Candida albicans Biofilms in Denture Wearers

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Dedication

This thesis is dedicated to my late Auntie Jane whose ambition, drive and achievements have always inspired me, and to my late Granddad Jackson whose elation at my acceptance onto this course of study has given me the drive to see it through to completion.

Declaration

I declare that this work has not already been accepted for any degree and is not being currently submitted in candidature for any other than the degree of Doctor of Philosophy of the Manchester Metropolitan University

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Abstract

The term denture stomatitis describes an inflammation of the oral mucosa in contact with the fitting surface (usually maxillary) of the denture. Although considered to have a multi-factorial aetiology, the incidence of denture stomatitis is strongly associated with poor denture hygiene and the presence of *Candida albicans,* in denture plaque. Dentures provided an ideal abiotic substratum for microbial attachment, retention and growth, providing hard, non-shedding of variable topography. Although a wide range of denture hygiene products and procedures are available for use, targeted activity towards Candida is not typically reported. In addition, denture cleansing protocols often use abrasive pastes and/or brushes which can alter denture topography and potentially increase the susceptibility to plaque accumulation and reduce cleanability.

The aim of this work was to investigate interactions between denture surface topography and *C.albicans*, with a view to identifying factors that would enhance denture hygiene.

Two sets of denture acrylic test surfaces were abraded in a linear direction; one using emery papers of increasing grit size, and the other by abrasion using toothbrush and dentifrices. All test surfaces were characterised using white light profilometry, enabling derivation of roughness parameters (Ra/Sa) and measurement of feature dimensions (width, depth). A method was developed that allowed biofilm growth from adherent blastospores or hyphae on these surfaces.

Initial retention on the abraded surfaces increased with increasing roughness values, but there was no effect on topography on the resultant biofilm. Biofilms developed from hyphae had significantly higher biomass than those from blastospores, and also presented a more open network structure. After biofilm removal, surface roughness significantly affected retention of remaining cells: rougher surfaces retained more cells, and retention was increased by the presence of hyphae. The orientation of hyphae on the abraded surfaces did not seem to be effected by topographic features, nor by the proximity of other cells. This latter observation was confirmed using an optical tweezer method, which enabled the specific placement of individual cells, with subsequent monitoring of germ tube formation.

The production of quorum sensing (QS) molecules was investigated as a factor influencing biofilm development. Using gas chromatography and mass spectrometry, volatile compounds were collected from planktonic and biofilms of *C.albicans* over time. Ethanol and the QS molecule farnesol were amongst the molecules identified, being produced at 4hour and 10hours respectively during the 24 hour incubation period. Farnesol was also shown to inhibit hyphal production by attached cells, when applied to the surfaces as a conditioning film, as well as in vapour form.

A denture cleanser with improved anti-Candida activity was effective against blastospore and hyphal biofilms during extended 1 and 16 hour soak times. Using confocal scanning laser microscopy (CSLM) live-dead staining of Candida biofilm indicated that the denture cleanser inactivated cells throughout the biofilm, with increased effect and penetration over time, but no difference between hyphal and blastospore biofilms was observed. When mixed biofilms were generated with *C.albicans* and *Streptococcus oralis*, the presence of the bacteria appeared to increase the effect of the cleanser on Candida biofilm. It is proposed that the inactivation of the bacteria may have disrupted the biofilm and enhanced its susceptibility. However this requires further investigation.

In order to reduce the accumulation of denture plaque and *C.albicans*, there is a need to limit substrate abrasion, reduce retention on the surface, prevent hyphal growth and ideally kill and remove denture plaque. Strategies towards these aims include targeted but gentle cleaning and the potential use of quorum sensing molecules.

Structure of Thesis

This thesis describes the investigation of *Candida albicans* retention, biofilm formation and removal from abraded denture acrylic surfaces. The thesis structure begins with chapter 1 which includes a brief overview and introduction to the subject area. The three chapters that follow include the work contributing towards the thesis. These chapters consist of more detailed relevant introductions, an aims section including research questions and hypotheses, materials and methods, results, discussion and conclusions. The final chapter of the thesis includes an overall discussion and conclusions from this work.

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List of Abbreviations

AD	Analogue to digital
AFM	Atomic force microscopy
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BDA	British Dental Association
CF	Cystic fibrosis
CFU	Colony forming units
COPD	Chronic obstructive pulmonary disease
CSLM	Confocal scanning laser microscopy
EPS	Extracellular polymeric substances
GC-MS	Gas chromatography – Mass Spectrometry
GDH	Glasgow Dental Hospital
GIT	Gastrointestinal tract
H0	Null Hypothesis
N	Newton (force)
NCTC	National Collection of Type Cultures
NCYC	National Collection of Yeast Cultures
OD	Optical density
PBS	Phosphate buffered saline
PMMA	Polymethyl Methacrylate
Ra	Roughness average (2 dimensional profilometry)
RPM	Repetitions per minute

Sa	Average surface roughness (3 dimension profilometry)
SAB	Sabouraud's dextrose media
SAPs	Secreted aspartyl proteinases
SD	Standard deviation
SPME	Solid phase micro extraction
Spp.	Species
T0	Time zero / baseline
TAED	Tetraacetylethylenediamine
TSB	Tryptone soya broth
WLP	White light profilometry

Chapter 1

Introduction to *Candida albicans* biofilms in denture wearers

1.1 Introduction

1.1.1 Biofilms

Biofilms are defined as complex communities of microbial cells, irreversibly attached to surfaces/interfaces or to each other and embedded in extracellular polymeric substances of their own origin that have altered gene expression and growth rates compared to their planktonic counterparts (Donlan and Costerton, 2002).

1.1.1.1 Biofilm history

Biofilms were first referred to by Anton Van Leeuwenhoek who microscopically examined scrapings from his own tooth surface and observed what he described then as the 'animalculi' (Costerton *et al*., 1999, Donlan 2002). It was not however until the 1970's that biofilms began to be recognised as important and regularly occurring components of the environment. Characklis for example, (1973) studied microbial 'slime' in water systems and demonstrated that it was strongly adherent and resistant to disinfectant. And in 1978, Costerton *et al*. reported theories on how microorganisms adhere to living and non-living materials and the survival benefits this niche provides. In the 1980s-1990s biofilm research continued to progress and further evidence emerged that alluded to the now well appreciated knowledge that these groups of adhered microbial cells are in fact structurally organised and complex communities that are ubiquitous in the environment. Advances in microscopic techniques, and increased interest over the decades has allowed for the investigation of biofilm formation and has enabled researches to gain valuable insights into how biofilms form.

1.1.1.2 Stages of biofilm formation

The formation of biofilms on surfaces is now thought to progress in five major stages (Figure 1.1). In the first stage, cells come into contact with and form initial attachments to a surface or interface. At this stage cells are reversibly attached and surrounded by only a small amount of EPS and many are therefore capable of independent movement (O'Toole and Kolter, 1998) by pilus-mediated twitching or gliding (Stoodley *et al*., 2002). During this initial stage cells exhibit species specific behaviours such as rolling, creeping and aggregate formation (Korber *et al*., 1995) before progressing into stage two. In stage two, cells proliferate and begin to produce extracellular polymeric substances (EPS) that increase the strength of attachment and make up the beginnings of the biofilm matrix. During stage three the primary development of the biofilm architecture progresses and water channels begin to form. This architectural development continues into the fourth stage in which, the biofilm architecture matures, resulting in structurally organised cells interspersed with water channels for nutrient and water access. The fifth and final stage occurs once biofilms have reached maturity, as cells are dispersed from the biofilm, disseminating them into the surrounding environment (Stoodley *et al*. 2002).

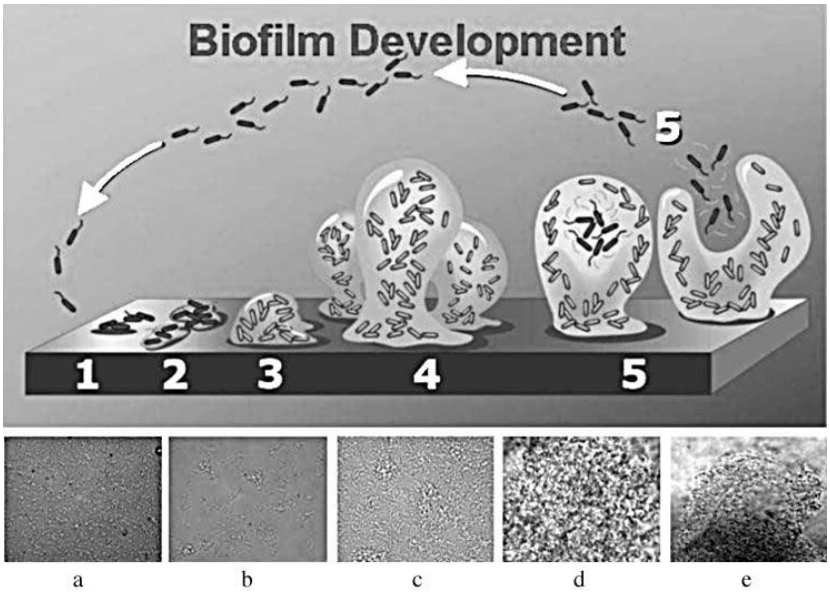


Figure 1.1 Image representing the 5 stage progression of biofilm formation where 1) cells attach to the surface, 2) and proliferate, producing EPS as they grow. 3) The biofilm architecture begin to form and 4) matures into organised columns of cells with interspersing water channels. 5) Upon biofilm maturation cells are dispersed into the surrounding environment to continue the cycle. Images a-e show photomicrographs of these this five stage progression in *P. aeruginosa* (Stoodley *et al*. 2002)

1.1.1.3 Biofilm phenotype

Biofilms are markedly different to planktonic cells in many ways, from the genes they express to the ways in which they grow and survive. Differential patterns of gene expression for example, have been reported on several occasions. Brözel *et al*. (1995), investigated levels of gene expression in attached *Pseudomonas aeruginosa* and noted eleven genes that were expressed in different levels during the various stages of development. Similarly, Whitley et al. (2001), investigated *P. aeruginosa* biofilms on chemostats and on submerged gravel. Their work demonstrated seventy three genes that were expressed differently during biofilm phase in comparison to when they were in panktonic phase. More recently in 2007, Yeater *et al*. investigated gene expression in *Candida albicans* biofilms on denture and catherter surfaces. They demonstrated that gene expression was very important for the regulation of morphology and phenotype. In particular, genes encoding glycolytic and non-glycolytic carbohydrate assimilation, amino acid metabolism and intracellular transport mechanisms were up-regulated during early biofilm development, processes that increased intracellular stocks of pyruvate, amino acids and pentoses which prepared the bifoilm comunity for the increase in cellular mass that followed. In comparison to earlier stages, gene expression was reduced in later development (48 hours), which was associated with the reduced level of metabolic activity in the mature phenotype.

As well as being different during biofilm formation and maturation, differentiation in gene expression is also found throughout mature biofilm life and is important for regulation, survival and adaptation of biofilm cells. Whitley *et al.* (2001), investigated biofilm and planktonic *P.aeruginosa* and found seventy genes that were expressed differentially in mature biofilm phase cells. These included genes that encoded proteins

involved in translation, metabolism, membrane transport, secretion and gene regulation. In a separate study, Sauer *et al*. (2002), investigated protein profiles of mature *P. aeruginosa* biofilms and found that over eight hundred proteins were expressed in much greater amounts than in planktonic cells. In addition, three hundred proteins were expressed that were not present during planktonic growth. These were identified and linked with various processes necessary for biofilm regulation including metabolism, phospholipids and lipopolysaccharide bio synthesis, membrane transport, secretion, adaptation and protective mechanisms. Differential gene expression is clearly an important adaptation for the biofilm mode of growth.

1.1.1.4 Biofilm growth rate and resistance

Biofilm cells grow and behave very differently to planktonic cells. Their growth rate for example has been shown to be far slower than planktonic cells. This is thought to be due reduced level of nutrient supply as a result of the dense population of cells within a biofilm structure which, in turn slows the cellular growth rate (Mah and O'toole, 2001). A reduced growth rate has been described in biofilm bacteria (Brown *et al.* 1988, Wetland *et al.* 1996) and linked to another quality which is increased resistance to antimicrobials (Evans *et al*. 1991, Toumanen et al. 1986). Biofilm cells are well known to exhibit increased resistance to antimicrobial agents and physical removal in comparison to planktonic and attached cells. This increased resistance has been linked to a number of different attributes of biofilms. The presence and structure of the exopolysaccharide rich biofilm matrix, for example is thought to reduce the ability of antimicrobial agents to penetrate and diffuse through the biofilm structure (deBeer *et al*. 1994, Suci *et al.* 1994, Hoyle *et al*. 1992). The biofilm phenotype may also convey antimicrobial resistance by increasing the presence of multidrug efflux pumps which can exude

antimicrobials from cells, and by altering the membrane protein composition of biofilm cells resulting in a reduction in cell permeability to antimicrobial agents (Brooun *et al.* 2000, Prigent-Combaret *et al*. 1999).

1.1.1.5 Clinical significance of biofilms

The biofilm phenotype clearly presents advantages to its inhabiting cells and although the majority of biofilms in the environment cause us little harm, there are many clinically significant biofilms that are of concern. Biofilms readily form on medical devices such as catheters, dentures and contact lenses where they pose a great threat of infection to patients (Donlan, 2001, Hawser and Douglas, 1994, Tautner B W and Darouiche R O, 2004, Mclaughlin-Borlace *et al*. 1998, Imamura *et al*. 2008, lamfon *et al*. 2005). Due to the difficulty in treatment of these biofilms this often results in replacement of the device altogether which can be costly. Biofillms also form on living tissues such as the teeth and gums of the oral cavity and mucosal surfaces in the body where they can result in persistent infections. For example, *Pseudomonas aeruginosa* biofilms are known to persist in cystic fibrosis (CF) patients where the disruption to the lung mucosa commonly allows for this opportunistic pathogen to colonise. Once colonised these biofilms are highly resistant to treatment and the initial colonisation often results in a lifelong battle of CF patients with *P. aeruginosa* infection (Costerton *et al*. 1999). As previously mentioned, clinical biofilms exhibit increased resistance to antimicrobial agents and antibiotic therapies which make them extremely difficult to treat. Microbial biofilms increase the persistence of infections in patients and create costly problems for the healthcare system. As such combating biofilm growth and treating mature biofilms is an important area of focus for research.

1.1.2 Denture plaque

Denture plaque is a clinically important biofilm. Like natural dentition, Immediately after being placed into the mouth the denture surface begins to be coated with a layer of salivary glycoproteins and immunoglobulins (including salivary amylase, albumin, mucin and lysomzyme) termed the 'acquired pellicle' (Baier and Glantz, 1978; Edgerton and Levine, 1992. Edgerton *et al.,* 1993; Avon *et al.,* 2007; Marsh and Martin, 2009). This layer of salivary protein provides specific adhesion receptors for organisms including *Streptococcus* spp. and *C. albicans* (Scannapieco *et al.,* 1995, Nikawa *et al.,* 1993; Edgerton *et al.,* 1993*)* and appears to vary in proportion and composition of proteins, depending upon the surface it forms on (Edgerton and Levine, 1992; Lindh *et al.,* 1999; Svendsen and Lindh, 2009). Edgerton and Levine have demonstrated that pellicles forming on denture surfaces consist of high molecular weight mucins, amylase, lyzozyme, albumin and immunoglobulin A. More recently, Svendsen and Lindh (2009) suggested from their findings that acquired pellicles on PMMA surfaces primarily consisted of lyzozyme and histatins, in comparison to those forming on dentine which consisted of carbonic anhydrase, carbonate dehydrastase, cystatins and lyzozyme and histatins. *C. albicans* has been shown to adhere to salivary mucins, which may influence their adhesion to denture acrylic *in – vivo* (Hoffman and Haidaris, 1993; Nikawa *et al.,* 1992; Nikawa *et al.*, 1993).

1.1.2.1 Primary colonisers in denture plaque

The progression of plaque formation is not too dissimilar to that of dental and other oral plaque (Nikawa *et al.,* 1998a; Sumi *et al.,* 2002). The initial acquired pellicle coating is followed by the adhesion of primary colonising microbes. Over the years

gram positive *Streptococcus* spp. (*S.oralis, S.mutans, S.mitis, S. gordonii, S.sanguinis,* and *S.parasanguinis*) have been most associated with primary colonisation, but other bacterial species including *Veionella* spp., *Neisseria* spp., *Rothia* spp., *Abiotrophia* spp., *Gamella* spp. and *Granullicatella* spp. (previously known as variant streptococci), have also been implicated in early (<8hours) denture colonisation (Jenkinson 2011; Aas *et al.,* 2005; Diaz *et al.,* 2006).

1.1.2.2 Secondary colonisers in denture plaque

If undisturbed, secondary colonisers are able to adhere and co aggregate with primary colonising microbes eventually forming complex, attached denture plaque communities (Coulthwaite and Verran*, 2007*). Secondary colonising microorganisms vary in relation to the position of the plaque formation (Jenkinson, 2011). However, *Candida* spp. are generally acknowledged to be secondary colonisers of denture plaque, attaching to *Streptococcus* spp. and *Actinomyces* spp. on the denture surface (Bamford *et al.,* 2009). Although denture plaque is less well investigated than dental plaque, the predominant cultivable organisms from denture plaque have been investigated previously by several authors. Theilade and Budtz-Jorgensen (1988), examined the cultivable plaque from the fitting surface of dentures from 8 patients. Findings revealed *Streptococcus* spp. (in particular *S.mutans*, *S.mitis, S.salivarius and S.sanguis)* dominated, persisting in 17-76% of samples*.* Gram positive rods *Actinomyces* spp. (*A.israelii, A.naeslundii*, *A.odontolyticus*) and *Lactobacilli, and Veillonella* spp. were also present. Gram negative rods and yeasts were identified in lower amounts. These findings are also supported by other studies (Lamfon *et al.,* 2005; Walter and Frank 1985; Budtz-Jorgensen *et al.,*1981). *Staphylococcus* spp. and *Micrococcus* spp., commonly found on skin and in the environment are rarely found

in the oral cavity but have been isolated from denture plaque taken from clinical cases of denture stomatitis (Webb *et al*., 1998b; Kulak et al., 1997*)*. Further culture studies of denture plaque have shown that it is a diverse microbial biofilm, structurally similar to dental plaque (Walter and Frank, 1985; Budtz-Jorgensen *et al.,*1981), with a similar microbial composition (Nikawa *et al.,* 1998a) but with elevated levels of yeasts (primarily *Candida* spp.), *Lactobacilus spp*., streptococci and staphylococci (Marsh *et al.,* 1992; Theilade and Budtz-Jorgensen, 1988). These elevated levels have been shown to be particularly notable in cases of denture stomatitis (Theiladde and Budtz-Jorgensen, 1988), and have been found to increase with the increase in age of the denture (Budtz-Jorgensen, 1974; Theilade *et al*., 1983; Wright *et al.,* 1985; Sumi *et al.,* 2003; Mizugai *et al.,* 2007). Although *Candida* spp. are of particular concern in denture wearers due to their strong association with denture stomatitis, their reported proportion in denture plaque in comparison to bacterial isolates is relatively low (Theilade and Budtz-Jorgensen, 1988; Koopsmans *et al.,* 1988).

1.1.2.3 Denture plaque as a reservoir for infection

Denture plaque has been shown to harbour several bacterial species that may be involved in infection. In a culture study by Koopsman *et al.* (1988), the microflora of four healthy denture wearers and four with the mucosal inflammatory condition, denture stomatitis was compared. Higher levels of obligate anaerobic bacteria were detected in patients with denture stomatitis. Aditionally, *Streptococcus* spp., *Veillonella parvula, Lactobacillus* spp., bacteroids and *Actinomyces* spp. were noted in higher amounts than the low amount of *Candida* spp. detected in both healthy and diseased patients. From this work they suggested that bacteria harboured in

denture plaque were important in denture stomatitis. Due to the increase in use of molecular assays for microbial identification, many additional bacterial species have now been identified in denture plaque. Sachdeo *et al.* (2008) used molecular techniques to investigate the denture plaque microflora. They identified 41 bacterial species and noted the dominance of *Viellonella spp*. and *Streptococcus spp.* They also found commonly associated periodontal pathogens *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* in denture plaque. They did not look for *Candida* spp. A molecular study by Mantzourani *et al.* (2010) additionally demonstrated the presence of bifidobacteria, commonly associated with dental caries in denture plaque. Yasui *et al.* (2011) investigated the presence of periodontopathic bacteria in complete denture wearers and found that although present in denture plaque, these organisms were less common than in patients with natural dentition. This applied to all species except for *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis and Fusobacterium nucleatum* which were present in similar levels. They were further able to positively correlate the presence of *P. gingivalis* and *Candida* spp. *Candida* spp. in denture plaque have also been positively correlated with the presence of bacteria from the class Bacilli in a study by Kraneveld *et al.* (2012). In addition to this Kraneveld *et al.* demonstrated a negative correlation between the presence of *Candida* spp. with fusobacteria, flavobacteria and bacteroids. Their study highlighted that increased presence of *Candida* spp. on denture surfaces reduced the diversity of denture plaque, promoting the growth of more acidogenic bacteria, such as streptococci.

1.1.2.4 The denture fitting surfaces

Denture plaque harboured on the denture fitting surface is known to be highly acidic and thus supports the growth of acidogenic organisms and *Candida* spp. (Samaranayake *et al.,* 1983; Verran, 1988). Plaque growth in this region is additionally supported by a low salivary flow, increased nutrient availability and more varied and irregular surface topography, which has implications for the maintenance of denture hygiene. These relatively stagnant conditions at the denture fitting surface allow for mature denture plaque biofilms to form which are then difficult to eradicate from the denture (Figure 1.2; Coulthwaite and Verran 2007). For these reasons it is desirable to limit the accumulation of microorganisms on denture surfaces with an aim to reducing plaque development.

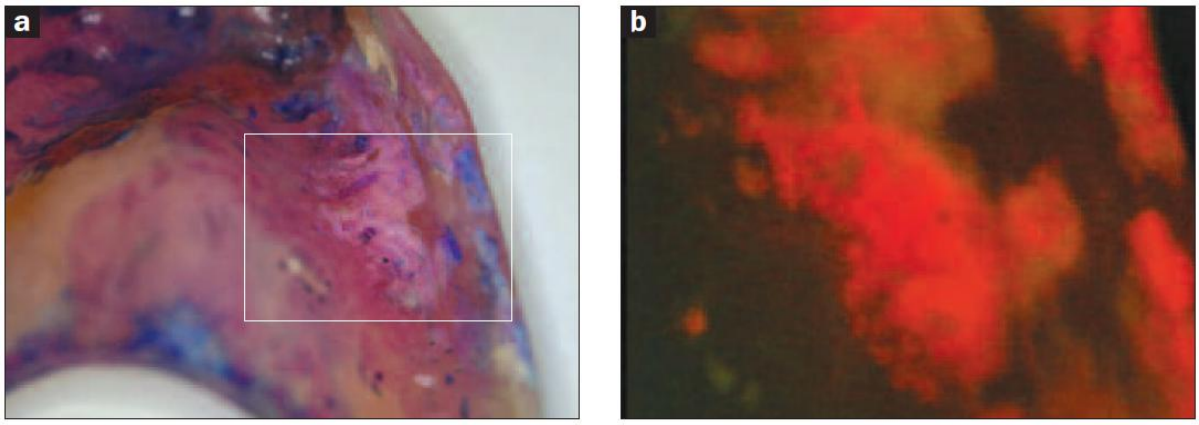


Figure 1.2 Images of the fitting surface of a used maxillary denture showing a) disclosed plaque and b) quantitative light induced fluorescence (QFL) image of dense areas of mature denture plaque formed on the fitting surface (as indicated by red fluorescence). Images are taken from Coulthwaite and Verran, 2007).

1.1.3 Denture Stomatitis

Denture plaque is a leading aetiologic factor in the oral mucosal inflammatory disorder, denture stomatitis (Catalan *et al*., 1987, Ableson, 1981). Denture stomatitis is a common and problematic disorder for denture wearers. Its progression is characterised by an inflammation and erythema of the oral mucosa in contact with the denture, where it is in close proximity to denture plaque. Affecting between 15 and 70% of denture wearers (Gendreau and Loewy, 2011), this condition causes discomfort and swelling, often resulting in dentures becoming ill fitting and thus difficult to wear. In turn, this can result in loss of functionality and influence dietary and lifestyle choices that can dramatically affect the quality of life for denture wearers. In addition to this, the onset and development of denture stomatitis can ultimately result in the denture having to be removed and replaced because treatments are often unsuccessful. This is a costly and demanding procedure for our health services (Polzer *et al.,* 2010).

1.1.3.1 Denture stomatitis aetiology and management

Over the years the aetiology and management of denture stomatitis has been of particular focus in research, with attempts being made to clarify the causes of this condition. Many contributing factors have been implicated. Bastiaan (1976) studied a population of 74 denture wearers. Half with denture related stomatitis and the other half, healthy denture wearers. Findings showed that denture trauma caused by poorly fitting dentures, higher prevalence of *C. albicans* and poor denture hygiene were strongly associated with the group reporting denture stomatitis symptoms. Ramage *et al.* (2004) utilised scanning electron microscopy to visualise *in-vivo* denture plaque biofilms from patients with denture stomatitis and noted a large

amount of established yeast and hyphal cells, indicating an important role for *C. albicans* biofilms in denture stomatitis. More recently a large cohort study by Figueiral *et al.* (2007) investigated the causative factors in 124 denture wearing patients (54 with clinical symptoms of denture stomatitis, 70 without). Their findings supported those of Bastiaan (1976) , showing that denture stomatitis is related to the trauma caused by poorly fitting dentures, and can be increased by the long term and overnight wearing of dentures as well as the denture age. They also supported the previous findings that the condition is strongly related to poor denture hygiene and the high prevalence of *Candida* spp., particularly *C. albicans* on denture surfaces. In addition to these factors, denture stomatitis mainly affects those who are predisposed to yeast infections due to underlying illness or disruption to natural defences e.g. immunosuppressive therapies/diseases (HIV/AIDS), diabetes and old age (Webb *et al.,* 1998b*;* Bartholomew *et al.,* 1987; Pires *et al.,* 2002). Thus denture stomatitis is a multifactorial condition. Poor denture hygiene however, appears to be one factor of particular significance. Without the maintenance of regular and effective denture hygiene routines, the susceptibility of denture surfaces to microbial colonisation is increased. If allowed to continue, this accumulation will lead to the development of the denture plaque biofilm, which like all biofilms, is notoriously difficult to remove from the denture surface due to the presence of the exo-polysaccharide rich matrix and the inherent resistance of biofilm microorganisms to antimicrobial agents (Budtz-Jorgensen, 1974; Mah and O'Toole, 2001; Lamfon *et al.,* 2005; Flemming and Wingender, 2010). Denture hygiene and the development of effective antimicrobial and plaque removal cleansing regimes is therefore an important area of focus for research.

1.1.4 Denture plaque and systemic infections

As well as posing a threat to the mucosal tissues of the oral cavity, plaque forming on dental and denture surfaces has been linked to serious systemic infections. Oral bacteria have been linked with conditions such as aspiration pneumonia, gastrointerstinal infection and chronic obstructive pulmonary disease (Coulthwaite and Verran, 2007).

1.1.4.1 Respiratory infections

The oral cavity may act as a reservoir for pathogens that are either shed from the oral plaque biofilm into small saliva droplets and inhaled, or inhaled directly into the lungs where they can colonise and cause infection (Scannapieco, 1999, Coulthwaite and Verran, 2007). This is especially true in susceptible patients such as those with lung disease and immune-compromised patients. Lindemann *et al*.(1985), isolated strains of *P. aeruginosa* from the tongues of 14 out of 20 cystic fibrosis (CF) patients compared to zero of 20 healthy control patients. In a similar study, Fourrier et al. (1998), found high correlation between dental plaque colonised with respiratory pathogens and the colonisation of saliva and tracheal samples. These were additionally found to be useful predictors of nosocomial infection in intensive care patients. These studies focussed on the oral surfaces on the tongue and natural dentition, but links have also been made in edentulous patients and respiratory disease.

In 2003, Senpuku *et al*. reported on an epidemiological study of institutionalised elderly, with 10-19 teeth, indicating a strong link between bacteria cultured from the oral cavity and respiratory and heart disease. They revealed significantly higher levels of opportunistic pathogens including *C. albicans*, *Pseudomonas* spp,

Staphylococcus spp. and *Serratia marcescens* in the oral cavity and pharynx of patients with severe systemic infections. They additionally found that *Pseudomonas* spp, *Klebsiella pnemonaie* and/or *C. albicans* were significantly higher in plaque samples taken from elderly patients with heart disease. Pulmonary pathogens such as *Prevotella spp*. have also been correlated with edentulism (Theilade and Budtz-Jœrgensen, 1988). And *C. albicans* (commonly isolated from denture plaque) has been isolated from trans-tracheal aspirates of pleuropulmonary infection and pulmonary samples (Emori and Gaynes, 1993).

The composition of denture and dental plaque In Chronic obstructive pulmonary disease (COPD) may also influence the frequency and occurrence of infections. *Haemophilus influenza* is a persistent cause of exacerbations in COPD patients (Murphy *et al*. 2004). *H. influenza* has been isolated from dental plaque on numerous occasions (Sumi et al. 2002, Scannapieco et al. 1992) and has also been reported in denture wearers. Fouche *et al*. (1986) identified *H. influenza* and *N. meningitidis* samples taken from the oral cavity of denture stomatitis patients. It may be that the persistence of these infections in the more susceptible COPD patients is enabled due to the colonisation of oral plaque by these pathogens, providing a reservoir for reoccurring infection.

1.1.4.2 Gastrointestinal infection

In 2003, Sumi et al. investigated the microbial flora of the dentures and the pharyngeal mucosa in 50 dependant elderly patients. Their findings demonstrated a high correlation (68.5%) between the two sites, indicating towards the denture surfaces acting as reservoirs for the colonisation of the pharynx in these cases. The oropharynx is known to be a primary site for the further colonisation of the

gastrointestinal tract (Hayland and Mandell, 1992), indicating a possible link between dentures and microorganisms colonising the gut. Dental and denture plaque is therefore considered to be a source of GIT colonization due to repeated swallowing of oral microorganisms (Fourrier *et al*, 1998). One example of the potential pathogens harboured in oral plaque is described in a study by Nguyen et al. (1992). Their work examined a total of 25 patients, and found the GIT pathogen *Helicobacter pylori* in supra-gingival and sub-gingival plaque samples. 18 of the patients investigated had *H. pylori* gastritis. 38% of these patients were found to have *H. pylori* in their dental plaque where as none of the healthy patients plaque was found to be positive for *H. pylori*. *H. pylori* has been linked with gastric carcinoma (Namura et al. 1991, Khalifa *et al.* 2010) and this possible connection between oral plaque and GIT colonisation is therefore of increasing concern.

The removable nature of dentures means that they may also spend a significant amount of time outside of the oral cavity where they may encounter less hygienic environments and as such introduce potential pathogens such as *Streptococcus pnemoniae*, *Haemophilus influenza, Neisseria meningitides*, *Pseudomonas* spp, *E.coli* and *Klebsiella* spp. (Sumi et al 2002) not usually associated with the normal flora, into the mouth. These factors are especially important in elderly or institutionalised denture wearers, whom are more likely to be susceptible to infection and to come into contact with pathogens with increased resistance (Coulthwaite and Verran 2007).

It is apparent that there is a need to limit the load of oral plaque biofilms such as denture plaque in the oral cavity, especially in the vast proportion of more

susceptible elderly denture wearers in order to limit the risk of localised and systemic infections.

1.1.5 Denture hygiene

As well as being essential to prevent the accumulation of denture plaque that may harbour potential pathogens, good denture hygiene is also required to limit malodour, and maintain good aesthetics in denture wearers (Jagger and Harrison*, 1995*). There is a wide range of denture hygiene products and protocols available for denture cleaning. The British Dental Association (BDA) recommends that dentures are washed every day using a soft to medium brush and either toothpaste or soap and water, supplementing this with soaking in commercial cleansers on a regular basis and rinsing thoroughly, before returning dentures to the mouth (*www.bdasmile.org/fiftyplus*). If performed regularly, these processes are usually adequate to maintain good denture hygiene. However many denture wearers are elderly, and as a result often suffer from medical conditions such as arthritis and dementia, that can impair their ability to carry out these procedures effectively (Gornitsky *et al.,* 2002). For these patients who may also be additionally predisposed to infections due to low immunity and conditions such as diabetes (Bartholemew *et al*., 1987), it is essential that simple but effective denture cleaning regimes are available.

1.1.5.1 Physical characteristics of dentures

Polymethyl-methacrylate (PMMA) is a synthetic polymer commonly used for the fabrication of denture bases. As opposed to metal materials, PMMA is cheaper, more available and easy to manipulate (Phoenix *et al.* 2004). PMMA is prepared by mixing a powder polymer with a liquid monomer to provide malleable dough that

can subsequently be moulded to patient specifications and hardened during a curing/polymerization process (Blagojevic and Murphy, 1999). The polymerization of the denture base resin can be achieved in several ways including heat curing (in a water bath or oven), cold curing/ autopolymerisation, curing with microwave irradiation, and intensive light (Blagojevic and Murphy, 1999; Rahn *et al*. 2009). These polymerisation processes can alter the mechanical properties of the denture. Microwave polymerisation has been reported to produce similar surfaces to that of heat cured resin (Compagnoni *et al.* 2004) but the polymerisation process is much quicker (De Clerk *et al*. 1987; Hayden *et al*. 1986), can increase denture hardness (Pheonix *et al* 2004) and can enhance the adaptation of the denture material to the patients mould (Sanders *et al.* 1987; Takamata *et al* 1989), producing a better fitting denture which may in turn may reduce the susceptibility to denture stomatitis (Budtz-jɔrgensn, 1981). Microwave curing has however, also been reported to cause increased porosity in denture base materials (Alkhatib *et al*. 1990), decreasing the mechanical properties of the denture and reducing aesthetic appearance (Keller *et al* 1985), as well as increasing the susceptibility to colonisation by oral microorganisms (davenport, 1970). Light curing of denture base resins was introduced in the 1980's and is also described as being a more rapid polymerisation method in comparison to conventional heat curing. This method as well as autopolymerisation protocols has however, been reported to result in higher levels of residual monomers being retained which can have cytotoxic effects and may cause allergic reactions in patients (Jorge *et al*. 2003; Barron *et al*. 1993; Schuster *et al* 1995; Tsuchiya *et al*. 1994; Cimpam *et al*. 2000). Conventional heat cured PMMA has been shown to be superior to alternative materials with increased flexural strength, and lower residual

monomer release (Jorge et al 2003; Lai et al. 2004), as such it remains to be the most popular material used for the fabrication of denture base resins (Lai *et al*. 2004).

Once fabricated, as with natural dentition, denture materials provide hard, non-shedding surfaces that have a naturally irregular surface topography due to the irregularities of the patients mouth mould. Additionally, PMMA is relatively soft and is therefore subject to wear through use and cleaning protocols that may further alter the denture surface topography. Newly fabricated and used dentures provide an ideal environment with variable niches that will support a wide range of oral microorganisms, making denture surfaces highly conducive to the formation of denture plaque. As this plaque formation is undesirable, finding a balance between removing denture plaque microorganisms and limiting changes to the physical characteristics of the denture should be the aim of denture cleaning protocols.

1.1.5.2 Abrasive denture cleansing and denture plaque

The use of dentifrices and toothbrushes for cleaning dentures may seem logical as a normal protocol for cleaning natural dentition. Dentures however are much more susceptible to abrasion than natural teeth and as such, this form of cleaning protocol may alter the surface topography over time (Oliveira *et al.,* 2008). This 'wear' of the surface has significant hygienic implications since the attached microorganisms can become protected in grooves, scratches and pits from both chemical and abrasive cleaning regimes (Whitehead *et al*., 2005). The roughness of denture substratum (and other surfaces) is indicated by the parameter R_a which can be defined as the mean arithmetic average of the absolute values of the roughness profile and relates to the relatively fine spaced irregularities that establish a predominant surface

pattern (Taylor *et al*., 1998). This measurement is used widely in the investigation of roughness on the adhesion and retention of microorganisms to surfaces (Radford *et al*., 1999; Gadelmawla *et al.,* 2002), and provides an overview (an arithmetic average), but not a detailed description of the surface profile. The effect of surface roughness in terms of Ra, on microbial adhesion has been examined extensively over the years and it is now generally accepted that, within specific limits related to cell size, an increase in Ra results in an increase in adhesion/retention of microorganism (Verran and Maryan, 1997; Lamfon *et al*., 2003; Morgan and Wilson, 2001; Taylor *et al.*, 1998; Verran and Whitehead, 2005). However some studies have provided more detailed accounts of the effect of surface roughness and topography on microbial retention indicating that this relationship may be more complicated than first appears. A 1998 study by Taylor *et al.* observed that small increases in surface roughness (Ra) had a significant effect, resulting in maximal microbial adhesion. However, surfaces roughened to a larger degree with deeper pits and grooves (although still resulting in more adhesion than smooth surfaces), resulted in less adhesion due to the reduced protection from shear forces (Taylor *et al*., 1998). These studies highlight the need to better characterise surfaces used in adhesion/retention studies, but this level of detail is not commonly reported. More detailed characterisation of surfaces would help to assess how cleaning protocols affect surface topography and the retention of microorganisms in more detail.

1.1.5.3 Chemical denture cleansers

Denture cleansing tablets with effervescing capabilities are another method of denture hygiene. These provide a means of both chemical and mechanical cleansing that is relatively easy to carry out. There are reports that these types of denture

cleanser are preferable for the maintenance of denture hygiene due to their low abrasive activity and ability to remove organic debris (Harrison *et al.,* 2004). They may also be beneficial for use by elderly denture wearers due to the low requirement for manual brushing they potentially provide. Reports in literature are however varied in their findings towards the success of these chemical denture cleaning products. A study by Dills *et al*. (1988) compared the efficacy of brushing with a denture paste to the use of a chemical cleanser for the microbial decontamination of dentures in vivo. They found that there was a significant difference between the two cleaning protocols, with the chemical denture cleanser soak being far superior to the paste and brush method. The brushing technique was reported as being inconstant in levels of plaque disruption, where as the use of chemical soaks was reported to have a broad range antimicrobial activity against gram negative anaerobic rod, gram positive facultative cocci and gram negative cocci, and greatly reduced the total bacteria load in denture plaque. In a more recent report by Glass *et al.* (2011), investigated the use of effervescing denture cleanser on denture plaque and showed that their use was sufficient for reducing the microbial load on denture surfaces but would not result in complete sterilisation. Their study also indicated that in order to improve cleaning, the soak time and temperature of the soak had to be increased compared to that recommended by the manufacturer. These factors are undesirable as extended soak times in chemical cleanser may reduce the hardness of denture acrylic resin and alter the denture aesthetics. Neppelenbroek *et al*. 2005 investigated soaks in disinfectants commonly used in chemical denture cleansers (including 3.78% sodium perborate, 4% chlorhexidine gluconate, or 1% sodium hypochorite) and water, on the hardness of denture acrylic

pieces. They reported significant reductions in the acrylic resin hardness following soaks in the disinfection solutions in comparison to water soaking. In 2009, Hong *et al.* described a study looking into the effect of nine commercially available denture cleansers and a water control on the colour stability of denture acrylic resin, polymerised in three different ways (heat polymerised, auto-polymerised and light polymerised). They observed significant colour changes in denture acrylic resins soaking in chemical cleansers and a high degree of variation between the differently polymerised acrylic resins used. Peracini et al. examined changes in colour, surface roughness and flexural strength of heat cured denture acrylic resin following denture cleanser soaks. They also found significant changes in these three denture properties following denture cleanser soaks, and additionally reported that the level of change over time was variable between the two types of cleanser tested. The findings from these studies indicate the need for fast acting; effective anti-microbial/anti-Candida cleansers that perhaps need to be tailored to the patient needs and to the type of denture in use.

1.1.5.4 Denture cleanser activity against *Candida* spp.

Of particular concern in relation to denture stomatitis is the reports from some studies of the low activity of commercial denture cleansers against *C. albicans* biofilms (Ramage *et al.,* 2005, Vierira *et al*., 2010). Nikawa *et al.* (1995) investigated the efficacy of 11 denture cleansing products on biofilms of *C. albicans* and noted a high amount of variable success at significantly reducing the viability of biofilm cells between the different cleansers, depending upon their chemical component. In a later study by Gornitsky *et al*. (2002) the efficacy of three commercially available denture cleansers in a population of geriatric patients in a long term care institution

was investigated. There study found that the three effervescent cleansers tested were all successful in significantly reducing denture plaque and staining but only two of the three cleansers tested significantly reduced the level of *Candida* spp., indicating a difference in the level of anti-Candida activity between denture cleanser brands. More work is required to investigate denture cleanser efficacy, especially against *Candida* spp. in denture plaque biofilms, in order to provide recommendations for future hygiene protocols and help to determine an effective and simple method, more accessible to elderly denture wearers.

1.1.6 *Candida albicans*

Although there has been a recent shift in focus onto bacteria in denture wearers (compensating for the skew towards *C. albicans* in the majority of early literature), *Candida* spp. and *C. albicans* in particular are still widely acknowledged to be of clinical importance and associated with denture stomatitis. *C. albicans* is described as being polymorphic as it is capable of switching between various forms, including, blastospores, pseudohyphae, true hyphae and chlamydospores (figure 1.2). These forms are exhibited by the yeast in response to different environmental conditions, and this morphological switching is thought to be significant for virulence, affecting its ability to adhere to various surfaces and aiding its success and survival as an opportunistic pathogen (Sudbery *et al.,* 2004; Molero *et al.,* 1998). The polymorphic nature of *C. albicans* has been shown to influence its ability to form biofilms on surfaces. In one study, mutant *C. albicans* strains, without the ability to exhibit polymorphic growth were tested against normal polymorphic strains for their ability to form biofilms on surfaces. The results showed an increase in the amount of adherent cell populations in the biofilm grown from the polymorphic *C. albicans*

strains, resulting in biofilms that consisted of highly adherent basal layers that were more difficult to remove from the test surfaces (Baillie and Douglas, 1999).

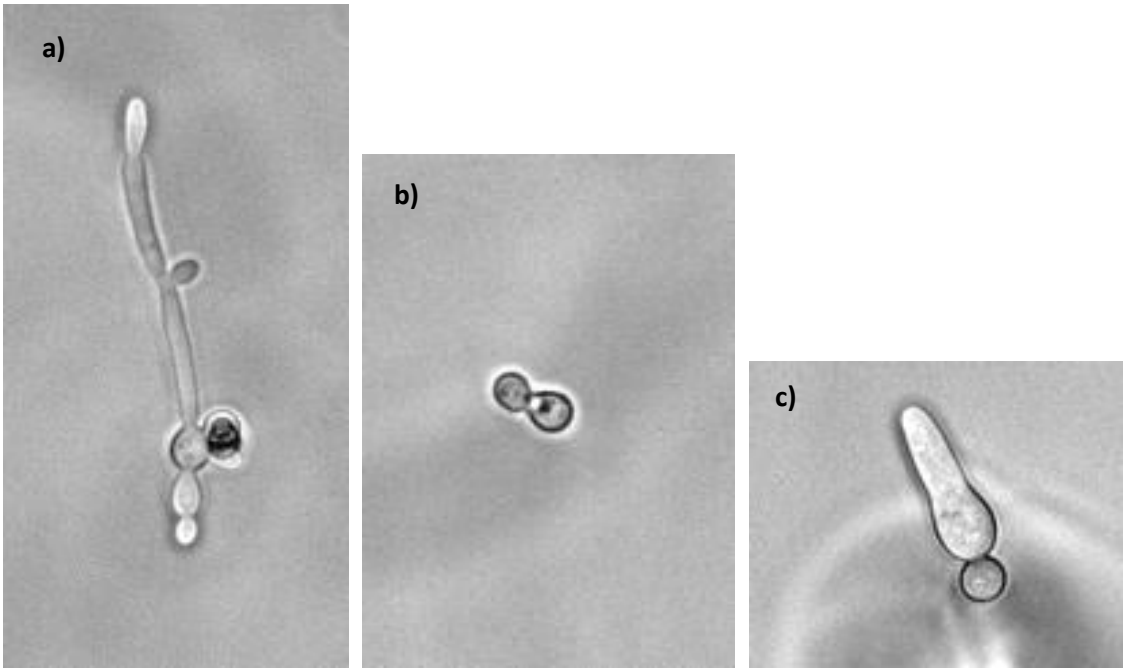


Figure 1.3 ***Candida albicans*** **a) true hyphae, b) budding yeast cells and C) pseudohyphae**, taken following examination of planktonic cultures after 12 hours of growth in SAB broth (orbital incubation at 37˚C).

Filamentous forms of *C. albicans* (figure 1.2) are thought to be significant for the biofilm formation of *C. albicans* (Ramage *et al.,* 2002a; Kumamoto and Vinces, 2005) and host invasion (Phan *et al.,* 2000; Davies *et al*., 1999) and as such the mechanisms behind their development and guidance have been extensively studied. Hyphal growth in *C. albicans* can be initiated by a number of environmental stimuli including, presence of serum, low pH, carbon dioxide concentration and temperature changes (Webb *et al.,* 1998a). Hyphal proliferation is regulated by the products of several genes in *C. albicans* including EFG1 (Stoldt *et al.,* 1997), CPH1 (Liu *et al.,* 1994; Brown and Gow, 1999) and TUP1 (Phan *et al*., 2000). Of these genes the genetic transcription factor Efg1p from the gene EFG1 is one of the best characterised for its involvement in hyphal growth (Kumamoto and Vinces, 2005). This regulator has been reported to both promote and suppress the expression of proteins associated with hyphal growth. Studies show that *C. albicans* mutants not in possession of the EFG1 gene are defective in hyphal formation (Lo *et al*., 1997). EFG1 is has also been shown to be linked to the function of the cyclin, hypha specific G1 cyclin – HGC1. HGC1 is expressed in hyphal forms of *C. albicans* only but is not expressed in strains that are deficient in the Efg1p gene (Zheng *et al.,* 2004). In certain conditions however, this gene has been shown to negatively regulate filamentation. Giusani *et al.* (2002) showed that in low temperatures and in a matrix the Efg1p repressed filamentation. The study demonstrated the complexity of genetic interactions in the morphological changes in *C. albicans*, highlighting them as a mechanism for its versatility and abilty to adapt and survive.

Hyphal forms of *C. albicans* have been shown to grow directionally in response to environmental stimuli including chemical signals (chemotropism), electrical fields

(galvanotropism) and in response to contact sensing (thigmotropism). The direction of hyphal growth is established by two key features of the hypha itself, the polarisome and the Spitzenkorper. The polarisome can be found in both budding yeast and hyphal forms of *C. albicans*, and drives the direction of budding and hyphal tip extension. The Spitzenkorper however is only found in hyphal forms and is thought to work in conjunction with the polarisome to direct hyphal growth (Crampin *et al.,* 2005). More recently (2010), Jones and Sudbery developed a potential model for hyphal tip extension involving the accumulation of secretary vesicles in the Spitzenkorper which are then mediated by the polarisome to promote tip extension. The polarity of the yeast *Saccharomycese cerevisiae* is determined by positioning markers encoded by the BUD genes. In *C. albicans* however the BUD regulation is relaxed and hyphal orientation is therefore open to external influences (Brand and Gow, 2009). The galvanotropic and thigmotropic responses observed in hyphae have been linked to calcium ion influx (Brand *et al.,* 2007). It has been proposed that electrical fields depolarise the plasma membrane on the mother cell initiating the voltage gated Ca^{2+} channel, Cch1. This initiation is thought to increase the Ca^{2+} availability at the hyphal tip and this increased concentration has been used to predict the site of outgrowth in other filamentous fungi (Brand and Gow, 2009; Brand *et al.,* 2007; Pu and Robinson, 1998). In turn, thigmotropism is reduced by decreased Ca^{2+} availability. Deletion of the Cch1 plasma membrane channel, or mechanisms that activate calcium influx, results in decreased sensitivity of hyphae to topographical features of a surface (Brand and Gow, 2009). The activation of calcium channels therefore seems important in determining the site and orientation of tip growth. But this phenomenon has not been investigated on abraded denture

surfaces, where topographical features may introduce barriers, blocking the access of potential signalling and chemical stimuli.

1.1.7 Adhesion

Adhesion of *Candida* spp. can occur in the oral cavity to both epithelial tissue and biomaterials such as denture acrylic, conditioned or not. This attachment can either be specific, involving cell surface molecules known as adhesins (e.g. mannoproteins and fibrillar adhesins), or non-specific, involving electrostatic or hydrophobic interactions (Radford *et al.,* 1998).

1.1.7.1 Adhesion and denture topography

As well as specific and non-specific interactions, the initial attachment and adhesion and subsequent retention of *Candida* spp. on the denture surface is thought to be favoured by the irregular surface topography of the fitting surface of denture materials, which increase the area available for microbial attachment and the cell surface contact, as well as increasing the number of niches in which microorganisms can elude the cleansing action of the saliva, tongue and external cleansing/chemical measures (Shay, 2000). This is of particular concern in dental prostheses fabricated from PMMA: even on smooth surfaces, the microscopic surface is frequently and irregularly pockmarked, often due to bubble formation from un-polymerised monomers forming during the course of denture processing (Shay, 2000). Denture soft lining materials (mainly silicone), designed to improve comfort for denture wearers may also enhance these effects as they are more permeable and porous (Coulthwaite and Verran, 2007).

1.1.7.2 *C. albicans* adhesins

The composition of the *C. albicans* cell surface is dynamic, and the yeast has been shown to modulate its expressed surface macromolecules *in vivo* (De Barnardis *et al.*, 1994), a property which may can be related to adherence to different natural and synthetic receptors (Cannon *et al.,* 1995). Mannoproteins make up approximately 40% of the cell wall biomass. β-1,3-glucans form the major stress bearing components of the cells wall. β1,6-glucans and chitin interconnect mannoproteins and β-1,3-glucans (Cate *et al.,* 2009; Chaffin *et al.,* 1998). In addition to these cell wall constituents, there is thought to be twenty or more cell wall proteins expressed by *C. albicans* at any one time. These determine overall cell surface properties including hydrophobicity, immunogenicity, charge, and permeability (Yin *et al.,* 2008). They are also involved in binding to salivary proteins on the denture acquired pellicle, adhesion to epithelia cells, teeth and denture surfaces, adhesion and co-aggregation to other adhering micro-organisms and biofilm formation (Klotz *et al.,* 2007). Some adhesins specific to *C. albicans* have been identified. CaEap1 is a cell wall adhesion found only in *C. albicans* and mediates adhesion to hydrophobic surfaces and is therefore likely to be highly involved in the adhesion to denture acrylic (Li and Palecek, 2003; Radford *et al.,* 1999). Similarly, the cell wall protein, hyphal wall protein 1 (HWP1), is highly expressed in hyphal forms of *C. albicans* and has been found to be necessary for its ability to form biofilms (Nobile *et al.*2006; Sandstorm, 2002).

1.1.7.2 Enzymes of *C. albicans*

In addition to morphological virulence determinants and adhesins, hydrolytic enzymes secreted by microbial pathogens are often involved with disease pathogenesis and *C. albicans* has been reported to secrete several enzymes including phospholipases and proteinases, which may contribute towards infection (Cannon *et al*., 1995). Of the known proteases, secreted aspartyl proteinases (SAPs) have been identified, the expression of which can be induced under different environmental stimuli. Eight of these SAPs are secreted into the extracellular environment while SAPs 9 and 10 are membrane anchored proteins. SAPs 4, 5 and 6 are expressed during hyphal formation (Schaller *et al.,* 2005). SAPs are restricted to activity in acidic conditions and have been found to contribute towards host tissue invasion by digesting or destroying cell membranes and degrading host surface molecules (Schaller *et al.,* 2005). This may be a contributing factor to the success of *C. albicans* in denture plaque on the denture fitting surface, where acidic condition and the proximity to the oral mucosa enhance these attributes. Sanglard *et al.,* (1997) used animal models to show how mutant strains of *C. albicans*, deficient in genes coding for SAPs 4, 5 and 6 were attenuated in virulence and less invasive to infected organs. SAPs 1, 2 and 3 have been implicated in the inhibition of terminal complement formation (Gropp *et al.,* 2009), aiding its survival from host immune defences. Acidic proteases secreted by *C. albicans* have been shown to cleave secretary immunoglobulin (IgA), allowing colonisation of mucosal surfaces (Akiyama *et al.,* 1993) thus contributing towards *C. albicans* colonisation. Two studies in 2008, using an experimental model involving a reconstructed human epithelium, showed that only SAP 5 was significantly expressed at the infectious stage. Both studies indicated

that SAPs 1-6 played no role in invasion (Lermann and Morschäuser, 2008; Naglik *et al.,* 2008). However both studies used only mutant strains of *C. albicans* that had been shown in prior years to have low virulence against mammalian cells (Taylor *et al.,* 2000). The role of SAPs in the virulence of *C. albicans* remains important.

Phospholipases of *C. albicans* include lysophospholipase-transacylase and phospholipases A, B,C and D (Schaller *et al.,* 2005; Mukherjee *et al.,* 2001; Niewerth and Korting, 2001). In a study by Hanel *et al.* (1995) involving mice infected with a lethal dose of *C albicans*, it was demonstrated that the treatment with fluconazole and β blockers that inhibited the action of phospholipases prevented death in the test subjects. Tissue samples additionally showed the inhibition of phospholipases by the drug combination used, prevented tissue invasion by *C. albicans*. Phospholipase B1 has been correlated with oral candidoses during genetic monitoring of expressed genes in *C. albicans* infected mice (Ripeau *et al.,* 2002). The identification of the various SAPS and phospholipases indicates the complicated and diverse ability of *C. albicans* to adjust and respond to its environment, an aspect which makes it an excellent opportunist pathogen and further highlights the need to combat its incidence in denture plaque. Increased sophistication of experimental approaches over recent decades has contributed significantly to our understanding of virulence of *C. albicans*.

1.1.8 *C. albicans* in denture plaque

The development of *C. albicans* biofilms on denture acrylic has been described as a process that occurs in three distinct stages; early (0-11 hours), intermediate (12-30 hours) and maturation (38-72 hours) phase (Chandra *et al.,* 2001). Early stage biofilms were described as adhering blastospores that develop into distinct colonies

and communities that appear as thick fungal growth across the surface by 11 hours. By the end of the intermediate stage *C. albicans* biofilms are layered structures comprising different morphological forms including blastospores, young hyphae and germ tubes; this stage is further distinguished by the presence of a film of extra polymeric substances (EPS) covering the fungal communities. Microbial EPS has been shown to consist of mostly polysaccharides (Costerten *et al.,* 1981) but proteins, nucleic acids (Nielsen *et al.,* 1997; Dignac *et al.,* 1998) and phospholipids (Takeda *et al.,* 1998) have also been identified. EPS has been implicated in several studies to be important for co-adhesion between microorganisms and their adhesion to synthetic surfaces such as denture acrylic (Wingender *et al.,* 1999; McCourtie and Douglas, 1984; Tsuneda *et al.,* 2003; Flemming and Wingender, 2010). Indeed, mature *C. albicans* biofilms are a dense and organised network of blastospores, pseudohyphae and true hyphae embedded in thick layers of EPS.

As with biofilms in general, the biofilm phenotype conveys advantages to *C. albicans*. Of particular clinical concern is the increased resistance to antifungal agents, fluconazole and amphotericin B (Ramage *et al.*, 2001a; Ramage *et al.,* 2006) and the difficulty in removing biofilms from surfaces (Seneviratne and Samaranayake, 2008). In addition to this, the ability of *C. albicans* to utilise quorum sensing also presents its benefits. Quorum sensing describes the polymicrobial coordination within a biofilm based on secreted molecules that are capable of triggering genetic responses when present in sufficiently high concentrations (Pereira-cenci *et al.,* 2008a). Quorum sensing has been demonstrated in *C. albicans* biofilms, with the majority of focus being on the quorum sensing molecule, farnesol. In 2002, Ramage *et al.* reported on the role of farnesol in the prevention of the germination of *C. albicans*, speculating

on its potential in the prevention of biofilm formation, and its use in improving denture hygiene. The study found that in high concentrations (300µM), farnesol prevented successful germination and ultimately resulted in scant or non-existent biofilms. However this activity was only effective before hyphal formation had been initiated suggesting a potential use for farnesol as a prevention strategy but not a treatment. In contrast to the effects observed of farnesol in *C. albicans* biofilms, research by Chen *et al*. in 2004 described an opposing quorum sensing molecule, tyrosol. The study examined the effects of tyrosol on *C. albicans* that had been diluted into fresh minimal medium. In these conditions the fungus normally enters a lag phase where exponential growth ceases and does not resume for some time. The findings from this study demonstrated that addition of tyrosol caused the cells to resume exponential growth immediately, bypassing the lag phase. From their findings, Chen *et al*. concluded that there is likely to be a reciprocal relationship between farnesol and tyrosol which reflects a complex system of secreted regulatory molecules that are responsible for the adaptation and responses of *C. albicans* to environmental changes (Chen *et al*., 2004). This opens up the prospect of the potential use of these quorum sensing molecules for use in the regulation and control of *Candida* biofilms. Farnesol would be a good target for experimental work, but further work is required to establish its effectiveness on roughened denture surfaces. Current knowledge of factors related to oral health of denture wearers focuses on maintaining good denture hygiene. Increased surface damage by aggressive protocols may enhance plaque accumulation and reduce removal, encouraging colonisation and phenotypic switching in *C. albicans*.

1.1.9 Aims

The aims of this work are to further the investigation into the retention, removal and biofilm development of the opportunistic pathogen *C. albicans* on abraded denture acrylic surfaces.

Chapter 2

Denture surface topography and the retention of *Candida albicans*

2.1 Introduction

2.1.1 Factors affecting the adhesion of *C. albicans* to denture acrylic surfaces

The adhesion of microorganisms to denture surfaces is a prerequisite for denture plaque formation and as such is an important focus for research. *C. albicans* adhesion to denture materials has been a focus of research for several decades and as such is known to be influenced by a number of factors. Surface hydrophobicity and surface free energy have been shown to influence adhesion of *Candida* spp., where cells with surface free energy similar to that of the acrylic surfaces results in greater adhesion (Minagi *et al.*, 1985; Miyake *et al.,* 1986). Samaranayake and Samaranayake and Luo and Samaranayake (1995, 2002), demonstrated a high level of intraspecies variation in hydrophobicity and also showed a significant positive correlation between cell surface hydrophobicity and adhesion in oral isolates of *C. albicans*, *C.glabrata* and *C.krusei*. Hydrophobic interactions are therefore considered to be important contributors to initial cell-surface adhesion but other factors also play an integral role. The presence of sucrose or glucose has been found to enhance the adhesion of *Candida* spp. to acrylic resin (Samaranayake *et al*., 1980; Samaranayake and MacFarlane, 1980). This finding was additionally supported by work published a year later that identified a cell surface layer of cells following incubation in sugars that enhanced their ability to adhere (McCourtie and Douglas, 1981). Similarly the presence of salivary proteins and serum has been shown to increase the adhesion of *Candida* spp. to denture acrylic surfaces (Vasilas *et al*., 1992; Nikawa *et al*., 1996; Edgerton *et al*., 1993; Nikawa *et al.,* 2000). The presence of whole human saliva on the other hand had the opposite effect, decreasing

adhesion (McCourtie *et al*., 1986; Maza *et al*., 2002; Moura *et al*., 2006). These findings suggest that although components of saliva such as dietary sugars and proteins such as mucins (Edgerton *et al.,* 1993) may promote adhesion, whole human saliva is more beneficial to the host, helping to prevent colonisation by *Candida* spp. The type of denture materials and polymerisation method may also have a significant effect on adhesion. Moura *et al* (2006) used heat polymerized and microwave polymerized denture acrylic to test for differences in surface free energy values and found them to be increased for surfaces cured using heat. As previously discussed, these differences in surface free energy can greatly affect adhesion, although no effect was investigated in this study. He *et al* (2006) demonstrated that there were significant differences observed in adhesion of *Candida* spp. to different denture acrylic resins. They also demonstrated that adhesion of *Candida* spp. was greater to cold cured acrylic as opposed to those cured by heat. These findings are supported by those of a previous study that investigated the adhesion of bacteria to cold and heat cured denture acrylic. In addition, cold cured acrylic was more susceptible to an increase in surface roughness upon application of abrasive papers and this increase in roughness correlated with an increase in adhesion (Morgan and Wilson, 2001). Thus the curing of the denture materials gave rise to a difference in their susceptibility to abrasion but it was the surface roughness that influenced the amount of microbial contamination observed. Similarly studies by Quirynen *et al* (1990) and Quirynen and Bollen (1995), have suggested that surface roughness can overrule other factors affecting adhesion. Their studies showed that increasing surface roughness resulted in a dramatic increase in bacterial colonisation whereas no impact was seen with a change in surface free energy on the same surfaces.

Although there are several factors that can influence adhesion, surface roughness and topography are clearly fundamental areas of focus for the consideration of denture surface hygiene.

2.1.2 Denture surfaces and surface topography

A complete newly fabricated denture comprises various surfaces and environments providing niches for colonising oral microorganisms including the smooth, polished surfaces of the 'teeth', crevices in-between and the surfaces of the denture base. Dentures have been shown to accumulate plaque in a similar way to natural teeth and gingival surfaces (Neill, 1968). However, unlike the natural oral environment the introduction of dentures results in occlusion of many of the natural surfaces and instead presents hard, non shedding surfaces that support the retention of microorganisms. In addition to this, the denture fitting surface comes into close proximity with the oral mucosa, providing a sheltered area with irregular topography that further promotes growth and maturation of denture plaque (Coulthwaite and Verran, 2007). The introduction of dentures to the oral cavity and the physical properties of the denture surface can therefore present several problems in terms of the maintenance of adequate oral hygiene.

During use, dentures are subjected to different mechanical and chemical stresses, through normal usage as well as cleansing methods, which may alter their topography. Mechanical cleaning of dentures such as the use of a toothbrush and abrasive dentifrices greatly increases surface roughness of dentures (Lira *et al*., 2012; Machado *et al*., 2012; Pisani *et al*., 2010; Heath *et al*., 2007; Harrison *et al*., 2004). In addition to this chemical (Neppelenbroek *et al.,* 2005) and microwave irradiation

cleaning methods (Campanha *et al.,* 2011) can decrease the hardness of denture acrylic resins by dehydrating them, causing them to become increasingly brittle. This in turn may make them more susceptible to topographical change via mechanical forces.

2.1.3 Microbial contamination of dentures

Microbiological studies into how change in denture topography affects retention of oral microorganisms and *Candida* spp. have been active areas of research for many years. Various abrasive methods have been adopted to produce denture surfaces with different degrees of standardised surface roughness for microbial testing. Radford *et al*., (1998) used surfaces that had been abraded with processing machinery commonly used in the fabrication of dental prostheses to test the effect of different surface finishes on adhesion. Similarly, Nevzatoglu *et al.,* (2007) used denture surfaces processed in different ways including; processed against ultra smooth glass surface, finished with a tungsten carbide bur or processed against dental plaster. These processing methods gave rise to surfaces with increasing degrees of roughness which were examined for their effect on the adherence of *C. albicans*. Other studies have used different grit size abrasive papers to create abrasion to denture surfaces and examine this change in surface roughness on *C. albicans* adhesion (Verran and Maryan, 1997). All of the above studies concluded that an increase in surface roughness results in an increase in retention of *C. albicans* on denture acrylic surfaces. However, the relationship between roughness and retention is less simple than this. In 1998 Taylor *et al*., investigated the adherence of two bacterial species to denture acrylic samples abraded with different grit sized

silicon carbide papers, and with bead and shot blasting, to produce surfaces with different grades of roughness. All roughened surfaces had enhanced retention in comparison to smooth controls but there appeared to be an optimum roughness threshold, above which the amount of adhesion began to decrease. This has also been reported in other studies including studies looking at *C. albicans* adhesion and can be attributed to the fact that increasing abrasion above this threshold decreases the available niches for colonising cells (by making features larger) to evade shear forces, allowing them to be more easily removed (Verran *et al*., 1991; Yamauchi *et al*., 1990). A minimum level of roughness (Ra = 0.2µm) for microbial retention has also been reported (Quirynen *et al.,* 1996). These studies imply that there is an optimal roughness for microbial adhesion. In addition to this, the shape and size of the microorganisms as well as the size and shape of the surface abrasions will affect how well cells are able to adhere and be retained on denture surfaces.

