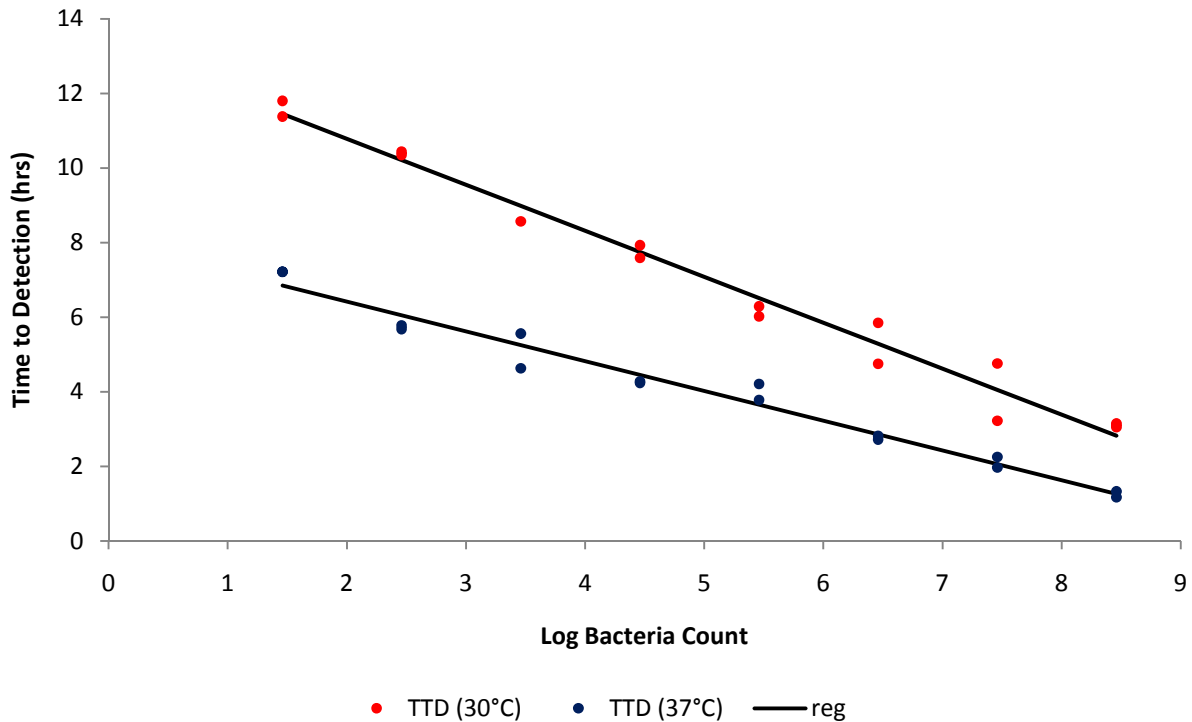
Figure 4.6 *Linear regression analysis of the results from the monitoring of P. verrucosum growth in MEB at 30°C using the indirect impedance method (table 8.11).*

	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	127.12	6.01	113.72	140.52	0.96	12
Log Spore Count	-16.81	1.07	-19.19	-14.44		

Table 4.6 *Statistical output for the regression analysis of the results from the monitoring of P. verrucosum growth in MEB at 30°C using the indirect impedance method*

Figure 4.7 *Linear regression analysis of the results from the monitoring of P. verrucosum growth in MEB at 30°C using the indirect impedance method and three different thresholds to define time to detection (table 8.11, table 8.12 and table 8.13)*

a	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	79.11	7.19	63.09	95.12	0.90	12
log spore count	-11.82	1.28	-14.66	-8.98		

b	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	127.12	6.01	113.72	140.52	0.96	12
log spore count	-16.81	1.07	-19.19	-14.44		

c	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	137.14	5.66	124.53	149.75	0.96	12
log spore count	-16.62	1.00	-18.86	-14.38		

Table 4.7 *Statistical output for the regression analysis of the results from the monitoring of P. verrucosum growth in MEB at 30°C using the indirect impedance method and the thresholds (a)-5% (b) -25% and (c) -50% to define time to detection*

Figure 4.8 *Linear regression analysis of the results from the monitoring of the radial growth of P. verrucosum on MEA at 25°C (table 8.10).*

30°C	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	-0.10	0.19	-0.49	0.28	0.98	48
Time	0.036	0.00084	0.035	0.038		

Table 4.8 *Statistical output for the regression analysis of the results from the monitoring of the radial growth of P. verrucosum growth on MEA at 25°C.*

4.2.3 *Fusarium oxysporum*

Figure 4.9 (see page 58) presents the indirect impedance analysis of the effect of incubation temperature and inoculum size on *Fusarium oxysporum* growth. Detection times were defined as the time at which the conductivity of the KOH solution decreased by 25%. Once again as with *P. verrucosum* no direct impedance analysis was performed.

In a similar result to *P. verrucosum,* as shown in Table 4.9 (see page 58) a linear relationship between *F. oxysporum* concentration and detection time was obtained (correlation coefficient: $R^2 \geq$

0.78), with the rate of response found to be significantly ($p \leq 0.05$) dependent on inoculum size (length of the impedance lag period inversely proportional to the size of the inoculum). However the results do show a greater degree of scattering and variability between replicate inocula at each inoculum level, especially at 30°C in comparison with results for *P. verrucosum*. This suggests that *F. oxysporum* may not be entirely suitable for this type of analysis possibly due to the apparent unpredictability of its metabolism and so CO_2 production in the given conditions of this experiment. In comparison results from the radial growth analysis of *F. oxysporum* presented in Figure and Table 4.10 showed a greater degree of reproducibility between plates ($R^2 = 0.988$). Despite it being a lengthier technique time wise the standard plate technique is more accurate and therefore it has to be concluded would be the preferred method for the monitoring of *F. oxysporum* growth.

Regardless of these inconsistencies in the data as expected the incubation temperature was found to have a significant impact ($p \leq 0.05$) on the growth rate of *F. oxysporum*. The specific generation time calculated from Figure 4.9 was 4.21 hours for 25°C and 2.94 hours for 30°C. The slope of the regression lines were then used to calculate the *y* intercept and therefore the detection time of a single *F. oxysporum* spore; this was found to be 100.94 hours (4.2 days) at 25°C and 74.88 hours (3.12 days) at 30°C. Therefore it can be concluded the optimum incubation temperature for growth is 30°C.

For radial growth analysis (Table 4.10) the growth rate of *F. oxysporum* was calculated as 0.106mm per hour from a starting inoculum of 1.6×10^6 spores/ml (log 6.2) at 25°C. At this rate visual growth was identified within 24 hours and 1cm of growth took approximately 96 hours (4 days) to achieve. In comparison with the impedance analysis, despite the standard plate technique being the more common method used, as with *P. verrucosum* it is a lot slower at detecting growth. However similarly both techniques do identify *P. verrucosum* as the slower growing organism, suggesting as seen with bacteria the differences in metabolic activity mirror differences in physical growth, though still in the case of *F. oxysporum* the standard plate technique does seem to be the more reliable method.
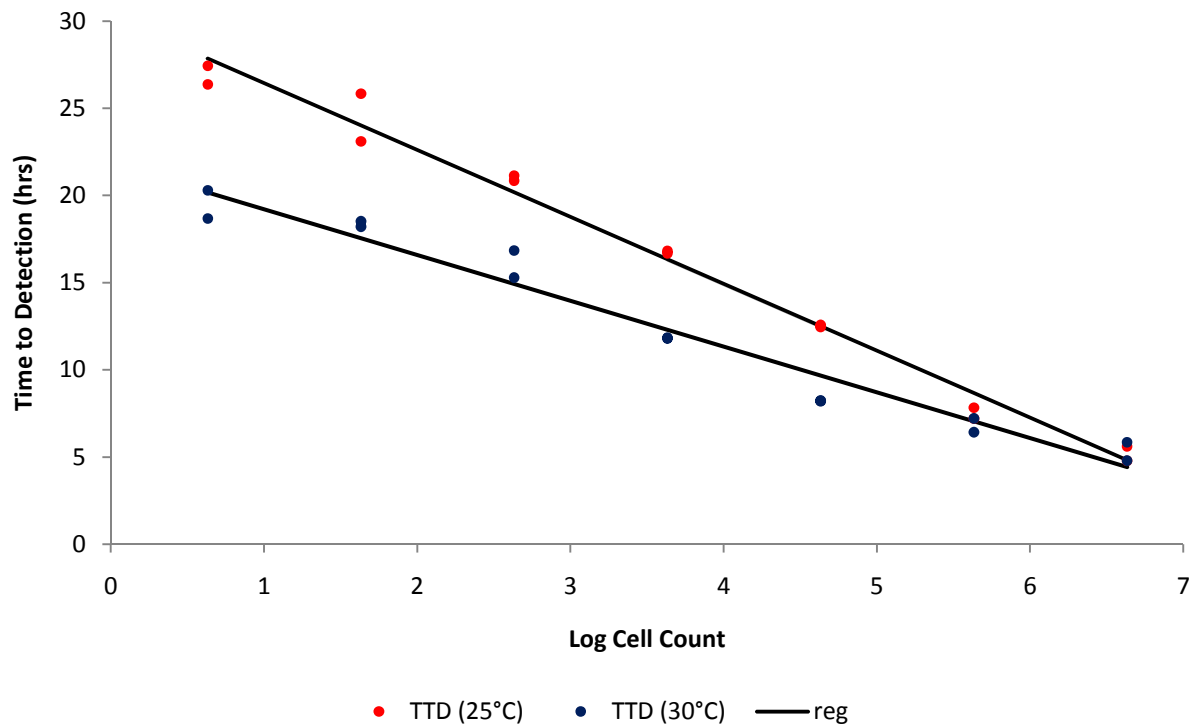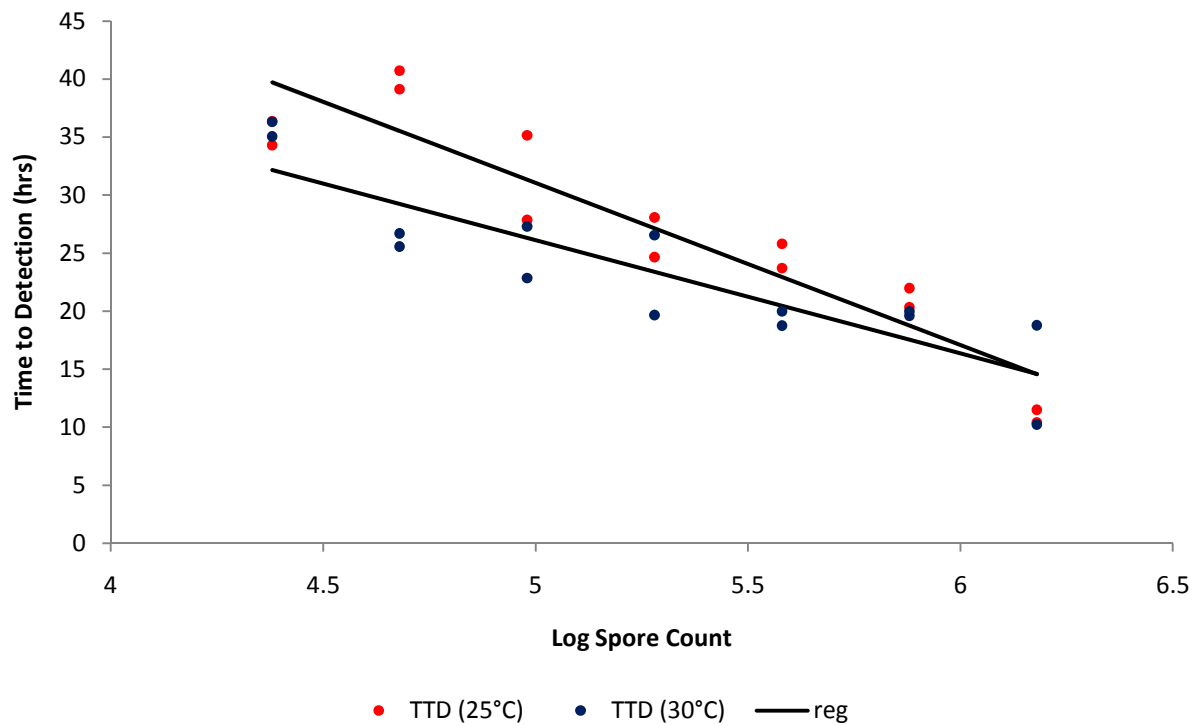
Figure 4.9 Linear regression analysis of the results from the monitoring of F. oxysporum growth in MEB at 30℃ and 25℃ using the indirect impedance method (table 8.14 and table 8.15).

a	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	74.88	7.85	57.77	91.99	0.78	14
log spore count	-9.75	1.48	-12.97	-6.53		

b	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	100.94	8.72	81.94	119.93	0.86	14
log spore count	-13.98	1.64	-17.55	-10.40		

Table 4.9 Statistical output for the regression analysis of the results from the monitoring of F. oxysporum growth in MEB at (a) 30℃ and (b) 25℃ using the indirect impedance method.
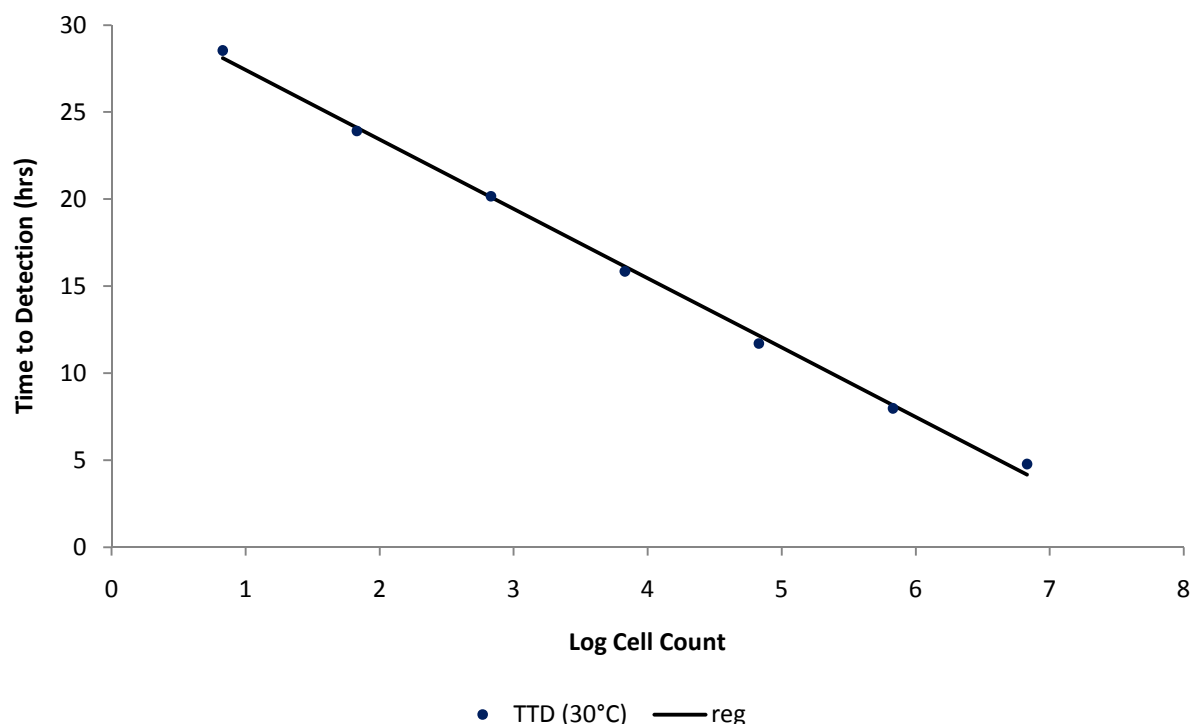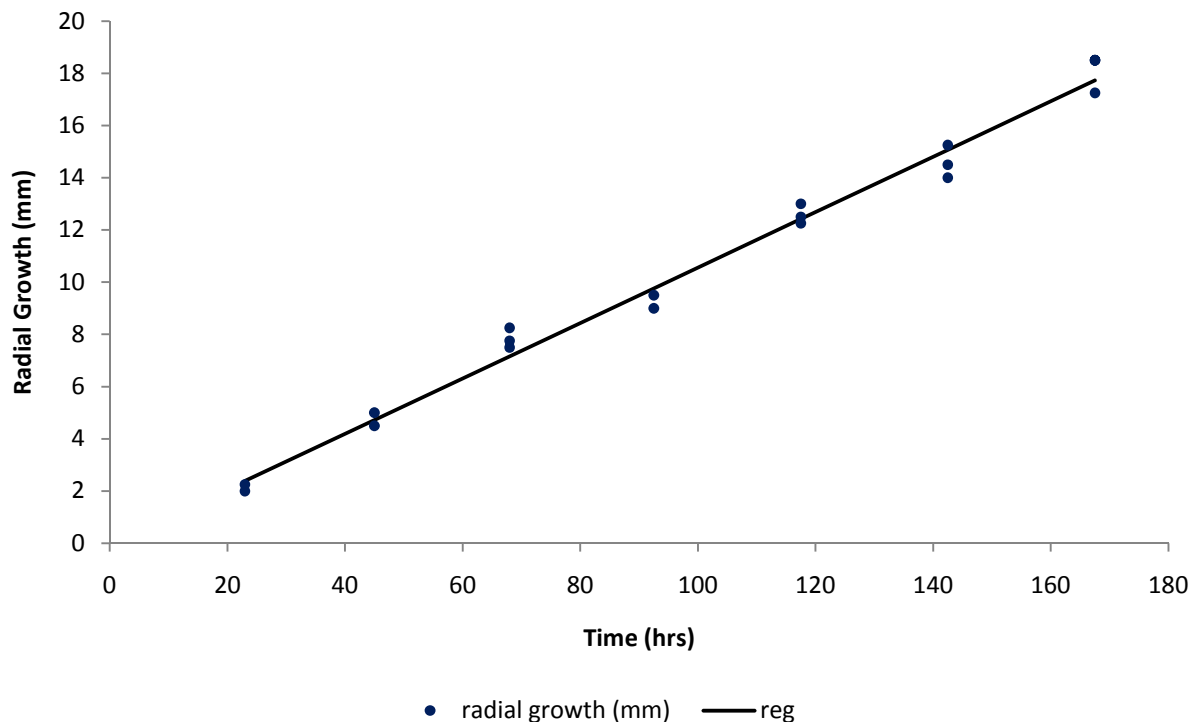
Figure 4.10 *Linear regression analysis of the results from the monitoring of the radial growth of F. oxysporum on MEA at 25°C (table 8.16).*

	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	-0.056	0.280	-0.641	0.529	0.988	21
Time	0.106	0.003	0.101	0.112		

Table 4.10 *Statistical output for the regression analysis of the results from the monitoring of the radial growth of F. oxysporum growth on MEA at 25°C.*

4.2.4 *Aspergillus niger*

4.2.4.1 Growth in optimum conditions

Figure 4.11 (see page 61) presents the indirect impedance analysis of the effect of incubation temperature and inoculum size on *Aspergillus niger* growth. Detection times were defined as the time at which the conductivity of the KOH solution decreased by 25%. Once again as with other filamentous fungi tested no direct impedance analysis was performed.

