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The Morphology and Molecular Physiology of *Zygosaccharomyces* Spoilage Yeasts

Thesis presented in candidature for the degree of *Philosophiae Doctor*

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Dedicated to my children,
Christopher Andrew and Dylan James
Love Dad

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Summary

Spoilage is a major problem for the food industry ultimately resulting in economic loss. Among the most prominent spoilage yeasts are members belonging to the *Zygosaccharomyces* genus. This research focuses on differences in organic acid resistance and the physiological basis of these differences between *Zygosaccharomyces bailii, Zygosaccharomyces kombuchaensis* and *Saccharomyces cerevisiae.*

Z. bailii, Z. kombuchaensis and *S. cerevisiae* differ in resistance to short, medium and longer chain organic acids. Organic acid resistance was shown to be effected by alterations to growth conditions. *S. cerevisiae* was the most sensitive to organic acids followed by *Z. kombuchaensis. Z. bailii* was overall the most resistant to organic acids. Organic acid inhibition was shown to increase with increasing chain length.

Electron microscopy was used to determine the effects of organic acids on yeast cell structure. Evidence is presented for short, medium and longer chain organic acids differing in their mode of inhibition. The cell wall was highlighted as differing between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* and as having a role in yeast organic acid resistance.

Protoplast fusion was successfully applied to *Z. bailii* and *Z. kombuchaensis* with *S. cerevisiae* to study the role of mitochondria in yeast organic acid resistance. Differences in sensitivity to ethidium bromide and petite forming capabilities were demonstrated. Hybrids were characterized in terms of morphology, physiology and organic acid resistance.

The ability of *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* to form pseudohyphae in the presence of isoamyl alcohol was assessed. Isoamyl alcohol was shown to induce an osmotic stress with a role for the high osmolarity glycerol pathway being demonstrated.

Z. bailii was shown to contain a subgroup based on differences in organic acid resistance, morphology, physiology and molecular composition. This is the first time that a subgroup with increased sensitivity to organic acids has been reported for *Z. bailii.*

Contents

Abbreviations

The abbreviations given below are those used in section 2 to 9. The list does not include atomic and chemical symbols or names of genes.

A	adenine
ABC	ATP binding cassette
ADI	acceptable daily intake
ADP	adenosine diphosphate
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
cAMP	cyclic adenosine monophosphate
CAP	controlled atmosphere packaging
c.f.u	colony forming units
CIP	cleaning-in-place
CWPs	cell wall proteins
DNA	deoxyribonucleic acid
D/O	drop-out
EDTA	ethylenediaminetetraacetate
EM	electron microscopy
EtBr	ethidium bromide
g	gravity
G	guanine
GC-MS	gas chromatography-mass spectrometry
GPI-CWPs	glycosylphosphatidylinositol-CWPs
G418	geneticin
h	hour
HCl	hydrochloric acid
HOG	high osmolarity glycerol
HPLC	high performance liquid chromatography
HSE	heat shock element
Hsp	heat chock protein
IAA	isoamyl alcohol
ITS	internal transcribed spacer
JEFCA	joint expert committee on food additives
L	litre
LB	Luria-Bertani medium
MAP	modified atmosphere packaging
MBTH	3-methylbenzothiazol-2-one hydrazone hydrochloride
µg	microgram
µl	microlitre
µm	micrometer
µM	micromolar
µmol	micromoles
mg	milligram
ml	millimetre
mM	millimolar
MIC	minimum inhibitory concentration
min.	minute

MM	minimal medium
MPa	megapascals
MPN	most probable number
nm	nanometre
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced)
OD	optical density
PCR	polymerise chain reaction
PD	peptone dextrose
PEG	polyethyleneglycol
Pf	protoplast fusant
Pir-CWPs	protein with internal repeats-CWPs
QUACS	quaternary ammonium compounds
rDNA	ribosomal deoxyribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
r.p.m	revolutions per minute
rRNA	ribosomal ribonucleic acid
s	second
Sc	*Saccharomyces cerevisiae*
SD	standard deviation
SDS	sodium dodecyl sulphate
SE	standard error
SEM	scanning electron microscopy
SHAM	salicylhydroxamic acid
SMT	sorbitol magnesium chloride Tris-HCl
STRE	stress responsive elements
T	thymine
TAE	Tris-acetate EDTA
Taq	*Thermus aquaticus*
TE	Tris-HCl EDTA
TEM	transmission electron microscopy
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
Uni	Unilever
UHP	ultra high purity
UV	ultra-violet
v/v	volume:volume ratio
w/v	weight:volume ratio
wt	weight
WT	wild-type
YD	yeast extract dextrose
YP	yeast extract peptone
YPA	yeast extract peptone acetate
YPD	yeast extract peptone dextrose
YPE	yeast extract peptone ethanol
YPG	yeast extract peptone glycerol
Zb	*Zygosaccharomyces bailii*
Zk	*Zygosaccharomyces kombuchaensis*

List of Tables

List of Figures

1. General Introduction

1.1 Food spoilage

Food spoilage can be considered as the undesirable changes caused by the presence or activities of microorganisms, which result in a food becoming spoiled and unacceptable for human consumption. Spoilage is a problem for the food industry as it ultimately results in economic loss (Loureiro, 2000). Economic loss as a result of spoilage may be incurred in several ways: loss of product, product recall, product disposal, customer compensation and loss of future sales. The actual number of food spoilage cases remains underestimated due to commercial confidentiality (Fleet, 1992). The prevention of food spoilage is, therefore, of major industrial concern.

1.2 Sources of food spoilage

Food spoilage is known to occur during pre- and post-harvesting of food and during preservation, packaging, transportation and storage (Neves *et al.*, 1994; Roller, 1999). The microorganisms, which cause spoilage, can be introduced from an external source during processing of food. Harmless commensal microorganisms can also become the source of spoilage, depending upon the changes, which take place during harvesting (Adams and Moss, 2000). Spoilage from raw materials including fruits is believed to be one of the main sources of spoilage (Fleet, 1992). The means by which foods become spoilt is an area of considerable debate, and can often lead to costly legal disputes between suppliers and manufacturers.

1.3 Food spoilage microorganisms

The types of microorganisms that cause food spoilage are vast in comparison with those, which cause food poisoning (Russell and Gould, 1991). Table 1.1 summarises the food spoilage microorganisms of major industrial concern. Bacteria comprise the

Table 1.1 Food spoilage microorganisms of major industrial concern (adapted from Russell and Gould, 1991).

Microorganism	Food
Gram-negative, catalase-positive, oxidase-positive rods e.g. *Pseudomonas*	Fish, meat, poultry and other protein rich foods stored at chilled temperatures
Gram-negative, catalase-positive, oxidase-negative rods e.g. *Acinetobacter*	Fish, meat, poultry and other protein rich foods
Gram-positive, catalase-positive, non-sporing rods e.g. *Corynebacterium*	Cured meats and sausages, vegetables and fresh meats in modified atmosphere packaging
Gram-positive, catalase-positive, cocci e.g. *Staphylococcus*	Cured meat products and milk-based products
Gram-positive, catalase-negative, non-sporing rods e.g. *Lactobacillus*	Milk and milk-based products, vegetables and meat in vacuum or low oxygen packs
Gram-positive, catalase-negative cocci e.g. *Streptococcus*	Cured meats, particularly if low in salt
Gram-positive, catalase-positive, spore-forming rods e.g. *Bacillus*	Heated foods with pH values above pH 4 e.g. rice
Gram-positive, catalase-negative, spore-forming rods e.g. *Clostridium*	Heated foods with pH values above pH 4 and with restricted oxygen availability
Yeasts e.g. *Zygosaccharomyces*	Low pH and low water activity foods independent of oxygen availability
Moulds e.g. *Penicillium*	Low pH and low water activity foods exposed to air

majority of food spoilage microorganisms, causing the spoilage of almost all food types including meats, vegetables and dairy products. Yeasts and moulds only become important as food spoilage microorganisms in conditions that do not permit bacterial growth: yeasts and moulds, therefore, predominant in foods at low pH and with low water activity.

1.4 Food spoilage yeasts

The application of yeasts in baking and brewing means that man regularly consumes large numbers of viable yeast with no apparent adverse consequences (Fleet, 1992). In addition, the pathogenic yeasts associated with human infections, such as *Candida albicans* are not transmitted through foods (Hurley *et al.,* 1987). Research into food spoilage has, therefore, largely focused on that caused by bacterial contamination, with yeast attributed food spoilage receiving relatively little attention (Fleet, 1992).

Research into yeast attributed food spoilage has recently been increased due to several factors. Firstly, consumers are demanding a more natural product, thereby, forcing manufacturers to find alternative strategies of food preservation. Secondly, European laws on both the types and concentrations of food preservatives are becoming restrictive. Thirdly, there is a growing trend for yeast-free diets as consumers become more concerned about possible allergic reactions. Finally, both the numbers and types of yeasts isolated from foods are increasing (Pitt and Hocking, 1997; Steels *et al.,* 1999a, b). Yeasts commonly isolated from foods include abnormal *Saccharomyces* strains and members belonging to *Brettanomyces, Kluyveromyces* and *Candida* genera (Fleet, 1992).

Davenport (1996; 1997) proposed the existence of three groups of yeasts, which could be isolated from a soft drinks factory. Group 1 yeasts, were true spoilage yeasts, being able to proliferate in soft drinks thereby causing their spoilage. Group 2 yeasts could cause spoilage as a result of a mistake in manufacturing and were termed hygiene spoilage yeasts. Group 3 yeasts were not capable of spoilage themselves, but were indicators of hygiene. Among the most prominent spoilage yeasts in group 1 are members belonging to the *Zygosaccharomyces* genus. Members of the *Zygosaccharomyces* genus share many similarities with the *Saccharomyces* yeasts and were originally classified under this genus (Steels *et al.,* 1999a). Barker introduced the genus *Zygosaccharomyces* at the beginning of the twentieth century for yeasts in which the conjugation of two (zygo) individual cells resulted in the formation of asci, which contained smooth ascospores (van der Walt & Johannsen, 1975). *Zygosaccharomyces* spoilage yeasts until recently were regarded as a phylogenetically heterogeneous group being intermixed with species of *Kluyveromyces, Saccharomyces* and *Torulaspora* (James *et al.,* 1994). Figure 1.1 shows the latest phylogenetic classification of the *Zygosaccharomyces* yeasts as proposed by Kurtzman (2003). In the new taxonomy the *Zygosaccharomyces* genus includes only six species with *Z. cidri, Z. fermentati, Z. mrakii, Z. florentinus* and *Z. microellipsoides* now being classified as *Lachancea cidri* and *Lachancea fermentati, Zygotorulaspora mrakii* and *Zygotorulaspora florentinus,* and *Torulaspora microellipsoides,* respectively (Kurtzman 2003).

Characteristics shared by the *Zygosaccharomyces* yeasts include being osmotolerant, fructophilic, highly fermentative, vitamin requiring and preservative resistant. However, individual species of this genus have distinct characteristics, which allow them to proliferate in their own environment. *Z. rouxii* is the type species of this genus and is one of the most osmotolerant yeasts known, growing in environments of low water

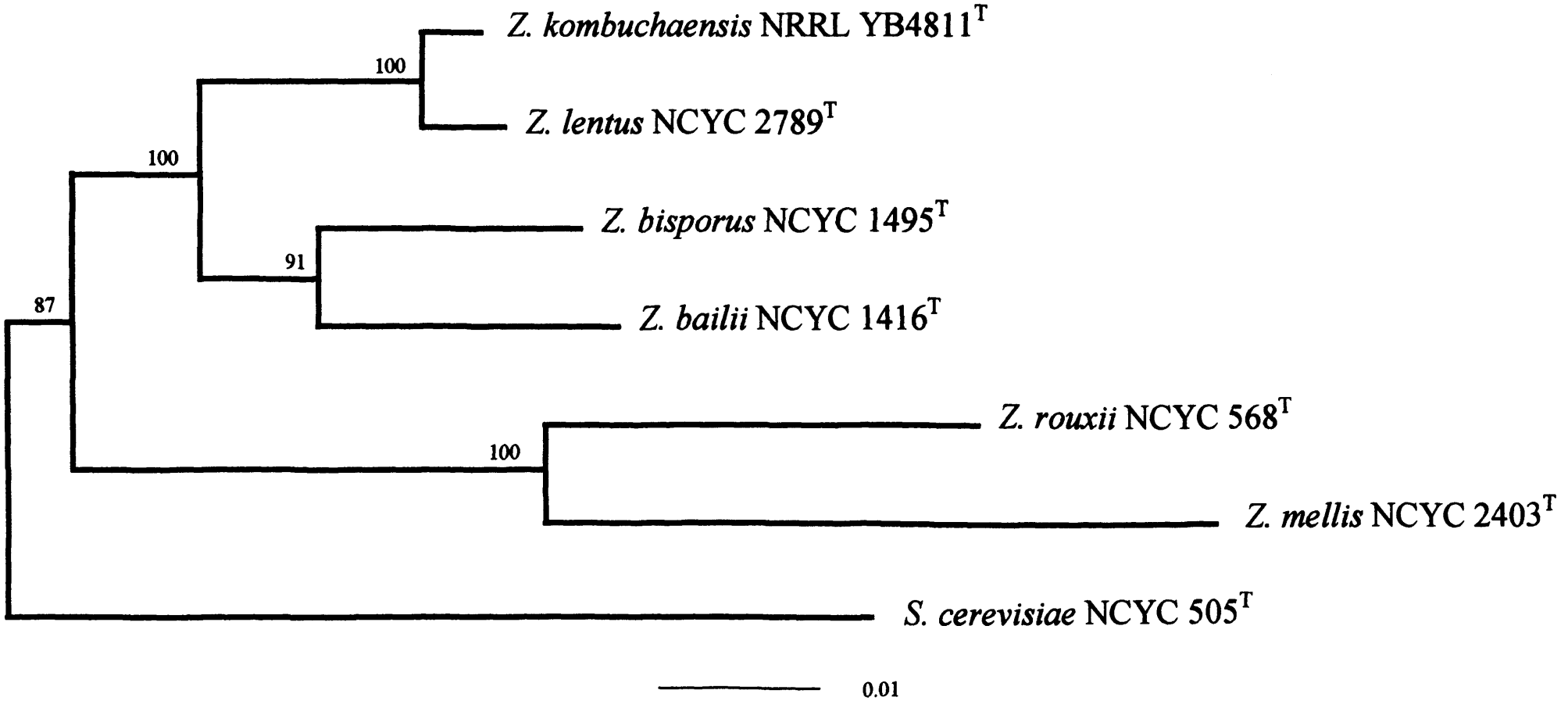


Figure 1.1 Phylogenetic tree of the *Zygosaccharomyces* genus as proposed by Kurtzman (2003)
The phylogenetic tree is based on 26S rDNA D1/D2 sequences with *S. cerevisiae* being used as the outgroup species. Bar is representative of one substitution per 100 nucleotides. Bootstrap values ≥50% are given. T = type strain. NCYC National Collection Yeast Cultures, Norwich UK, NRRL ARS Culture Collection, National Centre for Agricultural Utilization Research, Peoria, IL, USA. Figure reproduced from Steels *et al.* (2002)

activity and being found at a water activity of 0.62 in fructose (Tilbury, 1980). *Z. rouxii* therefore causes the spoilage of foods with low water activity including sugar syrups (Steels *et al.*, 2000). *Z. mellis* is also osmophilic and is believed to be responsible for the spoilage of honey (Scarr and Rose, 1966). *Z. mellis* was distinguished from *Z. rouxii* by DNA relatedness (Kurtzman, 1990). *Z. kombuchaensis* has recently been isolated from 'Kombucha tea' (Kurtzman *et al.*, 2001). *Z. kombuchaensis* has been identified as a potential spoilage yeast tolerating low pH conditions and is very closely related to *Z. lentus* (Steels *et al.*, 2002). The species of *Z. lentus* was identified from yeasts previously mis-classified as *Z. bailii* (Steels *et al.*, 1999a, b). *Z. bailii* itself is not a new species, having been identified as *Saccharomyces bailii* in 1895 by Linder and having its current nomenclature adopted in 1975 (Davenport, 1976). *Z. bailii* is very closely related to the slightly more osmotolerant, but more preservative sensitive *Z. bisporus*. *Z. bailii* and to a lesser extent *Z. bisporus*, are responsible for spoilage of preserved food (Thomas and Davenport, 1985). *Z. bailii* causes the spoilage of mayonnaise, pickles, marzipan, fruit concentrates, soft drinks, wines and several other foods and beverages (Thomas and Davenport, 1985). The most damaging of the *Zygosaccharomyces* yeast from a manufacturing point of view is often considered to be *Z. bailii*. *Z. bailii* is particularly problematic due to its ability to tolerate high levels of weak-acid preservatives under low pH conditions that inhibit the growth of most other food spoilage microorganisms.

1.5 Characteristics of yeast attributed food spoilage

Over the years, there have been a number of reviews into yeast attributed food spoilage (Deak, 1991; Fleet, 1992; In't Veld, 1996; Brul & Coote, 1999; Loureiro and Querol, 1999; Roller, 1999; Loureiro, 2000; Piper *et al.*, 2001; Brul *et al.*, 2002). In general large numbers of yeast in excess of 10^6 cells per gram are required before spoilage is

apparent. *Z. bailii* has been reported to cause spoilage at as little as one cell per litre, making it very difficult to prevent spoilage (Thomas and Davenport, 1985). Food spoilage attributed to yeasts often manifests itself as distorted packaging due to carbon dioxide production (Fleet, 1992). *Z. bailii* has the ability to proliferate under high carbon dioxide levels (Malfeito-Ferreira *et al.,* 1997) and can produce sufficient carbon dioxide to explode bottles, which may cause serious injury (Grinbaum *et al.,* 1994). The other major products of yeast growth (all of which have the potential to spoil a product) include alcohols, organic acids and esters (Berry and Watson, 1987). The ability of *Zygosaccharomyces* yeasts to produce secondary products remains largely undetermined, even though *Z. rouxii* has been manipulated to enhance formation of isoamyl alcohol (Yoshikawa *et al.,* 1995). Modifications associated with *Z. bailii* spoilage are haze formation and flavour impairment in the form of hydrogen sulphide (Thomas and Davenport, 1985).

1.6 Factors affecting the growth and survival of yeasts in food

Yeasts as unicellular eukaryotes are exposed to various forms of stress, which have a direct and indirect effect on their growth and survival. The factors that affect yeast growth in food include: intrinsic factors (nutrients, water activity, pH and redox potential), extrinsic factors (temperature, oxygen and carbon dioxide) and implicit factors (properties of the microorganisms present in the food and their interactions). These growth factors often interact in various combinations making the exact effects of each stress difficult to quantify.

1.6.1 Nutrients

The inability of a microorganism to utilize a major component of a food can limit growth and act as a severe disadvantage (Adams and Moss, 2000). Carbohydrates are

the most important nutrients for yeasts acting as both an energy and growth source (Deak, 1991). Differences in aerobic (assimilation) and anaerobic (fermentation) uses of carbon sources amongst yeasts, not only limits the food which they spoil but also acts as an important criterion in their taxonomic characterisation (Barnett *et al.,* 2000). In addition to a carbon source, yeasts also require a source of nitrogen, microelements and vitamins, all of which are normally provided in natural substances and foods (Deak, 1991). *Z. bailii* is known to require a source of B-group vitamins, including biotin and thiamine for growth (Thomas and Davenport, 1985).

The problem food manufacturers' encounter between a desirable product, which will be appealing to customers, and a product that is free from spoilage is illustrated by the following example. The addition of fruits containing sucrose and various other sugars to yoghurt can lead to a more appealing product for consumers but also increases the amount of carbohydrates available, and can lead thereby to a more diverse spoilage microflora (Adams and Moss, 2000).

1.6.2 Water activity (a_w)

Water activity can be defined as the amount of water available for microbial growth, which relates to the concentration of solutes in a food (Deak, 1991). Bacteria are sensitive to reduced water activity only being able to spoil foods with an a_w of 0.90 or greater. Yeasts in general are more tolerant to reduced water activity, normally preferring an a_w of 0.85 for growth. However, some yeasts, described as osmotolerant or xerotolerant, can grow at values of water activity as low as 0.62. The absolute limit for microbial growth in foods is a water activity of 0.61. Any spoilage that occurs below this level is the result of chemical rather than microbial spoilage (Garbutt, 1997).

1.6.3 pH

pH (defined as -log[H^+]) has a major influence on the growth and survival of microorganisms in foods. The pH of pure water is pH 7.0. The majority of foods are slightly acidic (pH <7.0), with alkaline (pH >7.0) foods generally having an unpleasant taste (Adams and Moss, 2000). Yeasts in general have a pH range between pH 3-8, with an optimum of around pH 4.5, and are less sensitive to pH than bacteria (Garbutt, 1997; Adams and Moss 2000). *Zygosaccharomyces spp.* grow at low pH, examples of which are *Z. bailii, Z. kombuchaensis* and *Z. lentus* with a minimum pH for growth of between pH 1.5-2.0 (Steels *et al.,* 2002). The pH tolerance of a yeast is dependent upon several factors, including temperature, nutrient availability, water activity and the presence of organic acids (Deak, 1991). Many organic acids lower the pH of foods and are therefore applied to many food substances as preservatives as discussed in section 1.9.

1.6.4 Redox potential (E_h)

Redox potential is a measure of the tendency of a substance to become oxidised (lose electrons) or become reduced (gain electrons). The redox potential of a food is dependent upon a number of factors including oxygen concentration, density of food, processing of food, pH, and presence of reducing substances (Garbutt, 1997). Microbial growth in food reduces its redox potential either by using any oxygen present or by producing reducing substances such as hydrogen (Adams and Moss, 2000). The relationship between redox and microbial growth in foods is complex and far from understood.

1.6.5 Temperature

Temperature is one of the most important factors affecting growth of all microorganisms. The vast majority of yeasts are mesophiles having an optimum

temperature of between 20-30°C. Few types of yeasts are psychrophiles with an optimum temperature for growth below 20°C e.g. *Leucospoirdium spp.* Some *Candida spp.* are thermophiles with a minimum temperature of growth of 20°C (Walker, 1998). The majority of food spoilage yeasts are mesophiles.

At temperatures exceeding the maximum for growth, yeast viability declines at an exponential rate (Walker, 1998). It has been speculated that the cell membrane is the primary site of action for most thermal damage, with changes in fluidity, permeability and ion leakage being evident (Walker, 1998). Fatty acids and lipids present within cell membranes play an important part in determining the range of temperatures at which growth is possible (Swan and Watson, 1997). Secondary, consequences of thermal damage include irregular cell growth, decline in intracellular pH, ribosome breakdown, DNA strand breakage (Dawes, 1976; Piper 1997; Walker, 1998) and induction of respiratory-deficient petites (Sherman, 1959).

1.6.6 Oxygen and carbon dioxide

Oxygen can be considered as an important growth factor, as yeasts grow poorly in its complete absence (Walker, 1998). The requirement for oxygen differs amongst yeasts, with the majority being facultative anaerobes, switching from aerobic (respiration) to anaerobic (fermentation) metabolism depending upon the availability of oxygen (Pasteur effect) and glucose (Crabtree effect) (Deak, 1991; Walker, 1998). Fermentative spoilage is very common, as fermenting yeasts can grow under low oxygen conditions such as those frequently encountered in foods and beverages (Deak, 1991). The presence of oxygen within a cell leads to the generation of reactive oxygen species: which are powerful oxidising agents that can react with and destroy most cell constituents, including phospholipids in cell membranes (Garbutt, 1997; Dawes, 1999).

The restriction of oxygen in foods and beverages that are rich in complex nutrients has recently been reported not to be an effective strategy for the inhibition of spoilage by *Z. bailii* due to the complex nutrients providing everything for growth (Rodrigues *et al.*, 2001a).

The inhibitory effect of carbon dioxide on yeast is due to several factors, whose individual contributions are yet to be fully determined (Adams and Moss, 2000). In yeast, growth and metabolic activity are normally impaired at carbon dioxide pressures of 0.3 MPa (Jones and Greenfield, 1982). However, *Z. bailii* can proliferate at carbon dioxide levels around 0.5 MPa. Carbon dioxide has the ability to act as a weak organic acid (section 1.9), and is, therefore, capable of penetrating the plasma membrane and acidifying the interior of a cell (Adams and Moss, 2000). The factors relating to the resistance or sensitivity of yeasts to carbonation are not fully understood (Deak, 1991).

1.6.7 Implicit factors

These are the factors that arise from the microorganisms themselves and their responses to the environment and one another. The implicit factors determine the rate of growth within the given environment, they originate from the following microbial interactions: mutualism (both species benefit), antagonism (one species benefits at the detriment of the other), and commensalism (one species benefits while the other is not affected) interactions. An example of antagonism is as follows: yeasts in general are slow growing in comparison to bacteria, but can produce ethanol which is inhibitory to bacteria, thereby, allowing the proliferation of the yeast.

1.7 Yeast Stress Responses

In response to adverse environmental changes yeasts undergo a series of stress responses, the aims of which are to protect the cell from the detrimental affects of the stress and to repair molecular damage (Mager and Hohmann, 1997). The stress conditions encountered by yeasts are largely those, which affect their ability to survive and grow in foods (section 1.6), and include starvation, heat, osmolarity, oxidative, low pH and weak-acid stress (Dawes, 1999). A consequence of a stress response is the acquisition of stress tolerance; therefore, a mild stress can lead to improved tolerance to a more severe stress (Mager and Hohmann, 1997). The stress response of yeasts can be divided into various sections; firstly, the sensing of the stress and the activation of signalling pathways, this is followed by either specific responses to that stress or a general response in which resistance to a number of stresses is gained. Both the specific and general responses result in changes in gene expression and in enzyme activities. The general response results in cross-protective mechanisms. The basis of the general stress response has been attributed to a large number of (general) stress responsive element (STRE) genes (Mager and Hohmann, 1997). The rationale behind the STRE genes is that under general stress conditions changes in the expression of a number of genes involved in stress responses will be beneficial to the cell. The outcome of the stress response, whether specific or general, is the acquisition of stress tolerance and the resumption of growth (Mager and Hohmann, 1997).

1.7.1 Starvation

Nutrient starvation can be regarded as the utilisation of one or more essential nutrient sources so that it becomes limited resulting in the cell entering stationary phase. Stationary phase is when the cell enters G_0 of the cell cycle and includes a number of specific characteristics: elevated levels of storage carbohydrates, elevation of STRE

genes (under the control of the cAMP-dependent protein kinase A), repression of ribosomal genes, and an increase in resistance to stress, in particular heat stress and resistance to cell wall lytic enzymes (Mager and Hohmann, 1997). A consequence of nutrient limitation is the induction of morphological change in the form of pseudohyphae. The nature of pseudohyphal development and the signalling pathways remain to be fully elucidated (Lorenz *et al.,* 2001).

1.7.2 Heat stress

The response of yeasts to heat shock is regarded as the best characterised stress response (Piper, 1997). Heat shock genes are induced on exposure to a shift in temperature via the activation of Hsf1p (a heat shock-specific transcription factor), which promotes transcription through the heat shock elements (HSEs), which are present in the promoter of target genes. A consequence of the induction of heat shock genes is the transient accumulation of heat shock proteins. Heat shock proteins (Hsp) thus far identified include the Hsp70 family, Hsp60, Hsp90, Hsp104 and Hsp26 (Dawes, 1999). The functions of heat shock proteins are to act as molecular chaperones in protein folding and transport and thus play an important role in protecting other proteins against thermal denaturation (Piper, 1997). The actions of the heat shock response result in the restoration of normal biological activity.

1.7.3 Osmolarity

Osmotic stress in which changes in osmolarity takes place can be described as either hyper-osmotic stress in which cells are transferred to a high salt or sugar medium, or hypo-osmotic stress in which cells are transferred to a low salt or sugar medium. The hyper-osmotic response is the better characterised of the osmotic responses (Nevoigt and Stahl. 1997; Mager and Siderius, 2002). A hyper-osmotic shock results in a rapid

loss of water and cell shrinkage and the recruitment of water from the vacuole (Hohmann, 1997; Mager and Siderius, 2002). The response to hyper-osmotic stress is firstly the temporary arrest of growth, followed by the closure of the glycerol channel Fps1p (Hohmann, 1997). The closure of Fps1p allows the intracelullar accumulation of glycerol, which as a major compatible solute helps to retain cell turgor (Mager and Varela, 2002). The high-osmolarity glycerol (HOG) mitogen activated protein (MAP) kinase pathway is also triggered (section 7.1). The HOG pathway regulates transcription of the glycerol-3-phosphate dehydrogenase (*GPD1*) gene, which results in the synthesis of glycerol. Once the cell has adapted to hyper-osmotic stress normal growth resumes.

1.7.4 Oxidative Stress

Oxidative stress can be induced by reactive oxygen species (ROS) that can react with and destroy various constituents of the yeast cell (Dawes, 1999). The forms of ROS derived from oxygen include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical (HO^-). These ROS are known to cause a wide range of biological damage including damage to lipids, proteins and DNA (Storz *et al.*, 1987). To combat the dangers of ROS generation, yeasts have developed a number of defence mechanisms. The aim of the defence mechanisms, which include both enzymatic and non-enzymatic systems are to maintain cellular redox state and protect cellular constituents (Jamieson, 1998). It is the balance between the ROS and the defence mechanisms of a cell, which determines the extent of the oxidative stress. The role of signal transduction pathways in the oxidative stress response of *S. cerevisiae* remains to be elucidated, but cAMP levels are known to be of importance (Jamieson, 1998). Recently, a role for the HOG pathway in oxidative stress has been shown (Navarro-Garcia *et al.*, 2003), indicating overlap between osmotic and oxidative stress pathways.