Panagoda and Samaranayake (1998) noted a positive correlation between cell length and adhesion of *Candida* spp. to acrylic surfaces. This observation was also noted in the earlier paper by Minagi *et al.,* (1985) highlighting that two species, differing in their cell size and shape also differed in the cell area available for adhesion. Whitehead *et al., (*2005) produced surfaces with defined topographies, and the dimension of micro-architecture (pores of varying sizes, 0.2, 0.5, 1 and 2µm) were used together with microorganisms of different sizes and shapes (*Staphylococcus aureus, Pseudomonas aeruginosa* and *C. albicans*) to investigate the relationship between topography and retention. Results showed that the cell size and shape in relation to the size of the surface feature greatly influenced the amount of retention.

Small cocci (*S.aureus* 0.5-1 µm) were observed to be best retained on surfaces with features of similar diameter to themselves. Similarly the rod shaped bacteria *P.aeruginosa* (1-2 µm in length) was preferentially retained in 1 µm surface abrasions. *C. albicans* retention was similar across all of the surfaces and was lower than that of the bacteria tested, perhaps due to the fact that all of the features used here were too small to significantly enhance retention of the larger (2-8 µm) yeast cells. Thus, the area of contact between a microorganism and the surface, influences retention (Flint *et al.,* 2000; Medilanski *et al.,* 2002). A surface micro-architecture with similar dimensions to microbial cells is likely to result in a greater amount of contact between the cell and surface feature allowing for a greater bond and increasing resistance to removal (Whitehead and Verran, 2006).

It is clear that there are optimal conditions for microbial retention to denture acrylic surfaces; but the exact dimensions of features on abraded and naturally worn denture acrylic surfaces are not commonly investigated in retention studies. In addition to this, *C. albicans*, commonly isolated from clinical cases of oral candidoses and denture stomatitis (Davenport, 1970; Kulak *et al*., 1994; Barbeau *et al*. 2003; Coco *et al.* 2008), is able to switch between morphological forms that vary in shape and size. Hyphal forms of *C. albicans* in particular are thought to be important in virulence (Kumamoto and Vinces, 2005; Calderone and Fonzi, 2001) and are suggested to be integral to the maturation of biofilms on denture acrylic (Lamfon *et al*., 2003). However, to the author's knowledge no work has yet been done to investigate how this morphological form of *C. albicans* interacts with different denture micro-architecture and topography.

2.1.4 Measuring roughness of denture surfaces

In the UK and USA the roughness parameter Ra, defined as the arithmetic mean deviation of the surface height from the centre line (Radford *et al*., 1999) is the most commonly reported measurement of surface roughness, generated by profilometers that scan across one length of a surface. Three Dimensional (3D) profilometry is also used to scan not just a length, but a given area of a surface providing an average deviation of surface from the centre, known as the mean arithmetic height, which is reported as Sa. Both Ra and Sa measurements have been used to enable comparisons of microbial contamination of different surfaces with different levels of roughness to be made. However, although the Ra/Sa measurements provide a useful general indication of surface roughness they are derived from averaging the deviations across a given length/area of a surface and as such are not sensitive to large peaks or valleys that may be present (Gadelmawla *et al.,* 2002). As previously discussed, the retention of microorganisms to denture surfaces appears to be influenced not only by overall surface roughness but by the size and shape of the organism and the specific surfaces features present. The Ra/Sa parameters do not provide details of the surfaces features present. In addition to this, reported Ra/Sa values are taken by calculating the mean from of a number of given scans of a surface as opposed to the entire surface area being tested. Infrequent surface defects occurring at intervals on test substrata may therefore be missed, but could greatly affect the subsequent retention of organisms (Verran and Whitehead, 2005). Roughness parameters are determined by the use of surface profiling instruments. Studies investigating adhesion and surface roughness report the use of different

profilometers, for example; Lamfon *et al.*, 2003 and Morgan and Wilson, 2001 and Taylor *et al.*, 1998, used laser profilometers to acquire the roughness data for their surfaces. Whitehead *et al.,* (2005) reported the use of atomic force microscopy and Verran and Maryan (1997) used a solid stylus profiler to establish roughness data. Variation in the ability of these surface profilometric techniques to accurately measure surface profiles has been reported (Poon and Bhushan, 1995) making it difficult to compare different studies. Surface measurements are limited by the ability of the profiling instrument probe to access micro-architecture. This will depend on the finite dimension of the stylus tip of the profiler and will affect how accurately it can measure the true surface profile (Poon and Bhushan, 1995). Different surface profilers will therefore provide different resolution levels in measurements of surfaces which may be important in microbial retention studies. Even micro-architecture of nanometer proportions can affect microbial retention (Whitehead and Verran, 2006). The literature therefore implies that better and more detailed characterisation of surfaces (in terms of feature profile and dimensions) with different topographies and roughness levels may be required to better explain and understand cell-substratum interactions.

2.1.5 Aims

To investigate the adhesion and retention of *C. albicans* yeast and hyphal cells to abraded denture acrylic.

The specific objectives included:-

- To produce abraded denture acrylic surfaces and fully characterise their surface topography.
- To investigate the adhesion and retention of *C. albicans* yeast and hyphal cells to denture acrylic surfaces with defined topography.
- To examine the growth and behaviour of *C. albicans* hyphae on abraded denture acrylic surfaces.

Research Question

Does surface topography and roughness affect the adhesion, retention and growth/behaviour of *C. albicans* yeast and hyphal cells on dention acrylic surfaces with defined topogrpahy?

H0

Surface topography and roughness have no effect on the retention and growth and behaviour of *C. albicans* yeast and hyphal cells.

2.2 Materials and Methods

2.2.1 Maintenance of cultures

A stock culture of *C. albicans* NCYC 1467/ GDH 2346 was stored at -80°C. Subcultures were prepared on Sabourauds dextrose agar (Oxoid ltd, Hampshire, UK) and refrigerated at 4°C prior to use. These cultures were replaced every four weeks.

2.2.2 Preparation of cell suspensions

C. albicans GDH 2346 *c*ell suspensions were prepared by inoculation of 100 ml SAB broth with one colony of the *C. albicans* strain (from stock culture), which was subsequently incubated at 37°C for 24 hours in an orbital shaker. Culture purity of each cell suspension was checked by streaking on nutrient and SAB agar, incubating at 37°C for 24 hours and examining for contaminant colonies.

2.2.3 Production of heat cured Polymethy methacrylate (PMMA)

1 cm^2 and 2 cm^2 pink heat cured Polymethyl methacrylate pieces were used in all studies. The 1 cm^2 test pieces were created and sourced in-house (Manchester Metropolitan University). Pink heat cure polymer (Meadway heat cure polymer - Bracon Dental Laboratory Products) was added to a glass jar containing 42 ml of the monomer methyl-methacrylate (Meadway universal heat cure liquid - Bracon dental Laboratory Products, East Sussex, UK) in a 3:1 ratio. The mixture was left to partially set for 10-15 minutes with the jar lid on, following which the resulting malleable putty was removed from the glass jar and mixed by manual manipulation until fully combined (approximately two minutes). A mould (Figure 2.1, 20 cm x 20 cm x 0.4 cm) made from polished stainless steel (Manchester Metropolitan University, Manchester, UK) was coated liberally with sodium alginate. The subsequent mixed

polymer was pressed firmly and evenly into the prepared mould to which the lid was applied, forced down and stabilised with bolts, which spread the mixture evenly across the mould. Excess polymer was manually removed from the sides of the mould, which was then placed in a heat curer in a hot air bath at temperatures up to 100˚C overnight. Following manufacturing the 20 cm x 20 cm x 0.4 cm sheet of pink heat cured denture acrylic (polymethyl-methacrylate) was removed from the heat curer and cut into approximately 400 1 cm^2 pieces using a guillotine (DDPG1000, Morgan Rushworth, Stourbridge, UK).

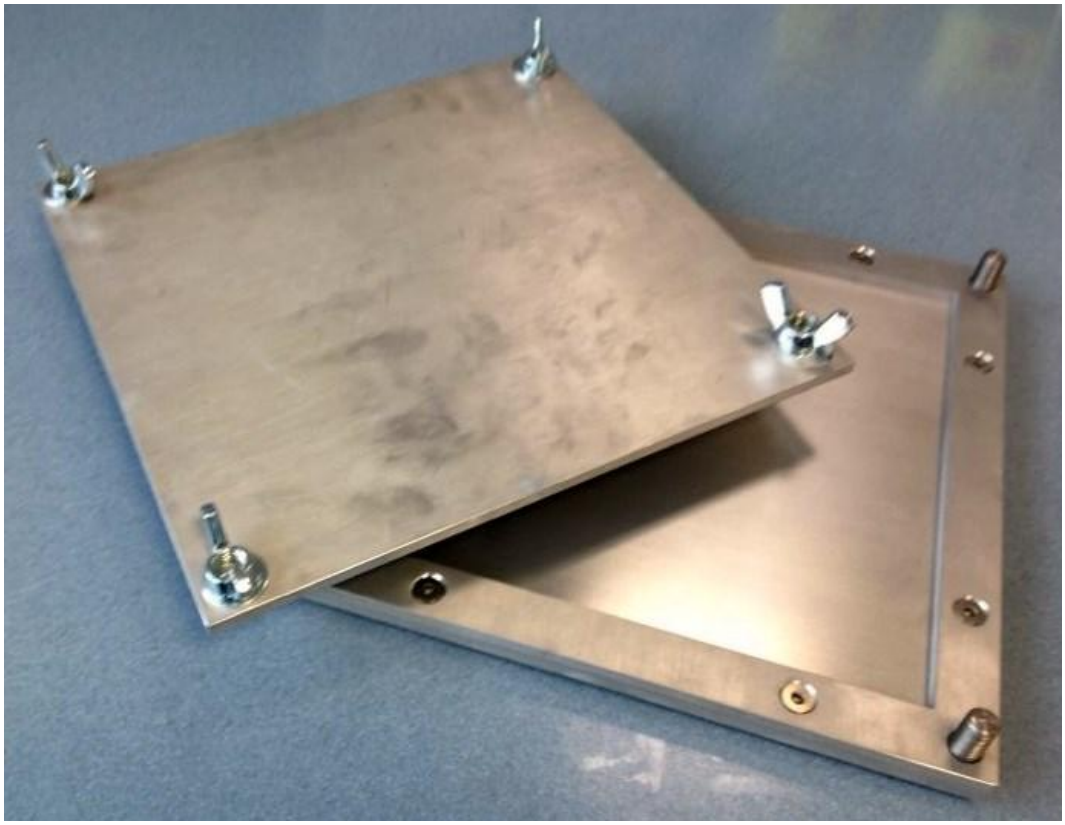


Figure 2.1. Stainless steel mould used for the production of PMMA sheets for the investigation of *C. albicans* adhesion to denture acrylic surfaces. Internal mould measured 20cm x 20cmx 0.4cm with highly polished surfaces to limit surface irregularities on fabricated PMMA sheets.

2.2.4 Abrasion of 1cm^2 heat cured PMMA with emery paper

The 1cm^2 pieces of pink heat cured acrylic were divided into six test groups which were subjected to different degrees of linear surface abrasion (un-abraded, P600/grit size 25.8µm, P400/grit size 35µm, P240/grit size 58.5µm, P100 Glass/ grit size 141µm and P100 Al_2O_3/ grit size 162µm; all papers manufactured by 3M Wetordry™, St Paul, MN, USA). The linear scratches were created by manual abrasion in a smooth motion, applying constant force parallel to the edge of a ruler in one direction. This abrasion stroke was repeated 10 times for each 1cm^2 PMMA piece. Test pieces were then ultrasonically cleaned in 70% ethanol for 1 minute to remove any surface debris, washed in running distilled water and allowed to dry in a class II laminar flow cabinet.

2.2.5 Abrasion of heat cured PMMA with abrasive dentifrices

Pieces of abraded pink PMMA (2cm^2) were prepared and provided by an external organisation (GlaxoSmithKline).

These surfaces were prepared using 800 abrasion strokes with blocks rotated 180 degrees after 400 strokes under 300g of pressure (2.9N force) (Charman *et al.,* 2009). There were four test groups; un-abraded (washed with water), low abraded (washed with Colgate (Colgate-palmolive, Surrey, UK) cavity protection 25:40 paste/water ratio), medium abraded (washed with Colgate total whitening 25:40 paste/water ratio) and high abraded (washed with neat Colgate luminous).

2.2.6 Characterisation of surface topography

Three replicates for each type of abraded 1cm² piece of acrylic were examined using white light interferometry (MicroXam surface mapping microscope, ADE Corporation/KLA Tencor, California, USA) with an AD phase shift controller (Omniscan, Wrexham, UK). Surface measurements were also taken using a solid stylus profilometer (Dektak IID, Veeco Instruments Inc, Plainview, NY) for comparison. On both profilometers, measurements of the average surface roughness (Sa/Ra), commonly used as an indicator of the degree of roughness in past and present adhesion studies (Verran and Maryan 1997; Radford *et al.,* 1998; Pereira-cenci *et al.,* 2006; Zamparini *et al*., 2010; Wady *et al.,* 2012), were recorded for five fields of each of three replicates of each test surface. Both profilometers produced surface profiles (Figure 2.2) that were printed off so that individual features could be measured manually with a ruler to provide detail of the depths and widths (Figure 2.3). For the solid stylus profilometer, the scan length was set to 5mm (to determine roughness data) and was subsequently lowered to 1mm to improve resolution when looking at the surface feature dimensions. Every profile generated varied in the number of features present, therefore each feature within a single profile was measured as seen in Figure 2.3 and pooled with feature data from replicate surfaces, this corresponded >100 surface feature measurements per test group.

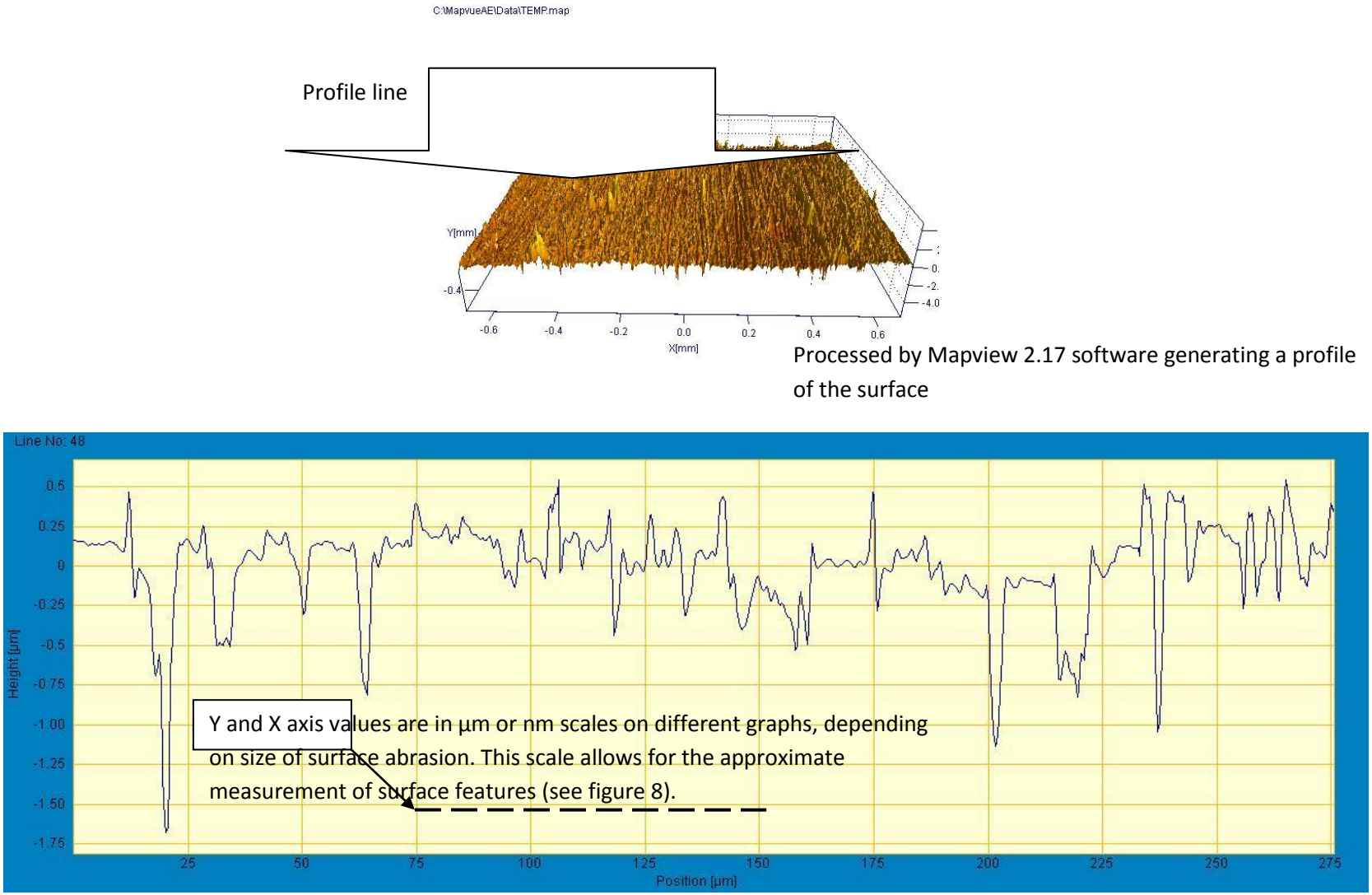
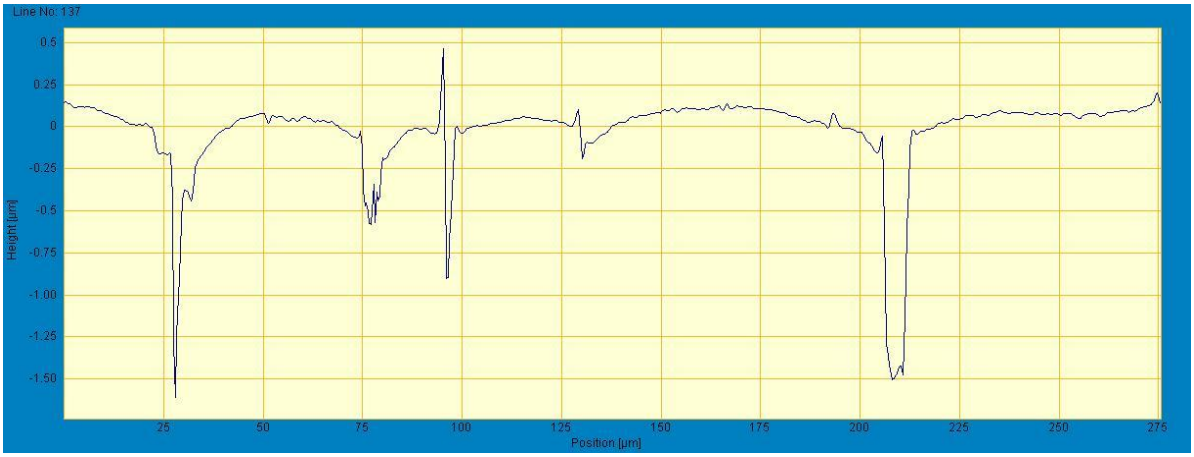


Figure 2.2 The generation of surface profiles from white light profilometry maps. Maps (top) are generated following a scan of the surface and profiles are then generated from these maps by drawing a profile line across the area or the map to be measured. The Mapview AE 2.17 software then creates a representative graph/profile of the deviation in the surface map (bottom).



Profile was printed and the width and depth of the surface deviations measured with a ruler (mm).

1) The Y and X axis scale were measured with a ruler and this measurement was used to determine how many µm/nm were represented per 1mm.

E.g. If X = 15mm and represents 25µm, 1mm = 15/25 = 0.6µm.

2) All of the main features on surface profiles were measured using a ruler and the size in µm/nm was determined -

E.g. The width of a feature was measured at 6mm. If 1mm = 0.6µm, 6mm = (0.6x6) = 3.6µm in width.

Figure 2.3. **The determination of topographical feature dimensions (width and depth) from profiles of WLP maps by analysis of generated surface profiles**. The y and x axis were measured to reveal mm/ µm, which were then used to calculate the µm dimensions of individual measured grooves and ridges.

2.2.7 Preliminary retention assay

Following preparation of the cell suspension, cells were harvested by centrifugation at 3000 rpm for 10 minutes, and subsequently washed in sterile phosphate buffered saline (PBS), three times. Cells were re-suspended in PBS to an optical density of 1.0 at 540nm which corresponded to 2.26± 0.14 X 10^5 cells/ml. 25 ml of the standardised cell suspensions were added to Petri dishes each containing 3 x 1 cm² PMMA test materials (three replicates of each different surface including; un-abraded, P600/grit size 25.8µm, P400/grit size 35µm, P240/grit size 58.5µm, P100 Glass/ grit size 141µm and P100 Al_2O_3/ grit size 162µm). All specimens were then incubated for 1hour at 37^oC without agitation. After 1hour the test materials (with adherent cells), were removed and washed by gently rinsing with running distilled water for three seconds with the test piece held at an angle to allow the water to run off, with the linear surfaces abrasions running parallel to the water. Subsequently, replicates were dried and stained by flooding with acridine orange in 2% glacial acetic acid (Sigma Aldrich, St Louis, USA) for 2 minutes and rinsing with distilled water. Test pieces were examined using light microscopy at x40 objective for adhered cells. Three replicates for each abrasion treatment were used; cell counts were taken in 15 fields per replicate and the number of cells per mm^2 was determined. The adhesion assay was repeated twice.

2.2.8 Standardising methods for retention assay

The results of the preliminary adhesion studies (2.2.7) had produced very low and in some cases no observable retained cells on all test substrata, which raised some concerns. After comparing the protocols used by different teams, a number of

differences were noted that provided reasons for the lower numbers of adhering cells witnessed. Variables identified within our laboratory included; the use of two different wash bottles for the wash step (Figure 2.4). One wash bottle had a thinner spout with a pointed tip (Figure 2.4a) and the other had a wider spout which was the same width along its entire length (Figure 2.4b); the use of different containers for the incubation step (large round Petri dish or a deeper square container – Figure 2.5), both containing 150 ml cell suspension but differing in depth and shape resulting in different volumes of cell suspension above the test surfaces

In order to standardise subsequent methods, the effect of the aforementioned differences in protocol was investigated. The retention method (2.2.7) with *C. albicans* was repeated with additional variables standardised; the force used to eject water from the wash bottle during the wash step, the wash technique and the volumes of cell suspension above the test pieces. Three replicates of the high abraded (2.2.5) 2 cm^2 test substrata were incubated in 150 ml of *C. albicans* cell suspension (1.94± 0.18 x 10^5 cells/ml) for one hour at room temperature, in two different plastic containers (large round Petri dish 15 cm x 1.5 cm, Sigma-Aldrich and deep square container 7 cm x 7 cm x 5 cm, Agar scientific, Essex, UK) to examine the effect of the depth of cell suspension on adhesion. Additionally three replicates of the same test substrata were incubated in 150 ml cell suspension in the square container and were subsequently washed as stated in 2.2.6 with two different wash bottles (figures 2.5a and 2.5b). All surfaces were subsequently removed, stained with 0.03% acridine orange, in 2% glacial acetic acid (Sigma Aldrich, St Louis, USA) and

examined with epi-fluorescent microscopy (Nikon Eclipse 6000, Burgerweeshuispad, Amsterdam), for adhered cell counts.

The results were analysed using a two tailed independent T-test and showed a significant difference ($P<0.01$) between the numbers of adhered *C. albicans* cells on the PMMA surfaces that were washed with the different wash bottles (Figure 2.6). Larger numbers of cells were retained on the surfaces washed with the bottle with the wide spout wash bottle, (Figure 2.4b). The wider spout of this wash bottle allowed water to flow more evenly, and the force applied was found to be easier to control in comparison to the thin spouted wash bottle, which had a more powerful water flow and removed more cells (Figure 2.6). In addition, higher numbers of retained cells were observed on PMMA incubated in the deeper square container compared with those incubated in the large (15cm x 1.5cm) Petri dish (Figure 2.7). Although this difference was not significant ($P=0.6$), There was a greater depth of cell suspension above surfaces incubated in the deep square Petri dishes in comparison to the depth of cell suspension above surfaces in the large round Petri which, could affect count data in further experiments.

In preliminary work, it was also noted that on some of the abraded surfaces, cells were not able to fully occupy linear surface abrasions and instead, in many cases, were positioned on top of scratches with less cell-surface area contact (Figure 2.8). It was therefore likely that holding the surfaces at an angle during rinsing and rinsing with the linear surface abrasions parallel to the water flow would maximise the removal of cells in these cases. Thus, seemingly minor changes in methods may

significantly affect results obtained. It is essential to describe methods in detail to enable comparisons between findings to be made with confidence.

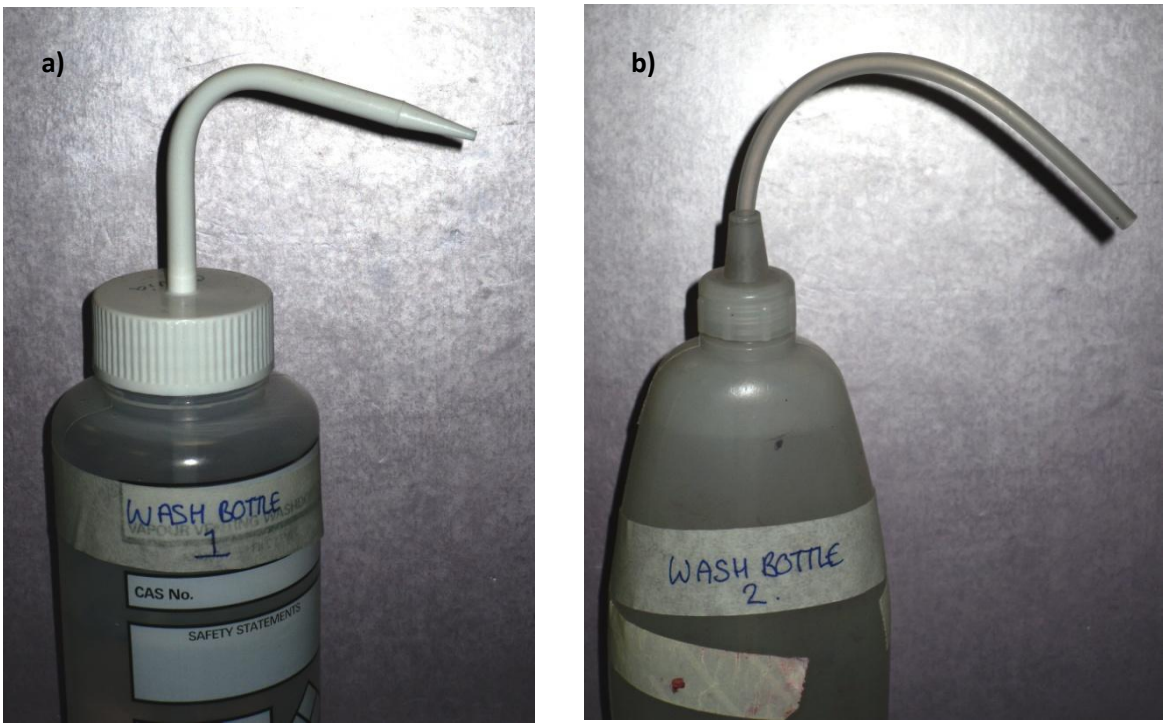


Figure 2.4. Different Wash Bottles used in the retention assays. a) Thin spout wash bottle, producing a more forceful water flow b) Wide spout wash bottle, spout is wider resulting in a less forceful stream of water.

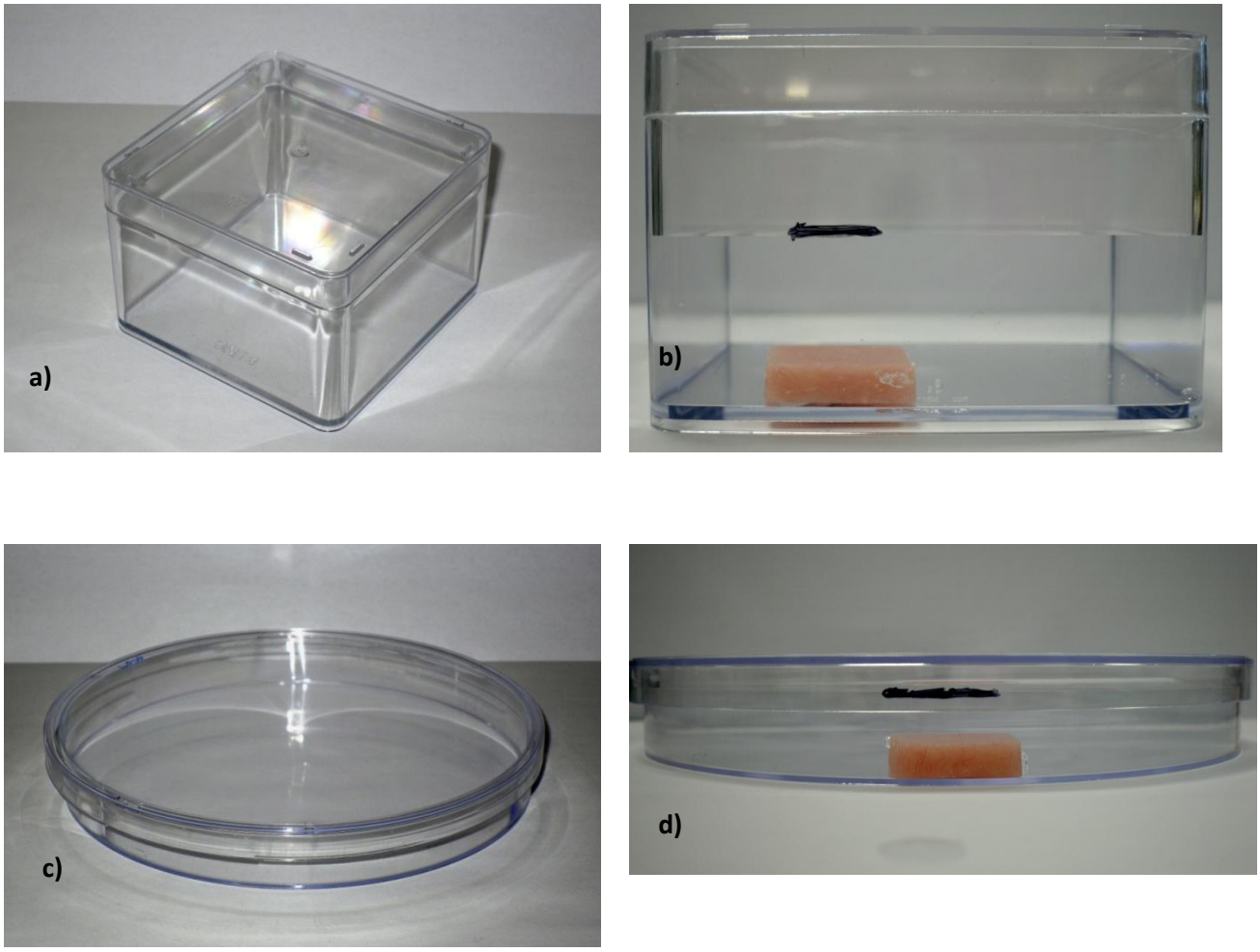


Figure 2.5. Different containers used during incubation of PMMA surfaces with cell suspensions in retention assays. a) Deep square container 7cm x 7cm x 5cm. b) Depth of suspension above PMMA surface in deep Square container (16mm) c) Image of large round Petri dish, 15cm x 1.5cm b) Depth of suspension above the surface of PMMA in large round Petri (6mm).

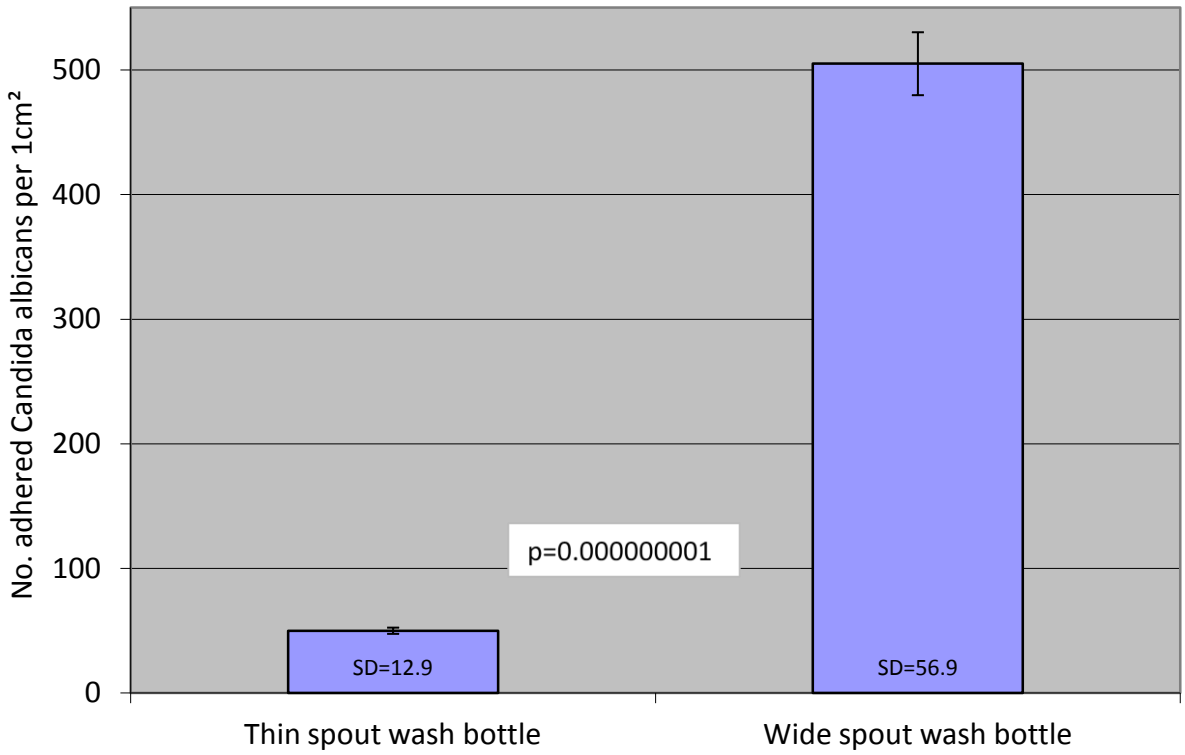


Figure 2.6. ***C. albicans*** **retention on abraded denture acrylic surfaces following rinsing with two different wash bottles**; (thin spout, and wide spout). Results based on three replicate surfaces analysed in three independent experiments (n=9), statistic significance determined using two tailed independent T-test.

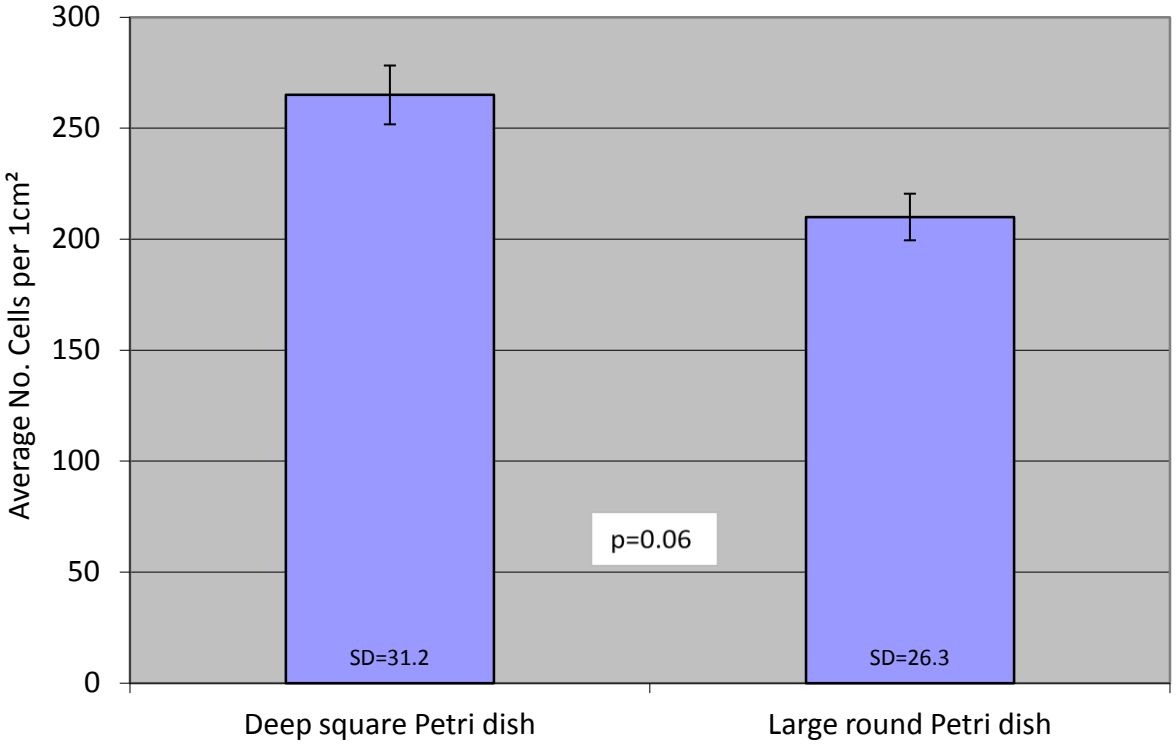


Figure 2.7. The difference in numbers of retained *C. albicans* cells on abraded denture acrylic surfaces following incubation in different shaped and sized containers. The results are based on three separate experiments each consisting of 3 replicate test surfaces (n=9). The same volume of cell suspension (150 ml) was used in each container on each occasion, statistical significance determined using two tailed independent T-test .

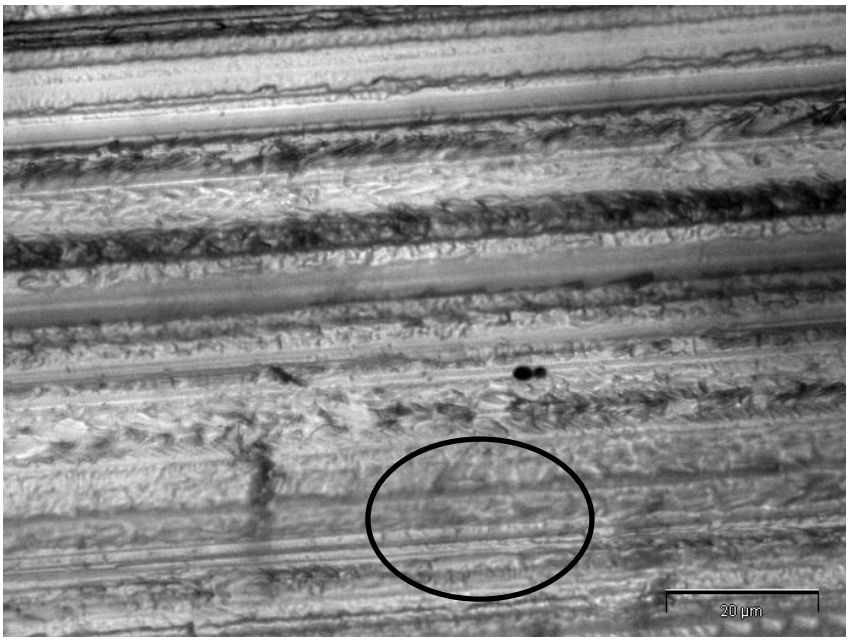


Figure 2.8. ***C. albicans*** **retained on linear scratches on abraded PMMA surface.** Surface was abraded with P600 emery paper (25.8µm grit size). Cells are aligned with surface scratch but appear to not be able to fully occupy it. Cell width exceeds width of feature and may therefore be less strongly attached. Scale bar represents 20µm.

2.2.9 Effect of abrasion on retention; defined assay

A reproducible defined method for adhesion/retention was established. 150 ml of the standardised cell suspension (2.10 ± 0.20 x 10^6 cfu per ml) was added to a large Petri dish (15 cm x 1.5 cm, Sigma-Aldrich) containing three replicates of each of either the 1cm² test materials (2.2.4) or the 2 cm^2 test substrata (2.2.5), which were then incubated for 1 hour at room temperature without agitation. After 1 hour the test materials (with adherent cells), were removed and washed by gently rinsing with running sterile water from a gentle even flowing wash bottle (wide spouted wash bottle) for three seconds with the surface held horizontal to the ground and the water directed to the centre of the PMMA pieces so that the excess liquid flowed across the surface and off the edge.

The replicates were air dried (for approximately 30 minutes) and stained by flooding with 0.03% acridine orange for 2 minutes and rinsing with distilled water, after which they were air dried again (30 minutes) and examined under epi-fluorescent microscopy. Cell counts and percentage coverage data were obtained for 10 fields per PMMA coupon.

2.2.10 Inducing hyphal growth in adhered cells

An initial investigation involving the use of the diagnostic germ tube test for *C. albicans* was performed using different concentrations of horse serum in order to determine the minimum concentration and incubation time required to induce hyphal growth in adhered cells. *C. albicans* GDH 2346 was incubated in 0%, 12.5%, 25%, 50% and 100% horse serum (diluted with sterile distilled water), in a 37°c water bath. Cells from each treatment were sampled every hour, over 6 hours, and

examined using light microscopy of wet mounts at x400 magnification. Hyphae were seen in all concentrations of serum after every hour in varying abundances and lengths. Incubation in 25% horse serum for 3 hours (Figure 2.9) induced hyphal protrusions that were long enough to see clearly, and that were not as clumped together as was noted with higher concentrations. It was decided that these conditions were appropriate for inducing hyphal growth in adhered cells. Thus, to induce hyphal growth in adhered *C. albicans* cells, cell suspensions were prepared and adhesion assays carried out as described in 2.2.9, using the same abraded surfaces. Immediately following the adhesion assay 3 replicates of each test piece were removed and analysed for adhered cells, and 3 replicates, with adherent *C. albicans* cells were placed in test tubes containing 2 ml of 25% horse serum and incubated for 3 hours to enable attached cells to produce true hyphae. After incubation, test materials were removed from the horse serum and gently washed with running distilled water (as detailed in 2.2.8), to remove excess serum and loosely adherent cells. Test pieces were stained with 0.03% acridine orange, and examined using light microscopy, to assess the effect of the linear scratches on hyphal growth of attached *C. albicans*. The assay was repeated twice with three replicates of each test piece from which, ten fields of view per test piece were examined and the number of cells and cells with hyphae were recorded.

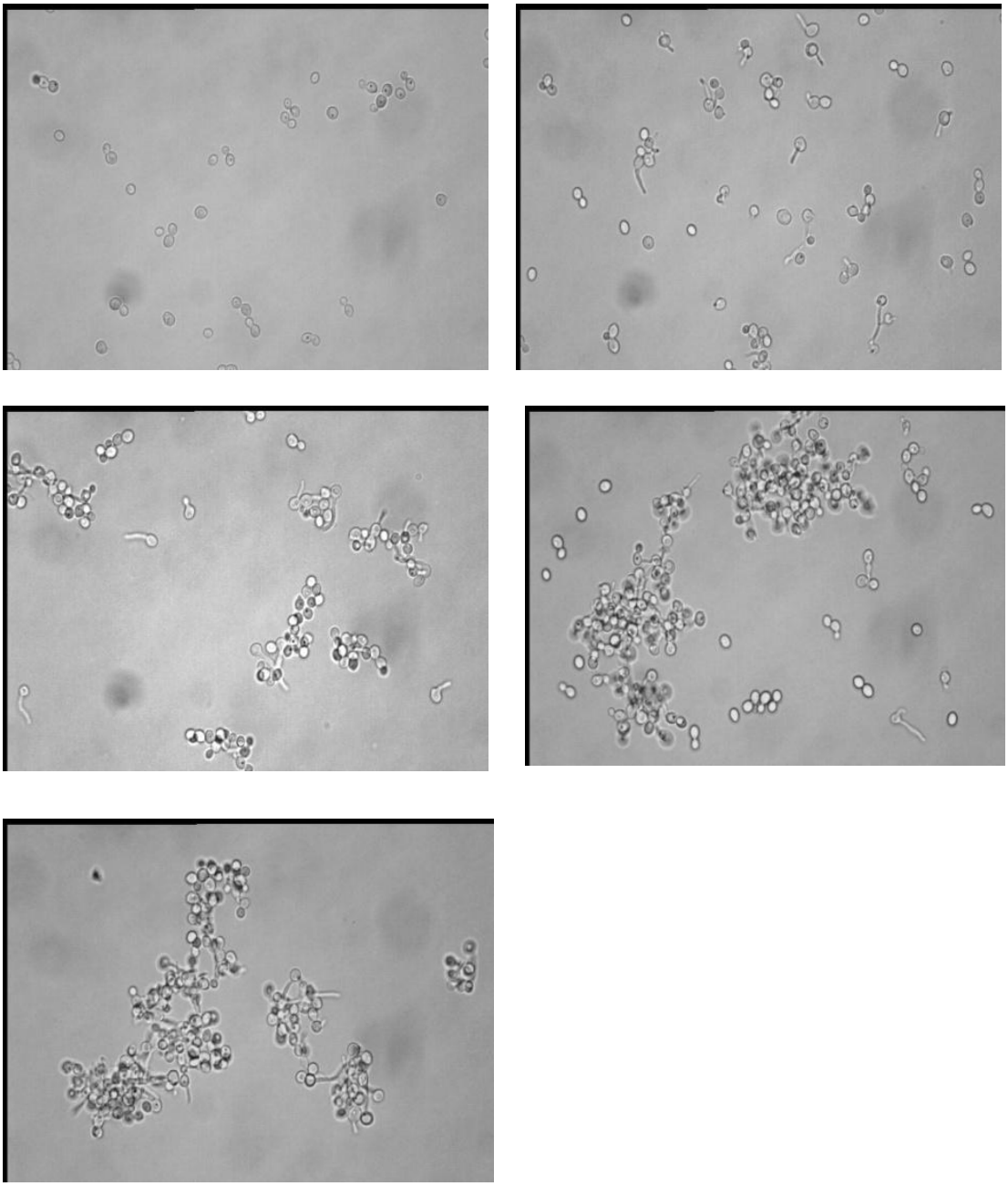


Figure 2.9. The growth of *C. albicans* hyphae in different concentrations of horse serum. Images are of **h**yphae of *C. albicans* after three hours incubation in a) 0% horse serum / control, b) 12.5% horse serum, c) 25% horse serum, d) 50% horse serum and e) 100% horse serum.

2.2.11 Statistical analysis

Analysis of cell counts

Due to the high degree of variance within the collected cell counts, the data did not fit normal distribution. As such, the usual model for cell counts (the Poisson log-linear model) had to be discounted. However, because repeated count measurements were made on replicates and the counts showed a considerable degree of over-dispersion compared to that of a Poisson distribution, the negative Binomial distribution was tested and found to adequately fit the data in this respect. Additionally, the repeated nature of the counts made it necessary to estimate parameters using a Generalized Estimating Equation approach in order to take into account, possible dependences between observations.

For these reasons the count data was analysed by firstly taking logs of the counts to stabilize the variance and then using a repeated measures model to account for the possible dependence between successive counts. This was found to successfully stabilise the count variance. Several covariance structures were fitted to the data in order to account for the repeated measures and a simple autoregressive model was found to provide an adequate fit.

Surface roughness and feature dimensions.

Average surface roughness data as determined by different profilometers were compared using a two way ANOVA. Measurements of widths and depths from profiles generated by the two different profilometers were compared using two sample t-tests.

2.3 Results

2.3.1 Measuring roughness parameters and feature dimensions of abraded denture surfaces

All test substrata (abraded with grit papers and abraded with dentifrices) were examined under white light profilometry, which generated maps of each surface (Figures 2.12 and 2.13). These were measured by the computer software (Mapview AE 2.17) providing roughness parameters including the Sa measurement for the average deviation of the surface within a given area (Table 2.1).

In comparison to the 2Dimensional roughness parameter Ra, The Sa measurement (mean arithmetic height) is derived from a scan of a given area of the surface, providing 3 dimensional information on the deviations and average surface roughness on an X,Y and Z plane. The Sa values generated in this work allowed for comparisons to be made between the different abrasion treatments used in the production of the surfaces. As may be expected, the un-abraded surfaces for both sets of test substrata had the lowest Sa values and these increased as the level of surface abrasion increased (Figures 2.12 and 2.13).

The dimensions of features on the abraded denture surfaces were determined by evaluation of the profiles generated from the surface maps (see 2.2.9). The profiles of five areas of three replicate test pieces for each test group were examined. The Average Sa values (Table 2.1) and range of feature dimensions (Tables 2.2 and 2.3) were much larger for surfaces abraded with emery papers (1.0-82.0µm in width) in comparison to those abraded with dentifrices (1.0-35.0µm in width, Tables 2.4 and 2.5). Larger features were generated when abrasive papers with larger grits were

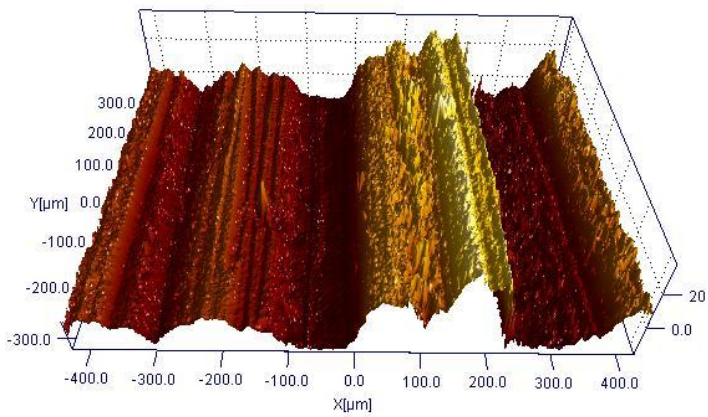
used. The most frequently occurring feature dimensions will presumably have a greater effect on adhesion and retention that is most representative of these surfaces. However this is based on the assumption that the surfaces present a standardised topography, which would not be applicable *in-vivo*, where large single features and presumably more varied topography, will have a dramatic effect.

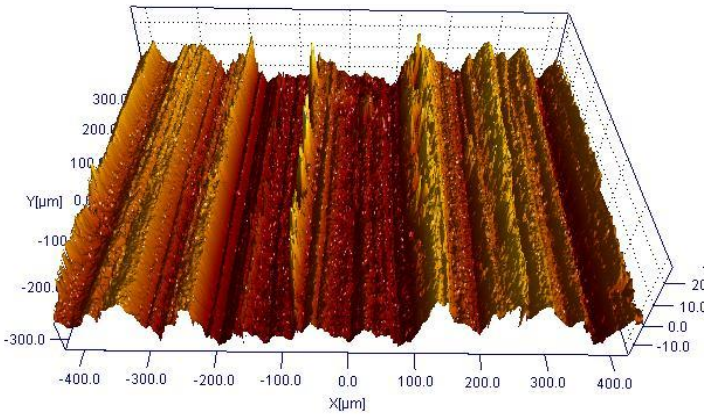
Features on surfaces abraded with abrasive emery paper were larger and of a greater range compared to those on dentifrice abraded surfaces. Feature widths ranged from 1.0-82.8µm, and depths from 0.02-6.8µm. the most frequently occurring micro-architecture ranged from2.5 – 9.0µm in width and 0.08-1.8µm in depth. The un-abraded surfaces had the lowest reported numbers for feature sizes but the most frequently occurring feature (2.5µm in width) approximated to *C. albicans* cell size (Tables 2.2 and 2.3).

The feature measurements of profiles generated for the surfaces abraded by brushing with different dentifrices revealed similar minimum (1.9µm) and maximum widths (32.3-39.9µm) apart from control surfaces where the maximum width of features was around half that of the abraded surfaces (15.2µm). The numbers/frequency of features on these surfaces increased with the increase in abrasiveness of the dentifrice used, and very few scratches were found on the un-abraded surfaces in comparison. The depth of features on these surfaces ranged from 3.5-1675nm (0.0035-1.675µm) with fewer of the deeper abrasions seen on surfaces abraded with the low abrasion method (Tables 2.4 and 2.5). The most frequently occurring features on these surfaces ranged from 1.9-3.5µm in width and 0.010-0.290µm in depth.

b) Sa=4.1µm

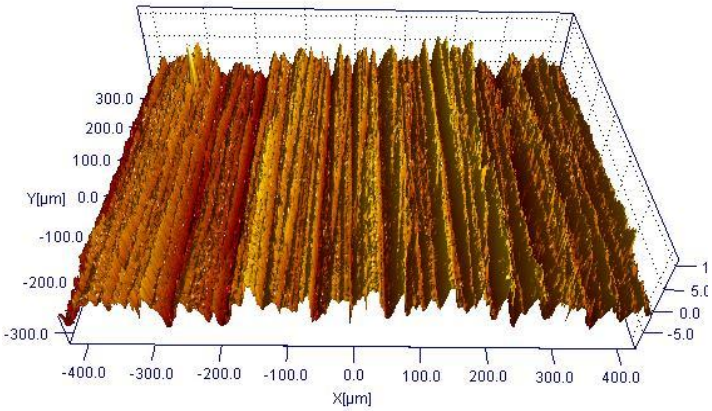
a) Sa=2.8µm

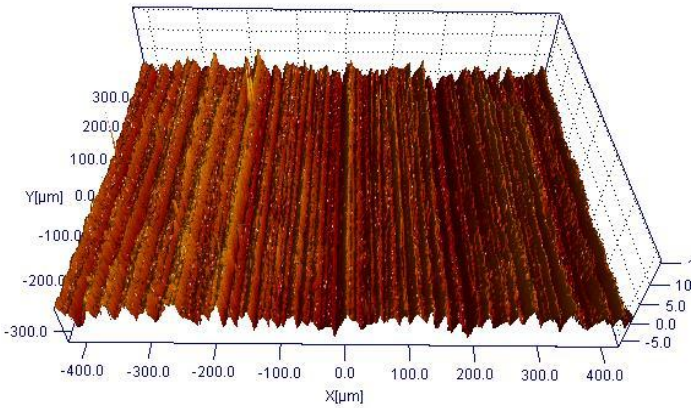




e) Sa=0.2µm

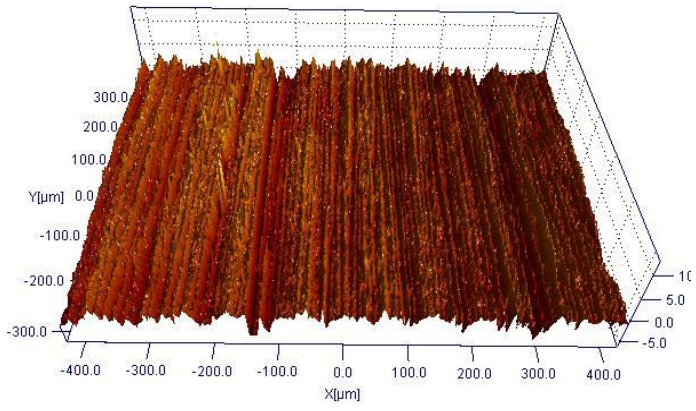
f) Sa=0.8µm





d) Sa=1.7µm

c) Sa=2.7µm



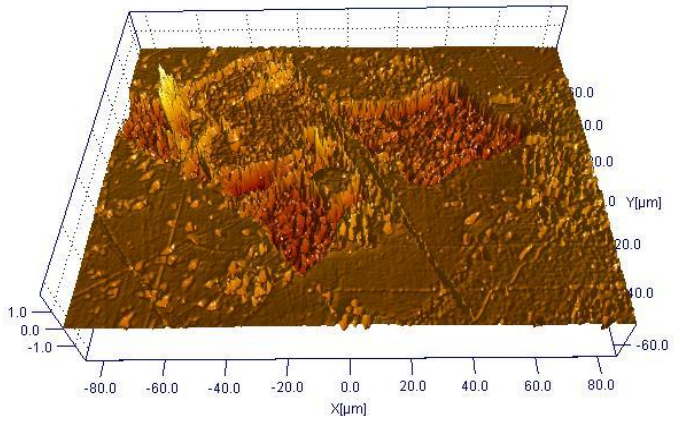
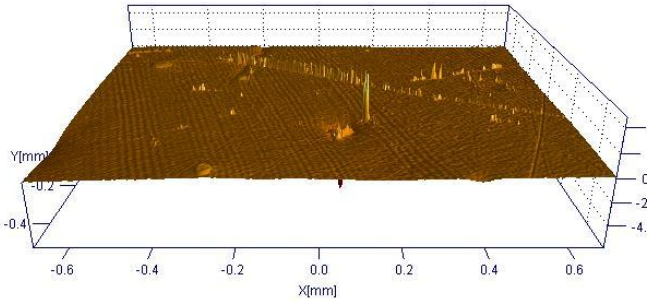
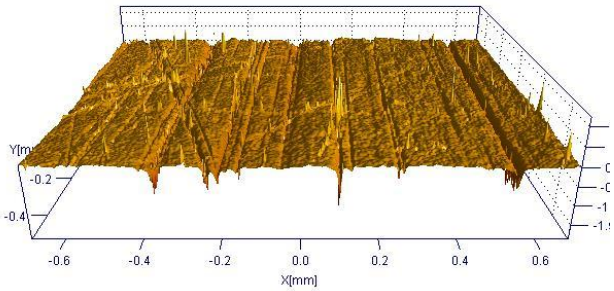


Figure 2.10. **WLP maps of test substrata abraded with different grit sized emery papers** including; a) P100 Al203 paper (grit 162µm), b) P100 glass paper (grit 142µm), c) P240 (grit 58.5µm), d) P400 (grit 35.0µm), e) P600 (grit 25.8µm) and f) Un-abraded.

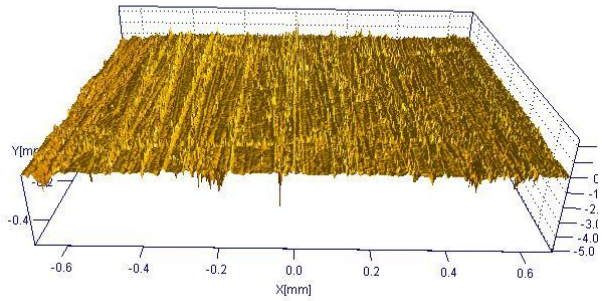
a) Sa = 0.03µm



b) Sa = 0.08µm



c) Sa = 0.28µm



d) Sa = 0.51µm

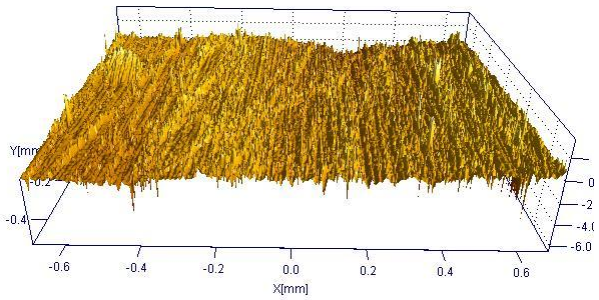


Figure 2.11. WLP maps of denture acrylic surfaces abraded with different dentifrices with varying levels of abrasive polish including a) Control surfaces (washed with water), b) Low abraded surfaces washed with dilute Colgate cavity protection (25:40 paste/water ratio), c)medium abraded washed with dilute Colgate total whitening (25:40 paste/water ratio) and d) high abraded washed with neat Colgate luminous.

	Average surface roughness / Sa (µm)	Standard deviation
1cm² test substrata		
Un-abraded	0.2	0.1
P600 Grit Paper	0.8	0.3
P400 Grit Paper	1.7	0.2
P240 Grit Paper	2.7	0.7
P100 Glass Paper	2.8	1.1
P100 Al_2O_3Paper	4.1	1.2
2cm² test substrata		
Control	0.03	0.01
Low abraded	0.08	0.02
Medium abraded	0.28	0.08
High abraded	0.5	0.19

Table 2.1. Mean values of surface roughness (Sa) measurements generated by white light profilometry of 1cm² and 2cm² PMMA surfaces that had been abraded with different grit sized emery papers or dentifrices. Five replicate surfaces were measured in total, and Sa was determined in ten fields per test piece, n=50.

1cm² test substrata Widths (µm)	Type of abrasion and size of abrasive grit used					
	Un-abraded	P 600 - 25.8µm	P 400 – 35.0µm	P 240 – 58.5µm	P 100 glass – 142.1µm	P 100 AL203-162µm
Smallest feature	1.0	2.6	3.4	1.5	3.3	3.3
Largest feature	9.8	28.0	30.8	27.5	82.8	54.0
Mean	3.5	10.1	11.0	9.4	15.2	13.7
Most frequently occurring feature	2.5	8.5	8.0	6.0	9.0	10.8

Table 2.2 Summary of feature widths of surfaces abraded with different grit sized emery papers used in retention studies. Table show the largest, smallest, average and most frequently found surface width dimensions from these surfaces (n>100).

1cm² test substrata Depths (µm)	Type of abrasion and size of abrasive grit used					
	Un-abraded	P 600 - 25.8µm	P 400 – 35.0µm	P 240 – 58.5µm	P 100 glass – 142.1µm	P 100 AL203-162µm
Smallest feature	0.02	0.2	0.3	0.4	0.2	0.4
Largest feature	0.64	5.7	6.0	6.7	6.8	8.3
Mean	0.18	1.4	1.8	2.4	1.3	1.9
Most frequently occurring feature	0.08	0.6	1.2	1.8	0.9	1.1

Table 2.3. Summary of feature depths of surfaces abraded with different grit sized emery papers used in retention studies. Table shows the largest, smallest, average and most frequently occurring surface depth dimensions from these surfaces (n>100).

2cm² test substrata Widths (µm)	Level of surface abrasion			
	Control	Low abraded	Medium abraded	High abraded
Smallest feature	1.900	1.900	1.900	1.900
Largest feature	15.200	32.300	39.900	34.400
Mean	5.800	6.600	9.194	8.260
Most frequently occurring feature	3.800	1.900	1.900	3.800

Table 2.4. Summary of feature widths of surfaces abraded with different abrasive dentifrices. Table shows the largest, smallest, average and most frequently occurring surface depth dimensions from these surfaces (n>100).

2cm² test substrata Depths (µm)	Level of surface abrasion			
	Control	Low abraded	Medium abraded	High abraded
Smallest feature	0.0035	0.033	0.033	0.060
Largest feature	0.075	1.200	1.320	1.675
Mean	0.016	0.300	0.293	0.333
Most frequently occurring feature	0.010	0.290	0.170	0.114

Table 2.5. Summary of feature depths of surfaces abraded with different abrasive dentifrices. Table shows the largest, smallest, average and most frequently occuring surface depth dimensions from these surfaces (n>100).

2.3.2 Characterisation of topography with different profilometers

As well as measuring surfaces for adhesion studies, four of the abraded surfaces (abraded with P100, P250, P400 and P600 grit emery papers, see 2.2.4) were examined with two different profilometers in order to compare their ability to determine roughness data and feature dimensions. The roughness parameters determined by the two separate profilometers for the differently abraded surfaces did not differ significantly ($P = 0.32$), with similar trends particularly in Ra/Sa values, being generated by both instruments (Figure 2.14). For both profilometers, the average surface roughness was found to increase with the increase in size of abrasive grit paper.

The surface feature widths measured (as described in 2.2.6) from the profiles generated by each profilometer differed significantly ($p<0.01$, table 2.6). Profiles generated by the solid stylus profilometer provided significantly higher ($p<0.01$) average values for width measurements. There was no significant difference found in the values generated for the depths of the micro-architecture by the two profilometers (Table 2.7).