In a similar result to *P. verrucosum* and *F. oxysporum,* As shown in Table 4.11 (see page 61) a good linear relationship between *A. niger* concentration and detection time was obtained

(correlation coefficient: $R^2 \geq 0.86$), with the rate of response found to be significantly ($p \leq 0.05$) dependent on inoculum size (length of the impedance lag period inversely proportional to the size of the inoculum). Though as with *F. oxysporum* but to a lesser extent there was slight variability in the detection times of replicate inocula at each inoculum level. Sawai *et al* (2003) performed a similar experiment on *A. niger*, but instead of using liquid medium, they utilised various solid media. Parallel to results shown in Figure and Table 4.11, a good linear relationship between microbial concentration and detection time was recorded but with a better correlation ($R^2 \geq 0.98$). This could suggest that the fluctuations and lack of reproducibility in some of the results recorded for both *A. niger* and *F. oxysporum* could be possibly because liquid medium is not ideal for the indirect impedance analysis of these organisms, causing unpredictable growth responses.

As expected the incubation temperature was found to have a significant impact ($p \leq 0.05$) on the growth rate of *A. niger*. The specific generation time calculated from Table 4.9 was 2.51 hours for 25°C and 2.25 hours for 30°C. The slope of the regression lines were then used to calculate the *y* intercept and therefore the detection time of a single *A. niger* spore; this was found to be 81.75 hours (3.4 days) at 25°C and 65.37 hours (2.72 days) at 30°C. Therefore it can be concluded the optimum incubation temperature for growth is 30°C.

For radial growth analysis ((Table 4.12) see page 62) the growth rate of *A. niger* was calculated as 0.17mm per hour from a starting inoculum of 1.6×10^6 spores/ml (log 6.2) at 25°C. At this rate visual growth was identified within 24 hours and 1cm of growth took approximately 65 hours (2.7 days) to achieve. In comparison with the impedance analysis, despite the standard plate technique being the more common method used, as with *P. verrucosum* and *F. oxysporum* it is a lot slower at detecting growth. A similar result was obtained by Sawai *et al.* (2003). Similarly both techniques show that *A. niger* is the faster growing fungus in comparison to *F. oxysporum* and *P. verrucosum*, showing that impedance analysis despite it being slightly less reproducible, could replace the standard plate technique at rapidly and easily estimate the initial microbial concentration based on detection time values.
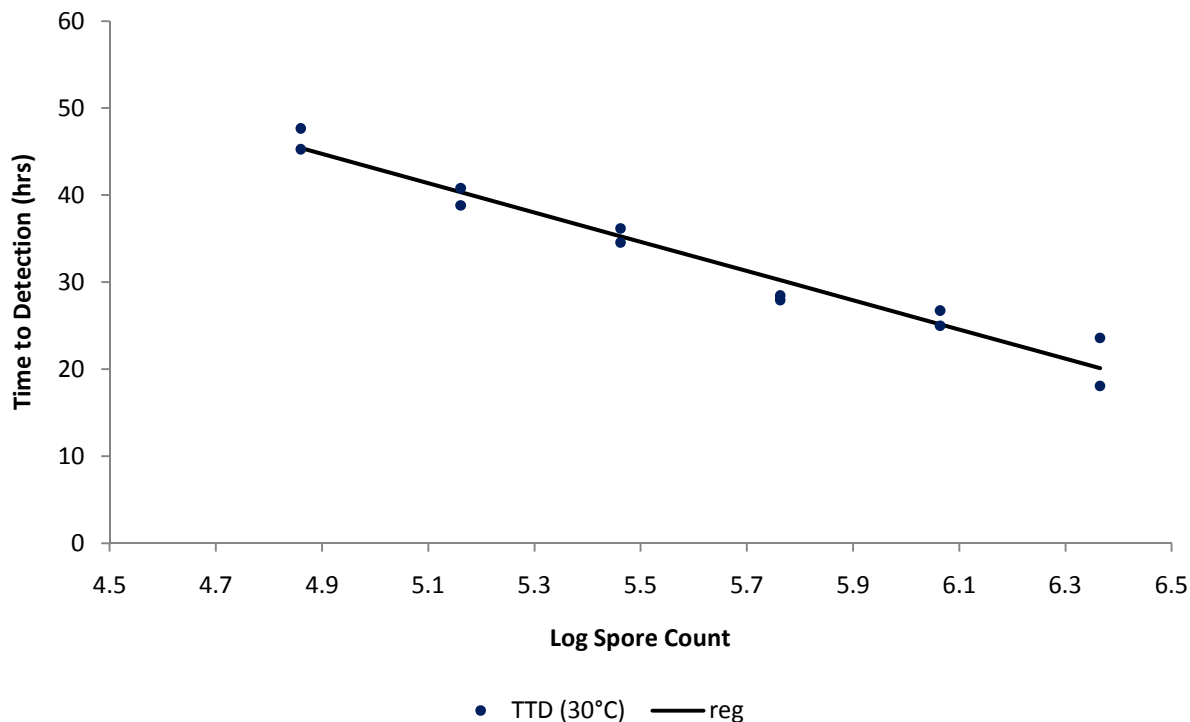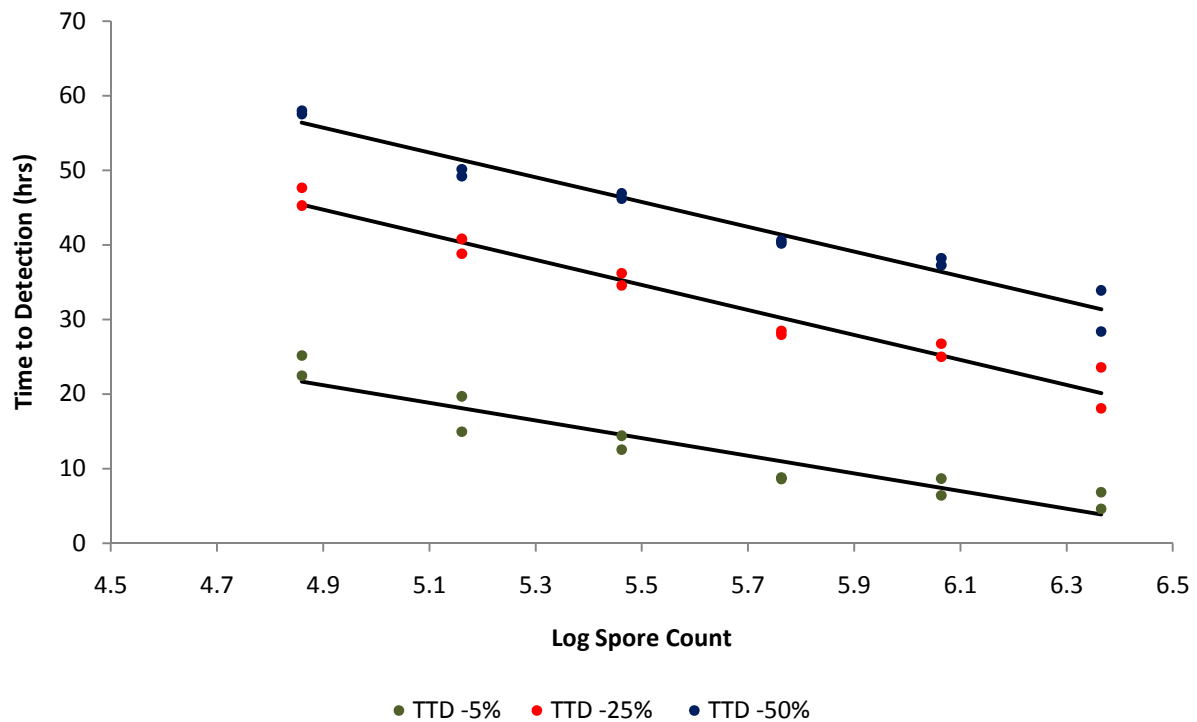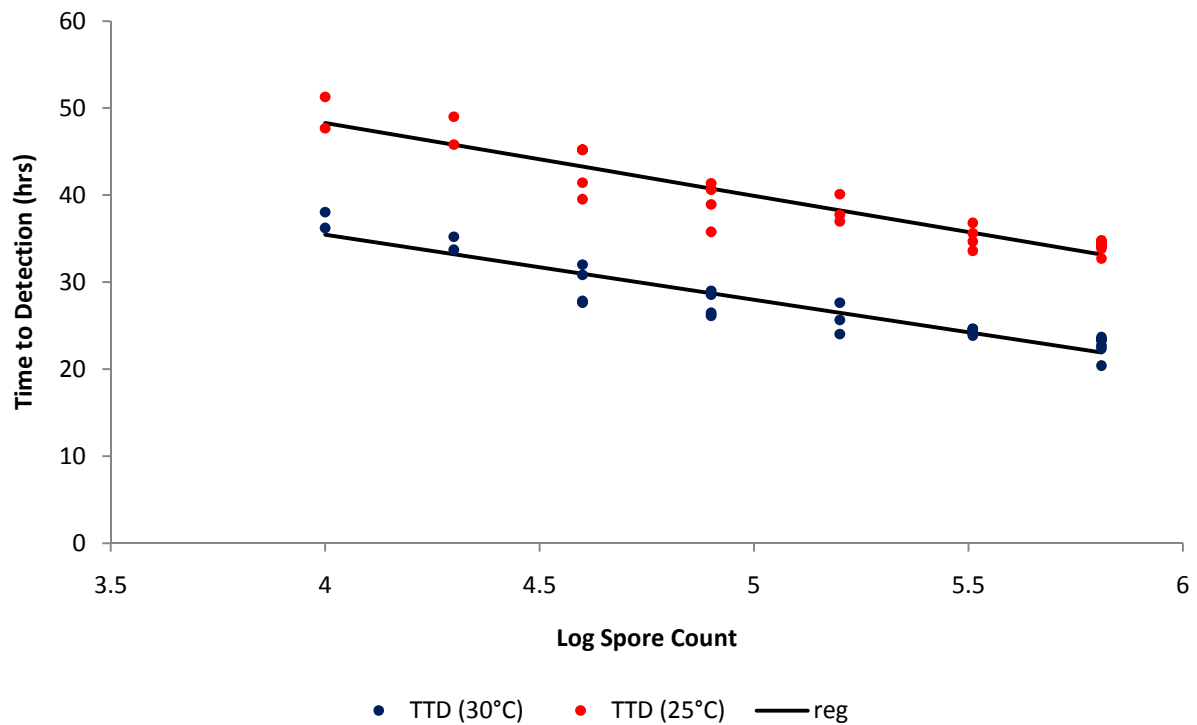
Figure 4.11 *Linear regression analyses of the results from the monitoring of A. niger growth in MEB at 30℃ and 25℃ using the indirect impedance method (table 8.17 and table 8.18).*

a	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	65.37	3.01	59.14	71.60	0.88	25
log spore count	-7.48	0.59	-8.70	-6.26		

b	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	81.75	3.47	74.59	88.92	0.86	26
log spore count	-8.36	0.68	-9.76	-6.97		

Table 4.11 *Statistical output for the regression analysis of the results from the monitoring of A. niger growth in MEB at (a) 30℃ and (b) 25℃ using the indirect impedance method.*
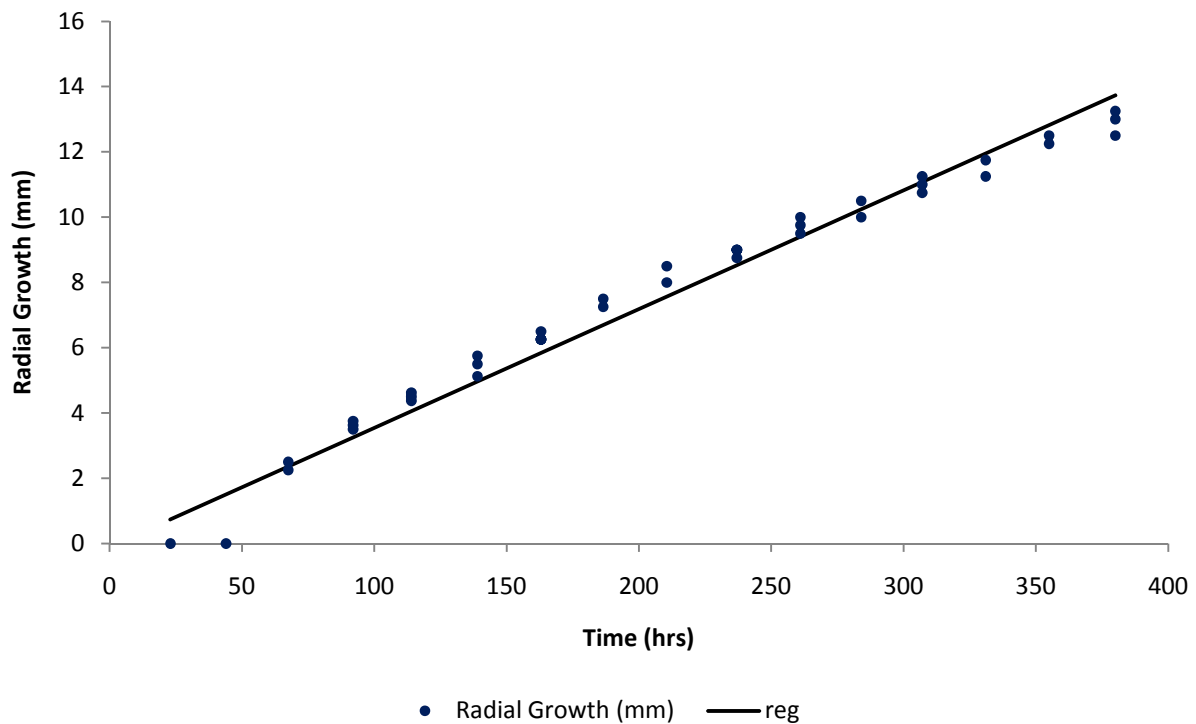
Figure 4.12 *Linear regression analysis of the results from the monitoring of the radial growth of A. niger on MEA at 25°C (table 8.19).*

	Coefficients	**Standard Error**	**Lower 95%**	**Upper 95%**	**R Square**	**Observations**
Intercept	-1.41	0.30	-2.04	-0.77	0.99	21
Time	0.17	0.0029	0.16	0.18		

Table 4.12 *Statistical output for the regression analysis of the results from the monitoring of the radial growth of A. niger growth on MEA at 25°C.*

4.2.4.2   Growth in the presence of sorbic acid

Figure 4.13 (see page 65) presents the results for the indirect analysis of *A. niger* inhibition by sorbic acid at pH 4.6 and pH 2.5. As with standard growth analysis detection times were defined as the time at which the conductivity of the KOH solution decreased by 25%.