1.7.5 Low pH and weak-acid Stress

The ability of yeasts to tolerate high levels of weak-organic acid at low pH has been the subject of intense research (Krebs, *et al.*, 1983; Warth, 1990; Piper, 1997; Steels *et al.*, 1999; Stratford and Anslow, 1996, 1998; Piper *et al.*, 2001). The possible resistance mechanisms utilised by yeasts and in particular by *Z. bailii* and *S. cerevisiae* to high concentrations of organic acids are discussed in section 1.10. However, at least in *S. cerevisiae* there is increasing evidence for the stress response to weak-organic acids being that of a previously uncharacterised stress response (Hatzixanthis *et al.*, 2003; Kren *et al.*, 2003). The induction of the weak-organic acid stress response, which manifests itself through the induction of *PDR12* in *S. cerevisiae*, is not a response to oxidative stress, hypotonic stress, calcium/calmodulin signalling, organic alcohols or acetate (Hatzixanthis *et al.*, 2003). The strongest inducers of *PDR12* are unbranched monocarboxylic acids with chain length C_3-C_8 (Hatzixanthis *et al.*, 2003). In summary, the stress response of *S. cerevisiae* to weak-organic acid stress is increasingly being found to represent a unique stress response.

1.8 Methods of food preservation

Modern food preservation procedures are based on the manipulation of factors affecting microbial growth (section 1.6). Food preservation in one form of another has been used for thousands of years. Smoking and the addition of salt to foods are some of the earliest food preservation methods (Adams and Moss, 2000). Food preservation techniques in current practice are summarised in Table 1.2. The majority of food preservation techniques focus on the delay or inhibition of microbial growth, with few acting on the direct inactivation of microorganisms. The application of several preservation methods to prevent microbial spoilage is called the 'hurdle effect'; each method on its own will not inhibit microbial growth, but do so when applied collectively (Leistner and Rodel,

Table 1.2 Modern methods of food preservation (Modified from Adams and Moss, 2000)

Procedure	**Factor affecting growth or survival**
Cooling, chill distribution and storage	Low temperature to retard growth
Freezing, frozen distribution and storage	Low temperature and reduction of water activity to prevent growth
Drying, curing and conserving	Reduction in water activity sufficient to delay or prevent growth
Vacuum and oxygen-free 'modified atmosphere' packaging	Low oxygen tension to inhibit aerobes and delay growth of facultative anaerobes
Carbon dioxide enriched 'modified atmosphere' packaging	Specific inhibition of some microorganisms by carbon dioxide
Addition of acids	Reduction of pH value and additional acid inhibition
Emulsification	Compartmentalisation and nutrient limitation; water-in-oil emulsion foods
Addition of preservatives	Inhibition of specific groups of microorganisms
Pasteurisation and Appertisation	Application of heat to inactivate target microorganisms
Radurisation and radicidation	Application of ionising radiation to inactivate target microorganisms
High hydrostatic pressure	Pressure inactivation of vegetative microorganisms

1976). An example of this approach includes lowering water activity, decreasing pH, restricting temperature and the addition of a preservative, all at sub-inhibitory levels, which collectively prevent microbial growth. Another advantage of the 'hurdle technology' is that consumers may find the product more acceptable as lower concentrations of preservatives are used.

1.8.1 Pasteurisation and Appertisation

Pasteurisation involves heating foods to temperatures between 60-80°C. Pasteurised foods include milk, ice cream, fruit juices and pickles. The temperature and duration of pasteurisation is dependent upon the food (Garbutt, 1997; Adams and Moss, 2000). Appertisation is the heating of foods to temperatures above 100°C; examples include canning of low pH foods and UHT treatment of milk (Garbutt, 1997). The two aforementioned preservation techniques represent the main application of heat food preservation. Heat is primarily applied to kill bacterial pathogens, and their spores with a secondary application directed towards the prevention of spoilage microorganisms (Adams and Moss, 2000).

1.8.2 Refrigeration and freezing

Refrigerated or chilled foods are stored at temperature between 0-5°C; frozen foods are stored at temperatures of -10 to -18°C (Russell and Gould, 1991). Psychrophiles, (including some yeasts), are capable of growth at chilled and refrigerated temperatures; therefore spoilage will eventually occur. Chilling or refrigerating foods increases the storage life of a product by increasing the lag phase of microbial growth. Storage life of chilled and refrigerated foods is dependent on several factors including composition of food, potential spoilage microflora and the use of complementary preservation

techniques (Garbutt, 1997). Due to the absence of microbal growth, no spoilage of frozen foods takes place at temperature below around -10°C (Russell and Gould, 1991).

1.8.3 Curing

Curing as a preservation technique involves the removal of liquid water by salting, smoking or drying, leading to a reduction in water activity. The removal of water from foods is the oldest known preservation method. The techniques of water removal may have been modernised to become more reliable but the principles are the same (Adams and Moss, 2000).

1.8.4 Food preservatives

Food preservatives are defined as 'substances that are capable of inhibiting, retarding or arresting the growth of microorganisms or any deterioration relating to the presence of microorganisms' (UK Food Regulations, 1989). Food preservatives can be divided into the following classes: organic acids and their esters (section 1.9), mineral acids (phosphoric acid), inorganic anions (sulphite and nitrite), carbon dioxide, sodium chloride, antibiotics (nisin and pimaricin) and smoke. Natural food preservatives (including vanillin, oregano and eugenol) are receiving increasing attention due to consumer pressure for more natural methods of food preservation. The regulations governing food preservatives do not include a number of substances, which are added to foods for other reasons but also provide broad preservative-resistance including food additives (Adams and Moss, 2000).

1.8.5 Radiation

Radiation can act on microorganisms indirectly (as do microwaves) or directly as with ultraviolet light and ionizing radiation. Microwave radiation (10^9 Hz) acts on

microorganisms indirectly in the presence of water by generating heat. Microwave ovens are used for cooking and re-heating foods domestically and throughout the catering industry. Microwave radiation has also been shown to have an application in the pasteurisation of milk; however, this has not been adopted on a commercial scale (Garbutt, 1997). Ultraviolet light (10^{15} Hz) can have a direct effect on microorganisms by damaging DNA replication: if sufficient damage is caused, cells will die. Applications of ultraviolet light to the food industry include: control of mould spores in bakeries and sterilisation of UHT milk packaging. Ionizing radiation (10^{18} Hz) is used on a limited scale for the decontamination of microorganisms in packaged foods. At the moment only foods that have received low-level irradiation including vegetables and poultry are commercially available (Garbutt, 1997).

1.8.6 Aseptic and modified atmosphere packaging

The aseptic packaging of foods is vital for food manufacturers' in order to prevent contamination. The packaging of foods in modified atmospheres is aimed at the inhibition of fast growing microbes that could quickly cause spoilage (Adams and Moss, 2000). Modified atmosphere packaging can be classified as three different procedures; all of which are carried out in gas phase, which excludes atmospheric oxygen and retains moisture. In vacuum packaging, air is removed from the packaging causing it to collapse around the food. In modified atmosphere packaging (MAP), packaged food is flushed through with a gas containing a mixture of carbon dioxide, oxygen and nitrogen. Finally, in controlled atmosphere packaging (CAP) the product environment is maintained constant throughout storage (Adams and Moss, 2000).

1.9 Organic acids as food preservatives

The major organic acid preservatives and their uses are listed in Table 1.3. Figure 1.2 provides details of the structures of common organic acids. Organic acids employed as food preservatives in the main act to prevent the growth of yeasts and moulds with some antibacterial activity (Russell and Gould, 1991). The most common food preservatives are those classed as 'weak-acid preservatives' including acetic, propionic, sorbic and benzoic acid. Weak-acid preservatives in aqueous solution exist in a pH dependent equilibrium between undissociated uncharged acid molecules (HA) and a dissociated state with charge anions (A^-) and protons (H^+). The pH at which the proportions of undissociated acid and dissociated charged anions/protons are equal is defined as the pK_a. This can be represented as an equilibrium expression

$$pK_a = [H^+]\,[A^-] / [HA]$$

The pK_a of an acid can be used to calculate the proportions of dissociated and undissociated forms of weak-acid preservatives at a given pH with the following formula:

$$pH = pK_a + \log \frac{[A^-]}{[HA]}$$

The equation is referred to as the Henderson-Hasslebach equation.

1.9.1 Weak-acid preservative theory

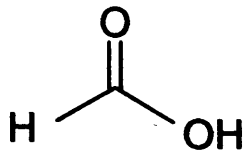
The 'weak-acid preservative theory' proposes that the highly lipophilic undissociated acid molecules, which predominate at low pH, diffuse across the plasma membrane into the cell. Once inside the cell the neutral pH of the cytoplasm causes the undissociated form to dissociate. The dissociation liberates protons and anions that cannot cross the plasma membrane and therefore accumulate in the cytoplasm. The result of dissociation is intracellular acidification, which inhibits microbial growth. Weak-acids known to

Table 1.3 Major organic acid food preservatives and their uses (data compiled from Russell and Gould, 1991; Adams and Moss, 2000)

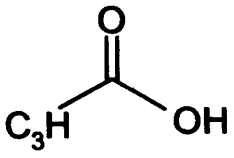
Organic acid	Typical uses	Concentrations (mg/kg)
Formic acid	Pickles and mustard	100
	Fruit juice concentrates	4000
Acetic acid	Pickles	% levels
	Chutneys	
	Salad dressings	
	Sauces	
	Vinegars	
Propionic acid	Bread	2000-5000
	Flour confectionery	1000-3000
	Jam and tomato puree	1000
Sorbic acid	Non-alcoholic beverages	100-1000
	Alcoholic beverages	200
	Processed fruit and vegetables	500-2000
	Bakery products	1000-2000
	Mayonnaise and salad dressings	1000-2000
	Mustard	250-1000
Benzoic acid	Non-alcoholic beverages	100-500
	Alcoholic beverages	200
	Fruit products	500-2000
	Sugar and flour based confectionery	1000
	Mayonnaise and salad dressings	250-2500
	Mustard	1000
Lactic acid	Fermented meat and dairy products	% levels
	Carbonated drinks	
Citric acid	Non-alcoholic drinks	% levels
	Bakery products	

Acceptable daily intake *ADI* (mg kg^{-1} body wt) sorbic acid 25; benzoic acid 5, propionic acid 10, acetic and lactic acid no limit

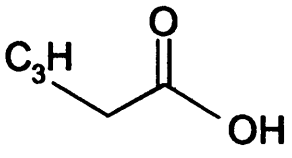
Figure 1.2 Structure of common organic acids



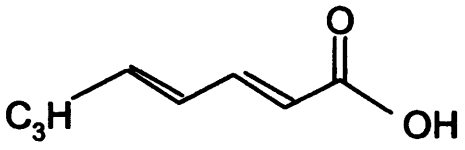
Formic acid



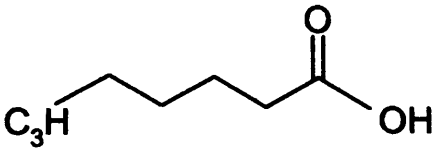
Acetic acid



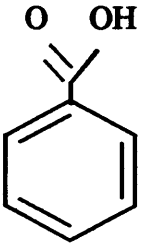
Propionic acid



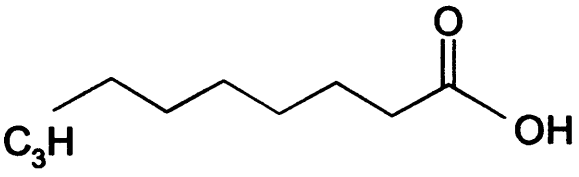
Sorbic acid



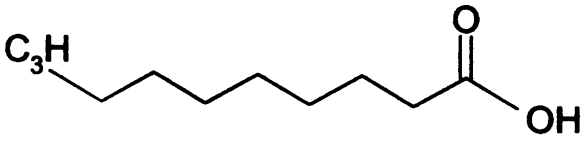
Hexanoic acid



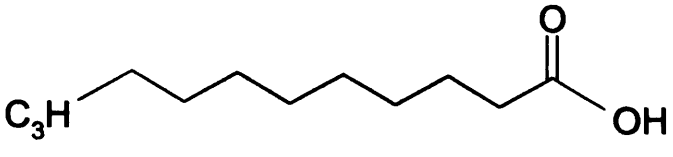
Benzoic acid



Octanoic acid



Nonanoic acid



Decanoic acid

Cause a drop in intracellular pH include acetic acid (Neal *et al.*, 1965; Arneborg *et al.*, 2000), sulphite (Pilkington and Rose, 1988) and benzoic acid (Krebs *et al.*, 1983). A number of mechanisms have been proposed for the inhibition of microbial growth: membrane disruption (Freese *et al.*, 1973; Stratford and Anslow, 1996, 1998; Bracey *et al.*, 1998), inhibition of the enzymes required for glycolysis (Krebs *et al.*, 1983), stress on intracellular pH homeostasis (Cole and Keenan, 1987a; Holyoak *et al.*, 1996; Bracey *et al.*, 1998) and/or the direct accumulation of toxic anions (Eklund, 1983).

1.9.2 Acetic acid (E260)

Acetic acid can be added in sufficient amounts to exert an effect on both flavour and product pH. Acetic acid in the main acts as a 'classic weak-acid preservative': inhibiting growth by reducing intracellular pH. Studies by Freese *et al.* (1973) have shown that acetic acid inhibits oxygen uptake and ATP production by over 70% in *Bacillus subtilis*. Acetic acid has also been shown to interact with the cell membrane to neutralise the electrochemical potential in bacteria (Sheu *et al.*, 1972). In *Escherichia coli* acetic acid inhibition has been shown to be due to a problem with methionine biosynthesis, which leads to the accumulation of the toxic intermediate homocysteine (Roe *et al.*, 2002). Recently, acetic acid has been shown to induce programmed cell death in *S. cerevisiae* (Ludovico *et al.*, 2001) and *Z. bailii* (Ludovico *et al.*, 2003), possibly as a result of oxidative stress. Interestingly, sugar induced apoptosis in *S. cerevisiae* has recently been reported (Granot *et al.*, 2003). Therefore, the ability of sugar to induce cell death in the highly fermentative *Z. bailii* would prove of interest.

1.9.3 Propionic acid (E280)

Propionic acid occurs naturally in a number of plants and in some cheeses through the activity of propionibacteria. Propionic acid is primarily used as an inhibitor of moulds

with some bacterial and yeast inhibition. The inhibitor action of propionic acid has been shown to be predominantly that of a 'classic weak-acid preservative'. A secondary more general inhibitory mechanism involving the dissociated form of propionic acid may also be present (Moon, 1983).

1.9.4 Sorbic acid (E200)

Sorbic acid is an unsaturated fatty acid 2-4-hexadienoic acid found as a precursor in the berries of the mountain ash. Sorbic acid and sorbates have a wide spectrum of action inhibiting fungi and bacteria. Sorbic acid has been shown to inhibit a number of enzymes including alcohol dehydrogenase, aspartase and succinic dehydrogenase (Martoadiprawito *et al.,* 1963; York and Vaughn, 1964). Recently, research has questioned the action of some 'classic weak-acid preservatives'. Stratford and Anslow (1998) propose that sorbic acid acts as an inhibitor of membrane function in a similar manner to ethanol because it releases insufficient protons to inhibit as a 'classic weak-acid preservative' (Stratford and Ueckert, unpublished). The majority of studies on the action of sorbic acid have been conducted in bacteria. Freese *et al.* (1973) reported that sorbic acid inhibited amino acid uptake resulting in loss of membrane potential. Finally, sorbic acid has been shown to act on the cell wall, inhibiting the cell division of germinated spores of bacilli (Seward *et al.,* 1982).

1.9.5 Benzoic acid (E210)

Benzoic acid and sodium benzoate were the first antimicrobials permitted by the U. S. Food and Drug Administration (Jay, 1992). Benzoic acid occurs naturally in cranberries, plums, cherry bark and tea and is prepared synthetically for use in the food industry. It is believed to act via a multi-factorial mechanism, with its dominant aspect being as a 'weak-acid preservative'. Benzoic acid has been proposed to influence the membrane,

either by interfering with membrane proteins, or by changing the membrane fluidity (Gomez and Herreo, 1983). It has also been shown to inhibit acetic acid metabolism (Bosund, 1962).

1.9.6 Human consumption of organic acid food preservatives

Consumer concerns over the use of food preservatives have increased in recent years. The main reason for the concern is that there are an increasing number of unsubstantiated reports of allergies, and other irritant-related problems to food preservatives (Parke and Lewis, 1992; Anon, 2001). Sorbic acid has been indicated as a possible skin irritant, while propionic acid has been speculated to cause migraines (Piper, 1999). Despite benzoic acid being regarded as safe as it is excreted in the urine as benzoylglycine (hippuric acid), the possibility of foods containing benzoic acid being toxic by inhalation could not be ruled out in a recent report (Anon, 2001). The main consumer concerns regarding food preservatives relate to the use of nitrites and sulphites. Nitrites are used in the preservation of meat because they reduce the chance of spoilage and also give the appearance of a red colour, which consumers find appealing. The problem with nitrites is that when the meat is cooked the nitrites form nitrosamines, which are carcinogenic (Parke and Lewis, 1992). In a bid to alleviate fears, some producers have lowered the amount of nitrites in meat and added ascorbic acid to prevent the formation of nitrosamines. Sulphites have been reported to cause respiratory problems for asthmatics with those on steroids being particularly at risk (Piper, 1999).

The main problem with ascertaining the potential of food preservatives to be detrimental for human consumption is that there is simply a lack of primary literature. Studies into the safety of food preservatives are not only scarce but are often inconclusive. The

legislation on food preservatives also complicates matters with some food preservatives being banned from certain countries while permitted with restrictions in other countries, as is the case with formic acid (Pollard, 1991). The *ADI* values listed in Table 1.3 are compiled by the joint expert committee on food additives (JEFCA) and represent an area of debate, with not only the values being questioned but the means by which they are derived also raising some concern (Pollard, 1991). To add to the complications, organic acid food preservatives have recently been reported to cause oxidative stress (Piper, 1999). Therefore, the ability of these acids to generate oxidative stress within the human body needs addressing as oxidative stress has been implicated in genetic diseases (Church *et al.,* 1993) and cancer formation (Dreher & Junod, 1996). In summary, until the effects of food preservatives on the human body are addressed, speculation relating to their safety will continue.

1.10 Yeast organic acid resistance

The protective response(s) invoked by yeasts in the presence of high concentrations of organic acids at low pH are believed to be focussed on maintaining intracellular pH (Krebs *et al.,* 1983; Cole and Keenan, 1987a). Despite recent research efforts, the mechanism(s) by which some yeasts are able to tolerate higher levels of organic acids than others remains speculative (Brul and Coote, 1999). Recently, it was speculated that the organic acid resistance mechanism(s) adopted by *S. cerevisiae* and the spoilage yeast *Z. bailii* differ (Piper *et al.,* 2001). A number of theories have been proposed to account for the resistance of yeast to organic acids and are summarised in Figure 1.3.

1.10.1 Reduction of acid influx

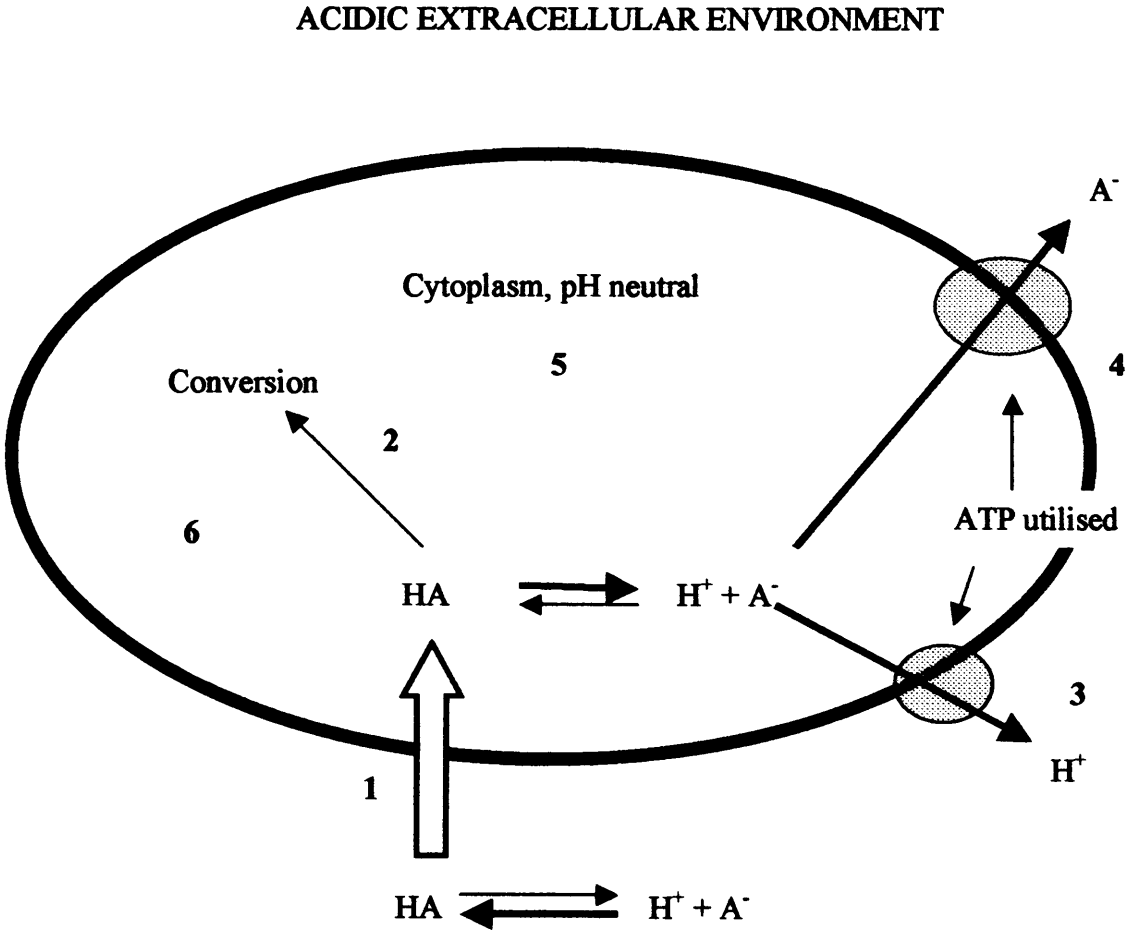
The ability of yeasts to reduce initial acid influx and thereby restrict intracellular damage through differences in the cell wall or membrane could prove crucial in their

ability to tolerate high levels of organic acids (Piper *et al.*, 2001). *S. cerevisiae* is known to change its membrane composition in response to a wide variety of stress conditions including high levels of organic acids (Thomas *et al.*, 1978; Sajbidor and Grego, 1992; Guerzoni *et al.*, 1993; Alexandre *et al.*, 1996; Fernandes *et al*, 2000). The ability of *Zygosaccharomyces* yeasts and in particular *Z. bailii* to change membrane composition under stress remains largely undetermined (Hosno, 1992; Balerias-Couto and In't Veld, 1995). The emphasis of research into cell membrane mediated mechanisms has led to a neglect of the cell wall as a means of restricting permeability. A number of yeasts have been shown to differ in their cell wall composition (Nguyen *et al.*, 1998). The potential involvement of the cell wall in yeast organic acid resistance is supported by the findings of Bom *et al.* (2001). These researchers demonstrated that the combination of a membrane-perturbing compound with an agent that interfered with GPI-cell wall protein layers represented an effective strategy to prevent the growth of spoilage yeasts including *Z. bailii*.

1.10.2 Conversion of the acid into a metabolic product

The ability of yeasts to metabolise organic acids represents another possible mechanism of yeast organic acid resistance. The metabolism of acetic acid by both *Z. bailii* and *S. cerevisiae* has received extensive attention. In *S. cerevisiae* the utilisation of acetic acid is inducible and subject to glucose repression (Cassio and van Uden, 1987; Cassio *et al.*, 1993; Casal and Leáo, 1995). Therefore, *S. cerevisiae* is unable to utilise acetic acid in the presence of glucose: acetic acid therefore enters the cell via simple diffusion and causes intracellular acidification (Casal *et al.*, 1996). In *Z. bailii* the intracellular metabolism of acetic acid is not subject to glucose repression and this compound is metabolised simultaneously with glucose; this could allow the intracellular free acetic acid to be maintained at values below which they exert toxic effects (Sousa *et al.*, 1996).

Figure 1.3 Schematic of the possible resistance mechanisms adopted by yeasts to weak-acid preservatives. Undissociated acid (HA) enters the cell via simple diffusion where the higher cytoplasmic pH causes the acid to dissociate liberating hydrogen ions (H^+) and anions (A^-) which accumulate causing intracellular acidification. Adapted from Loureirio (2000).



Mechanisms of yeast resistance may include one or more of the following: (1) Reduction in acid influx, (2) Conversion of the acid into a metabolic product, (3) Extrusion of hydrogen ions (H^+) (4) Extrusion of anions (A^-) (5) Increased intracellular buffering capacity and (6) Non-specific mechanisms.

The metabolism of other organic acids (including propionic and formic acids) has been shown to be metabolised differently between yeasts, but are generally less well understood (Casal *et al.,* 1996; Sousa *et al.,* 1996). Merico *et al.* (2003) recently conducted a complete analysis of aerobic sugar metabolism in *Z. bailii* in a bid to elucidate more about its carbon and energy metabolism. *Z. bailii* has been shown to use benzoate and sorbate as sole carbon sources, a property not shown by *S. cerevisiae* (Mollapour and Piper, 2001a, b). The oxidative degradation of sorbate and benzoate by *Z. bailii* is dependent upon a gene *ZbYME2,* which when heterologously expressed in *S. cerevisiae* confers the ability to utilise sorbate, benzoate and phenylalanine (Mollapour and Piper, 2001a, b).

1.10.3 Extrusion of hydrogen ions

An increase in hydrogen ion efflux would reduce the extent of intracellular acidification in the presence of organic acids at low pH. In yeast the plasma membrane H^+-ATPase creates an electrochemical potential across the cell membrane thereby driving nutrient uptake and regulating pH through hydrogen efflux. The H^+-ATPase pump has been shown to become activated in the presence of organic acids (Viegas and Sá Correira, 1991; Holyoak *et al.,* 1996). In theory, the H^+-ATPase pump can maintain intracellular pH by driving the expulsion of hydrogen ions, therefore, preventing intracellular acidification in the presence of organic acids. Research into the ability of H^+-ATPase to provide organic acid resistance and to act as a general stress response is extensive for *S. cerevisiae* (Panaretou and Piper, 1990; Rosa and Sá Correira 1991; Holyoak *et al.,* 1996). The H^+-ATPase system is energetically expensive consuming 2 ATPs for each weak acid molecule that enters (Piper *et al.,* 2001). Thus, organic acid resistance results in a diminished biomass (Stratford and Anslow, 1996; Piper *et al.,* 1997). The ability of the H^+-ATPase pump to provide organic acid resistance is therefore energy-dependent.

The importance of the H^+-ATPase pump in *Z. bailii* organic acid resistance remains to be determined.

1.10.4 Extrusion of anions

Exposure of *S. cerevisiae* cells to benzoic acid results in an efflux of accumulated anions via an energy-dependent mechanism (Henriques *et al.*, 1997). Piper *et al.*, (1998) have shown that the plasma membrane Pdr12 ATP binding cassette (ABC) transporter is required for the development of sorbate and benzoate organic acid resistance in *S. cerevisiae*. Recently, the transcription factor War1 has been identified as the regulator of *PDR12* in *S. cerevisiae* (Kren *et al.*, 2003). Two other plasma membrane proteins Gpr1 (Augstein *et al.*, 2003) and Azr1 (Tenereiro *et al.*, 2000) have been implicated in resistance to organic acids and in particular to acetic acid. The expulsion of anions through the Pdr12 transporter or any plasma membrane transporter coupled with hydrogen ion expulsion via H^+-ATPase pump has the potential to create a futile cycle: anions and hydrogen ions could recombine outside the cell and simply diffuse back into the cell. Research into how cells prevent a futile cycle of anion and hydrogen ion expulsion and re-entry is now required. However, it should be remembered that studies thus far using *Z. bailii* have shown no evidence for plasma membrane transporters being responsible for its exceptional organic acid resistance (Piper *et al.*, 2001). In addition, the acetate sensitive phenotypes reported for Pdr12 and Azr1 mutants have been shown to be the result of using *trp1* mutant strains (Bauer *et al.*, 2003).

1.10.5 Increased intracellular buffering

Increased buffering capacity by yeasts as a means of responding to organic acid stress has received little attention. Cole and Keenan (1987a) reported an increase in buffering capacity for *Z. bailii* in the presence of sorbic and benzoic acid. The reasons for the

increased buffering capacity were attributed to a slower growth rate due to organic acid stress, which decreased protoplast volume and thereby increased the concentration of cellular components and their buffering capacity. A detailed comparison of the buffering capacities of *S. cerevisiae* and *Z. bailii* under organic acid stress conditions would prove of interest.

1.10.6 Non-specific mechanisms

The possibility of a number of non-specific mechanisms being utilised by yeasts to combat organic acid stress also exists. *Z. bailii* has been shown to secrete an antifungal toxin zygocin, which has promise in combating fungal infections (Weiler *et al.*, 2002; Weiler and Schmitt, 2003). The secretion of zygocin by *Z. bailii* may, therefore, aid its proliferation in foods by killing of competitors. Overproduction of glycerol in *S. cerevisiae* resulted in double the amount of acetic acid being produced under anaerobic conditions (Eglinton *et al.*, 2002). It is therefore conceivable that if *Z. bailii* and *Z. kombuchanesis* had a greater production of glycerol they would be able to withstand higher concentrations of acetic acid. Pearce *et al.* (2001a) have shown benzoic acid resistance in *S. cerevisiae* to be effected by the genetic manipulation of 6-phosphofructo-1-kinase (PF1K) or fructose 2, 6 bisphosphate levels. Therefore, if a yeast were to contain differing levels of the enzymes involved in glycolysis it may have an altered resistance to organic acids. This is supported by the work of Krebs *et al.* (1983) which has shown organic acids to inhibit glycolysis. The application of proteome and transcriptome analysis to *S. cerevisiae* under sorbic acid stress has indicated the involvement of the heat shock protein Hsp26 (de Nobel *et al.*, 2001). Hsp26 is induced by a range of stresses including osmotic stress (Blomberg, 1997), exposure to hydrogen peroxide (Godon *et al.*, 1998) and heat shock. de Nobel *et al.* (2001) propose that in the case of sorbic acid stress, Hsp26 prepares proteins for degradation or refolding. Sorbic

acid also caused the induction of genes involved in oxidative stress (de Nobel *et al.*, 2001). The ability of organic acids to induce oxidative stress has been previously reported (Piper, 1999). Therefore, yeasts with the ability to tolerate oxidative stress may be able to withstand higher levels of organic acid stress.