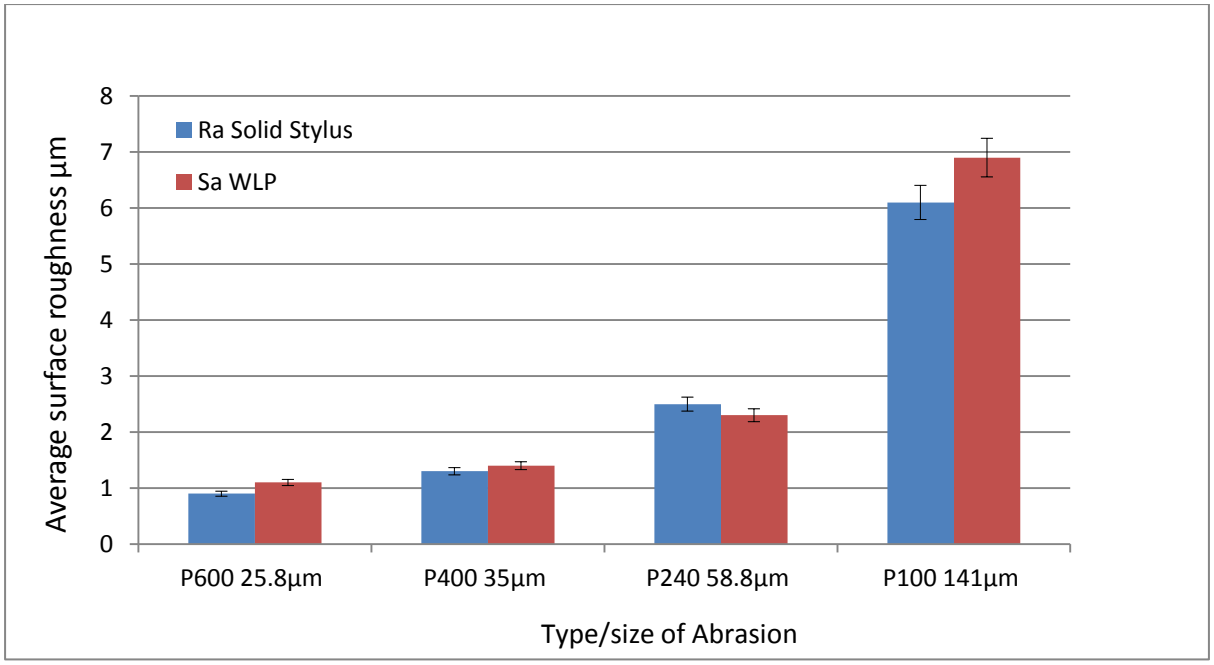


Figure 2.12. Comparison of solid stylus profilometry and white light profilometry for measuring surface roughness of PMMA abraded with different grit sized emery papaers. The Ra and Sa measurements generated by a solid stylus and a white light profilometers were taken from 5 scans of each of three replicate test surfaces. These test substrate were analysed in the same manner on three separate occasions (n=45).

	P100 (141µm grit)	P240 (58.5µm grit)	P400 (35µm grit)	P600 (25.8µm grit)
Solid Stylus profilometer	68	41.2	33.3	20.3
White light profilometer	45	19.1	17.2	12.7
P Value	0.004	0.001	0.001	0.001
Standard deviation	8.6	6.1	5.9	5.3

Table 2.6. **Mean widths of features on surface abraded with different grit sized emery papers.** Mean depth of features (µm) was calculated from measurements of fifteen fields of three replicates of surfaces abraded with different grit size emery paper calculated with two different profilometers, performed on two separate occasions (n=90).

	P100 (141.2µm grit)	P240 (58.5µm grit)	P400 (35µm grit)	P600 (25.8µm grit)
Solid stylus profilometer	6	2.8	2.6	1.3
White light profilometer	5.1	2.9	1.8	1.4
P Value	0.244	0.559	0.089	0.071
Standard deviation	4.4	1.3	1.6	0.5

Table 2.7. **Mean depth of features on surface abraded with different grit sized emery papers.** Mean depth of features (µm) was calculated from measurements of fifteen fields of three replicates of surfaces abraded with different grit size emery paper calculated with two different profilometers, performed on two separate occasions (n=90).

2.3.3 Retention of *C. albicans* to 1cm^2 denture acrylic surfaces abraded with different grit sized emery papers

The 1cm^2 PMMA coupons fabricated and abraded in-house initially revealed unexpected results in terms of adhesion/retention on control surfaces. The un-abraded surfaces which had been termed 'smooth controls' retained significantly higher numbers ($P<0.05$) of *C. albicans* than those that had been manually roughened (Figure 2.15). These surfaces had the lowest Sa values of the 1cm^2 test substrata but surface profiles + images generated by white light profilometry revealed that these 'smooth' surfaces consisted of irregular roughened patches (Figure 2.16). Features in these irregular areas were found to range from 1 to 9.8 microns in width with the most commonly occurring feature around 2.5µm. *C. albicans* cells can range from 2-8µm in diameter and these features may therefore have provided ideal niches for single cells to full occupy with a large amount of cell-surface interface enabling a greater bond. Thus this was not an appropriate control and was replaced in subsequent assays by the use of 2cm^2 abraded surfaces.

Otherwise, the numbers of *C. albicans* adhering to the abraded surfaces increased significantly ($P<0.01$), as the level of abrasion increased up to the second most abrasive paper used (P100 glass paper, 141 µm grit), (Figure 2.9, Table 2.1). The numbers of adhered cells decreased slightly on the surfaces abraded with the largest grit paper used (P100 AL203, 162µm grit paper) although this difference was not significant. The Sa of the lowest grit abraded surfaces was 4.4µm in comparison to an Sa of 2.8 µm of the surfaces abraded with the 141µm grit paper.

The lowest numbers of adhered cells were retained on PMMA abraded with the smallest grit size emery papers, P600 (25.8µm) and P400 (35.0µm).

Across all of the test substrata, there was variation in surface roughness and numbers of retained cells in different fields of the same test pieces (Figure 2.17). Areas consisting of more roughened micro topography retained more cells.

Standard deviation increased with the increase in surface roughness, with the highest standard deviations found on the Al_2O_3 (141.2µm grit emery paper) abraded surfaces. These surfaces consisted of scratches with smooth gullies with little or no retained cells and rough irregular topography with increased retention (Figure 2.17), which may account for this variation.

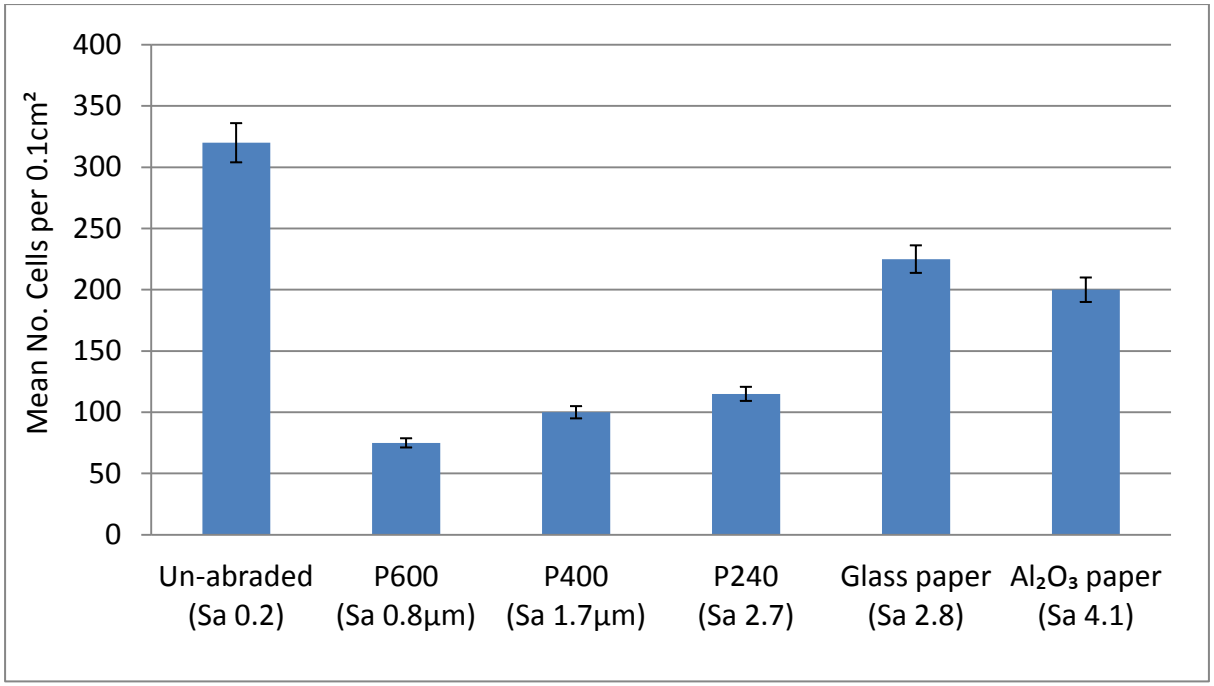


Figure 2.13. Retention of *C. albicans* on 1cm² PMMA coupons abraded with different grit sized emery papers to produce variable surface roughness (Sa). Graphical data based on means of 10 measurements of separate areas on three replicate test pieces, performed in two repeat experiments (n=60).

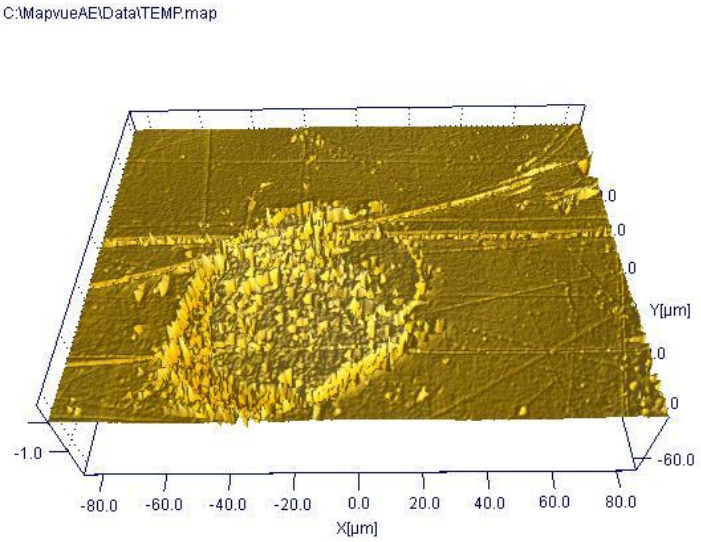


Figure 2.14. White light profilometry map of un-abraded/ 'smooth' 1cm² PMMA coupon. Image shows an example of an irregular patch of roughness noted on un-abraded surfaces following white light profilometry.

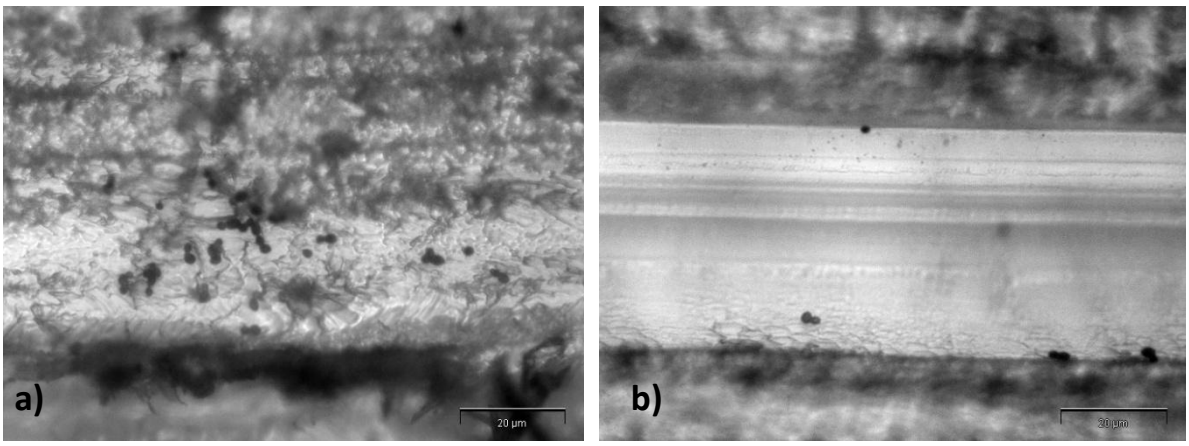


Figure 2.15. Visible difference between retention of cells (stained with 0.3% acridine orange) on smoother and rougher areas of the same abraded PMMA coupon. (a) Larger numbers of *C. albicans* cells retained in areas with rougher micro-topography on PMMA abraded with P100 emery paper. (b) Lower numbers of cells found adhering to the smoother groove on the same PMMA test piece. Scale bars represent 20µm.

2.3.4 Retention of *C. albicans* to 2cm^2 PMMA subjected to dentifrice abrasion

The 2cm^2 denture acrylic specimens that had been abraded during cleaning with varying grit sized dentifrices (2.2.5) were also compared for retention. The numbers of retained *C. albicans* increased as the level of abrasion and surface roughness parameter (Sa) increased (figure 2.18). Cells were preferentially retained in micro-architecture on abraded surfaces (figure 2.19). These surfaces had much lower average surface roughness parameters (Sa in particular) than surfaces that were abraded with emery papers, for which much higher numbers of retention were reported (Figures 2.15 and 2.18).

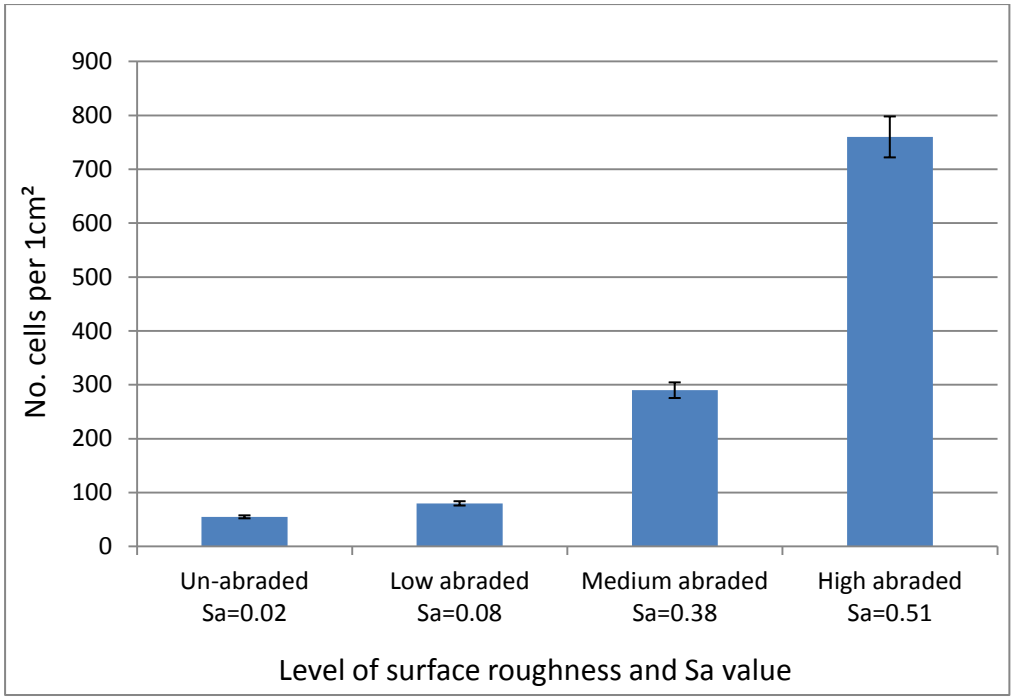


Figure 2.16. The mean retention of *C. albicans* on denture acrylic surfaces abraded during brushing with different dentifrices. Un-abraded surfaces (control) were washed with water, Low abraded washed with diluted Colgate cavity protection (25:40 paste/water ratio), medium abraded washed with diluted Colgate total whitening (25:40 paste/water ratio) and high abraded washed with neat Colgate luminous to produce surfaces with increasing surface roughness. Retention was measured in fifteen fields per test piece, three replicates of each test pieces were used in three separate experiments (n=135).

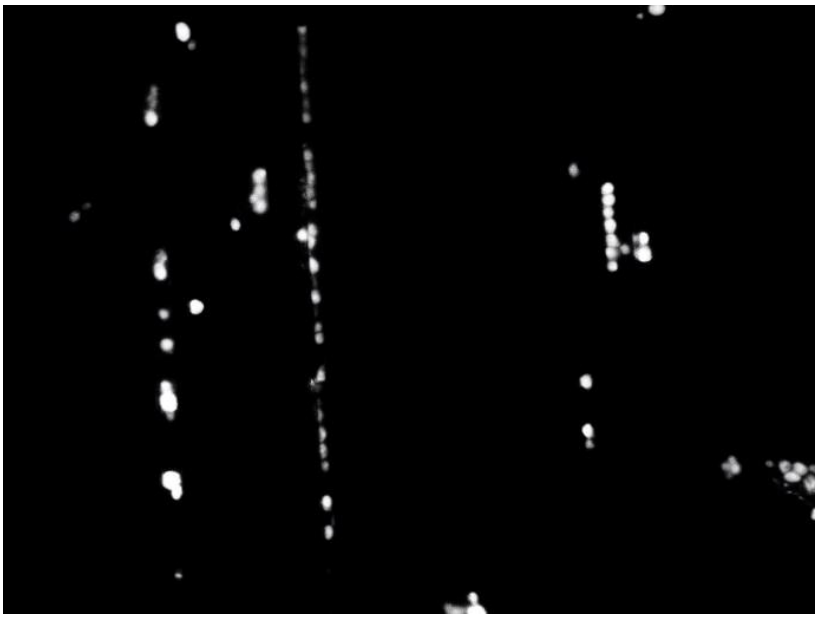


Figure 2.17. Cells of *C. albicans* retained on denture acrylic surfaces washed by brushing with a commercial dentifrice (High abraded – Colgate luminous neat). Cells (stained with 0.3% acridine orange) are preferentially retained in linear surface abrasions caused by abrasive wash technique.

2.3.4 Measuring cell retention

The retention of *C. albicans* to surfaces abraded with dentifrices was originally measured by two means; 1) by cell counts and 2) by percentage coverage. In the latter it was noted that the use of percentage coverage for determining amount of adhesion/retention may not be as accurate as actual counts. Two examples of this are demonstrated in Table 2.8. In one instance two different numbers of cell counts are represented by the same percentage coverage reading. In the other, a lower number of cells have higher representative percentage coverage than that of a higher number of cells. The percentage coverage measurement is related to the amount of fluorescence given off from stained cells. This varies depending upon the amount of stain taken up by individual cells and their size.

	Data set 1			Data set 2	
	No. cells	**% Coverage**		**No. Cells**	**% Coverage**
	70	1.46		264	9.97
	117	3.65		103	3.70
a)	185	7.20		233	6.54
	161	4.91		172	4.58
	325	9.91		174	3.95
	78	2.02		87	2.05
	89	3.14		233	7.57
	67	2.83		65	1.95
b)	92	3.75		188	6.76
	105	3.22		88	1.90
	111	4.40		221	7.32
	74	2.19		195	5.12

Table 2.8. **Discrepancies in percentage coverage results compared with cell counts**. Table shows two data sets of cell counts and percentage coverage readings taken from 12 fields of two identically abraded test pieces. Highlighted in example a) is a case where different cell counts are represented by very similar percentage coverage values. Similarly, b) indicates an example of where a lower number of cells (67) are represented by a higher percentage coverage value than a greater number of cells (88).

2.3. Hyphal growth of adhered *C. albicans* on abraded surfaces

The number of adhered *C. albicans* producing hyphae on abraded PMMA surfaces was counted in 10 fields on three replicate test pieces. On average, 81% of all adhered *C. albicans* produced germ tubes when incubated in 50% horse serum. More than one germ tube was observed branching out from individual cells (Figure 2.20 d). In some cases hyphal protrusions were seen to be following surface contours (figures 2.20 a) and b). In some instances hyphae also appeared to be growing towards one another (Figure 2.20 c). Overall, there did not seem to be an effect of topography on the direction of hyphal growth.

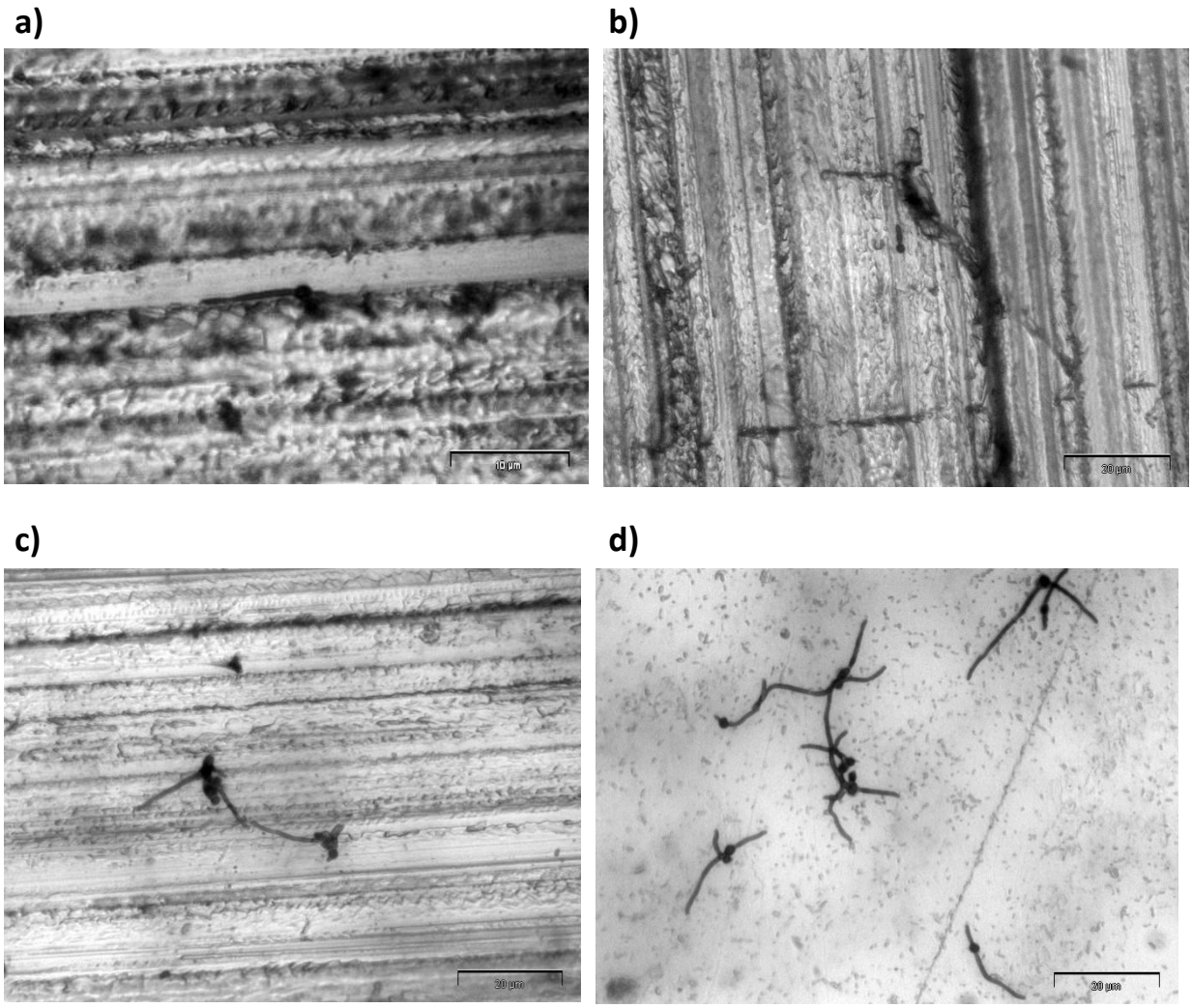


Figure 2.18. **Hyphal outgrowth and behaviour on surfaces abraded and unabraded PMMA surfaces.** (a) close up of hypha following a groove in the PMMA, scale bar represents 10µm. (b) hypha following linear groove in PMMA, (c) hyphae growing towards one another, (d) hyphal branching on un-abraded PMMA. Scale bar represents 20µm.

2.4 Discussion

2.4.1 Adhesion and retention of *C. albicans* on abraded denture surfaces

C. albicans cells were retained on denture acrylic surfaces (abraded with emery paper and dentifrices) in higher numbers as the surface roughness increased, as was anticipated (Verran and Maryan, 1997; Morgan and Wilson, 2001). On the more roughened emery paper abraded surfaces (Ra ranging from 0.2-4.1µm in comparison to 0.03-0.5µm for dentifrice abraded), the numbers of retained cells were noted to decrease slightly when the Sa increased from 2.8 (P100 glass paper abraded) to 4.1 (P100 Al_2O_3 abraded). Although this was not significant, this finding could support reports in literature, indicating that there is a threshold for *C. albicans* cell retention above which cells will be less well retained due to increased exposure to shear forces i.e. larger feature sizes implied by higher Ra/Sa values resulting in decreased retention.

In retention assays with the dentifrice abraded substrata, adhesion increased with the increase in level of surface roughness with the highest numbers found on the highest abraded surfaces. These surfaces had much lower Sa values than those abraded with emery papers, with the Sa of the high dentifrice abraded surface (0.5µm) being far below that of the P600 emery paper abraded surface (0.8µm, lowest emery abrasion used). Features on these high dentifrice abraded surfaces occurred much more frequently than those on medium and low abraded surfaces, the numbers of which decreased accordingly. A higher number of features would increase the surface area on denture surfaces, providing more area/attachment sites for microbial adhesion (Whitehead *et al.,* 2005), explaining why high abraded

surfaces, with the largest number of surface abrasions/scratches resulted in the higher numbers of adhesion. In addition to the amount of attachment, the strength of attachment to the surfaces assessed in this work has also been investigated in unpublished work (submitted for review, see Appendix 1) from our laboratory. This work used atomic force microscopy (AFM) to examine at the strength of retention of *C. albicans* and *Streptococcus oralis* on control, low medium and high abraded surfaces. The process involved the application of increasing force by the AFM cantilever, to detach cells from the surfaces (Whitehead *et al.,* 2006; Verran *et al.* Submitted for review, 2012). It was found that *C. albicans* cells formed stronger attachments to scratches on high-abraded surfaces whereas the smaller *S. oralis* cells had the strongest attachment to features on low abraded surfaces. Additionally cells were more easily removed when pressure was applied across rather than along surface scratches.

The smaller probe of the AFM was able to determine details of the surface feature sizes and indicated that scratches on low abraded surfaces were between 1-3 µm in width with an average depth of 1300nm. Medium abraded surfaces consisted of more scratches that ranged from 0.7-7µm in width with an average depth of 2300nm, and the high abraded surfaces had deeper scratches with an average depth of 3300nm but similar feature widths ranging from 0.5-5µm. The low and high abraded surfaces comprised features proportionate to *S. oralis* and *C. albicans* cells respectively which enhances their strength of attachment and retention. Although differences have been noted in the features widths and depths calculated by the different profilometers (where smaller probe sizes appear to provide data on smaller features and therefore more detail), no significant difference was found between the

Sa values determined for these surfaces using either the white light profilometer, the solid stylus profilometer or the atomic force microscope. This is interesting as it would appear feature sizes do not affect the overall surface roughness and highlights how Sa/Ra values may not provide enough detail of the underlining surface topography. Thus more data are needed in order to adequately describe the profile, width, height and frequency of a large number of topographic features on test surfaces in order to fully understand their interaction with cells in terms of amount and strength of retention.

2.4.2 Measuring cell retention

Two methods were employed for the quantification of cell retention on acrylic test substrata; one was a direct count of stained cells, and the other was a measurement of the percentage of surface covered by cells. Previous work have reported the use of both percentage coverage data (Verran and Motteram, 1987; Verran *et al*., 1991; Verran and Shakespear, 1991; Ellepola and Samaranayake, 1998) and cell counts (Samaranayake and MacFarlane, 1980; Verran and Maryan, 1997; Henriques *et al.,* 2004), to establish the amount of cell retention on denture acrylic surfaces, but the two methods of quantification do not seem to have been compared before. The percentage coverage measurement reported in this work revealed discrepancies (as described in section 2.3.3) in comparison to the direct count data. Percentage coverage was calculated using image analysis software (Olympus soft imaging software - Cell^{f}); this tool works by measuring the fluorescing areas in a given field and is useful in estimating the coverage of cells when there are too many cells in an area to count manually, providing an alternative method of analysing surface coverage. However, the areas measured can be affected by a number of variables. For example; cells may differ in size, some may fluoresce more than others and cells that occur in clumps will fluoresce differently from those that are separated, giving off a brighter fluorescent signal. In addition to this the areas to be measured are set manually by the operator and this level of human involvement introduces opportunity for a large amount of subjectivity in obtaining percentage coverage results which can lead to discrepancies within data sets. Although still subject to human error, the direct counting of retained cells on surfaces may therefore be a more reliable method for use (when possible), in retention studies.

2.4.3 Measuring roughness profilometry

Roughness parameters calculated by profilometers are valuable descriptors of an overall pattern in roughness of abraded surfaces and allow for the comparison of the degree of roughness of different surfaces. Ra/Sa values have been used in previous research to investigate the effect of denture surface roughness, and have led to the well accepted knowledge that increasing roughness increases cell retention (Quiryen and Bollen, 1995; Verran and Maryan, 1997; Radford *et al.,* 1998; Pereira-cenci *et al*., 2006; Zamparini *et al.,* 2010; Wady *et al*., 2012). Other work however, has indicated that surface roughness alone may not be sufficient for the analysis of microbial retention. Taylor *et al.,* (1998) demonstrated that there was an optimal threshold for the adhesion of bacteria to silicon surfaces, above which cell retention was reduced. This was also witnessed in this work, where, in some cases there were slightly increased numbers of reported retained cells on medium abraded ($2cm^2$) substrata surfaces compared to high abraded surfaces, although in general, an increase in roughness resulted in increased numbers of retained cells. Whitehead *et al*., (2005) demonstrated how surfaces features of different size and shape affected the retention of cells, whereby cells of similar dimensions were better retained. In these studies and those of unreported work from our laboratory, it has been demonstrated that roughness parameters alone may not be sufficient for the characterisation of surfaces used in retention studies. Surface micro-architecture plays a key role in the surface area available for microbial attachment and the strength of adhesion, which will affect the retention of different microorganisms (of variable sizes) in different ways. It would therefore be recommended that future

work investigating the microbial retention to surfaces incorporates the use of profilometry techniques to fully characterise surface profiles for comparison with cell retention.

The Ra and Sa roughness parameters for the differently abraded denture surfaces, obtained from the two different profilometers did not differ significantly indicating that both profilometers are similar in the analysis of surface roughness. However, profiles generated by the white light profilometer were more detailed, consisting of features of both µm and nm proportions, enabling more accurate measurement. In addition to this there was a significant difference in the widths of the micro-architecture calculated from profiles generated using the two profilometers, with the average width of features determined by the solid stylus profilometer being significantly higher than those determined by the white light profilometer. The white light profilometer utilises a laser beam to measure features whereas the solid stylus uses a solid probe. The beam diameter of the white light profilometer is smaller than that of the probe diameter of the solid stylus allowing it to fit into and measure both larger and smaller features resulting in a greater range of surface feature measurements and reducing the overall average width calculated (Figure 2.24).

The depths of the surface micro-architecture were not found to differ significantly suggesting that all features (large for solid stylus and large and small for white light profilometer) were of similar depth. However data determined by use of the AFM (see section 2.4.1) indicated differences in average feature depths (low abraded - 1300nm; Medium abraded - 2300nm; high abraded surfaces - 3300nm), indicating that the white light and solid stylus profilometer used in this work may not have

been optimal for measuring feature depths on these surfaces. However these two profilometers were used as the 1cm^2 PMMA surfaces (abraded with emery papers) were too rough for analysis using AFM. Roughness parameters have been shown to differ between different measurement techniques in the past and this is thought to be due to several contributing factors. The size of the stylus or magnification of the objective lens used (in the non-contact profilometers) has been reported to affect subsequent roughness measurements. Smaller stylus tips are considered to be able to follow surface profiles more accurately, picking up the finer surface detail (*Poon and Bhushan, 1995*). Future work could incorporate the use of the AFM, with white light profilometry and solid stylus profiometry to investigate these and other PMMA surfaces in order to determine the most appropriate instrument for use with surfaces of different levels of roughness.

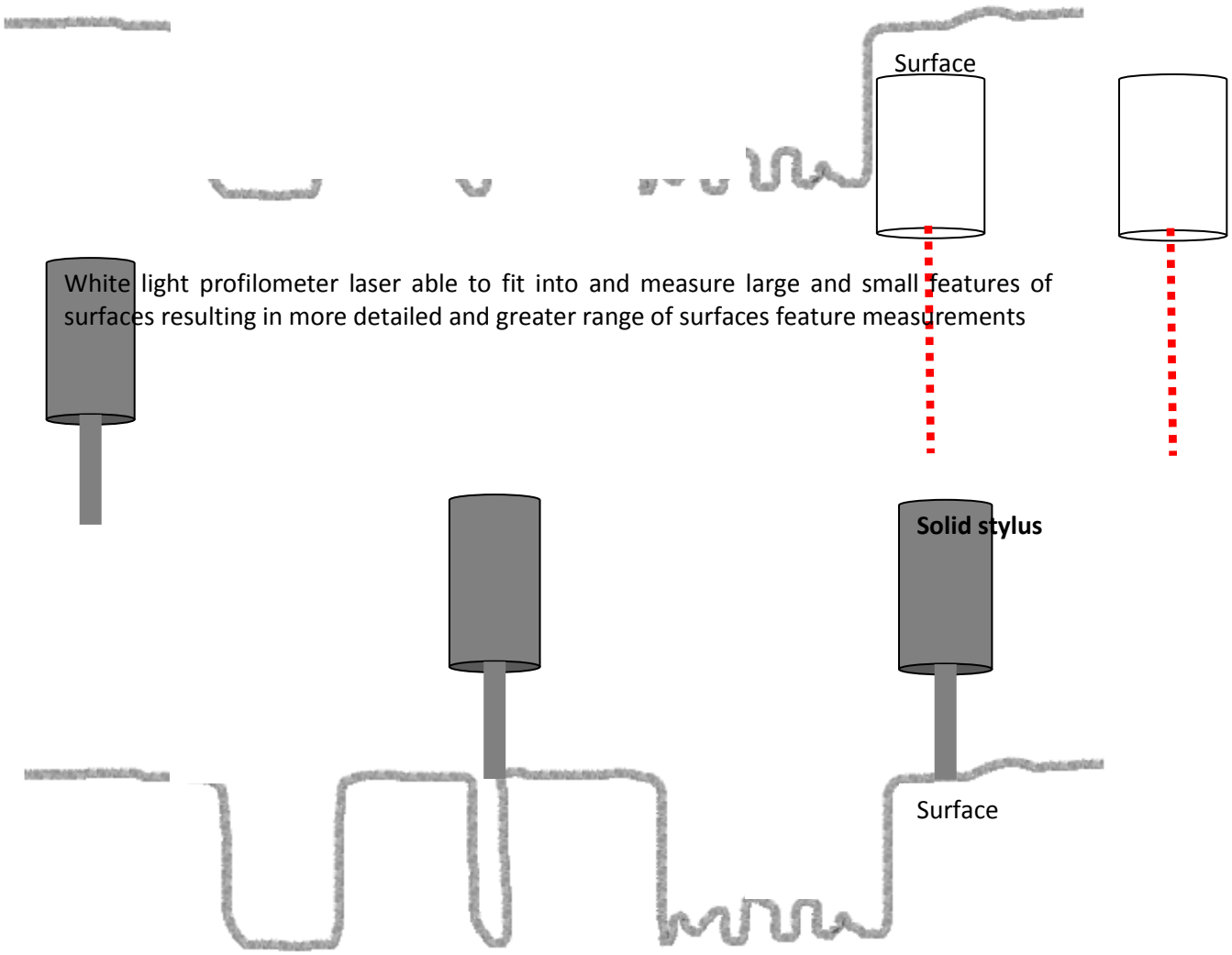


Figure 2.19. Illustration of the potential differences that different probes (of different diameters/sizes) may produce when analysing small features on abraded surfaces.

2.4.3 Hyphal growth of adhered *C. albicans* on abraded surfaces

The majority of attached *C. albicans* grew hyphae on all test substrata with different hyphal growth directions observed. Some hyphae were seen branching in all directions but no overall effect of surface topography on the directional growth of hyphae was found. Previous studies have also reported the multiple branching of hyphal protrusions from *C. albicans,* and have explained it as a means of maximising exploration of the surrounding environment (Gow, 1994a and 1994b).

Some hyphae in this study appeared to be growing towards one another, a phenomenon that does not seem to have been reported previously. This apparent attraction of hyphae to neighbouring cells may indicate a chemical influence on the direction of hyphal growth. The ability of hyphae to respond to chemical signals was debated in a review paper by Davies *et al.*, in 1999. The paper looked at the invasion of host tissues by *C. albicans* hyphae in an attempt to determine if hyphal responses were influenced by chemotropic or thigmotropic interactions, neither of which could be discounted. Hyphae have been implicated in the penetration of epithelial cells in other studies (Sherwood *et al.,* 1992; Watts *et al*., 1998), and in these reports, thigmotropic as opposed to chemotropic responses of hyphae were implicated. So far, studies have investigated the tropic growth of *C. albicans* hyphae on epithelial, agar and nucleopore/cellophane surfaces (Rotrosen *et al.,* 1985; Sherwood *et al*., 1992; Gow *et al*., 1994b; Watts *et al*., 1998). The work carried out here appears to be this first to report on *C. albicans* hyphal growth on denture acrylic surface with varying topographies.

Germ tubes (early hyphae) of *C. albicans* have been shown to be able to respond to chemical signals. Rotrosen *et al*., 1985 investigated the influence of chemical signals

released by endothelial cells, on the directional growth of germ tubes. They showed that chemical signals caused hyphae to grow downwards, directly into underlying tissue demonstrating chemotaxis. In addition to this, hyphal growth has been shown to be affected by chemical molecules; Ramage *et al*. (2002a), demonstrated how the introduction of farnesol to biofilms of *C. albicans* (attached to polystyrene tissue culture microtitre plates), inhibited biofilm growth and hyphal growth at high concentrations. This inhibition of hyphal growth by farnesol was also described by Enjalbert and Whiteway (2005). An opposing chemical to farnesol has also been identified. Like farnesol, tyrosol is released by *C. albicans* during growth and in the correct conditions, has been shown to induce hyphal growth (Chen *et al*., 2004; Alem *et al*., 2006). The chemotropic growth phenomenon in *C. albicans* might provide possible explanations for the observed hyphal behaviour in this study, where cell-cell signalling between cells within range of one another could cause positive chemotropic response. This was investigated further in studies using optical tweezers, placing yeast cells in various proximities to one another in order to see how hyphae grew once induced with horse serum. However, no pattern was observed. This may be due to insufficient concentrations of signalling molecules being produced in order to trigger an effect. These concentrations could be much higher in biofilms and would be expected to increase with incubation time. The impact of quorum sensing molecules on directional growth of hyphae could be investigated further using optical tweezers as is discussed in section 2.4.4.

2.5 Conclusions

Previous research has reported that increased substratum roughness, based largely on the Ra roughness parameters (average deviation from the centre line), is related to increased adhesion and retention of micro-organisms. For all of the abraded surfaces used in the adhesion assays with *C. albicans*, the same trend was observed. Cells were preferentially retained in surface scratches and features caused by abrasion with both the grit papers used and the different dentifrices. This has obvious hygienic implications as any retained cells will be able to proliferate and contribute to subsequent denture plaque formation once the denture is returned to the mouth. Minimising the amount of abrasion a denture is subjected to may help to lower the numbers of *C. albicans* cells and other micro-organisms being retained on its surface and as a result could help to reduce the susceptibility of denture wearers to oral candidoses, and improve oral health status.

The combination of findings across all of the work described suggests that the relationship between surface topography and microbial adhesion /retention is not merely proportional to surface roughness. There appears to be an optimal level of surface roughness for microbial retention that is proportional to cell dimensions and the variation in feature dimensions on surfaces can substantially affect the retention of cells. This work emphasizes the importance of fully characterising surfaces used in retention and adhesion studies, not only in terms of surface roughness parameters but also in terms of the size and shape of features present. This would enable more detailed investigation of topography and retention and provide the basis for better comparison of retention across different studies.

Results from this combination of studies also demonstrates the importance of standardised methods for adhesion and retention assay, specifying precise methods and taking into account the equipment used. Small differences in protocol can significantly affect results. Such differences should be noted when comparing findings to other published work.

From the studies performed here, there does not seem to be an effect of topography of the extension and orientation of *C. albicans* hyphae.

The next stage of this work will explore the growth of hyphae in adhered *C. albicans* further, investigating how the presence of hyphae influence subsequent biofilm development and their release of and response to quorum sensing molecules.

Chapter 3

The development and removal of *Candida albicans* biofilms on abraded denture surfaces

3.1 Introduction

3.1.1 Biofilms of *C. albicans*

Dentures provide hard non-shedding surfaces that allow for the build-up of plaque biofilms (including *Candida* spp.), over time. The development of biofilms containing *Candida* spp. on denture acrylic resin begins with cell adhesion, which can either occur directly to the conditioned surface or via a layer of pre-existing denture plaque (Pereira-cenci *et al*., 2008b). The surface topography of the denture has been shown to greatly influence adhesion and subsequent retention, with more roughened surfaces retaining more organisms (Pereira-cenci *et al*., 2008a, 2008b; Verran and Maryan 1997; Taylor *et al.,* 1998; Radford *et al.,* 1999). The topography of denture surfaces is difficult to regulate. Newly fabricated dentures present a topography reflecting the mucosa of the patient, and are potentially additionally abraded during fabrication and use. Cleaning regimens involving the use of hard brushes or abrasive cleansers may also alter the surface topography causing undesirable changes (Rathee *et al*., 2010).

The development of *Candida* spp. biofilms on denture acrylic has been described as a process that occurs in three distinct stages; early (0-11hours), intermediate (12-30 hours) and maturation (38-72 hours) phases (Chandra et al 2001). Early stage biofilms are described as adhering blastospores that develop into distinct colonies and communities that appear as thick fungal growth across the surface by 11hours. By the end of the intermediate stage biofilms are layered structures comprising different morphological cell forms including blastospores, young hyphae and germ tubes; this stage is further distinguished by the presence of a film of extracellular

polymeric substances (EPS) covering the fungal community. Likewise, mature *C. albicans* biofilms consist of a dense and organised network of blastospores, pseudohyphae and true hyphae embedded in thick layers of EPS (Ramage *et al*., 2001b). Biofilms grown from *C. albicans* GDH 2346 have been described as a basal layer of tightly packed yeast cells beneath a thicker more open section in the outer biofilm consisting largely of hyphal forms (Baille and Douglas 1999; Chandra *et al*., 2001). As with other organisms, the biofilm mode conveys advantages to *Candida* spp. including increased resistance to and shelter from antifungal agents, making it increasingly difficult to eradicate from surfaces (Seneviratne and Samaranayake, 2008).

3.1.2 Morphogenesis *in C. albicans*

The ability of *C. albicans* to alter its morphology is considered an important contributor to its virulence (Phan *et al*., 2000), with particular focus on hyphal forms. *C. albicans* hyphae have been reported to enhance adhesion to surfaces (Sundstorm, 2002) and are known to bind specifically to several human proteins, including fibrinogen, c3d, and laminin (Bouchara *et al.,* 1990*;* Trochin *et al.,* 1991). *C. albicans* hyphal formation has also been suggested as being important for the invasion of the host epithelium, allowing dissemination of the organism and aiding infection (Lopez – Ribot *et al*., 1996; Monteagudo *et al.,* 2003; Kumamoto and Vinces, 2005).This may be of particular concern for denture-wearing patients as the region of the denture from which *C. albicans* is most frequently isolated (the denture fitting surface) is in close proximity to the oral mucosa. Lamfon et al (2003) investigated the formation of *C. albicans* biofilms on different dental surfaces including enamel, dentine and

denture acrylic and found indications that the presence of hyphal cells at the substratum interface may be of importance for the formation and success of *Candida* spp. biofilm structures. They suggested that the organisation of *C. albicans* biofilms is influenced by the underlying surface topography and its interaction with hyphae, which may enhance the retention of biofilms on surfaces. *C. albicans* hyphae respond to contact (thigmotropism) and chemical stimuli (chemotropism) (Sherwood *et al.,* 1992; Watts *et al*., 1998; Davies *et al.,* 1999) which are also likely to be influenced by an exaggerated surface topography, providing contact surfaces for directional growth and possibly introducing barriers for chemical signals, thus affecting subsequent biofilm development. Although previous studies (Chapter 2) found no significant effect of substratum topography on the directional growth of hyphae, the effect of topography on the subsequent development of biofilms from different morphological forms of attached cells may affect overall plaque structure, integrity and retention.

3.1.3 Denture hygiene

Denture cleansing is essential in order to prevent malodour, improve aesthetics and reduce plaque development, which can irritate the oral mucosa (Jagger and Harrison, 1995). A brief glance along any chemist or supermarket shelf will reveal a wide range of denture hygiene products and protocols available, with little guidance as to the benefits of any given product. The British Dental Association (BDA) recommends that denture wearers wash their dentures every day using a soft to medium brush and either toothpaste or soap and water, supplementing this with soaking in commercial cleansers on a regular basis and rinsing thoroughly, before

returning dentures to the mouth (*www.BDAsmile.org*). These processes are usually adequate to maintain good denture hygiene. Harrison *et al.* (2004) tested a range of cleansers including conventional toothpaste, denture cleaning pastes and an immersion cleanser, against adhered *C. albicans* on denture surfaces. They found that all of the cleansers tested removed almost all of the adhered cells from the denture specimens; they did not however investigate the effectiveness of these cleansers against biofilms. Ramage *et al.*, (2005) found that a range of commercial denture cleansers were ineffective in complete killing of *C. albicans* biofilms. Similarly Nikawa *et al.* (1995) reported variable levels of activity against fungal biofilms from denture cleansing products. Additionally, Paranhos *et al.* (2009) tested the efficacy of mechanical, chemical, and a combination of both, cleansing methods for the successful decontamination of denture surfaces. As might be expected, findings indicated that the use of a chemical denture cleansing soak product alone was not as efficient at decontamination of the surfaces as when combined with brushing (with a dentifrice). However, brushing dentures with dentifrice pastes and toothbrushes has been shown to alter the surface topography (creating scratches and increasing surface roughness) before (Heath *et al.,* 1983*;* Haselden *et al.,* 1998*;* Harrison and Johnson, 2004) and as a result, has an effect on the re-colonisation of dentures once they are returned to the oral cavity, accelerating re-colonisation and reducing cleanability. In more recent work and with the improvement of chemical denture cleansers there have been contradictory reports. Ramage *et al.* (2012) reported the regular use of a novel chemical cleanser to be a successful and superior cleansing method to that of a combination of chemical and abrasive (by use of dentifrice) techniques. The findings from their work demonstrated that abrasive

cleansing resulted in residual viable cells in surface scratches that were able to repopulate after treatment had ceased. A chemical cleanser and mechanical cleaning with low abrasive activity but high efficacy for the reduction of viable microorganisms such as *C. albicans* would be preferable in order to minimise denture surface damage whilst maximising decontamination. For many elderly denture wearers, the issue of maintaining good denture hygiene is additionally complicated by chronic diseases such as arthritis, memory loss and disability which can impair their physical ability to carry out adequate cleansing (Shimazaki *et al.,* 2001; Chalmers and Pearson, 2005; Saarela *et al.,* 2013). For this reason denture cleansing protocols should be simple and effective.

3.1.4 Aims

To investigate how the presence of adhered *C. albicans* hyphae affects biofilm development, and removal.

The specific objectives included:-

- To grow biofilms from adhered *C. albicans* yeast and hyphal cells and investigate their morphology and structure.
- To identify any differences in biofilms grown from *C. albicans* hyphae or blastospores
- To test a potential anti-*Candida* denture cleanser for its efficacy to treat *C. albicans* biofilms and mixed culture biofilms (*C. albicans* and *Streptococcus oralis*) on abraded denture surfaces (in the absence of mechanical cleansing).

Research Question

Does the presence of Hyphae in early *C. albicans* biofilms effect subsequent biofilm growth, structure treatment and removal from denture acrylic surfaces?

H0

The early presence of hyphae will have no effect on the subsequent growth, structure, treatment and removal of *C. albicans* biofilms, grown on denture acrylic surfaces.

3.2 Materials and Methods

3.2.1 Maintenance of cultures

A stock culture of *C. albicans* NCYC 1467/ GDH 2346 was stored at -80°C. Subcultures were prepared on Sabourauds dextrose agar (Oxoid ltd, Hampshire, UK) and refrigerated at 4°C prior to use. These cultures were replaced every four weeks.

3.2.2 Preparation of cell suspensions

C. albicans GDH 2346 was cultured on Sabouraud's dextrose (SAB) agar (Oxoid). Cell suspensions were prepared by inoculation of 100 ml SAB broth with one colony of the *C. albicans* strain, which was subsequently incubated at 37°C for 24 hours in an orbital shaker. Culture purity of each cell suspension was checked every time, by streaking on nutrient and SAB agar, incubating at 37°c for 24 hours and examining for any contaminant colonies.

3.2.3 Retention assay

C. albicans GDH 2346 were harvested by centrifugation (3000 rpm for 10 minutes) from prepared cell cultures, washed twice in sterile water and re-suspended in sterile water to an optical density of 1.0 at 540 nm corresponding to approximately $1.10 \pm 0.18 \times 10^6$ cfu per ml. 150 ml of the standardised cell suspension was added to four large Petri dishes (15 cm x 1.5 cm, Sigma-Aldrich), each containing six replicates of either control, low, medium and high abraded 2 cm^2 test substrata (chapter 2, 2.2.8). These were subsequently incubated for 1hour at room temperature without agitation. After 1hour the test materials (with adherent *C. albicans* cells), were

removed and washed by gently rinsing with running sterile water from a gentle even flowing wash bottle (wide spouted wash bottle) for three seconds with the surface held horizontal and the water directed to the centre of the PMMA pieces, so that the excess liquid flowed across the surface and off the edge.

3.2.4 Inducing hyphal growth in adhered cells

To induce hyphal growth in adhered *C. albicans* cells, immediately following the adhesion assay three replicates of each test piece with adherent *C. albicans* cells were placed in small Petri dishes (60mm x 15mm) containing 20 ml of 25% horse serum and incubated for 3 hours at 37 °C to enable attached cells to produce true hyphae. After incubation, test materials were removed from the horse serum and gently washed with running distilled water to remove excess serum and loosely adherent cells.

3.2.5 Biofilm development from adhered yeast or hyphal cells

Following the retention assay and hyphal induction, 3 replicates of each test material (control, low, medium and high), with adherent yeast cells or adherent hyphae, were placed, using sterile forceps, horizontally into sterile 250 ml glass beakers containing 200 ml of sterile SAB broth and subsequently incubated statically at 37 ^{o}C for 48 hours (with the broth aseptically changed after 24 hours), to allow biofilm development.

3.2.6 Confocal Microscopy of *C. albicans* biofilms

Following 48 hour incubation all samples were removed from the beakers using sterile forceps and washed by gentle immersion once into sterile distilled water (with the surface held horizontally to the water interface). Test pieces with biofilms were

allowed to dry in a class II laminar flow cabinet (BH-EN 2003, Safe lab systems ltd, Bristol, UK) and were subsequently secured onto glass slides with double sided tape. Biofilms were stained with 0.05% Calcofluor white diluted with 10% potassium hydroxide (SigmaAldrich) and incubated at room temperature in the dark for 45 minutes according to the manufacturer's staining protocol. Following staining, samples were examined using a Confocal scanning laser microscope, (Leica DM 2500, LCS SPE 1000, Wetzlar, Germany) (CSLM) for differences in morphology between the biofilms formed from adhered cells and those formed from adhered cells with hyphae. Biofilms were visualised with the x 40 oil immersion lens (Type F immersion liquid, Leica, Wetzlar, Germany). Ten fields per test piece were examined and details of 3-dimensional composition and organisation were recorded. For each field a Z stack was developed (scanning every 2μm). The total height (μm) of the Z stack (figure 3.1) was recorded in order to investigate any differences in biofilm thickness.

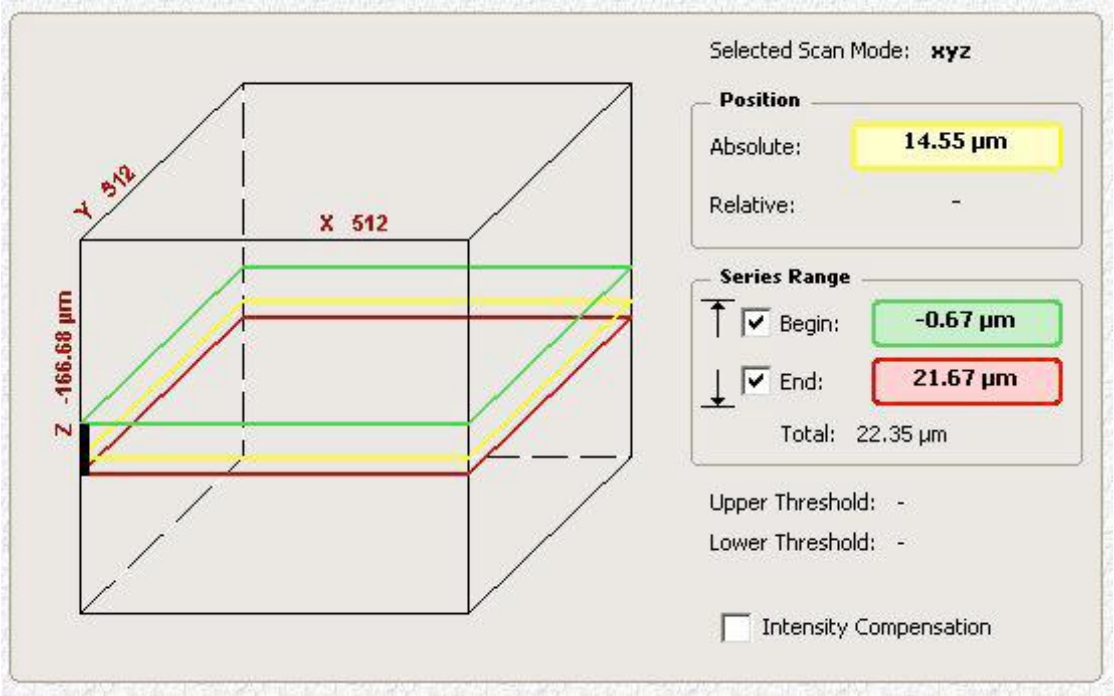


Figure 3.1 Acquisition window of the LAS AF Confocal software package where the Z stack settings are defined. This function enables the user to scan through layers of a sample by setting the beginning and end points (whilst visualising the sample on screen) and defining the height of each scan section (i.e. every 2µm).

3.2.7 Measuring biofilm mass

In order to compare the development of biofilm on the abraded denture surfaces from yeast or hyphal *C. albicans*, the mass in the form of dry weight was calculated. Biofilms were grown on three replicate test materials from each test group (control, low medium and high abraded surfaces) from either yeast or hyphal *C. albicans*. Following this, each individual test piece (with biofilm) was placed into sterile 25 ml bottles containing 10 ml of sterile phosphate buffered saline (PBS, Sigma Aldrich) and vortex mixed for 30 seconds. The re-suspended cells were subsequently filtered through 0.2 µm pore filter paper disks (Whatman international ltd, Maidstone, UK) and allowed to dry in a 37°C incubator for 24 hours. All dehydrated detached biofilm cells were weighed against a sterile control filter paper disk *(*Chandra *et al.,* 2001*)*.

3.2.8 Retention of cells following biofilm removal

Following removal of the hyphal or yeast cell biofilms for dry weight measurement the test surfaces were examined for retained cells. Subsequent to biofilm removal, all test substrata were placed in a dark class 2 laminar flow cabinet and stained with 0.03% Acridine orange [(Sigma Aldrich) diluted with 2% glacial acetic acid (BDH laboratories, Poole,UK)] for two minutes before being rinsed in sterile distilled water and allowed to dry. Stained test pieces were examined using epifluorescence microscopy (Nikon Eclipse 6000, Burgerweeshuispad, Amsterdam). The number (counts) of retained cells/hyphae and the percentage coverage (area of microscopic field covered by cells) were determined for each replicate sample in five different areas.

3.2.9 Denture cleanser testing

Preliminary studies were carried out to determine the efficacy of 2 versions of an effervescing denture cleansing product (figure 3.2) ['Polident 1' and 'Polident 2'] (Polident, GlaxoSmithKline, Surrey, UK) on planktonic *C. albicans.* 'Polident 1' was a 3 minute cleanser consisting of two oxidising agents; sodium perborate and potassium monopersulphate, a proteolytic enzyme, detergent and an effervescent base (Ferreira *et al.* 2009). 'Polident 2' consisted of the addition to these products of Tetraacetylethylenediamine (TAED), an activating agent for oxidisers. 'Polident 2' was hypothesized to have increased efficacy, possibly due to its increased oxidising potential (Ramage *et al.* 2012) with the addition of TAED.

A standardised cell suspension was prepared to an optical density of 1.0 at 540nm corresponding to approximately $1.23 \pm 0.14 \times 10^6$ cells/ml (as described in 4.2.2). 150 ml of the prepared *C. albicans* cell suspension was transferred aseptically into three separate sterile 200 ml glass beakers. At this stage, 1 ml of the cell suspension was removed from each beaker and placed into sterile 25 ml universal bottles each containing 9 ml of sterile water for a time zero measurement of colony forming units. Following this, one of each denture cleanser tablet was added to one of the beakers containing the 150 ml of the prepared cell suspension and a timer was started. 1 ml of each of the suspensions (either with denture cleanser 1, denture cleanser 2 or without cleanser) was removed after 3 minutes (denture cleanser 1 recommended soak time), 5 minutes (denture cleanser 2 recommended soak time) and 1 hour (extended soak time) and transferred to sterile universals containing 9 ml of sterile water. These were immediately diluted down to 10^{-4} and 100 µl of each dilution was plated out on to SAB agar (3 replicates) and incubated at 37°C for 24-48

hours for viable counts. From this preliminary work denture cleanser 2 was found to be effective against planktonic *C. albicans* after 1hour but not 3 or 5 minutes. A 1 hour time soak was therefore carried forward to investigate denture cleanser activity (against a water control) in further studies.

Figure 3.2 The two denture cleansers tested in preliminary investigations against planktonic *C. albicans*. a) 'Polident 1' containing sodium perborate and potassium monopersulphate, a proteolytic enzyme and detergent, and b) 'Polident 2' containing the above components with the addition of tetraacetylethylenediamine (TAED). These cleansers were both tested for their efficacy against *C. albicans*. 'Polident 2' was used in subsequent denture cleanser studies on *C. albicans* and mixed (*C. albicans* and *S.oralis)* biofilms.

3.2.10 Time kill of planktonic *C. albicans*

The preliminary study was repeated to determine a time of complete kill (mins) for the polident denture cleanser that had been found to be effective against *C. albicans* (denture cleanser 2). Sterile glass beakers containing 150 ml of the standardised *C. albicans* cell suspension were prepared as described previously. 1 ml of each suspension was transferred into 9 ml of sterile water (in sterile 25 ml universal bottles) to determine a time zero cell count. Following this a denture cleanser tablet was added to one of the beakers and a timer started. 1 ml of the cell suspensions were removed from each beaker every minute, for 20 minutes. These were immediately diluted to 10^{-4} in sterile water and 100 µl of each dilution was plated onto SAB agar (three replicates), incubated at 37℃ for 24-48 hours. Following incubation, time of complete kill for the denture cleanser was determined as the point where no viable colony forming units were seen on any of the repeat plates.

3.2.11 Polident denture cleanser activity against *C. albicans* biofilms

Two types of *C. albicans* biofilms were produced and used to investigate and compare the efficacy of the Polident denture cleanser selected in planktonic studies. These biofilms were either grown from adhered *C. albicans* blastospore or *C. albicans* hyphae (as described in 3.2.3-3.2.5). Replicates of each of these biofilm types were grown on either smooth/un-abraded (control) $2cm^2$ PMMA pieces or high abraded PMMA test pieces (chapter 2). 16 sterile 200 ml glass beakers were prepared, 8 containing 150 ml of sterile water and the other 8 containing 200 ml sterile water with 1 dissolved effervescent denture cleanser tablet. Immediately

after cleanser tablets had stopped effervescing (to exclude the mechanical properties of the denture cleanser and thus minimise biofilm disruption), three replicates of each biofilm on either control or high abraded surfaces were placed into each beaker. All beakers were left at room temperature for either 1 hour or 16hours (to simulate an overnight soak). Following this, test pieces with biofilms were removed and processed for viable counts as described below. Three replicates of each biofilm were also used before soak to acquire a time zero / base line viability value.

3.2.12 Denture cleanser activity against mixed biofilms

In order to further investigate the activity of the denture cleanser mixed biofilms of *C. albicans* GDH 2346 and *Streptococcus oralis* NCTC 11427 ACTC 35037 were generated. Cultures of each organism were prepared by inoculating 100 ml broth with one colony (SAB for *C. albicans*, Tryptone soya broth (TSB) for *S.oralis*) and incubating for 24 hours at 37°C in an orbital shaker. Both cultures were subsequently spun down (3000 rpm, 10 minutes), washed twice in sterile phosphate buffered saline (PBS) and re-suspended in PBS to an OD of 0.8 at 540nm (corresponding to approximately 2.18 ± 0.2 x 10^8 cells/ml for *S.oralis*, 6.9 ± 1.1 x 10^6 cells/ml for *C. albicans*). The two cultures were subsequently mixed in equal volumes (200 ml of each) in a sterile 500 ml glass conical flask, immediately prior to use. 150 ml of the mixed cell suspension was incubated with 18 replicates of the high abraded 2 cm^2 PMMA test pieces and 18 replicates of the control 2cm^2 surfaces separately, in two large Petri dishes for 1 hour at room temperature (25°C ± 2). Following 1 hour, 3 replicates of each test surface were removed (using sterile forceps) and placed

directly into 50% horse serum (diluted with sterile distilled water, in a unused sterile large Petri dish) and incubated at 37°C for a further 3 hours to induce hyphal growth in adhered *C. albicans*. Subsequently all test pieces with attached cells (mixed – with or without hyphae) were placed into sterile 250 ml glass beakers containing 200ml of tryptone soya broth and incubated at 37°C for 48 hours (broth changed aseptically after 24 hours). After 48 hours, mature mixed biofilms were removed from incubation and subjected to denture cleanser challenges of 1 and 16 hours. One denture cleanser tablet was dissolved in each of four separate sterile glass beakers containing 200 ml of sterile distilled water to which 3 replicates of each biofilm type were added immediately after effervescing had ceased. Three replicates of each biofilm type were removed from the denture cleanser after 1 hour and after 16 hours. Once removed from the denture cleanser, biofilms were processed for viable counts as described below (3.2.13). Three replicates of each biofilm were also processed at time zero to assess base line viability counts.

3.2.13 Viability testing of biofilms

Viable counts

Following treatment each individual test piece with treated hyphal/blastospore biofilm was placed into a 25 ml bottle containing 10 ml of sterile PBS and vortex mixed for 30 seconds to remove cells. These re-suspended cells were immediately diluted to 10^{-8} in sterile PBS and 100 µl of each dilution was plated out onto SAB/TSB agar (for *C. albicans* and *S.oralis* respectively), in duplicate for each individual test piece. Plates were incubated at 37°C for 24 hours, following which colony forming units were counted and recorded.

3.2.14 XTT Assay

The XTT assay was also employed in order to compare the susceptibility of the hyphal or blastospore biofilms to the denture cleanser (Polident 2). Biofilms were grown from both blastospores and hyphae of *C. albicans* (as described in 3.2.5) on P100 abraded 1 cm² denture acrylic surfaces (roughened with P100/141 µm grit size emery paper giving an average surface roughness/Ra of 2.8 µm, see chapter 2 for production and abrasion of surfaces). Following incubation, 6 replicate test pieces with either hyphal or yeast cell biofilms were placed into either the denture cleanser (1x Polident effervescent tablet dissolved in 200 ml sterile distilled water at room temperature as directed by manufacturers guidelines) or 200 ml sterile distilled water and left for one hour at room temperature. Following this, each 1 cm² acrylic specimen with attached biofilm was transferred to a 5 ml bottle to which 790 µl sterile PBS, 200 µl XTT reagent (Sigma Aldrich, dissolved in PBS to a final concentration of 1mg/mL and filter sterilised with a 0.2 µm pore size filter disc) and 10 µl of Menadione (Sigma Aldrich, in acetone to a 0.44 mM concentration immediately before each assay) was added (Silva *et al*., 2008; Ramage *et al.,* 2001c). The bottles were incubated at 37°C for three hours allowing the XTT components to react with metabolically active cells in the biofilms producing a coloured (orange-red) formazan by-product in the supernatant. After three hours 200 µl of supernatant from each bottle containing each replicate was transferred to a sterile 96 well microtitre plate (U bottomed) and analysed for optical density at 492 nm with a microplate reader (Multiskan Ascent, Thermo lab systems, Basingstoke, UK).