The detection times for *A. niger* at both pH 2.5 and pH 4.6 were delayed with increasing sorbic acid concentration. At pH 2.5 no conductivity change was observed over 300ppm, however in comparison at pH 4.6, sorbic acid displayed weaker antifungal activity, with changes in conductance observed at 500pm, the highest measured concentration of sorbic acid observed in

this experiment. This result was also reflected in the MIC (minimum inhibitory concentration: marks the concentration above which no growth is observed relative to the control) and NIC values (non-inhibitory concentration: the concentration above which the inhibitor begins to have a negative effect on growth) shown in Table 4.13 (see page 65). Similar results were seen in Figure 4.14 (see pages 66-68) and Figure 4.15 (see page 69) where the radial growth of *A.niger* in the presence of sorbic acid at pH 2.5 and pH 4.6 was monitored. It was found as with impedance analysis that there was an initial lag period that increased with the sorbic acid concentration, after which a linear increase in colony diameter in relation to incubation time was observed, a similar pattern to the conductance changes detected after the initial lag during impedance analysis. Furthermore there was also greater antifungal activity observed at pH 2.5, with no growth visualised over 200ppm and the radial growth rate at 100ppm calculated to be 0.177mm per hour. In comparison at pH 4.6 growth was seen at 800ppm, the highest concentration of sorbic acid tested in this experiment and the radial growth rate calculated to be 0.26mm per hour a 68% increase on the rate calculated at pH 2.5. Many studies have also demonstrated the importance of pH in the efficacy of sorbic acid. For example, Park and Marth, (1972) showed that *S.* typhimurium would grow in nutrient broth (pH 6.7) fortified with 3000ppm sorbic acid when the media was not acidified. However, when the pH was reduced to 5.0, growth did not occur. Bell *et al.* (1959) and Lambert and Stratford (1999) also observed a similar pattern of inhibition for filamentous fungi and yeasts. The results seen in other studies and both radial growth and impedance analysis are because as with other organic acids, it is the undissociated molecule that provides the antifungal activity of sorbic acid (Eklund, 1983). The amount of molecule in the undissociated form is determined by the pH. The antimicrobial effectiveness of sorbic acid increases as the pH value approaches its dissociation constant (pKa), which is 4.76. At this pH value, 50% of sorbic acid is in the effective undissociated form. So at pH 4.6 the undissociated fraction would be 59% of total sorbic acid concentration, though in comparison at pH 2.5, 99% of the total sorbic acid concentration would be undissociated. Hence more inhibition would be observed at lower total concentrations of sorbic acid at pH 2.5

In comparison with the MIC calculated at pH 2.5 (567ppm) and those from other studies, for example Liewen and Marth, (1984) where for *A. niger* the MIC was deduced to be 500ppm, the MIC calculated at pH 4.6 (2747ppm), is extremely high (nearly 3 times the legally permitted limit of use). The ability of filamentous fungi to metabolise sorbic acid is well established, several authors have reported that some fungi can metabolise and thereby detoxify it, resulting in the high MIC and

low NIC values seen in this study. GC/MS analysis of the headspace gas from *A. niger* cultures grown in the presence of sorbic acid at pH 4.6 were monitored to confirm the biotransformation of sorbic acid was the cause of this lack of antifungal activity, the results are shown in Figure 4.16 (see page 70). 1, 3-pentadiene (peak 7.77 and 7.80), the end-product of the decarboxylation of sorbic acid was detected in all samples, except for those where *A. niger* was grown in the presence of no sorbic acid. Furthermore the concentration of 1, 3-pentadiene detected rose with every increase in sorbic acid concentration. Many other studies have also demonstrated that filamentous fungi and yeasts are capable of degrading sorbic acid to 1, 3-pentadiene in particular (Casas *et al*. 1999; Kinderlerer and Hatton, 1990). Sorbic acid is also thought to undergo auto-oxidative degradation in aqueous systems, forming mono-aldehydes and carbonyls (Arya, 1980). However, no carbonyls or mono-aldehydes were detected in sufficient quantity to suggest that any auto-oxidation of sorbic acid took place and results from Plumridge *et al*. (2004) who observed in the absence of inoculum, the concentration of sorbic acid remained constant for up to 72 hours, support these findings. Furthermore Plumridge *et al* (2004) found no evidence in their study that suggested the bioavailability of sorbic acid was reduced by the possible binding or adsorption of the acid by cell walls or lipids of spores or mycelia. Therefore it has to be concluded that *A. niger* degrades sorbic acid to 1, 3 pentadiene through a decarboxylation process, and allowing growth at higher concentrations.
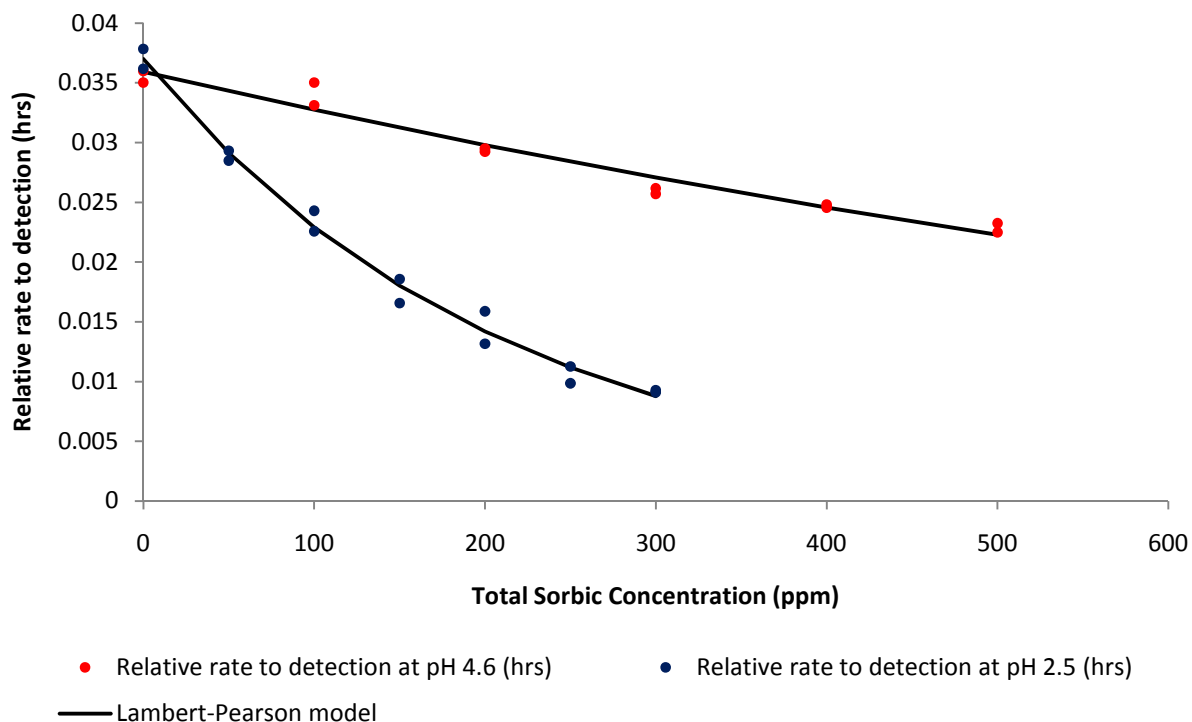
Figure 4.13 *analysis of the results from the monitoring of A. niger growth in MEB at 25°C in the presence of sorbic acid at pH 4.6 and pH 2.5 using the indirect impedance method (table 8.20 and table 8.21).*

(a)

Parameter	Estimate	Standard Error	Lower CL	Upper CL	MIC	NIC
P1	1032.3	133.33	823.12	1453.78	2747.32	195.85
P2	1.02	0.15	0.76	1.37		
P0	0.036	0.001	0.034	0.038		

(b)

Parameter	Estimate	Standard Error	Lower CL	Upper CL	MIC	NIC
P1	209.26	7.084	194.35	225.74	567.01	37.47
P2	0.995	0.063	0.86	1.14		
P0	0.037	0.001	0.035	0.039		

Table 4.13 *Statistical output for analysis of the results from the monitoring of A. niger growth in MEB at 25°C in the presence of sorbic acid at (a) pH 4.6 and (b) pH 2.5 using the indirect impedance method*
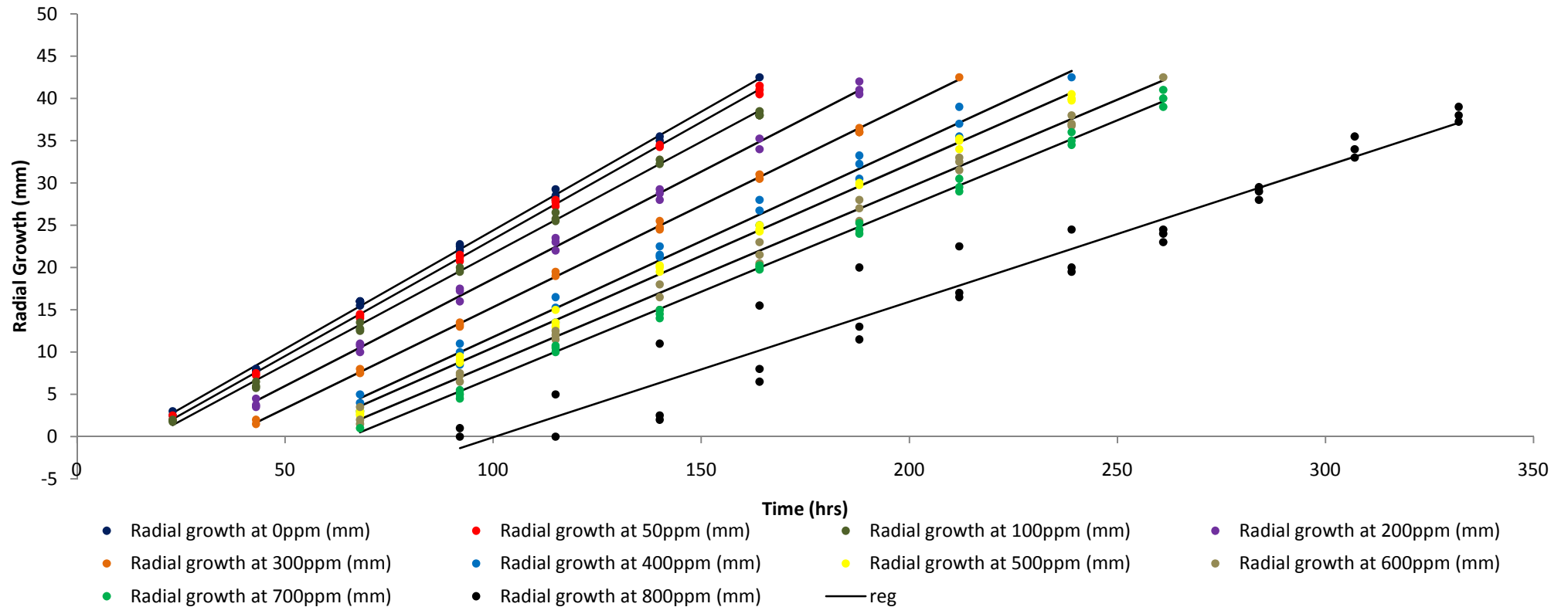
Figure 4.14 *Linear regression analysis of the results from the monitoring of the radial growth of A. niger on MEA at 25°C in the presence of sorbic acid at pH 4.6 (table 8.22, table 8.23, table 8.24 and table 8.25).*

a	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	-3.67	0.21	-4.11	-3.23	0.999	21
Time	0.28	0	0.28	0.28		

b	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	-4.314	0.161	-4.651	-3.978	0.999	21
Time	0.277	0.002	0.273	0.28		

c	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	-4.7	0.24	-5.21	-4.19	0.999	21
Time	0.26	0.002	0.26	0.27		

d	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	-6.7	0.37	-7.48	-5.92	0.997	21
Time	0.25	0.003	0.25	0.26		

e	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	-8.69	0.16	-9.03	-8.35	0.999	24
Time	0.24	0.001	0.24	0.24		

Table 4.14 Statistical output for the regression analysis of the results from the monitoring of the radial growth of A. niger growth on MEA at 25°C in the presence of (a) 0ppm (b)50ppm (c)100ppm (d)200ppm and (e)300ppm sorbic acid at pH 4.6

a	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	-10.83	0.7	-12.29	-9.37	0.99	24
Time	0.23	0.004	0.22	0.24		

b	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	-11.16	0.39	-11.97	-10.34	0.997	24
Time	0.22	0.002	0.21	0.22		

c	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	-12.07	0.47	-13.03	-11.11	0.996	27
Time	0.21	0.003	0.2	0.21		

d	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	-13.29	0.34	-13.99	-12.59	0.998	27
Time	0.2	0.002	0.199	0.21		

e	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	-16.09	1.43	-19.01	-13.17	0.95	33
Time	0.16	0.01	0.15	0.17		

Table 4.15 *Statistical output for the regression analysis of the results from the monitoring of the radial growth of A. niger growth on MEA at 25°C in the presence of (a)400ppm (b)500ppm (c)600ppm (d)700ppm (e)800ppm sorbic acid at pH 4.6.*
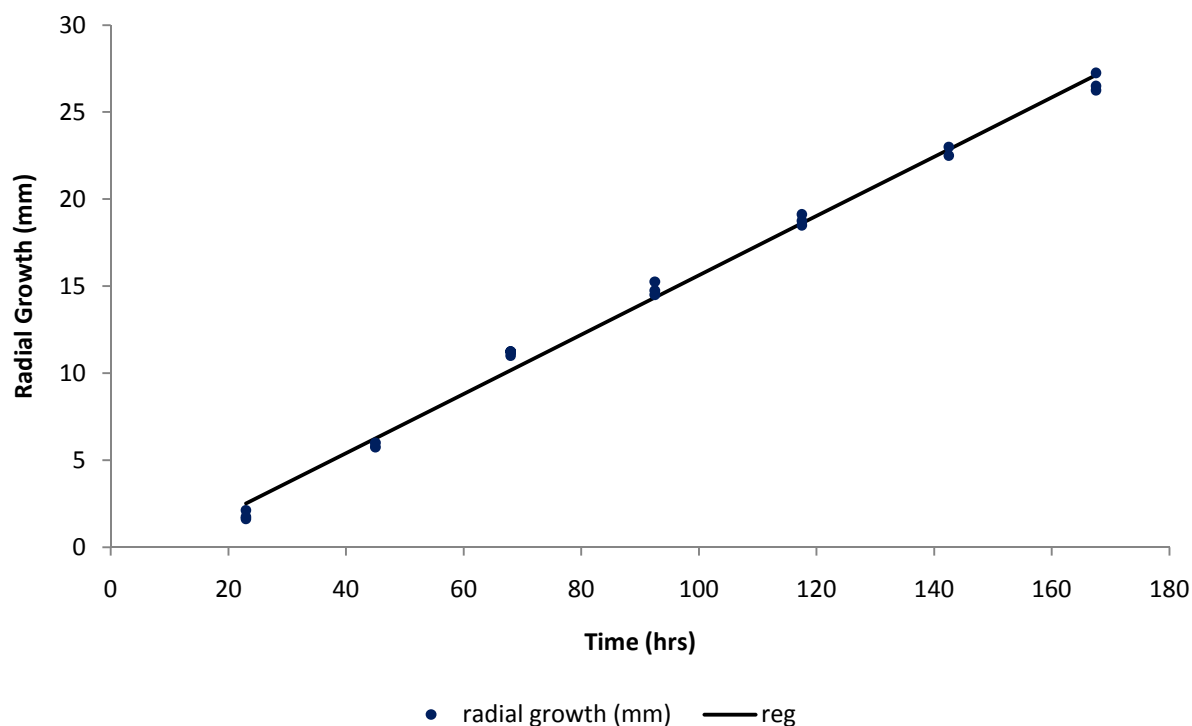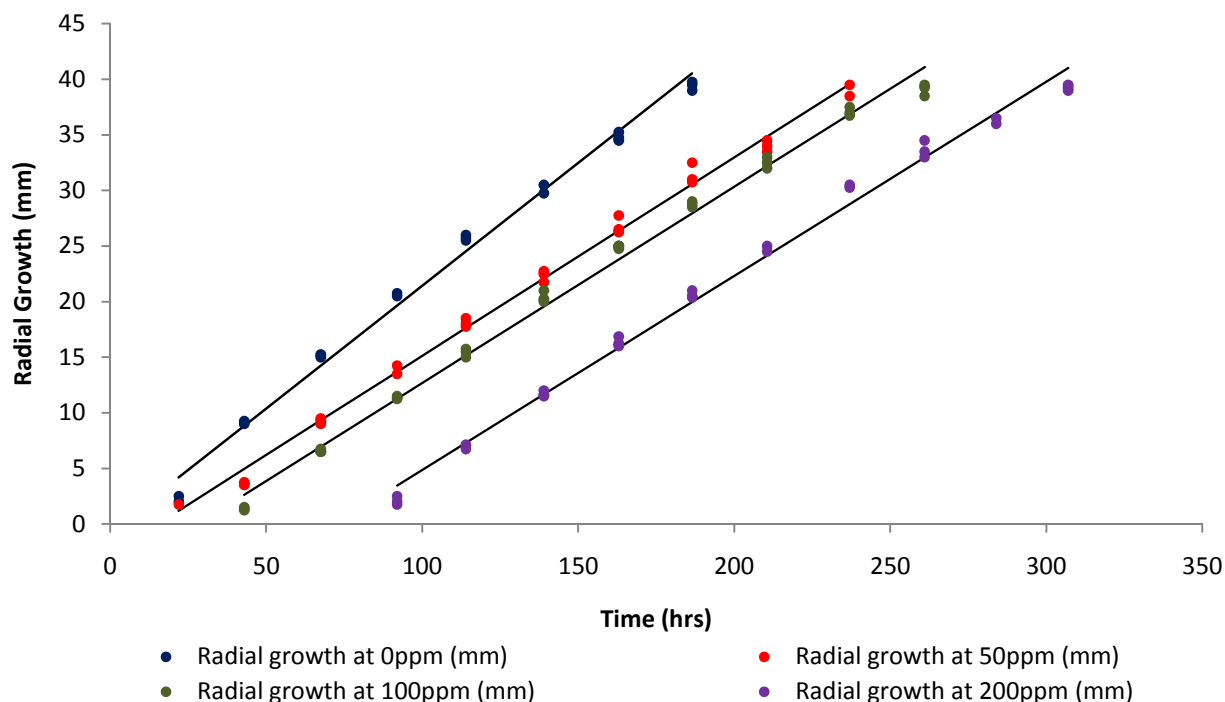
Figure 4.15 *Linear regression analysis of the results from the monitoring of the radial growth of A. niger on MEA at 25°C in the presence of sorbic acid at pH 2.5 (table 8.26 and table 8.27).*

a	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	-0.69	0.49	-1.71	0.34	0.992	24
Time	0.22	0.0042	0.21	0.23		

b	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	-2.73	0.31	-3.36	-2.10	0.996	30
Time	0.179	0.0021	0.17	0.18		

c	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	-4.97	0.44	-5.88	-4.06	0.994	30
Time	0.177	0.0027	0.17	0.18		

d	Coefficients	Standard Error	Lower 95%	Upper 95%	R Square	Observations
Intercept	-12.62	0.60	-13.85	-11.38	0.993	30
Time	0.175	0.0029	0.17	0.18		