1.11 Assessment of food spoilage

The assessment of food spoilage forms a fundamental part of food production. Microbiological analysis will be conducted on raw materials, line samples and final products to determine the presence or absence of microorganisms. The total numbers and types of any microorganisms present will then be determined (Garbutt, 1997). In the processing environment tests are conducted on the microbiological quality of the air, water and microbial contamination of surfaces (Garbutt, 1997). The following sections describe the methods used in the assessment of yeast attributed food spoilage.

1.11.1 Direct microscopic examination

The direct microscopic examination of foods provides one of the quickest and most inexpensive methods of assessing yeast spoilage. The limitation of the method is that only large numbers of organisms in excess 10^6 cells ml^{-1} will be detectable. Fluorescence microscopy provides a variation on this technique and allows the distinction between viable and non-viable cells. Methylene blue is also widely used in the brewing industry as an indicator of yeast viability (Willetts *et al.*, 1997; Smart *et al.*, 1999).

1.11.2 Plate counts

Plate counts (spread or pour plate) represent the most common method for isolating and enumerating yeasts involved in food spoilage (Fleet, 1992). Variations on the plate

count technique include membrane filtration. In this method a known volume of sample is passed through a membrane, which is then placed on an agar plate to assess growth. Most probable number (MPN) is used in the detection of low numbers of microorganisms. A recent development, which is based on the plate count technique, is the use of selective differential plating media; these are used to assess target organisms. Several types of selective differential plating media have been designed specifically for the identification of *Z. bailii* (Hocking, 1996; Makdesi and Beuchat, 1996).

1.11.3 Electrical impedance

Microbial growth will change the electrical properties of their environment, and these charges can be detected. The application of impedance technology is widely used for detecting bacterial populations and is now receiving greater attention in yeast food spoilage assessment (Fleet, 1992).

1.11.4 Measurement of ATP

Measurement of ATP in viable yeast cells provides good correlation with plate counts. Determination of bioluminescence provides the most common method for ATP assessment. However, the measurement of ATP has not been applied on a wide scale in the assessment of yeast food spoilage due to several factors including the cost of reagents, interference and difficulty in distinguishing between bacterial and yeast spoilage without further assessment (Fleet, 1992).

1.11.5 Predictive modelling

An alternative to direct assessment of food spoilage is that of predictive modelling. Predictive modelling focuses on the development of a mathematical model to predict the probability of microbial contamination of a food or beverage. The model uses various

physico-chemical properties of the food including pH, redox potential and nutrient availability to predict spoilage. The application of predictive modelling to determine the probability of a yeast causing spoilage has been reported, with many models focusing on the spoilage yeast *Z. bailii* (Cole *et al.*, 1987b; Deak and Beuchat, 1994; Battey *et al.*, 2002). Predictive modelling, therefore, represents an alternative to direct assessment of spoilage as it can provide rapid information about the microbial stability of a product which may reduce overall costs (Battey *et al.*, 2002).

1.12 Identification of food spoilage microorganisms

The confirmation of food spoilage must be followed by the identification of the spoilage microorganism(s). This allows the exact nature of the spoilage to be assessed and aid the prevention of potential future spoilage. The ability to identify microorganisms in foods must therefore be both reliable and quick.

1.12.1 Physiological identification

In addition to the differential selective plating media discussed in section 1.10.2 physiological tests used in the identification of yeasts can include aerobic growth tests, fermentation tests, mode of sexual reproduction, microscopic appearance of cells and biochemical characteristics (Barnett *et al.*, 2000). There are also commercial kits available that allow for the identification of yeasts. The analytical profile index (API) strips (BioMérieux, France) are probably the best well known. The ability of these kits to lead to accurate identification of yeasts is largely dependant on the skills of the scientist conducting the analysis.

1.12.2 Molecular identification

The molecular identification of yeasts has largely focused on differences in rDNA sequences (Scorzetti *et al.*, 2002). The highly conserved 18S, 5.8S and 28S rRNA domains are separated by internal transcribed spacer (ITS) regions (Figure 1.4). The 26S rDNA D1/D2 variable domains (large subunit) and the 18S rDNA (small subunit) represent coding regions used in yeast systematics. The 26S rDNA (large subunit) and ITS regions have received more attention in yeast systematics than 18S rDNA (small subunit) due to their smaller size (~600 bp) and higher rate of divergence. The extensive use of all three molecular chronometers has lead to the establishment of several databases for the classification and identification of yeasts (Takashima *et al.*, 1995; Kurtzman & Robnett, 1998; Sugita *et al.*, 2000). Mitochondrial DNA and cytochrome oxidase II (COX II) genes are also being increasingly used in yeast systematics (Esteve-Zarzoso *et al.*, 2003; Kurtzman and Robnett, 2003). Molecular methods are also used to assess yeast populations in food (Andrighetto *et al.*, 2000; Sujaya *et al.*, 2003).

Figure 1.4 Organisation of nuclear rRNA genes.

1.13 Cleaning processes in the food industry

The processing of foods to a high and consistent standard free of microbial spoilage requires the application of efficient and vigorous cleaning processes. A cleaning process can be considered to consist of two steps: the physical cleaning of surfaces directly and secondly, microbiological cleaning (disinfection) to remove any microorganisms, which survive physical cleaning. Chemical disinfectants have a broad spectrum of mechanisms

including the generation of oxidative stress and the disruption of cell integrity. Several common disinfectants used in the food industry include chlorine, iodophors, amphoterics, acid anionic surfactants, peracetic acid, QUACS (quaternary ammonium compounds) and hydrogen peroxide. All the aforementioned methods of disinfection are active against yeasts; the application of each is dependent on several factors including cost, nature of the food, spoilage micro-flora and the acid (Adams and Moss, 2000). The majority of cleaning in modern food processing is practised as cleaning-in-place (CIP) systems, which are automated.

1.14 Aims

The broad aim of the work presented in this thesis was to provide further insights into the morphology and physiology of *Zygosaccharomyces* spoilage yeasts, and how these may relate to organic acid resistance. Despite recent research efforts, the differential mechanisms utilised by yeasts to tolerate high levels of organic acid stress remains unknown. The research presented in this thesis focuses on the preservative resistant and notorious spoilage yeast *Z. bailii*, the recently identified potential spoilage yeast *Z. kombuchaensis* and the model yeast *S. cerevisiae*.

The specific aims of this research were:

- Determination of the differences in organic acid resistance between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* (Section 3).
- Investigation into the effects of growth conditions on yeast organic acid resistance (Section 4).
- Exploration into differences in morphology and physiology between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* (Sections 5, 6 and 7).
- Re-examination of *Z. bailii* classification (Section 8).

2. Materials and Methods

2.1 Chemicals

The chemicals used in this study were obtained from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, UK) unless otherwise stated. Yeast extract, Bacteriological peptone and Yeast Nitrogen Base without amino acids and ammonium sulphate were purchased from Becton-Dickinson (BD Biosciences, Oxford, UK). HPLC consumables were obtained from Supelco (Sigma-Aldrich Company Ltd, Dorset, UK). All organic acids were of HPLC grade. Electron microscopy consumables were supplied by Agar Scientific (Agar Scientific Limited, Stansted, UK).

2.2 Strains and media

Yeast strains used in this study are as listed in Table 2.1, and plasmids in Table 2.2. The media used for the cultivation of microorganisms are as detailed in Table 2.3. Yeast strains were maintained on YPD plates re-streaked monthly for short-term storage. For long-term storage strains were maintained on YPD agar slopes at 4°C or in 10% (v/v) glycerol at -80°C.

2.3 Growth Conditions

Starter cultures consisting of 20 ml medium in 50 ml conical flasks were incubated at 25°C without shaking. The OD of cultures was monitored at 600 nm with a Pye-Unicam SP8-400UV/VIS spectrophotometer. Samples were diluted in sterile medium to keep readings below 0.7 and allow for linear determination. Mid-exponential phase starter cultures (1-5 $\times 10^8$ cells ml^{-1}) were used to inoculate cultures containing 40 ml medium in 100 ml conical flasks. Cultures were incubated under the same conditions as starter cultures. Bacteria were grown in 3 ml medium in 14 ml falcon tubes at 30°C, with shaking at 220 r.p.m. Any variations to these conditions are as indicated in the text.

Table 2.1 Yeast strains used in this study.

Strain	Habitat/Genotype	Source
Z. bailii NCYC 1766	Grape and blackcurrant juice	NCYC *
Z. bailii NCYC 1416 T	Fermentation associated	NCYC
Z. bailii 11	Soft drinks factory	M. Stratford ¶
Z. bailii NCYC 385	Fermentation associated	NCYC
Z. bailii NCYC 1520	Unknown	NCYC
Z. bailii 20	Orange concentrate	M. Stratford
Z. bailii 80	Mexican topping sauce	M. Stratford
Z. kombuchaensis (102) NRRL YB4810	Russian, Kombucha tea	C. P. Kurtzman #
Z. kombuchaensis (198) NRRL Y27163	USA, Kombucha tea	C. P. Kurtzman
Z. kombuchaensis (199) NRRL Y27162	USA, Kombucha tea	C. P. Kurtzman
Z. kombuchaensis (200) NRRL YB4811 T	Russian, Kombucha tea	C. P. Kurtzman
S. cerevisiae (X2180-1B) NCYC 957	*MATα SUC2 mal gal2 CUP1*	NCYC
S. cerevisiae NCYC 1324	Polyploidy brewing (lager) strain	NCYC
S. cerevisiae NCYC 1119	Brewing (ale) strain	NCYC
S. cerevisiae BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF †
S. cerevisiae CDN1	Diploid; *S. cerevisiae* 957 × *S. cerevisiae* BY4741	J. R. Dickinson§
S. cerevisiae JRD895	*MATa his3Δ1 leu2Δ0 LYS2 MET15 ura3Δ0 ADH3*	EUROSCARF
S. cerevisiae YLR342W (*fks1* mutant)	BY4741 *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YLR342w::kanMX4*	EUROSCARF
S. cerevisiae YPR159W (*kre6* mutant)	BY4741 *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YPR159w::kanMX4*	EUROSCARF
S. cerevisiae YPL050C (*mnn9* mutant)	BY4741 *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YPL050c::kanMX4*	EUROSCARF
S. cerevisiae YBR023C (*chs3* mutant)	BY4741; *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YBR023c::kanMX4*	EUROSCARF
S. cerevisiae (X2180-1B) NCYC 957x	Petite	This study
S. cerevisiae NCYC 1324x	Petite	This study
S. cerevisiae NCYC 1119x	Petite	This study
S. cerevisiae BY4741x	Petite	This study
S. cerevisiae CDN1x	Petite	This study
S. cerevisiae JRD895x	Petite	This study

Table 2.1 cont Yeast strains used in this study.

Strain	Habitat/Genotype	Source
Pf1*Z. bailii* NCYC 1766 – *S. cerevisiae* JRD895x	Protoplast fusion	This study
Pf2*Z. bailii* NCYC 1766 – *S. cerevisiae* JRD895x	Protoplast fusion	This study
Pf3*Z. bailii* NCYC 1766 – *S. cerevisiae* JRD895x	Protoplast fusion	This study
Pf1*Z. bailii* NCYC 1416 ^{T} – *S. cerevisiae* JRD895x	Protoplast fusion	This study
Pf2*Z. bailii* NCYC 1416 ^{T} – *S. cerevisiae* JRD895x	Protoplast fusion	This study
Pf3*Z. bailii* NCYC 1416 ^{T} – *S. cerevisiae* JRD895x	Protoplast fusion	This study
Pf1*Z. kombuchaensis* (200) NRRL YB4811 ^{T} – *S. cerevisiae* JRD895x	Protoplast fusion	This study
Pf2*Z. kombuchaensis* (200) NRRL YB4811 ^{T} – *S. cerevisiae* JRD895x	Protoplast fusion	This study
Pf3*Z. kombuchaensis* (200) NRRL YB4811 ^{T} – *S. cerevisiae* JRD895x	Protoplast fusion	This study
S. cerevisiae IWD72	*MATα*	I. W. Dawes [¥]
S. cerevisiae L5528	*MAT***a** *ura3-52 his3*	S. Hohmann [Φ]
S. cerevisiae YSH1137	*MAT***a** *leu2::hisG trp1::hisG ura3-52 hog1::TRP1*	S. Hohmann
S. cerevisiae YSH1137-pRS426	*MAT***a** *leu2::hisG trp1::hisG ura3-52 hog1::TRP1+ pRS426 (URA3)*	This study
S. cerevisiae YSH1137-pRS426-HOG1	*MAT***a** *leu2::hisG trp1::hisG ura3-52 hog1::TRP1+ pRS426-HOG1 (URA3)*	This study

^{T} = Type strain.

[*] National Collection of Yeast Cultures, Institute of Food Research, Colney, Norwich, NR4 7UA.
[¶] Food Processing Group, Unilever R & D Colworth, Colworth House, Sharnbrook, Bedford, MK44 1LQ, UK.
[#] Agricultural Research Service Culture Collection, Microbial Genomics and Bioprocessing Unit, National Center for Agricultural Utilization Research, 1815 N. University Street, Peoria, Illinois 61604, USA.
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[†] EUROSCARF, Institute for Microbiology, Johann Wolfgang Goethe-University Frankfurt, Marie-curie-Strasse 9, Building N250, D-60439, Frankfurt, Germany.
[¥] School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, 2052 NSW, Australia.
[Φ] Cell and Molecular Biology, Göteborg University, Box 462, S-405 30, Göteberg, Sweden.

Table 2.2 Plasmids used in this study.

Plasmid	Details	Source
pRS426	A 2μm based yeast episomal plasmid	Christianson *et al.* 1992
pRS426-HOG1	A 2μm based yeast episomal plasmid containing *S. cerevisiae HOG1* gene with its own promoter.	S. Hohmann [Φ]

[Φ] Cell and Molecular Biology, Göteborg University, Box 462, S-405 30, Göteberg, Sweden.

Table 2.3 List of all media used in this study. All percentages are (w/v). For solidified media, agar was added at a concentration of 2% (w/v) and 3% (w/v) for regeneration agar. All media was sterilized by autoclaving at 121°C 1.3 MPa for 15 min. These recipes are the basic ingredients any additions or modifications are as described in the text.

Medium	Recipe
YPD	2 % D-Glucose, 2 % Bacto peptone, 1 % Bacto yeast extract
MM	2 % D-Glucose, 0.5 % Ammonium sulphate, 0.17 % Yeast Nitrogen Base without amino acids and ammonium sulphate
Regeneration	3 % Glycerol, 18.2 % Sorbitol, 0.5 % Ammonium sulphate, 0.17 % Yeast Nitrogen Base without amino acids and ammonium sulphate
Sporulation	0.06 % D- Glucose, 0.22 % Bacto yeast extract, 2 % Potassium acetate
LB	1 % D-Glucose, 1 % Bacto tryptone, 0.5 % Sodium chloride
SOC	0.18 % D-Glucose, 2 % Bacto tryptone, 0.5 % Bacto yeast extract, 0.05 % Sodium chloride, 0.05% $MgCl_2$

"Drop-out" solutions consisting of 40 mg of each amino acid and the bases adenine and uracil, (except for the amino acid or base required to confirm auxotrophic requirements) were added to 10 ml distilled water and autoclaved at 121°C 1.3 MPa for 15 min. A 100 μl aliquot of the required drop-out solution was spread over a solidified MM plate. For liquid MM cultures 200 μl of each drop-out solution was added per plate resulting in a concentration of 20 μg ml^{-1} for each amino acid and base.

2.4 Assessment of Inhibitor Toxicity

2.4.1 Preparation of inhibitor stocks

The organic acids used in this work are as detailed in Table 2.4. Short chain organic acids (formic, acetic, propionic) were added directly to sterilized pH-corrected growth media. Medium (hexanoic, sorbic, benzoic) and longer (octanoic, nonanoic, decanoic) chain organic acids were prepared as stock solutions in ethanol (to ensure solubility) and added to sterilized media. Salt (NaCl) was added to the growth media prior to sterilization. Hydrogen peroxide was added to cultures immediately after yeast inoculation to avoid inhibitor decomposition.

2.4.2 Culture conditions

Starter cultures consisting of 20 ml medium (adjusted to pH 4.0 with concentrated HCl) in 50 ml conical flasks were grown until mid-exponential phase at 25°C without shaking. Cell numbers were determined using a Neubauer Haemocytometer and cell suspensions prepared in sterile peptone water (Bacto peptone 0.1% w/v). Susceptibility to inhibitors was determined as described previously (Steels *et al.*, 1999b; 2000; 2002; Stratford *et al.*, 2002; Fitzgerald *et al.*, 2003). In brief, inhibitors were added to 10 ml medium (pH 4.0) in 30 ml universal bottles and inoculated with 1×10^3 cells ml^{-1}. Cultures were incubated at 25°C for 14 days without shaking. Minimum Inhibitory Concentrations (MICs) were defined as the lowest concentration of inhibitor, which inhibited visible growth. Control cultures containing 3% (v/v) ethanol were set up for medium and longer chain organic acids. Effects of inhibitors on growth were assessed by measuring culture OD600 nm. Results are presented as the mean of at least two independent experiments (four replicates), ± the standard error. Alterations to the culture medium are as indicated in text.

Table 2.4 Organic acids used in this study.

Systematic name	Common name	Number of Carbons	Chain Classification	pK_a
Methanoic acid	**Formic acid**	1 Carbon	Short Chain	3.75
Ethanoic acid	**Acetic acid**	2 Carbons	Short Chain	4.76
Propanoic acid	**Propionic acid**	3 Carbons	Short Chain	4.87
Hexanoic acid	Caproic acid	6 Carbons	Medium Chain	4.87
2, 4-hexadienoic acid	**Sorbic acid**	6 Carbons	Medium Chain	4.74
Benzoic acid	**Benzoic acid**	7 Carbons	Medium Chain	4.19
Caprylic acid	**Octanoic acid**	8 Carbons	Longer Chain	4.89
Pelagronic acid	**Nonanoic acid**	9 Carbons	Longer Chain	4.88
Capric acid	**Decanoic acid**	10 Carbons	Longer Chain	4.90

The name most used for the organic acid is in bold. pK_a values were obtained from Freese *et al.*, 1973; Adams and Moss 2000.

2.5 Electron Microscopy

2.5.1 Transmission Electron Microscopy (TEM)

All EM preparation was carried out in a fume cupboard due to the harmful nature of the reagents. Cells were pelleted at 1000 × *g* for 10 min. in an MSE2 Centaur centrifuge and washed twice with sterile distilled water. Washed cells were fixed in a 0.1 M sodium cacodylate buffer (pH 6.9) containing 3.2% (v/v) paraformaldehyde, 3.0% (v/v) glutaraldehyde, 4% (w/v) sucrose and 0.02% (w/v) calcium chloride at 4°C for 24 h. Cells were washed twice in 0.1 M sodium cacodylate buffer and then osmicated in 1% osmium tetroxide (OsO_4) at 4°C for 1 h. Pellets were embedded in 2% (w/v) agar. Solid samples were cut into small sections and dehydrated with successive ethanol washes (30, 50, 70 and 90% v/v) for 15 min. at 4°C. Two final ethanol washes of 100% (v/v) for 30 min. one at 4°C and the other at room temperature were carried out. Samples were then infiltrated in Spurr resin with ethanol (at the following ratios 25:75, 50:50, 75:25 v/v) in a rotor mixer for 1 h. Samples were then placed in 100% Spurr resin overnight followed by fresh 100% Spurr resin for 8 h at room temperature. Samples were transferred to casts and placed in an oven at 60°C for 48 h to polymerize. Ultrathin (approximately 60-90 nm) sections were obtained with an LKB Ultratome III (Stockhom, Sweden) and mounted onto 0.5% pioloform coated copper grids. Sections were stained with uranyl acetate for 10 min. and Reynolds lead citrate (pH 12) for 5 min. and washed three times with UHP water. Stained grids were viewed and photographed using a Jeol 1210V transmission electron microscope (Tokyo, Japan). Variations to this standard protocol are as detailed in the text.

2.5.2 Scanning Electron Microscopy (SEM)

Cells were processed and fixed as section 2.5.1. After treatment with 1% OsO_4 samples were taken through the following successive ethanol washes (30, 50, 70 and 90% v/v)

for 15 min. at 4°C. Samples were then placed in Beem capsules and washed twice in 100% ethanol at 4°C. All the ethanol in the samples was replaced with liquid CO_2, by placing samples into a critical point dryer (BAL-TEC AG, Balzers CPD 030, Liechenstein) as per manufacturer's instructions. Samples were placed onto aluminium stubs and gold coated (Edwards Sputter Coater S150B, Crawley UK). Samples were viewed under a Philips XL20 scanning electron microscope (Eindoven, The Netherlands).

2.6 Chitin analysis

2.6.1 Chitin distribution

Cells were grown in YPD pH 4.0 at 25°C without shaking for 48 h and centrifuged at 1000 × *g* for 10 min. in a MSE2 centaur centrifuge. Cells were washed twice with sterile distilled water and re-suspended in 0.5 ml sterile distilled water containing 20 µl of a 1 mg ml^{-1} Calcofluor white (Fluorescent Brightener, Sigma) stock. Samples were incubated at room temperature for 15 min. Samples were then washed twice in sterile distilled water and re-suspended in 200 µl sterile water. Samples were mounted onto slides and viewed using an Olympus BH2 triocular fluorescent microscope (Olympus UK Ltd, Middlesex, UK). Images were taken with a digital camera (HC 3002 Fujitsu, Tokyo, Japan).

2.6.2 Chitin content

A method based on that of Ride and Drysdale (1972) of converting chitin to chitosan with nitrous acid was employed. Yeast cells were grown as described in section 2.6.1 and homogenized to get a uniform suspension. A sample of 0.5 ml of homogenized cells was added to 9.5 ml of 0.24 M sodium hydroxide. Suspensions were centrifuged at 1000 × *g* for 10 min. and washed in 10 ml distilled water. To the pellet was added 3 ml

potassium hydroxide and heated at 100°C for 1.5 h with even mixing. Samples were cooled and 8 ml of 75% (v/v) ethanol added for 15 min. on ice followed by 0.9 ml of a Celite 545 (Acros, Fisher Scientific Ltd, Loughbrough, UK) suspension (1 g in 20 ml 75% v/v ethanol). The sample was centrifuged at 1000 × *g* for 10 min. and the supernatant discarded and the pellet washed once in 40% (v/v) ethanol and twice in distilled water. The supernatant was then discarded and the pellet re-suspended in 1.5 ml sterile distilled water and chitosan content determined. For chitosan determination 1.5 ml 5% (w/v) sodium nitrite and 1.5 ml 5% (w/v) potassium hydrogen sulphate (generating nitrous oxide) was added to the samples. Samples were shaken at 30°C and 160 r.p.m for 30 min. and then centrifuged for 10 min. at 1000 × *g*. Ammonium sulphamate 12.5% (w/v) was added and shaken for 5 min. followed by 0.5 ml of 0.5% (w/v) MBTH (prepared fresh each day). Sample was heated at 100°C for 3 min. and cooled. Finally, 0.5 ml of 0.5% (w/v) iron (III) chloride was added to the samples and shaken for 30 s. Samples were allowed to stand at room temperature for 30 min. for the colorimetric conversion (clear to blue) and the OD650 nm measured with reference to a blank containing 1.5 ml water only and taken through the chitosan conversion protocol. OD values were converted to chitosan and then to chitin levels with reference to calibration curves. The chitin and chitosan calibration curves obtained were conducted in triplicate and produced R^2 values greater than 0.95, over the range of concentrations examined. Results are the means of three independent cultures each performed in duplicate and converted to % chitin per mg dry weight, ± the standard error.

2.7 Yeast Protoplast formation

2.7.1 β-glucoronidase

Starter cultures were set up as in section 2.3 and used to inoculate 40 ml YPD in 100 ml conical flasks. Cultures were incubated for 48 h at 25°C without shaking and harvested

at 1000 × *g* for 10 min. A cell suspension of 1 × 10^8 cells ml^{-1} for each, yeast, was prepared in peptone water as in section 2.4.2. Cells were centrifuged at 1000 × *g* for 10 min. and washed twice in sterile distilled water. Pellets were re-suspended in 5 ml β-glucoronidase buffer containing 1 M sorbitol, 500 µl β-glucoronidase and 10 µl 2-mercaptoethanol in 5 mM Tris-HCl, pH 7.5. Suspensions were incubated at 30°C with gentle shaking (120 r.p.m). Protoplast formation was monitored by change in % OD600 nm and by the examining cells microscopically. Results are presented as the mean of an experiment performed in triplicate, ± the standard error.

2.7.2 Lysozyme

As a negative control the bacterial active lysozyme was examined. Protoplast formation was performed as described in 2.7.1 replacing the β-glucoronidase buffer with 5 ml 0.10 g lysozyme (5000 Units/g solid), 10 µl 2-mercaptoethanol in 50 mM Tris-HCl, pH 8.0.

2.7.3 Zymolyase

Protoplast formation was performed as 2.7.1 replacing β-glucoronidase with Zymolyase (Lyticase, Sigma). The β-glucoronidase buffer was replaced with 5 ml SMT buffer consisting of 1.2 M sorbitol, 10 mM $MgCl_2$, 0.10 g Zymolyase (200 Units/mg solid), 10 µl 2-mercaptoethanol in 2 mM Tris-HCl, pH 7.2.

2.8 Yeast Protoplast Fusion

Protoplasts from Zymolyase treatment were harvested at 1000 × *g* for 10 min. and washed twice in 10 ml SMT buffer. Cells from each of the donor yeasts were re-suspended in 3 ml SMT buffer mixed in equal proportions and harvested at 1000 × *g* for 10 min. Pellets were re-suspended in 2 ml 60% (w/v) PEG 3350 and 0.2 ml 1.0 M calcium chloride at room temperature for 5 min. A control replacing the 2 ml 60% (w/v)

PEG 3350 with 2 ml SMT buffer was also set up. To both suspensions 6 ml SMT buffer was added and left at room temperature for an additional 10 min. Cells were harvested at 1000 × *g* for 10 min. and re-suspended in 1 ml SMT buffer. Protoplasts were plated out on to selection plates as follows: 2 × 100 µl undiluted suspension and 2 × 100 µl of a 10 fold serial dilution were plated out onto regeneration agar containing 200 µg/ml of the aminoglycoside G418 (Melford Laboratories Ltd, Ipswich, UK). A 100 µl aliquot of undiluted suspension was plated out onto YPD as a control for protoplast formation. Plates were incubated at 25°C for 3-10 days. The frequency of protoplast fusion was calculated by comparing the number of c.f.u on selective and non-selective agar. Results are the mean of at least two independent experiments.

2.9 High-performance-liquid-chromatography (HPLC)

2.9.1 Preparation of samples

Cultures consisting of 10 ml MM pH 4.0 in 30 ml universal bottles with either 50 mM acetic or 25 mM propionic acid were set up as in section 2.4.2. Samples of 1 ml were taken at the indicated time intervals and centrifuged in 1.5 ml microfuge tubes at 13500 × *g*. Samples were then filtered through a 0.20 µm Millipore filter directly into a 2 ml analytical vial. Samples were stored at -20°C prior to analysis.

2.9.2 Sample analysis

A Dionex HPLC system (Dionex Ltd, Surrey, UK) was used for the detection and quantification of acetic and propionic acid. Samples of 20 µl were injected via an auto-sampler onto a Supelco C-610H organic acid column (30cm × 7.8mm ID). The mobile phase was 0.2% (v/v) orthophosphoric acid at a flow rate of 0.7 ml/min. The run time was 20 min. and a UV detector at 210 nm was used for the detection of acids. The

PRIME™ HPLC software (HPLC Technology Ltd, UK) was used for data analysis. Eluted peaks were compared to the retention times and peak areas of authentic standards. The retention times for acetic and propionic acids were 11.44 min. and 13.67 min., respectively. All concentrations of acids examined produced calibration graphs with R^2 values greater than 0.98. Results are the mean of two independent experiments (four replicates), ± the standard error.

2.10 Determination of total glycerol content

2.10.1 Preparation of samples

Starter cultures were set up as in section 2.3 and used to inoculate 40 ml YPD in 100 ml conical flasks with and without 0.5% (v/v) isoamyl alcohol. At various time intervals, 1.5 ml of culture were removed, placed in sterile microfuge tubes and boiled for 15 min. to stop enzymatic reactions. The samples were then centrifuged at 13500 × *g* to remove cell debris and the supernatant used as source for total glycerol measurements. Samples were stored at -20°C prior to analysis.

2.10.2 Sample analysis

Enzymatic analysis of total glycerol content was conducted according to the manufacturer's instructions (Glycerol determination kit, r-biopharm, Rhóne Ltd, Glasgow, UK). The principle of the assay is as follows: Glycerol is phosphorylated in the presence of ATP to L-glycerol-3-phosphate by glycerol kinase, giving ADP. Coupling ADP with *phosphenolpyruvate* in the presence of pyruvate kinase regenerates the ATP lost in the initial reaction. This leads to the formation of pyruvate. Pyruvate is reduced to L-lactate using L-lactate dehydrogenase. NADH is oxidised to NAD in this reaction, which is directly proportional to the amount of glycerol present. Therefore, the greater the change in absorbance at 340 nm the more NADH being oxidised and the

more glycerol present in the sample. Results were converted to µmol glycerol/mg dry weight/l and are presented as the mean of two independent experiments (four replicates), ± the standard error.