3.2.15 The penetration of *C. albicans* biofilms by denture cleansers

Biofilm Development

As described in previous chapter's biofilms developed from hyphal cells or blastospores of *C. albicans* GDH 2346 were used to investigate the penetration of denture cleansers. Cultures of *C. albicans* GDH 2346 were prepared by inoculating 100 ml SAB broth with 1-2 colonies of *C. albicans* and incubating in an orbital shaker overnight, as previously described. The following day cells were harvested by centrifugation (3000 rpm for 10 minutes), washed twice in sterile PBS and re-suspended in PBS to an optical density of approximately 1.0 $\pm$ 0.14 x 10^7 cells per ml. The prepared cell suspension was incubated with 18 high abraded 2 cm^2 test surfaces (see chapter 2 for surface preparation) to allow cells to adhere. These test substrata were chosen as they were found in previous studies to result in large amounts of retained cells which allowed for the development of biofilms that were less easily removed from the substrata, this was thought to be important as it was desired that the biofilm structures were kept as in tact as possible in order to investigate their penetration by cleansers. Following the adhesion of cells to surfaces, half of the test pieces (9 in total) were incubated with 50% horse serum for 3 hours To induce hyphal growth (described previously, chapter 2). All surfaces with attached blastospore or hyphal phase cells were subsequently incubated in 200 ml of SAB broth at 37°C for 48 hours (broth changed aseptically following 24 hours), to allow biofilm development.

3.2.16 Live dead staining and confocal microscopy

Three replicates of both hyphal and blastospore origin were subjected to soaks in either water or dissolved denture cleanser (1 x Polident in 150 ml sterile distilled water) for 0, 5 or 60 minutes. Following this, all biofilms attached to acrylic surfaces were stained and examined using confocal scanning laser microscopy (DM 2500, LCS SPE 1000, Leica, Milton Keynes, UK). The live dead staining was achieved using BacLight viability stain (Invitrogen, Life sciences, Paisley, UK), which utilizes two different nucleic acid stains; Syto 9- green fluorescence and Propidium iodide-red fluorescence. Syto 9 is able to penetrate and stain both viable and non-viable cells, propidium iodine acts only on cells with disrupted membranes (dead cells). Therefore when used in conjunction with one another these two dyes allow for the differentiation of living (green) and dead (red) cells within a cell population. This stain was prepared on the day of use and stored in a sterile 25 ml universal wrapped in tin foil to prevent its deterioration. Biofilms on test substrata were placed into sterile Petri dishes and gently flooded with BacLight viability stain before being wrapped in tin foil and left for 2 minutes.

Following staining replicate test pieces were examined individually using CSLM with a water immersion lens (HCX APO L40×/0.80 W U-V-I objective; Leica). The water immersion lens allowed for the visualisation of biofilms in their hydrated state through an excess of the live dead stain left on the test surface (Figure 3.3). For each test piece a Z stack was taken (section 3.2.6) in five different microscopic fields. The confocal microscope settings were altered to accommodate the two separate dyes

used (excitation approx 485 nm, detection at around 530 nm for syto 9 (green) and around 630nm for propidium iodide (red).

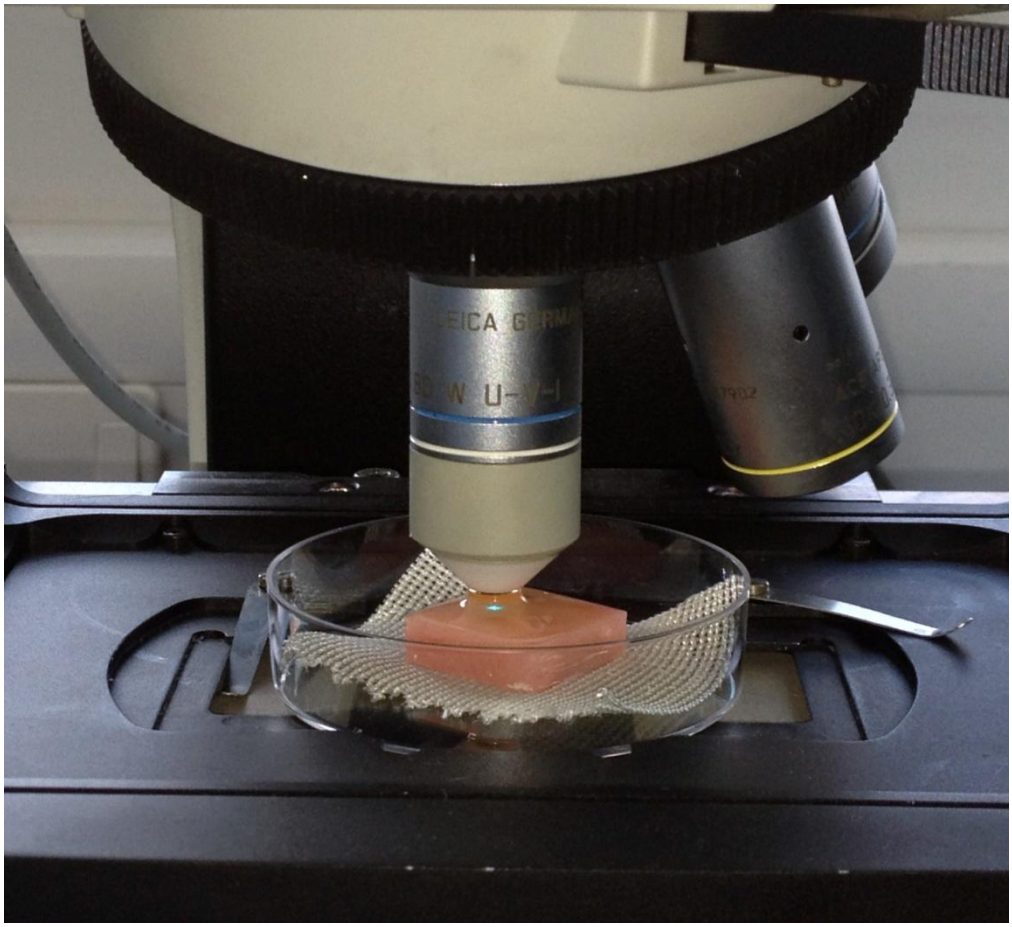


Figure 3.3 Water immersion lens (HCX APO L40×/0.80 W U-V-I objective) of the confocal scanning laser microscope (Leica DM 2005) used to investigate biofilm penetration by denture cleansers. Image illustrates the method adopted for the examination of living/hydrated biofilms. Microscopic observations are made through an interface of excess live dead stain on the test substrata.

3.2.16.1 Preliminary findings and method development

A preliminary run of the experiment highlighted some issues with the experimental design that affected the results. Individual scans of test pieces took between 1 and 2 minutes and therefore there were large time gaps between the analyses of test pieces from different test groups highlighting a need to stagger the incubation times to ensure that different replicates were examined after approximately the same amount of time in soak. Thus a 30 minute gap was left between placing each three replicates into soak in either cleanser or water in repeat experiments.

Secondly, the initial observations raised concerns about how stable the biofilms were. In addition, the exposure of *C. albicans* cells to the live dead stain may have reduced cell viability over time. For this reason and to limit these effects, each set of three replicates were processed separately and the stain was applied to each individual test piece immediately prior to microscopic examination.

Lastly problems were encountered during the microscopic examination of each individual test piece due to rapid evaporation of the stain from the surface. To counteract this, additional stain was gently pipetted onto the surface between examinations of each field of view. For each test piece ten fields were examined, in which Z stack images were acquired every 2 μm. Z stack images were ultimately combined to produce one image for each field (10 images per surface) which were subsequently visually compared and quantitatively analysed using Image J software for the intensity of red and green pixels.

3.2.17 Statistical analysis

Repeated observations on each replicate used in biofilm mass and viability work were checked for any evidence of independence and since none was found a normal level of distribution was assumed.

Biofilm thickness and mass

Differences in biofilm thickness and mass data were determined using two-way ANOVA to compare the effect of both the level of surface roughness and the presence of hyphae on these factors.

Cell retention following biofilm removal

The retention of yeast and hyphal cells following biofilm removal on surfaces were analysed in similar ways to the cell counts described in chapter 2. Counts of retained cells were highly variable and therefore did not fit patterns of normal distribution. A log of the counts was taken to accommodate for the variance and subsequent data were analysed using a two-way ANOVA.

Viability of *C. albicans* following denture cleanser treatment

Viable counts (CFU) and XTT assay data to determine the effect of denture cleanser soaks on hyphal and blastospore biofilm viability on abraded and un-abraded surfaces were analysed using two-way ANOVA.

Penetration of hyphal and blastospore biofilms by the 'Polident' denture cleanser.

Live/dead fluorescence intensity measurements from hyphal and blastospore biofilms after different lengths of soaks in denture cleanser were analysed individually, comparing the two groups and time zero, 5 minutes and 1 hour. This was done using a two tailed independent t-test to compare the two independent groups.

3.3 Results

3.3.1 Biofilm development from adhered *C. albicans* blastospores or hyphae

C. albicans biofilms grown from adhered blastospores and adhered cells with hyphae were compared for differences in morphology. From initial observations biofilms grown from the two cell types both consist of networks of budding yeast cells, hyphae and pseudohyphae (Figure 3.4). Biofilms grown from adhered cells with hyphae were more abundant in branching pseudohyphae that ran through the biofilm layers (figure 3.5). The resultant biofilm structure appeared to be less densely packed containing more open spaces and water channels. The hyphae in these biofilms were longer and travelled across the biofilm structure (horizontal to the surface) in comparison to those of blastospore biofilms which were much shorter and orientated downwards/inwards (towards the substratum interface) (figures 3.4-3.5).

As well as the differences in morphology, there were visible differences in biofilms grown from hyphal cells compared to those grown from blastospores. Biofilms grown from hyphal phase *C. albicans* were visibly much thicker in their wet forms compared to those grown from blastospores across all test substrata (figure 3.6).

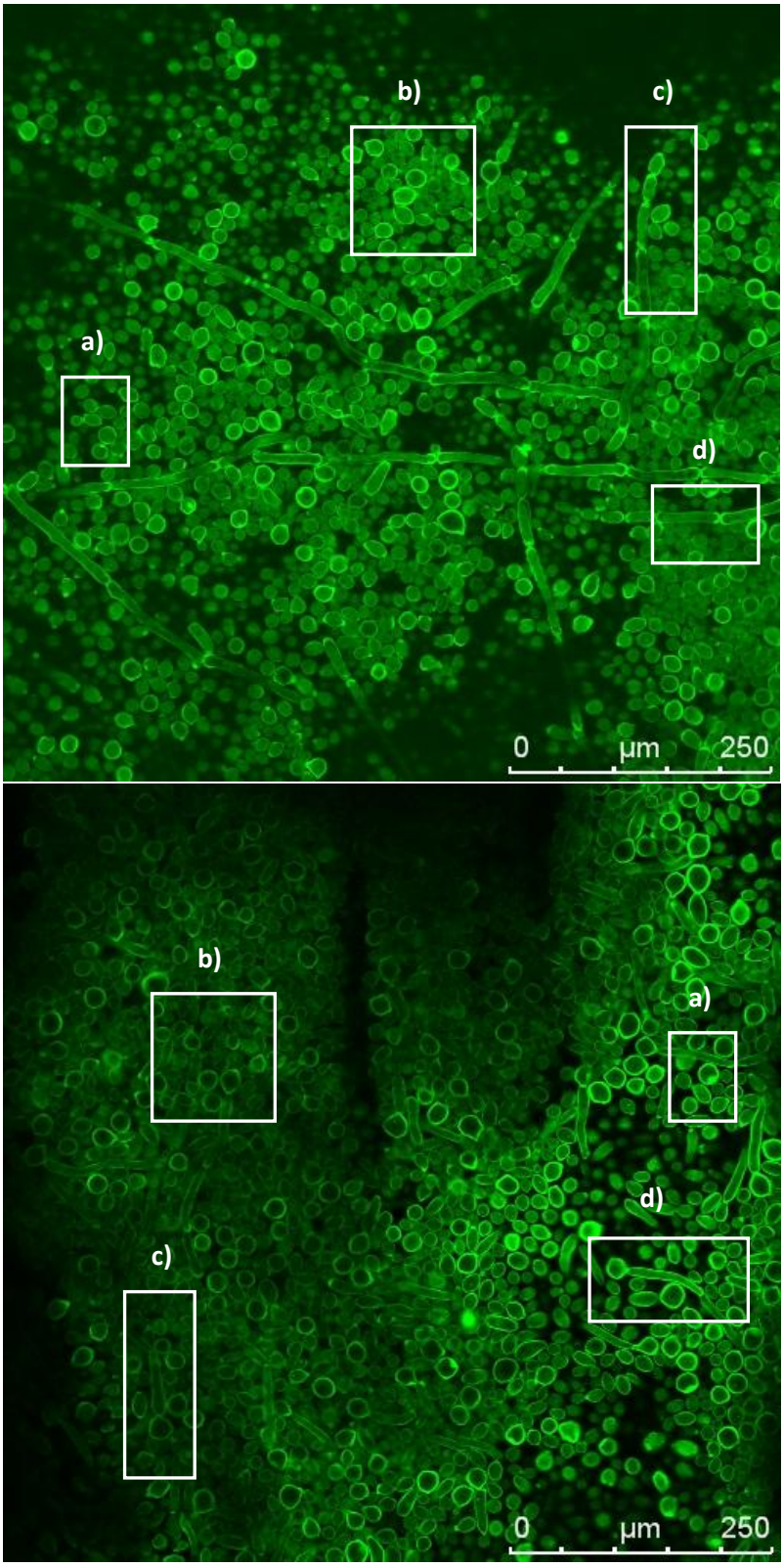


Figure 3.4. **CSLM images of *Candida albicans* biofilms grown from adherent hyphal (top) and blastospore cells (bottom).** Biofilms were grown on a typical 2cm² PMMA piece (high abraded) and stained with 0.5% calcofluor white. Images show a) budding yeast cell, b) blastospores, c) branching pseudohyphae and d) constrictions in septa of branching hyphae. Blastospore biofilms (bottom) appear more densely packed compared with hyphal biofilms (top).

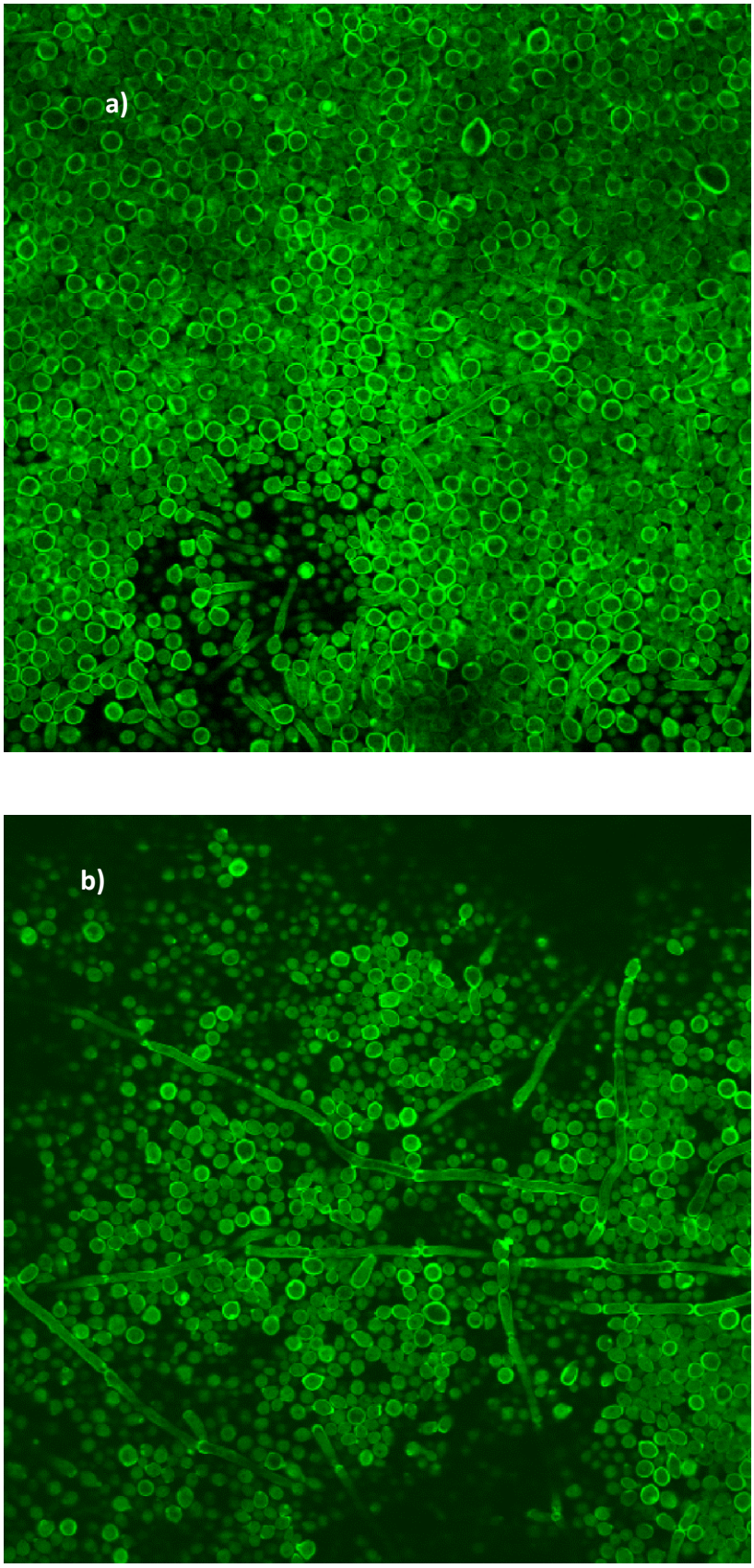


Figure 3.5. **CSLM images of *Candida albicans* blastospore and hyphal biofilms of *C. albicans.*** Biofilms (stained with 0.5% calcofluor white), grown on two typical high abraded 2cm² PMMA pieces from a) adhered blastospores, are tightly packed with organised layers of blastospores and hyphal protrusions orientated inwards. Biofilms grown from b) adhered *C. albicans* hyphae are less densely configured creating more channels and open

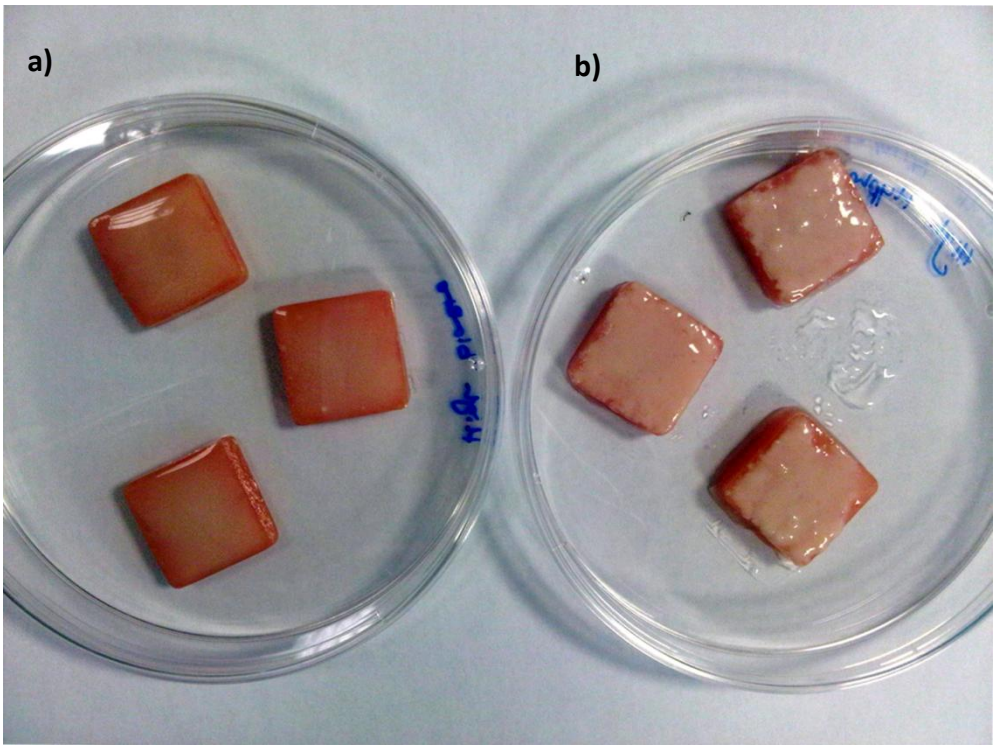


Figure 3.6 Macroscopic appearance of biofilms grown on high abraded denture acrylic surfaces from either a) blastospores or b) hyphae of *C. albicans*. Biofilms grown from hyphal cells are visibly thicker and more textured than those grown from blastospores.

3.3.2 Measuring biofilm thickness and mass

The thickness of biofilms was measured with CSLM by noting the thickness of the z stack that was taken in each confocal scan of the two biofilm types. The Z stack heights of the two different biofilm types grown on the same abraded surfaces were compared for differences using a two sample t-test assuming unequal variance. There was no significant difference in the Z height biofilm depths measured for the two biofilm types on the high and medium abraded surfaces. The depths of the blastospore and hyphal biofilms grown on the un-abraded surface were however found to differ significantly $p<0.05$ (figure 3.7).

Biofilms grown from hyphal phase cells had significantly higher biomass ($P \leq 0.005$) than those grown from adhered blastospores on control, low, medium and high abraded test surfaces (figure 3.8). The degree of surfaces roughness did not significantly affect the biomass of mature 48hour biofilms.

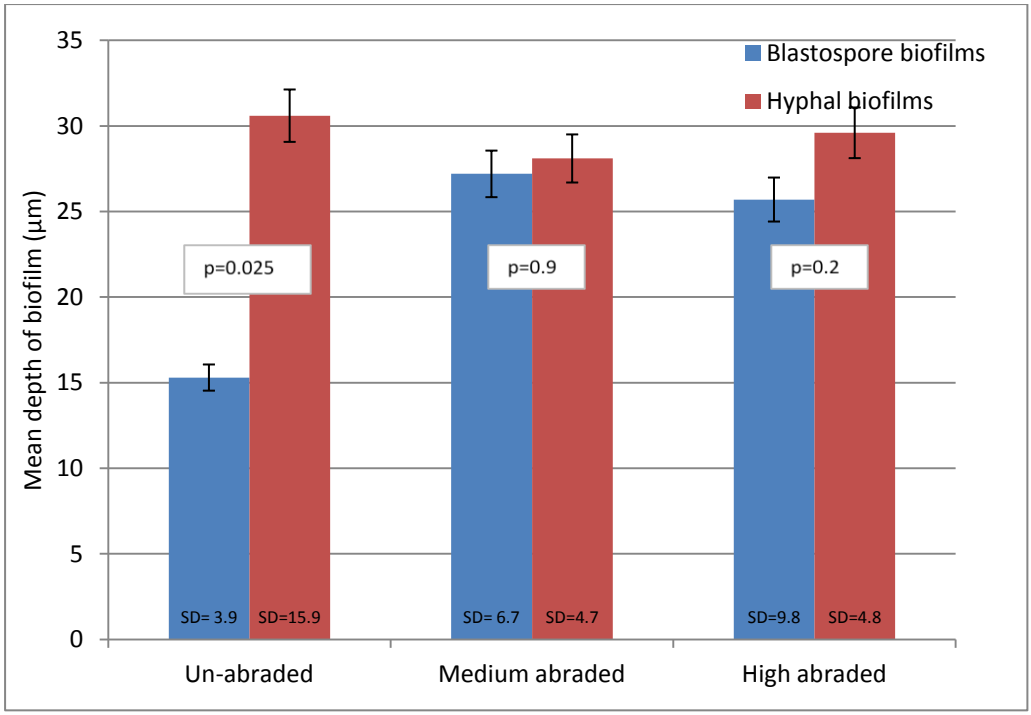


Figure 3.7 Average depths (µm) of *C. albicans* biofilms grown from either adhered blastospore or hyphal cells, on 2cm² denture acrylic surfaces that had been abraded using dentifrices with increasing abrasive qualities. Measurements taken on two separate occasions from five areas of three replicate test surfaces (n=30). Statistical significance determined using two way ANOVA.

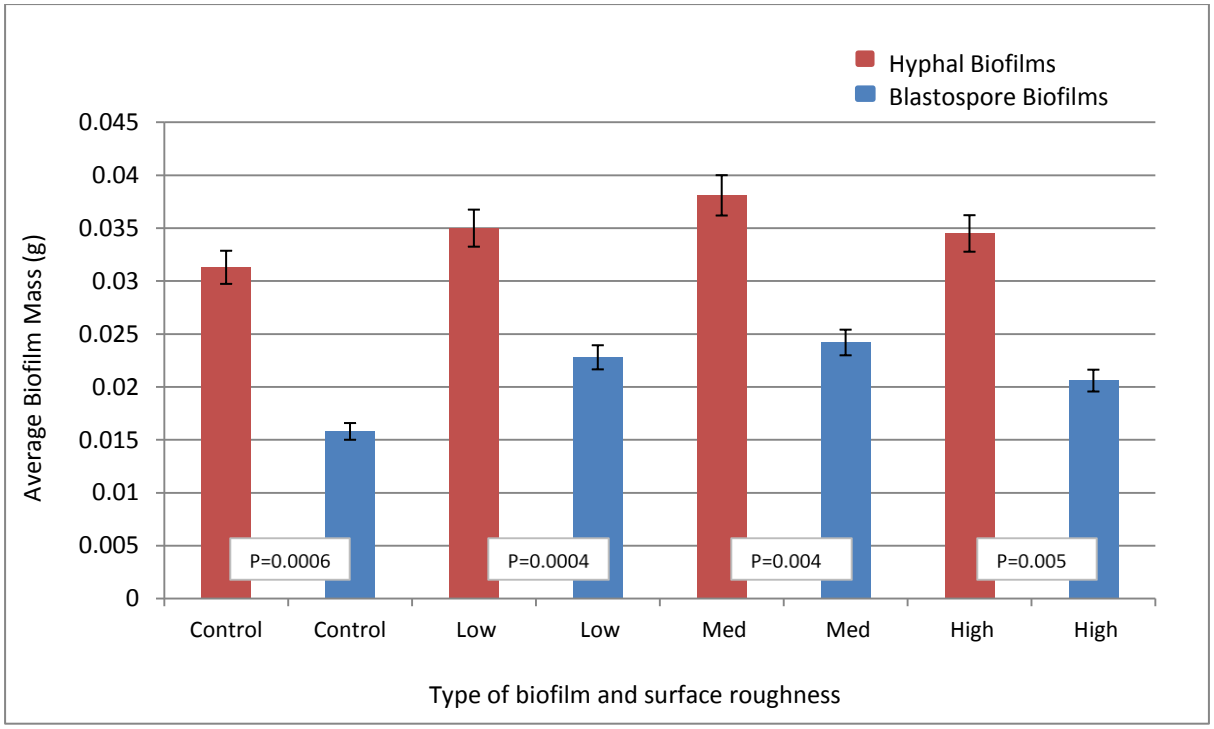


Figure 3.8 Mass (g) of biofilms grown from adhered blastospores and hyphae of *C. albicans* on denture acrylic pieces abraded using dentifrices with increasing levels of abrasive polish (control, low, medium and high). Results based on five biofilm mass measurements, on five replicate test surfaces taken on three separate occasions (n=15). Statistical significance determined using two way ANOVA.

3.3.3 Cells retained on surfaces following biofilm removal

Following removal of hyphal and blastospore biofilms, test surfaces were examined for retained cells. Significantly higher numbers of cells were retained on all abraded test substrata following removal of the hyphal biofilms in comparison to the numbers of cells remaining after removal of the blastospore bioflms (figure 3.9). The difference between hyphal and blastospore cell retention increased as the level of surface roughness increased, with the lowest numbers of retained cells found on the un-abraded/control surfaces. Cells were retained preferentially in linear surface scratches (figure 3.10). These images also revealed large amounts of long branching hyphae at the surface - biofilm interface on the surfaces that had been used to grow biofilms from hyphal forms of *C. albicans*. These hyphae were branching across surface micro-architecture, and interlacing with other hyphae and cells (figure 3.10). No hyphae were observed on surfaces used to grow biofilms from adhered blastospores of *C. albicans*.

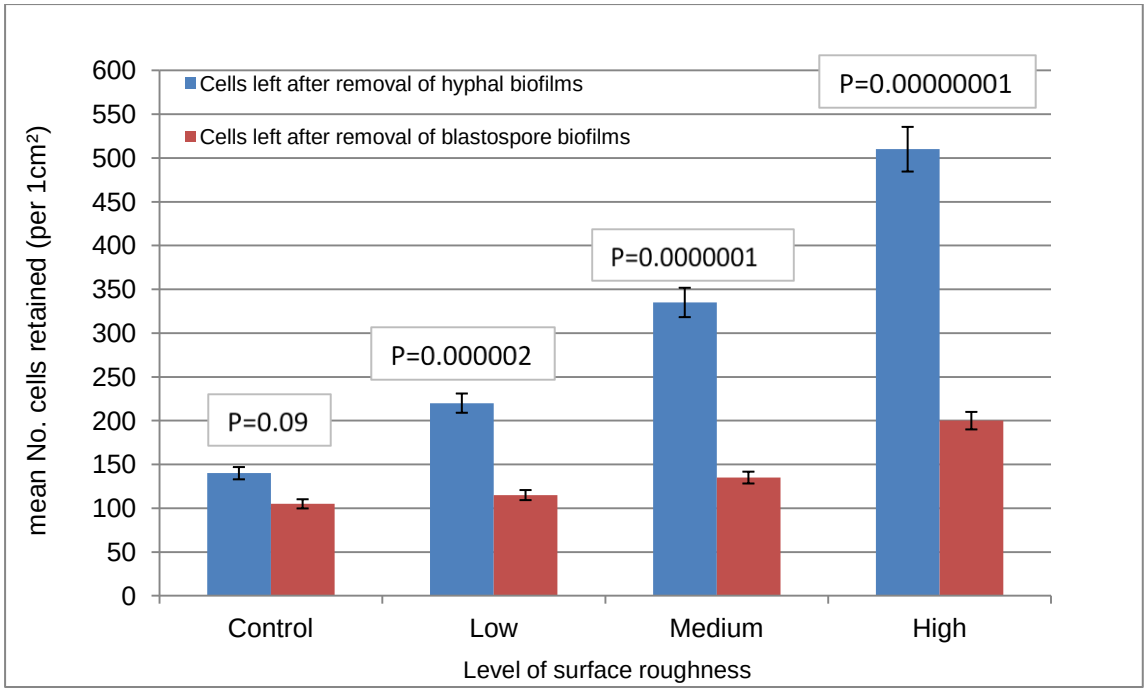


Figure 3.9 Number of *C. albicans* cells retained on abraded denture acrylic surfaces (abraded using dentifrices with increasing levels of abrasive polish) following removal of *C. albicans* blastospore or hyphal biofilms by vortex washing in sterile water. Retention of cells was measured in fifteen fields of three replicate test surfaces on three separate occasions (n=135). Statistical significance determined using two way ANOVA .

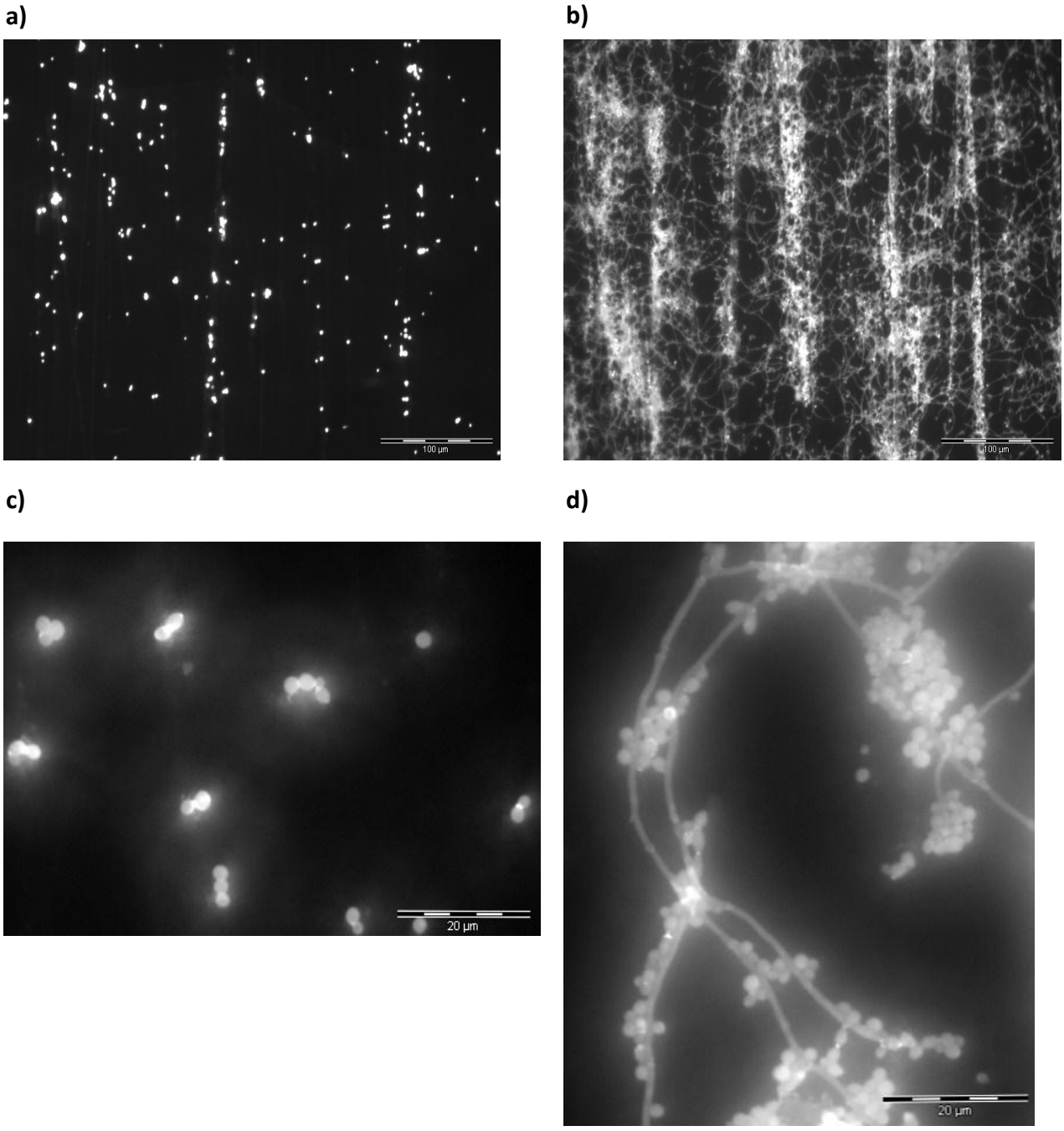


Figure 3.10 Epi fluorescence microscopy images of retained cells a) after blastospore biofilm removal; b) after removal of hyphal biofilm at x100 magnification; c) after removal of blastospore biofilms at x 400 magnification; d) after removal of hyphal biofilm at x400 magnification. All images are of retention on high abraded surfaces (scale bar represents 20µm).

3.3.4 Denture cleanser tests with *C. albicans*

Preliminary study

Initially two different Polident denture cleansers were tested against each other for their efficacy against planktonic *C. albicans*. Neither cleaner significantly reduced the numbers of viable cells following the 3 or 5 minutes denture cleanser challenge. However 'Polident 2' was effective at completely killing all viable *C. albicans* between 5 and 60 minutes (figure3.11).

The effectiveness of Polident 2 was tested further by determining the time of complete kill for planktonic *C. albicans*. The cleanser was tested against a water control, with sampling for viable counts taken every minute. There was a significant reduction in viability between 12 and 15minutes ($P<0.05$) and no viable cells were retrieved from 18minutes onwards (figure 3.12).

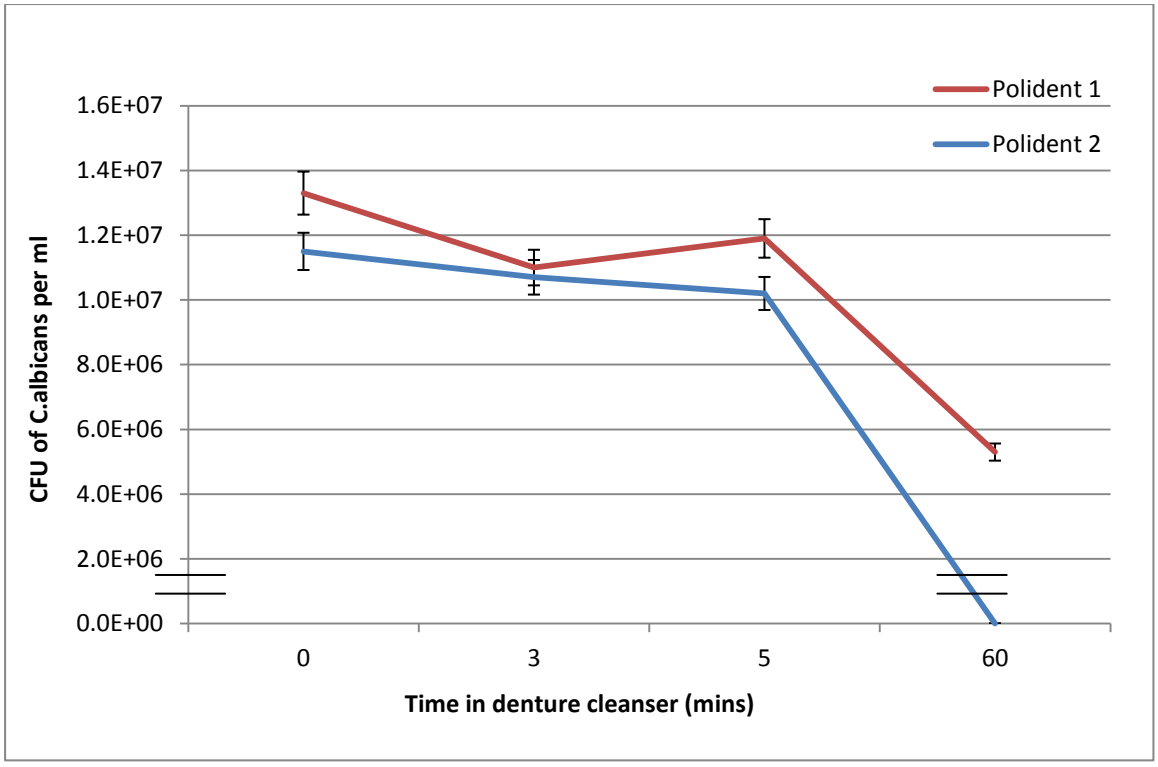


Figure 3.11 The viability of planktonic *C. albicans* after exposure to two different Polident denture cleansers ('Polident 1' and 'Polident 2') for 0, 3, 5 and 60 minutes. Mean viable counts were taken from three replicate plates of CFU counts taken in two separate experiments (n=6).

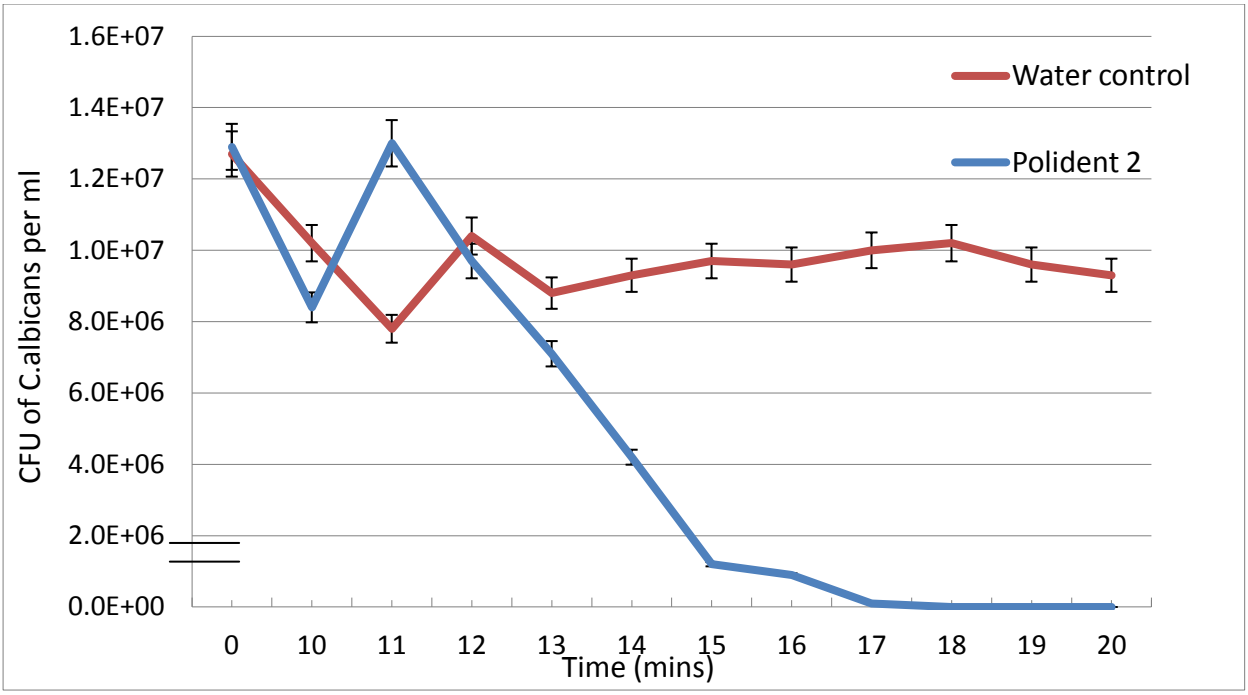


Figure 3.12 Viability of planktonic *C. albicans* over time (minutes) whilst being treated with either a denture cleanser (Polident 2) or a sterile water control. Mean viable counts were taken from three replicate plates of CFU counts taken on three separate experiments (n=9).

3.3.5 Denture cleanser activity against *C. albicans* biofilms.

Biofilms grown from adhered *C. albicans* hyphae and blastospore on high and control test surfaces were challenged with Polident 2 for 1 hour and 16hours before being sampled for viable counts. The viability of hyphal and blastospore biofilms were not significantly reduced after 1 hour soak in denture cleanser (Table 3.1). There was a significant reduction ($P<0.01$) in biofilm viability following 16 hours soaking in denture cleanser and no viable cells were retrieved from hyphal or blastospore biofilms grown on control/smooth surfaces (Table 3.1). Viable cells were however retrieved from biofilms grown on the high abraded surfaces (figure 3.13).

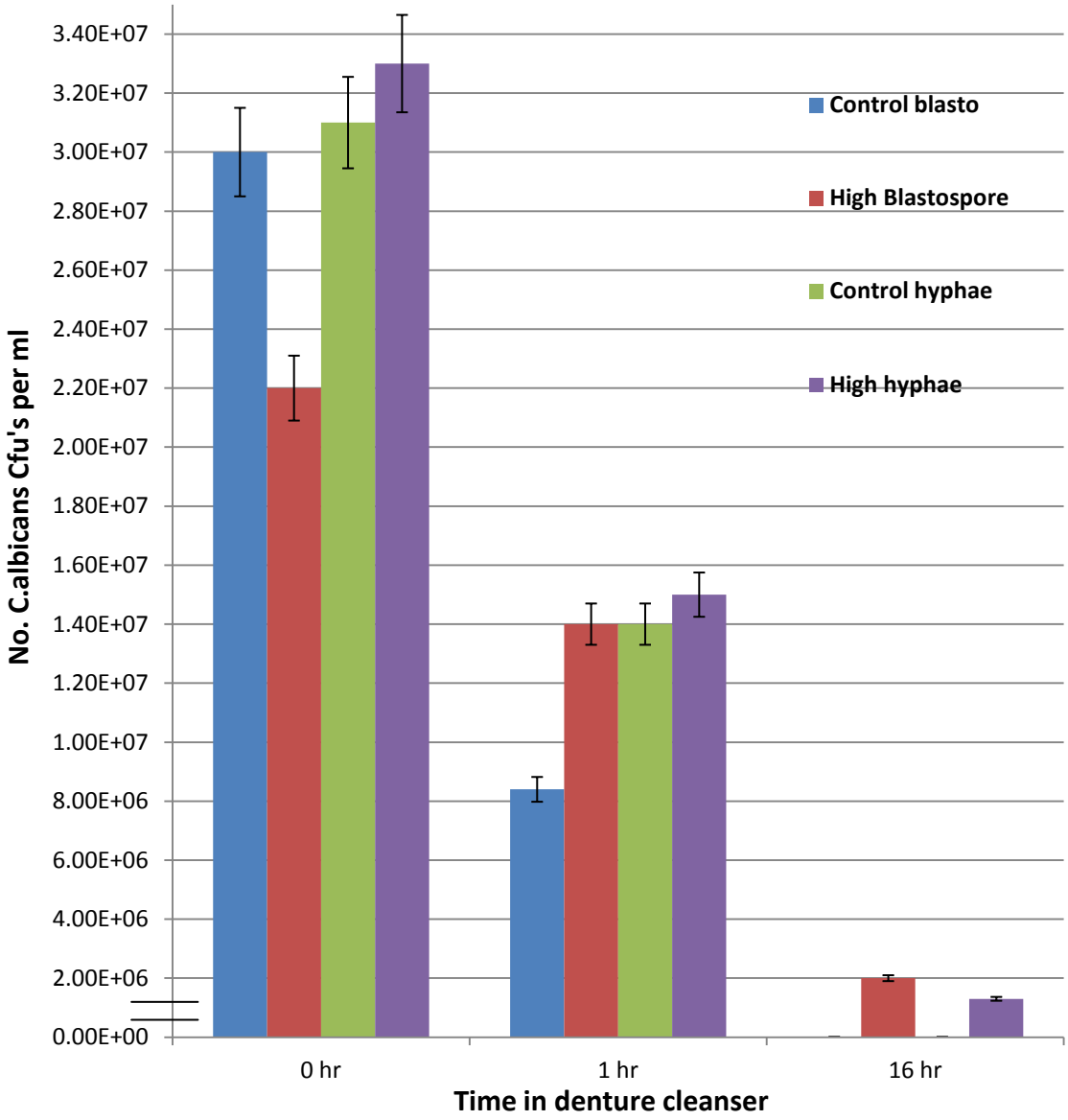


Figure3.13 The viability of *C. albicans* in hyphal and blastospore biofilms grown on either control (washed with water) or high abraded denture acrylic surfaces and treated with Polident 2 denture cleanser for 0, 1 and 16hours. Data based on three repeat experiments each consisting of five replicate test surfaces (n=15).

3.3.6 Denture cleanser activity against mixed biofilms

Mixed biofilms of *C. albicans* and *S.oralis* were also challenged with the Polident 2 denture cleanser and the effect on their viability was assessed. The viability of both *C. albicans* and Streptococcus were significantly reduced following 1 hour exposure to the denture cleanser ($P<0.05$). No viable cells were found following the 16 hour (overnight) denture cleanser soak (Table 3.1). The majority of viable cells that were cultured at 1hour were found on the high abraded surfaces.

Candida albicans				
	Control Blasto	**Control Hyphae**	**High Blasto**	**High Hyphae**
0 hour	1.40E+09 (SD=7.2)	6.40E+07 (SD=2.5)	1.70E+09 (SD=5.7)	9.50E+07 (SD=4.1)
1 hour	7.00E+03 (SD=1.3)	0 (SD=0)	4.00E+03 (SD=3.5)	3.30E+04 (SD=3.1)
16 hours	0 (SD=0)	0 (SD=0)	0 (SD=0)	0 (SD=0)
Streptococcus oralis				
	Control Blasto	**Control Hyphae**	**High Blasto**	**High Hyphae**
0 hour	2.90E+09 (SD=1.8)	3.10E+09 (SD=4.7)	2.00E+09 (SD=3.6)	1.80E+09 (SD=5.3)
1 hour	0 (SD=0)	0 (SD=0)	0 (SD=0)	1.10E+04 (SD=2.7)
16 hours	0 (SD=0)	0 (SD=0)	0 (SD=0)	0 (SD=0)

Table 3.1 The viability of *C. albicans* and Streptococcus oralis cultured from mixed biofilms (*C. albicans* and *S. oralis* ± hyphae) following soaking at room temperature in Polident denture cleanser for 0, 1 and 16hour. Data based on three repeat experiments each consisting of five replicate test surfaces with mixed biofilms (n=15).

3.3.7 XTT viability assay

The viability of hyphal or blastospore biofilms on abraded denture surfaces was additionally investigated via the XTT assay. The hyphal biofilms produced higher spectrophotometer readings ($p \leq .05$) than the blastospore biofilms (Figures 3.14-3.15) after exposure to either the denture cleanser or water after 1 or 16 hours. The higher readings correspond to higher metabolic activity and thus a higher number of viable cells (or more cell biomass as indicated in dry weight measurements) in the hyphal biofilms than in the blastospore biofims after treatment. After 16 hours incubation, both the blastospore and hyphal biofilms that had been soaked in denture cleanser had reduced activity ($P \leq .05$), compared to 1 hour. The corresponding biofilms that had been soaked in water remained the same and were higher than those soaked in denture cleanser (Figures 3.14 – 3.15).

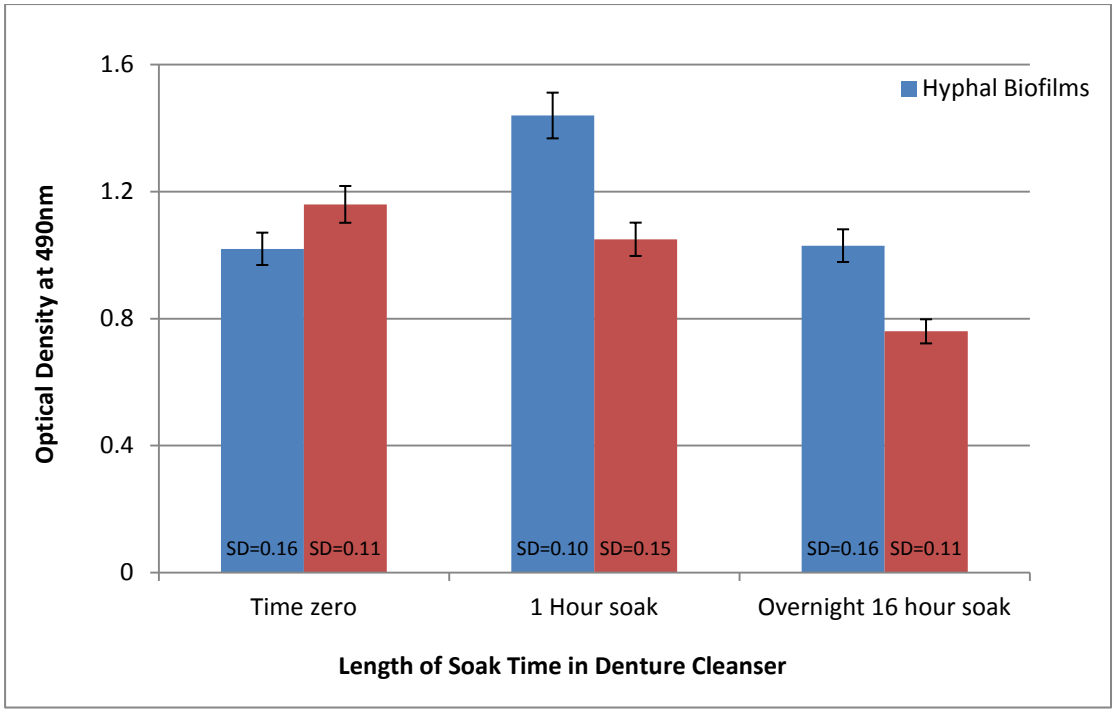


Figure 3.14 Effect of soaking in 'Polident' denture cleanser (0,1 and 16 hours) on the viability of *C. albicans* biofilms grown from either adhered Blastospore or Hyphal cells. Viability was determined using XTT assay and measured by optical density (greater OD = greater viability). Five replicate surfaces with either hyphal or blastospore biofilms were tested at each time point, the experiment was repeated on three separate occasions (n=15).

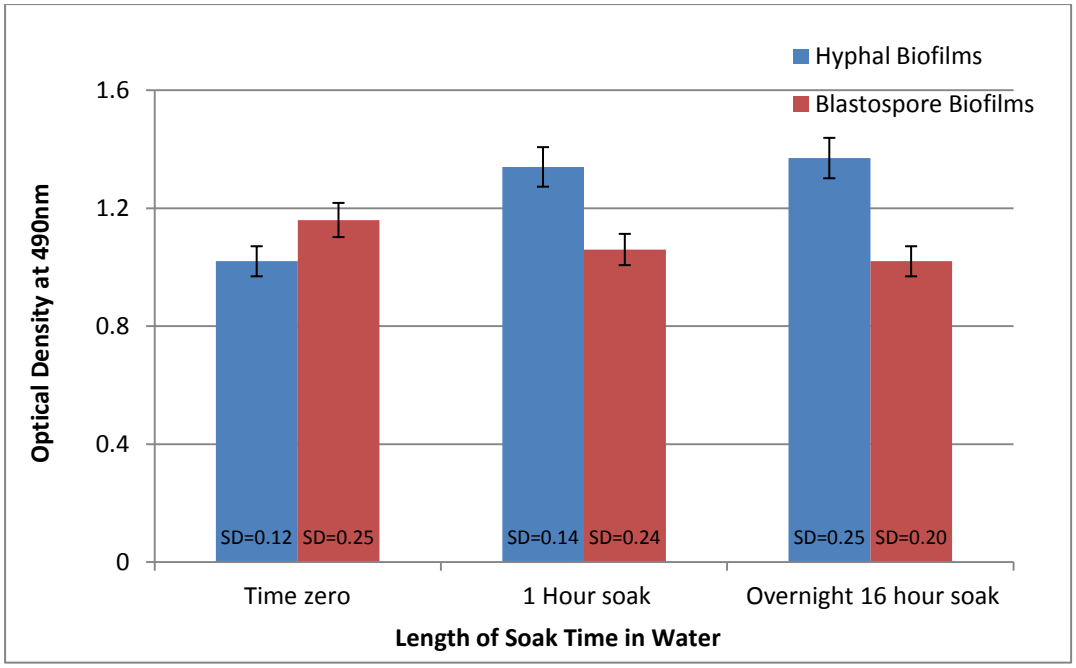


Figure 3.15 Effect of water soak (0,1 and 16 hours) on the viability of *C. albicans* biofilms grown from either adhered Blastospore or Hyphal cells. Viability was determined using XTT assay and measured by optical density (greater OD = greater viability). Five replicate surfaces with either hyphal or blastospore biofilms were tested at each time point, the experiment was repeated on three separate occasions (n=15).

3.3.8 The penetration of *C. albicans* biofilms by denture cleansers

The amount of live and dead *C. albicans* in blastospore and hyphal biofilms was determined using live dead staining (where live cells are detected as green fluorescent and dead cells fluoresce red) and confocal microscopy before treatment (at baseline/T0) and following 5 minute and 1 hour soaks in either denture cleanser or water. 16 hours was excluded as it was initially preferred to examine the effect of the denture cleanser over a short time period due to the recommended soak time of 5 minutes.

At baseline (T0) both hyphal and blastospore biofilms were viable, with the majority of fluorescence detected being green (figure 3.16). After 5minutes and 1 hour soaks in water this viability of both biofilm types was not significantly ($P>0.1$) reduced indicating little effect of water on biofilm viability. Following 5 minute (figure 3.17) and 1 hour (figure 3.18) soaks in denture cleanser, the viability of both hyphal and blastospore biofilms were significantly reduced ($P<0.05$). Although intensity analysis revealed a significant ($P<0.05$) increase in red (dead) fluorescence following five minutes soak in denture cleanser, visually biofilms continued to comprise of high numbers of viable cells. After 1 hour however this viability was, visually clearly reduced, which is consistent with results reported in sections 4.3.5 and 4.3.6.

From visual analysis and determination of the red and green fluorescent intensity there was no difference between the two biofilm types (hyphal/blastospore) under all test conditions in terms of the decrease in viability over time, although it was noted that the structure of the hyphal biofilms was more open and irregular than that of the blastospore biofilms which had been described in section 4.3.1 (figure 4.7).

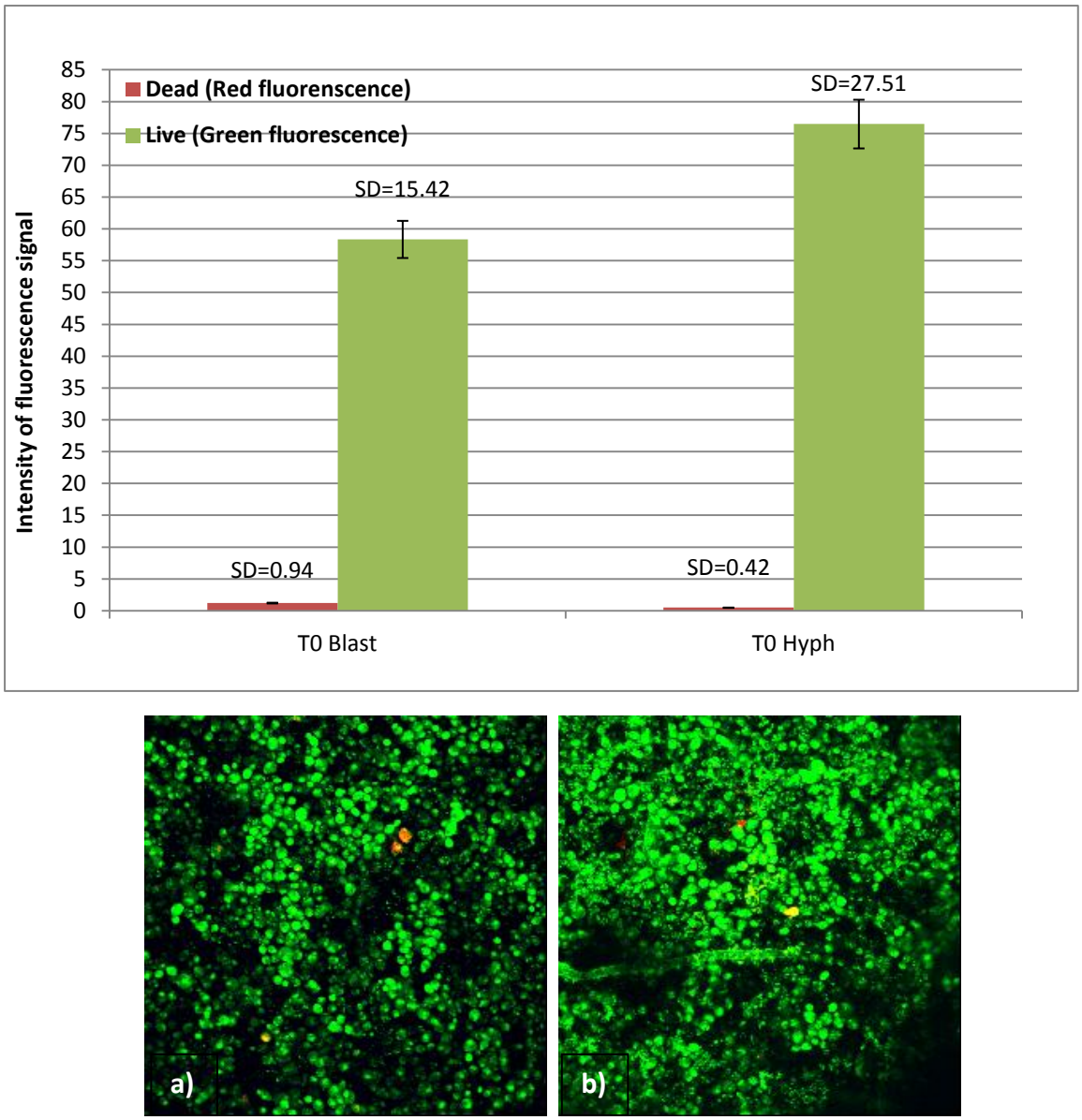


Figure 3.16 The amount and intensity of Live (green) and dead (red) *C. albicans* in biofilms grown from a) Candida blastospores or b) Candida hyphae. Graph (top) represents the average intensity of red and green fluorescence detected from biofilms at baseline (T0) before soaking in cleanser or water. Data based on fluorescence measurements taken from three replicate test pieces, in ten fields per pieces (n=30).

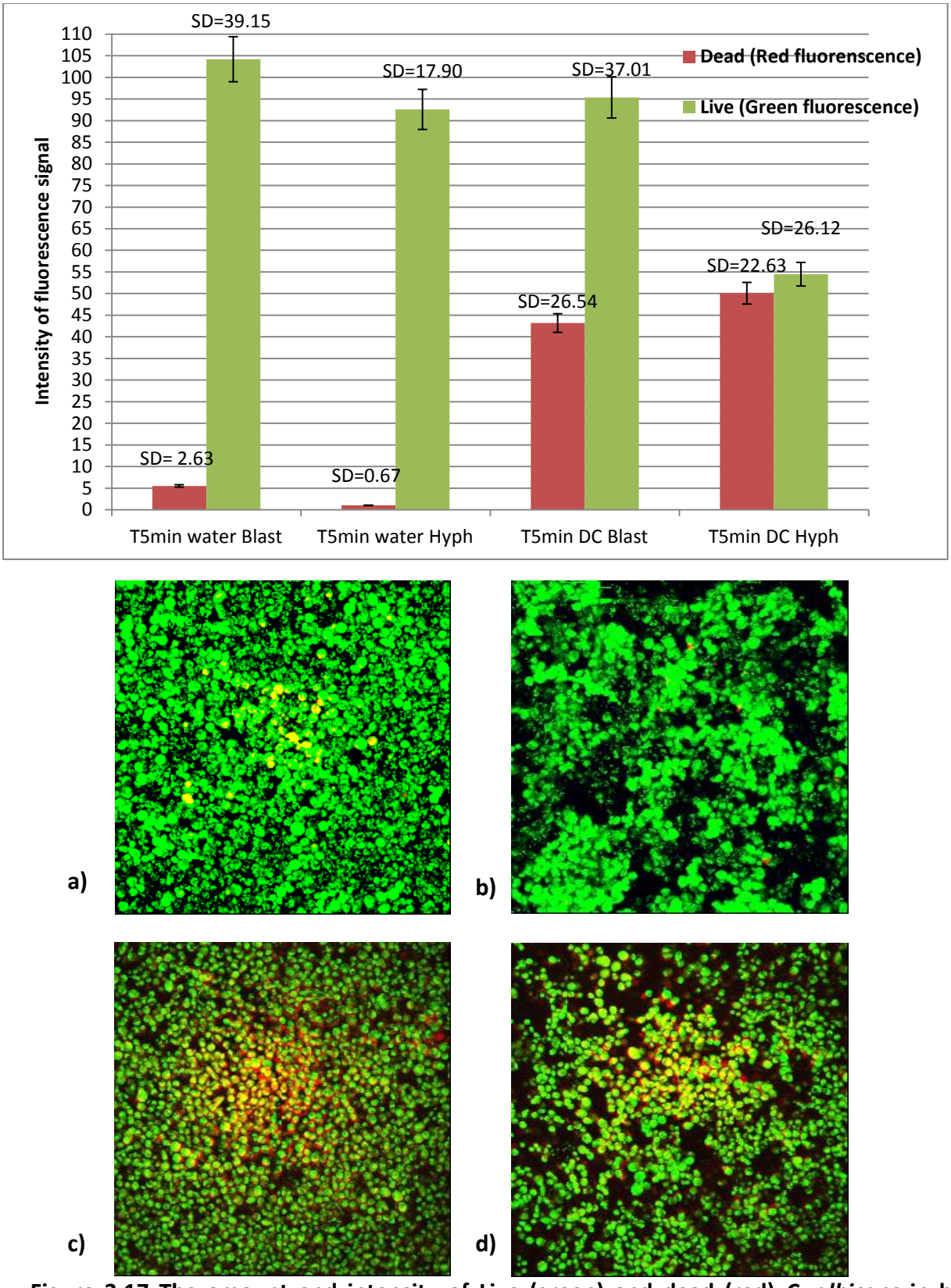


Figure 3.17 The amount and intensity of Live (green) and dead (red) *C. albicans* in biofilms treated for 5 minutes by soaking in either water of denture cleanser. Graph (top) represents the average intensity of red and green fluorescence detected from biofilms following a) 5 minute soak in water of blastospore biofilm; b) 5 minute soak in water of hyphal biofilms; c) 5 minutesoak in denture cleanser of blastospore biofilms and d) 5 minute soak in denture cleanser of hyphal biofilms (n=30).