Table 4.16 *Statistical output for the regression analysis of the results from the monitoring of the radial growth of A. niger growth on MEA at 25°C in the presence of (a) 0ppm (b)50ppm (c)100ppm and (d)200ppm sorbic acid at pH 2.5.*
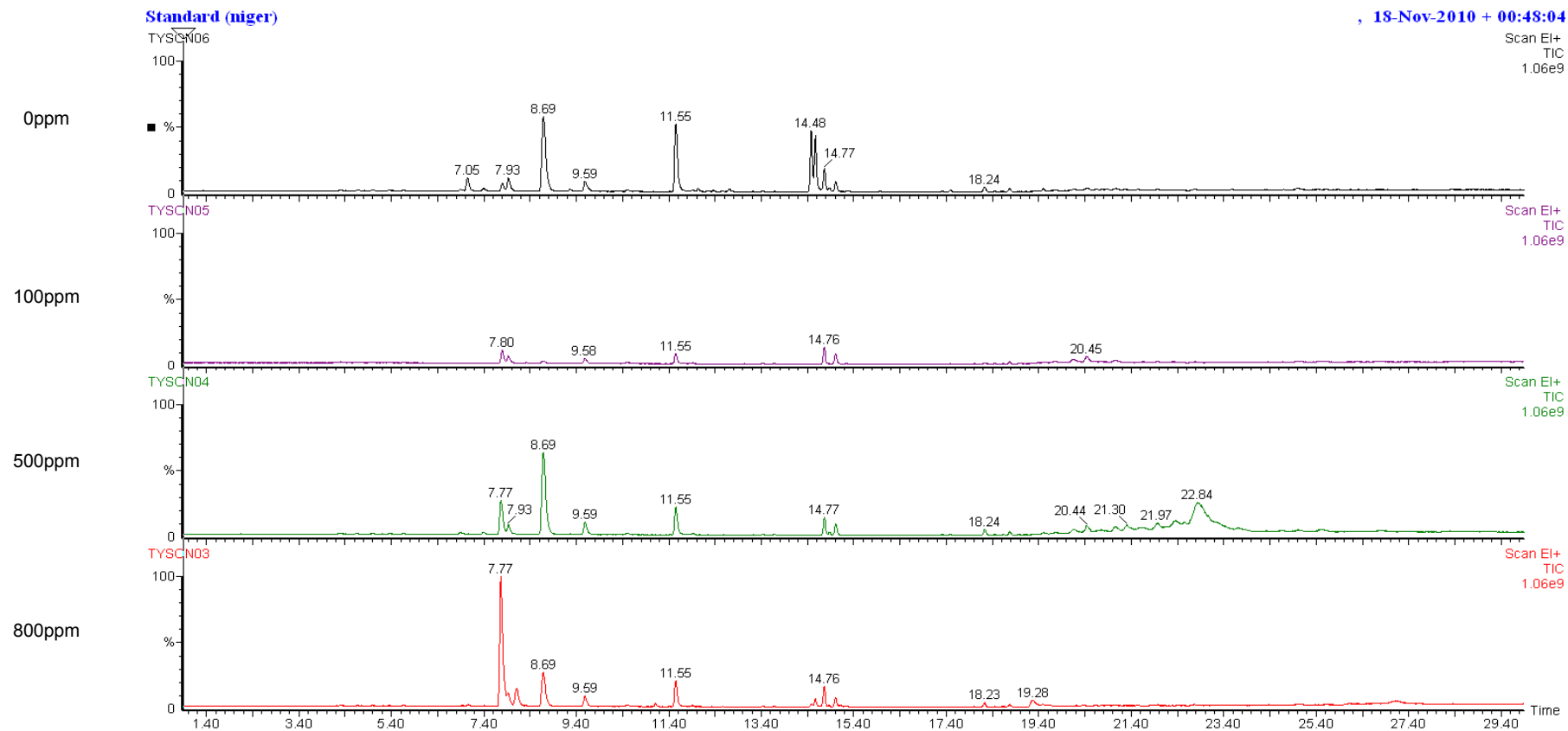
Figure 4.16 *GC/MS analysis of head space gas from A.niger cultures grown in the presence of sorbic acid at pH 4.6: peak 7.77 and 7.80 correspond with the production of 1, 3-pentadiene*

4.3 TURBIDIMETRY ANALYSIS OF FUNGAL GROWTH

4.3.1 *Aspergillus niger*

4.3.1.1 Growth in optimum conditions

Figure 4.17 presents the Bioscreen analysis of the effect of inoculum size on *Aspergillus niger* growth. The detection time was determined from a point where there was a rapid increase in optical density and can be related to the cell number. It was taken to be the time to achieve an optical density equalling 1 at 600nm.

As shown in Table 4.17 a good linear relationship between *A. niger* concentration and detection time was obtained for both (correlation coefficient: R^2 = 0.95), with the rate of response found to be significantly ($p \leq 0.05$) dependent on inoculum size (length of the impedance lag period inversely proportional to the size of the inoculum). There was also little variability in the detection times of replicate inocula at each inoculum level. Many other studies have also demonstrated using the Bioscreen the link between detection time and inoculum size but with bacteria (Johnston, 1998; Robinson *et al*. 2001) not filamentous fungi, making similarities between results difficult to assess. Though comparing results obtained using the impedance technique, a similar response to inoculum size is observed, however the Bioscreen did provide a higher degree of reproducibility.

The specific generation time calculated from Table 4.17 was 2.61 hours at 25°C. The slope of the regression line was then used to calculate the *y* intercept and therefore the detection time of a single *A. niger* spore; this was found to be 81.44 hours (3.4 days) at 25°C. The generation time and *y* intercept calculated from the impedance analysis of *A. niger* at 25°C are almost equal to those calculated from the bioscreen results, suggesting that the CO_2 production of *A. niger* is most likely directly proportional to the cellular growth. However, once again the lack of other studies directly comparing turbidimetry and impedance analyses makes confirmation of this difficult. Though Chorianopoulos *et al* (2006) and Sawai *et al* (2002) showed that the MICs of certain bacteria to various antimicrobials calculated using the impedance method were almost identical to that obtained by the turbidity method. These results demonstrate that the turbidity and impedance (indirect) are directly comparable and can be effectively used to evaluate filamentous fungal growth in replacement of the slower more labour intensive plating technique.

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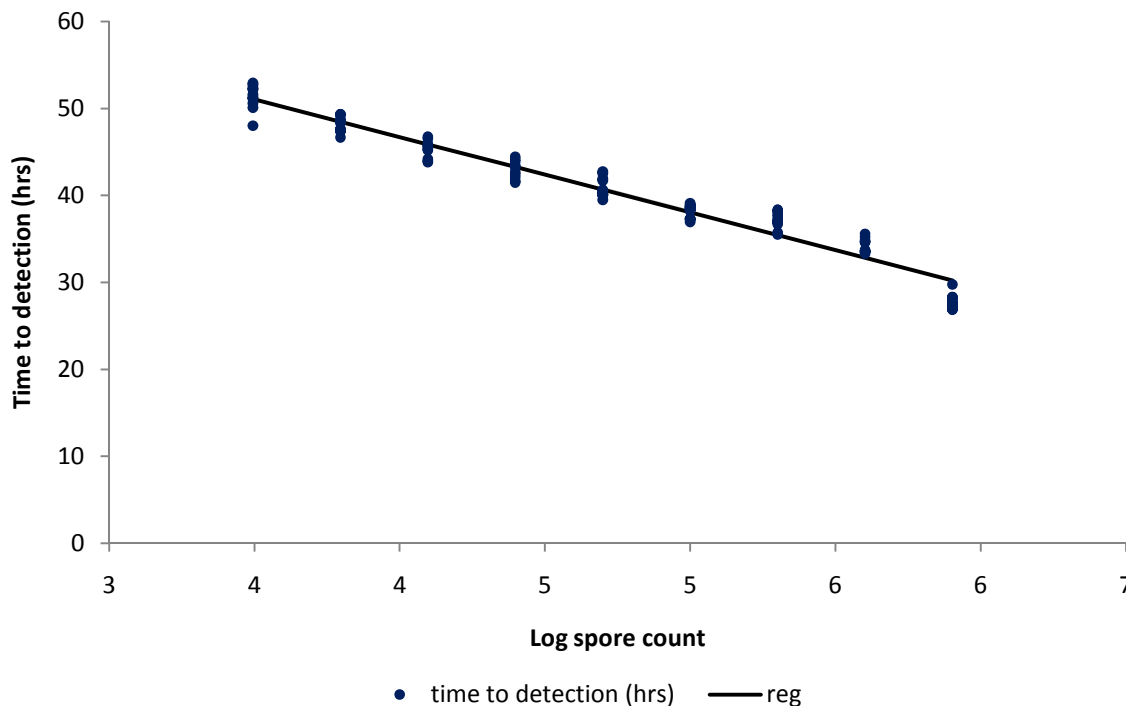
Figure 4.17 Linear regression analysis of the results from the monitoring of A.niger growth in semi-solid MEA at 25°C using the Bioscreen C Microbiological Analyser (table 8.28 and table 8.29)

	Coefficients	Standard Error	Lower 95%	Upper 95%	R square	Observations
Intercept	81.44	0.98	79.50	83.38	0.95	90
log spore count	-8.68	0.20	-9.09	-8.27		

Table 4.17 Statistical output for the linear regression analysis of the results from the monitoring of A.niger growth in semi-solid MEA at 25°C using the Bioscreen C Microbiological Analyser.

4.3.1.2 Growth in the presence of sorbic acid

Figure 4.18 (see page 74) presents the results for the bioscreen analysis of *A. niger* inhibition by sorbic acid at pH 4.6 and pH 2.5. The detection time was determined from a point where there is a rapid increase in optical density and can be related to the cell number. It was taken to be the time to achieve an optical density equalling 0.6 at 600nm. The detection threshold is lower than that utilised during standard growth analysis because the starting optical density was lower during the sorbic acid experiment, due to the different growth medium volume used.

The detection times for *A. niger* at both pH 2.5 and pH 4.6 were delayed with increasing sorbic acid concentration. At pH 2.5 no optical density change was observed over 250ppm, however in comparison at pH 4.6, sorbic acid displayed weaker antifungal activity, with changes in optical density observed up to 1100ppm. This result is also reflected in the MIC and NIC values shown in Table 4.18. Similar results were seen for both radial growth and impedance analysis. However in comparison the MIC values calculated for the impedance method were higher than those for the bioscreen. Chorianopoulos *et al* (2006) and Sawai *et al* (2002) showed that the MICs of certain bacteria to various antimicrobials calculated using the impedance method were almost identical to that obtained by the turbidity method. This does seem to suggest that sorbic acid has a greater effect on physical cellular growth than metabolic activity, a strange conclusion since sorbic acid's inhibitory action is thought to be via intracellular acidification of the cell cytoplasm resulting in the inhibition of key metabolic activities involved in glycolysis and active transport. Stratford and Anslow, (1998) hypothesised that sorbic acid in fact did not act as a classical weak acid preservative, releasing far fewer protons than other weak acid preservatives and thereby causing minimal change in the intracellular pH and therefore metabolic activity. It is thought, based on the relationship of inhibition with partition coefficient, that sorbic acid could have an effect on the plasma membrane in a manner similar to that of ethanol, affecting membrane fluidity and possibly causing leakage (Stratford and Anslow, 1998), which could explain the differences in MIC values between impedance and turbidimetry. However, Plumridge *et al* (2004) showed sorbic acid delayed *A. niger* spore germination and mycelia growth in a concentration dependant manner (as seen in this study) via causing intracellular acidification and thereby reducing intracellular ATP pools, levels of sugar-phosphomonoesters and phosphodiesters. Therefore without further analysis it is uncertain what the exact cause of this difference in MIC values or indeed the true mode of action of sorbic acid. However it can be concluded that these results demonstrate that the bioscreen can be used to evaluate antimicrobial efficacy.

As with the impedance analysis results the MIC calculated at pH 2.5 (328ppm) was far lower than that of the MIC calculated at pH 4.6 (951ppm). Along with the distinct kerosene scent (indicative of 1, 3-pentadiene production), which could be detected at the conclusion of the experiment, does suggest that as hypothesised with the impedance results that *A. niger* was able to degrade sorbic acid.
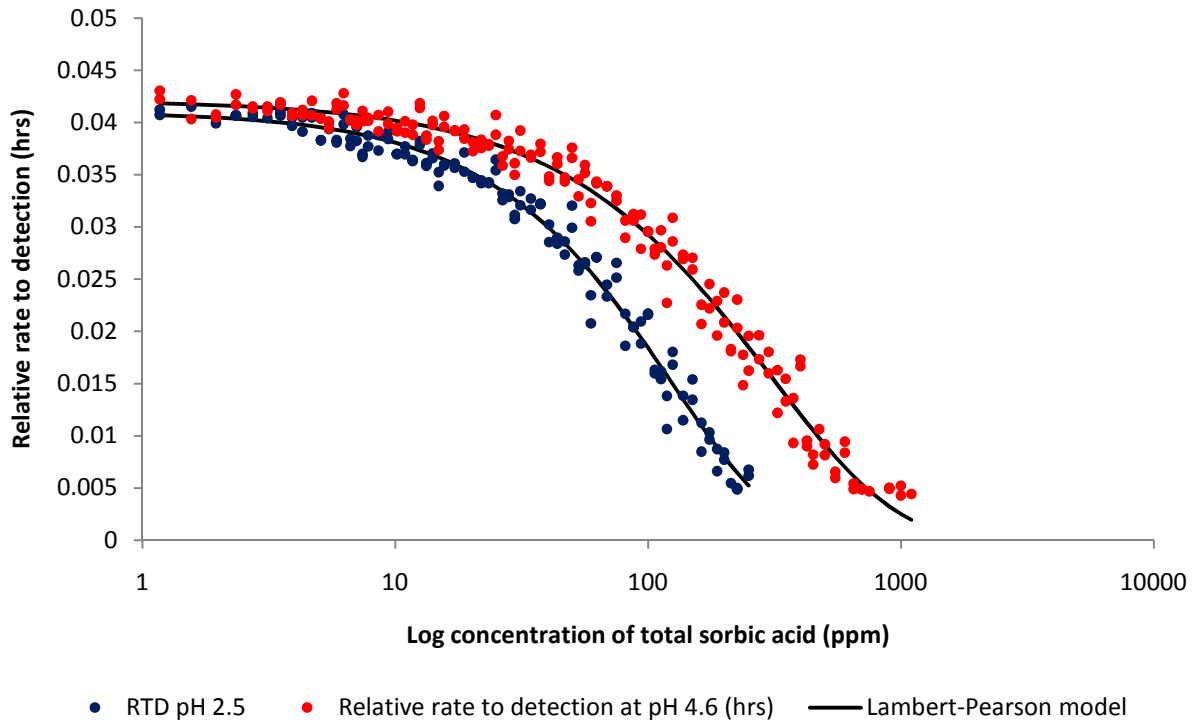
Figure 4.18 *analysis of the results from the monitoring of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid at pH 4.6 and pH 2.5 using the Bioscreen C Microbiological Analyser (table 8.30, table 8.31, table 8.32, table 8.33, table 8.34, table 8.35, table 8.36, table 8.37 and table 8.38).*

(a)

Parameter	Estimate	Standard Error	Lower CL	Upper CL	MIC	NIC
P1	319.18	6.31	307.05	331.8	951.19	39.81
P2	0.84	0.02	0.79	0.9		
P0	0.04	0.0003	0.04	0.04		

(b)

Parameter	Estimate	Standard Error	Lower CL	Upper CL	MIC	NIC
P1	124.87	2.12	120.75	129.16	328.09	23.92
P2	1.01	0.03	0.95	1.07		
P0	0.04	0.0003	0.04	0.04		

Table 4.18 *Statistical output for the analysis of the results from the monitoring of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid at (a) pH 4.6 and (b) pH 2.5 using the Bioscreen C Microbiological Analyser*

4.3.1.3 Growth in the presence of vanillin

Figure 4.19 presents the results for the bioscreen analysis of *A. niger* inhibition by vanillin at pH 4.6. The detection time was determined from a point where there is a rapid increase in optical density and can be related to the cell number. It was taken to be the time to achieve an optical density equalling 0.6 at 600nm.