2.11 Molecular genetic techniques

2.11.1 Transformation of *E. coli*

Escherichia coli DH5α competent cells (gift from H. Rogers, Cardiff University, Cardiff, UK) were thawed on ice from -80°C storage and a 100 µl aliquot transferred to a round bottomed 14 ml sterile tube. A 2 µl volume of plasmid DNA (typically 0.5 µg μl^{-1}) was added and the mixture inverted several times. The sample was incubated on ice for 20 min. and heat shocked for 45 s in a water bath at 42°C and incubated on ice for a further 2 min. A 900 µl aliquot of SOC medium (Table 2.3) was added and the sample incubated at 37°C for 1 h with gentle shaking. A 100 µl volume of transformation mixture was then spread onto plates LB containing 50 µg ml^{-1} ampicillin. Plates were incubated at 37°C overnight and transformants picked.

2.11.2 Plasmid preparations from *E. coli*

Transformed *E. coli* DH5α cells were grown overnight in 3 ml LB medium containing 50 µg ml^{-1} ampicillin in a 14 ml sterile tube and harvested for 3 min. at 13500 × *g* in a bench top microcentrifuge. Plasmid preparations were performed using the Q1Aprep Miniprep kit (Qiagen Ltd., Crawley, UK). In brief, pellets were re-suspended in 250 µl re-suspension buffer (buffer P1; 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg ml^{-1} RNase A), followed by 250 µl lysis buffer (buffer P2; 200 mM NaOH, 1% (w/v) SDS) and inverted 4-6 times to mix. To the viscous and now slightly clear preparation 350 µl of neutralization buffer (buffer N3; 25-50% (w/v) guanidium chloride, 10-25% (v/v) acetic acid) was added and the tube inverted as described previously. Preparations were

centrifuged at 13500 × *g* and supernatants decanted into Q1Aprep spin columns. The columns were centrifuged at 13500 × *g* for 1 min. and the eluate discarded. The Q1Aprep spin columns were washed twice in 750 µl wash buffer (buffer PE: 80% (v/v) ethanol) and the eluate discarded. Samples were centrifuged at 13500 × *g* for an additional 1 min. to remove residual wash buffer. Q1Aprep spin columns were transferred to clean microfuge tubes and the DNA was eluted by adding 50 µl elution buffer (buffer EB: 10 mM Tris-HCl, pH 8.5 warmed to 60°C). Samples were allowed to stand at room temperature for 5 min and centrifuged at 13500 × *g* for 1 min. Plasmid preparations were stored at -20°C.

2.11.3 Restriction digests and agarose gel electrophoresis

Restriction endonucleases and buffers were purchased from Promega (Southampton, UK). Restriction digests were performed in sterile microfuge tubes. Each restriction digest consisted of 2 µl plasmid DNA (typically 0.5 µg μl^{-1}), 2 µl of an appropriate 10 × buffer, 0.2 µl BSA (10 mg ml^{-1}), 0.5 µl restriction endonuclease (*EcoR I* 12 Units/µl and/or *Pst I* 10 Units/µl) made up to 20 µl with sterile UHP water. Digests were incubated at 37°C for 1 h. A 5 µl aliquot of each digest mixed with 1 µl loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 15% (w/v) ficoll) was run on a 1% (w/v) agarose gel in 1 × TAE (40 mM Tris-acetate, 2mM EDTA) and stained using ethidium bromide. A 5 µl volume of Hyper ladder 1 (Bioline Ltd, London, UK) was added to estimate fragment sizes. DNA was visualized using a Gene Genius gel documentation analysis system under UV light (Syngene, Cambridge, UK).

2.11.4 Transformation of yeast

A modified version of the Ito *et al.*, (1983) lithium acetate whole cell transformation protocol was used. In brief, a 20 ml overnight YPD culture incubated at 30°C 160 r.p.m

to an OD 600 nm 1-2 ($1 \times 10^7 - 5 \times 10^7$ cells ml^{-1}) was harvested at 1000 × *g* for 10 min. The pellet was washed twice in 4 ml TE buffer (10 mM Tris-HCl pH 7.6, 1mM EDTA) and re-suspended in 400 µl of lithium acetate solution (0.1 M lithium acetate in TE buffer) and incubated at 30°C 160 r.p.m for 60 min. A 5 µl volume of plasmid DNA (typically 0.5 µg μl^{-1}) was added to 150 µl of competent cells. Next 350 µl of a 50% (w/v) PEG 4000 solution in water was added to the transformation mixture and inverted several times. The transformation mixture was heated at 30°C for 1 h in a water bath and then heat shocked at 42°C for 5 min. and cooled immediately on ice. Finally, 100 µl of the transformation mixture was plated onto MM plates containing 100 µl uracil "drop-out" solution and incubated at 25°C for 2-4 days. Transformants were taken through several rounds of auxotrophic confirmation.

2.11.5 Colony polymerase chain reaction (PCR) to confirm transformants

A single colony grown at 25°C for two days on YPD was used for the colony PCR. A small amount of colony was transferred to the PCR reaction using a sterile toothpick. The reaction consisted of 45 µl 1.1 × Reddymix™ (ABgene, Surrey, UK) PCR master mix (1.25 units *Taq* DNA polymerase, 75mM Tris-HCl pH 8.8 at 25°C, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% (v/v) Tween 20, 0.2 mM each of dATP, dCTP, dGTP and dTTP and red dye for electrophoresis), 1µl P1 and 1µl P2 at a concentration of 50 pmoles μl^{-1} (Table 2.5) sterile UHP water was added to give a total volume of 50 µl per reaction. The PCR program was 94°C for 4 min. to denature followed by 35 cycles at 94°C for 30 s, 55°C for 45 s and 72°C for 80 s with a final extension at 72°C for 7 min. Amplification product size was assessed by resolving on a 1% (w/v) agarose gel which was stained with ethidium bromide and visualised under UV light as described in section 2.11.3.

Table 2.5 Primers used for HOG colony PCR

Primer	Target	Binding site	Sequence (5 -3)
P1	*S. cerevisiae* HOG1	389-411	GGGGTTTAAAATACGTTCACTCC
P2	*S. cerevisiae* HOG1	764-786	AATTGGATCTCTGTGTGGTAACG

Primers were designed using the *HOG1* gene sequence as a template (appendix) with oligonucleotides being produced by Sigma-Genosys (Sigma-Aldrich Company Ltd, Dorset, UK).

2.11.6 26S rDNA D1/D2 sequence analysis

The variable D1 and D2 domains of 26S rDNA were PCR-amplified directly from individual yeast colonies using the method as described in James *et al.*, (1994). The conserved fungal oligonucleotide primers NL1 (5' GCATATCAATAAGCGGAGG AAAAG) and NL4 (5' GGTCCGTGTTTCAAGACGG) were used (O' Donnell., 1993). Amplified 26S rDNA D1/D2 PCR products were purified using a Qiagen QIAquick PCR purification kit (Qiagen Ltd., Crawley, UK), and sequenced directly using a *Taq* DyeDeoxy terminator cycle sequencing kit (PE Biosystems, Warrington, UK) and an Omnigene thermal cycler (Hybaid). The amplification primers NL1 and NL4 were used to determine 26S rDNA D1/D2 sequences. Purified sequence reaction mixtures were electrophoresed in a PE Biosystems model 373A automated DNA sequencer. The 26S rDNA D1/D2 sequence alignment and the phylogenetic tree were constructed using the program DNAMAN version 5.1.5 (Lynnon BioSoft, Quebec, Canada). The 26S rDNA D1/D2 sequence analysis was kindly conducted by S. A. James (NCYC, Norwich, UK)

2.12 General physiological techniques

2.12.1 Cell wall/membrane phenotypic screens

A method based on that used by Ram *et al.*, 1994 and Martin-Yken *et al.*, 2001 was used. In brief, a cell suspension of 2×10^7 cells ml^{-1} was prepared in peptone water as described in section 2.3 and used to prepare 3 subsequent 10 fold serial dilutions. A 3 µl

volume of each dilution (neat, 10^{-1}, 10^{-2} and 10^{-3}) was spotted on agar plates containing the relevant chemical and incubated at 25°C for 5 days. Chemicals were prepared as follows: Calcofluor white (Fluorescent Brightener, Sigma) and congo red were prepared as 1% (w/v) stock solutions in water and filter sterilized. The aminoglycoside G418, caffeine, sodium orthovanadate (Na_3VO_4) and calcium chloride ($CaCl_2$) were prepared as 10 fold stock solutions in sterile UHP water. All chemicals were added to sterilized agar just before solidification. Assays were performed at least in duplicate and photographs of the plates were taken after 5 days incubation at 25°C.

2.12.2 Determination of petite forming capabilities using ethidium bromide

Cell starter cultures were set up as described in section 2.3. A cell suspension of 2×10^5 cells ml^{-1} was prepared and 100 µl of each suspension was spread plated onto a YPD agar plate containing 10 µl of 10 mg ml^{-1} ethidium bromide in the centre. Plates were incubated at 25°C for 3 days. Zones of inhibition were based on two independent experiments (four replicates) and standard errors calculated. Small colonies patched on to YPD plates to determine viability. Viable patches were then replica plated on to YPG, YPE and YPA plates to assess petite forming capabilities.

2.12.3 Isoamyl alcohol-induced pseudohyphal formation

Induction of pseudohyphal formation was carried out by addition of 0.5% (v/v) isoamyl alcohol (4.6 mM) as described previously (Dickinson, 1996). In brief, cultures were set up as described in section 2.3. To main cultures isoamyl alcohol was added to give a final concentration of 0.5% (v/v) by placing the tip of a pipette into the sterilized media and dispensing the isoamyl alcohol. The tip was subsequently washed three times in the media to ensure that all the isoamyl alcohol was dispensed. At various time intervals growth and pseudohyphal formation was assessed by measuring the culture OD600 nm

and scoring 400 cells for ability to form pseudohyphae. Results shown are the mean of at least three independent experiments ± the standard error.

2.12.4 Assessment of total cell viability

Citrate methylene blue was used for the determination of total cell viability as described by Smart *et al.* (1999). In brief, methylene blue was dissolved in 1 ml ethanol and mixed with 2% (w/v) sodium citrate solution to give a final concentration of 0.01% (w/v). A volume of 0.5 ml of yeast culture was mixed with 0.5 ml of the citrate methylene blue solution and the cells were examined microscopically after 5 min. The viability of 400 cells was determined by scoring stained blue cells as non-viable and unstained cells as viable. To serve as a control, 1 ml of culture for each yeast was heat killed at 70°C for 15 min. and the viability determined by methylene blue staining, which all cells appeared blue.

2.12.5 Colony forming unit (c.f.u) counts

Cultures were set-up as described in section 2.3 and serial dilutions prepared in peptone water at 24, 48 and 72 h incubation. A 100 µl aliquot of each dilution was spread onto a YPD plate and incubated at 25°C. After 3 days incubation the number of colonies on each plate were counted and the plates, which yielded counts between 30-300 colonies were used to determine the number of c.f.u per ml of the original sample. Results are presented as the means of at least two independent experiments with two or more determinations being made at each time interval.

2.12.6 Determination of dry weight

Culture samples of 1 ml were removed at various time intervals and centrifuged at 13500 × *g* for 3 min. in pre-weighed 1.5 ml microfuge tubes. Supernatants were removed and tubes placed in a hot oven until they reached constant weight. Dry weights are the mean of three independent determinations with standard errors <10%.

2.12.7 Assessment of flocculation capabilities

Flocculation was tested by dispersal with EDTA and aggregation with calcium chloride (Stratford, 1996a). Cultures were set up as described in section 2.3. Cultures were harvested at 1000 × *g* for 10 min. and washed twice in 10 ml distilled water. Pellets were re-suspended in a buffer of 50 mM sodium succinate (pH 4.0) containing 10 mM calcium chloride. Suspensions were agitated at 30°C 120 r.p.m and after 1 h sample supernatants were removed and dispersed in EDTA. The ability of cells to flocculate was then scored microscopically at each stage in comparison to the flocculent yeast *S. cerevisiae* NCYC 1119.

2.12.8 Ascospore formation

Strains were streaked onto YPD plates for two days at 25°C and replica plated to sporulation medium for the same period of time. Microscopic examination was employed to assess sporulation capabilities. Strains were scored as either positive or negative for ascospore formation.

3. Differences in organic acid resistance between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

Nunn C. D., Stratford, M. & Dickinson, J. R. (2002) Differences in organic acid resistance between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*. Poster Presentation at British Yeast Group Meeting, Swansea, UK.

3.1 Introduction

The ability of certain yeasts to spoil foodstuffs has been widely reported (Deak, 1991; Fleet 1992). Among the most high-profile spoilage yeasts is *Z. bailii* with its ability to proliferate in millimolar rather than micromolar levels of food preservatives (Piper *et al.,* 2001). The ability of *Z. bailii* to grow at high levels of preservatives causes considerable problems for the food and drink manufacturing industries. However, the factors contributing to yeast food spoilage remain far from fully understood.

Previous research has largely been conducted on the mechanisms of yeast organic acid resistance (Krebs *et al.,* 1983; Stratford and Anslow, 1996; Piper *et al.,* 1998; Cheng *et al.,* 1999; Mollapour and Piper, 2001b), with few studies focusing on the concentrations of organic acids required to inhibit growth. In this section, the aim was to further characterize the concentration of organic acids required to inhibit yeast growth. Several strains of *Z. bailii* from both foodstuffs and culture collections were selected along with the four currently know strains of the recently identified potential spoilage yeast *Z. kombuchaensis* (Kurtzman *et al.,* 2001). Several strains of the organic acid sensitive *S. cerevisiae* were also included. The acids selected were short (formic, acetic, propionic), medium (hexanoic, sorbic, benzoic) and longer (octanoic, nonanoic, decanoic) chain organic acids. The organic acids were selected on the basis that many of them are used in the food industry as 'weak-acid preservatives'. The experiments reported in this section were conducted in the complex media YPD at pH 4.0 at 25°C under static conditions with MICs and the effects of organic acids on growth being recorded after 14 days incubation. The conditions selected represent the closest laboratory conditions in which yeast cause spoilage and are used by researchers within the food industry (Steels *et al.,* 1999b; 2002; Stratford *et al.,* 2002; Fitzgerald *et al.,* 2003).

3.2 Results

3.2.1 Differences in short chain organic acid resistance between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

Z. bailii, Z. kombuchaensis and *S. cerevisiae* differ in resistance to short chain organic acids (Table 3.1). *S. cerevisiae* proved the most susceptible to short chain organic acids, having MICs lower than the other two species. *S. cerevisiae* and *Z. kombuchaensis* exhibited little strain variation, with similar MIC results being obtained for all strains. *Z. bailii* showed strain variation. *Z. bailii* strains NCYC 1416 and NCYC 385 having the lowest MICs of the *Z. bailii* strains examined for both acetic and propionic acid. *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* required higher levels of acetic acid to inhibit growth than either formic or propionic acid. *Z. bailii* and *S. cerevisiae* showed a similar pattern of sensitivity to formic acid as they did to propionic acid. *Z. kombuchaensis,* however, proved more resistant to propionic acid than to formic acid; having MICs higher than those of the *Z. bailii* strains. *Z. kombuchaensis* strains showed MICs for butyric acid (C4) and for valeric acid (C5) intermediate between *Z. bailii* and *S. cerevisiae* (data not shown).

Measuring culture optical densities assessed the effects of short chain organic acids on *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* growth. Figures 3.1-3.3 show that all three short chain organic acids caused a decrease in growth with increasing concentration. The *S. cerevisiae* strains examined showed good growth for all three short chain organic acids, at concentrations below the MIC. *Z. bailii* and *Z. kombuchaensis* showed a similar growth pattern on exposure to formic acid, acetic and propionic acid with growth decreasing progressively. *Z. bailii* strains NCYC 1416 and NCYC 385 reached considerably lower OD values than the other *Z. bailii* strains, even in the absence of

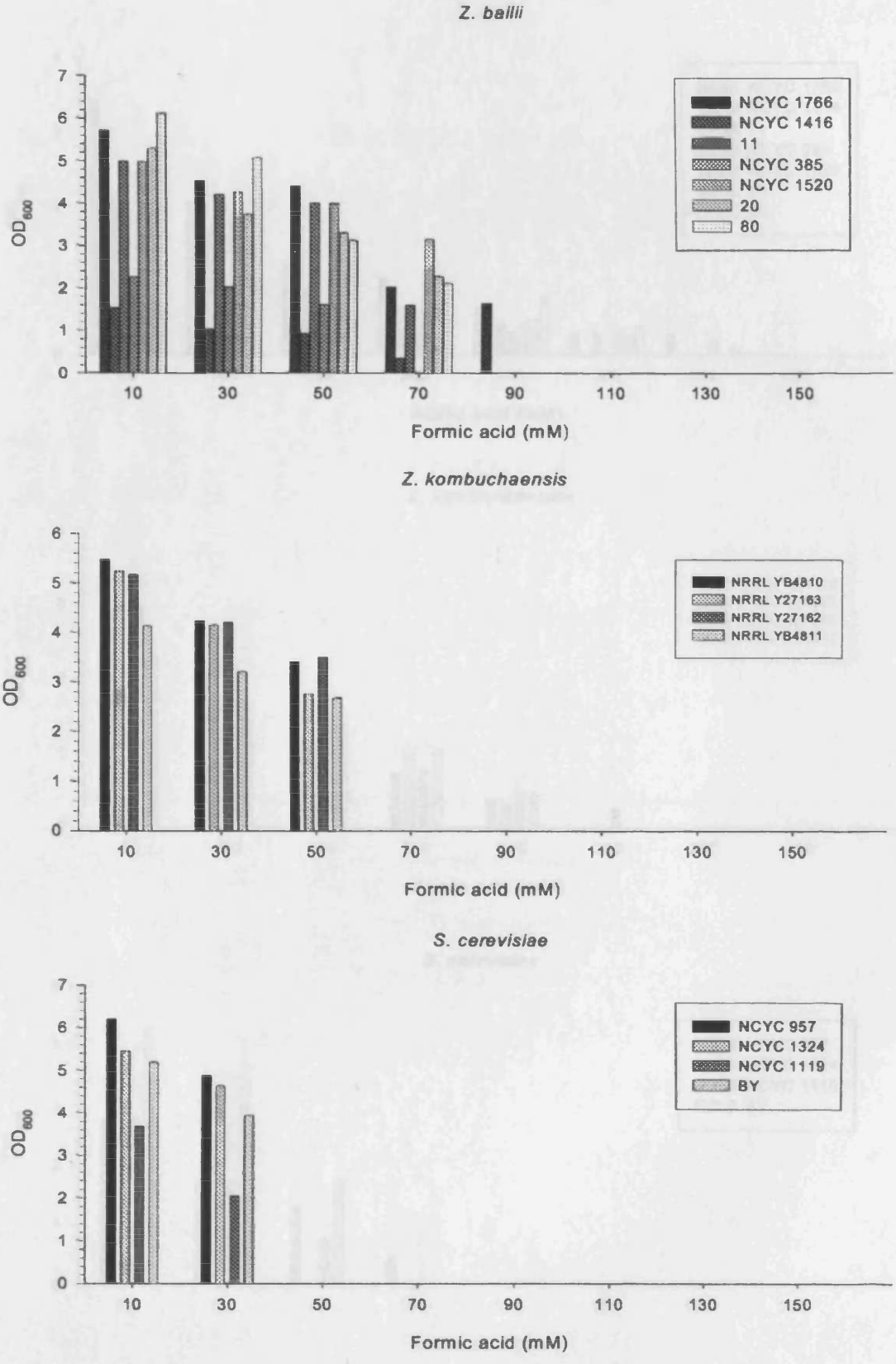
Table 3.1 Differences in short chain organic acid MICs (mM) between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

	Formic acid	Acetic acid	Propionic acid
Z. bailii NCYC 1766	110 ± 8.16	400 ± 20.41	100 ± 8.16
Z. bailii NCYC 1416^T	90 ± 14.14	300 ± 28.87	80 ± 11.55
Z. bailii 11	90 ± 8.16	350 ± 20.41	100 ± 0.00
Z. bailii NCYC 385	70 ± 9.57	300 ± 20.41	80 ± 11.54
Z. bailii NCYC 1520	90 ± 8.16	400 ± 20.41	100 ± 8.16
Z. bailii 20	90 ± 9.57	350 ± 28.87	100 ± 0.00
Z. bailii 80	90 ± 8.16	400 ± 0.00	100 ± 8.16
Z. kombuchaensis NRRL YB4810	70 ± 8.16	300 ± 0.00	120 ± 0.00
Z. kombuchaensis NRRL Y27163	70 ± 8.16	300 ± 20.41	120 ± 0.00
Z. kombuchaensis NRRL Y27162	70 ± 0.00	350 ± 28.87	120 ± 0.00
Z. kombuchaensis NRRL YB4811^T	70 ± 8.16	300 ± 0.00	120 ± 0.00
S. cerevisiae NCYC 957	50 ± 0.00	125 ± 10.20	50 ± 4.08
S. cerevisiae NCYC 1324	50 ± 0.00	75 ± 10.20	50 ± 0.00
S. cerevisiae NCYC 1119	50 ± 8.16	100 ± 0.00	50 ± 4.08
S. cerevisiae BY4741	50 ± 0.00	100 ± 10.20	50 ± 0.00

^{T} = Type strain

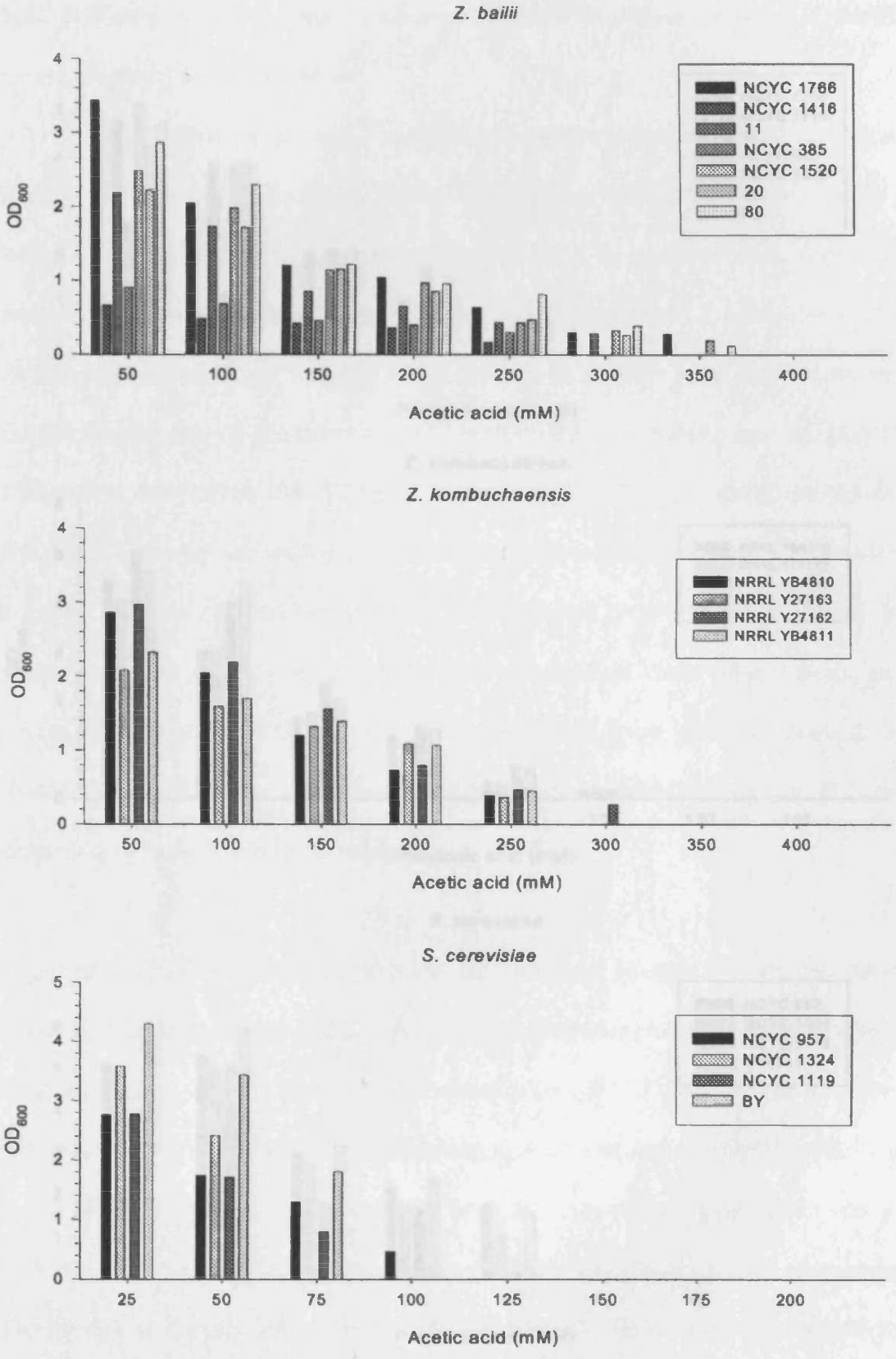
Values are mean MICs measured from at least two independent experiments (four replicates) in YPD pH 4.0 following 14 days incubation at 25°C, ± the standard error.

Figure 3.1 Effects of formic acid on *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* growth



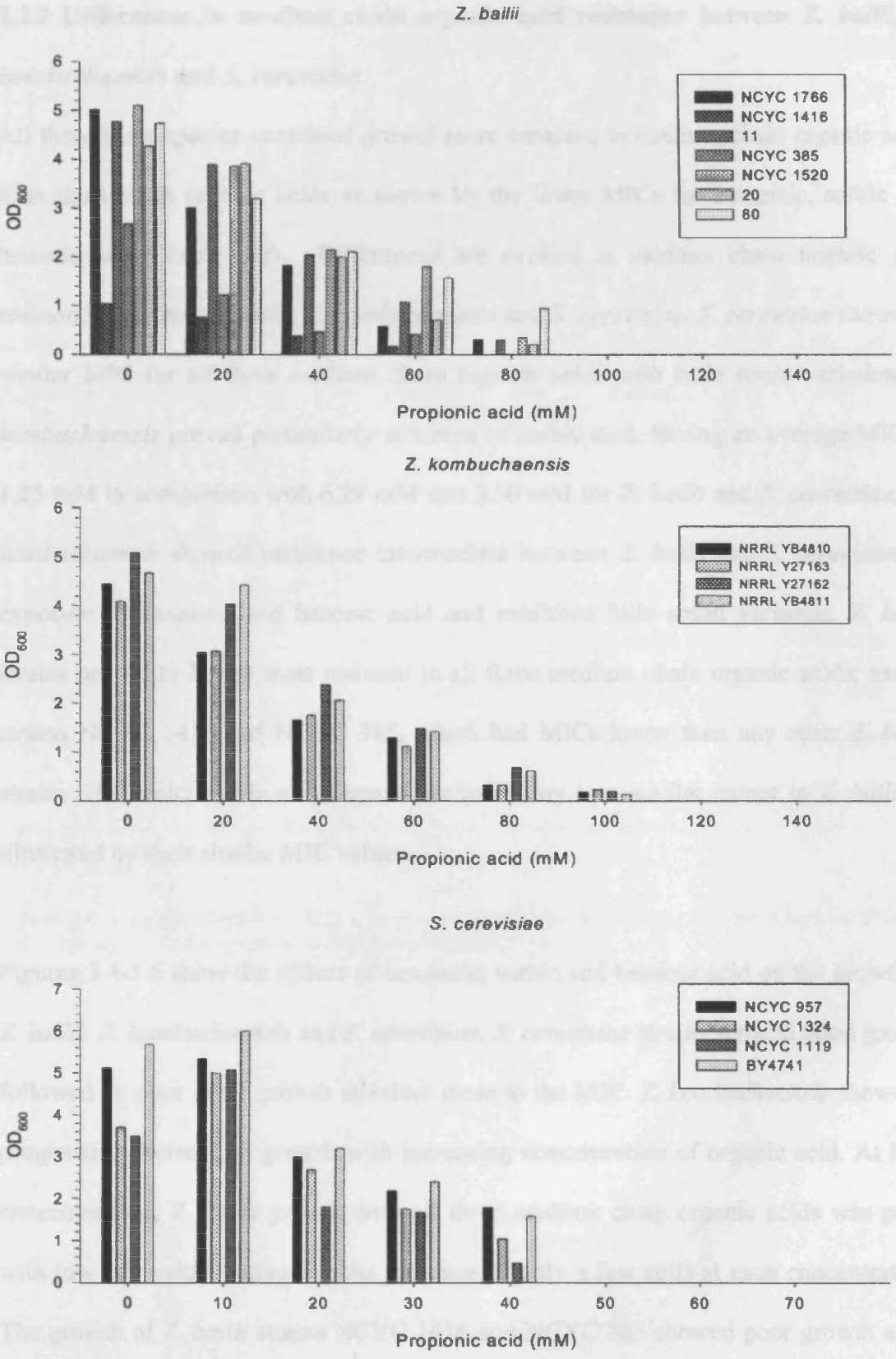
Results are representative of at least two independent experiments (four replicates) in YPD pH 4.0 following 14 days incubation at 25°C with standard errors <10%.

Figure 3.2 Effects of acetic acid on *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* growth



Results are representative of at least two independent experiments (four replicates) in YPD pH 4.0 following 14 days incubation at 25°C with standard errors <10%.

Figure 3.3 Effects of propionic acid on *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* growth



Results are representative of at least two independent experiments (four replicates) in YPD pH 4.0 following 14 days incubation at 25°C with standard errors <10%.

organic acids.