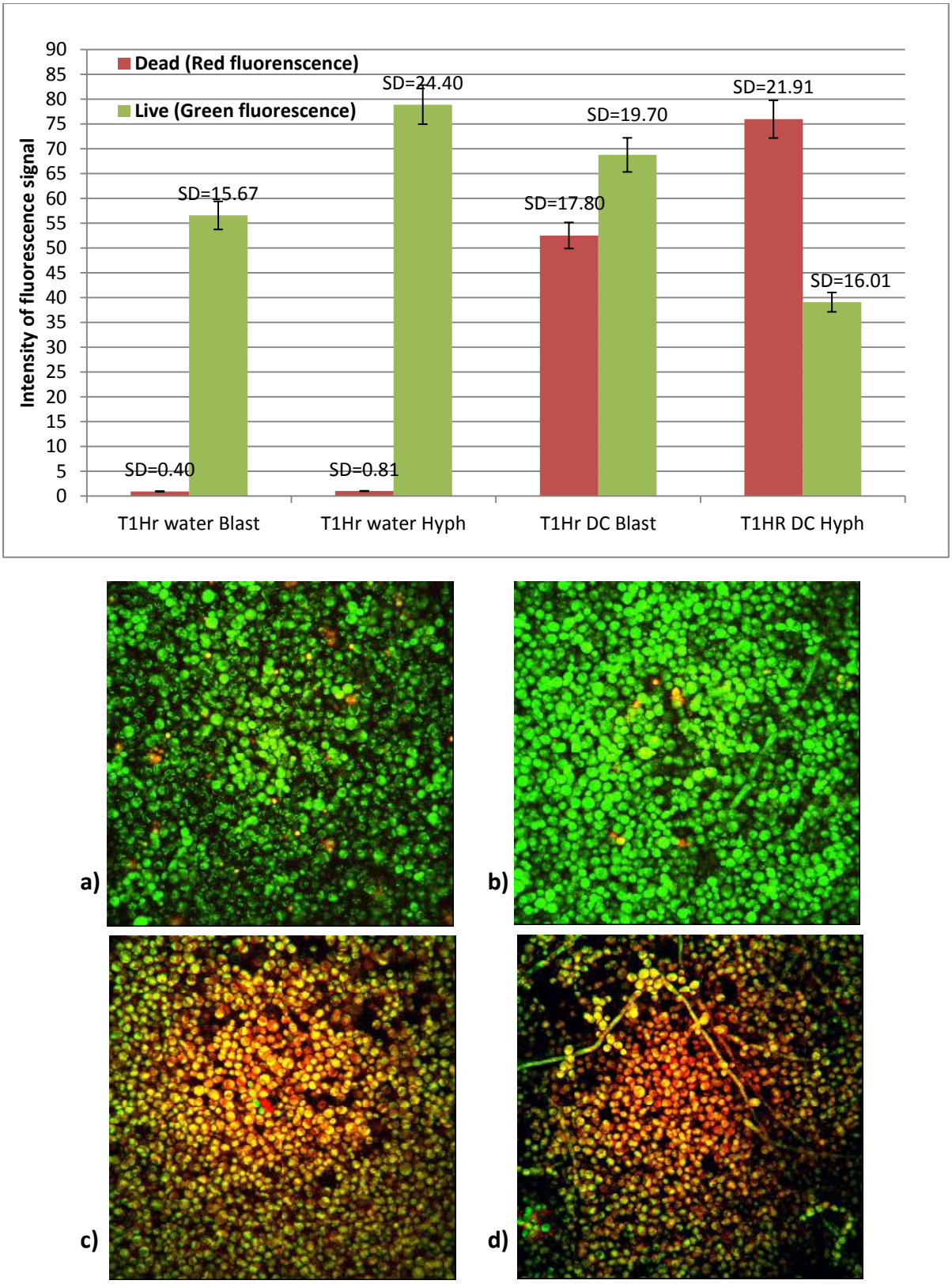


Figure 3.18 The amount and intensity of Live (green) and dead (red) *Candida albicans* in biofilms treated for 1 hour by soaking in either water of denture cleanser. Graph (top) represents the average intensity of red and green fluorescence detected from biofilms following a)1 hour soak in water of blastospore biofilm; b) 1 hour soak in water of hyphal biofilms; c) 1 hour soak in denture cleanser of blastospore biofilms and d) 1 hour soak in denture cleanser of hyphal biofilms (n=30).

3.4 Discussion

3.4.1 Morphology of *C. albicans* biofilms

The findings of this work indicated that the early presence of *C. albicans* hyphae has a dramatic effect on biofilm architecture, altering the densitiy, arrangement and orientation of biofilm cells. Biofilms of *C. albicans* have been investigated extensively, enabling description of their formation and morphology (Seneviratne and Samaranayake, 2008; Ramage *et al.,* 2001b). This work aimed to investigate how hyphae in particular, influence *C. albicans* biofilm development and sensitivity to cleanser. As has been reported in other studies (Ramage *et al.,* 2001b; Ramage *et al.,* 2005), after 48 hours, biofilms comprised complex and organized matrices containing all fungal cell morphologies. However In this study, biofilms grown from adhered hyphal cells contained more hypha-hypha contact, and hyphae were longer and more prevalent throughout the biofilm structure. Hyphal forms of *C. albicans* have been demonstrated to be important for the integrity and formation of spatially organised biofilm structures and have been shown to be important for the resistance of mechanical removal from surfaces in this and other work (Baille and Douglas, 1999). Additionally, hyphae have been shown to increase the compression strength of *C. albicans* biofilms (Paramonova *et al*., 2009) and are also thought to be more adherent than blastospores (Kimura and Pearsall, 1980); thus an advantage may be conveyed in terms of initial colonization as well as subsequent retention. The control of growth of hyphae in *C. albicans* has been shown to be influenced by quorum sensing molecules farnesol and tyrosol, whereby, at sufficient concentration, farnesol acts as a filamentation inhibitor (Ramage *et al.,* 2002a) and tyrosol acts as

an opposing molecule stimulating the filamentation pathway (Chen *et al.,* 2004). Thus it may be of interest to investigate the production and effects of these molecules on the growth of *C. albicans* biofilm from hyphal cells, in future work. This could be done by adding such molecules to adhered *C. abicans* hyphae at various stages of growth into biofilms, and examining the microscopic structures hourly, (using the confocal method described in this work) and numbers of viable cells, in order to investigate how farnesol in particular effects the formation of hyphal biofilms over time.

In addition to the differences observed in biofilm morphology, an increase in biofilm mass and reduction in the removal of cell biomass from denture surfaces was also observed for the biofilms grown from adhered *C. albicans* hyphae. The amount of biofilm was visibly different for the two biofilm types across all test substrata. This visible difference in the thickness of the biofilms however was not translated in the measurements of the Z stack height in most cases. This is likely to be due to the collapse in biofilm structure of the dehydrated biofilms (Paramonova *et al.,* 2007) during the staining protocol. It may therefore be preferable in future, to examine wet specimens with the CSLM using a water immersion objective. The use of dry weights for the different biofilms was a more successful method of measuring the difference in biofilm mass in these studies. The larger amount of biomass and the abundance of hyphae at the surface-biofilm interface and within the hyphal biofilms is likely to have conferred an advantage upon challenge by mechanical removal mechanisms and cleanser. This may help to explain why, in this study, the hyphal biofilms were less easily removed from the surfaces than blastospore biofilms. The long branching hyphae running through and across the hyphal biofilms appeared to

provide a strong structural framework that may have increased the resistance to removal of the biofilms and also demonstrated enhanced interactions between hyphae and underlying substratum topography. Oral bacterial species have been shown to adhere to *C. albicans* hyphae in *in-vivo* denture biofilm work (Nett *et al.,* 2010). The presence of hyphae may therefore also aid the retention of bacterial species in denture biofilms. Future work could utilise similar live dead staining and confocal microscopy methods in an aim to investigate the structure of mixed biofilms, the activity of denture cleansers on these, and the ease of removal of bacteria with the presence of *C. albicans* hyphae.

3.4.2 Retention of cells following biofilm removal

The numbers of cells retained on the denture acrylic resin surfaces after the removal of both hyphal and blastspore biofilms increased with increases in surface roughness, with the highest numbers seen on the most abraded surfaces. Similar findings have been reported in previous studies where surface roughness has been shown to affect initial adhesion and early colonisation (Verran *et al*., 1997*;* Radford *et al.*, 1999). However, the total amount of 48 hour biofilm on these surfaces was not affected by surface roughness. This has also been reported previously where following 4 hours incubation the surface roughness of test substrata had no additional effect on colonisation by *S. oralis* biofilms (Morgan and Wilson, 2001). Even with regular cleaning regimens, it is unlikely that the denture surface will ever be completely clear of microorganisms. If surfaces that are more roughened retain more cells following cleaning, these retained cells (if still viable) are able to proliferate upon returning to the oral cavity and the risk of developing mature

denture plaque biofilms and potentially subsequent denture stomatits is increased. Even If the retained cells lose viability, they may nevertheless provide additional attachment sites and nutrient sources for new colonizers (Wentworth *et al.,* 1991*;* Puckett *et al*., 2010), thus there is a requirement for the removal of cellular debris, with minimal disruption to surface topography for denture cleaning procedures, irrespective of their antimicrobial efficacy. These points are especially of importance to patients who may be more susceptible to infection, or those who are less able to effectively clean their dentures. In addition to this, in this study the difference in retention between the 2 types of *C. albicans* biofilm was greatest on the most abraded surfaces, suggesting an increased interaction between hyphae and the larger/more numerous features on that surface. This observation has been made previously (Lamfon *et al.,* 2003); thus, it may be that minimizing denture surface roughness by using non-abrasive or low abrasive cleansing regimens may be a step towards reducing *C. albicans* colonization of denture surfaces and denture plaque formation in general.

3.4.3 *C. albicans* and mixed biofilm viability

The denture cleanser was effective at reducing the viability of 48hour *C. albicans* biofilms following 16hours soaking on un-abraded and relatively smooth denture surfaces. There was also a significant reduction in viable cells after 16 hours on biofilms grown on high abraded/rough surfaces, but a large number of cells (approx 1.3-2.0 x 10^6 cfu/ml) remained viable. This is likely to be enhanced by the cells evading contact with the denture cleanser in niches created by the surface scratches.

As previously described this may be of concern for the consideration of subsequent contamination once dentures are returned to the mouth.

In the case of mixed biofilms, the denture cleanser was effective at reducing viable counts after one hour soak and no viable *C. albicans* or *S.oralis* cells were cultivated from surfaces following a 16hour soak. These findings indicate that *C. albicans* was more resistant to the chemical action of the denture cleanser in pure biofilms rather than mixed. In previous studies mixed biofilms have been shown to confer advantages to inhabiting organisms increasing resistance to antimicrobial agents. Adam *et al.* (2002) investigated mixed *C. albicans* and *Staphylococcus epidermidis* biofilms and found that extracellular matrix material of *S. epidermidis* was able to reduce the activity of anti fungal agents. Al-fattani and Douglas (2004) demonstrated how the bacterial matrix in mixed biofilms with *C. albicans* reduced the speed of penetration of cleanser into the biofilm. They also however indicated that this decrease in the rate of diffusion of antifungal agents did not account for the resistance seen in biofilm cells. In 2006 Al-fattani and Douglas continued their investigation into the resistance of *C. albicans* biofilms and revealed that the composition of the biofilm matrix on *Candida* spp. is an important determinant of resistance. It may therefore be that mixed biofilms tested in these studies were less viable after treatment than pure *C. albicans* biofilms due to a difference in this matrix composition caused by the presence of *Streptococcus oralis*. Alternatively, the *S. oralis* strain used in this work was quickly inactivated by the denture cleanser. *S. oralis* may have occupied spaces between and surrounding *C. albicans* cells, altering the overall organisational structure of the biofilm. The *S. oralis* cells may have been more rapidly killed upon exposure to the cleanser, disrupting the overall biofilm

structure and enhancing the accessibility of the cleanser, to *C. albicans* cells and in turn increasing their susceptibility (figure 3.19). It is not clear however whether or not the denture cleanser used here only killed the Streptococcus species or killed and removed them from the biofilm. Webb et al (1998c) examined microwave sterilisation of dentures contaminated with *Streptococcus gordonii* and *C. albicans* and found that although cells were killed, they remained attached to denture surfaces. They additionally used scanning electron microscopy to determine live and dead adhered cells based upon their morphology. They noted that microwave sterilisation resulted in morphological cell changes in both organisms indicative of cell death. This may be relevant to the investigations of this work, as the inactivation of *Streptococcus oralis* with the denture cleanser used, may also have altered cell morphologies, causing shrinkage, collapse and/or lysis which will have affected the biofilm structure. The morphology of *Streptococcus spp*. following denture cleanser treatment does not seem to have been investigated previously. It would therefore be a suitable extension of this work to perform scanning electron microscopy studies on adhered cells and biofilms of *Streptococcus oralis* and *C. albicans* in order to investigate any effects of the cleanser on the disruption of cell morphology. The effect of the inactivation of bacteria in mixed biofilms on the survival of *C. albicans* could also be investigated by exposing mixed (*C. albicans* and *S. oralis*) biofilms to antibiotics and examining the subsequent susceptibility of yeast cells after bacterial inactivation.

Live dead staining and confocal microscopy revealed the effect of the Polident denture cleanser on *C. albicans* biofilms and showed that the cleanser was

effectively able to penetrate and kill both hyphal and blastospore derived 48hour biofilms. This work should be repeated using mixed biofilms assessing the structural differences and penetration/activity of cleansers. This would enable comparison of the pure *C. albicans* and mixed biofilms structure and susceptibility to cleansers. Investigating the effects of other oral microorganisms on the activity of denture cleansers against *C. albicans* biofilms would also help to investigate this further, and help to clarify preliminary observations.

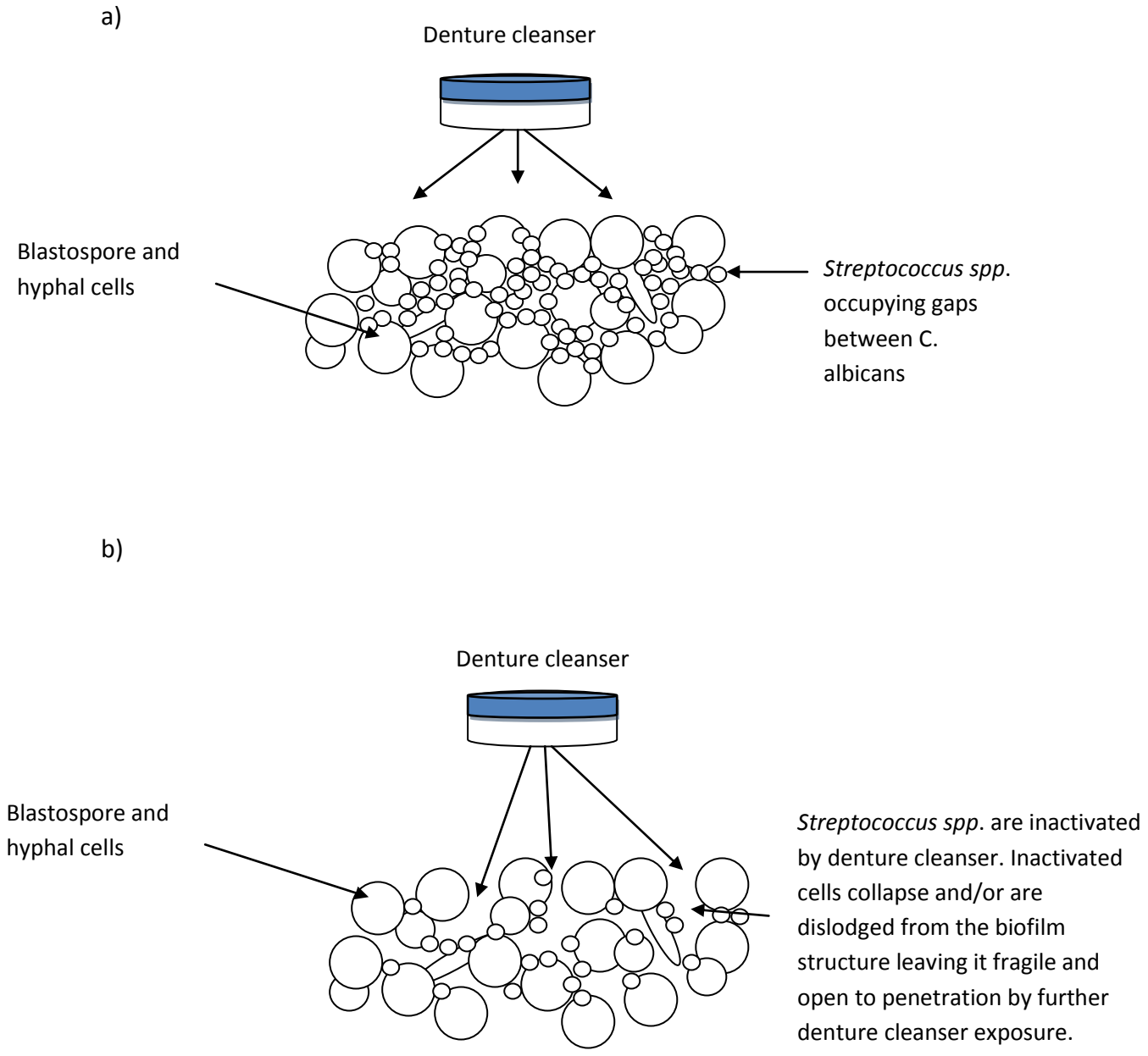


Figure 3.19 Hypothesized model for increased sensitivity of *C. albicans* in mixed (*C. albicans + S.oralis*) biofilms showing a) *C. albicans* and *Streptococcus* spp. biofilm at beginning of exposure to denture cleanser and b) following cleanser soak where susceptible Streptococcal cells have been rapidly inactivated and some removed by denture cleanser leaving *C. albicans* cells exposed. The biofilm structure is compromised, potentially decreasing its stability and providing better accessibility for the cleanser to *C. albicans* cells.

The XTT viability assay indicated higher metabolic activity in biofilms grown from hyphal phase *C. albicans* than those grown from adhered blastospores after exposure to both denture cleanser and water. However viable count data did not indicate any increased survival of hyphal biofilms. Hyphae are metabolically active (Kuhn *et al*., 2002) and therefore the large mass of hyphae within the hyphal biofilm structure is likely to have significantly influenced XTT results, with little effect on viable count data. The biofilm phenotype is known to confer various advantages by increasing resistance to antimicrobial challenges and by protecting cells from mechanical forces (Ramage *et al*., 2001b; Ramage *et al.,* 2002c; Chandra *et al.,* 2001). In comparison to the pure cultures used in this work, mixed biofilm have greatly increased complexity, the denture plaque biofilm, therefore, poses a challenge to hygiene procedures. There are many protocols available for denture cleaning (Rathee et al., 2010). Those involving the use of denture cleansers commonly consist of a simple 5 to15 minute soak, followed by a rinse. Brushing is also recommended, to ensure that plaque and debris are removed from the surface. As with these findings, some denture cleansers have been demonstrated to be effective in reducing, but not eradicating biofilms of *C. albicans* (Jose *et al*., 2010). In this work the cleanser used was tested solely for the effectiveness of its chemical formulation, without effervescence, since the focus of the assay was on viability of the cells in the *in-vitro* biofilm. This enabled comparison of the survival of hyphal and blastospore biofilms. Rinsing was also omitted as these mechanical mechanisms would clearly enhance biofilm removal and viability was the focus. The addition of a mechanical removal process such as the effervescent nature of the cleanser (Ramage *et al.,* 2012) and brushing would greatly enhance the effectiveness of the cleanser, and

regular cleansing of denture surfaces in this manner is likely to be sufficient for maintaining good denture hygiene. As well as excluding the effervescent activity of the denture cleanser, this work had additional limitations including the omission of a salivary conditioning film. Due to the different reported findings in literature, the effect of the salivary pellicle on the adhesion of yeast such as *C. albicans* is not clear. Some studies suggest the addition of a pellicle enhances adhesion. Edgerton *et al.* (1993) reported that the addition of submandibular-sublingual saliva promoted the adhesion of *C. albicans* to PMMA. Similarly, Nikawa *et al.* (1997) found that coating of acrylic surfaces with whole unstimulated saliva increased the activity of *C. albicans* biofilms. In contrast to these finding however, whole un-stimulated saliva has been reported in a number of studies to inhibit the adhesion of *Candida* spp. (McCourtie *et al.,* 1986; Samaranayake and MacFarlane, 1980; Samaranayake *et al.,* 1980). The difference in reported findings suggests that further work to clarify the effects of the salivary pellicle on the adhesion of *Candida* spp. to denture materials with different topographies is required and it may be interesting to investigate the effect of a salivary pellicle on the adhesion and subsequent biofilm formation of *C. albicans* hyphae and blastospores further in future studies.

In this work, extended soak times with the denture cleanser of 1 hour and 16 hours were used compared to the 5 minute soak recommended by the manufacturer. These extended soaks were incorporated in order to challenge the 48hour *C. albicans* biofilms, and although far removed from the recommended soak time, these parameters may be comparable to an overnight soak: in any case, the work enabled the viability of the different biofilms to be explored further.

3.4.4 Live dead staining of hyphal and blastospore biofilms of *C. albicans*

In this study Live dead staining coupled with confocal scanning laser microscopy was used to investigate the penetration and kill of hyphal and blastospore biofilms following soaks in either denture cleanser or water over set times (0,5 and 60 minutes). In terms of the activity of the cleanser against both biofilm types the results show a slight reduction in viability (as indicated by an increase in red fluorescence) following a 5 minute soak in denture cleanser compared to the water control and a further, enhanced reduction and contrast to control soaks following one hour soak in denture cleanser. These findings concur with XTT and viable count data (described previously): the denture cleanser was found to effectively kill but not remove *C. albicans* biofilm.

In previous work (section 4.3.6-4.3.7), the quantification of *C. albicans* viability has been achieved directly using viable cell counts and indirectly by spectrophotometer measurement of formazan coloured biproducts of metabolically active cells with the use of the XTT assay. These methods have been employed in similar studies investigating the efficacy of anti microbial treatments on *C. albicans* planktonic cultures and biofilms (Silva *et al.,* 2008; Ramage *et al.,* 2001a, 2001c) and have been proven to provide data that can be easily and accurately compared. These methods however do not allow for the direct visualisation and examination of antimicrobial activity and the effect on live biofilm structures, as was used in this work.

3.4.4.1 Evaluation of technical and software capabilities

Image J software allowed for the semi quantification of fluorescence in generated images by producing an intensity reading for red and green fluorescence in each. This enabled graphical representation of live and dead cells within biofilms grown from

the two cell types. Results indicated a small decrease in the proportion of live to dead cells (green to red fluorescence intensity) in hyphal biofilms compared with blastospore biofilms, which would in turn indicate that the hyphal biofilms were slightly more susceptible to the activity of the cleanser than the blastospore biofilms (in pure *C. albicans* cultures). The structure of these biofilms was notably more open due to the high hyphal content which may have allowed greater access of the denture cleanser into the biofilm mass. Hyphal biofilms were however also found to be more difficult to remove from denture surfaces, with more cells being retained following removal (described previously), which may be significant for re-colonisation. The increased susceptibility of hyphal biofilms as indicated by the Image J analysis of CSLM images is based on a fluorescence intensity reading but is not a quantification of the actual number of red and green fluorescing cells. The use of further image analysis software with the capability of quantifying red and green pixels in these images may help to provide more conclusive data. In addition to this similar limitations of the use of image J to those described for the use of percentage coverage analysis (as opposed to adhered cell counts) (chapter 2), were observed. The analysis of images using image J relied upon the manual setting of fluorescent thresholds for the red and green fluorescent channels. The software split the image into red and green fluorescing channels the intensity of which could then be manipulated. This factor of the use of image J for this type of analysis leaves the images open to interpretation by the operator and as such allows for the interference of human involvement. This is of particular focus in areas of image where both red and green fluorescence are detected, known as co-localisation (Roberts *et al*., 2010; Sommerfield *et al.,* 2012). Here dead or compromised cells take up both red and

green stains and as a result appear yellow. This result can easily be manipulated using the threshold settings for red and green cells and introduces susceptibility to human error.

The incorporation of the use of the water immersion lens allowed for biofilms to be viewed in their hydrated forms resulting in minimal disruption of their structure and arrangement. With the use of the water immersion lens, the image of biofilms was however, easily disturbed and highly sensitive to vibrations. With confocal scan times of around 60-90 seconds this often made image capture difficult. Additionally it was noted that in some captured images there was an increased detection of red fluorescence centrally, which indicated an effect of the laser on *C. albicans* viability. The photosensitization of *Candida* spp. has been described before (DeSouza *et al.,* 2006) and this contributing factor may have influenced results, especially over the course of 60-90 second scans. In future work it would be prudent to limit the exposure to laser emission as much as possible, minimising the time taken to find and focus a region of interest and perhaps increasing the Z stack scan heights from 2µm to 4µm to in turn reduce the scan times. Overall the method used in this work provided a means of visualising and interpreting the activity of a novel denture cleanser on *C. albicans* biofilms grown from hyphal and blastospore cells.

3.5 Conclusions

A method was developed for the analysis of the penetration and activity of denture cleansers on *C. albicans* biofilms. The results supported previous findings indicating that the method was useful for the gathering of qualitative data to support quantitative analysis of antifungal effects. This method could be improved in future work and used in other applications including the investigation on other antimicrobials and different microbial communities.

The early presence of hyphae influences the structure and organisation of subsequent *C. albicans* biofilm on denture acrylic surfaces. Biofilms developed from adherent hyphal cells have greater biomass and are less easily removed from denture acrylic surfaces than those developed from and more abundant in *C. albicans* blastospores.

In the case of both hyphal and blastospore biofilms, more cells were retained on surfaces that were more heavily abraded, post treatment/removal. The commercially available denture cleanser tested had good chemical activity against both *C. albicans* hyphal and blastospore biofilms but was not effective at completely eradicating live cells from the denture surface, especially in cases where there was increased surface roughness. These findings suggest the recommendation that hygiene protocols involve a low abrasive mechanical cleansing method in addition to the use of a chemical cleanser. This type of cleaning regime should be carried out regularly with a view to minimising denture surface contamination whilst limiting the change to surface topography. Denture cleansers should target physical removal of

organisms from the denture surface (with minimal surface damage) as well as having a chemical inactivation effect in order to maintain good denture hygiene.

Chapter 4

Quorum sensing in *Candida albicans*

4.1 Introduction

4.1.1 Quorum sensing in *C. albicans*

Quorum sensing in *C. albicans*, is an expanding area of research with the majority of focus currently being on the quorum sensing molecule farnesol. Farnesol is a naturally occurring organic compound described as being an acylic sesquiterpene alcohol, insoluble in water and isolated regularly from essential oils such as cintronella, rose, acacia and Seville orange oil (*Agarwal. Chemistry of organic natural products, 14 edition, 2010;* Derengowski *et al.,* 2009). In 2001, Hornby *et al.,* identified farnesol as a quorum sensing molecule in *C. albicans* highlighting its activity at certain concentrations above a threshold (30-35 μM) in the suppression of hyphal development in spite of the presence of hyphal inducing triggers, but with no effect on the growth rate of the yeast. This was supported by the work of Ramage *et al*., (2002a) that also described the reversible inhibitory effect of farnesol on hyphal production and additionally showed that although farnesol inhibits the initiation of hyphal growth it does not prevent the elongation of already existing hyphae. Since the identification of farnesol as a quorum sensing molecule in *Candida* spp. there has been increasing focus on its role and influence on other microorganisms. Farnesol produced predominantly by two *Candida* spp., *C. albicans* and *C. dubliniensis* (Martins *et al.,* 2007; Weber *et al.,* 2008) has been shown to have an inhibitory effect on other microbial species including yeasts (Machida *et al*., 1999), fungus (Semighini *et al.,* 2006; Derengowski *et al*., 2009) and bacteria (Koo *et al.,* 2003). This function has been implicated as a means of minimising the competition for growth conditions. Farnesol has been shown to enhance the activity of antibiotic treatments

on resistant strains of *Staphylococcus aureus* and *Escherichia coli*. Brehm-Stecher and Johnson (2003) demonstrated that the presence of farnesol disrupted the cytoplasmic membranes of these two species causing increased permeability and susceptibility to antibiotic therapies. This was further supported by work by Jabra-Rizk *et al.* (2006), which also showed membrane disruption of *S.aureus* by farnesol. The production of farnesol by *C. albicans* may therefore convey advantages, suppressing the growth and increasing the susceptibility of competitors. However at certain concentrations farnesol has also been identified as an inducer of apoptosis in *C. albicans* (Shirtliff *et al.,* 2009) and is also known to reduce biofilm formation (Ramage *et al.,* 2002a, 2002b; Jaba-Rizk *et al.,* 2006). Ramage *et al.,* (2002a), demonstrated that the addition of farnesol at high concentrations (300 µM) inhibited biofilm growth in *C. albicans*. They demonstrated that the effect of this molecule is concentration dependant and hypothesized that it may have a role in biofilm dispersal once the accumulation of cells reaches a threshold level. In addition to the effect on microbial cells, farnesol has been implicated in the enhancement of pathogenicity and the suppression of host defences by *Candida* spp. Navarathna *et al*. (2007a and 2007b) reported increased susceptibility to systemic candidiasis in mice infected with *C. albicans* that had an increased ability to produced farnesol. They further noted that administration of farnesol to mice suppressed lymphocyte cell functions. In a more recent study by Abe *et al*. (2009) the efficacy of murine macrophages to inhibit mycelia growth of *C. albicans* following their cultivation in 56-112 µM of farnesol was investigated. Their findings demonstrated decreased activity of macrophages against *C. albicans* and additionally demonstrated intracellular production of reactive oxygen species (linked with cell apoptosis),

nucleic change and DNA fragmentation indicating damage to the macrophages caused by farnesol. The apparent promotion of reactive oxygen species in macrophages by farnesol is interesting when put into context with other work. Westwater *et al*., (2005) reported a link between farnesol and resistance to oxidative stress by hydrogen peroxide and superoxide anion generating agents. Farnesol has also been shown to convey a protective response by increasing chlamydospore production in *C. albicans in-vitro*. This increased production may be linked to increased resistance to the effects of reactive oxygen species due to the robust nature of this cell morphology (Martin *et al*., 2005). The collective works on the influences and effects of farnesol production provide invaluable evidence that farnesol is an integral auto regulatory molecule for *C. albicans*, influencing and controlling a number of cellular and external activities including; overpopulation, morphogenesis, competition by other species, assistance in withstanding host defences and dissemination, and thus contributing to its ability for adaptation and survival.

4.1.2 Non-farnesol quorum sensing molecules

Although farnesol is evidently an important molecule, other compounds have also been associated with quorum sensing in *C. albicans*. The role of tyrosol is currently mainly associated with the resumption of growth to exponential phase from lag phase (Chen *et al.,* 2005) and the promotion of mycelial morphologies. Alem *et al.,* (2006) investigated the effects of both tyrosol and farnesol on *C. albicans* biofilms. They found that in early stages of biofilm development (1-6 hours), tyrosol stimulated hyphal growth. The presence of tyrosol and farnesol were variably

effective dependant on the age of biofilm. Tyrosol was reported to continue to have an effect up to 14 hours of growth following which the effects of farnesol became apparent. Farnesol effects were also noted to dominate in 24 and 48 hour biofilms, indicating increased activity of farnesol in later stages of biofilm growth. Farnesol and tyrosol thus appear to have opposing effects. Tyrosol stimulates hyphal growth in early cultures allowing for biofilms to become established, and as the concentration of farnesol increases in later stages of biofilm development the initiation of hyphal growth is inhibited reducing overpopulation. These findings contribute towards the evidence that these two molecules are produced and utilised as auto regulators, maintaining a homeostatic level of growth in the dimorphic yeast. Dodecanol, a 12 carbon alcohol, is structurally similar to farnesol and has been shown to have the same hyphal inhibiting influence, but this molecule is not deemed to be of physiological relevance (Davis-Hanna *et al.,* 2008; Hogan *et al.,* 2004; Hall *et al.,* 2011). Nerolidol, an ethyl alcohol, has also been implicated in the inhibition of hyphal production in *C. albicans*, especially in later stages of biofilm development (Hornby *et al*., 2001; Martins *et al*., 2007; Chauhan *et al.,* 2011), no additional roles for these molecules have yet been identified. Although not described as a quorum sensing molecule, ethanol is also readily produced by growing *C. albicans* cultures as a by product of fermentation (Pappagianis and Marovitz 1996; Chang and Kollman, 1998) and has been linked with the promotion of germ tube production (Pollack and Hashimoto, 1985).

4.1.3 Identification of quorum sensing molecules from *C. albicans*

In previous works different methods have been used for the identification of farnesol in microbial cultures. In 2001 Hornby *et al.* isolated a quorum sensing molecule from the supernatant of growing yeast cultures with an aim to identify it. Their initial work utilised thin layer chromatography where silica gel plates with a fluorescent indicator and liquid mobile phase allowed for the separation and identification of the organic functional groups within their isolated substance. The thin layer chromatography revealed clues about the compound indicating that it had multiple carbon-carbon bonds, a free hydroxyl group and no ester bonds (Hornby *et al.,* 2001). Further tests utilised gas chromatography coupled with mass spectrometry (GC-MS). Gas chromatography (section 4.2) used commonly in chemistry for the separation and identification of volatile organic compounds in a given sample. In their work, this method was invaluable. Gas chromatography of the isolated compound revealed three peaks at 9.8, 9.9 and 10 minutes. These peaks were analysed with mass spectrometry which indicated that they were isomers of one compound. Further comparison to database of known compounds identified these isomers as the 15 carbon sequiterpene, farnesol. This method for the identification of volatile compounds has also been applied in other studies. Buzzini *et al.,* (2003a) utilised Gas chromatography with solid phase microextraction (SPME) to identify volatile organic compounds. SPME (section 4.2.4) utilises a needle onto which volatile organic compounds are absorbed and concentrated before being placed into the gas chromatograph for identification. Using this extraction method, Buzzini *et al*. Identified volatiles produced by 98 ascomycetous yeast strains, finding an abundance of alcohols, aldehydes and esters generated over 72 hours of growth.

Other work by Buzzini *et al.,* in (2003b) utilised the same method (Gas chromatography with SPME) to analyse volatile organic compounds produced by *Candida oleophila*. In this study they noted how growth in different conditions resulted in different volatile organic compounds being produced and identified. Martins *et al.,* (2007), also adopted SPME GC-MS to analyse volatile molecules produced by *Candida* spp. (*C. albicans* and *C. dubliniensis*) and found that profiles generated following analysis were dependant on species, culture mode and growth time.

4.1.4 Aims

To investigate the production of quorum sensing molecules by *C. albicans* and their effect on adhesion and hyphal growth on abraded denture surfaces.

The specific objectives included:-

- To Investigate the quorum sensing molecules released over time by growing *C. albicans* cultures using gas chromatography (SPME GC-MS)
- To Investigate any potential differences in volatile quorum sensing molecules produced by blastospore and hyphal biofilms of *C. albicans*
- To investigate the effect of farnesol on adhesion and biofilm formation by *C. albicans*

Research questions

What volatile compounds are released from planktonic cultures and biofilms of *C. albicans* as they grow over time?

Can the known quorum sensing molecule farnesol be detected from *C. albicans* cultures using gas chromatography?

Does the presence of *C. albicans* hyphae effect what volatile compounds are given off by *C. albicans* biofilms and planktonic cultures?

H0

The presence of *C. albicans* hyphae in planktonic and biofilm cultures will have no effect on the volatile compounds given off and detected using gas chromatography

4.2 Materials and Methods

4.2.1 Maintenance of cultures

A stock culture of *C. albicans* NCYC 1467/ GDH 2346 was stored at -80°C. Subcultures were prepared on Sabourauds dextrose agar (Oxoid ltd, Hampshire, UK) and refrigerated at 4°C prior to use. These cultures were replaced every four weeks.

4.2.2 Optical tweezer microscopy

Optical tweezer manipulation was pioneered by A Ashkin *et al*., (1986) and first used on bacteria in 1987 (Ashkin and Dziedzic, 1987). It encompasses a method using forces exerted by strongly focussed beams of light to trap and manipulate small particles (Grier, 2003). Since its first use, optical tweezer research has revealed ever expanding applications in chemistry, physics and biology, where small particles, atoms and biological matter play integral roles. In the biological field, applications for optical tweezer microscopy have included the investigation and manipulation of DNA (Wang *et al.,* 1997; Bustamante *et al*., 2000), enzymes and motor movement (Svoboda *et al*. 1994), plant cell reorganisation (Ashkin and Dziedzic, 1989) and the manipulation of bacteria, viruses (Ashkin and Dziedzic, 1987; Block *et al*. 1989) and yeasts including the manipulation of intracellular organelles (Sacconi *et al*., 2005). Arneborg *et al* (2005) utilised optical tweezers to investigate the effect of confinement on the growth of yeast cells. Using a dual laser optical tweezer microscope they positioned cells of the yeast S*accharomyces cerevisiae* in a circle around single cells of the yeast *Hanseniaspora uvarum* and found that this confinement inhibited growth. A similar method was designed for this study, in order to assess whether there was any effect of neighbouring cells on the directional

growth of *C. albicans* hyphae. A dual beam optical tweezer microscope (BioPhotonics workstation, KU life, Copenhagen Denmark) was utilised in order to investigate the directional growth of *C. albicans* hyphae. The optical tweezer (Figure 4.1) utilises two opposing objectives that focus laser beams to create traps, allowing for the direct manipulation (picking up, repositioning, moving and rotating) of living cells (Arneborg *et al*. 2005; Aabo *et al*. 2010).

One colony of *C. albicans* GDH 2346 was inoculated into 50ml sterile Sabouraud's Dextrose broth and incubated at 37^{o}C in an orbital shaker (250rpm) overnight. 1ml of the resulting cell suspension was subsequently removed, spun down for 30 seconds in a desktop centrifuge (Sigma-Aldrich) and washed once in sterile water. Cell were then re-suspended in 1ml of sterile water and diluted down to 10^{-4} (approximately 1.02 ± 0.02 x 10^{2} cells/100µl, determined from plate counts of CFU). Approximately 70 µl of the prepared cell suspension was inoculated into a growth chamber which consisted of a glass perfusion chamber (CoverWell, Grace-Biolab) sealed against a glass coverslip (Menzel-Gläser #1, 0.15 x 24 x 40 mm). The glass cover slip forms the base of the chamber, onto which the cells fall and attach. Initially the glass slips were used as provided, but problems were encountered since cell adhesion to glass surfaces was difficult to attain. Thus, thin strips of a protein coating were placed on the surface to encourage cell adhesion. A thin line (1-2 mm), stencil was created on the glass slide with two strips of adhesive tape. A 0.8 mg/ml solution of Concanavalin A (Sigma-Aldrich) was inoculated onto the cover slip and allowed to dry for 30 minutes at room temperature. Subsequently the tape stencil was removed and the resultant coated strip on the cover slips was used. When used as part of the sealed perfusion chamber this presented a highly adherent centre

region but also areas where cells could be manipulated either side of the Concanavalin A line (Figure 4.2). A 100 µl pipette was used to inoculate the prepared perfusion chamber with 70 µl of the standardised suspension. This chamber was the secured onto the microscope stage with adhesive tape to prevent movement during manipulation. Cells were visualised through the computer interface, and laser manipulation was used to place cells in close proximity to one another, after which they were left to adhere to the bottom of the cover slip (<10 seconds). Once cells had been placed, the perfusion chamber was flooded with 25 % horse serum (diluted with sterile water) and then the two pores of the perfusion chamber (Figure 4.2) were sealed with adhesive seal tabs (Hybriwell™, Hybridisation sealing system, Invitrogen, USA). Following this, the microscope stage was heated to approximately 37°C and the computer image was adjusted accordingly in response to the stage expansion. The set up was observed over 2-3 hours with images taken every 5 minutes following the development of hyphae. In total 12 experiments were carried out, with 2-10 cells observed in each experiment.

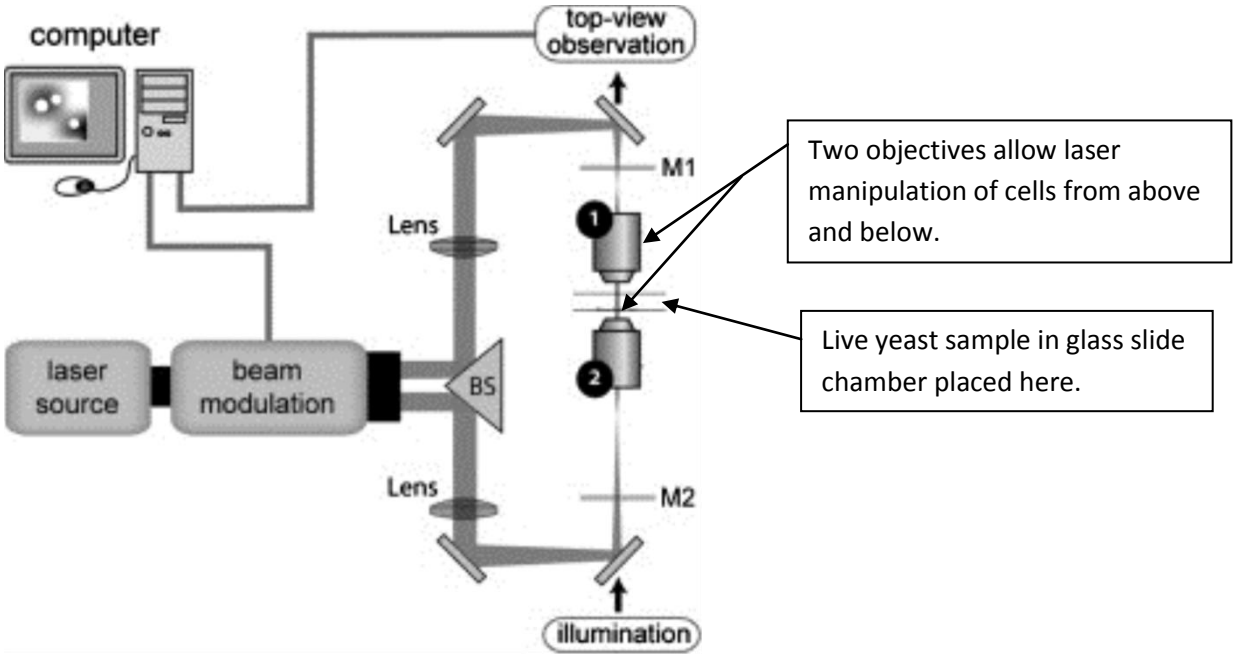


Figure 4.1. **Diagrammatic representation of BioPhotonics workstation, Aabo *et al*., 2010.**

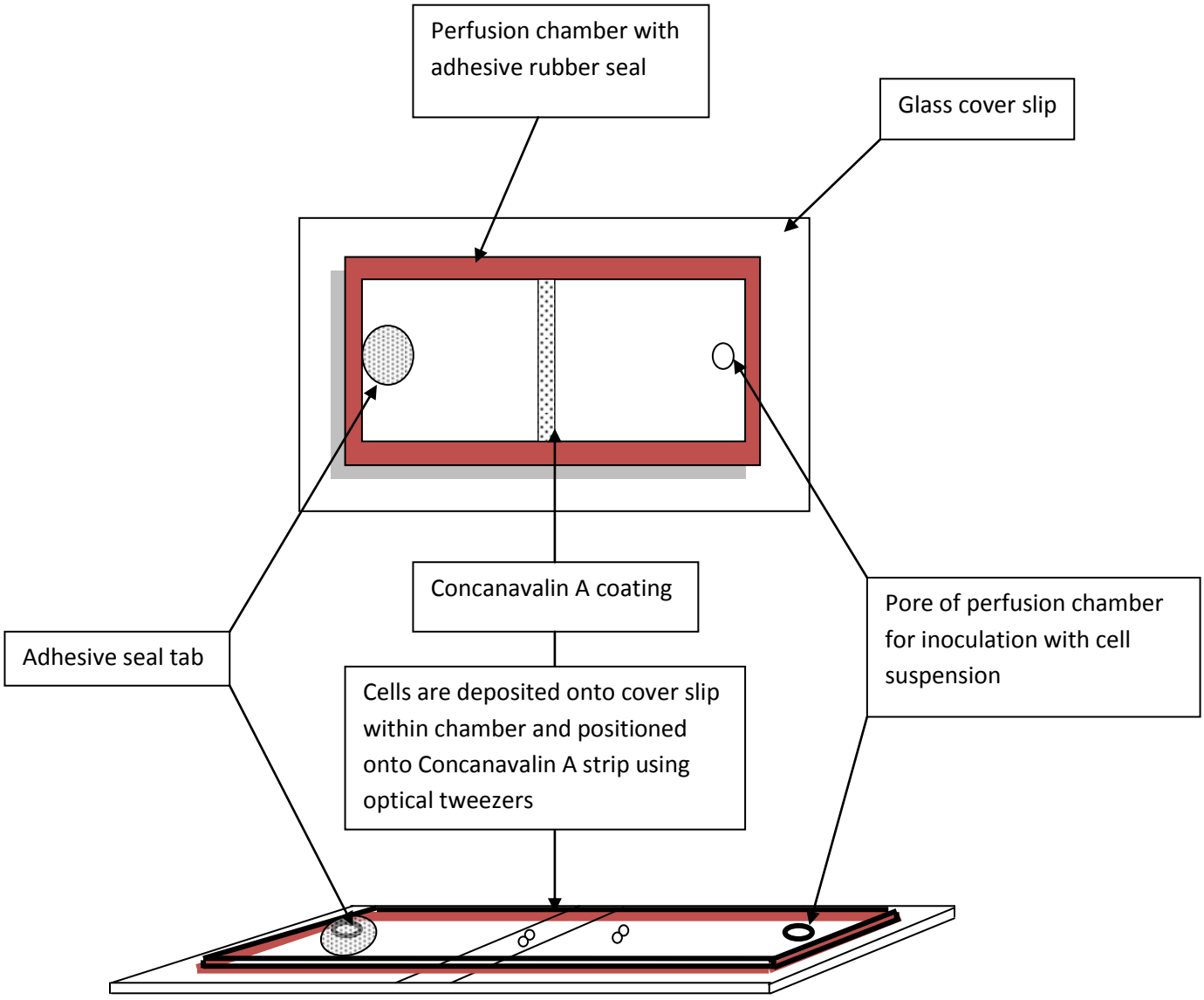


Figure 4.2. Diagrammatic representation of the glass slide and perfusion chamber set-up from above (top) and from the side (bottom), used for experimental studies with the optical tweezer microscope.

4.2.3 Detection of quorum sensing in *C. albicans* by gas chromatography

In order to investigate the quorum sensing molecules released by planktonic cultures and biofilms of *C. albicans* gas chromatography coupled with mass spectrometry and solid phase micro extraction (SPME GC-MS) was employed.

Gas chromatography coupled with mass spectrometry allows for the separation and detection of volatile substances within a mixture. A small amount of a sample can be injected by syringe directly into the head space of the chromatograph which is set to a higher temperature than the sample's boiling point and therefore causes its immediate evaporation within the injector port (Figure 4.3). An inert carrier gas such as helium or nitrogen (liquid phase) is then used to force the vaporised sample onto the GC column (solid phase). In this region the sample is separated as the different molecules within the sample partition between the carrier gas and the column at varying speeds/times depending on their size. They are carried and separated along the length of the column until they reach the detector which sends a signal to a chart recorder producing a peak that represents the component of the sample being detected. Molecules in a sample will be separated at varying rates and are therefore detected at different times providing a profile of peaks for a given sample. These peaks can then be compared to known standards for identification. The area of the peak represents the concentration of each molecule in a given sample being detected and therefore allows for some quantification in comparison to known standards (Luo *et al.,* 2007).

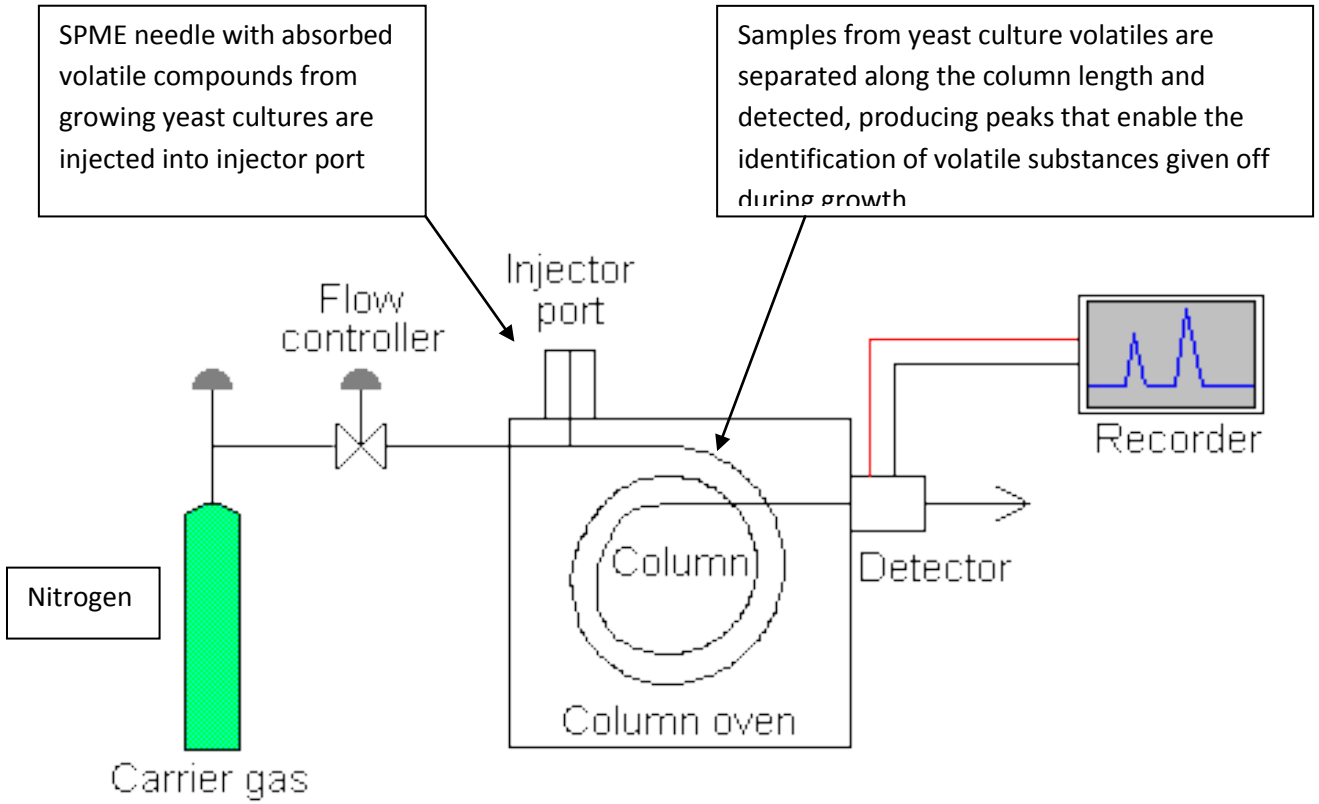


Figure 4.3 Diagrammatic representation of Gas chromatography system. Samples are injected into the injector port and transported by the carrier gas onto and through the column. Different molecules in a sample move through the column at different speeds until they reach the detector which sends a signal producing a peak for each molecule (*Sheffield Hallam University, teaching.sch.ac.u*k).

4.2.4 Gas chromatography programme for volatile identification

For the identification of volatiles produced by growing *C. albicans* cultures a pre-loaded settings programme was set up on the gas chromatograph. The column temperature was initially maintained at 70°C for 10 minutes to allow for the full desorption of volatile compounds from the fibre. It was then increased to 270°C at a rate of 10°C per minute, and held at this temperature for 3 minutes. The injector and detector temperatures were set to 270°C. The injector was split less with a flow rate set to 100 ml per minute. The resulting run time for each sample was 33minutes. This method was developed from a previous method used to identify volatile compounds produced by fungal isolates (Bingley *et al.*, 2012).

4.2.5 Solid phase microextraction (SPME)

Solid phase microextraction was the method employed for the capture and injection (into the chromatograph) of volatile compounds from yeast cultures in these studies. SPME utilises a polymer-coated fused fibre to absorb volatiles given off from a sample. These volatiles are then desorbed from the fibre upon injection into the heated chromatography injector port. Analytes are concentrated on the fibre, and are rapidly delivered to the column where they are separated out as described previously.

In these studies a SPME needle containing a 2 cm long fibre consisting of a polydimethylsiloxane bonded flexibe fused silica core coated with 50/30 divinylbenzene / carboxen (Supelco, Sigma Aldrich, Dorset, UK) was used. The needle was inserted into sealed vials containing sample aliquots and the needle fibre was exposed to the headspace above the sample for 15 minutes to allow any volatile

compounds from the sample to be absorbed and concentrated on the fibre. Immediately following absorption the fibre was retracted into the needle, the needle removed from the vial and injected into the gas chromatograph injector port, immediately followed by the commencement of the preloaded GC-MS programme.

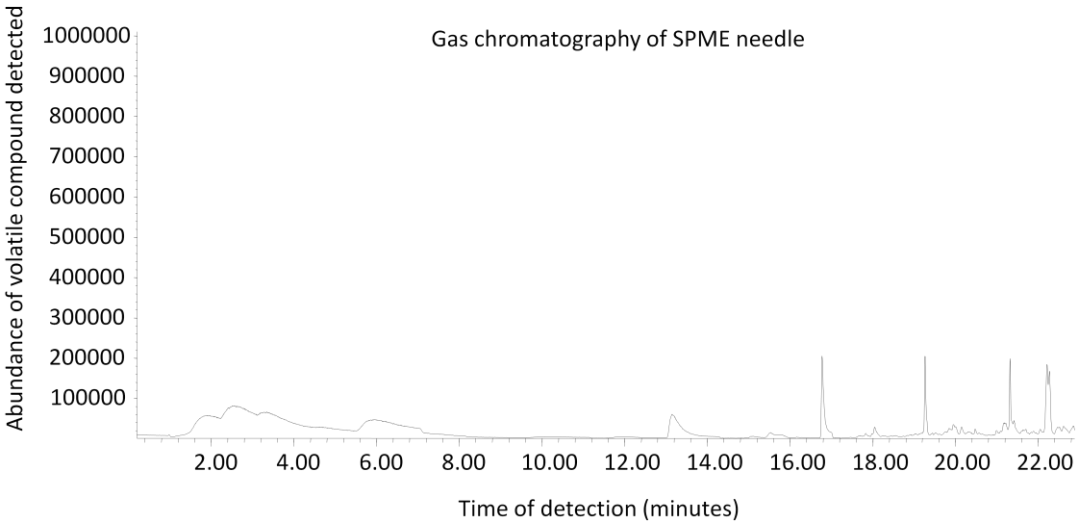
4.2.6 Preparation and analysis of *C. albicans* cultures

For planktonic cultures, one hundred millilitres of SAB broth in a 200 ml conical flask was inoculated with one colony of *C. albicans* GDH 2346, cultured on SAB agar freshly from frozen culture 48 hours prior to use. The inoculated medium was incubated in a 37°C orbital water bath set to 100 rpm. 2 ml of the growing yeast culture was removed from the conical flask every hour, 30 minutes prior to the hour time point, and placed into 10 ml head space vials (Sigma Aldrich) which were immediately sealed by crimping lids with thin butyl rubber septa. These sealed vials were then labelled by their time point and placed in a rack in the heated water bath allowing for volatile compounds to accumulate within the headspace. 15 minutes prior to the hour time point the SPME needle was pierced through the crimped seal of the vial and the fibre was exposed to the headspace above the sample for 15 minutes before being extracted and analysed using the GC program previously described. Gas chromatography was performed on aliquots of the yeast culture every hour from 0-12 and 18-24 hour time periods. At these time points 10 µl of the growing culture was removed and used to make a wet mount for the analysis of cell morphology using light microscopy. An additional 1000 µl was removed and placed into 9000 µl sterile PBS. This suspension was diluted to 10^{-6} (in PBS) and 100 µl of each dilution was plated onto SAB agar and incubated (37˚C, 24 hours) to determine

viable counts at each hour of growth. The experiment and analysis was repeated on three separate occasions to ensure reproducibility.

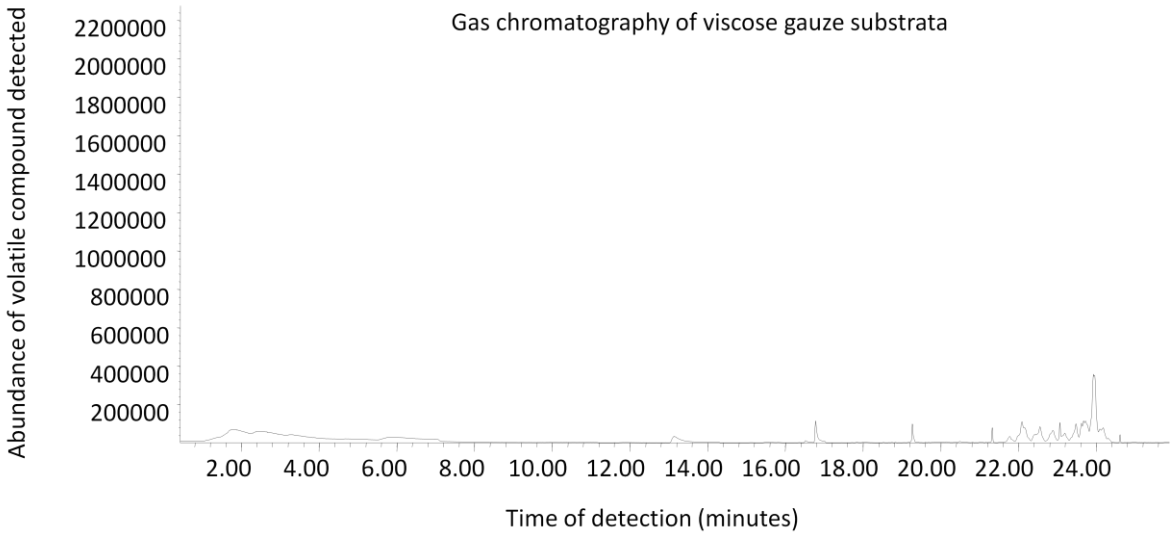
For analysis of volatiles produced by *C. albicans* biofilms, a slightly different method was employed. In previous work *C. albicans* biofilms have been grown on denture acrylic surfaces. Polymethy methacrylate (PMMA) surfaces are produced using volatile compounds which are generally removed from the material during polymerisation but residual volatile monomers have been reported (Kedjarune *et al.,* 1999). This was of concern when considering the gas chromatography method to be used. Any potential residual volatile compounds in acrylic surfaces could have interfered with the absorption of compounds from the *C. albicans* biofilms, onto the SPME needle. As an alternative 1cm^2 pieces of sterile gauze (Systagenix, North Yorkshire, UK) were used instead. This material was chosen for its mesh like structure (provided increased surface area for microbial attachements and biofilm growth), and its flexibility (allowing for it to be manipulated into gas chromatography vials easily). The gauze used in these experiments was an inert 100 % knitted viscose dressing material, commonly used in wound care. This substratum was tested using gas chromatography. One 1 cm^2 piece was placed into a GC vial containing 2ml or Sabouraud's dextrose broth and incubated in a water bath (37°C) for 30 minutes. SPME sampling and GC-MS revealed no organic volatile compounds released from the gauze sample indicating its suitability for use (Figures 4.4-4.5). Gauze substrata were prepared by cutting 9.5 cm x 9.5 cm sheets into 1 cm^2 pieces and autoclaving at 121°C for 15 minutes. Two pieces of 1 cm^2 gauze were added aseptically to 50 ml of SAB broth inoculated with one colony of *C. albicans* GDH 2346. These cultures were then incubated for 24, 48 and 72 hours following which gauze pieces with attached

biofilm were removed, placed and sealed into 10 ml gas chromatography vials and analysed with SPME GC as previously described. In order to examine any potential differences in compounds given off by different morphological forms of *C. albicans*, hyphal biofilms were also analysed in this way. Hyphal biofilms were produced on gauze samples by placing the gauze pieces into inoculated broth as before but this time the broth was 80 % SAB and 20 % horse serum to ensure and abundance of hyphal growth. Hyphal and blastospore biofilms were grown for 24, 48 and 72 hours (figure 4.6) and the experiment was repeated on two separate occasions to ensure reproducibility. Prior to the commencement of each GC run the column was standardised by running the program without any sample injected, to eliminate any residual volatiles on the column and needle. Controls were used to validate the identification of volatiles and to eliminate any interference from possible volatiles given off by additional components. The controls included 1 ml SAB broth, 1 piece of gauze in 1 ml SAB broth, 1ml neat farnesol and 1ml ethanol (100 ppm in distilled water).



Conditioning needle				
Volatile name	**% Quality**	**Peak area**	**Peak width**	**Ret time**
benzene	93	57406033	0.202	0.255
tetrasiloxane	80	105848984	1.138	2.373
cyclotetrasiloxane	91	137150266	1.074	5.714
cyclopentasiloxane	50	120907564	0.189	13.107
cyclotetrasiloxane	25	99926858	0.062	16.773
hexadecamethylcyclooctasiloxane	74	37518432	0.032	19.265
hexadecamethylcyclooctasiloxane	49	63145706	0.069	21.32
morphine	45	26177054	0.034	23.067
tetradecamethylcycloheptasiloxane	52	15685707	0.028	24.615

Figure 4.4. **Gas chromatograph (top) of volatile compounds detected during conditioning of the GC column and SPME needle**. Table (bottom) shows the compound detected with no sample present on the needle. The majority of compounds are siloxanes released from the SPME fibre coating during heating.



1 cm² viscose gauze in 2 ml SAB				
Volatile name	**% Quality**	**Peak area**	**Peak width**	**Ret time**
cyclotrisiloxane	64	11372940	1.115	5.935
cyclotetracyloxane	72	6362742	0.318	13.135
cyclopentasiloxane	91	5476224	0.08	16.777
cyclohexasiloxane	91	2502787	0.042	19.267
tetradecamethylcycloheptasiloxane	93	1275797	0.027	21.323
tetradecamethylcyclododecasiloxane	72	1791815	0.11	21.774
tetradecamethylcyclododecasiloxane	38	9897893	0.163	22.092

Figure 4.5. **Gas chromatograph (top) of volatile compounds detected from viscose gauze**. Table (bottom) shows the compounds detected are comparable with those given off by the SPME needle during conditioning (figure 3.2), indicating that the gauze material is inert.