The detection times for *A. niger* at pH 4.6 as with the results for sorbic acid were delayed with increasing vanillin concentration. Optical density changes were observed up to 700ppm, however the duration of the experiment was reduced to just 48 hours, because of light source failure. As shown in Table 4.19 despite this the MIC for vanillin was calculated to be 1413ppm, this falls within the inhibitory range reported (1,000-1,500ppm) for various Aspergilli, including *A. niger* (Lopez-Malo *et al.* 1995). Radial growth analysis of the affect of vanillin on *A. niger* growth by Lopez-Malo *et al.* (1998), also showed a similar affect. Many other studies have also demonstrated the antifungal activity of vanillin (Fitzgerald *et al.* 2002; Lopez-Malo *et al.* 1997; Lopez-Malo *et al.* 2005). In comparison with the results from Figure 4.18, sorbic acid showed a higher degree of antifungal activity since lower concentrations were required to inhibit mould growth than with vanillin. The MIC for sorbic acid at pH 4.6 was calculated to be 951ppm, 67% lower than the MIC for vanillin. Matamoros-Leon *et al.* (1999) also showed vanillin to be the inferior inhibitor. The high MIC of vanillin in conjunction with its strong olfactory qualities and weaker antifungal activity in comparison with sorbic acid makes it a rather unsuitable natural alternative and sole inhibitor.

The effect of pH on vanillin was not examined in this study because it was thought, with it being a phenolic compound; it would have little to no effect on its antifungal activity. However results from Lopez-Malo *et al.* (1997), state differently. The effect of pH on the antimicrobial activity of phenolic compounds is poorly understood. However, some authors reported a greater effect as the pH was lowered, similar to that seen with weak acids. Sykes and Hooper (1954) found greater effects of phenolics at acid pH values, and they attributed this to the increased solubility and stability of these compounds at low pH. Juven *et al.* (1994), showed that thymol (also a phenolic compound) had enhanced activity at low pH and that at low pH values, the thymol molecule is mostly undissociated, and may bind better to the hydrophobic regions of the membrane proteins and dissolve better in the lipid phase of the membrane. However it has also been hypothesised that the increase in antimicrobial action at reduced pH could be in part the result of the effect of low pH

alone. In general moulds achieve optimum growth at around pH 5, and are more resistant to inhibitory factors at that pH. Therefore if the pH is reduced the effect of other inhibitory factors such as vanillin could be enhanced (Skirdal and Eklund, 1993).The exact effect of pH on the efficacy of phenolics like vanillin is still unknown and until the exact mechanism of action of some of these compounds is determined it will remain difficult to ascertain.
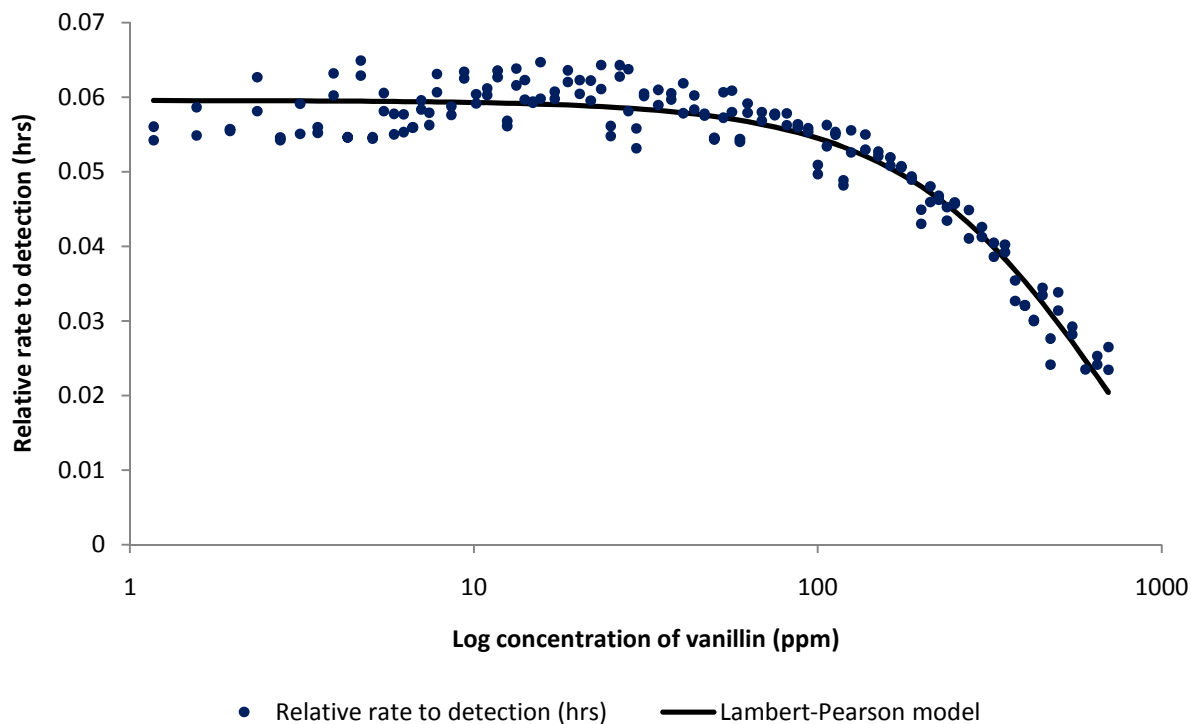
Figure 4.19 analysis of the results from the monitoring of A. niger growth in semi-solid MEA at 25°C in the presence of vanillin at pH 4.6 using the Bioscreen C Microbiological Analyser (table 8.44, table 8.45, table 8.46, table 8.47, table 8.48, table 8.49, table 8.50, table 8.51, table 8.52 and table 8.53)

Parameter	Estimate	Standard Error	Lower CL	Upper CL	MIC	NIC
P1	664.32	19.78	628.84	706.02	1413.15	215.04
P2	1.28	0.06	1.17	1.40		
P0	0.06	0.00035	0.06	0.06		

Table 4.19 Statistical output for the analysis of the results from the monitoring of A. niger growth in semi-solid MEA at 25°C in the presence of vanillin at pH 4.6 using the Bioscreen C Microbiological Analyser

4.3.1.4   Growth in the presence of sorbic acid and vanillin

Figure 4.20 presents the results for the bioscreen analysis of *A. niger* inhibition by a combination of sorbic acid and vanillin at pH 4.6 along with the RTD contour diagram from the modelled data.

The detection time was determined from a point where there is a rapid increase in optical density and can be related to the cell number. It was taken to be the time to achieve an optical density equalling 0.6 at 600nm.

As with previous results, when used as individual antimicrobials, sorbic acid proved to be the most effective, with a lower concentration required to inhibit growth, though it does have to be noted eventually for both sorbic acid and vanillin fungal growth was detected at 900ppm, the highest measured concentration observed in this experiment. When used in combination however, greater antifungal activity was observed at lower concentrations of sorbic acid, 900ppm vanillin with 500ppm sorbic acid was found to completely inhibit growth. This represents a near 50% reduction on the MIC values calculated for both these antimicrobials when utilised as sole inhibitors. A similar result was also observed by Matamoros-Leon *et al.* (1999), where a combination of 500ppm vanillin with 300ppm sorbic acid was found to inhibit various pencillia species for up to one month at 25°C and pH 3.5.

Matamoros-Leon *et al*. (1999), also go on to suggest that these results show synergistic effects on mould inhibition when vanillin and sorbic acid are applied in combination. However, for both vanillin and sorbic acid the actual mode of inhibition is still open for debate though it has been hypothesised both have an effect to varying degree on the cell membrane and enzymatic reactions. If the value of P_2 (from the Lambert Pearson model) is approximately 1 for each inhibitor, then in combination if there is a purely additive effect i.e. no synergy or antagonism present, then C_q will be approximately 1, and the modelled values of P_1 and P_3 will be equivalent to the values found individually (Lambert and Bidlas, 2007). From Table 4.20 it can be seen the C_q value is 0.859 and the concentration parameters for sorbic and vanillin in concentration were 523 and 602 ppm respectively; when analysed individually these were 319 and 664 ppm respectively. However, the MIC for sorbic in combination with vanillin was 1111.8 ppm vs. 951 found individually and the MIC for vanillin in combination with sorbic was 1488 ppm vs. 1413 ppm found individually. These results suggest that in combination at pH 4.6 sorbic acid and vanillin act additively.
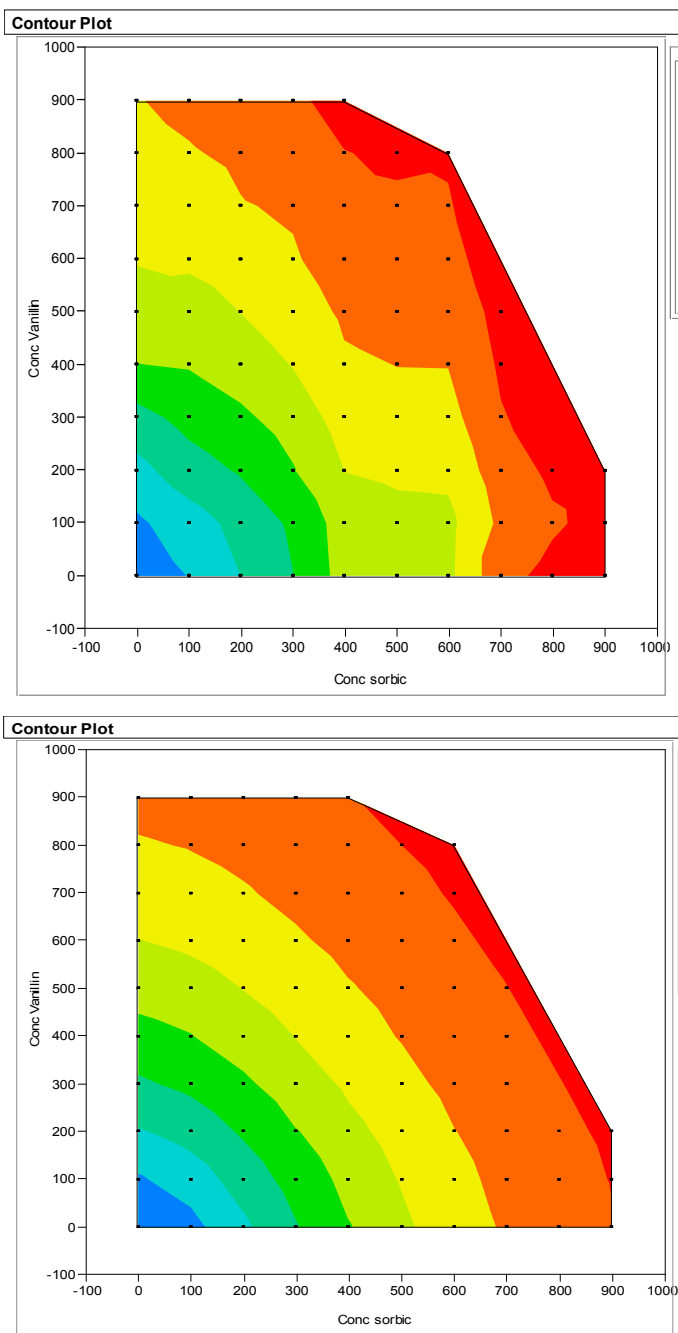
Figure 4.20 *Observed RTD (top) and Modelled RTD (bottom) Contour diagram for the growth of A. niger in semi-solid MEA at 25°C in the presence of sorbic acid and vanillin at pH 4.6. The experimental grid is shown by the black points. Contours are in increments of 0.000125min⁻¹, with a maximum (Blue) of 0.000875 and a minimum (Red) of 0.000125. (table 8.39, table 8.40, table 8.41, table 8.42 and table 8.43)*

| Parameter	Estimate	Standard error	Lower CL	Upper CL
P0	0.001022	2.05E-05	0.000981	0.001065
P1	523.55	13.33	496.67	550.68
P3	602.85	17.21	568.60	638.22
C2	1.55	0.08	1.41	1.70
C4	1.29	0.07	1.17	1.43
Cq	0.859	0.044	0.774	0.950

Table 4.20 *Table of parameters for the analysis of the results from the monitoring of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid and vanillin at pH 4.6 using the Bioscreen C Microbiological Analyser.*

5 Discussion

In all instances for the impedance analysis (direct and indirect) of bacterial growth there was a high correlation between log counts and detection times, both techniques also displayed the capability to detect low initial bacterial populations in a relatively short time frame (7.94 hours for a single *E.coli* cell incubated at 37°C). However, the application of the direct technique was found to be restricted. Due to growth being detected as a conductivity change of the growth medium, the range of media suitable for direct measurements was limited. In the present study malt extract broth was found to be overly conductive for effective impedance analysis, other commonly used selective media such as mannitol salt broth would pose a similar problem. The indirect technique however proved to be an adequate alternative, though longer detection times were recorded in comparison with the direct technique the overall growth patterns and reproducibility were the same. Therefore from the results in this study it is clear that both the direct and indirect methods are applicable for the rapid estimation of bacterial populations from the relationship between detection time and initial population. Many other studies have also come to a similar conclusion (Cady *et al.* 1978; Gibson, 1988; Sawai *et al.* 2002; Ur and Brown, 1975), and the method itself is already widely applied for rapid microbial detection in foodstuffs such as fish (Dupont *et al* 1994), meat (Firstenberg-Eden, 1983) and milk (Lanzanova *et al.* 1993). The key advantage is its applicability to highly turbid samples, such as foods and insoluble materials, making the technique a promising alternative to the standard plating in the detection of bacterial growth, especially within the food industry.

The direct impedance technique however is largely deemed unsuitable for detecting fungi such as moulds and yeasts, which produce electrolytes at a slow rate by metabolism and also adsorb electrolytes (Eden and Eden 1984; Suomalainen and Oura, 1971), meaning that they do not produce the large changes in conductivity required for this type of analysis. Additionally, despite their rapidity both techniques are considered to be largely unreliable and inconsistent especially in comparison with the widely utilised standard plate technique. However in the present study in most instances for the impedance analysis of fungal growth, both mould and yeast displayed a high correlation between log counts and detection times. The direct technique though, was found to produce inconclusive and negative results despite visual growth for the moulds tested (*Aspergillus niger*, *Fusarium oxysporum* and *Penicillium verrucosum*), this was not the case for *Saccharomyces cerevisiae*, where clear conductance changes similar to those seen for the indirect

technique, proportional to the initial inoculum population were observed. Sawai *et al*. (2003) also found that filamentous fungi caused no change in media conductance when measured directly but yeasts on the other hand had the ability to cause clear conductance changes for both the indirect and direct method. Other studies have observed similar findings for the impedance analysis of yeast growth (Deak and Beuchat, 1993; Owens *et al*. 1989; Owens *et al*. 1992). However in all instances where different media compositions were tested, the results for the direct technique were highly variable. For example, Sawai *et al*. (2003) showed that from a selection of four different media, for *S. cerevisiae* and *Candida albicans* a good conductivity curve could be obtained with glucose peptone broth only. This dependence on media composition is also observed in results obtained by Owens *et al.* (1992), whereby changing the nitrogen and carbon source of growing yeasts, they were able to influence the size of the conductance change. Despite this if the correct medium is utilised, the direct technique can provide reproducible results, and is a promising alternative to the standard plate method. However the degree of variability caused by its dependence on media composition would make it largely unsuitable for the growth analysis of yeasts, especially since the indirect technique can give equally accurate and rapid results without the disadvantage of being hindered by the type of media utilised in the experiment.

Although for *A. niger* and *P. verrucosum* the indirect technique proved to be a rapid and reproducible analysis method, for *F. oxysporum* this was not the case. Even though there was a correlation between the detection times and initial inoculum size for *F. oxysporum*, it was relatively weak in comparison with the other two moulds tested, displaying a far greater degree of variability. However due to the lack of other research concerning the impedance analysis of filamentous fungal growth, the cause of this variability is unknown. The indirect analysis of *A. niger* and *Rhizopus stolonifer* growth on potato dextrose agar by Sawai *et al*. (2003) yielded clear conductivity curves and detection times, similar to that seen in this study for *A. niger* and *P. verrucosum*, this could be evidence to suggest that *F. oxysporum* is the cause of this variability and not the technique. It could be speculated that *F. oxysporum* may simply not be entirely suitable for this type of analysis because of its apparent unpredictable metabolic activity and CO_2 production in the given conditions of this study (grown in liquid broth). Despite this the indirect technique (and the direct technique for yeasts) does appear to be a promising alternative to traditional plating procedures requiring colony development on agar media. Compared with the 3 to 7+ days of incubation required to obtain reliable counts on plating media, yeasts can be detected within 24

hours and moulds depending on the species and initial population count well within 72 hours. Furthermore its capability to detect low initial spore populations in a relatively short time frame (65.37 hours for a single *A. niger* spore incubated at 30°C), encompassed with the possibility to process solid and high turbid samples, makes indirect conductimetric analysis (and to a lesser extent direct analysis) a valuable future technique for practical application within the food industry for analysing fungal growth

Despite its widespread application in the monitoring of bacterial growth turbidimetric analysis as with conductimetric analysis is not readily utilised for the monitoring of filamentous fungal growth and has largely been defined as unreliable and error prone, also it is often argued results obtained do not give a true estimate of the amount of growth actually occurring. This is largely because filamentous fungi grow as hyphae, which are multi-cellular cylindrical, thread-like structures specifically adapted for growth on solid surfaces, therefore when grown in liquid medium filamentous fungi readily adhere to the walls of any container they are inoculated in. Therefore the fungus is not evenly distributed within the medium, which is required for this type of analysis to get accurate results. To overcome this problem in the present study semi-solid agar was utilised, this traps and holds fungal spores in suspension and therefore should in theory lead to a more even growth throughout the medium. Indeed a high correlation between log counts and detection times for *A. niger* was observed. Furthermore the technique's capability to detect low initial spore populations in a relatively short time frame (81.44 hours for a single *A. niger* spore incubated at 25°C) and the Bioscreen's ability to process 200 separate samples at any one time, make the turbidimetric technique a promising rapid alternative to traditional plating procedures for the analysis of filamentous fungal growth. However because it cannot be utilised for high turbidity and solid samples, its applicability within the food industry could be limited, especially with equally rapid and reliable analysis techniques already available that can handle such samples.