3.2.2 Differences in medium chain organic acid resistance between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

All three yeast species examined proved more sensitive to medium chain organic acids than short chain organic acids as shown by the lower MICs for hexanoic, sorbic and benzoic acid (Table 3.2). Differences are evident in medium chain organic acid resistance between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*. *S. cerevisiae* showed a similar MIC for all three medium chain organic acids with little strain variation. *Z. kombuchaensis* proved particularly sensitive to sorbic acid, having an average MIC of 1.25 mM in comparison with 6.29 mM and 3.50 mM for *Z. bailii* and *S. cerevisiae*. *Z. kombuchaensis* showed resistance intermediate between *Z. bailii* and *S. cerevisiae* on exposure to hexanoic and benzoic acid and exhibited little strain variation. *Z. bailii* strains proved to be the most resistant to all three medium chain organic acids; except strains NCYC 1416 and NCYC 385, which had MICs lower than any other *Z. bailii* strains. Hexanoic, sorbic and benzoic are inhibitory to a similar extent in *Z. bailii* as illustrated by their similar MIC values.

Figures 3.4-3.6 show the effects of hexanoic, sorbic and benzoic acid on the growth of *Z. bailli, Z. kombuchaensis* and *S. cerevisiae*. *S. cerevisiae* strains showed good growth followed by poor or no growth at values close to the MIC. *Z. kombuchaensis* showed a progressive decrease in growth with increasing concentration of organic acid. At high concentrations, *Z. bailii* growth with all three medium chain organic acids was poor, with low OD values indicating the presence of only a few cells at each concentration. The growth of *Z. bailii* strains NCYC 1416 and NCYC 385 showed poor growth at all concentrations as with short chain organic acids.

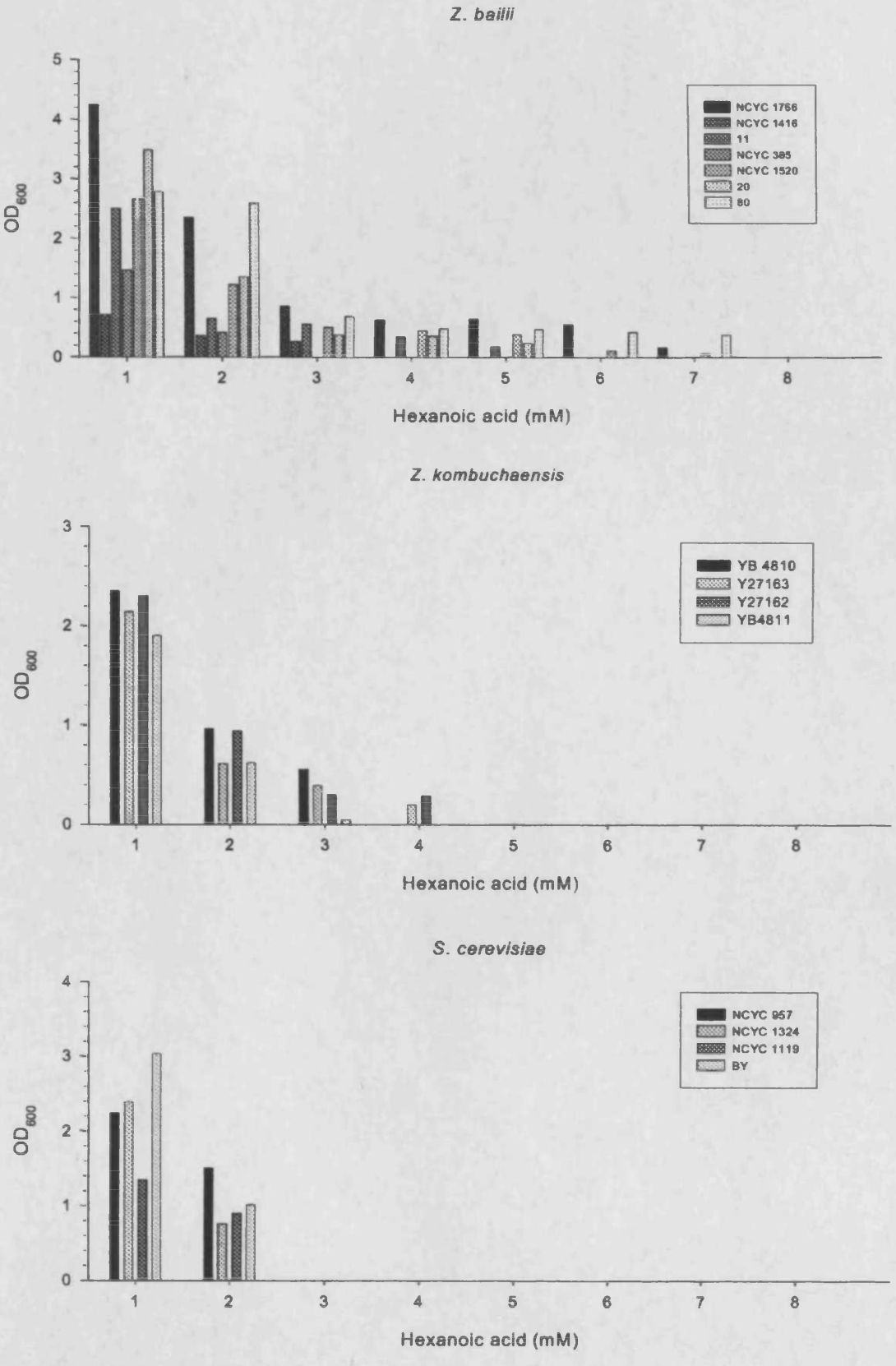
Table 3.2 Differences in medium chain organic acid MICs (mM) between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

	Hexanoic acid	Sorbic acid	Benzoic acid
Z. bailii NCYC 1766	8 ± 0.41	8 ± 0.71	9 ± 0.48
Z. bailii NCYC 1416^T	4 ± 0.58	4 ± 0.58	5 ± 1.22
Z. bailii 11	6 ± 0.00	5 ± 0.71	9 ± 1.08
Z. bailii NCYC 385	3 ± 0.41	3 ± 0.91	5 ± 0.71
Z. bailii NCYC 1520	8 ± 0.91	8 ± 0.33	9 ± 0.48
Z. bailii 20	6 ± 0.00	8 ± 0.58	8 ± 0.00
Z. bailii 80	8 ± 0.91	8 ± 0.33	9 ± 0.71
Z. kombuchaensis NRRL YB4810	4 ± 0.41	1.5 ± 0.13	6 ± 0.71
Z. kombuchaensis NRRL Y27163	5 ± 0.58	1.2 ± 0.14	6 ± 0.00
Z. kombuchaensis NRRL Y27162	5 ± 0.58	1.2 ± 0.82	6 ± 0.41
Z. kombuchaensis NRRL YB4811^T	4 ± 0.41	1.2 ± 0.18	6 ± 0.71
S. cerevisiae NCYC 957	3 ± 0.00	4 ± 0.58	3 ± 0.00
S. cerevisiae NCYC 1324	3 ± 0.00	3 ± 0.33	3 ± 0.41
S. cerevisiae NCYC 1119	3 ± 0.41	4 ± 0.58	3 ± 0.00
S. cerevisiae BY4741	3 ± 0.00	3 ± 0.00	4 ± 0.58

^{T} = Type strain

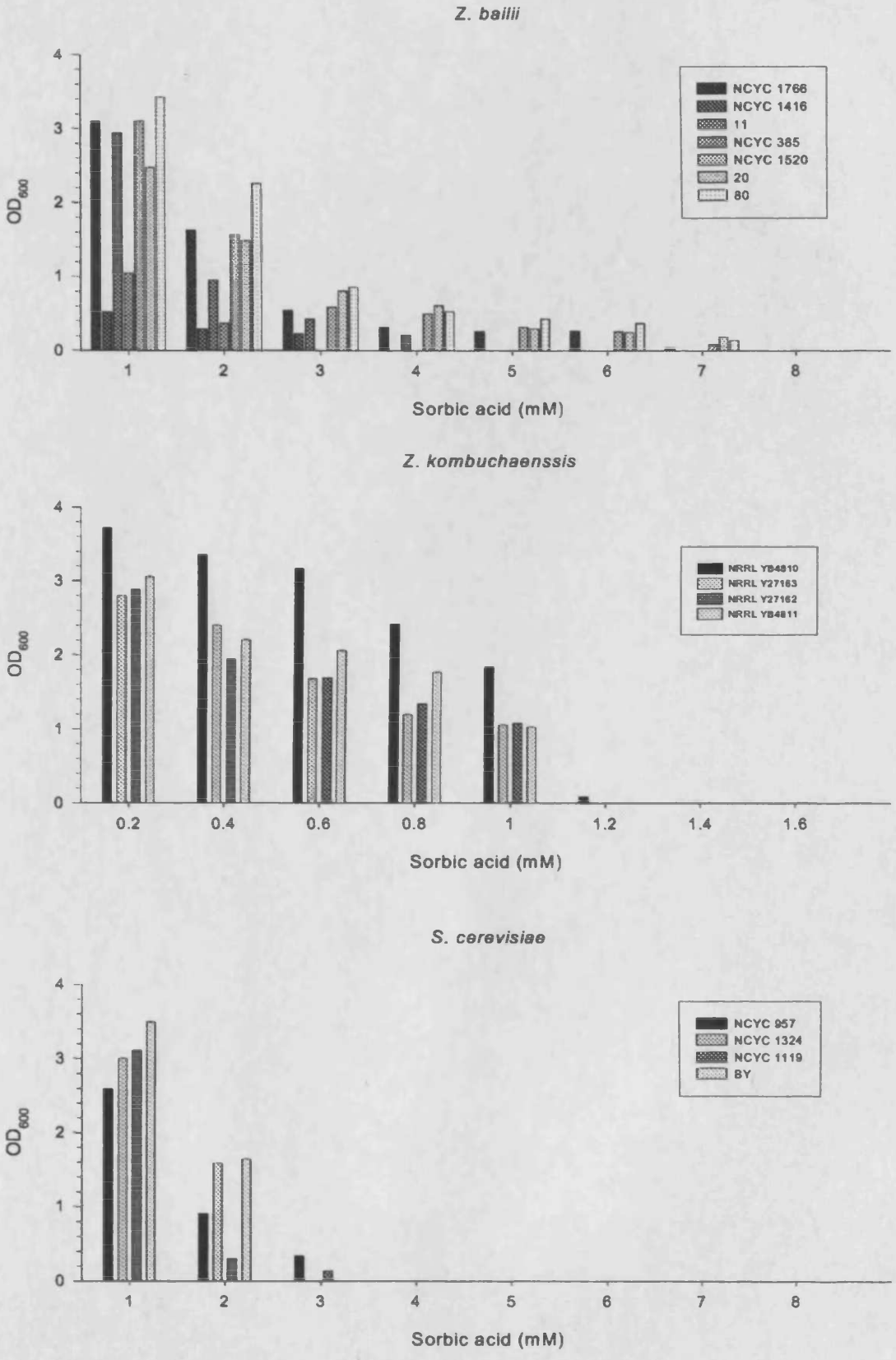
Values are mean MICs measured from at least two independent experiments (four replicates) in YPD pH 4.0 following 14 days incubation at 25°C, ± the standard error.

Figure 3.4 Effects of hexanoic acid on *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* growth



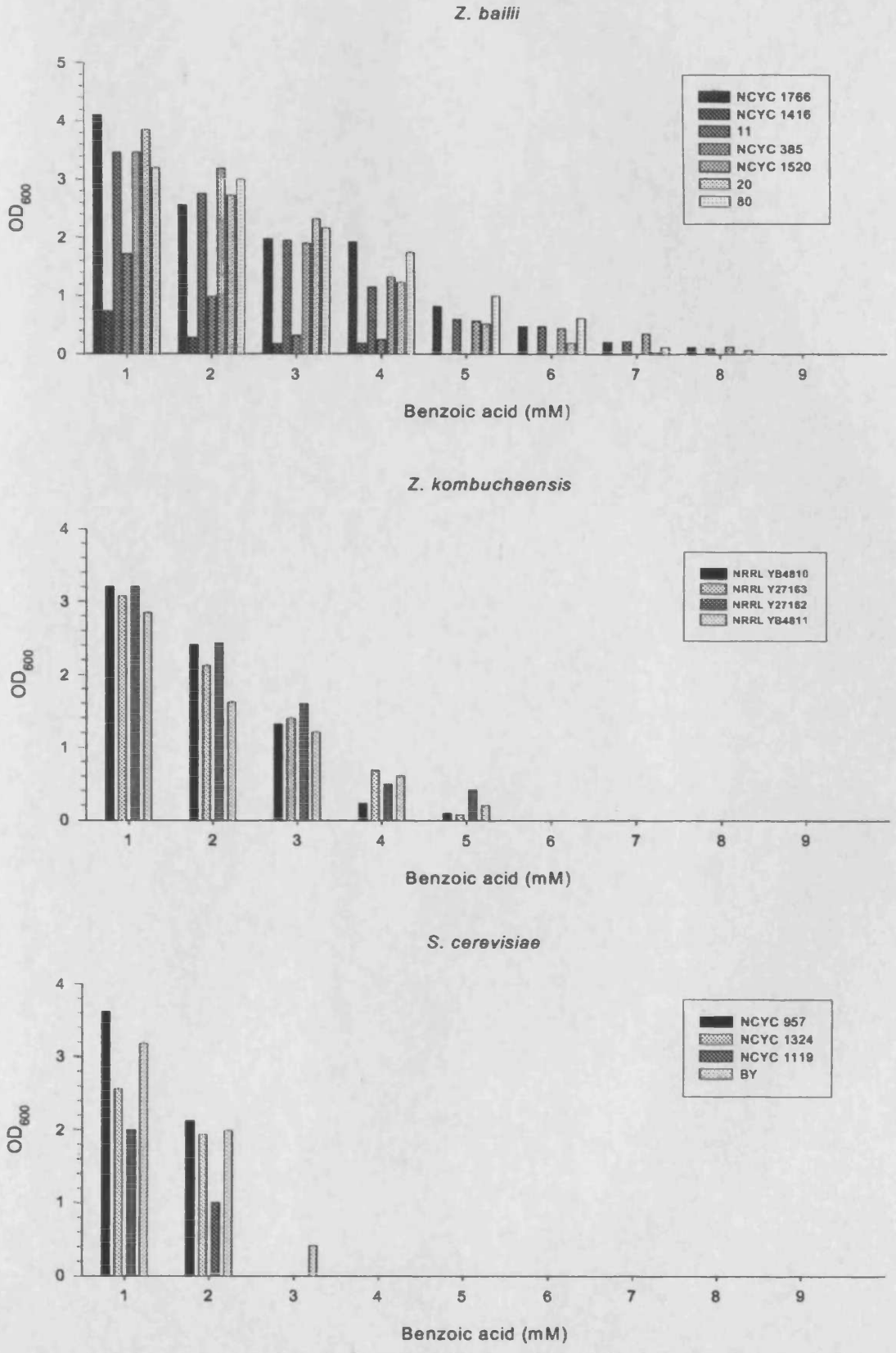
Results are representative of at least two independent experiments (four replicates) in YPD pH 4.0 following 14 days incubation at 25°C with standard errors <8%.

Figure 3.5 Effects of sorbic acid on *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* growth



Results are representative of at least two independent experiments (four replicates) in YPD pH 4.0 following 14 days incubation at 25°C with standard errors <8%.

Figure 3.6 Effects of benzoic acid on *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* growth



Results are representative of at least two independent experiments (four replicates) in YPD pH 4.0 following 14 days incubation at 25°C with standard errors <8%.

3.2.3 Differences in longer chain organic acid resistance between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

Table 3.3 shows the differences in longer chain organic acid resistance between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*. The ability of longer chain organic acids to inhibit yeast growth is greater than that of the short and medium chain organic acids examined. *S. cerevisiae* and *Z. kombuchaensis* showed similar levels of resistance to all three longer chain organic acids; with *S. cerevisiae* being slightly more resistant to nonanoic acid. *Z. bailii* strains were the most resistant to the longer chain organic acids examined. *Z. bailii* NCYC 1416 and NCYC 385 appear to be slightly more susceptible than the other strains as indicated by lower MICs to both octanoic and nonanoic acid. No strain variation in resistance to decanoic acid is evident for any strains examined.

The effect of longer chain organic acids on the growth of *Z. kombuchaensis* and *S. cerevisiae* is that of a progressive decrease in growth with increasing concentration of organic acid (Figures 3.7-3.9). *Z. bailii* strains show good growth at low concentrations of octanoic and nonanoic acid with poor growth at concentrations approaching the MIC, indicating the growth of only a few cells. In the presence of decanoic acid all *Z. bailii* strains show good growth at concentrations of 0.1 mM or below (except NCYC 1416 and NCYC 385) with poor growth at 0.15 mM.

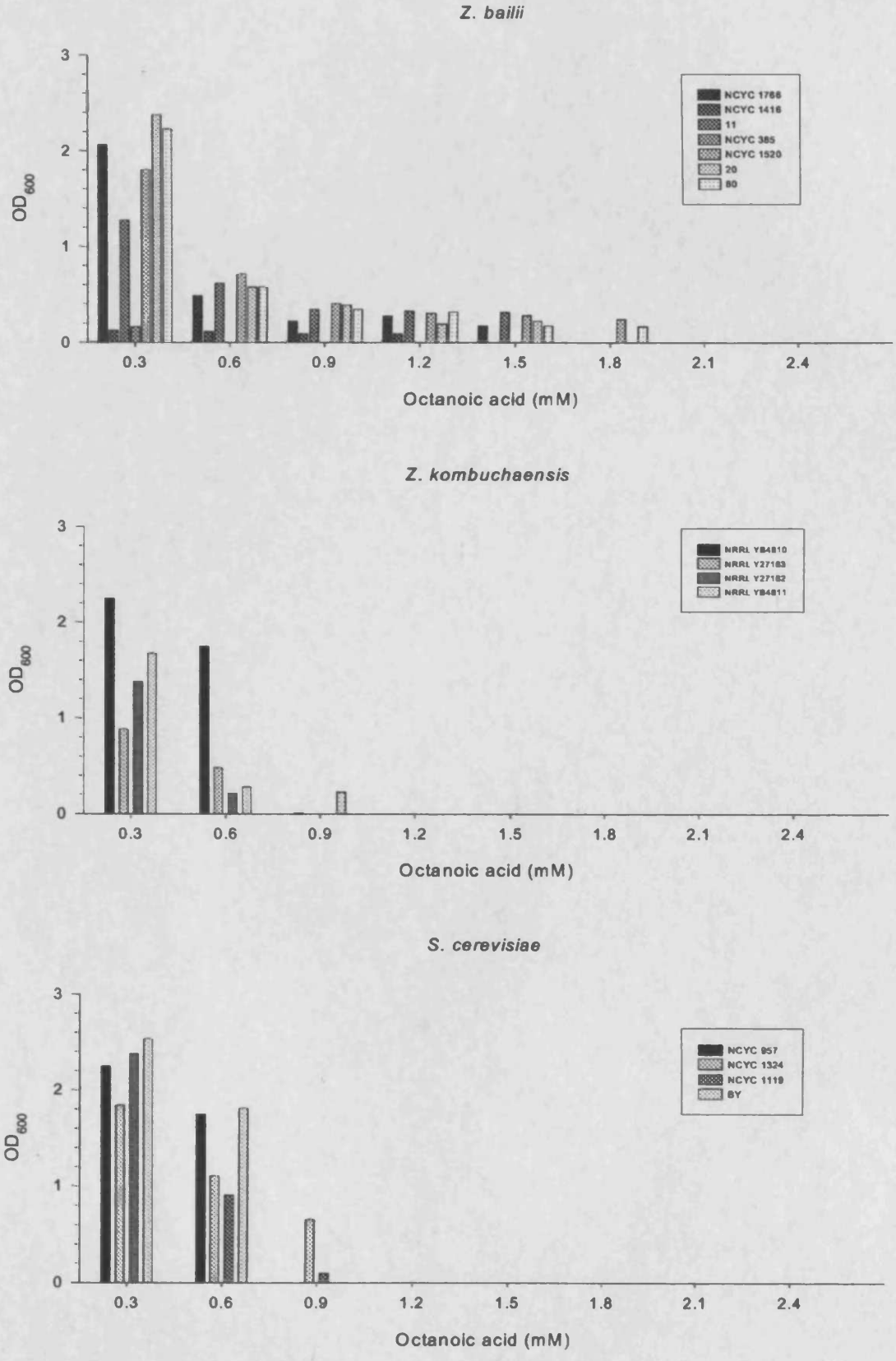
Table 3.3 Differences in longer chain organic acid MICs (mM) between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

	Octanoic acid	Nonanoic acid	Decanoic acid
Z. bailii NCYC 1766	2.1 ± 0.17	0.6 ± 0.00	0.20 ± 0.00
Z. bailii NCYC 1416^T	1.5 ± 0.21	0.5 ± 0.11	0.20 ± 0.04
Z. bailii 11	1.8 ± 0.00	0.6 ± 0.04	0.20 ± 0.00
Z. bailii NCYC 385	0.6 ± 0.00	0.4 ± 0.11	0.20 ± 0.04
Z. bailii NCYC 1520	2.1 ± 0.00	0.7 ± 0.09	0.20 ± 0.00
Z. bailii 20	1.8 ± 0.00	0.7 ± 0.04	0.20 ± 0.00
Z. bailii 80	2.1 ± 0.17	0.8 ± 0.00	0.20 ± 0.02
Z. kombuchaensis NRRL YB4810	1.2 ± 0.12	0.4 ± 0.00	0.15 ± 0.00
Z. kombuchaensis NRRL Y27163	0.9 ± 0.12	0.4 ± 0.04	0.15 ± 0.00
Z. kombuchaensis NRRL Y27162	0.9 ± 0.00	0.4 ± 0.04	0.15 ± 0.02
Z. kombuchaensis NRRL YB4811^T	1.2 ± 0.00	0.4 ± 0.00	0.15 ± 0.00
S. cerevisiae NCYC 957	0.9 ± 0.00	0.5 ± 0.00	0.15 ± 0.02
S. cerevisiae NCYC 1324	1.2 ± 0.00	0.5 ± 0.04	0.15 ± 0.00
S. cerevisiae NCYC 1119	1.2 ± 0.12	0.5 ± 0.04	0.15 ± 0.00
S. cerevisiae BY4741	0.9 ± 0.00	0.4 ± 0.00	0.15 ± 0.00

^{T} = Type strain

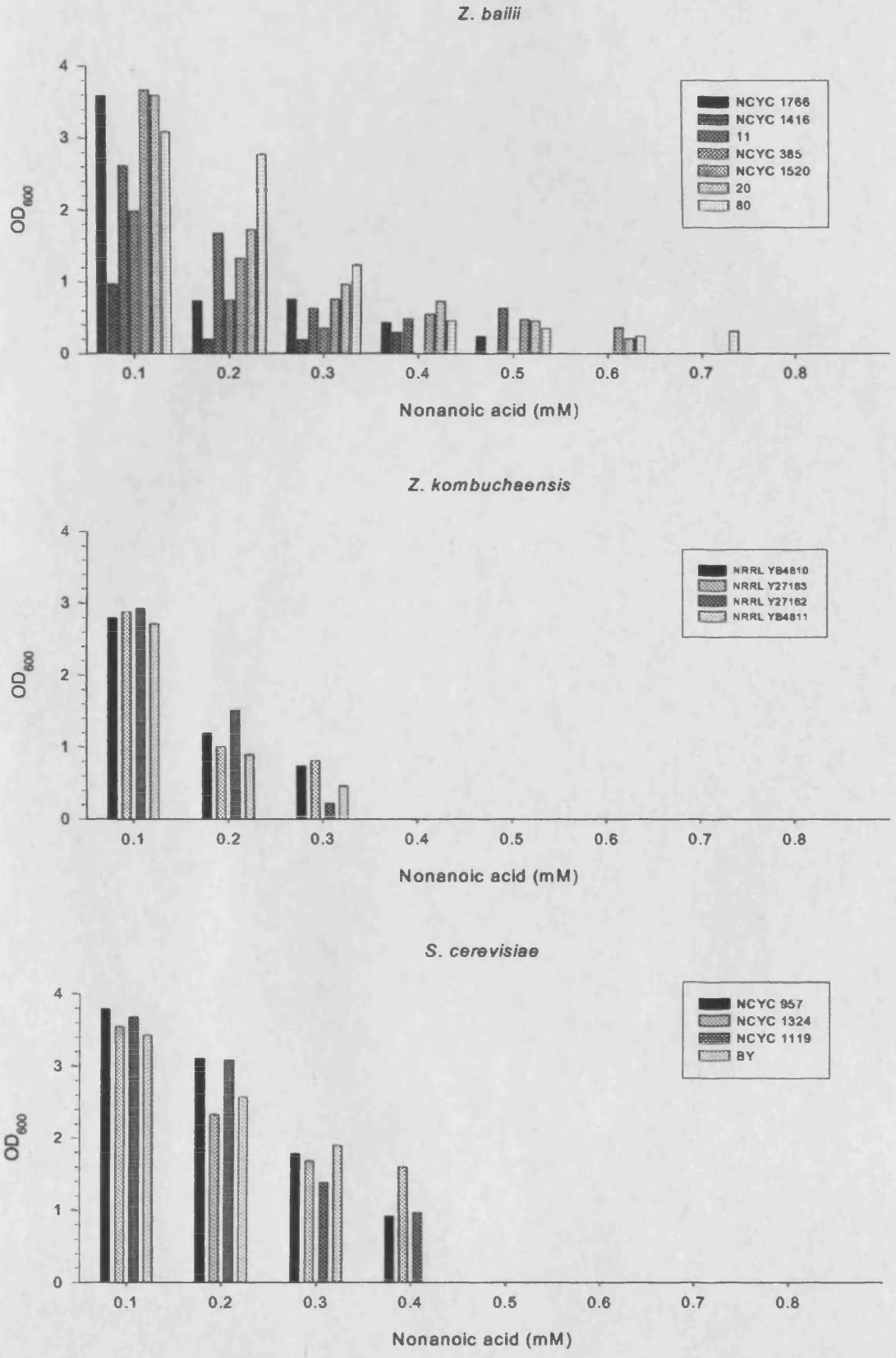
Values are mean MICs measured from at least two independent experiments (four replicates) in YPD pH 4.0 following 14 days incubation at 25°C, ± the standard error.

Figure 3.7 Effects of octanoic acid on *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* growth



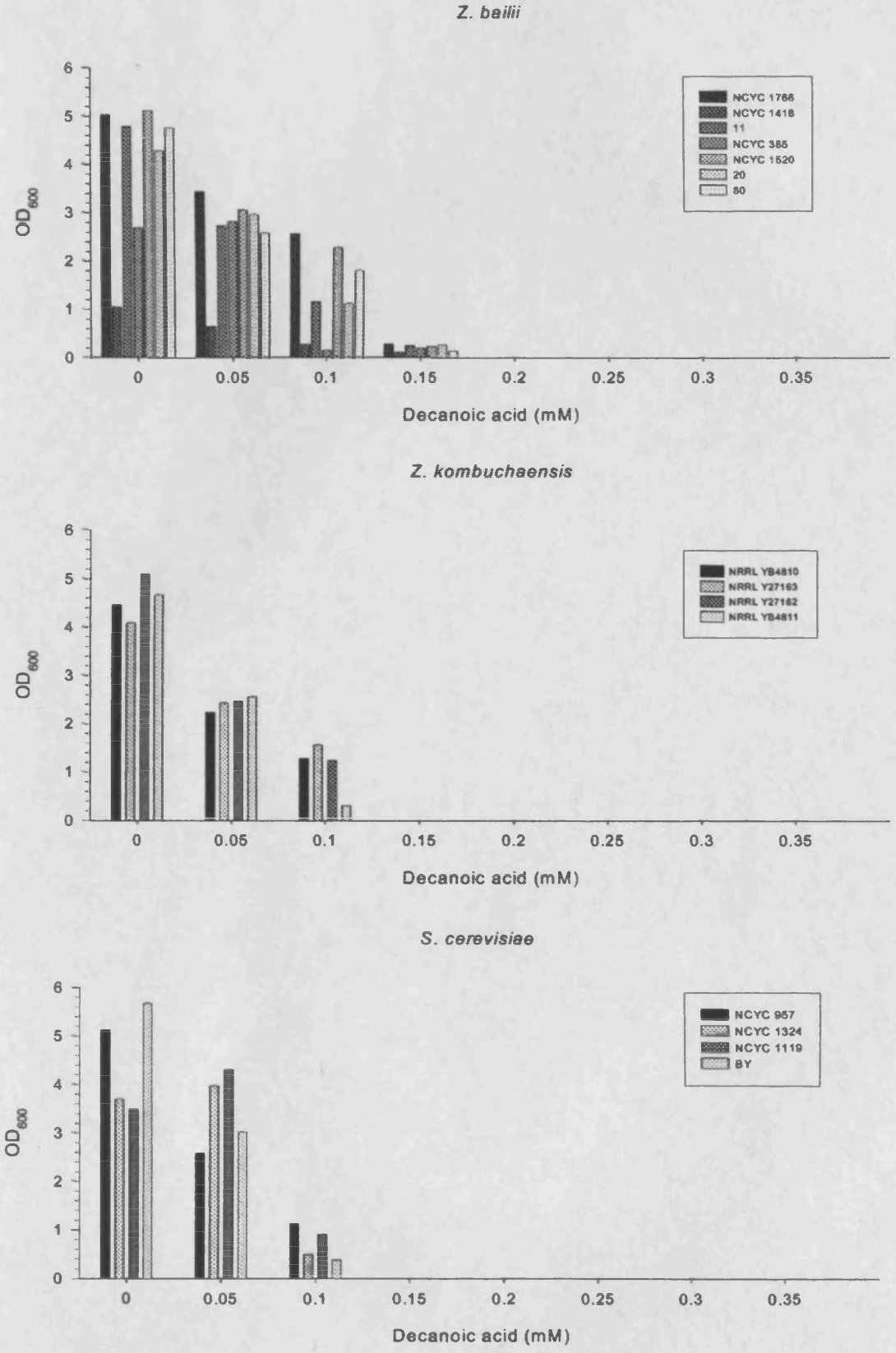
Results are representative of at least two independent experiments (four replicates) in YPD pH 4.0 following 14 days incubation at 25°C with standard errors <8%.