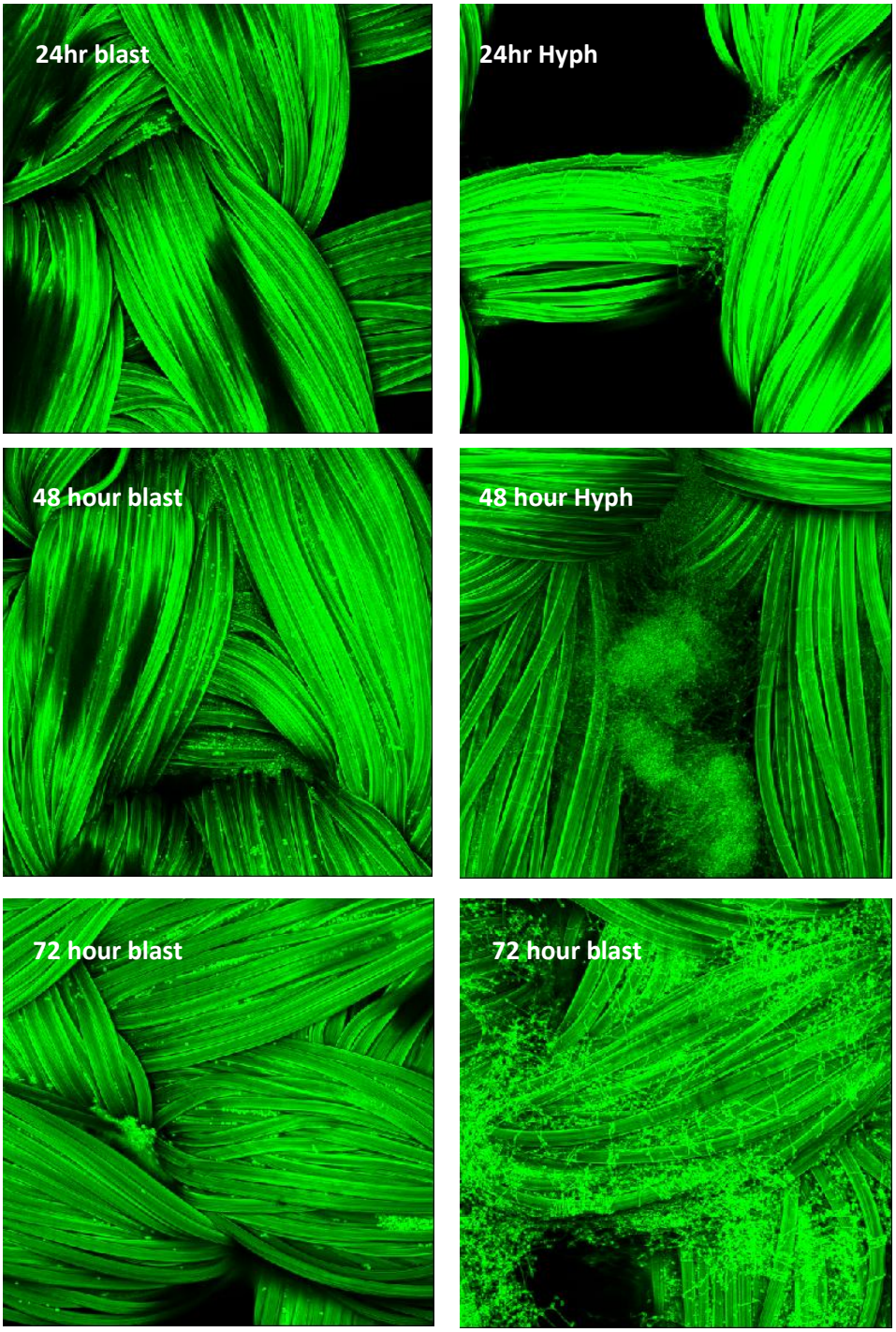


Figure 4.6 Gauze biofilms used for gas chromatography analysis. Biofilms were grown from blastospore of *C. albicans* (left) and *C. albicans* with induced hyphal growth (right) on 1 cm² pieces of gauze. Biofilms were grown for 24 (top), 48 (middle) and 72 (bottom) hours.

4.2.7 Effect of farnesol on the adhesion of *C. albicans*

The direct and indirect effect of farnesol on adhesion and hyphal formation of *C. albicans* on abraded denture acrylic surfaces was investigated. For analysis of the direct effect of farnesol, medium abraded 2 cm^2 PMMA test surfaces (abraded by washing with Colgate total whitening 25:40 paste/water ratio, Ra 0.38), were washed with farnesol (95 %, 3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol, Sigma Aldrich) by coating the surface with farnesol and rinsing with running distilled water for five seconds. Six replicate test surfaces were subsequently incubated with a standardised *C. albicans* GDH 2346 cell suspension for 1 hour at 37°C to allow for adhesion. Following this, three replicate surfaces were placed into 50 % horse serum and incubated at 37°C for three hours (as described previously) to induce hyphal growth in adhered cells.

For the analysis of indirect effects of farnesol (vapour), a disc diffusion method was employed. As before, surfaces were incubated with a standardised cell suspension, but in this case three absorbent sterile 6mm disks inoculated with 10 µl of farnesol (95 %) were place on the lid (with Vaseline) of the Petri dishes in which the surfaces were incubated with the cell suspensions (Edwards-Jones *et al.,* 2004). During the 1 hour incubation these Petri dishes were sealed with adhesive tape to avoid escape of farnesol vapour. After one hour three replicate test pieces were removed and placed into fresh Petri dishes containing 50 % horse serum and again three discs with 100µl of absorbed farnesol were attached to the Petri lid. These were sealed and incubated at 37°C for 3 hours to investigate any indirect farnesol effects on hyphal induction. In previous work farnesol has been shown to inhibit hyphal development, even in the presence of hyphal stimulating factors (Hornby *et al*., 2001). It was

therefore hypothesised that the direct contact with farnesol would inhibit hyphal growth in adhered cells. The effects of farnesol vapour on *C. albicans*, does not appear to have been investigated previously however other essential oils have been tested using similar vapour disc diffusion methods for their effects on bacterial growth (Lopez *et al*., 2005). All test substrata with attached cells were subsequently stained with 0.03% acridine orange and examined using epi-fluorescence microscopy. Ten fields per test piece were examined and in these areas the surface coverage and proportion of hyphal growth were examined. Percentage coverage results were analysed using a two way analysis of variance (ANOVA) to determine statistical significance.

4.2 Results

4.3.1 Optical tweezer microscopy

In examination of hyphal growth in adhered *C. albicans*, hyphae were observed to be growing towards one another in some cases (chapter 2, figure 2.18). This behaviour was investigated further using optical tweezer microscopy that allowed cells to be placed at different proximities, hyphal growth to be induced and the growth of hyphae to be observed in real time (time lapse photography). From the 12 studies carried out, various hyphal responses were observed, but no pattern could be deduced (Figure 4.7). In five of the studies, cells produced an early germ tube from one area which did not develop but instead a secondary germ tube formed and developed at an alternative site on the cell.

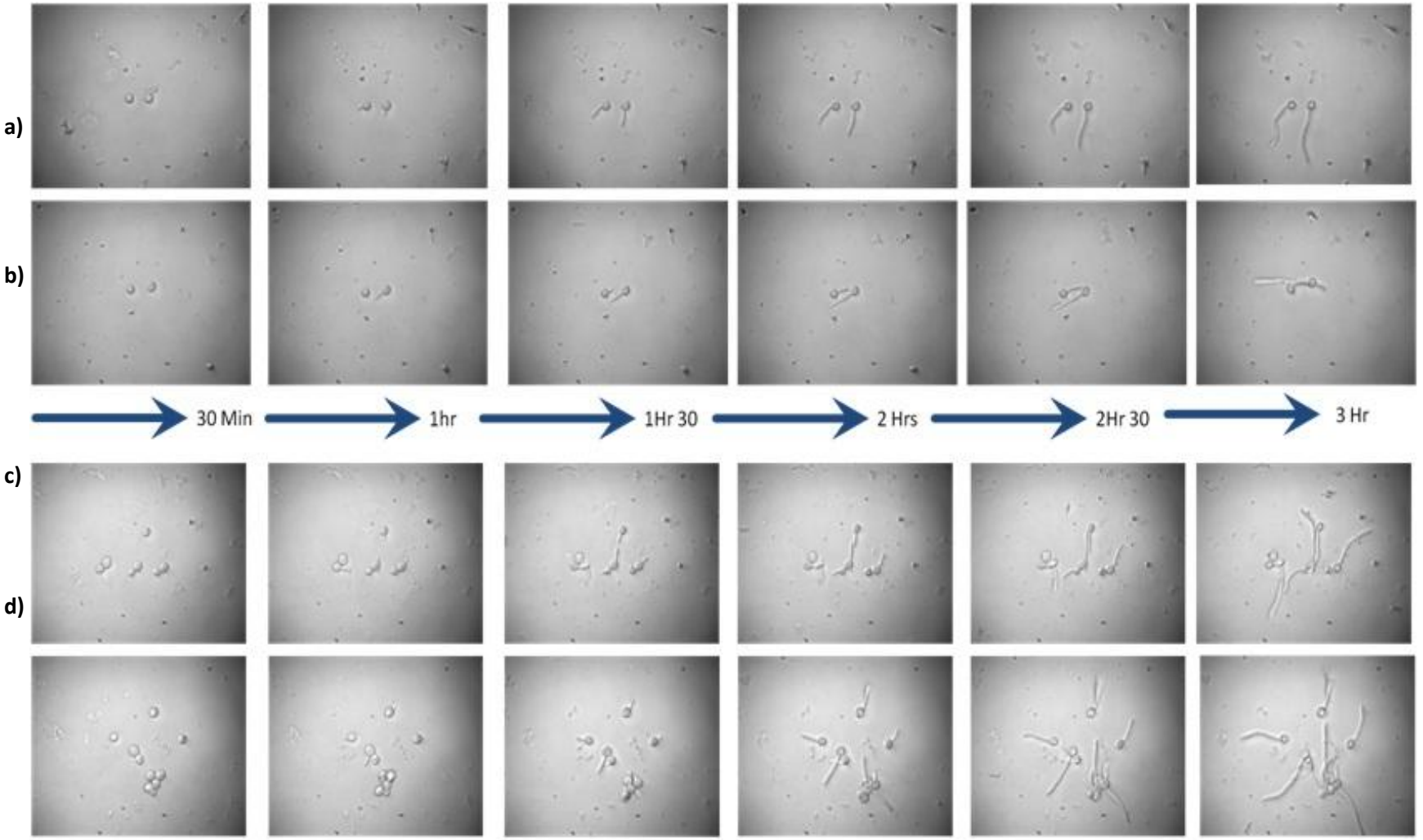


Figure 4.7. The growth of hyphae in relation to one another as observed using time lapse photography over 3 hours incubation (37°C) in horse serum using optical tweezer microscopy. Hyphae are seen to a) grow in a similar direction, b) grow towards the opposing cell, c) in a line format with opposing isolated cell, hyphae grew in different directions and in one case towards the isolated cell and d) in a circular grouped formation hyphae grew out in every direction

4.3.2 Gas chromatography of *C. albicans*

Gas chromatography analysis of *C. albicans* planktonic cultures in three separate repeat experiments allowed for the identification of volatile and potential quorum sensing compounds. Key volatile compounds detected and the morphology of *C. albicans* cells present in the culture was determined every hour from 0-12 and 18-24 hours of growth (Figures 4.8-4.28). Chromatographs revealed several peaks that were identified as siloxanes, compounds given off by the SPME needle fibre (as identified during conditioning, Figure 4.4). These siloxane compounds include a peak representing tetradecamethylcycloheptasiloxane, detected at around 18 minutes in each sample and a peak representing hexadecamethylcyclooctasiloxane, detected around 20 minutes. Because these compounds were known to be a product of the SPME needle fibre, only peaks representing volatile compounds produced by *C. albicans* were reported (Figures 4.8-4.28).

At baseline (T0) where the broth culture had only just been inoculated with *C. albicans* there were a small number of single and budding yeast cells in the 10µl sample extracted. Each hour following this, the number of budding yeast cells present increased. At three hours of growth the first pseudohyphal protrusions were noted and following this, the numbers of budding yeast cells, pseudohyphae and true hyphae increased every hour until the last time point at 24 hours. Hyphal and yeast cells were visibly longer/larger between 20 and 24 hours by which time these cells were in abundance, but in terms of morphology, blastospore cells and budding yeast dominated at every time point.

Over the course of three separate 24 hour experiments, the key volatile compounds found (Farnesol, farnesol synonyms, nerolidol and ethanol, (figure 4.8) were detected at relatively the same times of growth (± 2 hours) with the exception of solanesol which was only detected in two of the three repeats. These compounds were deemed to be key volatiles due to their reported effect on *C. albicans* (Section 4.1.1-4.1.2). Ethanol was first detected at 4 hours (38% accuracy, Figures 4.8 and 4.13) and continued to be detected in increasing amounts and with greater calculated percentage accuracy up to 24 hours (Figures 4.13-4.28). Farnesol was identified at 10 hours of growth and was subsequently detected in increasing amounts at 11, 12 and 18-24 hour time points (Figures 4.8, 4.19-4.28). The microscopy of cells at this stage revealed an abundance of clumped budding yeast cells and pseudohyphae. Nerolidol, a molecule structurally very similar to farnesol was detected at 11 hours onwards. Between 18 and24 hours of growth a number of peaks were produced for farnesol identified under different synonyms. These included 6, 10-dodecadien 1-ol, 3-7-11 trimethyl and farnesol 1, 2 and 3 (Figures 4.19-4.28). At 22 hours of growth, solanesol was detected (Figure 4.8/4.26) and continued to be detected at 23 and 24 hour time points (Figures 4.27-4.28). The quality of this identification was low at between 38-43 % accuracy.

4.3.3 Gas chromatography of *C. albicans* hyphal and blastospore biofilms

Blastospore and hyphal biofilms were examined at 24, 48 and 72 hours for the volatile compounds produced. At 24, 48 and 72 hours there was little difference between the volatile compounds detected from blastospore biofilms and those detected from hyphal biofilms. At 24 hours, both biofilm types produced ethanol and nerolidol (Figure 4.29-4.30). At 48 and 72 hours both biofilm types continued to yield peaks for ethanol and in addition to this peak representing farnesol type molecules were identified (Figures 4.31-4.34).

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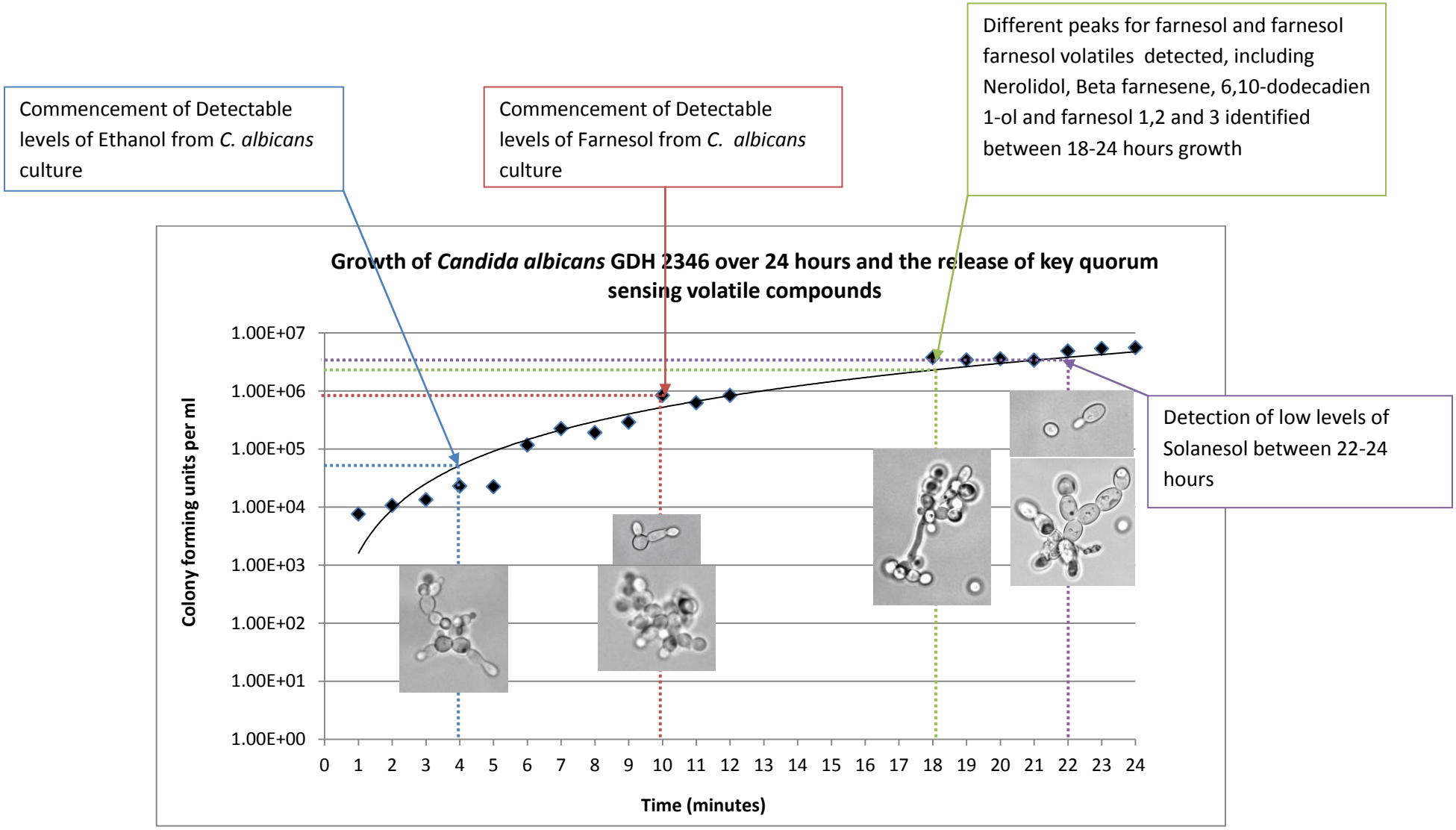
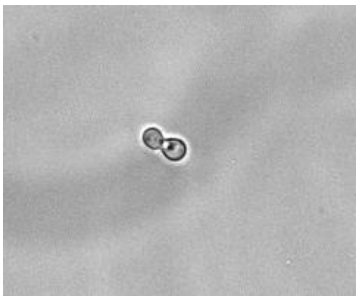


Figure 4.8 The growth and release of key quorum sensing volatile compounds from *C. albicans* planktonic cultures over 24 hours. Volatile compounds were detected and identified using gas chromatography, all compound were detected in three repeat experiments.

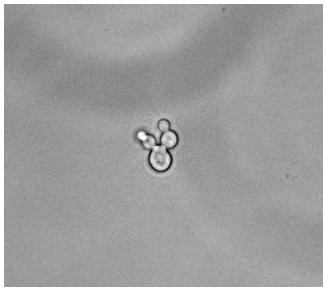
Time 0



Volatile name	% Quality	Peak area	Peak width	Ret time

Figure 4.9 *C. albicans* morphology and key volatile compound detected at Time of inoculation of medium (T0). At T0 no volatile compounds were detected in three repeat studies.

Time 1 hour



Volatile name	% Quality	Peak area	Peak width	Ret time
butanal	86	18589066	0.374	1.4
benzoic acid	47	435978	0.04	21.2

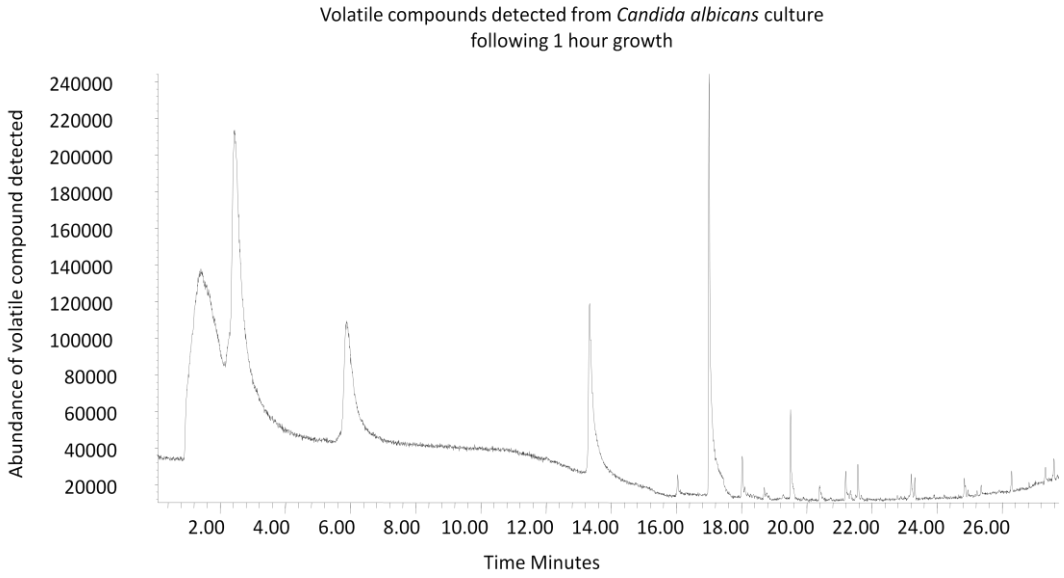
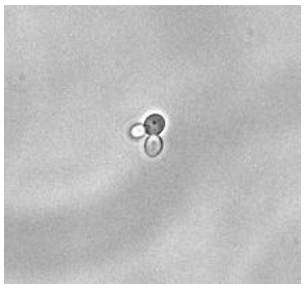


Figure 4.10 *C. albicans* morphology and significant volatile compounds detected following 1 hour of growth. Table of volatile compounds detected and image of Candida morphology (top). Chromatogram of planktonic *C. albicans* culture after 1 hour (bottom).

Time 2 hour



Volatile name	% Quality	Peak area	Peak width	Ret time
cyclobutanol	64	10551716	0.117	1.2
benzoic acid	37	465034	0.038	21.4

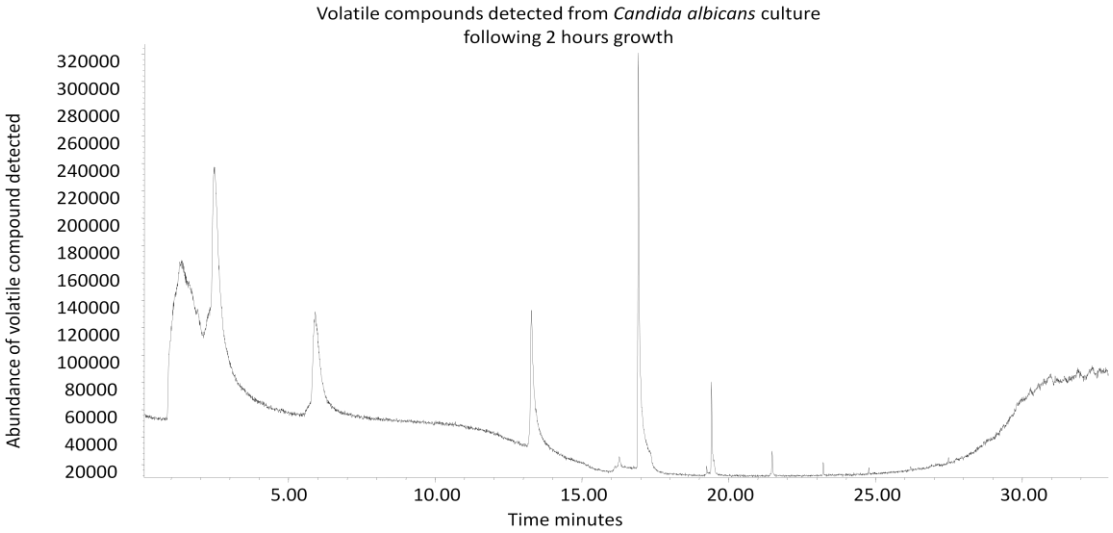
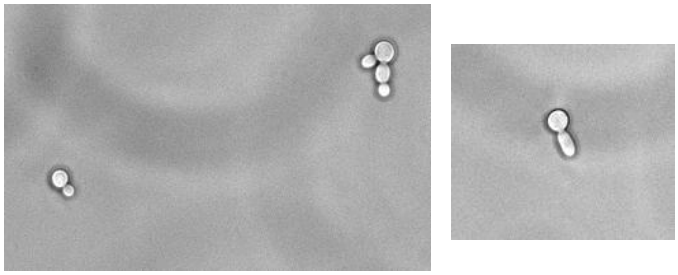


Figure 4.11 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 2 hours of growth and analysis of chromatogram (bottom).

Time 3 hour



Volatile name	% Quality	Peak area	Peak width	Ret time
cyclobutanol	64	10551716	0.117	1.2

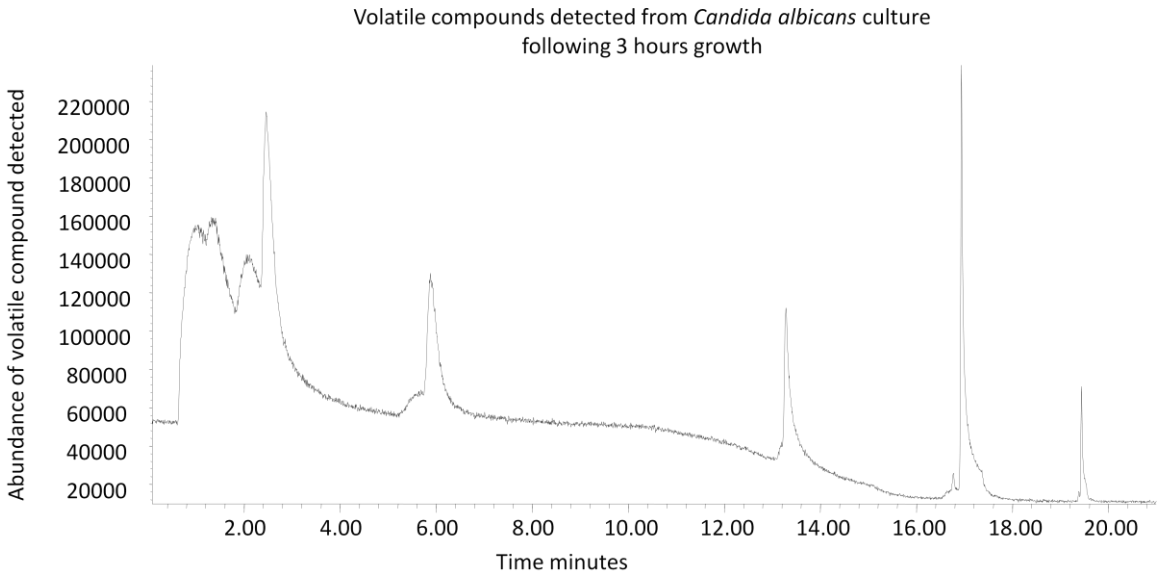
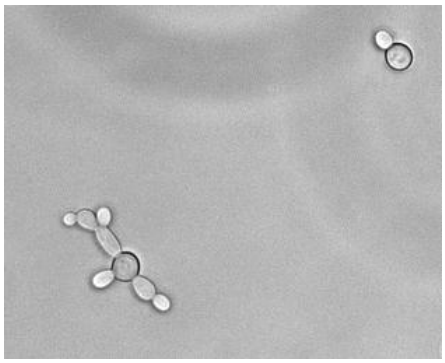


Figure 4.12 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 3 hours of growth and analysis of chromatogram (bottom).

Time 4 hour



Volatile name	% Quality	Peak area	Peak width	Ret time
Ethanol	38	14308980	0.207	1.24
Cyclobutanol	53	14149796	0.197	1.4
carbamic acid	50	4338008	0.1	1.5
Heptanol	50	7374735	0.134	1.6
1-Butanol	64	1353358	0.078	1.9
Benzoic acid	43	528634	0.036	21.5

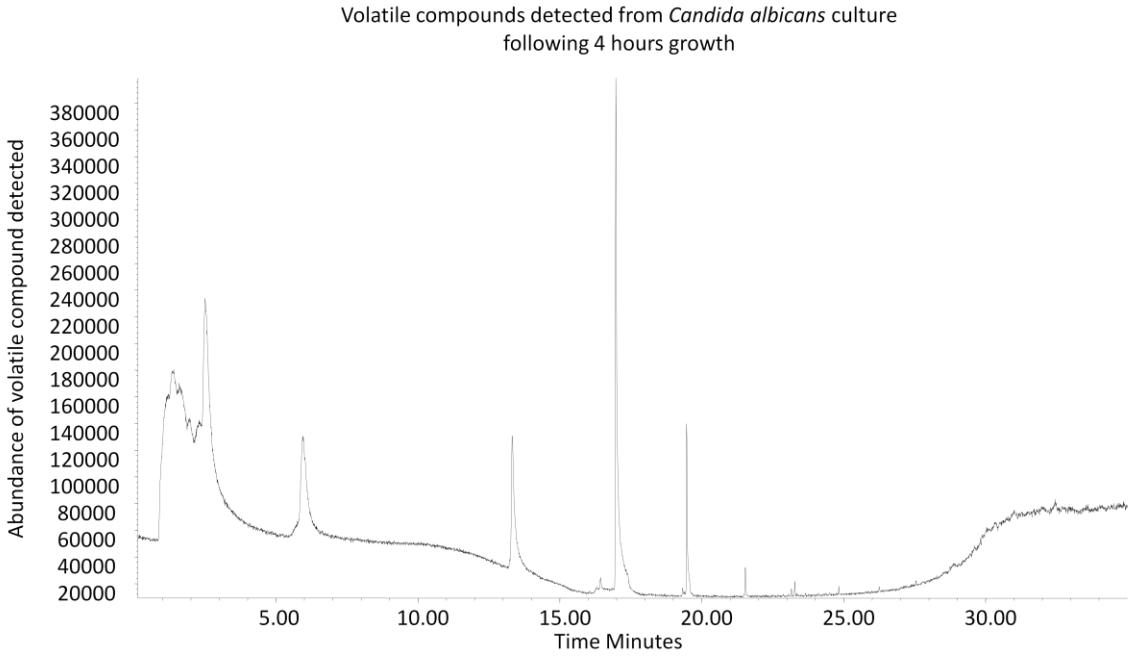
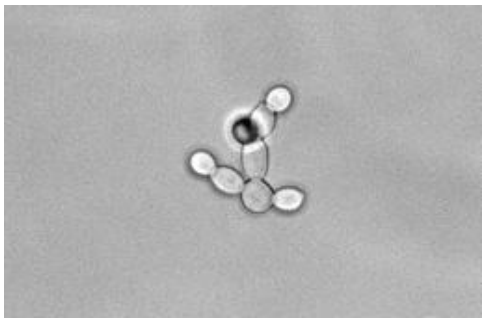


Figure 4.13 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 4 hours of growth and analysis of chromatogram (bottom).

Time 5 hour



Volatile name	% Quality	Peak area	Peak width	Ret time
Ethanol	38	13415412	0.19	0.5
Cyclobutanol	59	4782319	0.095	0.54
1-butanol	48	21608559	0.291	0.83
benzoic acid	38	623331	0.04	21.4
dianthramide	37	301331	0.03	23.2

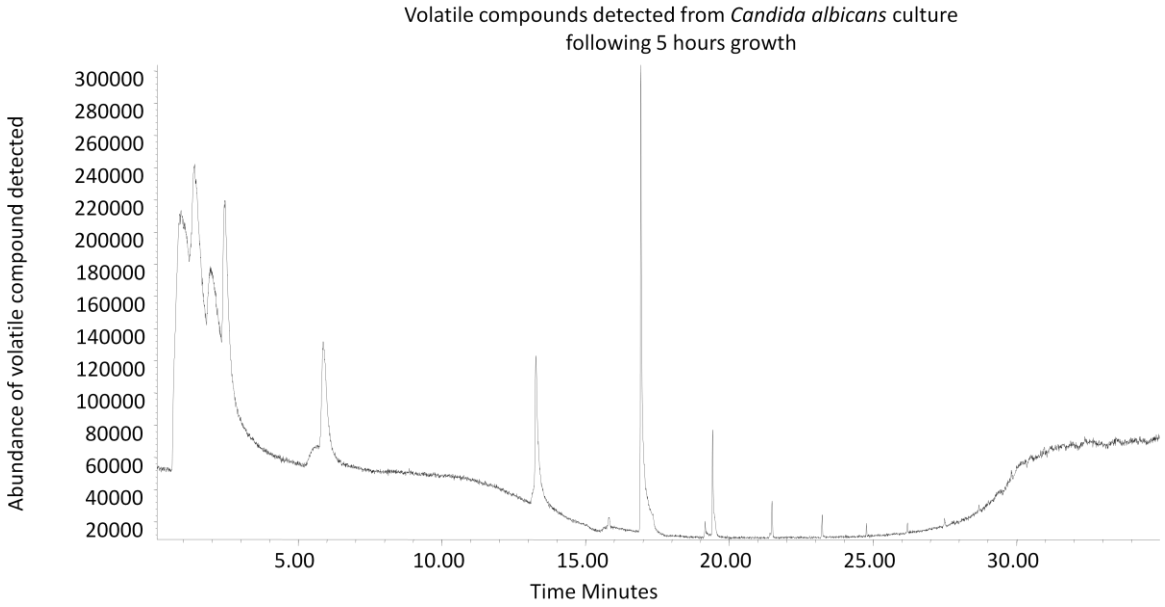
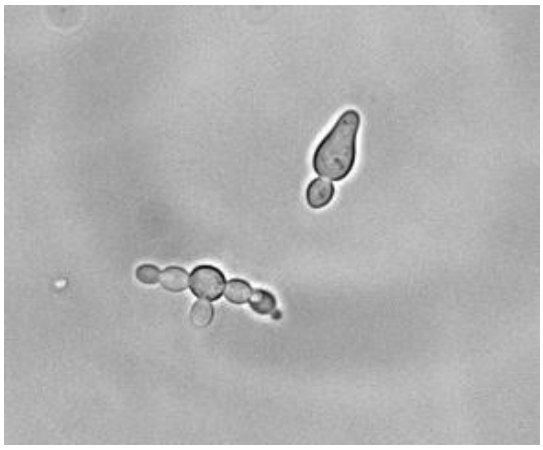


Figure 4.14 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 5 hours of growth and analysis of chromatogram (bottom).

Time 6 hour



Volatile name	% Quality	Peak area	Peak width	Ret time
Ethanol	50	5095145	0.25	0.9
1-butanol	43	7403757	0.2	1.3
benzoic acid	37	360055	0.03	21.4

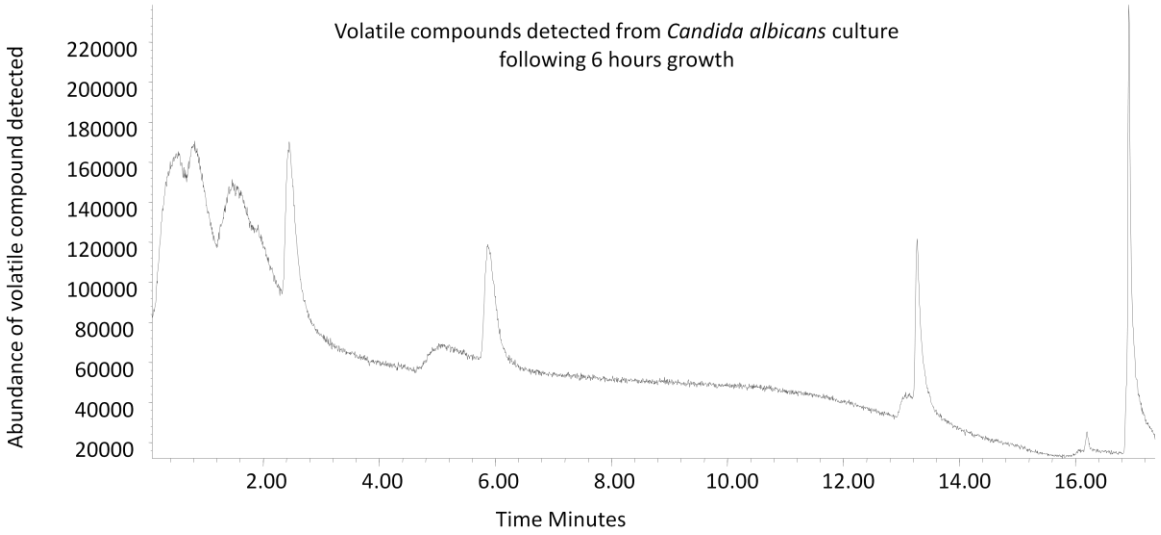
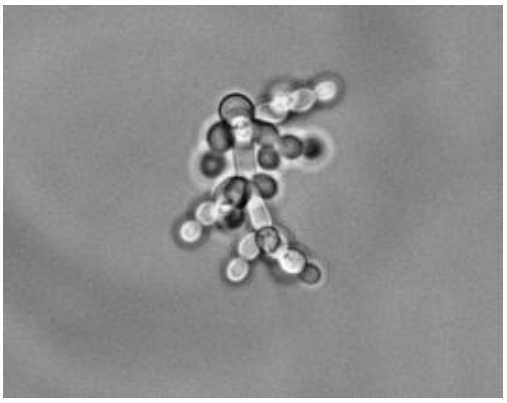


Figure 4.15 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 6 hours of growth and analysis of chromatogram (bottom).

Time 7 hour



Volatile name	% Quality	Peak area	Peak width	Ret time
Ethanol	50	30174446	0.379	1.3
1-butanol	49	2901043	0.117	1.67
1-pentanol	83	2919845	0.097	1.8
benzoic acid	53	411533	0.034	21.4
dianthramide	32	193000	0.025	23.2

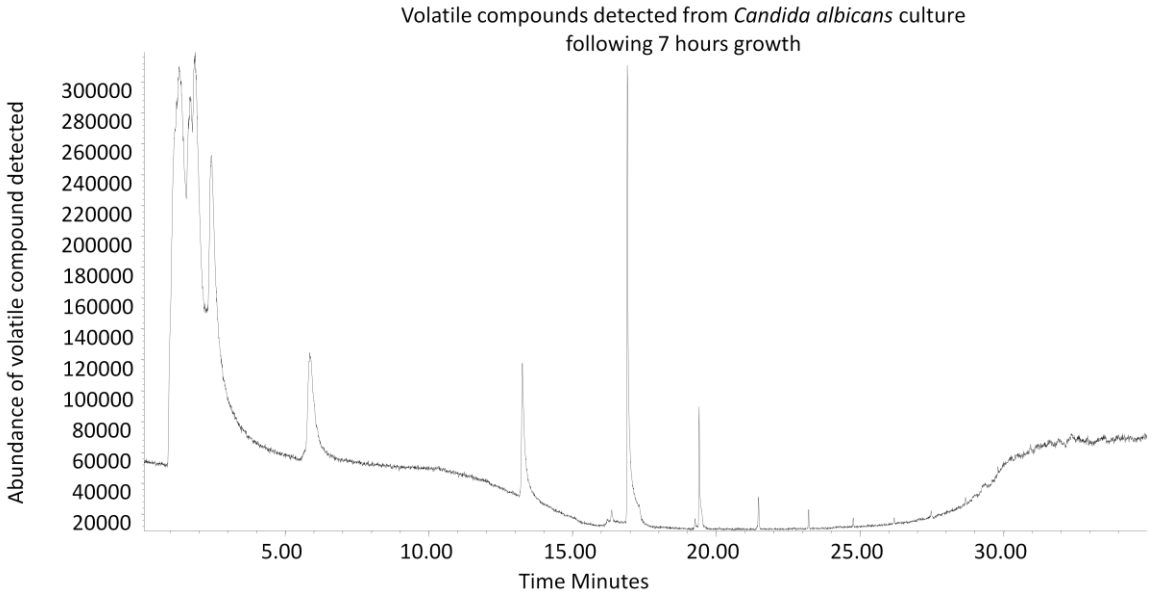
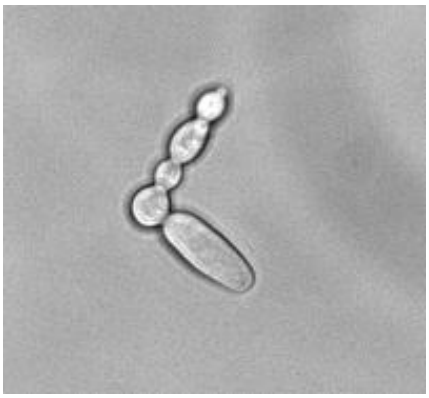


Figure 4.16 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 7 hours of growth and analysis of chromatogram (bottom).

Time 8 hour



Volatile name	% Quality	Peak area	Peak width	Ret time
Ethanol	47	43373174	0.333	0.36
1-pentanol	59	11448271	0.163	0.91
benzoic acid	43	693124	0.032	21.5
diantramide	32	281622	0.027	23.2

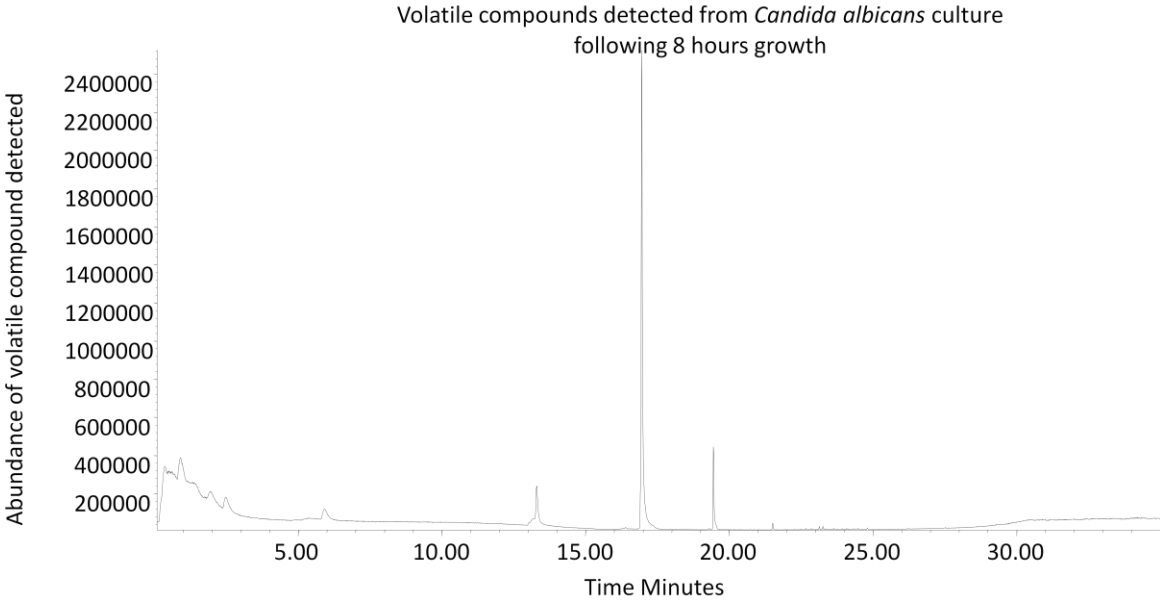
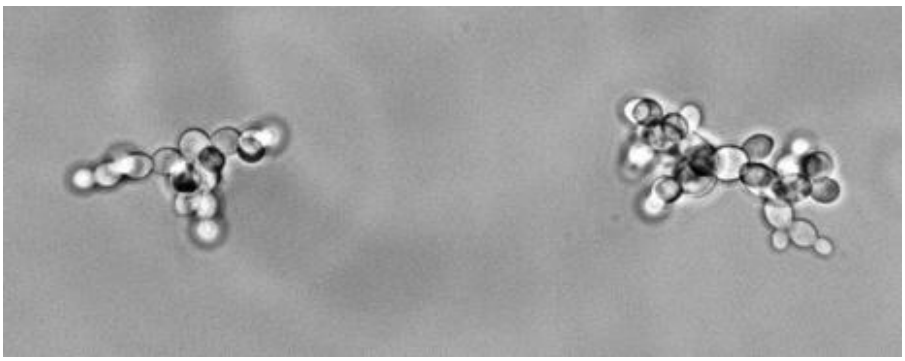


Figure 4.17 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 8 hours of growth and analysis of chromatogram (bottom).

Time 9 hour



Volatile name	% Quality	Peak area	Peak width	Ret time
Ethanol	72	65408222	0.3	0.9
1-pentanol	74	24840230	0.24	1.45
benzoic acid	50	395770	0.174	21.5

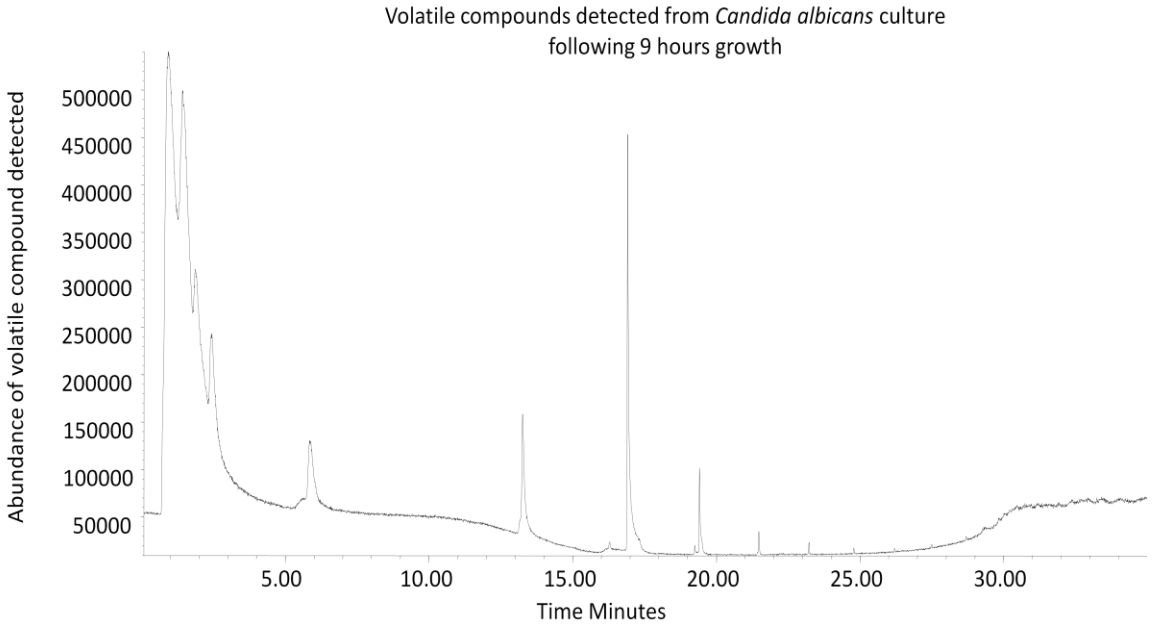
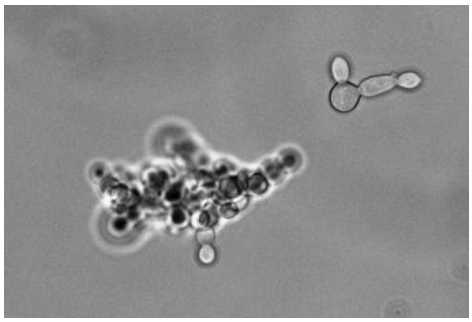


Figure 4.18 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 9 hours of growth and analysis of chromatogram (bottom).

Time 10hour



Volatile name	% Quality	Peak area	Peak width	Ret time
Ethanol	64	171453739	0.5	1.1
1-pentanol	83	6832490	0.14	1.9
benzoic acid	50	520619	0.032	21.5
Farnesol	38	341255	0.05	22.1

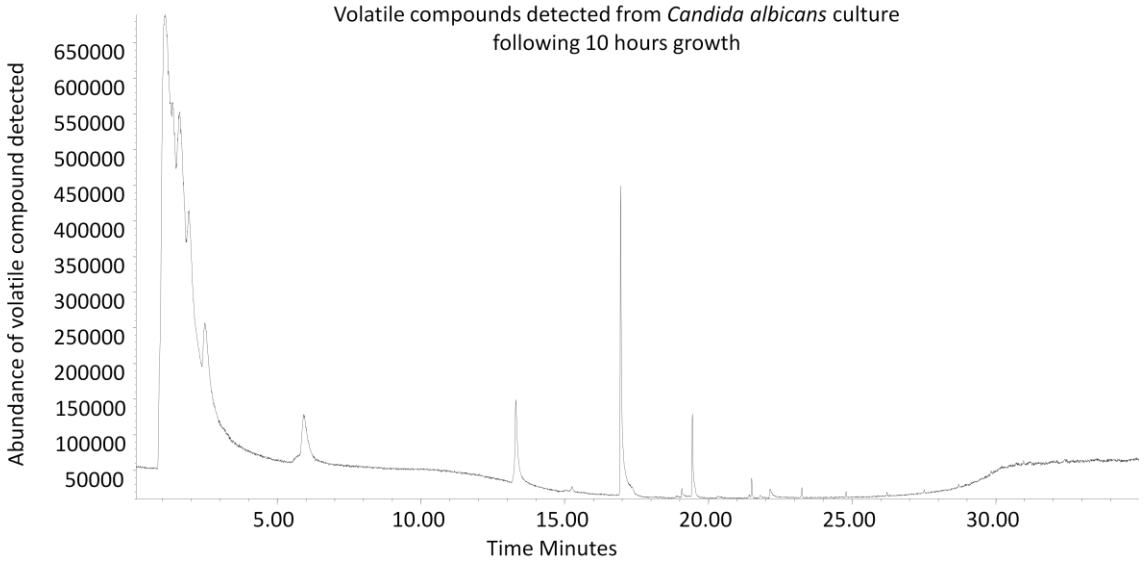
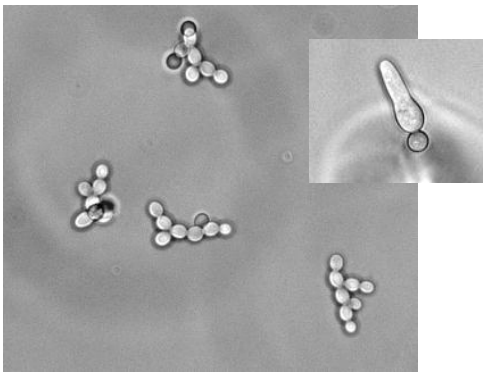


Figure 4.19 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 10 hours of growth and analysis of chromatogram (bottom).

Time 11hour



Volatile name	% Quality	Peak area	Peak width	Ret time
Ethanol	72	141033180	0.335	1.3
1-pentanol	83	7925185	0.128	1.8
Nerolidol	30	367624	0.048	20.2
Benzeneacetic acid	53	586829	0.03	21.4
Farnesol	80	1110432	0.056	22.1

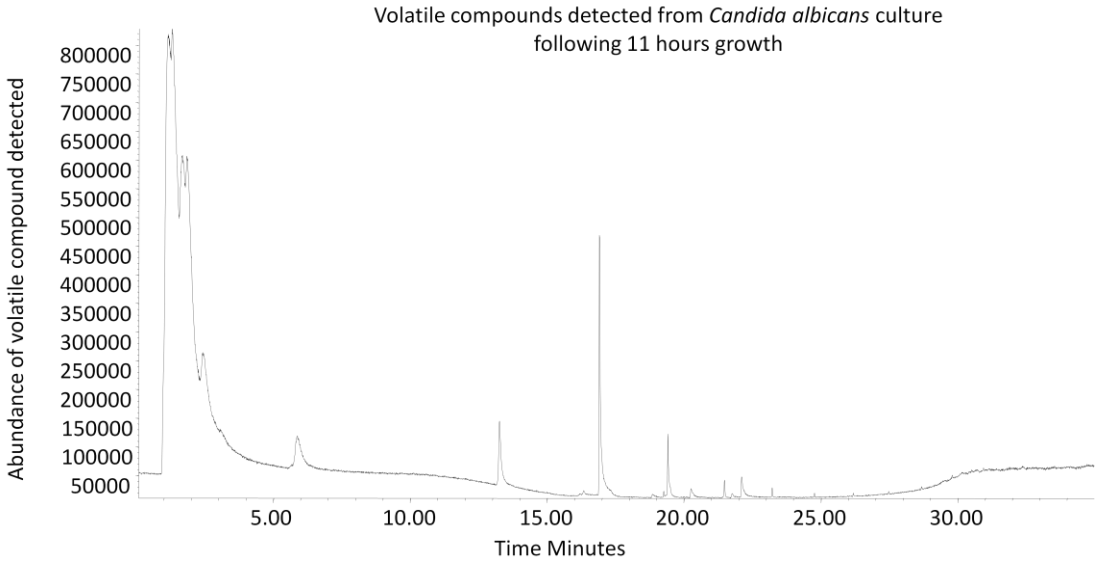
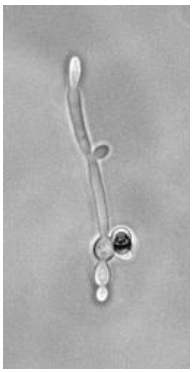


Figure 4.20 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 11 hours of growth and analysis of chromatogram (bottom).

Time 12 hours				
				
Volatile name	**% Quality**	**Peak area**	**Peak width**	**Ret time**
Ethanol	64	273768140	0.663	0.9
1-pentanol	74	6947256	0.149	1.8
Nerolidol	64	1622973	0.031	21.4
benzoic acid	50	381003	0.044	21.7
Farnesol	83	2419277	0.061	22.1

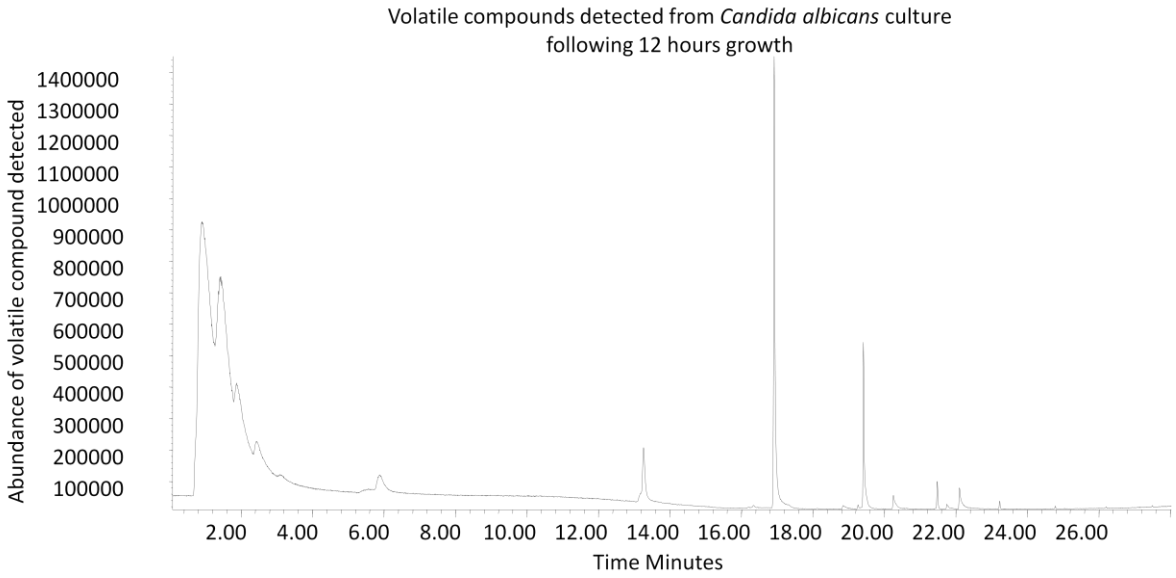
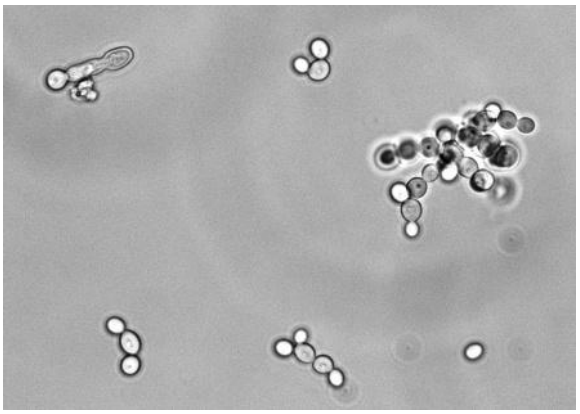


Figure 4.21 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 12 hours of growth and analysis of chromatogram (bottom).

Time 18 hours



Volatile name	% Quality	Peak area	Peak width	Ret time
Ethanol	83	128122088	0.328	1.1
1 pentanol	83	112013921	0.411	1.65
1 butanol	64	11025322	0.209	3.1
Nerolidol	95	8169233	0.064	20.24
6, 10-Dodecadien-1-ol, 3,7,11-trimethyl	91	3799953	0.069	21.74
Farnesol 3	86	1908057	0.083	22.1
Bis-Oxazepam	43	280932	0.026	23.23

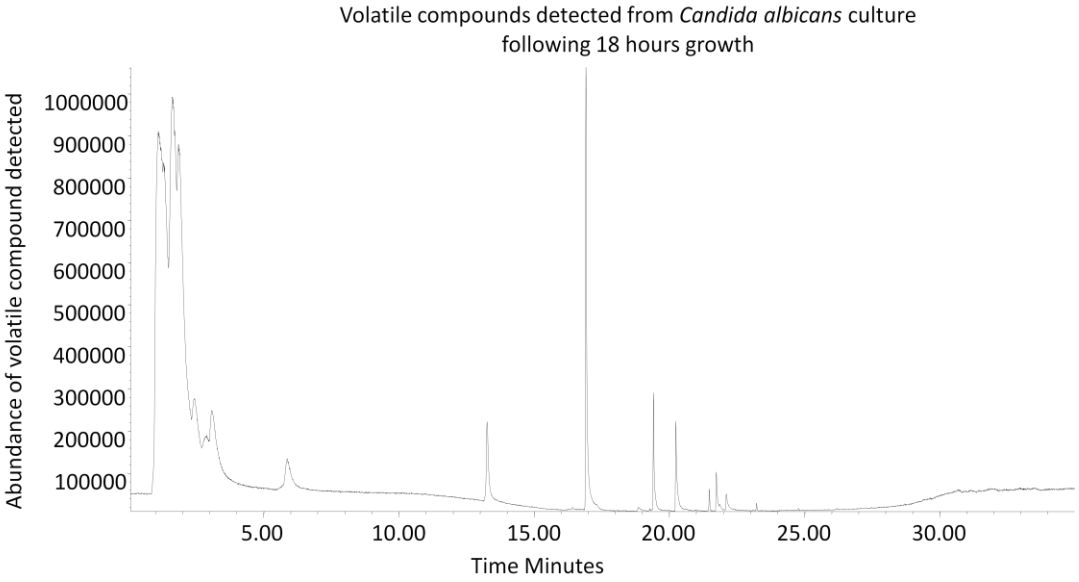
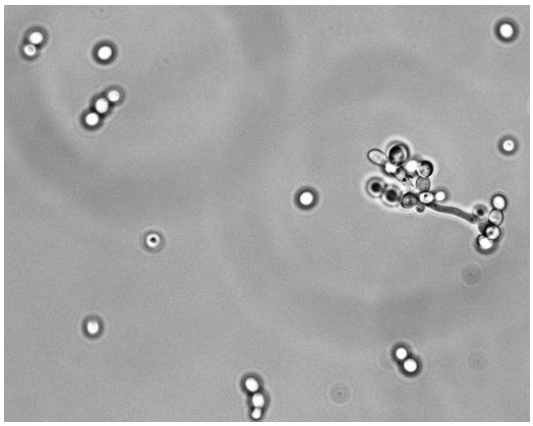


Figure 4.22 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 18 hours of growth and analysis of chromatogram (bottom).

Time 19 hours



Volatile name	% Quality	Peak area	Peak width	Ret time
Ethanol	80	138301521	0.368	1.32
1 pentanol	86	124311086	0.319	1.86
1 butanol	72	35198494	0.258	3.1
Trans-beta farnesene	95	2062034	0.111	18.8
Nerolidol	95	16155109	0.052	20.23
6, 10-Dodecadien-1-ol, 3,7,11-trimethyl	91	9416644	0.035	21.7
Farnesol 1	81	695026	0.041	21.8
Farnesol 3	91	7216646	0.049	22.1

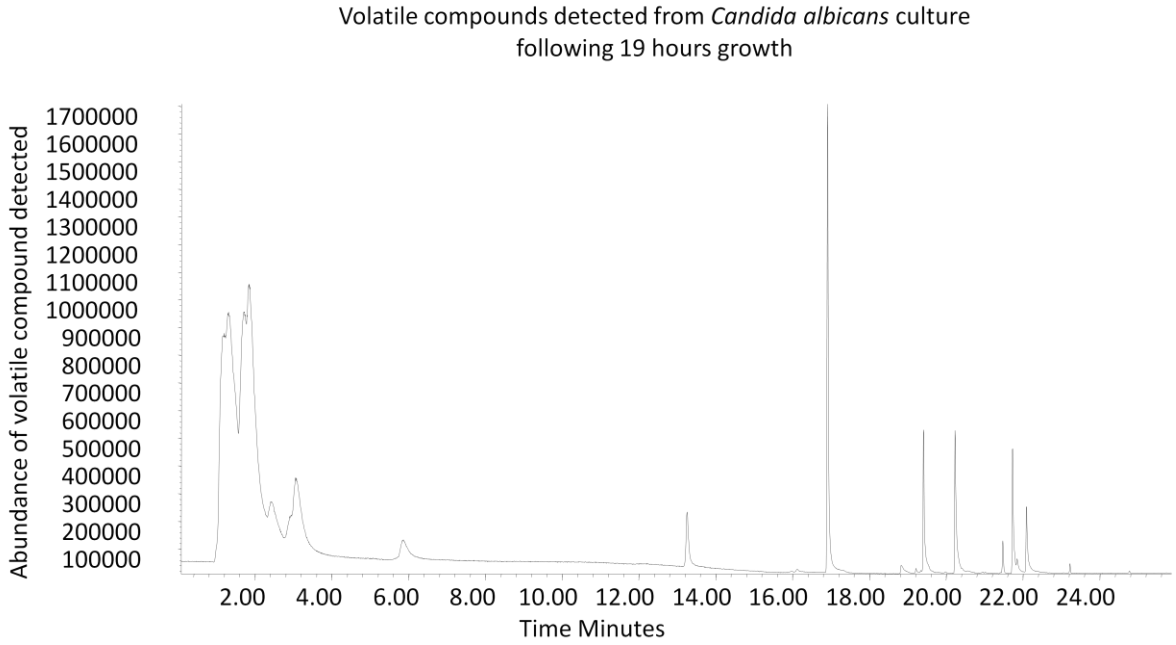
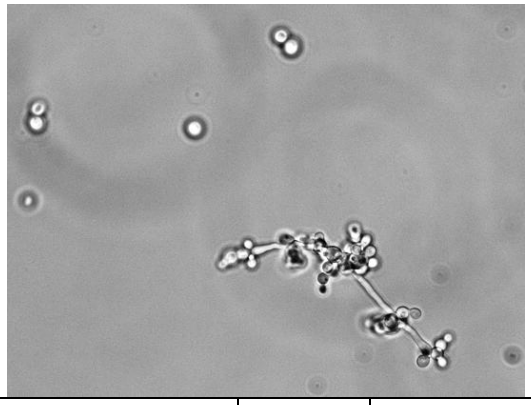


Figure 4.23 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 19 hours of growth and analysis of chromatogram (bottom).

Time 20 hours



Volatile name	% Quality	Peak area	Peak width	Ret time
Ethanol	78	127914115	0.387	1.3
1 pentanol	83	117930514	0.347	1.9
1 butanol / Iso-amylacetate	80	59496888	0.302	3.1
Nerolidol	95	19543236	0.043	20.23
6, 10-Dodecadien-1-ol, 3,7,11-trimethyl	91	16906551	0.032	21.7
Farnesol 1	68	10743077	0.04	21.8
Farnesol 3	91	1799312	0.025	22.1

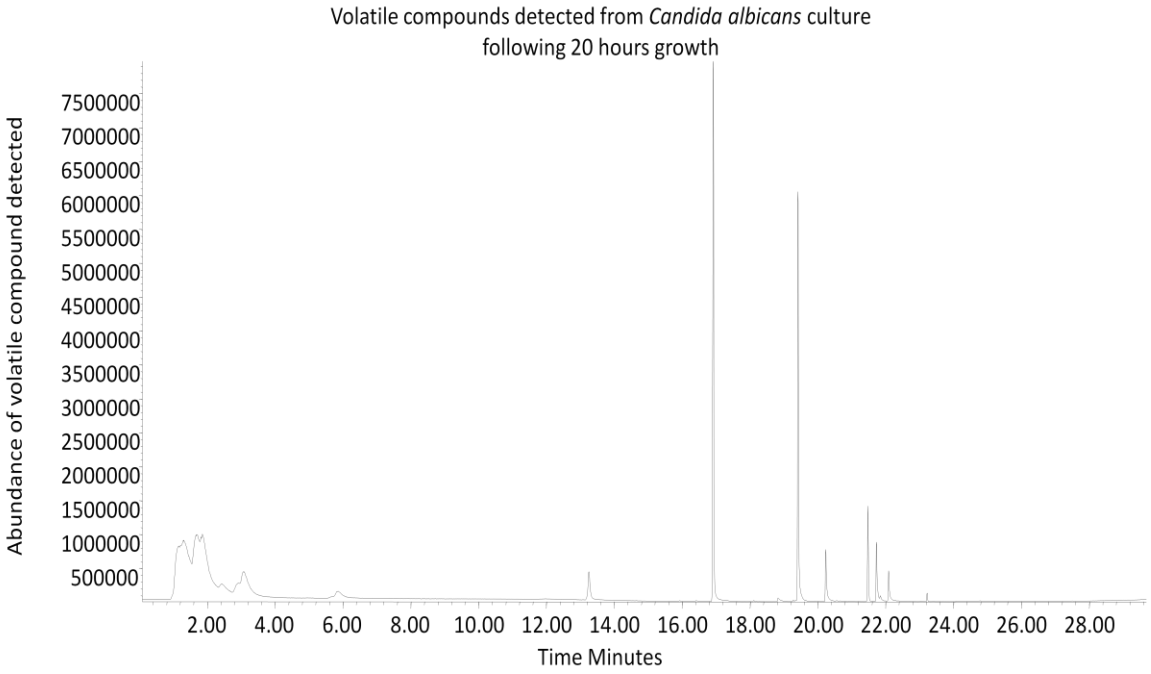
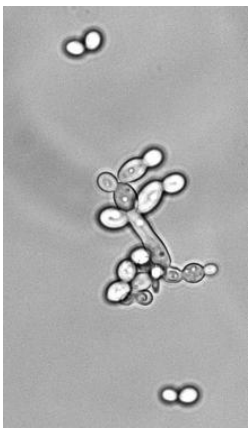


Figure 4.24 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 20 hours of growth and analysis of chromatogram (bottom).