In addition to determining the capability of the conductimetric and turbidimetric techniques in analysing filamentous fungal growth in optimum conditions, the effects of sorbic acid and vanillin were also assessed. It was found that sorbic acid displayed weaker antifungal activity at high pH (in the case of this study pH 4.6). This is an unsurprising result considering that for weak acids it is largely accepted that the undissociated molecule is the source of their inhibitory action, and that the amount of molecule in the undissociated form is determined by the pH. Therefore the

antimicrobial effectiveness increases as the pH value approaches its dissociation constant (pKa), which in the case of sorbic acid is 4.76. At this pH value, 50% of sorbic acid is in the effective undissociated form. So in the present study where pH 4.6 was used, the undissociated fraction would be just 59% of total sorbic acid concentration, in comparison at pH 2.5 the undissociated fraction would be 99% of the total sorbic acid concentration. Hence it is unsurprising that sorbic acid had less of an inhibitory effect at higher pH. Many other studies have also demonstrated the importance of pH in the efficacy of sorbic acid (Bell *et al*. 1959; Lambert and Stratford, 1999; Park and Marth, 1972).

These results would suggest that sorbic acid does inhibit microbial growth as a classical weak acid preservative contrary to the theory hypothesised by Stratford and Anslow (1998). Indeed, Plumridge *et al* (2004) showed sorbic acid delayed *A. niger* spore germination and mycelia growth in a concentration dependant manner (as seen in this study) via causing intracellular acidification and thereby reducing intracellular ATP pools, levels of sugar-phosphomonoesters and phosphodiesters. However comparing the MIC values calculated for the impedance and turbidimetry methods, it can be seen that those for the impedance analysis are in fact higher than those for the bioscreen. This is a peculiar result, considering that in optimum conditions results between the two methods were distinctly similar, suggesting that the CO_2 production (indicator of metabolic activity) is most likely directly proportional to the cellular growth. Therefore it has to be proposed that sorbic acid has a greater effect on physical cellular growth than metabolic activity. A strange conclusion since sorbic acid's inhibitory action is thought to be via intracellular acidification of the cell cytoplasm resulting in the inhibition of key metabolic activities involved in glycolysis and active transport. This result in fact supports the hypothesis of Stratford and Anslow (1998) that sorbic acid does not act as a weak acid preservative, releasing far fewer protons than other weak acid preservatives and thereby causing minimal change in the intracellular pH and therefore metabolic activity. In a study by Stratford *et al*. (2009), it was shown that the internal pH of *A. niger* conidia treated with 500ppm sorbic acid did not fall below pH 6.3, it has been reported substantial reduction in enzyme activity in *A. niger* does not occur until the internal pH falls below pH 6 (Legsia and Golic Grdadolnik, 2002). Therefore sorbic acid must possess additional mechanism(s) of cellular inhibition. Many have been suggested including specific action on enzymes and metabolic pathways, uncoupling of oxidative phosphorylation and inhibition of respiration but from these findings the most likely is membrane damage. It is thought, based on the relationship of inhibition

with partition coefficient, that sorbic acid could have an effect on the plasma membrane in a manner similar to that of ethanol, affecting membrane fluidity and possibly causing leakage (Stratford and Anslow, 1998). Though further analysis is required to fully determine the mode of action of sorbic acid, the most likely reasoning for the results seen in this study is sorbic acid does act as a classical weak acid preservative, but also has alternative mechanism(s) of inhibition.

The ability of filamentous fungi to metabolise sorbic acid is well established, early reports by Melnick *et al*. (1954) suggested that degradation of the acid occurs via β-oxidation, producing CO_2 and H_2O. However in the present study it was found *A.niger* was capable of metabolising sorbic acid via a decarboxylation reaction to 1, 3 –pentadiene, but only at pH 4.6 and not pH 2.5. Several other authors have also confirmed a similar reaction taking place (Casas *et al*. 1999; Kinderlerer and Hatton, 1990). Indeed, for *A. niger* the decarboxylase enzyme has been shown to be a phenylacrylic acid decarboxylase, encoded by the gene *padA1*, with the capacity for decarboxylation of sorbic acid greatest during conidial germination and outgrowth (Plumridge *et al.* 2004; Plumridge *et al*. 2008). This is a possible cause for the lack of 1, 3-pentadiene detected in samples grown at pH 2.5, because the inhibitory action of sorbic acid at this pH is at its peak, conidial germination and all round growth would be limited, therefore the likelihood of any significant decarboxylation taking place would be low. This does pose a problem to the food industry, where foods are often for multiple reasons kept at a higher pH and with restrictions on the amount of sorbic acid that can be combined with food, leaves the likelihood of spoilage high.

In comparison with sorbic acid, vanillin displayed weaker antifungal activity, with MIC values calculated to be much higher than would be organoleptically acceptable for use as a sole inhibitor in food. However in the present study the effect of pH on vanillin was not tested, because it is a phenolic compound, it was not anticipated that this variable would have much impact on its inhibitory action. However some authors have reported a greater inhibitory effect when pH is lowered, similar to that seen with classical weak acids (Juven *et al* 1994; Skirdal and Eklund, 1993). For example Sykes and Hooper (1954) found greater effects of phenolics at acid pH values, and they attributed this to the increased solubility and stability of these compounds at low pH. However because the exact mechanism(s) of action of vanillin is relatively unknown, it makes confirmation of this difficult, regardless of the effect of pH, the use of vanillin as a sole inhibitor and replacement for 'synthetic' additives is very unlikely due to its strong taste and aromatic qualities,

encompassed with its relatively poor antifungal activity. In addition Fitzgerald *et al.* (2003) showed that various yeasts had the ability to bio-convert vanillin to its respective alcohol and acid derivatives, thus further diminishing its antimicrobial effectiveness. Furthermore realistically for it to be incorporated as a preservative, its activity in real food systems would have to be examined, especially since it has been reported that the presence of lipids and/or proteins has a detrimental effect on it antimicrobial activity (Lopez-Malo *et al*. 1995), further limiting its possible application as a sole inhibitor.

The relatively poor antifungal activity and strong olfactory characteristics of vanillin means that any possible future application as a preservative, would be most likely be in combination with other antimicrobials or 'hurdles'. As shown in this study and others (Matamoros-Leon *et al* 1999), a greater antifungal effect was observed at lower concentrations when vanillin was combined with low doses of sorbic acid (in the present study 500ppm sorbic acid with 900ppm vanillin completely inhibited growth). Matamoros-Leon *et al*. (199) suggested a synergistic interaction as the cause of this increase in antifungal action; however for both vanillin and sorbic acid as stated previously the mode of action is still highly speculative. Also results from the present study do suggest that any combination of sorbic acid and vanillin at pH 4.6 act additively. Regardless the additive effect seen could be useful in reducing the amounts of inhibitor required to prevent mould growth, though it is likely there are more suitable natural antimicrobials to complement sorbic acid than vanillin.

6 Conclusion

In conclusion both the impedance and turbidimetry methods provided a sensitive and rapid means of detecting, and, under standardised conditions, measuring the activity of micro-organisms. The rate of response showed close correlation with the concentration of both bacteria and spores in the initial inoculum for each strain tested so correlation curves could be constructed to estimate the number of viable cells and spores in a suspension. Moreover, both methods can be used for the accurate screening of potential antimicrobial substances. However, because the impedance technique can be used to analyse both high turbidity and solid samples, it shows the most promise in terms of application within the food industry. In comparison with the turbidimetry method though, the impedance method did show a greater deal of variability and there is the possibility it is unsuitable for the analysis of certain fungal species. In addition the bioscreen holds the distinct advantage of being able to process 200 samples at any one time. Despite these disadvantages both are promising rapid alternatives to the standard plating technique.

In this study it was also found that sorbic acid is an effective agent that delays spore germination and retards mycelia growth. Consistent with other findings, the action of sorbic acid was also found to be highly pH dependant, seemingly proving that in does indeed act as a classical weak acid preservative. However discrepancies between Bioscreen and impedance analysis results do suggest the sorbic acid has a greater effect on physical cellular growth than metabolic activity, implying an alternative mechanism of action, possibly to the cellular membrane. Regardless of this growth-inhibitory properties are limited since *A. niger* at pH 4.6 was shown to have the ability to metabolise or degrade the acid via what is thought to be a decarboxylation reaction, largely because traces of 1,3 pentadiene were detected in the media after colonisation. The possible application of vanillin as a natural substitute for sorbic acid however is highly unlikely. It showed a lower degree of antifungal activity, along with its olfactory qualities and it previously being shown as with sorbic acid the compound is susceptible to degradation makes it unsuitable. Although when utilised in combination with sorbic acid a clear additive effect was observed which could prove useful to reduce the amounts needed to inhibit mould growth, though more suitable natural antimicrobials do exist which would better compliment sorbic acid in this respect.

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8   Appendix

log bacterial count (CFU/ml)	time to detection (hrs)
8.52	0.57
8.52	0.8
7.52	1.41
7.52	1.6
6.52	2.67
6.52	2.57
5.52	3.81
5.52	4.36
4.52	5.45
4.52	5.45
3.52	6.9
3.52	7.13
2.52	8.67
2.52	8.47
1.52	9.96
1.52	9.76

Table 8.1 results for a direct impedance analysis of E. coli growth in TSB at 30°C. The experiment was done in duplicate

log bacterial count (CFU/ml)	time to detection (hrs)
8.52	0.47
8.52	0.56
7.52	0.69
7.52	0.69
6.52	1.21
6.52	1.46
5.52	2.34
5.52	2.31
4.52	3.03
4.52	3.11
3.52	4.06
3.52	4.31
2.52	5.05
2.52	4.82
1.52	6.14
1.52	5.8

Table 8.2 *results for a direct impedance analysis of E. coli growth in TSB at 37°C. The experiment was done in duplicate.*

log bacterial count (CFU/ml)	time to detection (hrs)
8.52	1
7.52	2.09
6.52	3.75
5.52	4.55
4.52	6.69
3.52	8.28
2.52	9.47
1.52	11.51

Table 8.3 *results for an indirect impedance analysis of E. coli growth in TSB at 30°C.*

log bacterial count (CFU/ml)	time to detection (hrs)
8.52	1.82
7.52	1.46
6.52	2.1
5.52	3.07
4.52	4.1
3.52	5.29
2.52	6.21

Table 8.4 *results for an indirect impedance analysis of E. coli growth in TSB at 37°C.*

log bacterial count (CFU/ml)	time to detection (hrs)
8.46	3.15
8.46	3.05
7.46	4.76
7.46	3.22
6.46	5.85
6.46	4.75
5.46	6.29
5.46	6.02
4.46	7.59
4.46	7.93
3.46	8.57
3.46	0.43
2.46	10.35
2.46	10.44
1.46	11.38
1.46	11.8

Table 8.5 *results for a direct impedance analysis of S. thyphimurium growth in TSB at 30℃. The experiment was done in duplicate*

log bacterial count (CFU/ml)	time to detection (hrs)
8.46	1.33
8.46	1.17
7.46	1.97
7.46	2.25
6.46	2.82
6.46	2.72
5.46	3.78
5.46	4.21
4.46	4.24
4.46	4.28
3.46	4.63
3.46	5.56
2.46	5.78
2.46	5.68
1.46	7.22
1.46	7.21

Table 8.6 *results for a direct impedance analysis of S. thyphimurium growth in TSB at 37℃. The experiment was done in duplicate*

log cell count (CFU/ml)	time to detection (hrs)
6.63	5.61
6.63	4.78
5.63	7.83
5.63	7.2
4.63	12.57
4.63	12.45
3.63	16.82
3.63	16.68
2.63	21.13
2.63	20.84
1.63	23.1
1.63	25.83
0.63	26.36
0.63	27.43

Table 8.7 *results for a direct impedance analysis of S. cerevisiae growth in TSB at 25℃. The experiment was done in duplicate*

log cell count (CFU/ml)	time to detection (hrs)
6.63	5.85
6.63	4.79
5.63	7.21
5.63	6.42
4.63	8.2
4.63	8.24
3.63	11.84
3.63	11.8
2.63	16.84
2.63	15.29
1.63	18.52
1.63	18.21
0.63	20.29
0.63	18.68

Table 8.8 *results for a direct impedance analysis of S. cerevisiae growth in TSB at 30℃. The experiment was done in duplicate*

log cell count (CFU/ml)	time to detection (hrs)
6.83	4.78
5.83	7.98
4.83	11.71
3.83	15.85
2.83	20.16
1.83	23.92
0.83	28.54

Table 8.9 *results for an indirect impedance analysis of S. cerevisiae growth in MEB at 30℃.*

time (hrs)	radial growth (mm)	time (hrs)	radial growth (mm)
23	0	210.5	8
23	0	210.5	8
23	0	210.5	8.5
44	0	237	9
44	0	237	8.75
44	0	237	9
67.5	2.5	261	10
67.5	2.25	261	9.5
67.5	2.25	261	9.75
92	3.75	284	10.5
92	3.5	284	10.5
92	3.625	284	10
114	4.625	307	11.25
114	4.5	307	11
114	4.375	307	10.75
139	5.125	331	11.25
139	5.75	331	11.75
139	5.5	331	11.75
163	6.25	355	12.5
163	6.5	355	12.25
163	6.25	355	12.25
186.5	7.25	380	12.5
186.5	7.5	380	13.25
186.5	7.5	380	13

Table 8.10 *results from the monitoring of the radial growth of P. verrucosum on MEA at 25°C*

log spore count (spores/ml)	time to detection (hrs)
6.37	23.59
6.37	18.08
6.06	26.74
6.06	25
5.76	28.46
5.76	27.94
5.46	34.55
5.46	36.18
5.16	38.81
5.16	40.8
4.86	45.27
4.86	47.66

Table 8.11 *results for an indirect impedance analysis of P. verrucosum growth in MEB at 30 °C using a -25% detection threshold. The experiment was done in duplicate.*

log spore count (spores/ml)	time to detection (hrs)
6.37	6.83
6.37	4.6
6.06	8.67
6.06	6.42
5.76	8.81
5.76	8.61
5.46	12.56
5.46	14.41
5.16	14.94
5.16	19.69
4.86	22.45
4.86	25.16

Table 8.12 *results for an indirect impedance analysis of P. verrucosum growth in MEB at 30 °C using a -5% detection threshold. The experiment was done in duplicate.*

log spore count (spores/ml)	time to detection (hrs)
6.37	33.89
6.37	28.39
6.06	38.2
6.06	37.27
5.76	40.61
5.76	40.17
5.46	46.89
5.46	46.2
5.16	50.14
5.16	49.19
4.86	58
4.86	57.53

Table 8.13 results for an indirect impedance analysis of P. verrucosum growth in MEB at 30°C using a -50% detection threshold. The experiment was done in duplicate.

log spore count (spores/ml)	time to detection (hrs)
6.18	10.23
6.18	18.78
5.88	19.59
5.88	19.99
5.58	19.99
5.58	18.76
5.28	19.67
5.28	26.54
4.98	22.85
4.98	27.3
4.68	26.7
4.68	25.57
4.38	35.06
4.38	36.31

Table 8.14 results for an indirect impedance analysis of F. oxysporum growth in MEB at 30°C. The experiment was done in duplicate.

log spore count (spores/ml)	time to detection (hrs)
6.18	10.42
6.18	11.5
5.88	20.35
5.88	21.98
5.58	23.7
5.58	25.81
5.28	24.66
5.28	28.08
4.98	35.16
4.98	27.86
4.68	40.73
4.68	39.13
4.38	36.37
4.38	34.29

Table 8.15 *results for an indirect impedance analysis of F. oxysporum growth in MEB at 25°C. The experiment was done in duplicate.*

Time (hrs)	radial growth (mm)
23	2.25
23	2.25
23	2
45	4.5
45	5
45	5
68	7.75
68	8.25
68	7.5
92.5	9
92.5	9
92.5	9.5
117.5	12.5
117.5	12.25
117.5	13
142.5	14
142.5	14.5
142.5	15.25
167.5	18.5
167.5	17.25
167.5	18.5

Table 8.16 *results from the monitoring of the radial growth of F. oxysporum on MEA at 25°C*

log spore count (spores/ml)	time to detection (hrs)
5.81	34.17
5.81	34.63
5.81	32.72
5.81	34.38
5.81	33.86
5.81	34.81
5.51	33.6
5.51	36.81
5.51	34.68
5.51	35.62
5.2	36.97
5.2	36.99
5.2	37.74
5.2	40.11
4.9	35.78
4.9	38.93
4.9	41.36
4.9	40.61
4.6	41.44
4.6	39.54
4.6	45.23
4.6	45.19
4.3	49
4.3	45.8
4	51.29
4	47.69

Table 8.17 *results for an indirect impedance analysis of A. niger growth in MEB at 25°C. The experiment was done in duplicate. The experiment was repeated.*

log spore count (spores/ml)	time to detection (hrs)
5.81	22.36
5.81	23.37
5.81	22.77
5.81	23.68
5.81	23.39
5.81	20.41
5.51	24.65
5.51	24.08
5.51	23.87
5.51	24.59
5.2	25.64
5.2	24.03
5.2	27.65
4.9	26.14
4.9	26.48
4.9	29.01
4.9	28.55
4.6	27.84
4.6	27.63
4.6	32.01
4.6	30.83
4.3	35.22
4.3	33.72
4	38.04
4	36.23

Table 8.18 *results for an indirect impedance analysis of A. niger growth in MEB at 30°C. The experiment was repeated.*

Time (hrs)	radial growth (mm)
23	1.75
23	2.125
23	1.625
45	5.75
45	6
45	6
68	11.25
68	11
68	11.25
92.5	14.5
92.5	14.75
92.5	15.25
117.5	18.75
117.5	18.5
117.5	19.125
142.5	22.5
142.5	22.5
142.5	23
167.5	26.25
167.5	26.5
167.5	27.25

Table 8.19 *results from the monitoring of the radial growth of A. niger on MEA at 25℃*

Sorbic acid concentration (ppm)	Time to detection (hrs)	Relative rate to detection (hrs)
0	27.79	0.036
0	28.55	0.035
100	28.56	0.035
100	30.2	0.033
200	33.89	0.030
200	34.2	0.029
300	38.92	0.026
300	38.22	0.026
400	40.3	0.025
400	40.75	0.025
500	43.02	0.023
500	44.48	0.022

Table 8.20 results for an indirect impedance analysis of A. niger growth in MEB at 25°C in the presence of sorbic acid at pH 4.6. The experiment was done in duplicate.