Figure 3.8 Effects of nonanoic acid on *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* growth



Results are representative of at least two independent experiments (four replicates) in YPD pH 4.0 following 14 days incubation at 25°C with standard errors <8%.

Figure 3.9 Effects of decanoic acid on *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* growth



Results are representative of at least two independent experiments (four replicates) in YPD pH 4.0 following 14 days incubation at 25°C with standard errors <8%.

3.3 Discussion

This study shows that *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* differ in resistance to short, medium and longer chain organic acids. As many of the organic acids used in this study are routinely used within the food industry as food preservatives, the results are of direct relevance. For example on the basis of MICs *Z. bailii* and *Z. kombuchaensis* appear to have a means of tolerating high concentrations of short chain organic acids, which is absent in *S. cerevisiae*. This means that foods preserved with short chain organic acids are at a greater risk of spoilage from *Z. bailii* and *Z. kombuchaensis* than *S. cerevisiae*. The resistance of the potential spoilage yeast *Z. kombuchaensis* to propionic acid suggests foods preserved with this, including bread and cheese, are at a greater risk of spoilage while foods preserved with sorbic acid would be at a low risk of spoilage. Collectively, the inhibitory data gathered from this study can be used to conduct risk assessments on foodstuffs and could influence the preservation strategies of certain foodstuffs.

The *Z. bailii* strains unlike *Z. kombuchaensis* and *S. cerevisiae* showed variation in resistance to all organic acids. The results suggest that two groups exist within *Z. bailii,* one which is resistant and another relatively sensitive to organic acids. The two strains of *Z. bailii* that show increased sensitivity to organic acids are NCYC 1416 and NCYC 385. The existence of a more organic acid sensitive group in *Z. bailii* means has not been reported previously (Warth, 1991; Steels *et al.,* 2000; 2002; Mollapour and Piper, 2001a, b). The relevance of a more organic acid sensitive group for *Z. bailii* to the food industry means that not all *Z. bailii* strains are capable of exceptional organic acid resistance. Indeed, by studying the differences between organic acid resistance and sensitive strains the basis of the *Z. bailii* resistance to organic acids may be revealed.

The fact that *Z. kombuchaensis* is very sensitive to sorbic acid (MIC 1.25 mM) but resistant to acetic acid (MIC 300 mM) adds to the theory that a number of mechanisms may be involved in yeast organic acid resistance (Brul and Coote, 1999; Piper *et al.*, 2001). If sorbic acid (pK_a 4.74) and acetic acid (pK_a 4.76) acted as 'classic weak-acid preservatives' both would have similar inhibitory concentrations, the fact they do not supports the findings of Stratford and Anslow (1998) that sorbic acid does not act as a 'classic weak-acid preservative'. The ability of an acid to act as a 'classic weak-acid preservative' can be extended to propionic acid and hexanoic acid both of which have a pK_a of 4.87 and should therefore also have a similar inhibitory capacity, but were shown experimentally to have differing MICs. Therefore, there are a number of organic acids, which should have similar inhibitory capacities on the basis of the 'classic weak acid preservative' theory, but in reality do not. The application of weak-acid preservatives on the basis of the weak-acid preservative theory and pK_a values within the food industry without prior information about MICs could therefore lead to a higher incidence of spoilage.

Analysis of the growth of all three yeast species in the presence of organic acids showed differences. *S. cerevisiae* strains exhibit good growth to the MIC, at which growth ends abruptly for all classes of organic acids. The general poor growth of *Z. bailii* and *Z. kombuchaensis* at extreme organic acid concentrations could be the result of highly-resistant "super" cells, as reported for *Z. bailii* in the presence of sorbic acid (Steels *et al.*, 2000). The presence of "super" cells for *Z. bailii* and *Z. kombuchaensis* could be one of the most significant factors contributing to the ability of these yeasts to cause spoilage and means that spoilage could occur from only a few cells. The existence of "super" cells for *Zygosaccharomyces* yeasts warrants further investigation.

4. Effects of growth conditions on yeast organic acid resistance

4.1 Introduction

Numerous factors are known to influence yeast growth. The most common factors affecting yeast growth include temperature, pH, water activity and nutrient availability (section 1.6). The factors affecting growth of many yeasts including *S. cerevisiae* are well documented (Barnett *et al.,* 2000). However, the influence of growth conditions on the food spoilage yeast *Z. bailii* remains largely unknown. The only concern of many researchers towards *Z. bailii* until relatively recently has been to kill it as opposed to studying its uniqueness (Dickinson, 2000). In the previous section, differences in organic acid resistance were shown between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*. In a bid to further understand the interaction between growth conditions and organic acid resistance I examined the influence of YPD composition, glucose concentration, carbon source, nitrogen source, water activity and vitamin additions on MICs to short (acetic and propionic), medium (sorbic and benzoic) and longer (nonanoic) chain organic acids.

The experiments reported in this section were conducted at pH 4.0 in YPD except for the nitrogen source experiments, which were conducted in minimal medium. Strains of *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* were selected on the basis of results obtained in section 3. Two strains of *Z. bailii* were selected: one that is resistant to organic acids and the other more sensitive. Two strains of *Z. kombuchaensis* were also selected, as this potential spoilage yeast has only recently been identified with little being known about its spoilage capabilities (Kurtzman *et al.,* 2001; Steels *et al.* 2002). Only one strain of *S. cerevisiae* was included in this section as the strains examined in section 3 exhibited little strain variation. The strain selected for *S. cerevisiae* has been previously used in studies on preservative resistance (Stratford and Anslow, 1996; 1998).

4.2 Results

4.2.1 Effects of YPD composition on yeast organic acid resistance

The effects of changing the composition of YPD upon yeast organic acid resistance were analysed. Table 4.1 shows the effects of omitting one of the components from YPD on yeast organic acid resistance. Alterations to the YPD composition increased the sensitivity of all three yeast species to the organic acids examined. The omission of yeast extract had the greatest effect, while the omission of peptone had the smallest effect, on organic acid resistance, for all three yeast species. Table 4.1 shows that both strains of *Z. bailii* and *Z. kombuchaensis* produced some results which gave no growth over the concentration range examined, when grown in PD. *S. cerevisiae,* however, showed growth under all conditions examined for all three classes of organic acids. The longer chain organic acid exhibited the greatest inhibitory action to all three species; with acetic acid being the least inhibitory as shown by the high MICs. *Z. bailii* NCYC 1766 was the most resistant yeast to all classes of organic acids except propionic acid in YD, to which the two *Z. kombuchaensis* strains were the most resistant.

4.2.2 Effects of glucose concentration on yeast organic acid resistance

All three yeast species showed growth for all organic acids examined in YPD with 0.5, 5 and 10% (w/v) glucose (Table 4.2). The results show that the MICs obtained for each yeast under the three different concentrations of glucose remain relatively constant e.g. the MIC of acetic acid to *S. cerevisiae* in 0.5, 5 and 10% glucose is 90, 95 and 90 mM, respectively. The pattern of *Z. bailii* NCYC 1416 being the more sensitive of the two *Z. bailii* strains to organic acids remained constant. *S. cerevisiae* still proved the most sensitive to organic acids with *Z. kombuchaensis* giving results intermediate between *Z. bailii* and *S. cerevisiae*; except for sorbic acid where it proved the most sensitive and propionic acid where it was the most resistant.

Table 4.1 Effects of complex medium composition on organic acid MICs (mM) in *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

	Zb 1766	*Zb* 1416	*Zk* Y27162	*Zk* YB4811	*Sc* 957
YPD					
Acetic acid	400 ± 20.41	300 ± 28.87	350 ± 28.87	300 ± 20.41	125 ± 10.20
Propionic acid	100 ± 8.16	80 ± 11.55	120 ± 0.00	120 ± 0.00	50 ± 4.08
Sorbic acid	8 ± 0.71	4 ± 0.58	1.2 ± 0.82	1.2 ± 0.18	4 ± 0.58
Benzoic acid	9 ± 0.48	5 ± 1.22	6 ± 0.41	6 ± 0.71	3 ± 0.00
Nonanoic acid	0.6 ± 0.00	0.5 ± 0.11	0.4 ± 0.04	0.4 ± 0.00	0.5 ± 0.00
YP					
Acetic acid	250 ± 28.87	200 ± 28.87	175 ± 20.41	175 ± 28.87	65 ± 6.64
Propionic acid	60 ± 4.08	30 ± 8.16	NG	NG	10 ± 1.21
Sorbic acid	7.0 ± 0.00	1.0 ± 0.04	0.9 ± 0.14	0.6 ± 0.18	1.5 ± 0.14
Benzoic acid	10 ± 1.22	4.5 ± 0.48	3.5 ± 0.71	2.0 ± 0.71	1.5 ± 0.18
Nonanoic acid	0.7 ± 0.21	0.4 ± 0.17	0.2 ± 0.17	0.2 ± 0.21	0.3 ± 0.12
YD					
Acetic acid	300 ± 20.41	275 ± 20.41	225 ± 28.87	225 ± 28.87	100 ± 10.41
Propionic acid	80 ± 0.00	65 ± 0.00	85 ± 8.16	95 ± 11.54	55 ± 4.08
Sorbic acid	6.5 ± 0.91	4.5 ± 0.91	1.2 ± 0.13	1.2 ± 0.13	3.5 ± 0.18
Benzoic acid	9.0 ± 1.08	4.5 ± 1.22	4.5 ± 1.22	3.5 ± 0.71	3.5 ± 0.58
Nonanoic acid	0.7 ± 0.11	0.5 ± 0.09	0.4 ± 0.04	0.4 ± 0.04	0.5 ± 0.11
PD					
Acetic acid	100 ± 10.41	NG	200 ± 20.41	150 ± 28.87	80 ± 10.20
Propionic acid	NG	NG	NG	NG	30 ± 1.21
Sorbic acid	4.0 ± 0.91	NG	NG	0.6 ± 0.18	0.5 ± 0.33
Benzoic acid	2.0 ± 0.58	NG	NG	1.0 ± 0.33	2.0 ± 0.33
Nonanoic acid	0.6 ± 0.09	0.3 ± 0.04	0.3 ± 0.02	0.3 ± 0.04	0.4 ± 0.09

NG = no growth

Values are mean MICs measured from at least two independent experiments (four replicates) following 14 days incubation at 25°C, ± the standard error.

Table 4.2 Effects of glucose concentration on organic acid MICs (mM) in *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

	Zb 1766	*Zb* 1416	*Zk* Y27162	*Zk* YB4811	*Sc* 957
YPD 2% (w/v) Glucose					
Acetic acid	400 ± 20.41	300 ± 28.87	350 ± 28.87	300 ± 20.41	125 ± 10.20
Propionic acid	100 ± 8.16	80 ± 11.55	120 ± 0.00	120 ± 0.00	50 ± 4.08
Sorbic acid	8 ± 0.71	4 ± 0.58	1.2 ± 0.82	1.2 ± 0.18	4 ± 0.58
Benzoic acid	9 ± 0.48	5 ± 1.22	6 ± 0.41	6 ± 0.71	3 ± 0.00
Nonanoic acid	0.6 ± 0.00	0.5 ± 0.11	0.4 ± 0.04	0.4 ± 0.00	0.5 ± 0.00
YPD 0.5% (w/v) Glucose					
Acetic acid	250 ± 20.41	200 ± 10.20	200 ± 10.20	200 ± 20.41	90 ± 10.20
Propionic acid	90 ± 11.55	60 ± 8.16	80 ± 4.08	80 ± 4.08	55 ± 4.08
Sorbic acid	7.0 ± 0.71	2.0 ± 0.00	0.9 ± 0.00	0.6 ± 0.13	3.5 ± 0.00
Benzoic acid	9.0 ± 0.91	3.0 ± 0.58	3.0 ± 0.71	3.0 ± 0.00	3.0 ± 0.00
Nonanoic acid	0.7 ± 0.09	0.4 ± 0.04	0.3 ± 0.04	0.3 ± 0.04	0.5 ± 0.04
YPD 5% (w/v) Glucose					
Acetic acid	350 ± 20.41	175 ± 20.41	250 ± 10.20	250 ± 10.20	95 ± 10.20
Propionic acid	100 ± 8.16	70 ± 8.16	120 ± 0.00	120 ± 4.08	55 ± 4.08
Sorbic acid	7.0 ± 0.91	4.0 ± 1.22	0.9 ± 0.12	0.9 ± 0.13	3.5 ± 0.18
Benzoic acid	9.5 ± 1.08	3.0 ± 1.22	4.0 ± 0.00	4.0 ± 0.58	3.5 ± 0.00
Nonanoic acid	0.8 ± 0.11	0.4 ± 0.04	0.4 ± 0.09	0.4 ± 0.04	0.5 ± 0.09
YPD 10% (w/v) Glucose					
Acetic acid	250 ± 20.41	200 ± 28.87	250 ± 20.41	200 ± 20.41	90 ± 10.20
Propionic acid	90 ± 0.00	70 ± 8.16	100 ± 0.00	100 ± 0.00	55 ± 4.08
Sorbic acid	6.0 ± 1.22	4.0 ± 1.22	1.2 ± 0.12	1.2 ± 0.12	4.0 ± 0.91
Benzoic acid	7.0 ± 0.00	3.5 ± 0.71	4.5 ± 0.58	4.5 ± 0.33	3.0 ± 0.33
Nonanoic acid	0.8 ± 0.09	0.4 ± 0.04	0.4 ± 0.04	0.4 ± 0.00	0.4 ± 0.00

Values are mean MICs measured from at least two independent experiments (four replicates) following 14 days incubation at 25°C, ± the standard error.

4.2.3 Effects of carbon source on yeast organic acid resistance

The effects of changing carbon source in complex medium upon yeast organic acid resistance were also analysed. Glucose was replaced with the similarly fermentable carbon sources fructose, sucrose and mannose plus the non-fermentable carbon sources ethanol and glycerol (at 2% w/v except glycerol, which was at 3% w/v). Glucose and glycerol (both at 1%) in the growth media were also used. Table 4.3 shows that the MICs obtained for the fermentable carbon sources are higher than those obtained for the two non-fermentable carbon sources, with the 1% glucose and 1% glycerol giving MICs intermediate between fermentable and non-fermentable carbon sources. The results show that the two *Z. bailii* strains had their highest MICs for all classes of organic acids examined in YPSucrose (except standard YPD). *Z. kombuchaensis* and *S. cerevisiae* exhibited greatest resistance to all organic acids in YPFructose media. In YPGlycerol media *Z. bailii* NCYC 1416, *S. cerevisiae* NCYC 957 and the *Z. kombuchaensis* strains showed no growth over the range of organic acid concentrations examined. *Z. bailii* NCYC 1766 gave MICs in the presence of YPglycerol which were considerably lower than those obtained using fermentable carbon sources (Table 4.3). For YPEthanol, some strains failed to grow at all concentrations of organic acids tested.

4.2.4 Effects of nitrogen source on yeast organic acid resistance

The effects of nitrogen source on *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* organic acid resistance were studied in minimal medium. Minimal medium normally contains ammonium sulphate as the nitrogen source at 0.5% (w/v), in these experiments the ammonium sulphate was compared to media containing two non-polar amino acids proline and leucine at the same 0.5% (w/v) concentration. *S. cerevisiae* exhibited similar MICs to all three classes of organic acids irrespective of the nitrogen source (Table 4.4). The two *Z. bailii* strains examined NCYC 1766 and NCYC 1416 also exhibited little

Table 4.3 Effects of carbon source on organic acid MICs (mM) in *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

	Zb 1766	*Zb* 1416	*Zk* Y27162	*Zk* YB4811	*Sc* 957
YPD					
Acetic acid	400 ± 20.41	300 ± 28.87	350 ± 28.87	300 ± 20.41	125 ± 10.20
Propionic acid	100 ± 8.16	80 ± 11.55	120 ± 0.00	120 ± 0.00	50 ± 4.08
Sorbic acid	8 ± 0.71	4 ± 0.58	1.2 ± 0.82	1.2 ± 0.18	4 ± 0.58
Benzoic acid	9 ± 0.48	5 ± 1.22	6 ± 0.41	6 ± 0.71	3 ± 0.00
Nonanoic acid	0.6 ± 0.00	0.5 ± 0.11	0.4 ± 0.04	0.4 ± 0.00	0.5 ± 0.00
YPFructose					
Acetic acid	325 ± 28.87	225 ± 20.41	325 ± 28.87	325 ± 28.87	110 ± 15.55
Propionic acid	100 ± 0.00	70 ± 8.16	110 ± 8.16	110 ± 4.08	60 ± 8.16
Sorbic acid	8.0 ± 0.71	4.5 ± 0.58	1.8 ± 0.18	1.8 ± 0.18	3.5 ± 0.33
Benzoic acid	9.0 ± 0.58	4.0 ± 1.22	6.5 ± 1.22	5.5 ± 0.91	4.0 ± 0.00
Nonanoic acid	0.8 ± 0.09	0.6 ± 0.04	0.6 ± 0.00	0.6 ± 0.04	0.5 ± 0.00
YPSucrose					
Acetic acid	375 ± 20.41	280 ± 28.87	250 ± 20.41	275 ± 10.20	95 ± 10.20
Propionic acid	100 ± 8.16	85 ± 8.16	105 ± 4.08	105 ± 0.00	55 ± 4.08
Sorbic acid	8.5 ± 1.22	5.0 ± 0.91	1.2 ± 0.12	1.2 ± 0.13	3.5 ± 0.18
Benzoic acid	10.0 ± 1.22	6.0 ± 0.58	6.0 ± 0.33	5.0 ± 0.58	3.5 ± 0.33
Nonanoic acid	0.8 ± 0.11	0.5 ± 0.09	0.4 ± 0.09	0.5 ± 0.04	0.5 ± 0.09
YPMannose					
Acetic acid	350 ± 20.41	250 ± 28.87	250 ± 20.41	250 ± 20.41	95 ± 10.20
Propionic acid	95 ± 4.08	80 ± 8.16	105 ± 0.00	105 ± 8.16	60 ± 0.00
Sorbic acid	6.5 ± 0.71	4.5 ± 0.58	1.4 ± 0.18	1.4 ± 0.18	2.5 ± 0.58
Benzoic acid	7.5 ± 0.00	3.5 ± 0.58	5.0 ± 0.00	4.5 ± 0.71	3.5 ± 0.33
Nonanoic acid	0.8 ± 0.11	0.6 ± 0.09	0.6 ± 0.04	0.6 ± 0.00	0.4 ± 0.04

Values are mean MICs measured from at least two independent experiments (four replicates) following 14 days incubation at 25°C, ± the standard error.

Table 4.3 cont Effects of carbon source on organic acid MICs (mM) in *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

	***Zb* 1766**	***Zb* 1416**	***Zk* Y27162**	***Zk* YB4811**	***Sc* 957**
YPEthanol					
Acetic acid	225 ± 20.41	100 ± 20.41	75 ± 10.20	75 ± 10.20	NG
Propionic acid	75 ± 4.08	NG	NG	NG	15 ± 1.21
Sorbic acid	5.0 ± 0.48	2.5 ± 0.33	NG	NG	1.0 ± 0.09
Benzoic acid	5.0 ± 0.58	1.0 ± 0.11	NG	0.5 ± 0.04	NG
Nonanoic acid	0.7 ± 0.04	0.3 ±	NG	NG	0.2 ± 0.02
YPGlycerol					
Acetic acid	150 ± 10.20	NG	NG	NG	NG
Propionic acid	60 ± 0.00	NG	NG	NG	NG
Sorbic acid	5.0 ± 1.22	NG	NG	NG	NG
Benzoic acid	6.0 ± 0.71	NG	NG	NG	NG
Nonanoic acid	0.7 ± 0.11	NG	NG	NG	NG
YP1% Glucose + 1% Glycerol					
Acetic acid	200 ± 20.41	175 ± 20.41	225 ± 10.20	225 ± 10.20	105 ± 10.20
Propionic acid	75 ± 4.08	65 ± 8.16	75 ± 4.08	75 ± 0.00	45 ± 4.08
Sorbic acid	5.5 ± 1.22	3.5 ± 0.71	0.9 ± 0.09	0.9 ± 0.06	3.5 ± 0.00
Benzoic acid	6.5 ± 0.00	4.5 ± 0.58	4.5 ± 0.33	4.5 ± 0.33	3.5 ± 0.58
Nonanoic acid	0.7 ± 0.09	0.6 ± 0.09	0.5 ± 0.00	0.5 ± 0.04	0.6 ± 0.09

NG = no growth.

Values are mean MICs measured from at least two independent experiments (four replicates) following 14 days incubation at 25°C, ± the standard error.

Table 4.4 Effects of nitrogen source on organic acid MICs (mM) in *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

	Zb 1766	*Zb* 1416	*Zk* Y27162	*Zk* YB4811	*Sc* 957
YPD					
Acetic acid	400 ± 20.41	300 ± 28.87	350 ± 28.87	300 ± 20.41	125 ± 10.20
Propionic acid	100 ± 8.16	80 ± 11.55	120 ± 0.00	120 ± 0.00	50 ± 4.08
Sorbic acid	8 ± 0.71	4 ± 0.58	1.2 ± 0.82	1.2 ± 0.18	4 ± 0.58
Benzoic acid	9 ± 0.48	5 ± 1.22	6 ± 0.41	6 ± 0.71	3 ± 0.00
Nonanoic acid	0.6 ± 0.00	0.5 ± 0.11	0.4 ± 0.04	0.4 ± 0.00	0.5 ± 0.00
MM Ammonium sulphate					
Acetic acid	250 ± 20.41	250 ± 28.87	150 ± 10.20	150 ± 10.20	80 ± 5.97
Propionic acid	60 ± 0.00	40 ± 4.08	60 ± 4.08	70 ± 0.00	50 ± 4.08
Sorbic acid	5.0 ± 0.71	3.0 ± 0.58	0.6 ± 0.04	0.3 ± 0.04	2.0 ± 0.09
Benzoic acid	5.0 ± 0.58	3.0 ± 0.91	1.0 ± 0.12	1.0 ± 0.12	2.5 ± 0.00
Nonanoic acid	0.5 ± 0.09	0.4 ± 0.04	0.1 ± 0.02	0.2 ± 0.04	0.4 ± 0.09
MM Proline					
Acetic acid	250 ± 20.41	200 ± 28.87	250 ± 28.87	250 ± 20.41	80 ± 10.20
Propionic acid	70 ± 4.08	50 ± 8.16	60 ± 8.16	70 ± 4.08	50 ± 0.00
Sorbic acid	6.0 ± 0.91	3.0 ± 0.58	0.6 ± 0.09	0.6 ± 0.09	2.5 ± 0.33
Benzoic acid	4.0 ± 0.71	3.0 ± 0.71	3.0 ± 0.00	3.0 ± 0.58	2.5 ± 0.33
Nonanoic acid	0.5 ± 0.00	0.4 ± 0.09	0.2 ± 0.00	0.3 ± 0.00	0.4 ± 0.04
MM Leucine					
Acetic acid	250 ± 20.41	250 ± 20.41	300 ± 20.41	300 ± 20.41	80 ± 0.00
Propionic acid	60 ± 0.00	50 ± 4.08	70 ± 0.00	70 ± 0.00	50 ± 4.08
Sorbic acid	6.0 ± 1.22	4.0 ± 0.58	0.9 ± 0.00	0.6 ± 0.12	2.5 ± 0.33
Benzoic acid	6.0 ± 1.22	4.0 ± 0.71	3.0 ± 0.58	3.0 ± 0.00	3.0 ± 0.58
Nonanoic acid	0.6 ± 0.04	0.4 ± 0.09	0.3 ± 0.02	0.3 ± 0.04	0.4 ± 0.00

Values are mean MICs measured from at least two independent experiments (four replicates) following 14 days incubation at 25°C, ± the standard error.

variation in all organic acid MICs in media containing different nitrogen sources. The MIC for acetic acid against the two *Z. bailii* strains were around the 250 mM level in all three nitrogen sources; while for the remaining organic acids *Z. bailii* strain NCYC 1766 was the most resistant as indicated by the higher MICs. The results show that both strains of *Z. kombuchaensis* were the most sensitive to changes in nitrogen source. The lowest MICs for *Z. kombucahensis* to all organic acids were generally in minimal media containing ammonium sulphate, while the highest MICs were obtained in the presence of leucine. The MIC for acetic acid was double in leucine minimal medium than it was for ammonium sulphate, the propionic acid MICs, however, remained similar at around 60-70 mM for all three nitrogen sources for both *Z. kombuchaensis* strains. The pattern of longer chain organic acids being the most inhibitory remained constant in minimal medium as in complex medium.

4.2.5 Effects of water activity on yeast organic acid resistance

The altering of medium water activity in yeast organic acid resistance was explored by the addition of glycerol and the two non-metabolisable sugars sorbitol and mannitol to YPD pH 4.0 at a concentration 10% (w/v). Table 4.5 shows that the MICs obtained for all three yeast species in the presence of glycerol were similar to those obtained for mannitol and sorbitol additions. The biggest difference in MIC between growth conditions was obtained for *Z. kombuchaensis* on exposure to sorbic acid. The MIC for both strains of *Z. kombuchaensis* to sorbic acid was 1.8 mM in YPD containing sorbitol and mannitol in comparison to 2.4 mM with glycerol supplementation. The yeasts collectively were the most resistant to acetic acid and the most sensitive to nonanoic acid as indicated by MICs.

Table 4.5 Effects of water activity on organic acid MICs (mM) in *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

	Zb 1766	*Zb* 1416	*Zk* Y27162	*Zk* YB4811	*Sc* 957
YPD					
Acetic acid	400 ± 20.41	300 ± 28.87	350 ± 28.87	300 ± 20.41	125 ± 10.20
Propionic acid	100 ± 8.16	80 ± 11.55	120 ± 0.00	120 ± 0.00	50 ± 4.08
Sorbic acid	8 ± 0.71	4 ± 0.58	1.2 ± 0.82	1.2 ± 0.18	4 ± 0.58
Benzoic acid	9 ± 0.48	5 ± 1.22	6 ± 0.41	6 ± 0.71	3 ± 0.00
Nonanoic acid	0.6 ± 0.00	0.5 ± 0.11	0.4 ± 0.04	0.4 ± 0.00	0.5 ± 0.00
YPD + 10% Glycerol					
Acetic acid	350 ± 28.87	250 ± 28.87	250 ± 20.41	300 ± 28.87	110 ± 10.20
Propionic acid	110 ± 0.00	100 ± 8.16	110 ± 4.08	120 ± 0.00	80 ± 4.08
Sorbic acid	7.0 ± 0.33	6.0 ± 0.58	2.4 ± 0.24	2.4 ± 0.27	3.5 ± 0.33
Benzoic acid	10.0 ± 1.22	6.0 ± 1.22	8.0 ± 0.91	8.0 ± 0.58	4.0 ± 0.00
Nonanoic acid	0.4 ± 0.04	0.3 ± 0.09	0.3 ± 0.04	0.3 ± 0.00	0.3 ± 0.00
YPD + 10% Sorbitol					
Acetic acid	350 ± 20.41	300 ± 20.41	250 ± 15.55	300 ± 20.41	110 ± 10.20
Propionic acid	120 ± 0.00	100 ± 8.16	120 ± 0.00	120 ± 0.00	80 ± 4.08
Sorbic acid	7.0 ± 0.58	5.0 ± 0.58	1.8 ± 0.24	1.8 ± 0.18	4.0 ± 0.33
Benzoic acid	10.0 ± 1.22	6.0 ± .71	7.0 ± 1.22	7.0 ± 1.22	3.5 ± 0.58
Nonanoic acid	0.4 ± 0.00	0.3 ± 0.09	0.3 ± 0.00	0.3 ± 0.09	0.3 ± 0.00
YPD + 10% Mannitol					
Acetic acid	350 ± 20.41	300 ± 20.41	300 ± 28.87	300 ± 28.87	120 ± 15.55
Propionic acid	120 ± 8.16	90 ± 4.08	120 ± 0.00	120 ± 0.00	80 ± 4.08
Sorbic acid	6.0 ± 0.33	6.0 ± 0.58	1.8 ± 0.12	1.8 ± 0.21	4.5 ± 0.00
Benzoic acid	10.0 ± 1.22	7.0 ± 0.91	7.0 ± 1.22	7.0 ± 0.00	4.5 ± 0.58
Nonanoic acid	0.4 ± 0.00	0.3 ± 0.09	0.3 ± 0.04	0.3 ± 0.00	0.3 ± 0.04

Values are mean MICs measured from at least two independent experiments (four replicates) following 14 days incubation at 25°C, ± the standard error.

4.2.6 Effects of vitamin source on yeast organic acid resistance

Z. bailii is known to require certain B group vitamins for growth (Thomas and Davenport, 1985). Therefore, the effects of adding 10 times the concentration of the three B group vitamins riboflavin, pyroxidine and thiamine that are collectively found in YPD (appendix 1) was analysed in relation to yeast organic acid resistance. The vitamins were made in stock solutions and filter sterilised to avoid the pressures and temperatures of autoclaving. Table 4.6 shows that the MICs for all three classes of organic acids obtained did not vary considerably with the different vitamins added. *S. cerevisiae* proved the most susceptible to all organic acids except sorbic acid in which the two *Z. kombuchaensis* strains were the most sensitive. The two *Z. bailii* strains showed differences in organic acid resistance in all three-vitamin addition media, with *Z. bailii* NCYC 1416 being the more sensitive.