Time 21 hours



Volatile name	% Quality	Peak area	Peak width	Ret time
Ethanol	78	125098871	0.42	1.3
1 pentanol	83	129226435	0.388	1.87
1 butanol / Iso-amylacetate	72	36375298	0.314	3.1
Nerolidol	95	20266101	0.042	20.25
6, 10-Dodecadien-1-ol, 3,7,11-trimethyl	91	26172334	0.032	21.7
Farnesol 2	96	1441396	0.034	21.86
Farnesol 3	91	12299133	0.042	22.1

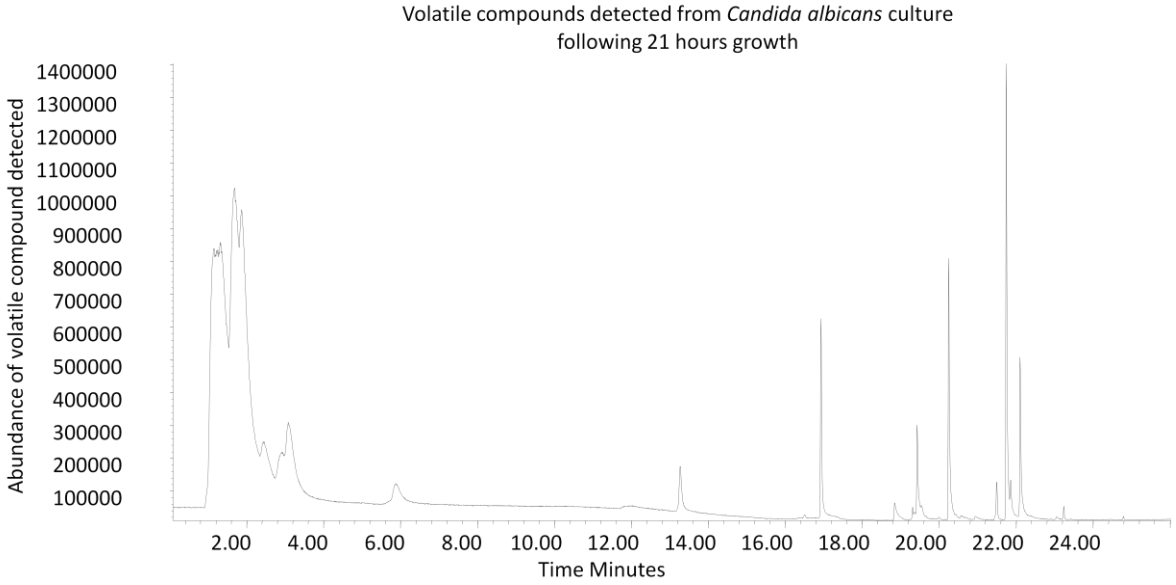
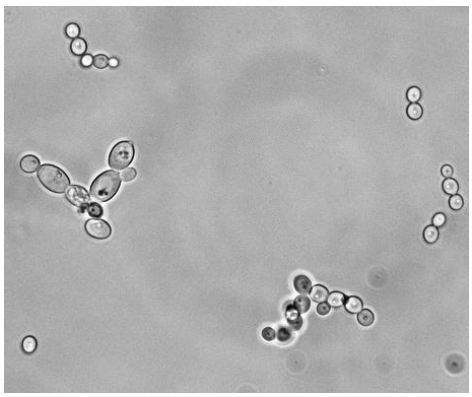


Figure 4.25 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 21 hours of growth and analysis of chromatogram (bottom).

Time 22 hours



Volatile name	% Quality	Peak area	Peak width	Ret time
Ethanol	78	127782118	0.375	1.3
1 pentanol	83	142799036	0.35	1.85
1 butanol / Iso-amylacetate	72	50644338	0.301	3.1
Trans-beta farnesene	97	2338696	0.077	18.8
Nerolidol	95	17734376	0.039	20.23
6, 10-Dodecadien-1-ol, 3,7,11-trimethyl	91	29559322	0.031	21.7
Farnesol 1	83	1618660	0.03	21.8
Farnesol	94	12973081	0.039	22.1
solanosol	38	424355	0.055	23.03

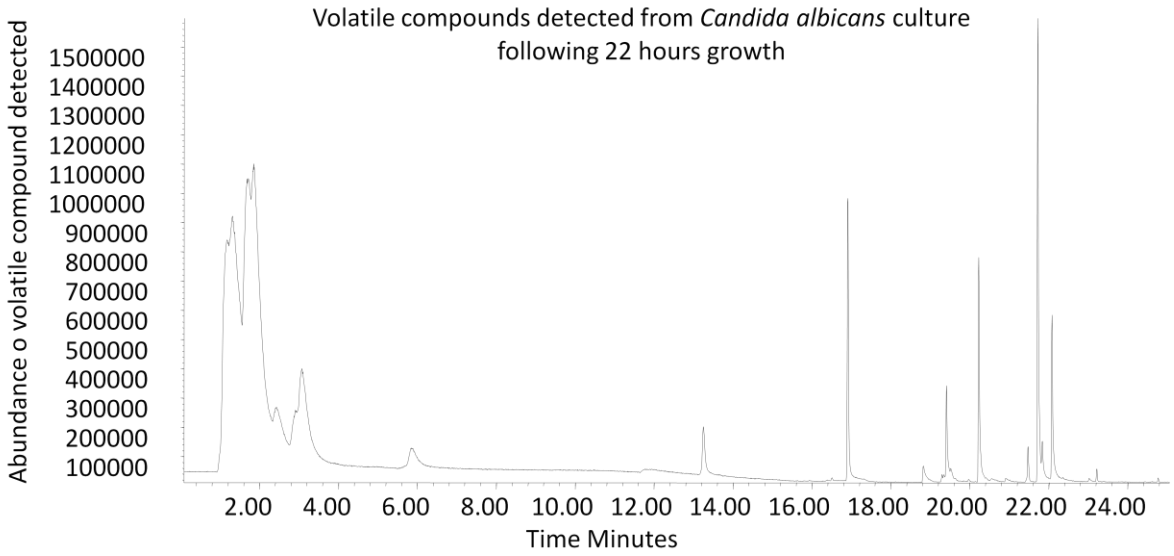
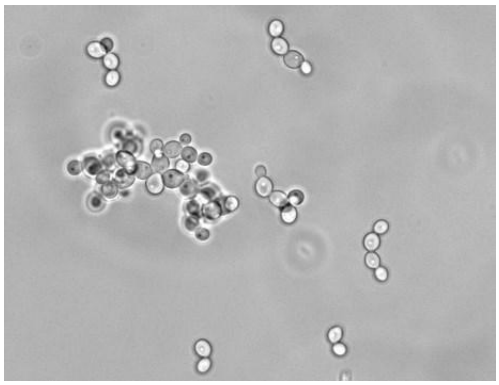


Figure 4.26 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 22 hours of growth and analysis of chromatogram (bottom).

Time 23 hours



Volatile name	% Quality	Peak area	Peak width	Ret time
Ethanol	72	101295052	0.31	0.95
1 pentanol	83	136300985	0.394	1.49
1 butanol / Iso-amylacetate	80	13539953	0.2	3.06
Trans-beta farnesene	96	2415131	0.085	18.8
Nerolidol	95	13220025	0.039	20.2
6, 10-Dodecadien-1-ol, 3,7,11-trimethyl	91	26193308	0.031	21.7
Farnesol 1	80	1614601	0.04	21.8
Farnesol	94	10651396	0.045	22.1
solanosol	43	533506	0.067	23.02

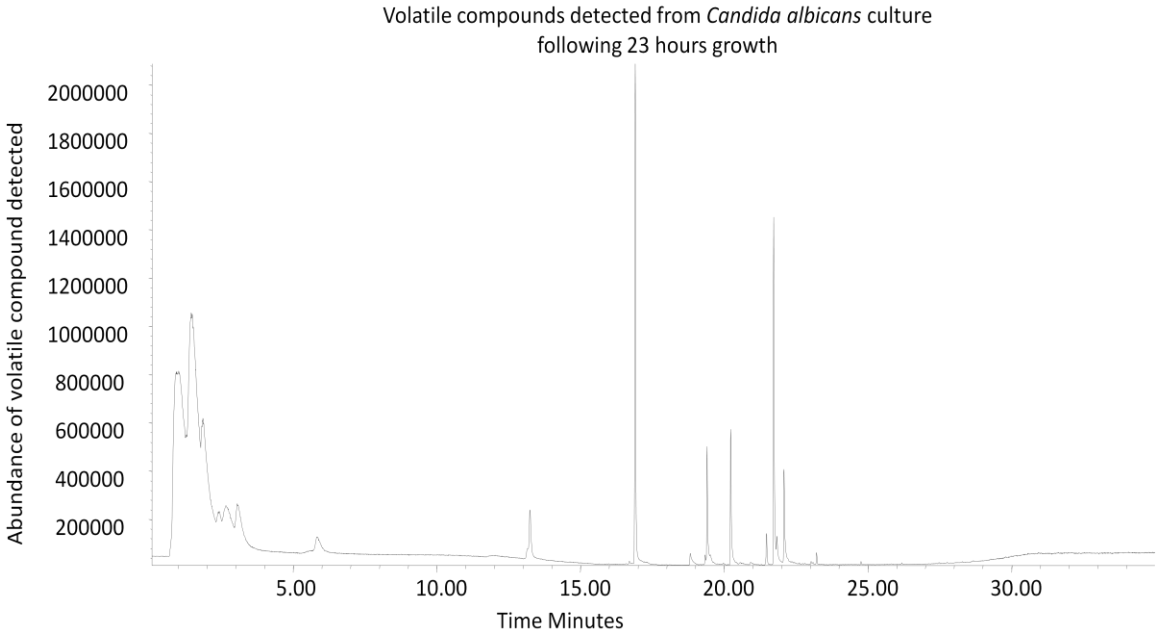
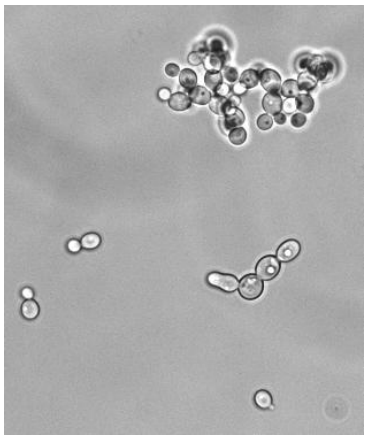


Figure 4.27 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 23 hours of growth and analysis of chromatogram (bottom).

Time 24 hours



Volatile name	% Quality	Peak area	Peak width	Ret time
Ethanol	72	58995024	0.415	1.3
1 pentanol	83	143878914	0.397	1.6
1 butanol / Iso-amylacetate	72	15849236	0.165	3.04
Nerolidol	93	13443195	0.038	20.24
6, 10-Dodecadien-1-ol, 3,7,11-trimethyl	91	26527882	0.03	21.7
Farnesol 2	86	2117111	0.41	21.8
Farnesol 3	94	10944259	0.044	22.1
Solanosol	35	848593	0.025	23.2

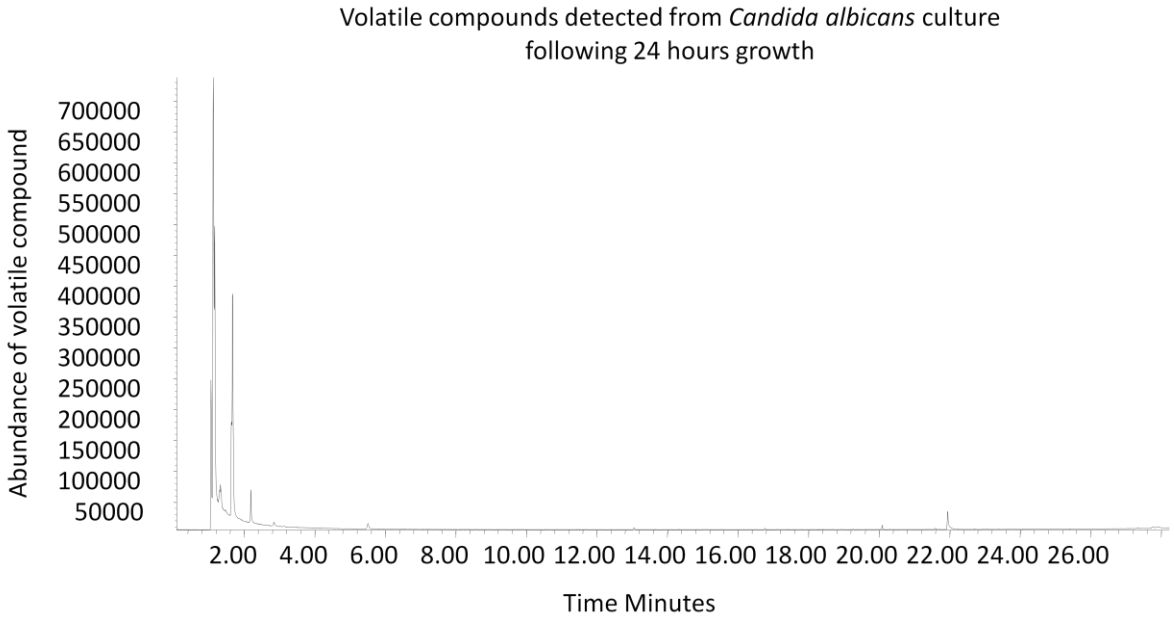


Figure 4.28 *C. albicans* morphology and key volatile compounds detected (top) following SPME-GC detection of planktonic culture after 24 hours of growth and analysis of chromatogram (bottom).

24 hour blasto biofilm				
Volatile name	**% Quality**	**Peak area**	**Peak width**	**Ret time**
carbon dioxide	4	1508538	0.022	1.05
Ethanol	90	18698306	0.052	1.12
acetic acid	38	1087187	0.063	1.33
1 pentanol	83	1540086	0.029	1.63
1 butanol	86	3615718	0.022	1.67
Nerolidol	87	1044025	0.046	21.94

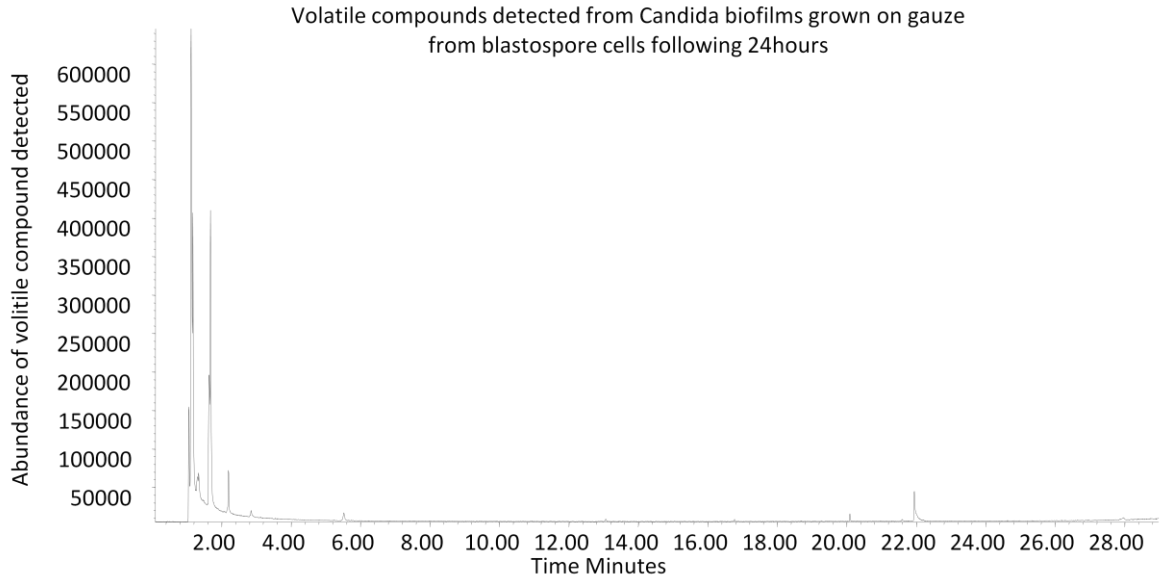


Figure 4.29 Volatile compounds and potential quorum sensing molecules detected (top) from 24 hour blastospore biofilms of *C. albicans* grown on gauze and analysed using gas chromatography and database referencing of chromatogram (bottom).

24 hour hyphal biofilm				
Volatile name	**% Quality**	**Peak area**	**Peak width**	**Ret time**
carbon dioxide	4	2665035	0.02	1.08
Ethanol	90	19441826	0.043	1.16
acetic acid	49	1543979	0.04	1.32
1-butanol	90	11955879	0.031	1.66
Nerolidol	91	805807	0.033	21.95

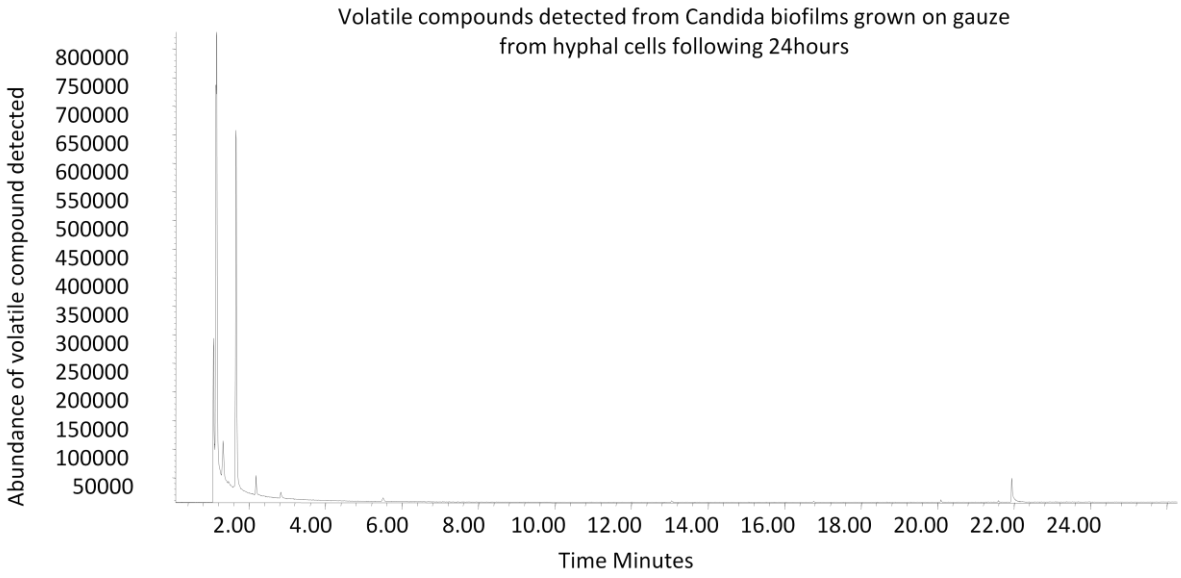


Figure 4.30 Volatile compounds and potential quorum sensing molecules detected (top) from 24 hour hyphal biofilms of *C. albicans* grown on gauze and analysed using gas chromatography and database referencing of chromatogram (bottom).

48 hour blasto biofilm				
Volatile name	**% Quality**	**Peak area**	**Peak width**	**Ret time**
carbon dioxide	4	2023580	0.018	1.07
ethanol	90	26083369	0.043	1.13
acetic acid	49	1972966	0.042	1.32
1-butanol	90	14155144	0.034	1.67
alpha farnesene	89	443835	0.026	20.09
Farnesol	40	478249	0.027	21.58
Nerolidol	90	1185719	0.027	21.94

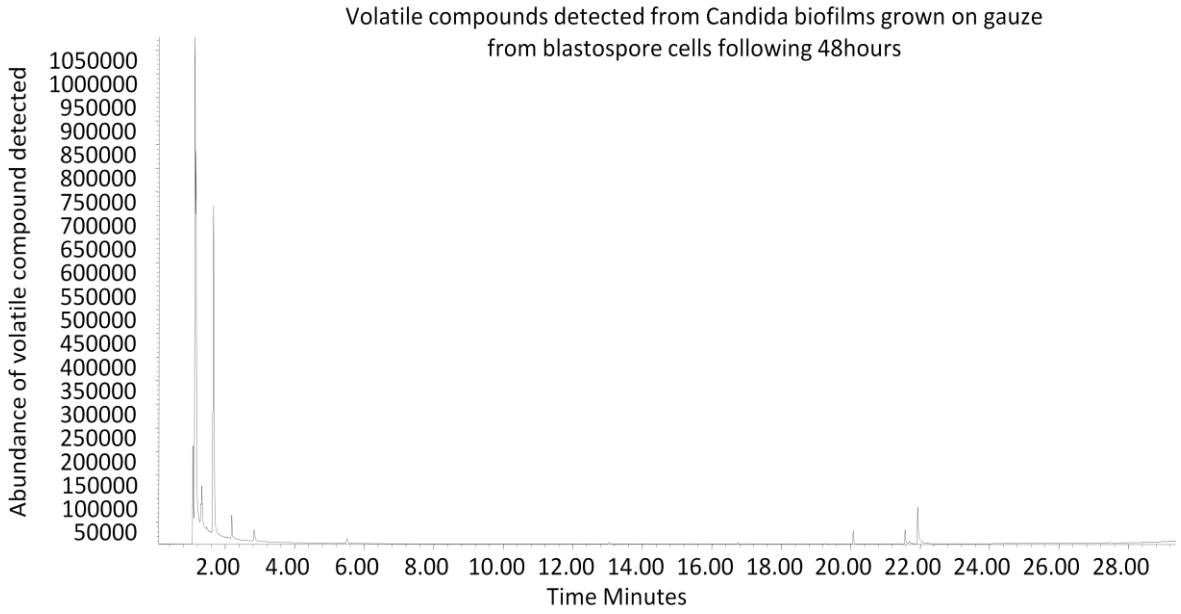


Figure 4.31 Volatile compounds and potential quorum sensing molecules detected (top) from 48 hour blastospore biofilms of *C. albicans* grown on gauze and analysed using gas chromatography and database referencing of chromatogram (bottom).

48 hour hyphal biofilm				
Volatile name	**% Quality**	**Peak area**	**Peak width**	**Ret time**
carbon dioxide	4	1677284	0.013	1.04
Ethanol	90	32135756	0.049	1.11
acetic acid	49	1824857	0.057	1.33
1-butanol	86	13345563	0.044	1.67
6,10 dodecatrien-3-ol	64	241520	0.025	20.09
6-Octen-1-ol	40	240901	0.027	21.58
beta bisabolene	76	591349	0.031	21.94

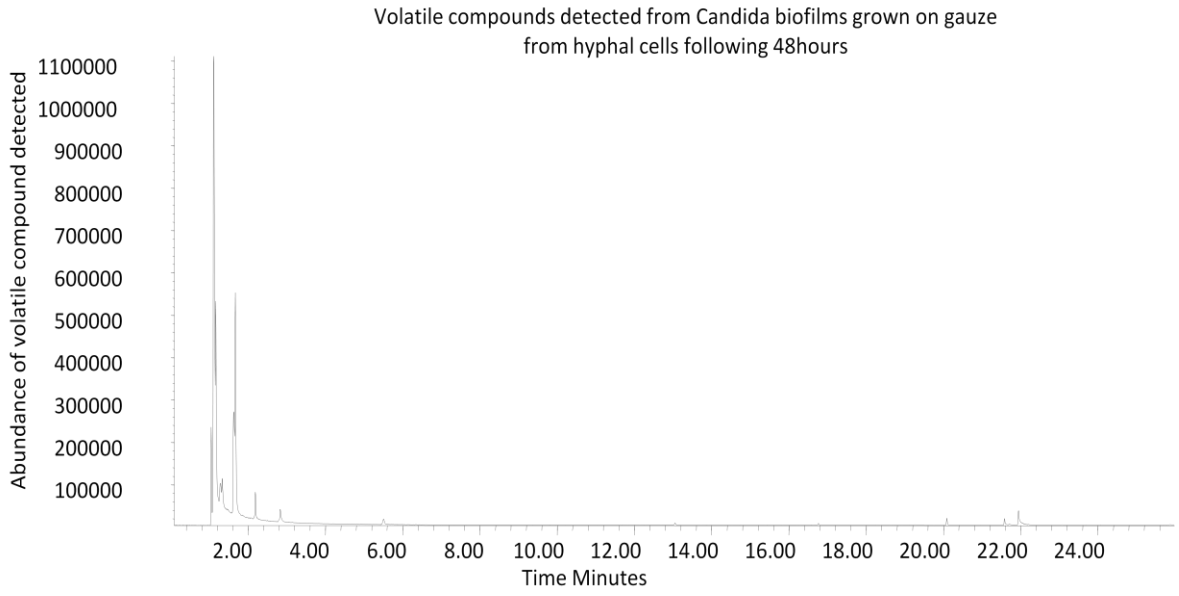


Figure 4.32 Volatile compounds and potential quorum sensing molecules detected (top) from 48 hour hyphal biofilms of *C. albicans* grown on gauze and analysed using gas chromatography and database referencing of chromatogram (bottom).

72 hour blast				
Volatile name	**% Quality**	**Peak area**	**Peak width**	**Ret time**
carbon dioxide	4	528874	0.016	1.067
Ethanol	90	33482420	0.041	1.127
acetic acid	72	2113560	0.052	1.329
1-butanol/ISOAMYLALCOHOL	90	15927669	0.04	1.668
nerolidol	78	169070	0.024	20.086
dodecadien 1-ol	54	934875	0.029	21.58

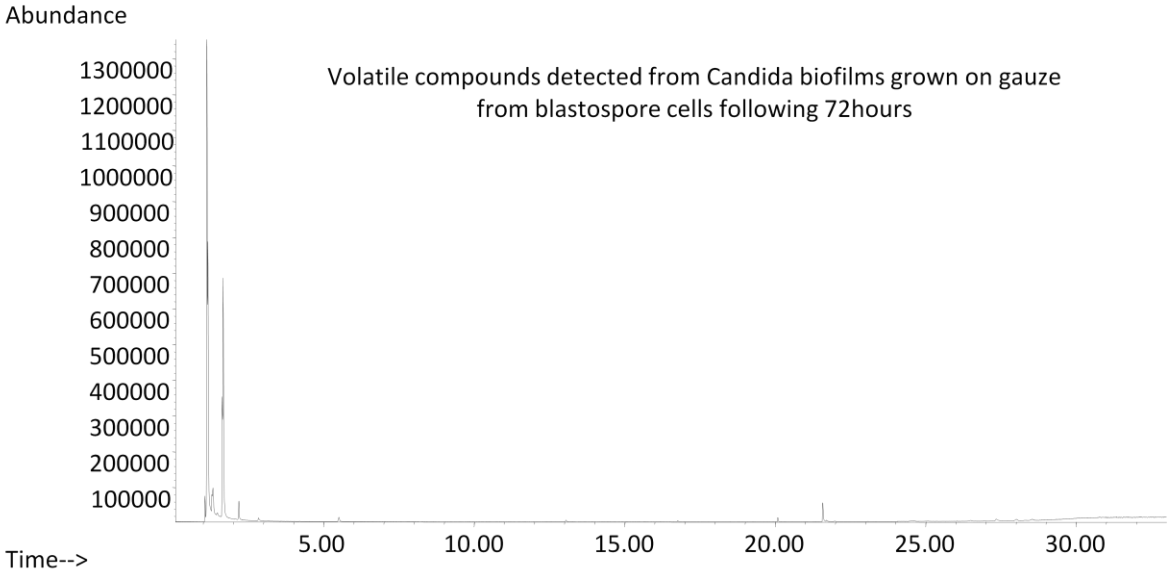


Figure 4.33 Volatile compounds and potential quorum sensing molecules detected (top) from 72 hour blastospore biofilms of *C. albicans* grown on gauze and analysed using gas chromatography and database referencing of chromatogram (bottom).

72 hour hyphal biofilm				
Volatile name	**% Quality**	**Peak area**	**Peak width**	**Ret time**
Ethanol	90	34890405	0.056	1.13
acetic acid	86	1761829	0.04	1.324
1-butanol/ISOAMYLALCOHOL	90	15286603	0.037	1.663
beta bisabolene/alpha farnesene	90	545578	0.024	20.082
6-10-Dodecadien-1-ol	90	2126771	0.031	21.578

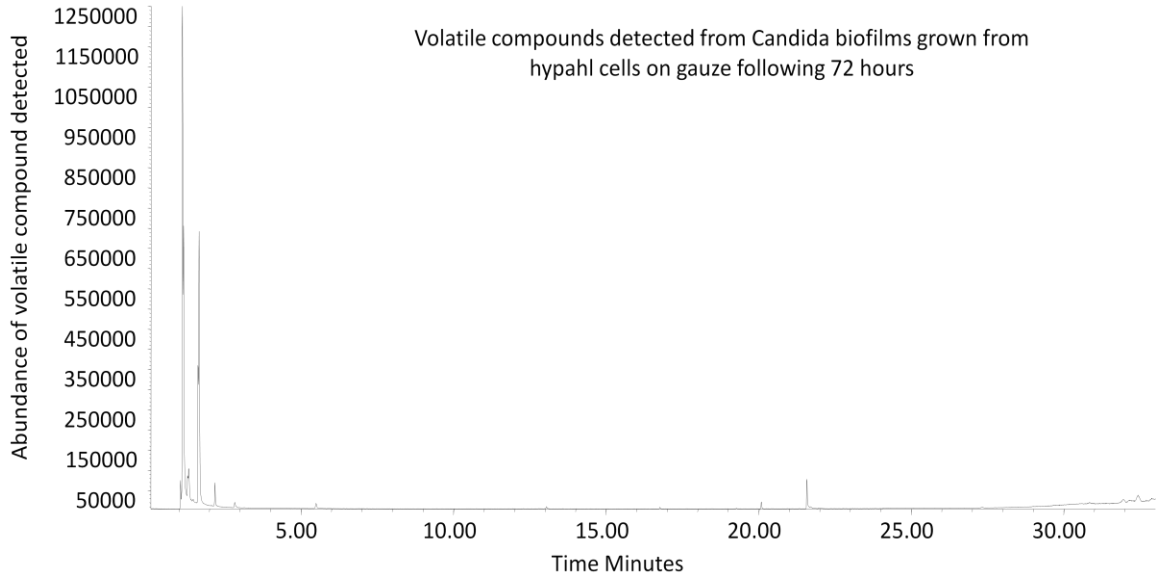


Figure 4.34 Volatile compounds and potential quorum sensing molecules detected (top) from 72 hour hyphal biofilms of *C. albicans* grown on gauze and analysed using gas chromatography and database referencing of chromatogram (bottom).

4.3.5 Direct and indirect effect of farnesol of adhesion of *C. albicans* to abraded denture acrylic

The effect of direct and indirect contact of farnesol on the adhesion of *C. albicans* to abraded denture acrylic and the serum induction of hyphae in adhered cells was investigated. For direct contact, surfaces were washed with farnesol by flooding the surfaces and rinsing with distilled water. Indirect contact was achieved using vapour diffusion from farnesol-inoculated discs (100µl) attached to the lid of the test container during adhesion hyphal induction. Three replicate test pieces were examined for both conditions, and 10 fields per test piece were visually compared.

In terms of adhesion, there was little difference in the amount and pattern of adhered cells between the normal adhesion test group and the test surfaces subjected to adhesion with indirect farnesol contact (disc diffusion). In both cases there was an abundance of adhered *C. albicans* which was preferentially noted in surface features and scratches and was consistent across the entire abraded surface (Figure 4.35). Test substrata that were washed with farnesol prior to adhesion however were less consistent. In these cases cells appeared in clumps (figure 4.35-4.36), and overall adhesion was significantly reduced $P<0.01$ (figure 4.37). These clumps of cells varied in size but occurred in irregular patches across the test substrata.

For both direct and indirect test groups farnesol was found to have an effect on the induction of hyphal growth with horse serum, reducing hyphal growth in adhered cells in both cases. In the control group (no farnesol present), the 50% horse serum used successfully induced hyphal growth in the majority of adhered *C. albicans*. Under exposure to farnesol vapour via disc diffusion however the proportion of cells

producing hyphae was reduced. This was further exaggerated in test surfaces that were washed with farnesol, in which no visible hyphal growth was found (figure 4.36).

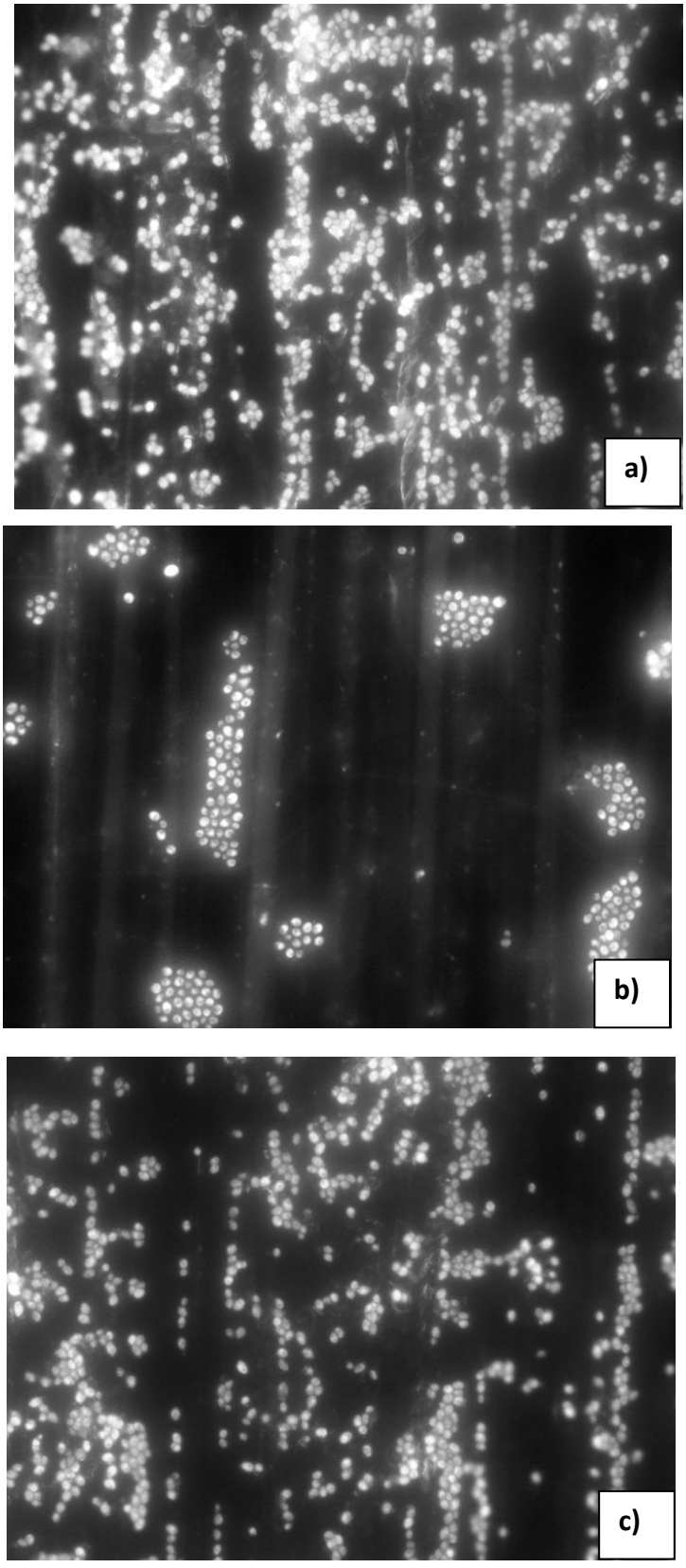


Figure 4.35 Adhesion of *C. albicans* to abraded denture acrylic surfaces (a) under normal conditions, (b) after wash with oily farnesol resulting in cell clusters and (c) after exposure to farnesol vapour.

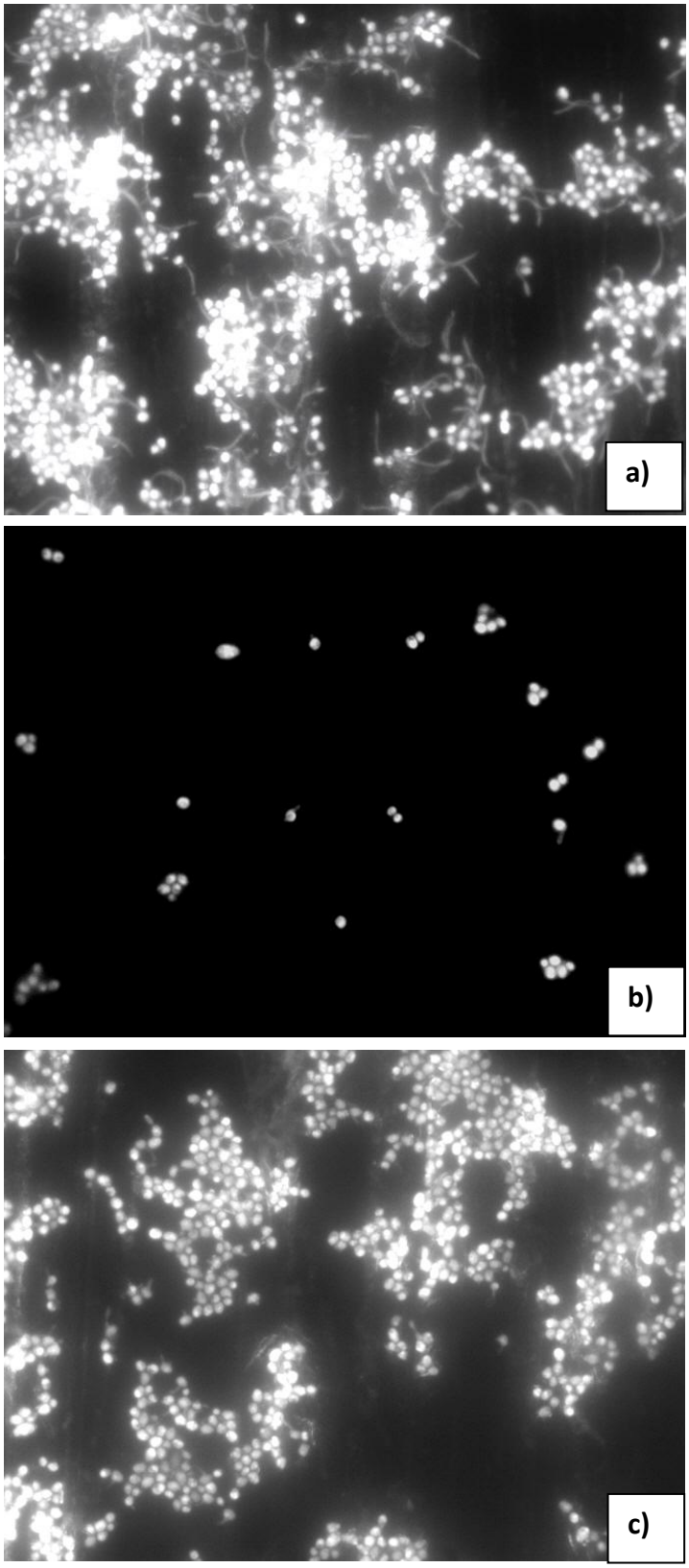


Figure 4.36 Hyphal formation in adhered *C. albicans* on abraded acrylic surfaces (a) under normal conditions, where hyphal growth is abundant, (b) after surface wash with farnesol where budding yeast cell are present in small clusters due to oily properties of farnesol and no hyphae have formed and (c) in the presence of farnesol vapour which has reduced the degree of hyphae growth in adhered cells.

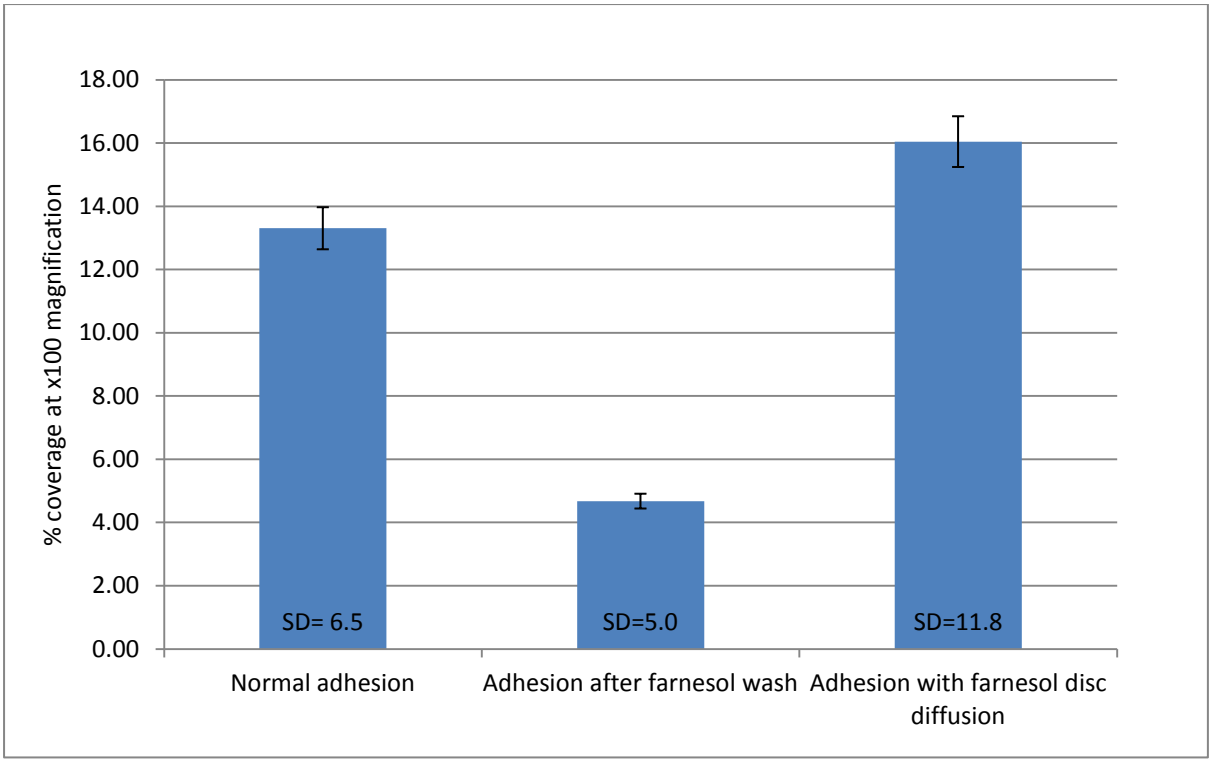


Figure 4.37 The percentage coverage of *C. albicans* on abraded denture acrylic surfaces, in normal conditions, following wash with farnesol and with exposure to farnesol vapour. Adhesion is significantly reduced ($P<0.01$, as determined using 2-way ANOVA) on surfaces washed with neat farnesol, but not after exposure to farnesol vapour.

4.4 Discussion

4.4.1 Optical tweezer microscopy

Preliminary work carried out in these studies utilised a novel method of investigating *C. albicans* hyphal growth and behaviour. Optical tweezers use forces generated by the interaction high intensity light beams with matter, to trap and manipulate objects within the focus of an objective lens. Since its introduction by Ashkin *et al*. (1986) there have only been a handful of reports of the use of this technique for the investigation of yeasts and filamentous fungi, several of which have utilised yeast to test the capabilities of different optical tweezer systems (Ashkin, 1991; Daria *et al*., 2004; Sacconi *et al.,* 2005; Lafong *et al.,* 2006). The work reported here appears to be the first account of the experimental use of optical tweezers for the investigation of *C. albicans* hyphae. Although findings of this preliminary work were inconclusive and reproducibility was poor, this may have been due to a range of contributing factors such as a concentration of cells too low to elicit an effect, no available nutrients and technical issues with temperature control. In order to expand the data collected and investigate this area more thoroughly it would be beneficial to initially increase the concentration of cells within the system. An initial set up placing a single cell adjacent to a large cluster of cells would be interesting to examine (Figure 4.38). It may be hypothesized that a higher concentration of cells would release more signalling molecules and may therefore cause more of a response in the hyphae of the nearby single cell, causing it to grow towards the cell cluster.

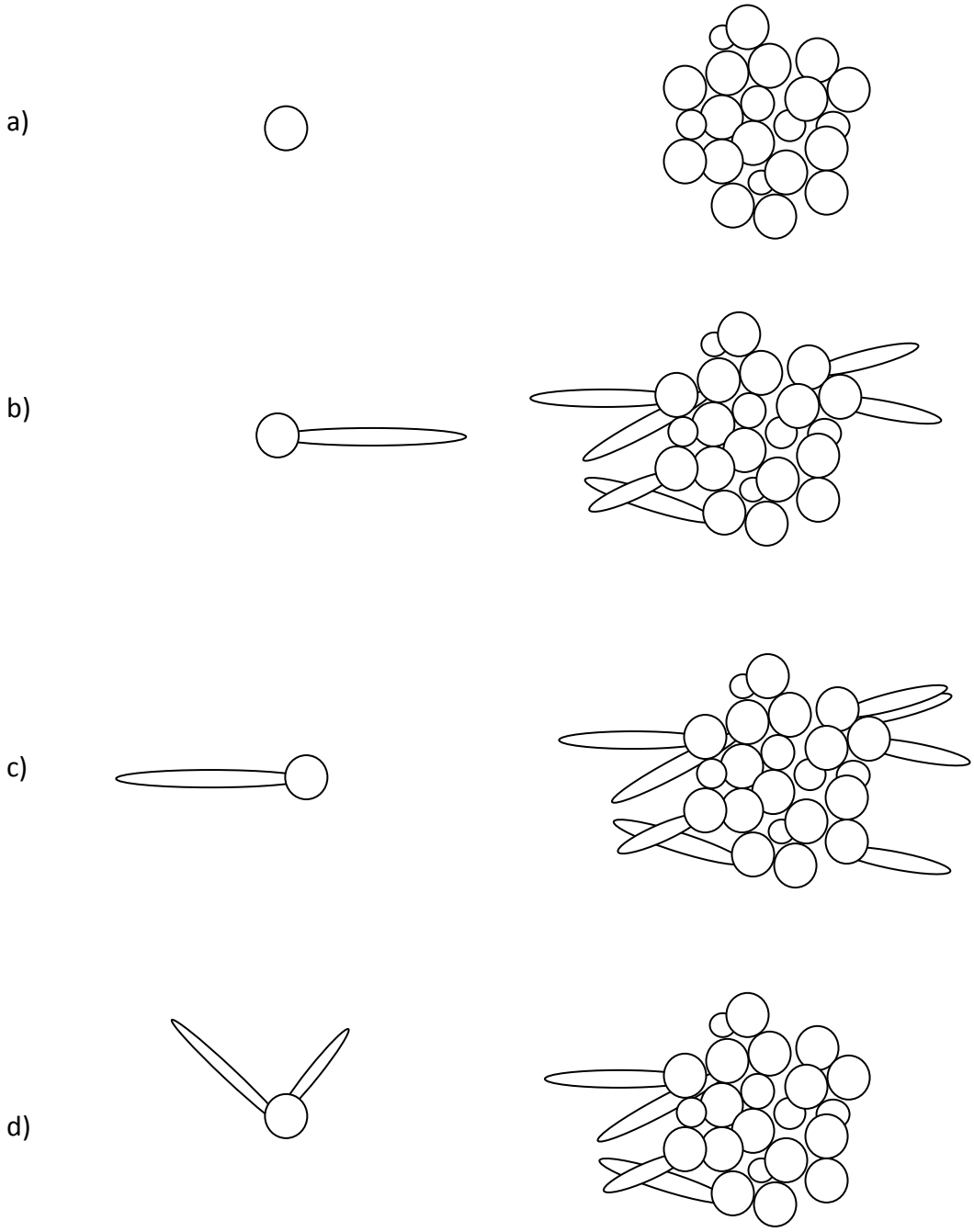


Figure 4.38. **The suggested placement of cells and the potential observations for future optical tweezer work.** a) Cells could be arranged with a single cell being placed near to a large cluster of cells. A larger group of cells would be hypothesized to result in a greater concentration of signalling molecules which may result in direction hyphal growth for example, b) Hypha of single cell may be attracted to and grow towards concentrated cell cluster, c) may be repelled to grow in the opposite direction or d) may grow out randomly as was seen in work reported here.

One observation made in the optical tweezer work carried out here was that many cells initiated germ tube growth in one region, which ceased shortly after and then proceeded from a different region of the cell. This phenomenon does not seem to have been reported previously but suggests some preference for the location of germ tube outgrowth by *C. albicans* cells. In previous work the emergence site of germ tubes has been reported from any surface location (Mackenzie, 1964) and in non-adjacent, random patterns from cells (Chaffin, 1984) providing inconclusive evidence for the preferred sites of germ tube emergence. Chaffin (1984) indicated that temperature may affect the site of germ tube growth. This could be significant to the results of this study as the temperature of the optical tweezer system was difficult to maintain and monitor. This was because the temperature was set to 37°C through the computer system and produced by the heated stage, which did not have a means of temperature gauge and may have been influenced by the temperature of the room from day to day. Another variable was found in the duration of manipulation of cells with the optical laser. Cells of the yeast *Saccharomyces cerevisiae* were shown to be affected by long term and pulsating exposure to lasers in this same system (Aabo *et al.*, 2010). In their work Aabo *et al.* showed that laser exposure affected the ability of *S.cerevisiae* to bud, and increased laser power reduced budding. In this study *C. albicans* cells were manipulated into position by the same lasers and this may therefore have affected hyphal growth. There is also evidence to suggest that the emergence sites of budding cells and germ tubes is influenced by electrical forces (Crombie *et al*., 1990), but this does not explain the appearance of two germ tubes on cells monitored in this work. It would therefore be necessary to investigate this phenomenon further in order to understand the purpose behind this growth. Future work could incorporate electrical

stimuli in an attempt to investigate the preferred site of germ tube emergence and direction of elongation further. Future studies could also seek to progress further with this work by incorporating known signalling molecules into the system and observing *C. albicans* yeast cell and hyphal growth behaviour. This work has highlighted the vast potential of the use of optical tweezer microscopy in the investigation of hyphal growth.

4.4.2 Identification of quorum sensing molecules in *C. albicans*

Gas chromatography identified time points at which key volatile compounds and known quorum sensing molecules were released from the *C. albicans* cultures. In three repeat studies volatile compounds (farnesol and ethanol) were detected within a two hour window, giving some indication of reproducibility. Ethanol began to be detected at 4 hours of growth followed by Farnesol at 10 hours, other farnesol synonyms, dodecanol and nerolidol were detected between 18-24 hours and solanesol at 22 hours. Ethanol production in *C. albicans* is well reported (Wilson *et al*., 1966; Iwata, 1976; Logan and Jones, 2000) and has been shown to initiate germ tube production (Pollack and Hashimoto, 1985; Zeuthen *et al.,* 1988). Pseudo hyphal growth was first noted at 3 hours in *C. albicans* cultures. This may indicate the production of ethanol in early stages of growth at levels below detection (i.e. not in high enough concentrations in the headspace to compete for absorption sites on the SPME fibre). Farnesol reached detectable levels at 10 hours. At this time the dominant cell morphology was budding yeast cells, although pseudohyphae and true hyphae were present. As described in section 4.1, farnesol appears to have a cumulative effect on *C. albicans* inhibiting hyphal growth in later stages of growth (Alem *et al.,* 2006) In the 24 hour observations

of planktonic cultures in this work therefore farnesol is not likely to have influenced cell morphologies greatly. The semi quantified amount of farnesol however (as with other detected compounds) was noted to increase over time. Between 18 and 24 hours of growth different peaks representing farnesol were detected. These included dodecanol and farnesol 1, 2, and 3. Dodecanol and nerolidol have been described in previous research (Davis-Hanna *et al.,* 2008; Hogan *et al.,* 2004; Hornby *et al.,* 2001; Martins *et al.,* 2007; Chauhan *et al.,* 2011) and are believed to have similar activity to farnesol. The detection of these compounds and other farnesol volatiles in the later stages of planktonic growth indicate evidence for the cumulative action of the increasing amount of farnesol, indicating a potential regulatory response on the yeast in preventing an over growth of hyphal morphologies (Kuhn *et al*., 2002), in the nutrient limited environment of the aging broth culture. In the latter stages of planktonic growth (22-24 hours) small amounts of a compound identified as solanesol was detected in 2 of the three repeat studies. This compound was detected with low accuracy (38-43%) and may therefore have been identified incorrectly when compared to the known compound database. Ethanol however, was also initially detected in lower concentrations and with low accuracy in the early stages of growth, its concentration and accuracy of detection increased over the 24 hour time period. Solanesol found in this work may therefore also have been in low concentrations in initial detection stages and if experiments had been continued over a further 24 hours the concentration and accuracy at which it was detected may have increased. The potential production of solanesol by *C. albicans* has not been reported previously. Solanesol is a trisequiterpenoid alcohol commonly isolated from tobacco leaves and is the starting component of vitamin K and Co enzyme-Q10 (Severson *et al.,* 1977). Solanesol has also been described as having a

potentiating effect on these molecules, increasing their activity. Co enzyme Q10 is produced commercially as a therapy by fermenting beets and sugar cane with yeast (Cooke, 2012). It is thought to be an endogenous antioxidant in humans (Frei *et al.,* 1990, Tomasetti *et al.,* 2001), and is an important contributor to oxidative phosphorylation in mitochondria. Perhaps the presence of solanesol (the starting product of these substances) in the later stages of planktonic growth of *C. albicans* cultures could provide a protective function in response to oxidative stress caused by the accumulation of waste products including reactive oxygen species over time. The identification of solanesol in this work is inconclusive and further work investigating any possible production and effects of this product in *C. albicans* is required if its presence and activity are to be elucidated.

Alem *et al.,* 2006, reported the identification of tyrosol from early stage *C. albicans* biofilms and planktonic cultures. Tyrosol was not isolated from planktonic or biofilm cultures in this work which may be a result of a number of factors. Firstly, biofilms analysed in this work were only measured for detectable compounds at 24 and 48 hours of growth. Alem *et al.,* detected tyrosol after 10 hours of growth in planktonic cultures and in 1-6 hour biofilms and noted that farnesol dominated in later stages of biofilm growth. In this study any tyrosol present and tyrosol production by cells at 24 and 48 hours may have been low in comparison to the amount of farnesol and therefore not detected. Alem *et al*., also utilised a method of detection, high performance liquid chromatography, different from that of the GC-MS used in this work, which may have resulted in differences in the compounds detected. An early study by Nykanen *et al.,* (1966) used gas chromatography to identify tyrosol but their methodology utilised different GC-MS settings to those of this study. Their

chromatography was performed at 190° C, with the injection port kept at 330° C, and flow rate 200 ml per min. In the work reported here, the column temperature was initially maintained at 70°C for 10 minutes and then increased to 270°C at a rate of 10°C per minute, the injector and detector temperatures were set to 270°C and the flow rate was set to 100ml per minute. These differences in methods used may have influenced the volatiles that were detected; tyrosol may have been present in *C. albicans* cultures but was not detected using this method. Furthermore their study examined different strains of *C. albicans* to that used in this study and also used different growth conditions, factors which have been shown to affect volatile compounds produced (Buzzini *et al.,* 2003a, 2003b; Martins *et al*., 2007). Future work could experiment with different settings of the GC-MS equipment in order to find optimal conditions for detection of tyrosol as well as ethanol and farnesol.

Overall The SPME GC-MS method used in this study was successful in the identification of volatile and potential quorum sensing molecules produced by *C. albicans*. It provides a basis for future work whereby repeat experiments could incorporate changes to growth conditions to investigate differences in volatiles released. It would be of interest to investigate the release and accumulation of compounds under stress conditions such as low oxygen or nutrient supply. Different growth conditions could be explored including growth in carbon dioxide rich environment or at different temperatures. Additionally it would be interesting to utilise this method to investigate the production of compounds from mixed cultures and other cell morphologies of *C. albicans*, during various growth phases. Another consideration for future work would be to explore the quantification of the volatile

compounds identified in this study. Although an excellent method of extraction, SPME provides results that are difficult to quantify due to the nature of the absorbance of the needle. The needle once placed into the headspace of the sample absorbs evaporated compounds readily and soon becomes saturated. There is no control of the proportion of different molecules absorbed and no measure of how much in total is analysed by the subsequent GC-MS. Peak areas give a semi quantitative analysis, whereby the most abundant molecule present in the sample absorb most frequently onto the SPME fibre and as such are desorbed and detected in the highest amounts upon GC analysis. This however cannot be compared to an amount per sample. Future work could investigate the use of headspace sampling and gas chromatography, where a syringe is used to directly sample the headspace above the sample. A known amount of this head space gas can be extracted and injected into the gas chromatograph allowing for a comparison of the amount detected with the amount injected in order to improve quantitative analysis. Alternatively internal standards could be used to determine peak areas at different concentrations of volatile compounds, which could be compared to the peaks detected from growing yeast cultures as an estimate of the concentration.

4.4.3 Effect of direct and indirect farnesol exposure on adhesion and hyphal formation in *C. albicans*

The investigation into the direct and indirect effects of farnesol on the adhesion and hyphal formation in *C. albicans* supported findings of previous work. Farnesol vapour (from 100 µl inoculated discs) had no effect on the amount or arrangement of adhesion of *C. albicans* to abraded denture surfaces. Direct contact of these surfaces with farnesol however resulted in clumped cell adhesion possibly due to the water insoluble hydrophobic nature of the oily farnesol (Derengowski *et al.,* 2009). Hyphal induction under serum conditions was inhibited by both directed contact with farnesol washed surfaces and incubation with discs giving off farnesol vapour. This effect of farnesol has been described previously (Hornby *et al*., 2001; Ramage *et al*., 2002a), although the identification of this action by farnesol vapour has not been previously described. Farnesol is known to reduce *C. albicans* biofilm formation but has also been implicated as an integral molecule for the survival of *Candida* spp. (section 4.1). Further investigations into its use, concentration effects and incorporation into cleansers and treatment may expand its capacity for use as a control or preventative measure for *C. albicans* biofilm growth on denture surfaces.

4.5 Conclusions

Gas chromatography analysis of *C. albicans* cultures detected known quorum sensing and effecter molecules that could be correlated with *C. albicans* morphologies and incubation time. These factors could potentially be developed and applied to the identification and diagnosis of candidiasis using gas chromatography analysis of patient samples including breath analysis for the identification of oral infections. Furthermore, the potential identification of solanesol from planktonic *C. albicans* may suggest that there are many more signalling and effector molecules yet to be identified for *C. albicans*.

As with other research, farnesol was found to prevent the filamentation of *C. albicans*, a process considered to be integral for pathogenesis in this species. Further investigation into this and other identified quorum sensing molecules may help to provide vital insights and novel treatments for *C. albicans* biofilm formation on denture surfaces.

Further work will look at denture cleanser assays for their efficacy against blastospore and hyphal biofilms of *C. albicans*. The incorporation of farnesol into such denture cleanser in order to prevent hyphal growth and reduce biofilm accumulation by *Candida* spp. on denture surfaces could be investigated in the future.

Chapter 5

Discussion and Future Work

5.1 Denture surface topography and the retention of *C. albicans*

The aims of this work were to investigate the retention, removal and biofilm development of the opportunistic pathogen *C. albicans* on abraded denture acrylic surfaces. Previous work has investigated the relationship between *C. albicans* retention and denture surface roughness (Quirynen *et al*., 1990, Verran *et al.,* 1991, Quirynen and Bollen, 1995; Radford *et al*., 1998, Morgan and Wilson 2001, Coulthwaite and Verran, 2007) aiming to identify factors that would reduce *Candida* spp. on dentures and thus the incidence of *Candida* associated denture stomatitis; a condition in which *C. albicans* is strongly implicated (Budtz-Jorgensen, 1974; Webb *et al*.1998a, 1998b, 1998c; Ramage *et al.,* 2004). Many previous studies have utilised surface roughness parameters, particularly the Ra value (average arithmetic surface roughness) to relate surface topography to cell retention. However, surface feature dimensions in relation to cell shape and size are also important. In this work Ra/Sa values within the ranges used (typically encountered via brush abrasion) were found to positively correlate with an increase in the amount of *C. albicans* retention on denture acrylic surfaces. Features on denture acrylic surfaces with similar dimensions to adhering cells are likely to result in an increased strength of attachment and cells will therefore be more difficult to eradicate from the denture surface. In this work features within the ranges of *C. albicans* cell sizes were found across all test substrata, and it is suggested that many more measurements should be made of features across a surface to be able to predict their interaction with microorganisms. To the author's knowledge, the topography and micro-architecture of typical denture surfaces has not yet been described in this context. *In-vivo*, the

roughness and topography of the denture fitting surface is related to that of the patient's tissue. Does this topography alter over time? It may be hypothesized that smoother surfaces of the denture, such as the gums, might become more abraded over time, whilst the irregular topography of the denture fitting surface might become smoother as it is abraded by brushing. In addition to this and as with all *in-vivo* work, a high amount of variability would be expected as different regular cleansing regimes and lifestyle factors would alter the topography of dentures on a patient to patient basis. This would make it difficult to design a study with sufficient numbers for statistical significance. Although the abrasion methods used in this work are likely to be exaggerated in comparison to those that can occur *in-vivo*, they allowed for the investigation of retention and accumulation of *C. albicans* in relation to a standardised roughness and topography. The nature of the abrasion methods used on these surfaces, however, resulted in linear features with dimensions within a standardised range. *In-vivo*, dentures may be exposed to a wide variety of abrasive processes such as different foods, handling damage and variable cleaning protocols is which are likely to generate a much wider range of features. There remains much to be done with regards to the characterisation of denture surface topography and its relationship with cell retention.

5.2 Quorum sensing in *C. albicans*

The investigation into quorum sensing molecules released by planktonic *C. albicans* combined a growth curve with gas chromatography, allowing for the correlation between cell numbers, morphology and the release of volatile compounds over time, in what we believe to be a unique study. It would be interesting to introduce quorum

sensing molecules such as tyrosol and farnesol into growing cultures at various stages of the growth curve and investigate effects on the progression of growth, hyphal development and detection of volatiles. Additionally, optical tweezer work carried out in this project provided a novel method which has potential for the future study of *C. albicans* hyphae. Farnesol and tyrosol could also be added to this set up at various stages of growth. This work would help to determine any effect of these molecules on planktonic and adhered (optical tweezer) cells. It may be expected that the introduction of tyrosol would enhance the growth and production of hyphal cells which may in turn affect the time of observable hyphal growth and the detection of volatile compounds with gas chromatography. Similarly, farnesol has been shown to inhibit the initiation of hyphal growth but, if already emerged, germ tubes continue to develop despite the presence of farnesol (Ramage *et al,* 2002a). If farnesol were added at different times to the growing planktonic or adhered *C. albicans*, it may effect when and if hyphal growth would be observed and the volatile compounds detected. The control of hyphal growth *in-vivo* may significantly affect the morphology of biofilms in which *C. albicans* is present.