Sorbic acid concentration (ppm)	Time to detection (hrs)	Relative rate to detection (hrs)
0	26.43	0.038
0	27.65	0.036
50	34.12	0.029
50	35.11	0.028
100	44.3	0.023
100	41.17	0.024
150	53.84	0.019
150	60.39	0.017
200	75.98	0.013
200	62.99	0.016
250	88.79	0.011
250	101.46	0.010
300	110	0.009
300	107.82	0.009

Table 8.21 results for an indirect impedance analysis of A. niger growth in MEB at 25°C in the presence of sorbic acid at pH 2.5. The experiment was done in duplicate.

Time (hrs)	Radial growth at 0ppm (mm)	Radial growth at 50ppm (mm)	Radial growth at 100ppm (mm)
23	2.5	2	2
23	3	2.5	1.75
23	3	2.5	1.75
43	8	7.5	5.75
43	7.75	7.25	6
43	8	7.25	6.5
68	16	14	12.5
68	15.5	14.25	12.75
68	16	14.5	13.5
92	22.5	21.5	20
92	22	21.25	19.5
92	22.75	20.75	19.5
115	28.5	27.25	26.5
115	28	28	25.5
115	29.25	27.75	25.75
140	35.5	34.25	32.25
140	35	34.5	32.75
140	35	34.5	32.75
164	42.5	41	38
164	42.5	41.5	38.5
164	42.5	40.5	38

Table 8.22 *results from the monitoring of the radial growth of A. niger on MEA at 25°C in the presence of 0ppm, 50ppm and 100ppm sorbic acid at pH 4.6*

Time (hrs)	Radial growth at 200ppm (mm)	Radial growth at 300ppm (mm)	Radial growth at 400ppm (mm)
23	0	0	0
23	0	0	0
23	0	0	0
43	4.5	1.5	0
43	3.5	1.5	0
43	3.75	2	0
68	10.75	8	4
68	10	7.5	3
68	11	7.5	5
92	17.5	13	11
92	16	13.25	8.5
92	17.25	13.5	10
115	23.5	19.5	16.5
115	22	19	15.25
115	23	19	13.5
140	28	24.5	22.5
140	29.25	24.75	21.5
140	28.75	25.5	21.25
164	34	30.5	26.75
164	34	30.5	25
164	35.25	31	28
188	41	36	33.25
188	42	36.5	30.5
188	40.5	36	32.25
212	-	42.5	39
212	-	42.5	35.5
212	-	42.5	37
239	-	-	42.5
239	-	-	42.5
239	-	-	42.5

Table 8.23 *results from the monitoring of the radial growth of A. niger on MEA at 25°C in the presence of 200ppm, 300ppm and 400ppm sorbic acid at pH 4.6*

Time (hrs)	Radial growth at 500ppm (mm)	Radial growth at 600ppm (mm)	Radial growth at 700ppm (mm)
23	0	0	0
23	0	0	0
23	0	0	0
43	0	0	0
43	0	0	0
43	0	0	0
68	2.5	2	1
68	3	1.5	1
68	2.75	3.5	1
92	9.5	7.5	4.5
92	9	6.5	5.5
92	8.75	7.25	5
115	13	12	10
115	13.5	12.5	10.5
115	15	11.5	10.75
140	20.25	18	14
140	19.5	16.5	15
140	20	15	14.5
164	24.5	20.5	20
164	24.25	23	19.75
164	25	21.5	20.25
188	29.75	25.5	24
188	30	27	24.5
188	30	28	25.25
212	34	33	29
212	35	32.5	29.5
212	35.25	31.5	30.5
239	39.75	38	36
239	40	37	35
239	40.5	36.75	34.5
261	-	42.5	39
261	-	42.5	40
261	-	42.5	41

Table 8.24 *results from the monitoring of the radial growth of A. niger on MEA at 25℃ in the presence of 500ppm, 600ppm and 700ppm sorbic acid at pH 4.6*

Time (hrs)	Radial growth at 800ppm (mm)	Time (hrs)	Radial growth at 800ppm (mm)
23	0	188	11.5
23	0	188	13
23	0	188	20
43	0	212	16.5
43	0	212	17
43	0	212	22.5
68	0	239	19.5
68	0	239	20
68	0	239	24.5
92	0	261	24
92	0	261	23
92	1	261	24.5
115	0	284	28
115	0	284	29.5
115	5	284	29
140	2.5	307	34
140	2	307	35.5
140	11	307	33
164	6.5	332	39
164	8	332	38
164	15.5	332	37.25

Table 8.25 *results from the monitoring of the radial growth of A. niger on MEA at 25°C in the presence of 800ppm, sorbic acid at pH 4.6*

Time (hrs)	Radial growth at 0ppm (mm)	Radial growth at 50ppm (mm)	Radial growth at 100ppm (mm)
22	2.5	1.75	0
22	2	1.75	0
22	2	1.75	0
43	9.25	3.5	1.25
43	9.25	3.75	1.25
43	9	3.75	1.5
67.5	15	9	6.5
67.5	15	9.25	6.75
67.5	15.25	9.5	6.5
92	20.75	13.5	11.25
92	20.5	13.5	11.375
92	20.5	14.25	11.5
114	26	18.5	15.5
114	25.5	17.75	15.75
114	25.75	18	15
139	29.75	21.75	20
139	30.5	22.5	20.25
139	30.5	22.75	21
163	35.25	27.75	25
163	34.5	26.5	24.75
163	34.75	26.25	25
186.5	39.5	32.5	28.5
186.5	39	31	29
186.5	39.75	30.75	28.75
210.5	-	34.5	32.5
210.5	-	33.5	32
210.5	-	34	33
237	-	39.5	36.75
237	-	39.5	37.5
237	-	38.5	37
261	-	-	38.5
261	-	-	39.25
261	-	-	39.5

Table 8.26 *results from the monitoring of the radial growth of A. niger on MEA at 25℃ in the presence of 0ppm, 50ppm and 100ppm sorbic acid at pH 2.5*

Time (hrs)	Radial growth at 200ppm (mm)	Time (hrs)	Radial growth at 200ppm (mm)
22	0	186.5	20.5
22	0	186.5	20.375
22	0	186.5	21
43	0	210.5	25
43	0	210.5	25
43	0	210.5	24.5
67.5	0	237	30.25
67.5	0	237	30.5
67.5	0	237	30.5
92	2.5	261	34.5
92	2	261	33.5
92	1.75	261	33
114	7	284	36
114	6.75	284	36
114	7.125	284	36.5
139	11.5	307	39.5
139	11.625	307	39
139	12	307	39.25
163	16.875	331	-
163	16	331	-
163	16.25	331	-

Table 8.27 *results from the monitoring of the radial growth of A. niger on MEA at 25°C in the presence of 200ppm sorbic acid at pH 2.5*

log spore count (spores/ml)	time to detection (hrs)	log spore count (spores/ml)	time to detection (hrs)
5.9	28.26	5	36.96
5.9	27.62	5	38.55
5.9	27.33	5	38.22
5.9	28.33	5	39.11
5.9	27.94	5	38.75
5.9	26.96	5	37.32
5.9	29.75	5	38.39
5.9	26.87	5	38.6
5.9	27.46	5	37.26
5.9	26.93	5	38.89
5.6	33.51	4.7	40.07
5.6	33.73	4.7	40.64
5.6	33.52	4.7	40.47
5.6	33.58	4.7	42.75
5.6	34.67	4.7	40.16
5.6	35.23	4.7	42.53
5.6	34.61	4.7	39.47
5.6	35.57	4.7	41.71
5.6	34.86	4.7	41.93
5.6	33.29	4.7	40.03
5.3	35.74	4.4	42.67
5.3	36.69	4.4	43.44
5.3	37.02	4.4	44.45
5.3	36.94	4.4	42.44
5.3	37.87	4.4	42.08
5.3	37.16	4.4	43.89
5.3	37.55	4.4	41.68
5.3	38.2	4.4	41.48
5.3	38.36	4.4	44.14
5.3	35.51	4.4	43.26

Table 8.28 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C*

log spore count (spores/ml)	time to detection (hrs)
4.1	43.81
4.1	46.5
4.1	44.18
4.1	46.74
4.1	45.69
4.1	45.22
4.1	45.89
4.1	45.56
4.1	45.3
4.1	43.94
3.8	46.67
3.8	49.29
3.8	49.35
3.8	47.63
3.8	47.37
3.8	48.52
3.8	47.58
3.8	48.3
3.8	48.79
3.8	47.44
3.49	51.26
3.49	52.93
3.49	52.33
3.49	48.01
3.49	51.13
3.49	52.7
3.49	50.10
3.49	50.58
3.49	52.17
3.49	51.58

Table 8.29 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C*

Sorbic acid concentration (ppm)	time to detection (mins)	Relative rate to detection (hrs)
0	1407.06	0.0426
0	1379.09	0.0435
1.17	1473.33	0.0407
1.17	1455	0.0412
1.56	1440	0.0417
1.56	1444.76	0.0415
1.95	1483.33	0.0404
1.95	1503.2	0.0399
2.34	1475.79	0.0407
2.34	1474.12	0.0407
2.73	1480	0.0405
2.73	1466.36	0.0409
3.13	1484.44	0.0404
3.13	1446	0.0415
3.52	1462.73	0.0410
3.52	1476	0.0407
3.91	1483.33	0.0404
3.91	1512.63	0.0397
4.3	1533.68	0.0391
4.3	1482.22	0.0405
4.69	1482.11	0.0405
4.69	1467.06	0.0409
5.08	1566.67	0.0383
5.08	1566.32	0.0383
5.47	1501.18	0.0400
5.47	1524.44	0.0394
5.86	1565.22	0.0383
5.86	1575.65	0.0381
6.25	1506.36	0.0398
6.25	1471.11	0.0408
6.64	1561	0.0384
6.64	1589.47	0.0377
7.03	1516.47	0.0396
7.03	1568.89	0.0382
7.42	1622.22	0.0370
7.42	1634.12	0.0367

Table 8.30 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid at pH 2.5*

Sorbic acid concentration (ppm)	time to detection (mins)	Relative rate to detection (hrs)
7.81	1548.42	0.0387
7.81	1590.67	0.0377
8.59	1546.67	0.0388
8.59	1608	0.0373
9.38	1535.56	0.0391
9.38	1561.9	0.0384
10.16	1624.44	0.0369
10.16	1621.18	0.0370
10.94	1591.43	0.0377
10.94	1623.16	0.0370
11.72	1647	0.0364
11.72	1652.63	0.0363
12.5	1584.21	0.0379
12.5	1569.33	0.0382
13.28	1673.33	0.0359
13.28	1661.11	0.0361
14.06	1620	0.0370
14.06	1640	0.0366
14.84	1702.35	0.0352
14.84	1768.75	0.0339
15.63	1672.94	0.0359
15.63	1667.5	0.0360
17.19	1663	0.0361
17.19	1682.35	0.0357
18.75	1615.56	0.0371
18.75	1700	0.0353
20.31	1728	0.0347
20.31	1727	0.0347
21.88	1741.05	0.0345
21.88	1755.56	0.0342
23.44	1755.56	0.0342
23.44	1750.53	0.0343
25	1693.33	0.0354
25	1646.67	0.0364
26.56	1842.22	0.0326
26.56	1806.67	0.0332

Table 8.31 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid at pH 2.5*

Sorbic acid concentration (ppm)	time to detection (mins)	Relative rate to detection (hrs)
28.13	1812	0.0331
28.13	1825.88	0.0329
29.69	1926.67	0.0311
29.69	1950.48	0.0308
31.25	1871.11	0.0321
31.25	1795.45	0.0334
34.38	1833.68	0.0327
34.38	1895.79	0.0316
37.5	1861.11	0.0322
37.5	1865.71	0.0322
40.63	2102.22	0.0285
40.63	1984.44	0.0302
43.75	2070	0.0290
43.75	2113.75	0.0284
46.88	2096.67	0.0286
46.88	2192.94	0.0274
50	1872.31	0.0320
50	2004.21	0.0299
53.13	2322.86	0.0258
53.13	2278.89	0.0263
56.25	2270.48	0.0264
56.25	2254.12	0.0266
59.38	2556.84	0.0235
59.38	2889.52	0.0208
62.5	2212.63	0.0271
62.5	2214.74	0.0271
68.75	2451.25	0.0245
68.75	2571.25	0.0233
75	2258.67	0.0266
75	2385.26	0.0252
81.25	3222.86	0.0186
81.25	2766.67	0.0217
87.5	2938.82	0.0204
87.5	2937.5	0.0204
93.75	2864.44	0.0209
93.75	3187.5	0.0188

Table 8.32 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid at pH 2.5*

Sorbic acid concentration (ppm)	time to detection (mins)	Relative rate to detection (hrs)
100	2780	0.0216
100	2765	0.0217
106.25	3756	0.0160
106.25	3679	0.0163
112.5	3708.24	0.0162
112.5	3889.41	0.0154
118.75	4341.11	0.0138
118.75	5631.11	0.0107
125	3327.78	0.0180
125	3567.50	0.0168
137.5	4335	0.0138
137.5	5221.18	0.0115
150	3895.56	0.0154
150	4460	0.0135
162.5	7071.25	0.0085
162.5	5332.38	0.0113
175	5809.33	0.0103
175	6223.75	0.0096
187.5	6871.76	0.0087
187.5	9056.25	0.0066
200	7148	0.0084
200	7794	0.0077
212.5	10960.67	0.0055
225	12013.33	0.0050
225	12303.33	0.0049
250	8876.25	0.0068
250	9703.57	0.0062

Table 8.33 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid at pH 2.5*

Sorbic acid concentration (ppm)	time to detection (mins)	Relative rate to detection (hrs)
0	1422	0.0422
0	1367.06	0.0439
1.17	1393.91	0.0430
1.17	1421.05	0.0422
1.56	1487.78	0.0403
1.56	1424.35	0.0421
1.95	1482.5	0.0405
1.95	1472	0.0408
2.34	1438.75	0.0417
2.34	1405.45	0.0427
2.73	1451.3	0.0413
2.73	1444.71	0.0415
3.13	1460	0.0411
3.13	1445.71	0.0415
3.52	1430.91	0.0419
3.52	1438.95	0.0417
3.91	1476.84	0.0406
3.91	1466.32	0.0409
4.3	1474.44	0.0407
4.3	1456	0.0412
4.69	1474.74	0.0407
4.69	1425.88	0.0421
5.08	1486.25	0.0404
5.08	1484.44	0.0404
5.47	1521.05	0.0394
5.47	1496.67	0.0401
5.86	1455	0.0412
5.86	1434.12	0.0418
6.25	1401.82	0.0428
6.25	1440.91	0.0416
6.64	1492.38	0.0402
6.64	1488.57	0.0403
7.03	1492.94	0.0402
7.03	1508.42	0.0398
7.42	1496.25	0.0401
7.42	1459.13	0.0411