Table 4.6 Effects of vitamin additions on organic acid MICs (mM) in *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

	Zb 1766	*Zb* 1416	*Zk* Y27162	*Zk* YB4811	*Sc* 957
YPD					
Acetic acid	400 ± 20.41	300 ± 28.87	350 ± 28.87	300 ± 20.41	125 ± 10.20
Propionic acid	100 ± 8.16	80 ± 11.55	120 ± 0.00	120 ± 0.00	50 ± 4.08
Sorbic acid	8 ± 0.71	4 ± 0.58	1.2 ± 0.82	1.2 ± 0.18	4 ± 0.58
Benzoic acid	9 ± 0.48	5 ± 1.22	6 ± 0.41	6 ± 0.71	3 ± 0.00
Nonanoic acid	0.6 ± 0.00	0.5 ± 0.11	0.4 ± 0.04	0.4 ± 0.00	0.5 ± 0.00
YPD + 10×Riboflavin					
Acetic acid	300 ± 28.87	200 ± 20.41	250 ± 20.41	250 ± 20.41	120 ± 10.20
Propionic acid	100 ± 0.00	70 ± 4.08	100 ± 0.00	110 ± 8.16	60 ± 0.00
Sorbic acid	8.0 ± 1.22	5.0 ± 0.91	1.2 ± 0.12	1.2 ± 0.18	4.0 ± 0.58
Benzoic acid	8.0 ± 0.71	5.0 ± 0.91	5.0 ± 1.22	5.0 ± 0.91	4.0 ± 0.71
Nonanoic acid	0.4 ± 0.02	0.3 ± 0.09	0.2 ± 0.04	0.2 ± 0.00	0.3 ± 0.00
YPD + 10×Pyridoxine					
Acetic acid	300 ± 20.41	200 ± 10.20	250 ± 20.41	250 ± 20.41	120 ± 10.20
Propionic acid	110 ± 8.16	90 ± 4.08	120 ± 0.00	120 ± 8.16	60 ± 4.08
Sorbic acid	8.0 ± 0.71	6.0 ± 1.22	1.2 ± 0.58	1.2 ± 0.58	4.0 ± 0.33
Benzoic acid	10.0 ± 1.22	6.0 ± 0.91	5.0 ± 0.00	5.0 ± 0.58	4.0 ± 0.71
Nonanoic acid	0.4 ± 0.00	0.3 ± 0.09	0.2 ± 0.04	0.2 ± 0.00	0.3 ± 0.09
YPD + 10×Thiamine					
Acetic acid	350 ± 20.41	200 ± 20.41	250 ± 0.00	250 ± 20.41	120 ± 10.20
Propionic acid	110 ± 8.16	80 ± 4.08	120 ± 8.16	120 ± 0.00	60 ± 8.16
Sorbic acid	8.0 ± 1.53	5.0 ± 0.91	1.2 ± 0.18	1.2 ± 0.24	4.5 ± 0.71
Benzoic acid	9.0 ± 0.00	5.0 ± 0.71	5.0 ± 0.71	5.0 ± 0.00	4.0 ± 0.58
Nonanoic acid	0.3 ± 0.09	0.3 ± 0.11	0.3 ± 0.09	0.3 ± 0.04	0.3 ± 0.00

Values are mean MICs measured from at least two independent experiments (four replicates) following 14 days incubation at 25°C, ± the standard error.

4.3 Discussion

This section shows that the alteration of growth conditions can have an effect on yeast organic acid resistance. All three yeast species were more susceptible to organic acids in minimal medium than in the complex growth media YPD. The omission of yeast extract from the complex growth media resulted in no growth for strains of *Z. bailii* and *Z. kombuchaensis* to some organic acids. The *S. cerevisiae* strain included in the study, however, did not show a similar pattern. Therefore, foodstuffs lacking complex nutrients such as those present in yeast extract would be at a greater risk of spoilage by *S. cerevisiae* than by *Z. bailii*. The results showed that the omission of Bacto peptone had little influence on yeast organic acid resistance. Both yeast extract and Bacto peptone contain similar components (appendix), but yeast extract contains a higher level of B group vitamins. *Z. bailii* has previously been reported to be dependent upon B group vitamins to cause spoilage (Thomas and Davenport, 1985). This study analyzed the effects of vitamin additions on yeast organic acid resistance but experiments in which vitamins were omitted from the growth media would be of interest as they could isolate the specific vitamins which *Z. bailii* and *Z. kombuchaensis* are dependent upon. This section highlights the need within the food industry to conduct analyses on the factors affecting yeast growth as these may allow new preservation strategies to be developed while providing an accurate risk assessment of spoilage.

Experiments performed with non-fermentable carbon sources showed the need of *Z. bailii* NCYC 1416, *Z. kombuchaensis* and *S. cerevisiae* for a fermentable carbon source to cause spoilage. The more organic acid resistant *Z. bailii* strain NCYC 1766 was able to grow in the presence of the organic acids examined with a non-fermentable carbon source. This strain, therefore, appears to be less dependent upon fermentation as a means of spoiling food than may have been thought previously. This suggests that this

highly resistant organic acid strain is dependent upon its mitochondria and a low level of respiration in order to cause spoilage. The importance of respiration in organic acid resistance is an avenue of investigation still remaining to be pursued.

Z. kombuchaensis had slightly higher MICs to medium chain organic acids in the presence of 10% (w/v) glycerol, mannitol and sorbitol than in their absence. A link between water activity and medium chain organic acid resistance could help explain the mode of action of this group of organic acids. Sorbic acid has been speculated to act primarily on the yeast membrane (Stratford and Anslow, 1998; Stratford and Ueckert, unpublished). If this were the case, then any change in the water activity of the growth media would have a direct influence on resistance. The fact that water potential had little effect on the resistance to acetic acid a known 'classic weak-acid preservative' supports this theory.

The results from this section provide some of the first evidence in detailing how growth conditions can affect yeast organic acid resistance. The information can be applied to the food industry in order to conduct risk assessments on the potential spoilage miroc-flora of a product depending on carbon, nitrogen source, the presence of key vitamins and the food preservative. The results may also aid the development of new products in which the potential incidence of spoilage must also be considered as a factor. An extension to the study would be to examine extrinsic factors including temperature and oxygen availability on yeast organic acid resistance. However, the restriction of oxygen in complex media has recently been shown not to be an effective strategy for the prevention of *Z. bailii* spoilage (Rodrigues *et al.,* 2001a). This section and the findings of Rodrigues *et al.,* (2001a) both highlight the dependence of the spoilage yeast *Z. bailii* on the intrinsic properties of the food to provide the essentials for growth.

5. Yeast cell structure and organic acid resistance

Nunn, C. D., Stratford, M., Turner, M. P. & Dickinson, J. R. (2003) Yeast cell structure and organic acid resistance. Oral Presentation at British Yeast Group Meeting, Leicester, UK.

5.1 Introduction

The cell structure of yeasts share many similarities to higher eukaryotes and have been used as model organisms for studying cell organization (Walker, 1998; Daum, 2000). Yeast cells exhibit great diversity in terms of cell size, shape and colour. Many of the morphological differences between yeasts are used in their classification (Barnett *et al.*, 2000). Yeast cells are highly organised consisting of the following structures: nucleus, mitochondria, golgi apparatus, secretory vesicles, endoplasmic reticulum, vacuoles and sometimes peroxisomes. All of the aforementioned structures are contained in the cytosol, which is surrounded by the cell envelope. The cell envelope consists of a plasma membrane, periplasm and cell wall. All of these structures interact and allow cells to adapt to various environmental conditions.

The cell envelope plays a major role in yeast cells by controlling osmotic and permeability properties of the cell (Walker, 1998). The plasma membrane provides the primary barrier for the passage of hydrophilic molecules and keeps the yeast unicellular. *S. cerevisiae* has provided much of the research focus for studying the plasma membrane. The plasma membrane consists primarily of a lipid bilayer with globular proteins. In the *S. cerevisiae* plasma membrane is located the ATP binding cassette (ABC) transporter proteins, including Pdr12 which has been shown to have a role in yeast organic acid resistance (Piper *et al.*, 1998). The plasma membrane also contains the ATPase proton pumps, which become activated in the presence of organic acids (Viegas and Sá-correira, 1991; Holyoak *et al.*, 1996). The periplasm functions to separate the plasma membrane and cell wall.

The cell wall of *S. cerevisiae* has been extensively studied with much being known about its structure and composition (Klis, 1994; Stratford, 1994; Klis *et al.*, 2002). The

function of the cell wall is primarily to provide physical protection and osmotic support from the external environment (Smith *et al.,* 2000). The cell wall is not a static structure and changes constantly with its environment (Popolo *et al.,* 1997). The yeast cell wall accounts for 15-25% of cell dry weight (Stratford, 1994). The main components of the cell wall are 1, 3 β-glucan, 1, 6 β-glucan, mannoproteins and chitin. Theses components account for 55, 5, 40 and 1-2% of cell wall dry weight (Kapteyn *et al.,* 2001). The molecular organisation of the *S. cerevisiae* cell wall is shown in Figure 5.1. The mannoproteins (highly glycosylated proteins rich in mannose) form the outer layer of the cell wall and determine the surface properties of the cell such as immunogenicity, hydrophobicity, flocculence and porosity (Klis *et al.,* in press). The mannoproteins are heavily glycosylated and consist of *N*-linked carbohydrate side-chains and shorter *O*-linked side chains (Cutler, 2001). The isolation of a number or mannan (*mnn*) mutants deficient in glycosylation showed the *MNN9* gene whose product is a transmembrane protein to be one of the most important genes in glycosylation; *mnn9* strains exhibiting serious defects in glycosylation and sensitivity to hygromycin B (Jaafar *et al.,* 2003). The mannoproteins consist of two classes of covalently linked cell wall proteins (CWPs): the first class consists of the glycosylphosphatidylinositol (GPI) dependent CWPs, which represent the most abundant class of CWPs (Klis *et al.,* in press). The majority of GPI-CWPs are located in the plasma membrane while the ones present in the cell wall are only expressed under specific growth conditions. The GPI-CWPS are linked indirectly to 1,3 β-glucan through the highly branched 1,6 β-glucan (Klis *et al.,* 2002). The GPI-CWPs form the external protein layer while the second more minor class of mannoproteins, protein with internal repeats (Pir)-CWPs through an alkali-sensitive direct linkage to 1,3 β-glucan, form the inner skeletal layer (Kapteyn *et al.,* 1999). Pir-CWPs have been identified in *Z. rouxii* (Toh-e *et al.,* 1993). The mechanical strength of the cell is mainly due to the highly elastic 1,3 β-glucan meshwork, which

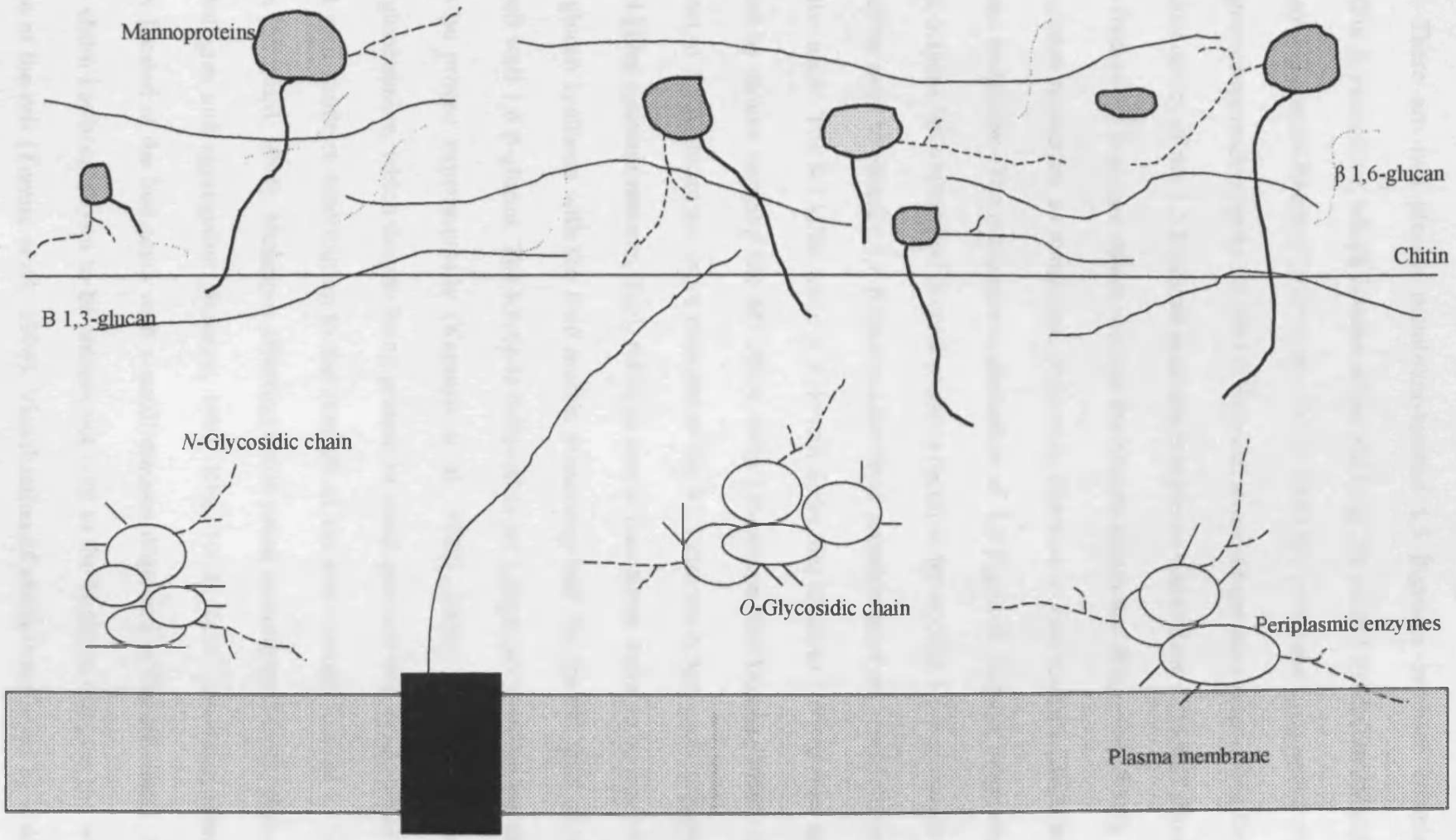


Figure 5.1 *S. cerevisiae* cell wall structure and composition. The cell wall is located adjacent to the plasma membrane and consists of two layers. The cell strength is provided by the complexes formed between chitin and 1,3/1,6 β-glucans. Mannoproteins are covalently linked to the inner glucan layer and are involved in determining surface properties of the cell. The inner skeletal layer contains the periplasmic enzymes. Adapted from Walker (1998).

forms around the entire cell and is maintained by hydrogen bonding (Klis *et al.*, in press). There are two plasma membrane-located 1,3 β-glucan synthase complexes formed in *S. cerevisiae*, which contain either Fks1p or the related Fks2p/Gsc2p and the regulatory G protein Rho1p (Cabib *et al.*, 2001). *FKS1* (so called due to its sensitivity to the immunosuppressive agent FK506) is believed to encode proteins responsible for the catalytic activity of the 1,3 β-glucan synthase complexes and to form a channel allowing newly formed 1,3 β-glucan chains through the plasma membrane (Klis *et al.*, 2002). The 1,6 β-glucan molecules as mentioned previously function to link the GPI-CWPs to 1,3 β-glucan molecules. The molecular organisation of 1,6 β-glucan remains unknown but genetic screens have identified several proteins required for normal 1,6 β-glucan levels. One screen used to identify 1,6 β-glucan associated proteins involves sensitivity to the K1 killer toxin. The K1 killer toxin is a protein toxin that binds to 1,6 β-glucan and is secreted by strains carrying the M1 RNA virus (Shahinian and Bussey, 2000). Cells deficient in 1,6 β-glucan are more resistant to the K1 killer toxin and such mutants are termed killer-resistant mutants (*kre*). *KRE6* is one of four genes know to be involved in 1,6 β-glucan synthesis with the *kre6* mutant possessing half the normal level of wild-type cell wall 1,6 β-glucan. The Kre6p is believed to be a β-glucan synthase but this is yet to be proven experimentally (Kapteyn *et al.*, 1999). Chitin is a polymer of *N*-acetylgluocasmine, which despite being present in small amounts has an important role in cell wall structure contributing to the strength of the cell through linkage to 1,3 β-glucan (Stratford, 1994). Mutations affecting chitin cause osmotic sensitivity, abnormal morphologies and aggregation (Bulawa, 1992; Klis, 1994). In *S. cerevisiae,* chitin is mainly located at the bud scars with a small amount dispersed in the cell walls. In *Z. rouxii* chitin has been shown to be present not only in the budscars but over the whole surface of the cell (Tomita *et al.*, 1996). Visualisation of chitin is achieved by staining with Calcofluor white which is a fluorescent anionic dye that binds to 1,4 β-glucans

such as chitosan, cellulose and chitin (Klis *et al.,* 2002). Chitin and 1,3 β-glucan synthesis takes place at the plasma membrane and there is increasing evidence for 1,6 β-glucan also being synthesized at the plasma membrane (Klis *et al.*, in press). Chitin synthesis in *S. cerevisiae* and *C. albicans* involves three chitin synthases each with a different function (Kim *et al.,* 2002). In *S. cerevisiae, CHS1* plays a role in response to the primary septum dissolving and has a repair function; *CHS2* synthesizes the primary septum separating the daughter and mother cell. *CHS3* roles include synthesis of chitin at the bud site forming a chitin ring, deposition of chitosan in the ascospore cell wall and depositing chitin in the lateral walls on exposure to cell wall damage (Klis *et al.,* in press). Collectively, all the evidence indicates that the assembly of the cell wall takes place entirely at the cell surface (Klis *et al.*, 2002).

Damage to the cell wall results in three main responses, firstly, there is an increase in chitin which alters the balance of cell wall polysaccharides. Secondly, the association between β-glucan, mannoproteins and chitin becomes altered. A consequence of the second response is an increase in cell wall proteins being linked to 1,3 β-glucan and chitin as a result of a lowering of 1,6 β-glucan. A third response of cell wall damage is a transient redistribution of cell wall synthesis and repair mechanisms, which ensures strengthening of the cell wall at the site of damage (Lagorce *et al.,* 2003). Cell wall damage can be induced by temperature, osmotic shock and chemical drugs (Smith *et al.,* 2000). Indeed, the application of chemical drugs known to interfere with the cell wall, proved invaluable in deciphering the genes involved in cell wall construction (Klis *et al.,* 2002). In *S. cerevisiae* sensitivity to these compounds identified cell wall mutants defected in all aspects of cell wall construction (Klis *et al.,* 2002). 1,3 β-glucanases, Calcofluor white, congo red, SDS, Hygromycin B and caffeine have all been applied in the detection of cell wall mutants (Ram *et al.,* 1994; van der Vaart *et al.,* 1995; Lussier

et al., 1997; Dean, 1999; Martin-Yken *et al.*, 2001). The application of such compounds to other yeasts may aid in the elucidation of their cell wall composition.

The yeast cell wall is a primary target for antifungal compounds as it represents a unique structure to these microorganisms (Klis *et al.*, in press). There is an increasing demand to elucidate more about the yeast cell wall in a bid to identify new targets for antifungals, particularly against the human pathogen *C. albicans*. The antifungal agents currently available include: polyenes, systematically active azoles and sterol synthesis inhibitors (Odds *et al.*, 2003). One of the major problems for the food industry is the lack of information about the cell wall of spoilage yeasts such as *Z. bailii*. Only one study has been reported which targets the cell wall of *Z. bailii,* which was based on disruption of GPI-CWPs (Bom *et al.*, 2001). In addition the cell wall of food spoilage yeasts may actually contribute to their resistance to organic acids contributing to spoilage capabilities and is an area that needs to be studied.

The application of EM to study the effects of stress agents or in deciphering the role of genes in yeast is extensive (Shimada *et al.*, 1993; Cappellaro *et al.*, 1994; Popolo *et al.*, 1997; Cid *et al.*, 1998; Osumi *et al.*, 1998; Granot *et al.*, 2003). The aim of TEM is to produce a sample representative of the actual structure of the yeast at harvesting. Yeast TEM is regarded as particularly difficult due to two main factors. The first being cell density, if too few cells are present then there will be insufficient sample, while if there are too many cells they may be insufficiently fixed. Secondly, the yeast cell wall is impermeable to many fixatives. The yeast EM procedure can be divided into three stages. The first stage is fixation. Chemical fixation is the most common method for yeast ultrastructure analysis and is achieved in two steps, the first of which is pre-fixation with an aldehyde crosslinker. Glutaraldehyde is a bifunctional crosslinker that

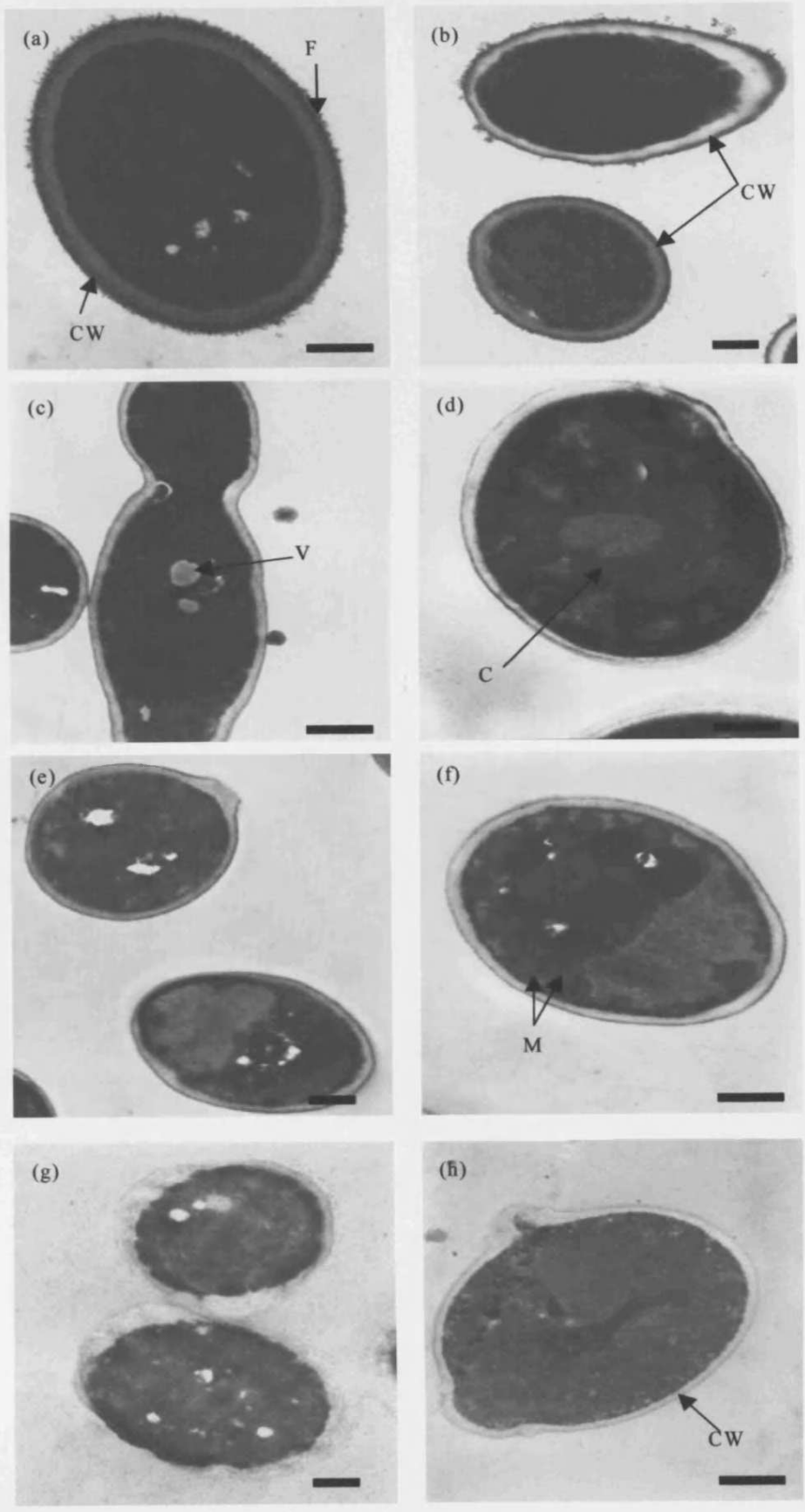
irreversibly cross-links proteins and this is often used with formaldehyde. Formaldehyde penetrates the sample quickly and temporarily stabilises structure until the entry of glutaraldehyde (Hayat, 1981). The second step in chemical fixation is termed post-fixation, which involves the application of osmium tetroxide or potassium permanganate. Osmium tetroxide reacts well with unsaturated fats, which provides good fixation of membranes and lipids. Potassium permanganate proves particularly useful in the fixation of membranes, but has been reported to result in a grey cytoplasm with little definition of organelles (Rambourg *et al.,* 1993). At this stage the yeast sample can be set in agar to minimise sample loss. *En Bloc* staining can also be included in the fixation protocol; it involves placing the sample in uranyl acetate, which is believed to aid the fixation of nucleic acid containing structures. Dehydration is the second stage of the yeast EM procedure and is carried out with increasing concentrations of ethanol (or acetone). The final stage of an EM procedure is to infiltrate the sample with resin. The aim of infiltration is to ensure that the thin section required for EM analysis does not contain large holes as this will lead to a loss of structural information. After polymerization of the resin the sample is cut into thin sections (60-90 nm) via a microtome, placed onto grids, stained and viewed under an electron microscope.

This section examines the effects of short, medium and longer chain organic acids on yeast cell structure using EM in a bid to elucidate more about their modes of inhibition. Differences in cell structure between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* are also examined via the application of TEM and SEM, sensitivity to cell wall chemicals and analysis of cell wall chitin. The final part of the section looks at whether differences in cell wall composition can contribute to yeast organic acid resistance by using organic acid challenge assays against *S. cerevisiae* cell wall mutants.

5.2 Results

5.2.1 Method development: Yeast electron microscopy

In a bid to improve upon the EM fixation protocol described in section 2.5.1, various modifications were attempted as shown in Figure 5.2. The first modification attempted was replacing post-fixation for 1 h with osmium tetroxide to 24 h with 1% (w/v) potassium permanganate (a-b). Permanganate treated cells have a well-defined cell wall, with dark staining throughout. However, permanganate caused a loss of cytoplasmic components, with the intracellular contents appearing grey and poorly defined. Another problem with permanganate treatment of yeast cells was that "flecks" were produced on the cell wall, which may be interpreted as damage when examining the effects of stress agents e.g. organic acids. Infiltration is one of the major problems in yeast TEM work, due to the yeast cell wall (Wright, 2000). One of the most common resins in yeast TEM work is the low viscosity Spurr resin. Figure 5.2 (c-d) shows the effect of Araldite resin a slightly higher viscosity resin on yeast TEM. Araldite generally, resulted in a greater number of holes in the sections, which limited the cell structure information that could be taken from each section. The fixative described in section 2.5.1 contained sucrose. The effect of omitting sucrose from the fixative with and without *En Bloc* staining was examined. Figure 5.2 (e-f) shows the results obtained with the yeast *Z. kombuchaensis*. Two possible consequences of omitting sucrose may have been slightly less definition of cytoplasmic components and perhaps not as efficient fixation as shown by the darkly stained band in (e). *En Bloc* staining appeared not to increase the resolution of the final TEM image obtained as shown by the similarity of images (e-f). Finally, the effect of calcium chloride on yeast TEM fixation was examined. One of the concerns regarding the inclusion of calcium chloride in the fixation protocol was that it might promote yeast flocculation, making the fixation of samples more difficult. Images (g-h) illustrate


(a)
F
CW
(b)
CW
(c)
V
(d)
C
(e)
(f)
M
(g)
(h)
CW

the effects of omitting calcium chloride from the fixative for *Z. bailii* NCYC 1766 and *Z. kombuchaensis*. The omission of calcium chloride had drastic effects on the TEM image obtained for *Z. bailii* NCYC 1766 with poor definition of cell structure arising; including lack of cell wall definition and no cytoplasmic components. The omission of calcium chloride for *Z. kombuchaensis* had little influence on cytoplasmic components but did result in lack of cell wall fixation. The modifications attempted appeared not to add to the original fixation protocol in terms of better quality TEM images.

5.2.2 Electron microscopy comparison of *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* cell structure

5.2.2.1 Transmission electron microscopy (TEM)

Figure 5.3 shows the results of a TEM comparison between an organic acid resistant *Z. bailii* strain NCYC 1766, a more organic acid sensitive strain NCYC 1416, *Z. kombuchaensis* and *S. cerevisiae*. The fixation protocol was as described in section 2.5.1. The TEM images illustrate that the cell structure of these three yeast species differ in a number of aspects. Firstly, cell size, *Z. bailii* NCYC 1416 contained the largest cells followed by *Z. bailii* NCYC 1766, then *Z. kombuchaensis* with *S. cerevisiae* having the smallest cells (Table 5.1). The cell shape of the yeasts differed in that *Z. bailii* (both strains) contain cells that are long oval, *Z. kombuchaensis* contains cells that are more spherical while the *S. cerevisiae* cells appeared to be round. Table 5.1 shows that the three *Zygosaccharomyces* yeasts have cells with a thicker cell wall than *S. cerevisiae*. The cell wall of *Z. bailii* and *Z. kombuchaensis* also appears more electron dense than *S. cerevisiae*. The TEM images obtained for all four yeasts show that the cell structures are well maintained and that cytoplasmic organelles are visible. The cell structures evident include cell membrane (CM), cell wall (CW), mitochondria with cristae (M), nucleus (N), endoplasmic reticulum (ER) and vacuole (V).