In-vivo C. albicans would exist within a denture plaque biofilm, consisting of a diverse community of microorganisms. Several bacterial species isolated from denture plaque are known to release and communicate via quorum sensing (Li *et al.*, 2001; Miller and Bassler, 2001; Waters and Bassler, 2005; Suntharalingam and Cvitkovitch, 2005). It would therefore be valuable to develop the methods used here to investigate the volatile compounds and potential quorum sensing molecules released from mixed *Candida* spp. and oral bacteria cultures and biofilms, with the

ultimate aim of encouraging a less pathogenic denture plaque that is easier to manage.

5.3 The development and removal of *C. albicans* biofilms on denture surfaces

Adherent hyphae of *C. albicans* altered the structure of subsequent biofilm, and reduced removal of residual cells from abraded denture surfaces, a phenomenon that has been previously reported (Baille and Douglas 1999). *C. albicans* hyphae have been implicated as important contributors to the virulence of *C. albicans* in terms of adhesion to surfaces (Kimura and Pearsall 1980), dissemination, and invasion of host tissues (Lopez-Ribot *et al*., 1996). Although *in-vivo*, it is unlikely that denture plaque comprises of the same extent of hyphal growth investigated in these studies, the presence of hyphal, pseudohyphal and germ tubes of *C. albicans* would appear to be undesirable in *Candida* spp. and denture plaque biofilms, due to the difficulty of removal and association with tissue invasion and virulence (Gow *et al.,* 2002; Felk *et al.,* 2002; Kummoto and Vinces, 2005). In this work hyphae were found to create a more open biofilm structure and contribute to the retention of *C. albicans* cells at the denture surface/biofilm interface. *In-vivo* the presence of hyphae may therefore also aid the retention and other microorganisms within the hyphal network.

In this work, mixed and pure *C. albicans* biofilms were tested against a Polident denture cleanser which was expected to have increased efficacy due to the addition of TEAD, an activator for oxidising agents. This cleanser was tested solely for its chemical activity (i.e. in absence of effervescence) and found to be highly effective against *Streptococcus oralis* biofilms and effective against *C. albicans* biofilms following extended soak times. An unexpected finding was that *C. albicans* in

biofilms appeared to be more susceptible to the activity of the cleanser when grown with *S.oralis* as opposed to when grown in pure biofilms. This result has not been reported previously but may be explained by further investigation of the mixed biofilm structure. The inactivation of *S.oralis* (which may have occupied spaces surrounding *C. albicans* cells) prior to *C. albicans* in these biofilms may have resulted in disruption to the biofilm structure (Chapter 4) and increasing the accessibility of the denture cleanser to *C. albicans* cells. Future work could use the confocal microscopy method developed in this work to investigate the structure of mixed biofilms, paying attention to the organisation of bacterial species in relation to *C. albicans* cells and investigating how their inactivation affects subsequent survival of *C. albicans* within these biofilms.

5.4 *Candida albicans* GDH 2346 NCYC 1467

In this work only one strain of *Candida albicans* (GDH 2346, NCYC 1467) was used across all experimental assays. This strain was chosen as it was originally isolated from a clinical case of denture stomatitis at Glasgow Dental Hospital and has been used in several other studies (Verran *et al*. 1991, Verran and Maryan, 1997, Bulad *et al*. 2004). The use of this strain alone however is a limitation of this work as other *C. albicans* strains may behave differently in terms of adhesion and biofilm and hyphal growth. A study by Vasalis et al. (1992) investigated the effect of morphological differences between different *C. albicans* strains on their adhesion to denture acrylic. They found that the different strains varied significantly in the level of adhesion to denture acrylic surfaces, which was enhanced in the presence of a salivary pellicle. They also observed that a morphological variant strain resulted in

lower levels of adhesion. Other studies have also shown that different strains of *C. albicans* can differ in the level of adhesion to denture acrylic surfaces (Waters *et al.* 1997, Calderone and Braun, 1991). Additionally, McCourtie and Douglas (1985) demonstrated differences in the extracellular polymers produced by different strains of *C. albicans.* As EPS makes up a large constituent of Candida biofilms, these differences may also influence the structure of biofilms and their removal from denture acrylic surfaces. Different strains of *C. albicans* may vary in the morphological forms they exhibit which will in turn alter the biofilms they form (Baillie and Douglas, 1998). It would be useful in future work to repeat experiments with different strains of *C. albicans* in order to compare them within the context of this work.

5.5 Conclusion

The results of the work carried out in this project indicate that in order to manage the accumulation of *Candida* spp. and other denture colonisers on denture surfaces, it is desirable to limit the amount of abrasion to which a denture is subjected to reduce the retention of cells, and to prevent hyphal growth of *C. albicans*. The resultant topography itself does not affect the way the cells grow, but does enhance cell retention. Low abrasive cleansers alongside a low abrasive mechanical protocol such as effervescence, vigorous rinse or brushing with a soft brush would be beneficial in order to reduce the level of microbiological contamination by minimising the amount of abrasion. Hyphal growth in denture plaque biofilms appears to be undesirable; it would therefore be beneficial for future work to investigate the incorporation of quorum sensing molecules such as farnesol into denture cleansers or denture materials in order to reduce the amount of hyphal growth and in turn contribute to the efficacy of hygiene regimes. The Polident denture cleanser tested in these studies was effective at reducing the viability of both *C. albicans* biofilms and mixed *C. albicans* and *S.oralis* biofilms. Coupled with its effervescent capability and a rinse and brushing, a successful, regular denture hygiene regime for the maintenance of good denture hygiene and reduction in the incidence of denture stomatitis may be anticipated.

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Appendices

Appendix I – Submitted journal article

Submitted to: Journal of Prosthetic dentistry under review process.

The effect of dentifrice abrasion on denture topography, and the subsequent retention of microorganisms on abraded surfaces

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Introduction

Denture acrylic resin is softer than tooth enamel, and as such is more susceptible to abrasion.[1] If inappropriate products are used to clean the denture, for example dentifrices intended for teeth, then the acrylic resin will become scratched.[2] It is well recognised that surfaces with increased roughness are better able to retain both microorganisms[3-5] and organic material,[6] thus cleanability is reduced. The application of increased force is then required to clean the surface, resulting in increased surface wear. Thus a cycle of wear and soiling is initiated, that is already well recognised in other applications,[6] but which has received relatively little attention in prosthetic dentistry.[2]

The literature on the effect of surface roughness on the retention of oral microorganisms on surfaces is significant, but some difficulties can be encountered in comparing results from different studies due to the number of variables used. These include different methods to produce and characterise the modified,[7] different experimental conditions[8,9] and different microorganisms[10-12] of different dimensions. It is likely that microbial cells will be retained within surface defects (such as scratches or pits) of comparable dimension to the cells themselves,[13] but the relationship between these two parameters has not been explored in prosthetic dentistry. It is also important to differentiate between methods used to assess the amount of retention – measured for example by assessing numbers of attached cells, or coverage of surface by cells – and the strength of retention: the latter perhaps being the more pertinent phenomenon.[14] No studies could be identified which compared these two phenomena.

The aim of this study was to investigate whether abrasion of denture acrylic resin that has been caused by dentifrice application affects the retention of microorganisms and thus surface cleanability and hygiene. Two microorganisms were used: *Candida albicans*, generally deemed to be a significant aetiological agent in denture stomatitis,[15] and *Streptococcus oralis,* an early coloniser of hard oral surfaces.[16] Two assays were utilised: 1 a 'retention assay', which compared the retention of cells on surfaces after 1h exposure and a standardised rinsing procedure, which gives an indication of 'amount of attachment', and a 'strength of attachment assay', which applies an increasing force onto attached cells in order to remove them.

Methods

Surface preparation and characterisation

Substrata

Polymethylmethacrylate (PMMA) coupons 2 cm^2 and 5 mm thick were abraded in a defined manner using 800 strokes with blocks rotated 180 degrees after 400 strokes under 2.9 N force [17] to provide linear, approximately parallel, abrasions. The abrasive used was a toothpaste slurry, with the dentifrice selected to provide different degrees of abrasion: Colgate Luminous (Colgate Ltd, Guildford Business Park, Surry, UK) was used neat to provide 'high abrasion' (HA); 'medium abrasion' (MA) used Colgate Total Whitening 25:40 paste/water ratio; and 'low abrasion' (LA) used Colgate cavity protection 25:40 paste/water ratio. A non-abraded control was included.

White light profilometry (WLP)

A MicroXAM (phase shift) surface mapping microscope (ADE Corporation address) with an AD phase shift controller (Omniscan, Wrexham, UK) was coupled with an image analysis system (Mapview AE 2.17 (Omniscan, Wrexham, UK) to characterise the surface.[18] Analysis was carried out using EX mode, with 10 different fields examined for 3 replicate coupons of each abraded surface. Surface maps were generated, along with surface profile and roughness values (S_a – average deviation of the roughness irregularities from the surface mean centre line, and S_q – standard deviation of the distribution of the surface peak heights), at a magnification of x 101.61 (86.14 x 64.07 µm). Measurements of representative feature width and depth were made.

Atomic Force Microscopy (AFM)

The AFM (Explorer, Veeco Instruments Ltd., Cambridge, UK) operated in contact mode using silicon nitride pyramidal shaped tips with a nominal spring constant of 0.05 N m^{-1}. Averages of R_a (average deviation of the roughness irregularities from the surface) and R_q (Standard deviation of the distribution of the surface peak heights, also known as RMS) values for the surface profile were obtained from three linear scans taken across individual samples over 50 µm x 50 µm fields[19] with a scan rate of 100.03 µm s^{-1} and 300 pixel resolution.

AFM data were also used to determine whether the surfaces could be used in subsequent strength of attachment assays, particularly for the yeast cells. Since *C. albicans* cells can measure up to 5 microns and the z height limit of the AFM is

around 6 microns, too rough a surface would result in the AFM being unusable either to visualise the cells or assess strength of attachment.

Retention assays

Maintenance of Candida albicans

Stock cultures of *Candida albicans* NCYC 1467 (also known as GDH 2346) were stored at −80˚C. Sub-cultures were prepared on Sabourauds agar (Lab M, UK) agar before use and were maintained at 4˚C. New stocks were prepared from the frozen stock cultures every 4 weeks. In preparation for assays, stock cultures were inoculated onto Sabouraud agar and incubated at 37˚C for 24 hours. Then a single colony of *C. albicans* was inoculated into 100 ml of Sabouraud broth (Lab M, UK) which was subsequently incubated at 37˚C for 24 hours in an orbital shaker.

Cells were harvested by centrifugation, following which they were washed in sterile water 2 times, then re-suspended in sterile water to an optical density of 1.0 at 540 nm, corresponding to a standardised cell suspension containing approximately 7.8 ± 0.18 x 10^4 cfu ml^{-1} .

Maintenance of S.oralis

Stock cultures of *S. oralis* NCTC 11427were stored at −80˚C. Sub-cultures were prepared before use and were maintained at 4˚C. New stocks were prepared from the frozen stock cultures every 4 weeks. In preparation for assays, stock cultures were inoculated onto blood agar and incubated at 37˚C for 48 hours under anaerobic conditions. Then a single colony of *S. oralis* was inoculated from a blood agar plate into 100 ml of BHI broth (Lab M, UK) and incubated for 18 hours without

shaking 37˚C in an anaerobic cabinet. Cells were harvested by centrifugation (3600 × *g* for 12 minutes) and washed once in 10 ml sterile distilled water. The resultant standardised cell suspension was adjusted to an optical density of 1.0 at 540 nm corresponding to a concentration of $1.32 \pm 0.81 \times 10^{9}$ cfu ml^{-1}.

Retention assay

Standardised cell suspension (150 mL) was added to a large (13.5 cm diameter) Petri dish, containing 3 replicates of each of the test materials, which was then incubated for 1 hr at room temperature (25C ± 2) without agitation. After 1 hour the test materials (with adherent cells), were removed and washed by rinsing with running sterile water (approx 7 ml ± 1) from a even flowing wash bottle for 3 seconds with the surface held horizontally and the water directed to the centre of the PMMA pieces. This allows pooling and flow of liquid over the surface, removing loosely adhered cells. The surfaces were then tilted to drain off excess liquid and left to dry in a class II laminar flow cabinet.

Cells retained on the surfaces were stained with acridine orange (0.03% in 2% glacial acetic acid): surfaces were rinsed and dried before examination using epifluorescence microscopy (x400) (Nikon Eclipse E600, Nikon UK Ltd, Richmond, UK). Ten fields of each replicate surface were examined. The percentage of an area of each microscopic field was calculated using cell^{F} software (Olympus Soft Imaging Solutions, Southend, UK); the number of cells in each field was also counted. The experiment was repeated.

Statistics

Statistical tests were carried out using a 2 tailed distribution *t*-test with 2 sample homoscedastic variances. The results are reported as mean ± standard deviation or mean ± standard error.

Strength of attachment

In order to determine the strength of attachment, a known force is applied to attached cells using the AFM cantilever tip, and this force is then increased until cells are dislodged. On the abraded surfaces, strength of attachment assays were carried out either along or across the linear features.

One hundred microlitres of cells were applied to the test surfaces and dried for 1 hour under a microbiological class 2 laminar flow hood and for an additional 23 hours at room temperature in a sterile container.

The AFM was operated in contact mode and measurements were carried out at a rate of 100.03 µm s^{-1} at a scan size of 50 µm ×50 µm. Substrata with dried cells were placed on the AFM stage and a dry scan of the sample was taken to ensure the presence of cells in the area of analysis. One hundred microliters of HPLC grade water (BDH, UK) were placed on the sample and the AFM laser was re-aligned. The cantilever was brought into contact with the surface under the water and a measurement of the force applied to the cantilever was obtained from force-distance curves,[19] and a perpendicular applied force was calculated.[19,20]

Replicate substrata were used enabling 4 detachment experiments per surface treatment. After each scan the remaining cells were counted manually in the field of

view and counts were plotted as a percentage value of the initial count against the lateral force applied.

In preliminary work, for *C.albicans* on the smooth surface and low abrasion surfaces, following wetting the cells were lifted straight from the surface within 2 scan passes. Therefore for subsequent experiments, following the first wet scan, cells were redried onto the surface which was then rescanned. This redrying process was repeated for every scan.

Results

Surface topography

For both the WLP and AFM (Figure 1), all roughness parameters increased with increasing abrasion. Differences in the S_a/R_a and S_q/R_q data were greater for the rougher surfaces.

WLP images revealed linear features (scratches) of decreasing number in a given area, from high to low abraded surfaces. The profiles reveal the width and depth of these scratches (Figure 2). The maximum width was similar for all abraded surfaces, at 30-35 μm, but around half that value for the few scratches noted on control surfaces. Scratch depths ranged from 3.5 nm to well over 1000 nm on all other surfaces. The number of scratches was higher with increasing abrasion. Fewer deep abrasions were apparent on the LA surfaces. For all abraded surfaces, smaller defects were evident within larger scratches.

The smaller AFM probe provided additional data. On the low abraded surface (data not shown), only occasional scratches could be seen, since, on this scale, they

were widely separated. These were 1 – 3 µm wide µm, with an average depth of 1300nm. The medium abraded surfaces presented more scratches which ranged in size from 0.5 – 7 µm in width with an average depth of 2300 nm. The high abraded surfaces, showed scratches with the greatest average depth (3300 nm), but also presented some features narrower (0.5 – 5 µm) than those observed with the medium abraded surfaces.

Using retention assays, for both microorganisms, cells were retained in highest numbers on the high abraded surfaces, followed by the medium abraded, low abraded and smooth surfaces (Figure 3). The standard deviation is higher for rougher surfaces, especially for the bacteria. Coverage by bacteria is higher than that for yeast (as it is in the initial inoculum).

Cell counts were also taken of each field for *C.albicans*, giving trends comparable to those of the coverage data, but with some differences due to different cell sizes (budding cells, pseudohyphae) giving similar counts but different (higher) coverage data (results not presented). Numbers of bacteria were too high to count.

Strength of attachment of Candida albicans

On the smooth surface very few yeast cells were retained, despite the application of low forces (<2.2nN). *C. albicans* were more easily removed from the low abrasion surface when the tip was moving across the surface features (Figure 4) than along them (Figure 5).

Data are presented in Figure 6, showing the relationship between increasing scan number (and thus increasing force) and percentage of cells remaining on the surface.

When strength of attachment was assessed by applying force *across* the scratches on the abraded surfaces, (Figure 6a) an increase in abrasion treatment (low to high) size increased the strength of cell attachment. In all cases, the first scan removed the majority of retained organisms: indeed, after 4 scans no cells remained on the low abraded surface. When the strength of attachment was assessed by applying force *along* the scratches (Figure 6b), cells persisted on the low abraded surface for up to7 scans. Retention was higher on both the medium and (particularly the) high abraded surfaces than that observed when the force was applied across the features. Thus overall, the yeast cells were harder to remove when force was applied along the scratches, and cells were more strongly retained on the more abraded surfaces.

Strength of attachment of S. oralis

As soon as *S. oralis,* was immersed in water, the cells appeared to swell (Figure 7), although there is some removal with increasing scan number. In contrast to *C. albicans* (Figure 6) on the smooth PMMA surfaces, *S. oralis* cells were harder to remove (Figure 8), with cells still attached to the surface at scan 7. When the strength of attachment was assessed by applying force *across* the scratches (Figure 8a), cells were strongly attached to low and medium abraded surfaces, with 50% of the initial cell number remaining after 7 scans. In contrast, on the high abraded surfaces (roughest), the majority of the bacteria were removed after only 3 scans. When the strength of attachment was assessed by applying force *along* the surface features (Figure 8b), cells could not be removed from the low abraded surface. More cells remained on the medium abraded than the high abraded surfaces, indicating that the bacterial cells, considerably smaller than the yeast, were most strongly held within the smaller scratches present on the low abraded surfaces.

Discussion

A range of methods have been employed to characterise denture acrylic resin surfaces that have been abraded in a standardised manner, in an attempt to simulate wear by usage of dentifrice. Increasing abrasion increased all roughness parameters, demonstrating the susceptibility of denture acrylic resin to abrasion, and potentially increased propensity for subsequent increased plaque accumulation[21] and more vigorous cleaning. The actual numerical values for roughness that were obtained via WLP and AFM were similar. Differences in probe size, numbers of replicate measurements made, size of area analysed are factors that may help account for differences, as will variability between individual features on the abraded surfaces. However, it is not the intention of this work to simulate exactly reproducible linear features,[19] rather to standardise the abrasion process in order to produce substrata of sufficiently different topographies to enable comparison. The variability observed between feature sizes in this study indicates that if it is intended to relate the size of surface features to interaction with microorganisms, more data should be obtained, describing the profile, width and height of a number of replicate features on these surfaces, enabling a more comprehensive characterisation, rather than relying on statistical indicators of the deviation of a profile.[22]

Data were obtained regarding the dimensions of linear features (scratches) created by abrasion, but findings indicated that more data should be obtained. AFM data revealed the depth of scratches increasing with increasing abrasion. The range of scratch widths was wide, but measurements did not alter to any large extent between the 3 abraded surfaces using either AFM or WLP. It was not possible to

determine whether there was increased width at the top of scratches on the more abraded surfaces due to lack of data. There were also more scratches on the more abraded surfaces, thus one might anticipate then that these surfaces would be more difficult to 'clean' due to the increased feature frequency, depth and more irregular profile.

The probe size of a given profilometer will affect the sensitivity of equipment used, the detail of the profile generated, and hence the data obtained.[7] Equipment that provides measurements most sensitive at the dimension of the microorganism of concern – in this case, AFM – should be selected as appropriate. However, both WLP and AFM revealed that topographic features that are particularly relevant to the retention and attachment of microorganisms (i.e. features of micrometer dimension) are often present within larger features – in this case, scratches.

For both yeast and bacterial cells, an increase in surface roughness, as indicated by Ra or Sa value, increased the numbers of cells retained. Additionally, at higher coverage/cell number levels, it is likely that the data would provide an underestimation of total contamination due to cells being beneath others, particularly on the more abraded surfaces with deeper features. This phenomenon could be explored further using confocal microscopy, along with investigation regarding the effect of shear on attachment and retention. There is little salivary flow on the occluded denture fitting surface, and plaque accumulates in this relatively stagnant environment, but flow cells enable additional exploration of the interactions occurring between cells and surface. The amount of cells retained is however, of little consequence if they are easily removed, and have merely settled

into the crevices. It is the strength of cell attachment data which is of more value, suggesting that cells are more strongly retained on surfaces where feature size approximates to cell size. Thus the differences between the bacteria and the yeast in terms of strength of attachment can be related to the cell size relative to feature size, hence the area of interaction between cell and feature.[12] When cells are firmly retained, application of a force across a linear feature is more effective at dislodging the cell than application of a force along the feature, presumably because of the resistance provided by the opposing wall of the feature. Differences between the across and in-direction measurements were most noticeable on the surfaces that had features similar to the size of the cell being investigated i.e. the low abraded surfaces for *S.oralis*, and the high abraded surfaces for *C.albicans*. Of course, the requirement that the cells needed to be dried onto the surface prior to assessment of strength of attachment puts some artificiality into the situation, since this will inevitably increase the retention. However, as noted, the drying stage is essential, and the relative ease of removal can still be determined. In addition, in vivo, it is the most recalcitrant plaque and the most strongly retained cells that are of concern with regards to denture hygiene.

There are some technical issues which preclude directly comparison of removal of the 2 microorganisms – although the different behaviours can be explained, as above. Firstly, the methodologies used were not identical. Because of the rapid removal of the *C. albicans* cells from the surface following wetting, the cells on the surface were dried between scans to encourage retention in order to obtain any cell counts. However, *S. oralis* was strongly attached and appeared to swell on

wetting. It was not easy to obtain data using this microorganism. It would be interesting, and perhaps beneficial, to compare these results with those obtained using other oral streptococci. An additional limitation is that the forces applied to the attached cells cannot be directly compared. This is because the forces cannot be set so that all measurements start at the same point, since they are affected by differences in cantilever spring constants and environmental conditions, which vary from slide to slide. In addition, the scan sizes are different because the size of the microorganisms is so different (50 μm^2 *C. albicans* and 10 μm^2 *S. oralis*). It must also be noted that this work focuses on cells attached onto a surface from a planktonic suspension. Subsequent colonisation and biofilm formation have not been addressed.

Finally, a number of technical difficulties need to be overcome when measuring the strength of attachment of microbial cells to polymer surfaces using AFM. A highly charged surface repels the scanning cantilever tip, thus a high force has to be used initially to overcome the inertia of the electrostatic interactions of the surface. Although strength of attachment assays have been carried out on polymer surfaces previously,[23,24] this study is the first reported using the atomic force microscope to measure strength of attachment of oral species on denture acrylic surfaces.

To the authors' knowledge, this is the first publication where the amount and strength of attachment of organisms to surfaces have been compared. In terms of removal of attached cells, it is the latter parameter which is of more concern. The AFM provides data on a scale which enables an assessment of the relationship

between the dimension of the cell and the topographic features with which it is interacting.

Acknowledgements

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List of Figures

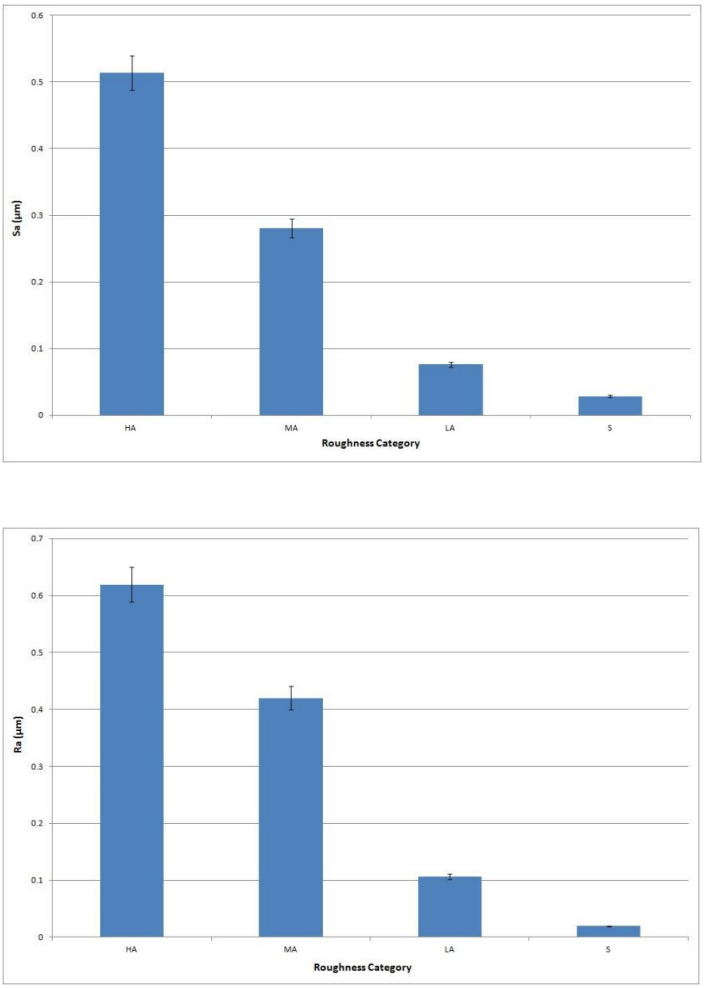


Figure 1. Surface roughness values (S_a and R_a) for the abraded substrata, using a) WLP and b) AFM. HA = high abrasion: MA = Medium abrasion: LA = Low abrasion: S = Smooth non abraded (control).

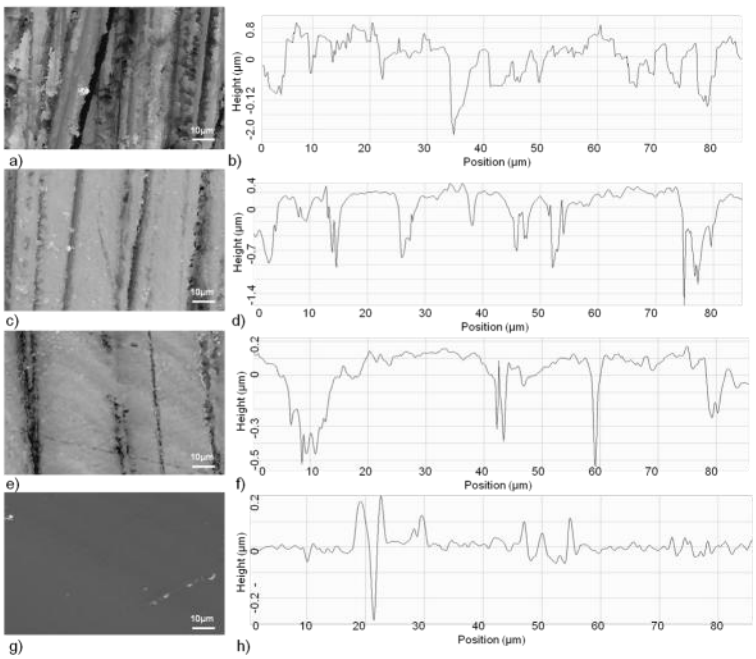
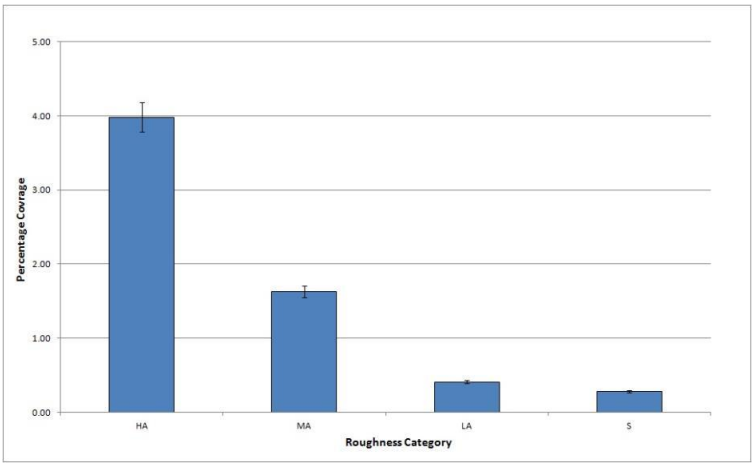


Figure 2. Image and surface profiles of a/b) highly abraded surface c/d) medium abraded e/f) low abraded surface and g/h) smooth, non- abraded surface demonstrating substratum topography and features



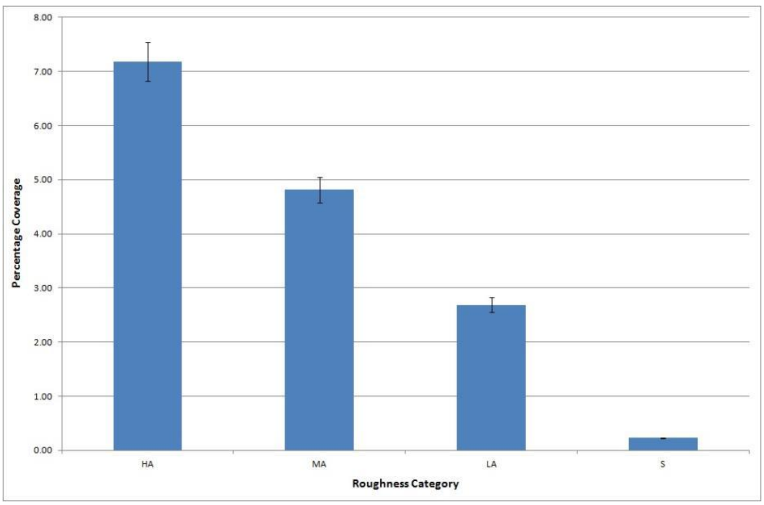


Fig 3. Coverage of the high abraded (HA), medium abraded (MA), low abraded (LA) and smooth (S) surfaces by a) *C.albicans* and b) *S.oralis* (n=30).

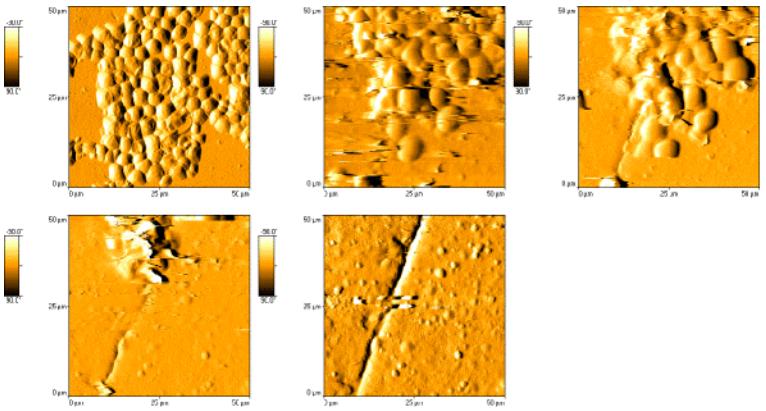


Fig. 4. *C. albicans* cell removal with cantilever tip moving across the surface features a) dry scan b) first scan under water (2.3 nN) c) second scan under water with increased force (3.7 nN) d) third scan under water with increased force (3.8 nN) e) fourth scan under water with increased force (4.3 nN).

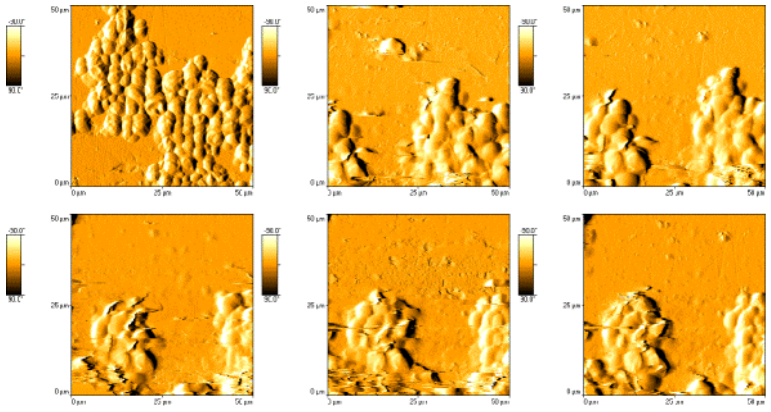


Fig. 5 Candida cell removal with cantilever tip moving along the surface features a) dry scan b) first scan under water (2.2 nN) c) second scan under water with increased force (3.0 nN) d) third scan under water with increased force (5.6 nN) e) fourth scan under water with increased force (8.42 nN) f) fifth scan under water with increased force (8.9 nN) g) sixth scan under water with increased force (10.6 nN) h) seventh scan under water with increased force (9.7 nN)

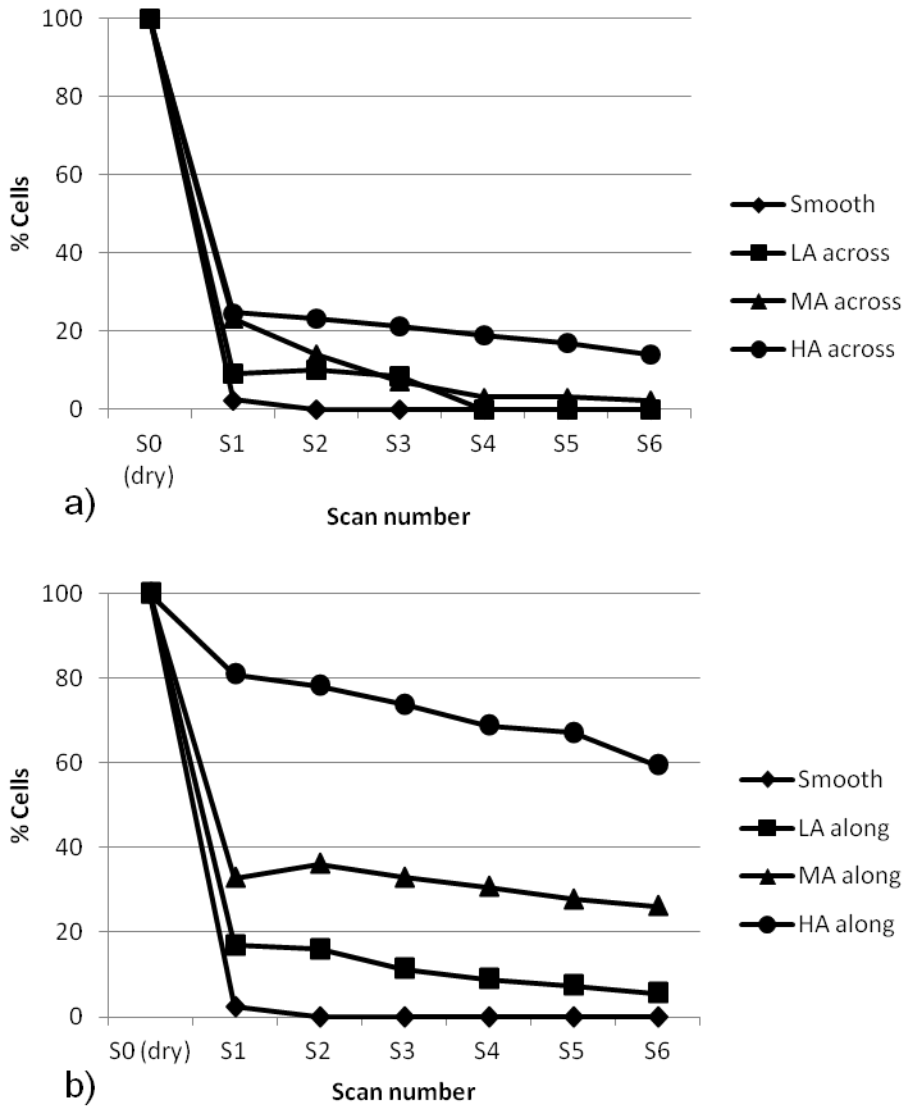


Fig. 6. The effect of increasing applied force on the removal of attached *C.albicans* cells from surfaces. The atomic force microscope probe was scanned (S) over surfaces with attached cells with increasing applied force (0 – 25nN). The percentage of cells remaining after each scan was assessed. Surfaces were smooth ,low abraded (LA), medium abraded (MA) and high abraded (HA) PMMA. The force was applied across (a) or along (b) abraded surface linear features (scratches). On the smooth surface very few yeast cells were retained. *C. albicans* were more easily removed from the low abraded surface when the tip was moving across the surface features. An increase in abrasion treatment (low – high) increased the strength of cell attachment. Cells were retained more on medium and high abraded surfaces with force applied along the features.

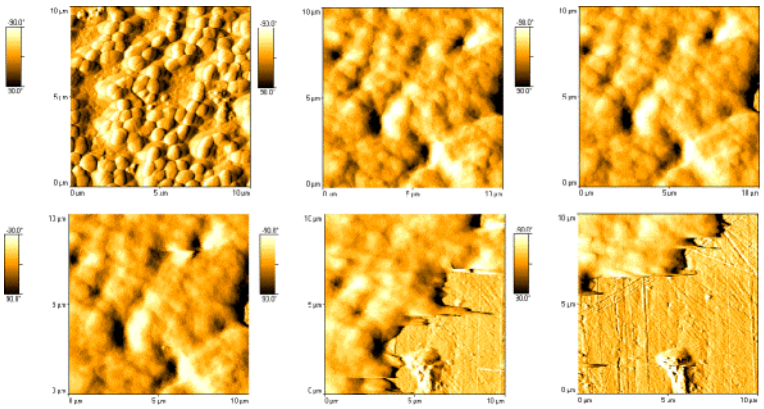


Fig. 7. A) a dry scan of *S. oralis* on the surface. With increasing (wet) scans (b-f), cells are removed, but some extracellular material is apparent when cells are wetted.

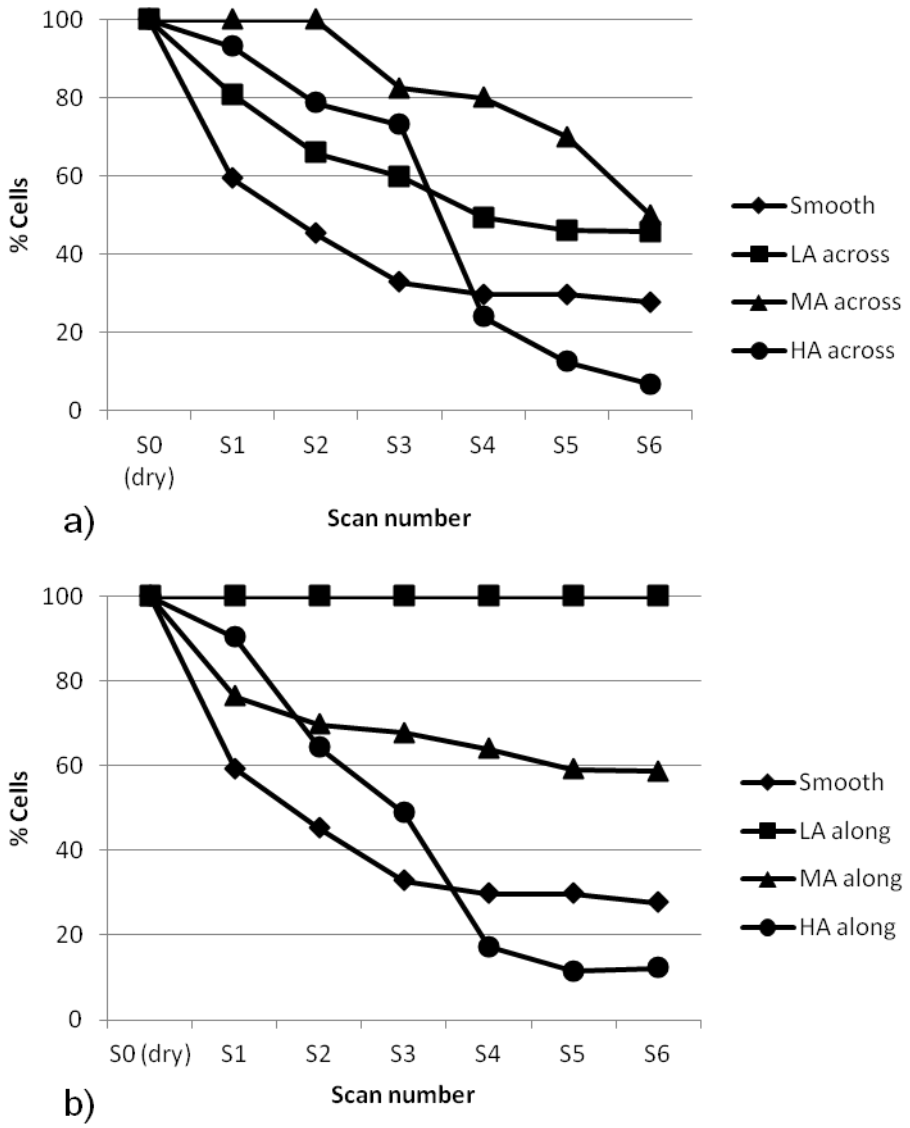


Fig. 8. The effect of increasing applied force on the removal of attached*S. oralis* from surfaces. The atomic force microscope probe was scanned (S) over the surfaces with attached cells with increasing applied force (0 – 25nN). The percentage of cells remaining on each scan was assessed. Surfaces were smooth, low abraded (LA) , medium abraded (MA) and high abraded (HA) PMMA The force was applied across (a) and along (b) abraded surface linear features (scratches).. By applying force across the surface features, cells were more difficult to remove from low and medium abraded surfaces than from the high abraded surfaces. By applying force along the surface features, cells could not be removed from the low abraded surface. More cells remained on the medium abraded surfaces than the high abraded surfaces.

Appendix II – Article in Press

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Biofilm development by blastospores and hyphae of *Candida albicans* on abraded denture acrylic resin surfaces

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ABSTRACT

Statement of a problem. *Candida albicans* is a known etiological agent of denture stomatitis. Candida hyphae exhibit the ability to respond directionally to environmental stimuli. This characteristic is thought to be important in the penetration of substrata such as resilient denture liners and host epithelium. It has been suggested that hyphal production also enhances adhesion and survival of Candida on host and denture surfaces. Surface roughness can additionally enhance adhesion where stronger interactions occur between cells and surface features of similar dimensions.

Purpose. It is known that cleaning regimens and general use may result in the abrasion of denture acrylic resin. Increased roughness can enhance retention of cells on surfaces. Therefore, the development of hyphal and blastospore biofilms on abraded denture acrylic resin specimens and the ease of removal of these biofilms were investigated.

Material and methods. Biofilms were grown for 48 hours on abraded 1 cm^2 denture acrylic resin specimens from adhered hyphal phase *C. albicans* or from adhered blastospores. Subsequently, all specimens were stained with Calcofluor White and examined with confocal scanning laser microscopy (CSLM). Biofilms were removed by vortex mixing in sterile phosphate buffered saline (PBS). Removed cells were filtered (0.2 µm pore size). Filters were dried at 37°C for 24 hours for dry weight measurements. Any cells remaining on the acrylic resin specimens were stained with 0.03% acridine orange and examined with epi fluorescence microscopy. Statistical significance was determined using a two way Anova ($P=<.01$, $\alpha = .01$).

Results. Biofilms grown from both cell types contained all morphological forms of *C. albicans*. Although the underlying surface topography did not affect the amount of biofilm produced, biofilms grown from hyphal phase Candida were visibly thicker and had greater biomass ($P<.01$). These biofilms were less easily removed from the denture acrylic resin, especially in the case of rougher surfaces, evidenced by the higher numbers of retained cells ($P\leq.01$).

Conclusion. The presence of hyphae in early Candida biofilms increased biofilm mass and resistance to removal. Increased surface roughness enhances retention of hyphae and yeast cells and will therefore facilitate plaque regrowth. Minimization of denture abrasion during cleaning is therefore desirable.

INTRODUCTION

Candida albicans is a known etiological agent of chronic erythematous candidosis (denture stomatitis). This inflammatory disorder affects approximately 60% of denture wearers, causing inflammation of the oral mucosa in close contact with the denture.[1] As with natural dentition, dentures provide hard non-shedding surfaces that enable the build-up of plaque biofilms over time. Candida biofilm development on denture acrylic resin begins with adhesion, which can either occur directly to the conditioned surface or via a layer of pre-existing denture plaque.[2] The surface topography of the denture has been shown to greatly influence adhesion and subsequent retention, with more roughened surfaces retaining more organisms.[3-5] The topography of denture surfaces is difficult to regulate. Newly fabricated dentures present a topography reflecting the mucosa of the patient, and are potentially additionally abraded during fabrication and use. Cleaning regimens

involving the use of hard brushes or abrasive cleansers may also alter the surface topography and thus may be undesirable.[6]

Candida biofilm formation on denture acrylic resin surfaces has been investigated and described previously.[7] In vitro, attached budding yeast cells (2 to 4 hours) begin to filament after 4 hours, forming pseudo and true hyphae until after 8 hours, neighboring cells and their filaments become entwined, forming spatially organized woven structures. After 24 to 48 hours of undisturbed growth the Candida biofilms increase in complexity, consisting of several different layers and all morphological fungal forms.[8] The ability of *C. albicans* to alter its morphology is considered to be an important contributor to its virulence, [9] with particular focus on hyphal forms. Candida hyphae have been reported to enhance adhesion to surfaces[10] and are known to bind specifically to several human proteins, including fibrinogen, c3d, and lamanin.[11-13] Candida hyphal formation has also been suggested as being important for the invasion of the host epithelium, allowing dissemination of the organism and aiding infection.[14] Recent attention to contact sensing, or thigmotropism, highlights a possible mechanism for this invasion, where hyphal tip extension is directed in response to contact.[15] The effect of substratum surface topography, specifically of denture acrylic resin surfaces, on this phenomenon has not been investigated. Implications for denture hygiene and oral health are also apparent.

Candida morphogenesis and specifically the transition from yeast to hyphal states are considered important factors in virulence. Candida biofilm development on denture acrylic resin is affected by surface topography, but the effect of topography on different morphological forms of Candida has not previously been

investigated. In this study surfaces of varying degrees of roughness were investigated for their effect on the growth of biofilms from two morphological forms of Candida; hyphae and blastospores. Four aspects were investigated including; biofilm morphology, mass, retention and viability following treatments with denture cleanser. The null hypothesis for the study was that the presence of adhered *Candida albicans* hyphae as opposed to blastospores will not affect the development and removal of subsequent Candida biofilm.

MATERIAL AND METHODS

Culture preparation.

One colony of *Candida albicans* GDH 2346 (NCYC 1467) cultured on Sabouraud's (SAB) Dextrose agar (Oxoid Ltd, Basingstoke, UK) was used to inoculate 100 mL of SAB broth, which was subsequently incubated overnight at 37°C in an orbital shaker (DMS 360; Fisher Scientific, Loughborough, UK) at 150 rpm. The cells were harvested by centrifugation, washed twice in sterile phosphate buffered saline (PBS) (Oxoid), and resuspended in PBS to an optical density of 1.0 at 540 nm, approximately $1.23 \pm 0.14 \times 10^7$ cells/mL.

Preparation of surfaces.

Two differently prepared sets of denture acrylic resin specimens were used for the investigation of biofilm growth**.** Two centimeter squared specimens of pink, heat-polymerized polymethyl methacrylate (PMMA. Meadway heat cure polymer/liquid monomer, Bracon dental laboratory products, Etchingham, UK) were produced and abraded by GlaxoSmithKline consumer healthcare (St George's Avenue, Weybridge, UK) using commercially available dentifrices with varying levels of abrasive activity.[16] These surfaces were subjected to the dentifrices with 800

strokes using a soft nylon bristle tooth brush (Oral B, GlaxoSmithKline consumer healthcare, Weybridge, UK) that was rotated 180 degrees after 400 strokes and compressed with a 2.9 N force. Four different degrees of roughened surfaces were used in the investigation: Control (washed with water), low abrasion (washed with Colgate cavity protection 25:40 paste/water ratio; Colgate Ltd, Guildford, UK), medium abrasion (washed with Colgate Total Whitening 25:40 paste/water ratio), and the high abrasion test group (washed with neat Colgate luminous). Following abrasion, these surfaces were rinsed for 30 seconds in running distilled water. These test substrata were used to compare biofilms visually and evaluate biofilm mass.

The second set of surface specimens were made in house (Manchester Metropolitan University) from pink, heat-polymerized PMMA (Bracon dental laboratory products, Etchingham, UK). These specimens were 1 cm^2 in size and were abraded manually with p100 (162 µm grit size) emery paper (Wetordry; 3M, Bracknell, UK) with 10 strokes in 1 direction (downwards) parallel to the edge of a ruler.

Hyphal induction and biofilm development.

Forty mL of the standardized cell suspension were added to 4 sterile Petri dishes, each containing 3 replicates of the test materials (PMMA set 1), which were then incubated for 1 hour at 37°C without agitation. After adhesion, test specimens were removed and washed gently by immersion in sterile water and agitation (while immersed) by raising and lowering (parallel to the base of the vessel) 10 times. With sterile forceps, half of the replicates were subsequently placed into sterile 25 mL bottles containing 10 mL SAB broth and incubated for 48 hours at 37°C to produce blastospore biofilms. The broth was replaced with fresh sterile medium after 24

hours. Hyphal production was induced in adhered cells on the remaining half of the test surfaces by incubating them in 50% horse serum ((Oxoid) diluted with sterile water) in a preheated 37°C water bath for 3 hours. Each test specimen was then washed, placed in SAB broth, and incubated at 37°C for 48 hours as above (fresh medium added after 24 hours) to produce hyphal biofilms.

Confocal Scanning Laser Microscopy (CSLM) to investigate biofilm morphology.

After 48 hours incubation, all specimens were removed from the 25 mL bottles and washed as described above, to remove non-adherent cells. Biofilms on surfaces were dried in a class II laminar flow cabinet (BH-EN 2003, Safe lab systems ltd, Bristol, UK) (for 1 hr), stained with 0.5% Calcofluor White diluted with 10% potassium hydroxide (Sigma Aldrich, Steinheim, Germany) and incubated at room temperature in the dark for 45 minutes according to the manufacturer's staining protocol. Biofilms were visualized with the ×40 oil immersion (Type F immersion liquid; Leica, Wetzlar, Germany) lens with Confocal scanning laser microscopy (Leica DM 2005, LCS SPE 1000). Three replicates of each biofilm type (hyphal/blastospore) on the 4 differently abraded test pieces (control, low, medium and high) were used and 10 fields were examined per test specimen.

Measuring biofilm mass and retention of cells following removal of biofilms.

In order to assess biofilm mass 3 replicates of hyphal and blastospore biofilms were grown on all abraded test substrata. Individual test surfaces with attached cells were vortex mixed for 30 seconds in 25 mL bottles containing 10 mL of sterile water. The resuspended cells were filtered through 0.2 µm pore filter paper disks (Whatman international ltd, Maidstone, UK), which were then dried for 24 hours in a 37°C incubator and weighed against a sterile filter control. After removal

of the biofilms, test surfaces were stained with 0.03% acridine orange (Sigma Aldrich, St Louis Mo) diluted with 2% glacial acetic acid (BDH laboratories, Poole, UK) and examined with epifluorescence microscopy (Nikon Eclipse 6000; Burgerweeshuispad, Amsterdam, Netherland) for any remaining attached cells. The number of retained cells /hyphae and the percentage of a microscope field covered by cells were determined for each replicate specimen in 10 fields per test piece. The experiment was repeated 3 times. A two way Anova was used to determine the level of significance in results of biofilm mass. The numbers of retained cells were also analyzed using a two way anova however due to the variability within the data it was first transformed using log function.

XTT assay.

In order to compare the susceptibility of hyphal or blastospore biofilms to denture cleansers, biofilms grown from both cell types on the 1 cm^2 PMMA surfaces (abraded with p100 grit emery paper) were prepared as previously described and incubated for 1 hour at room temperature in either a denture cleanser (1 tablet [Polident; GlaxoSmithKline consumer healthcare, Weybridge, UK] in 200 mL sterile water at room temperature as directed by manufacturer guidelines) or 200 mL of sterile water at room temperature. The 1 cm^2 acrylic specimens with attached biofilm were removed and placed into small 5 mL bottles to which 790 µL sterile PBS, 200 µL XTT (Sigma Aldrich) dissolved in PBS to a final concentration of 1mg/mL and filter sterilized with a 0.2 µm pore size filter, and 10 µL Menadione (Sigma Aldrich), prepared in acetone to a 0.44 mM concentration immediately before each assay, was added.[17,18] The 5 mL bottles were incubated at 37°C for 3 hours, allowing the XTT components to interact with the metabolically active cells in the biofilms,

releasing a colored formazan by-product into the supernatant. After 3 hours, 200 µL of supernatant from each 5 mL bottle containing each test specimen was transferred to a sterile 96 well microtiter plate (U bottomed) and analyzed for optical density at 492 nm with a microplate reader (Multiskan Ascent; Thermo Lab Systems, Basingstoke, UK). Five replicate biofilms (5 hyphal, 5 blastospore) were tested on 2 separate occasions by soaking in either cleanser or water as previously stated. For each replicate the supernatant was removed and measured for optical density.

RESULTS

Biofilms grown from blastospores or hyphae consisted of networks of budding yeast cells, hyphae, and pseudohyphae. However, the biofilms grown from hyphal phase Candida contained longer hyphae compared to the hyphae in biofilms grown from adhered blastospores, which were much shorter and tended to be oriented downwards toward the surface (Fig. 1). Most notably, the biofilms grown from hyphal phase Candida were abundant in branching hyphae that fed through the biofilm structure and often appeared to join at several points, a phenomenon which was not seen as frequently in the blastospore biofilms.

Biofilm Mass

Biofilms grown from adhered hyphal phase Candida had a significantly higher biomass ($P \leq .01$) than those grown from adhered blastospores on control, low, medium, and high abraded test surfaces (Fig. 2). The degree of surface roughness did not significantly affect the biofilm mass. This difference in biofilm mass is visible without magnification (Fig. 3).

Cells remaining on surfaces after washing.

Significantly higher numbers of cells were retained (P<.01) on all test substrata for the hyphal biofilms than on the blastospore biofilms (Fig. 4). The difference between hyphal and blastospore biofilm cell retention increased as the level of surface roughness increased.

XTT assay

The hyphal biofilms produced higher spectrophotometer readings (p≤.01) than the blastospore biofilms (Figs. 5, 6) after exposure to either the denture cleanser or water. The higher readings correspond to higher metabolic activity and thus a higher number of viable cells (or more cell biomass) in the hyphal biofilms than in the blastospore biofims after treatment. After 12 hours incubation, both the blastospore and hyphal biofilms that had been soaked in denture cleanser had reduced activity (P≤.01) where the corresponding biofilms that had been soaked in water remained the same (Figs. 5, 6).

DISCUSSION

The results of this study support rejecting the null hypothesis that the presence of adhered *Candida albicans* hyphae as opposed to blastospores will not affect the development and removal of subsequent Candida biofilm In this study the early presence of Candida hyphae was shown to affect biofilm architecture, increase biofilm mass and reduce the removal of biofilms from denture surfaces. Candida biofilms have been investigated extensively, enabling description of their formation and morphology.[7,18] Specifically, this study aimed to investigate how hyphae influence Candida biofilm development. As has been reported in other studies,[8,18] after 48 hours, biofilms comprise complex and organized matrices containing all

fungal cell morphologies. However In this study, adhered Candida hyphae gave rise to biofilms containing more hypha-hypha contact and hyphae were longer and more prevalent throughout the biofilm structure. Candida hyphae have been thought to be more adherent than blastospores[10]; thus an advantage is conveyed in terms of initial colonization as well as in terms of increased biomass. This greater bond and larger amount of mass is likely to confer an advantage, helping to explain why, in this study, the hyphal biofilms were less easily removed from the surfaces than blastospore biofilms. The long branching hyphae running through and across the hyphal biofilms appeared to provide a strong structural framework that increased the resistance to removal of the biofilms and also demonstrated enhanced interactions between hyphae and underlying substratum topography.

The numbers of cells retained on the denture acrylic resin surfaces after the removal of both hyphal and blastspore biofilms increased with increases in surface roughness, with the highest numbers seen on the most abraded surfaces. Similar findings have been reported in previous studies.[3,5] The amount of biofilm on these surfaces following maturation at 48 hours growth however, was not affected by surface roughness. In many healthy denture wearers the accumulation of this biomass is prevented by regular denture cleaning. However even with the most rigorous cleaning regimens, it is unlikely that the denture surface will ever be completely clear of microorganisms. If surfaces that are more roughened retain more Candida following cleaning, these retained cells are able to proliferate upon returning to the oral cavity, the risk of developing mature denture plaque biofilms and subsequent denture stomatits is increased. If the retained cells lose viability, they will nevertheless provide additional attachment sites and nutrient sources for

new colonizers. These points are especially of importance to patients who may be more susceptible to infection, or those who are less able to effectively clean their dentures. In addition to this, in this study the difference in retention between the 2 types of Candida biofilm was greatest on the most abraded surfaces, suggesting an increased interaction between hyphae and the larger/more numerous features on that surface. This observation has been made previously[19]; thus, it may be that minimizing denture surface roughness by using non-abrasive or low abrasive cleansing regimens may be a step towards reducing Candida colonization of denture surfaces and denture plaque formation in general.

Biofilms grown from hyphal phase Candida survived better (indicated by the metabolic assay XTT) than those grown from adhered blastospores after exposure to both denture cleanser and water, probably due to the presence of greater numbers of more established hyphae and/or increased biomass. The biofilm phenotype is known to confer various advantages by increasing resistance to antimicrobial challenges and by protecting cells from mechanical forces.[18,20] The denture plaque biofilm, therefore, poses a challenge to hygiene procedures. There are many protocols available for denture cleaning.[6] Those involving the use of denture cleansers commonly consist of a simple 5 to15 minute soak, followed by a rinse. Brushing is also recommended, to ensure that plaque and debris are removed from the surface. As with these findings, some denture cleansers have been demonstrated to be effective in reducing, but not eradicating Candida biofilms.[21] In this study the cleanser used was tested solely for the effectiveness of its chemical formulation, without effervescence, since the focus of the assay was on viability of the cells in the in vitro biofilm. This enabled comparison of the survival of hyphal and blastospore

biofilms. Rinsing was also omitted as these mechanical mechanisms would clearly enhance biofilm removal. Additional study limitations include the omission of a salivary conditioning film, which might affect initial attachment to the surface. In addition, in vivo, denture plaque comprises a complex mixed microbial population whose structure and physiology might well differ from those of the simple Candida biofilms investigated here. In this study, extended soak times with the denture cleanser of 1 hour and 12 hours were used compared to the 5 minute soak recommended by the manufacturer. Although far removed from the recommended soak time, our parameters might be comparable to an overnight soak: in any case, the work enabled the viability of the different biofilms to be explored further

CONCLUSION

The presence of hyphae increases the retention of Candida on denture acrylic surfaces and therefore increased its presence in subsequent plaque formed on the surface. More cells were retained on surfaces that were more heavily abraded. Findings have implications in terms of denture hygiene procedures.

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Figures

Fig. 1. CSLM images of *Candida albicans* biofilms (stained with 0.5% Calcofluor White), grown on abraded PMMA. A) biofilm grown from adhered blastospores, B) biofilm grown from adhered hyphal phase Candida. Biofilms grown from hyphal cells are more abundant in long, branching pseudohyphae.

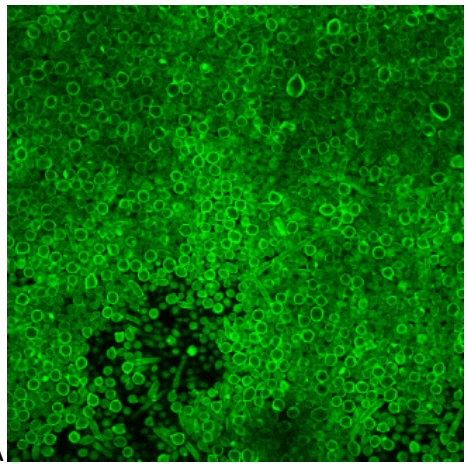
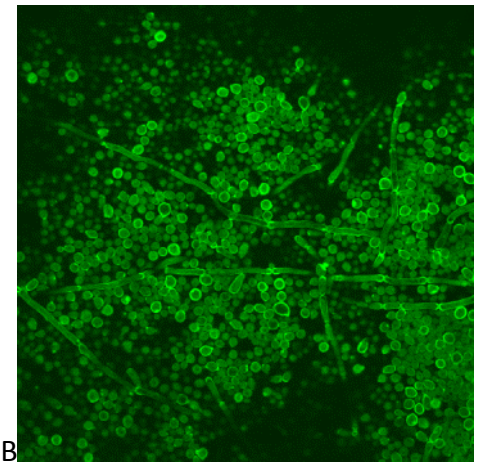
A  B 

Fig. 2. Mass (g) of biofilms grown from adhered blastospores and hyphal phase *Candida albicans* on PMMA with different levels of surface roughness (control, low, medium, and high abraded) (n=3).

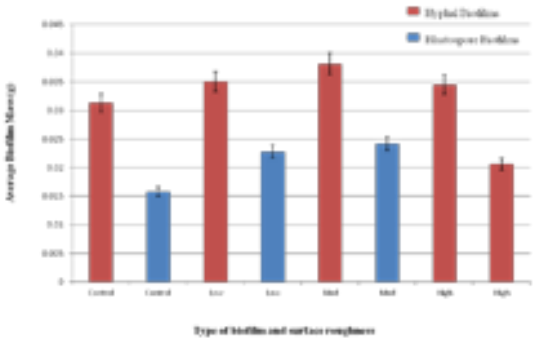


Fig. 3. Biofilms grown on abraded PMMA specimens from attached Candida blastospores (left) and attached Candida hyphae cells (right). Biofilms grown from adherent hyphae (right) are visibly thicker and more textured.

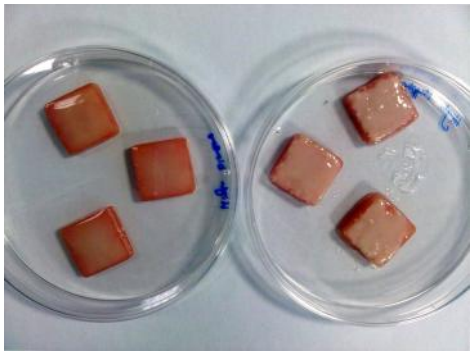


Fig. 4. Number of *Candida albicans* cells retained on differently roughened PMMA specimens (control, low, medium, and high test specimens abraded with dentifrices), after vortex washing for 30 seconds in sterile water (n = 30).

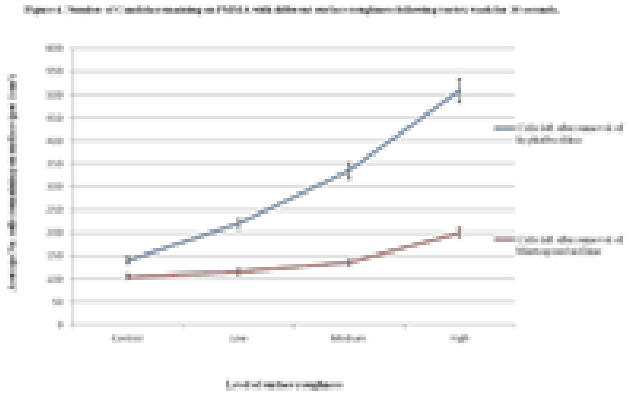


Fig. 5. Effect of soaking in denture cleanser at room temperature for different durations on viability of *Candida albicans* biofilms grown from adherent blastospores or hyphal phase cells (n=3).

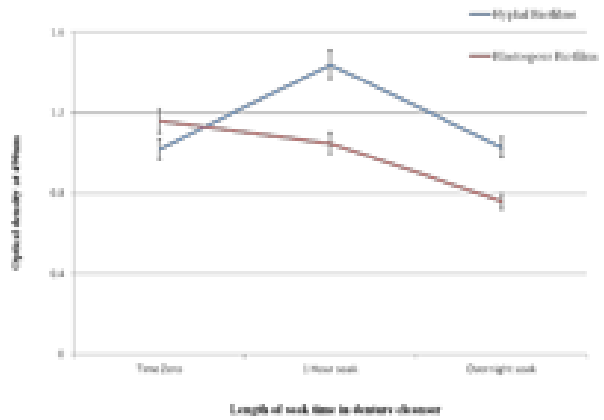


Fig. 6. Effect of soaking in water on viability of *Candida albicans* biofilms grown from adherent blastospores and hyphal phase cells (n=3).