Table 8.34 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid at pH 4.6*

Sorbic acid concentration (ppm)	time to detection (mins)	Relative rate to detection (hrs)
7.81	1493.75	0.0402
7.81	1484	0.0404
8.59	1532.5	0.0392
8.59	1473.68	0.0407
9.38	1505	0.0399
9.38	1462	0.0410
10.16	1520	0.0395
10.16	1532.63	0.0391
10.94	1537.5	0.0390
10.94	1495.79	0.0401
11.72	1545	0.0388
11.72	1507.5	0.0398
12.5	1433.64	0.0419
12.5	1449.41	0.0414
13.28	1548	0.0388
13.28	1563.53	0.0384
14.06	1494.67	0.0401
14.06	1508.42	0.0398
14.84	1605.56	0.0374
14.84	1570.59	0.0382
15.63	1477.27	0.0406
15.63	1515.79	0.0396
17.19	1528.75	0.0392
17.19	1530.59	0.0392
18.75	1560	0.0385
18.75	1525.88	0.0393
20.31	1574	0.0381
20.31	1611	0.0372
21.88	1597.5	0.0376
21.88	1564	0.0384
23.44	1582.5	0.0379
23.44	1588	0.0378
25	1545	0.0388
25	1473.68	0.0407
26.56	1632.22	0.0368
26.56	1672.63	0.0359

Table 8.35 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid at pH 4.6*

Sorbic acid concentration (ppm)	time to detection (mins)	Relative rate to detection (hrs)
28.13	1602.11	0.0375
28.13	1569	0.0382
29.69	1715	0.0350
29.69	1662.11	0.0361
31.25	1529.41	0.0392
31.25	1609.09	0.0373
34.38	1625	0.0369
34.38	1637.65	0.0366
37.5	1581	0.0380
37.5	1613.68	0.0372
40.63	1743.33	0.0344
40.63	1723	0.0348
43.75	1636.52	0.0367
43.75	1665.88	0.0360
46.88	1746.67	0.0344
46.88	1728	0.0347
50	1638.82	0.0366
50	1596.47	0.0376
53.13	1736.67	0.0345
53.13	1821.9	0.0329
56.25	1705.33	0.0352
56.25	1670	0.0359
59.38	1964.44	0.0305
59.38	1857.78	0.0323
62.5	1756	0.0342
62.5	1747.06	0.0343
68.75	1768.89	0.0339
68.75	1771.25	0.0339
75	1845.56	0.0325
75	1817.89	0.0330
81.25	1960	0.0306
81.25	2072	0.0290
87.5	1960	0.0306
87.5	1921	0.0312

Table 8.36 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid at pH 4.6*

Sorbic acid concentration (ppm)	time to detection (mins)	Relative rate to detection (hrs)
93.75	2150	0.0279
93.75	1924	0.0312
100	2028	0.0296
100	2032.22	0.0295
106.25	2193.75	0.0274
106.25	2150	0.0279
112.5	2140	0.0280
112.5	2021.25	0.0297
118.75	2640.95	0.0227
118.75	2280	0.0263
125	1943.33	0.0309
125	2097.65	0.0286
137.5	2229	0.0269
137.5	2194.74	0.0273
150	2218.1	0.0271
150	2314	0.0259
162.5	2660	0.0226
162.5	2898.82	0.0207
175	2702.67	0.0222
175	2445	0.0245
187.5	3062.35	0.0196
187.5	2620	0.0229
200	2876.19	0.0209
200	2529.52	0.0237
212.5	3280	0.0183
212.5	3313.33	0.0181
225	2602.86	0.0231
225	2950	0.0203
237.5	4043.33	0.0148
237.5	3377.5	0.0178
250	3694.12	0.0162
250	3067.5	0.0196
275	3460	0.0173
275	3055.29	0.0196

Table 8.37 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid at pH 4.6*

Sorbic acid concentration (ppm)	time to detection (mins)	Relative rate to detection (hrs)
300	3753.33	0.0160
300	3328	0.0180
325	3684.71	0.0163
325	4917	0.0122
350	3878.89	0.0155
350	4511.76	0.0133
375	6441.18	0.0093
375	4410	0.0136
400	3604	0.0166
400	3470	0.0173
425	6670	0.0090
425	6281.11	0.0096
450	8280	0.0072
450	7320	0.0082
475	5640	0.0106
500	7355.29	0.0082
500	6522.11	0.0092
550	10062.67	0.0060
550	9151.67	0.0066
600	7165.71	0.0084
600	6353.64	0.0094
650	11050.34	0.0054
650	12246	0.0049
700	12215	0.0049
700	12360	0.0049
750	12806	0.0047
900	12192.31	0.0049
900	11946.15	0.0050
1000	13966.67	0.0043
1000	11502.4	0.0052
1100	13532.5	0.0044

Table 8.38 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid at pH 4.6*

Vanillin concentration (ppm)	time to detection (hrs)	relative rate to detection (hrs)
0	1014.67	0.0591
0	1052.94	0.0570
1.17	1070.77	0.0560
1.17	1106.67	0.0542
1.56	1093.75	0.0549
1.56	1023.16	0.0586
1.95	1076.84	0.0557
1.95	1082.11	0.0554
2.34	1032.5	0.0581
2.34	957.65	0.0627
2.73	1098.67	0.0546
2.73	1106.25	0.0542
3.13	1089.41	0.0551
3.13	1015	0.0591
3.52	1087	0.0552
3.52	1072.22	0.0560
3.91	996.47	0.0602
3.91	949.47	0.0632
4.3	1098.46	0.0546
4.3	1098.89	0.0546
4.69	924.44	0.0649
4.69	954.29	0.0629
5.08	1098.46	0.0546
5.08	1102.35	0.0544
5.47	1032.63	0.0581
5.47	991.11	0.0605
5.86	1090.67	0.0550
5.86	1038.95	0.0578
6.25	1085	0.0553
6.25	1040	0.0577
6.64	1073.75	0.0559
6.64	1072	0.0560
7.03	1028.75	0.0583
7.03	1007.5	0.0596
7.42	1066.67	0.0563
7.42	1036	0.0579

Table 8.39 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of vanillin at pH 4.6*

Vanillin concentration (ppm)	time to detection (hrs)	relative rate to detection (hrs)
7.81	989.33	0.0606
7.81	951.11	0.0631
8.59	1041.54	0.0576
8.59	1021.25	0.0588
9.38	960	0.0625
9.38	946.67	0.0634
10.16	1014.44	0.0591
10.16	993.75	0.0604
10.94	981.18	0.0612
10.94	995.56	0.0603
11.72	957.33	0.0627
11.72	944.29	0.0635
12.5	1069.33	0.0561
12.5	1056.19	0.0568
13.28	974.44	0.0616
13.28	940	0.0638
14.06	1005.45	0.0597
14.06	963.53	0.0623
14.84	1010	0.0594
14.84	1012.5	0.0593
15.63	1003.53	0.0598
15.63	927.78	0.0647
17.19	1003.81	0.0598
17.19	987.78	0.0607
18.75	943.64	0.0636
18.75	967.62	0.0620
20.31	992.86	0.0604
20.31	963.33	0.0623
21.88	1008	0.0595
21.88	964.44	0.0622
23.44	982.35	0.0611
23.44	933.33	0.0643
25	1095.29	0.0548
25	1069.23	0.0561
26.56	956.25	0.0627
26.56	933.6	0.0643

Table 8.40 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of vanillin at pH 4.6*

Vanillin concentration (ppm)	time to detection (hrs)	relative rate to detection (hrs)
28.13	1032.5	0.0581
28.13	941.25	0.0637
29.69	1129.33	0.0531
29.69	1075.38	0.0558
31.25	997.33	0.0602
31.25	992.22	0.0605
34.38	1017.89	0.0589
34.38	984	0.0610
37.5	1005.26	0.0597
37.5	991.76	0.0605
40.63	1037.33	0.0578
40.63	970	0.0619
43.75	1028.75	0.0583
43.75	996.47	0.0602
46.88	1043.33	0.0575
46.88	1038.67	0.0578
50	1104.71	0.0543
50	1100	0.0545
53.13	1048.24	0.0572
53.13	989.33	0.0606
56.25	1034.67	0.0580
56.25	986	0.0609
59.38	1111.11	0.0540
59.38	1103.33	0.0544
62.5	1014.44	0.0591
62.5	1036.47	0.0579
68.75	1055.71	0.0568
68.75	1034.67	0.0580
75	1038.46	0.0578
75	1042.11	0.0576
81.25	1067.06	0.0562
81.25	1037.65	0.0578
87.5	1072.94	0.0559
87.5	1064.71	0.0564
93.75	1074.74	0.0558
93.75	1084.62	0.0553

Table 8.41 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of vanillin at pH 4.6*

Vanillin concentration (ppm)	time to detection (hrs)	relative rate to detection (hrs)
100	1178.75	0.0509
100	1207.78	0.0497
106.25	1123.53	0.0534
106.25	1066.67	0.0563
112.5	1091.76	0.0550
112.5	1085	0.0553
118.75	1228.57	0.0488
118.75	1246.15	0.0481
125	1080	0.0556
125	1141.18	0.0526
137.5	1091.43	0.0550
137.5	1133.33	0.0529
150	1152	0.0521
150	1138.75	0.0527
162.5	1181.18	0.0508
162.5	1155.79	0.0519
175	1186.32	0.0506
175	1182.50	0.0507
187.5	1226.25	0.0489
187.5	1215.24	0.0494
200	1395	0.0430
200	1336.25	0.0449
212.5	1306.25	0.0459
212.5	1248.89	0.0480
225	1297.89	0.0462
225	1283	0.0468
237.5	1326.32	0.0452
237.5	1381.11	0.0434
250	1314	0.0457
250	1307.78	0.0459
275	1461.18	0.0411
275	1337.5	0.0449
300	1454.12	0.0413
300	1409.33	0.0426
325	1483.08	0.0405
325	1555	0.0386

Table 8.42 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of vanillin at pH 4.6*

Vanillin concentration (ppm)	time to detection (hrs)	relative rate to detection (hrs)
350	1492	0.0402
350	1530	0.0392
375	1693.33	0.0354
375	1836.47	0.0327
400	1872.5	0.0320
400	1867.78	0.0321
425	2002.35	0.0300
425	1992.14	0.0301
450	1742	0.0344
450	1793.75	0.0334
475	2170.91	0.0276
475	2486.25	0.0241
500	1912.22	0.0314
500	1773.33	0.0338
550	2053.33	0.0292
550	2128.11	0.0282
600	2555.45	0.0235
600	2555.56	0.0235
650	2486.67	0.0241
650	2372.38	0.0253
700	2265	0.0265
700	2560	0.0234

Table 8.43 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of vanillin at pH 4.6*

Sorbic acid concentration (ppm)	Vanillin concentration (ppm)	Time to detection (mins)	Relative rate to detection (hrs)
0	0	931.2	0.00107
0	0	949.52	0.00105
100	0	1168.3	0.00086
100	0	1141	0.00088
200	0	1358.2	0.00074
200	0	1308.57	0.00076
300	0	1605.3	0.00062
300	0	1584	0.00063
400	0	2272.94	0.00044
400	0	2135.24	0.00047
500	0	2273.3	0.00044
500	0	2384.21	0.00042
600	0	2481.3	0.0004
600	0	2447	0.00041
700	0	6828.57	0.00015
700	0	5616.67	0.00018
800	0	11753.3	0.00009
800	0	10716	0.00009
900	0	12505	0.00008
900	0	12502.2	0.00008

Table 8.44 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid and vanillin at pH 4.6*

Sorbic acid concentration (ppm)	Vanillin concentration (ppm)	Time to detection (mins)	Relative rate to detection (hrs)
0	100	1111.58	0.0009
0	100	1114.74	0.0009
100	100	1263.16	0.00079
100	100	1219.13	0.00082
200	100	1409	0.00071
200	100	1402.86	0.00071
300	100	1673.3	0.0006
300	100	1630.91	0.00061
400	100	2362.5	0.00042
400	100	2155.71	0.00046
500	100	2457.5	0.00041
500	100	2370.59	0.00042
600	100	2500	0.0004
600	100	2422.5	0.000413
700	100	4478.75	0.000223
700	100	4384	0.000228
800	100	7933.3	0.000126
800	100	6350	0.000157
900	100	13217.14	0.000076
900	100	10975	0.000091

Table 8.45 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid and vanillin at pH 4.6*

Sorbic acid concentration (ppm)	Vanillin concentration (ppm)	Time to detection (mins)	Relative rate to detection (hrs)
0	200	1288.89	0.00078
0	200	1246.32	0.0008
100	200	1476	0.00068
100	200	1453.91	0.00069
200	200	1628.75	0.00061
200	200	1638	0.00061
300	200	1967.5	0.00051
300	200	1962.5	0.00051
400	200	2735.29	0.00037
400	200	2628.75	0.00038
500	200	2869.3	0.00035
500	200	2807.5	0.00036
600	200	2970	0.00034
600	200	2806.67	0.00036
700	200	5389.41	0.00019
700	200	5532.86	0.00018
800	200	9791.43	0.0001
900	200	13030	0.000077
900	200	12083.75	0.000083

Table 8.46 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid and vanillin at pH 4.6*

Sorbic acid concentration (ppm)	Vanillin concentration (ppm)	Time to detection (mins)	Relative rate to detection (hrs)
0	300	1451.67	0.00069
0	300	1530.48	0.00065
100	300	1724.62	0.00058
100	300	1722.86	0.00058
200	300	1878.67	0.00053
200	300	1937.65	0.00052
300	300	2250	0.00044
300	300	2265	0.00044
400	300	3162.67	0.00032
400	300	3015	0.00033
500	300	3405.3	0.00029
500	300	3382.86	0.00030
600	300	3456	0.00029
600	300	3336	0.0003
700	300	7934.55	0.00013
700	300	7142.67	0.00014

Table 8.47 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid and vanillin at pH 4.6*

Sorbic acid concentration (ppm)	Vanillin concentration (ppm)	Time to detection (mins)	Relative rate to detection (hrs)
0	400	2011.43	0.0005
0	400	1977.5	0.00051
100	400	1989.3	0.0005
100	400	2070	0.00048
200	400	2332.94	0.00043
200	400	2266.25	0.00044
300	400	2710.77	0.00037
300	400	2686.67	0.00037
400	400	3773.3	0.00027
400	400	3733.3	0.00027
500	400	4022.5	0.00025
500	400	4032.86	0.00025
600	400	4033.3	0.00025
600	400	4057.14	0.00025
700	400	10280	0.0001
700	400	8414	0.00012

Table 8.48 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid and vanillin at pH 4.6*

Sorbic acid concentration (ppm)	Vanillin concentration (ppm)	Time to detection (mins)	Relative rate to detection (hrs)
0	500	2578.95	0.00039
0	500	2258.57	0.00044
100	500	2377.3	0.00042
100	500	2280	0.00044
200	500	2754.29	0.00036
200	500	2614.12	0.00038
300	500	3268.89	0.00031
300	500	3100	0.00032
400	500	4281.43	0.00023
400	500	4406.15	0.00023
500	500	4806	0.00021
500	500	4714.29	0.00021
600	500	4735	0.00021
600	500	4621.3	0.00022
700	500	12780	0.000078
700	500	11781	0.000085

Table 8.49 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid and vanillin at pH 4.6*

Sorbic acid concentration (ppm)	Vanillin concentration (ppm)	Time to detection (mins)	Relative rate to detection (hrs)
0	600	2769.3	0.00036
0	600	2662.5	0.00038
100	600	2871.43	0.00035
100	600	2765.71	0.00036
200	600	3310	0.0003
200	600	3078.67	0.00032
300	600	3861.54	0.00026
300	600	3718.67	0.00027
400	600	5438.46	0.00018
400	600	5403.3	0.00019
500	600	6303.3	0.00016
500	600	6124.4	0.00016
600	600	5876	0.00017
600	600	5840	0.00017

Table 8.50 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid and vanillin at pH 4.6*

Sorbic acid concentration (ppm)	Vanillin concentration (ppm)	Time to detection (mins)	Relative rate to detection (hrs)
0	700	3264	0.00031
0	700	3026.15	0.00033
100	700	3216	0.00031
100	700	3190	0.00031
200	700	3927.27	0.00025
200	700	3853.3	0.00026
300	700	4521.54	0.00022
300	700	4038.3	0.00025
400	700	6504.4	0.00015
400	700	6460	0.00015
500	700	7018.46	0.00014
500	700	6898.57	0.00014
600	700	6994	0.00014
600	700	8060	0.00012

Table 8.51 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid and vanillin at pH 4.6*

Sorbic acid concentration (ppm)	Vanillin concentration (ppm)	Time to detection (mins)	Relative rate to detection (hrs)
0	800	3415.71	0.00029
0	800	3343.53	0.0003
100	800	3885.71	0.00026
100	800	3792.31	0.00026
200	800	4500	0.00022
200	800	4338.3	0.00023
300	800	5460	0.00018
300	800	5085	0.0002
400	800	7572.86	0.00013
400	800	7960	0.00013
500	800	9537.14	0.00010
600	800	9061.82	0.00011
600	800	8421.1	0.00012

Table 8.52 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid and vanillin at pH 4.6*

Sorbic acid concentration (ppm)	Vanillin concentration (ppm)	Time to detection (mins)	Relative rate to detection (hrs)
0	900	3805.3	0.00026
0	900	3957.14	0.00025
100	900	4700	0.00021
100	900	4594.29	0.00022
200	900	5430	0.00018
200	900	5570.77	0.00018
300	900	6815.38	0.00015
300	900	6852.73	0.00015
400	900	11856.92	0.000085
400	900	11966.15	0.000084

Table 8.53 *results for the bioscreen analysis of A. niger growth in semi-solid MEA at 25°C in the presence of sorbic acid and vanillin at pH 4.6*