Figure 5.3 Transmission electron microscopy comparison of (a, b) *Z. bailii* NCYC 1766 (c, d) *Z. bailii* NCYC 1416 (e, f) *Z. kombuchaensis* NRRL YB4811 (g, h) *S. cerevisiae* NCYC 957 grown in YPD pH 4.0 at 25°C without shaking and fixed at 48 h incubation. CW = Cell Wall, CM = Cell Membrane, M = Miotchondria, ER = Endoplasmic Reticulum, V= Vacuole, N = Nucleus. Bar = 1 μm.

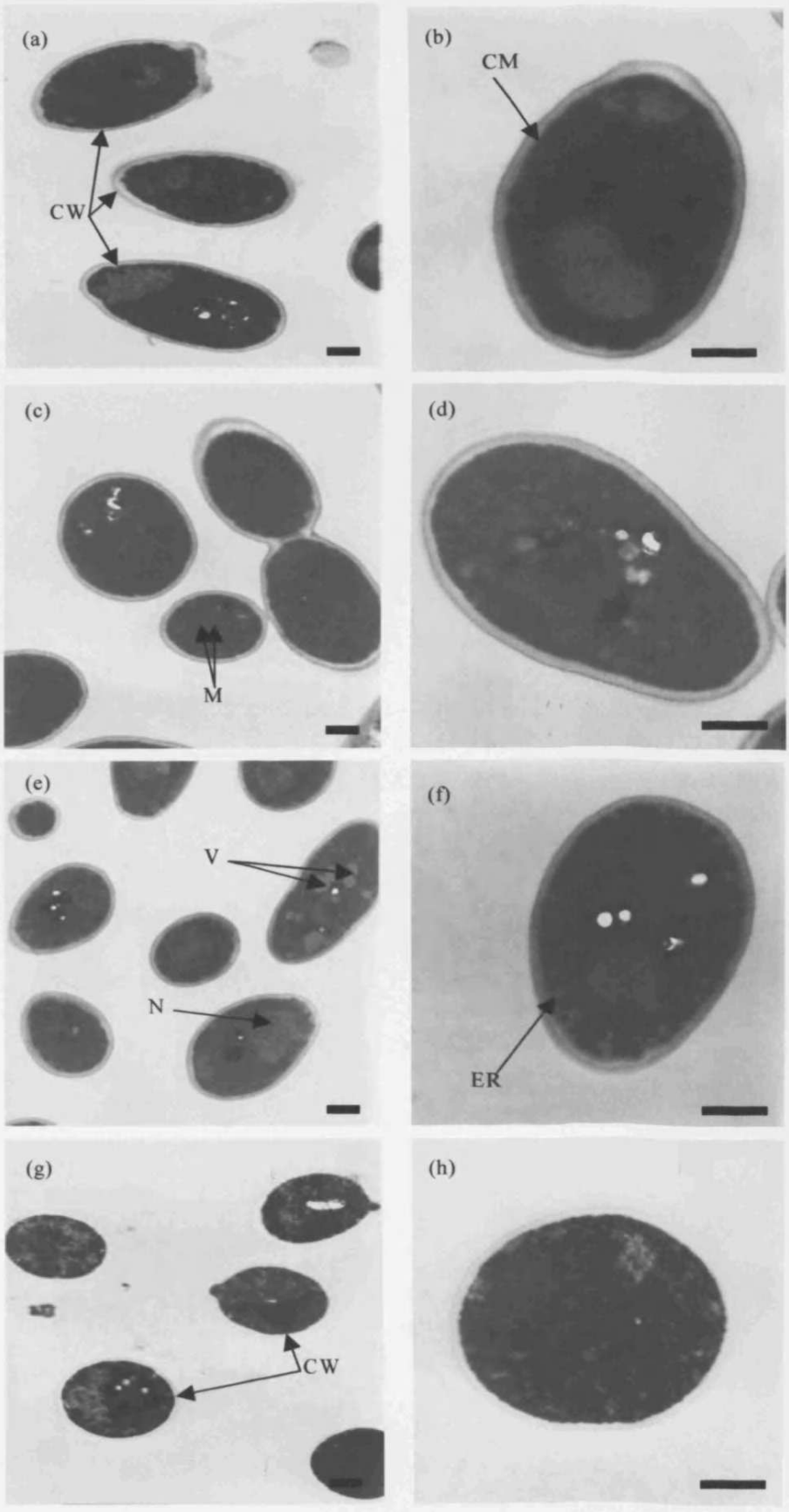

(a)
CW
(b)
CM
(c)
M
(d)
(e)
V
N
(f)
ER
(g)
CW
(h)

Table 5.1 Differences in cell length and cell wall thickness between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

Yeast	Cell length (μm) ± SD	Cell wall thickness (μm) ± SD
Z. bailii NCYC 1766	5.75 ± 0.500	0.191 ± 0.023
Z. bailii NCYC 1416	6.02 ± 0.423	0.196 ± 0.017
Z. kombuchaensis NRRL YB4811	4.16 ± 0.322	0.168 ± 0.022
S. cerevisiae NCYC 957	3.44 ± 0.489	0.111 ± 0.019

Results are the average lengths based on examining at least 20 cells that appeared to have been cut in the same plane ± standard deviation from TEM images.

5.2.2.2 Scanning electron microscopy (SEM)

A SEM comparison of the three yeast species was also conducted to compare cell topology (Figure 5.4). The SEM images illustrate that relatively small amounts of cell debris are evident under control conditions for *Z. kmobuchaensis* and *S. cerevisiae* (e-h). The differences in both cell size and shape detailed in 5.2.2.1 are reinforced by the SEM images. The images for *Z. bailii* NCYC 1416 confirm the aggregating nature of this yeast with chains of cells being clearly evident. *Z. bailii* NCYC 1416 is also the strain which appears to exhibit the most variation in cell morphology, having cells of numerous shapes and sizes (c-d). The SEM images show multiple bud scars (BS) for all the yeasts. Apart from the bud scars, the cell wall appeared intact, smooth and continuous, being indicative of little structural damage.

5.2.3 Effect of organic acids on yeast cell structure

5.2.3.1 Acetic acid

Initial EM attempts focussed on harvesting cells from inhibitor-treated cultures as described in section 2.4. However, these cultures yielded insufficient material to fix cells for EM analysis. Therefore, cultures were set up in 40 ml YPD pH 4.0 as in section 2.3 containing various concentration of organic acids. Growth was assessed by measuring culture OD600 nm at various time intervals. This method yielded a sufficient number of cells at 48 h incubation to be fixed for EM analysis. Figure 5.5 shows the growth curves obtained for *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* in YPD pH 4.0 with concentrations of acetic acid from 100 to 500 mM. *Z. bailii* NCYC 1766 was able to grow in acetic acid concentration of 400 mM, while the other yeasts could only grow in a maximum concentration of 300 mM. Despite the low OD values reached by *Z. bailii* NCYC 1416 even in the absence of acetic acid, sufficient cells were yielded to

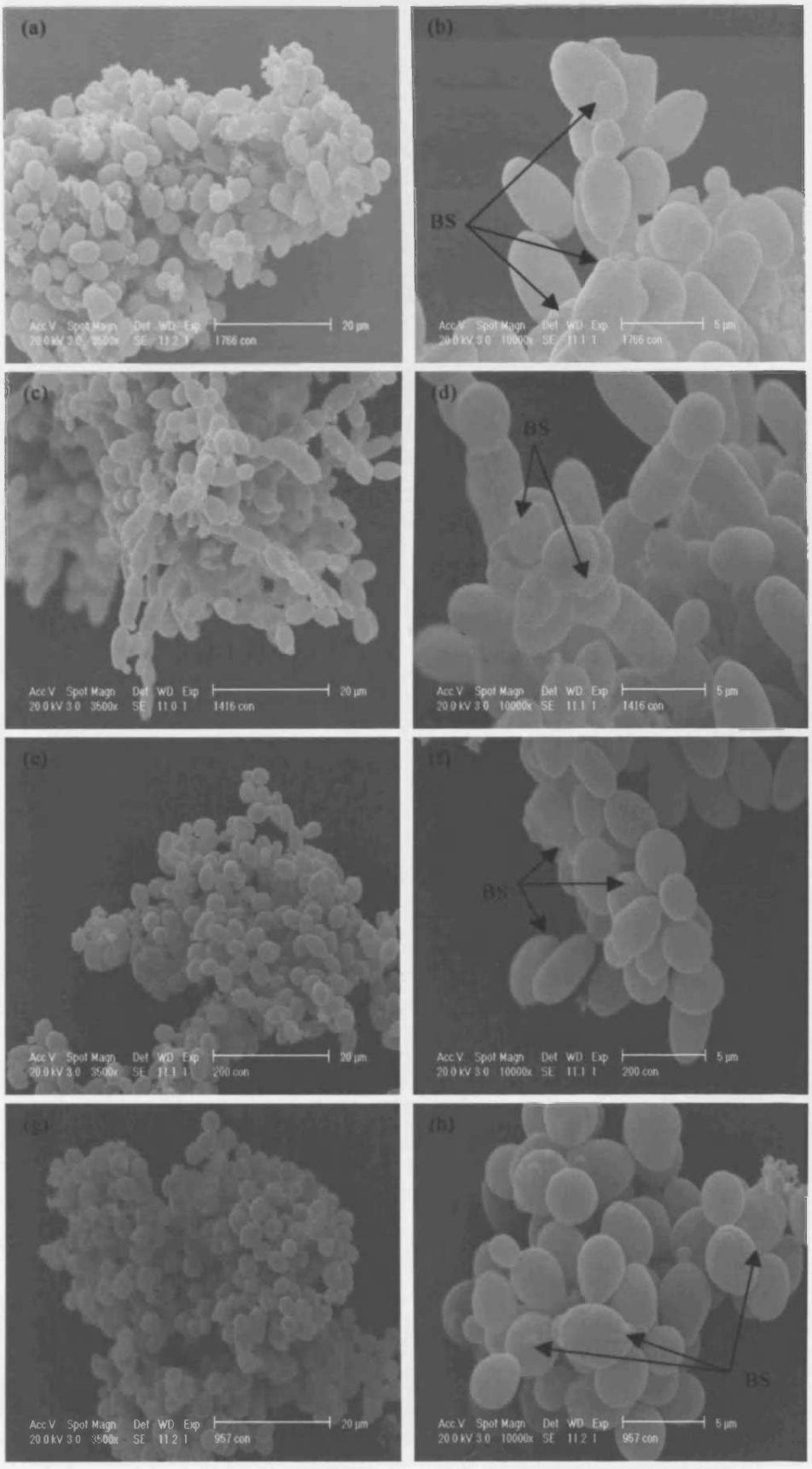
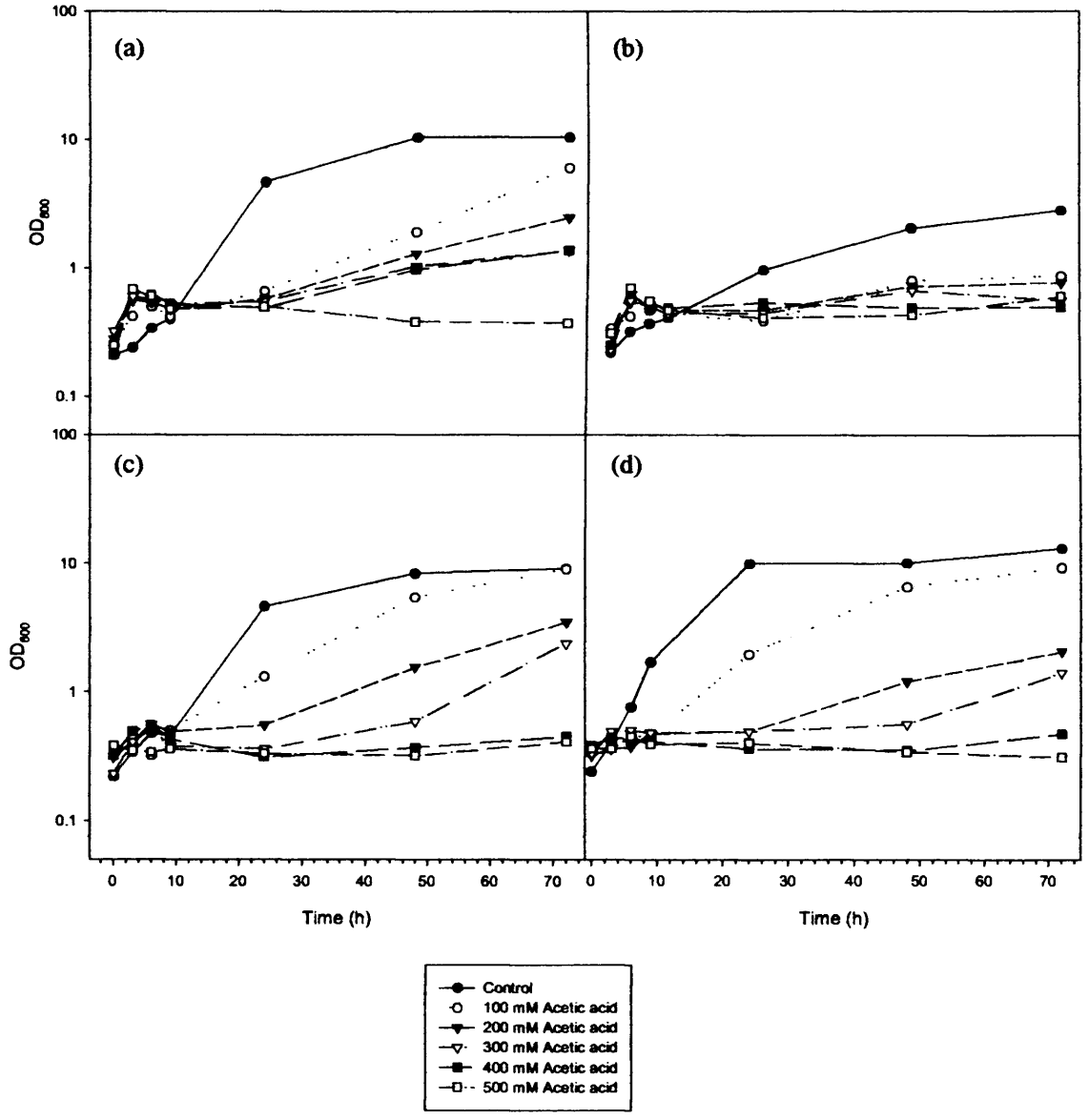

(a)
(b)
BS
(c)
(d)
BS
(e)
(f)
BS
(g)
(h)
BS

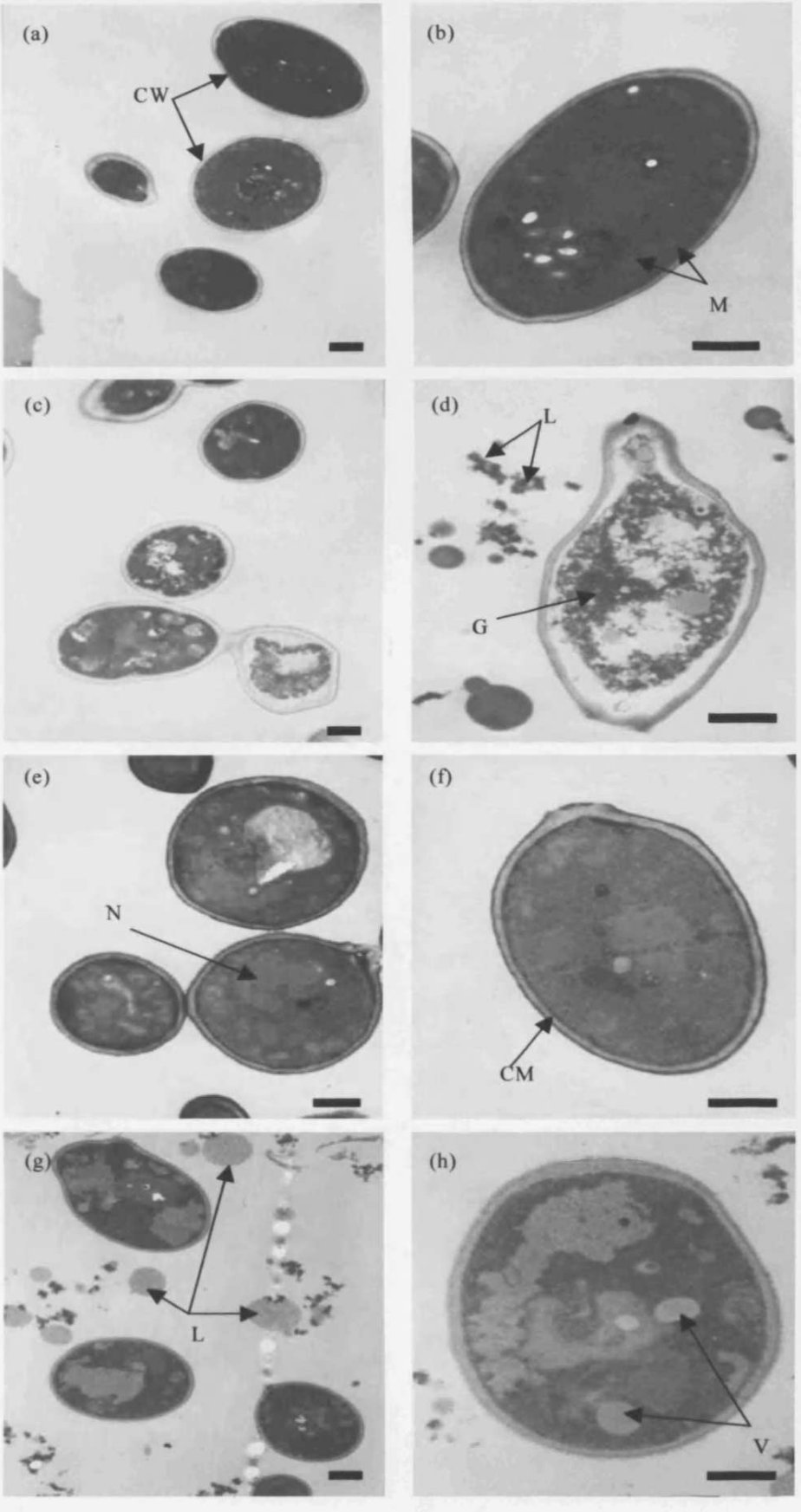
Figure 5.5 Growth curves for (a) *Z. bailii* NCYC 1766 (b) *Z. bailii* NCYC 1416 (c) *Z. kombuchaensis* NRRL YB4811 (d) *S. cerevisiae* NCYC 957 grown in YPD pH 4.0 at 25°C without shaking with concentrations of acetic acid as indicated in legend.

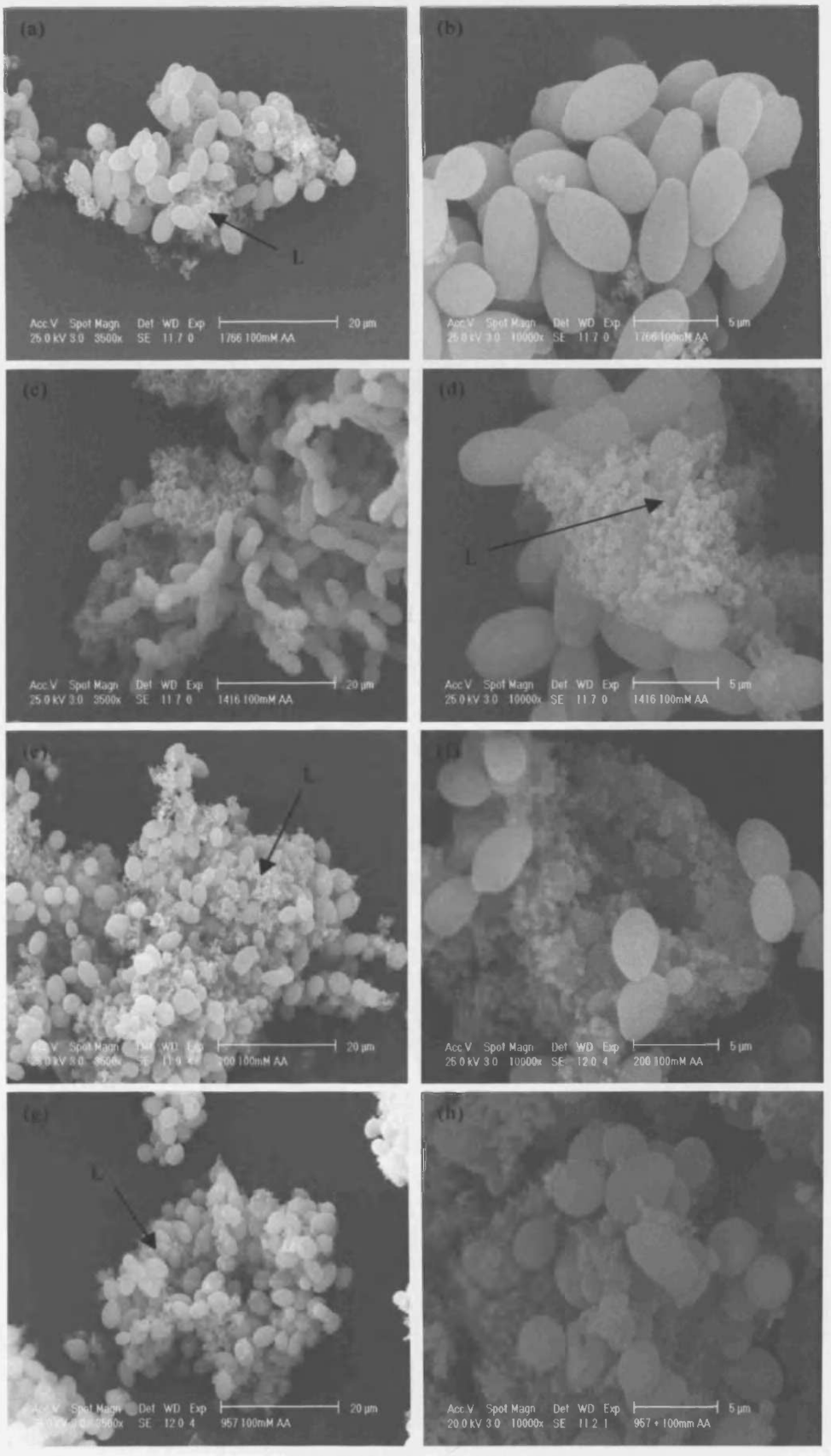


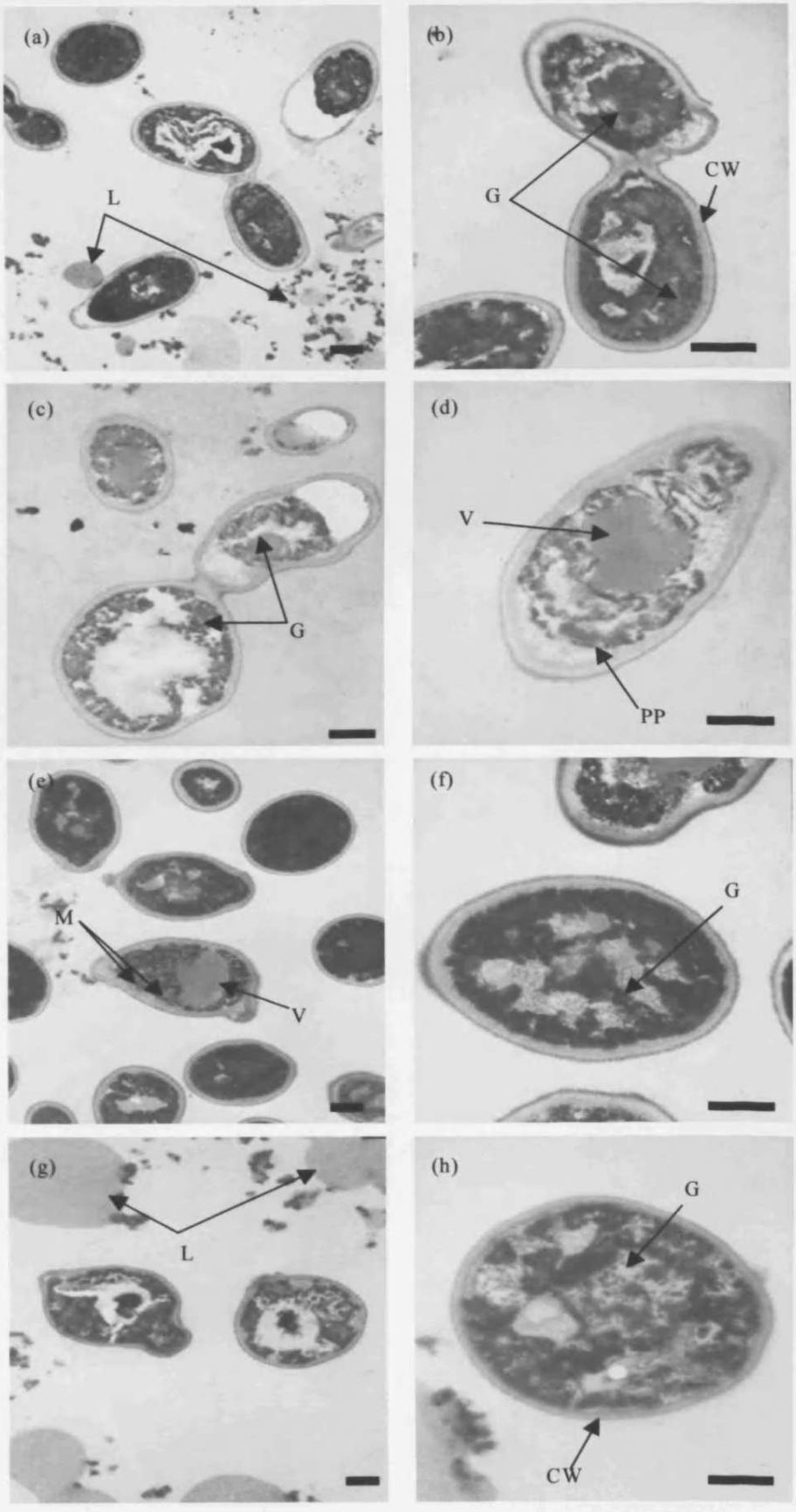
Results are representative of two independent experiments (four replicates) with standard errors <10%.

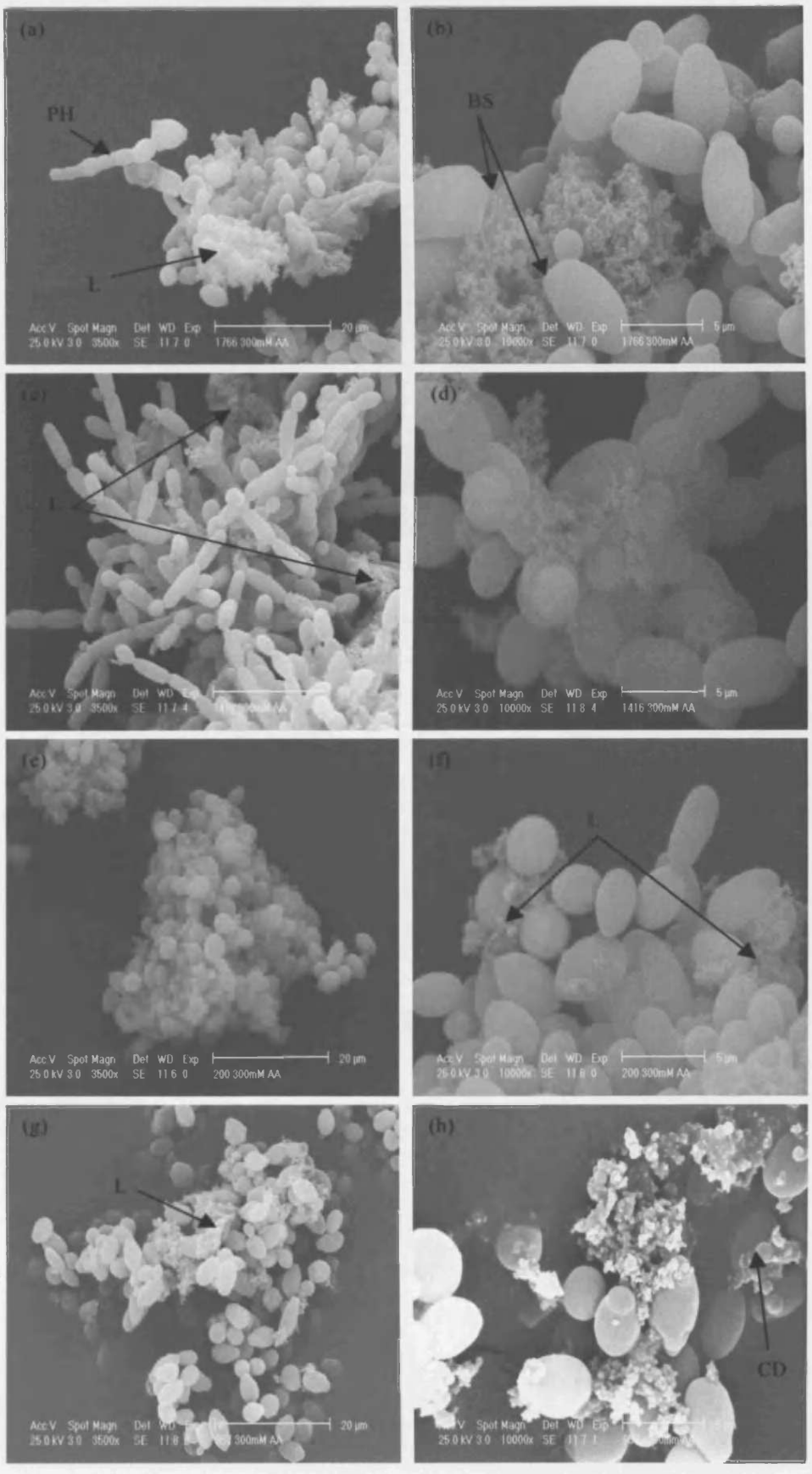
prepare EM samples. TEM and SEM analysis was conducted on acetic acid concentrations of 100 mM and 300 mM for all yeasts plus 400 mM for *Z. bailii* NCYC 1766.

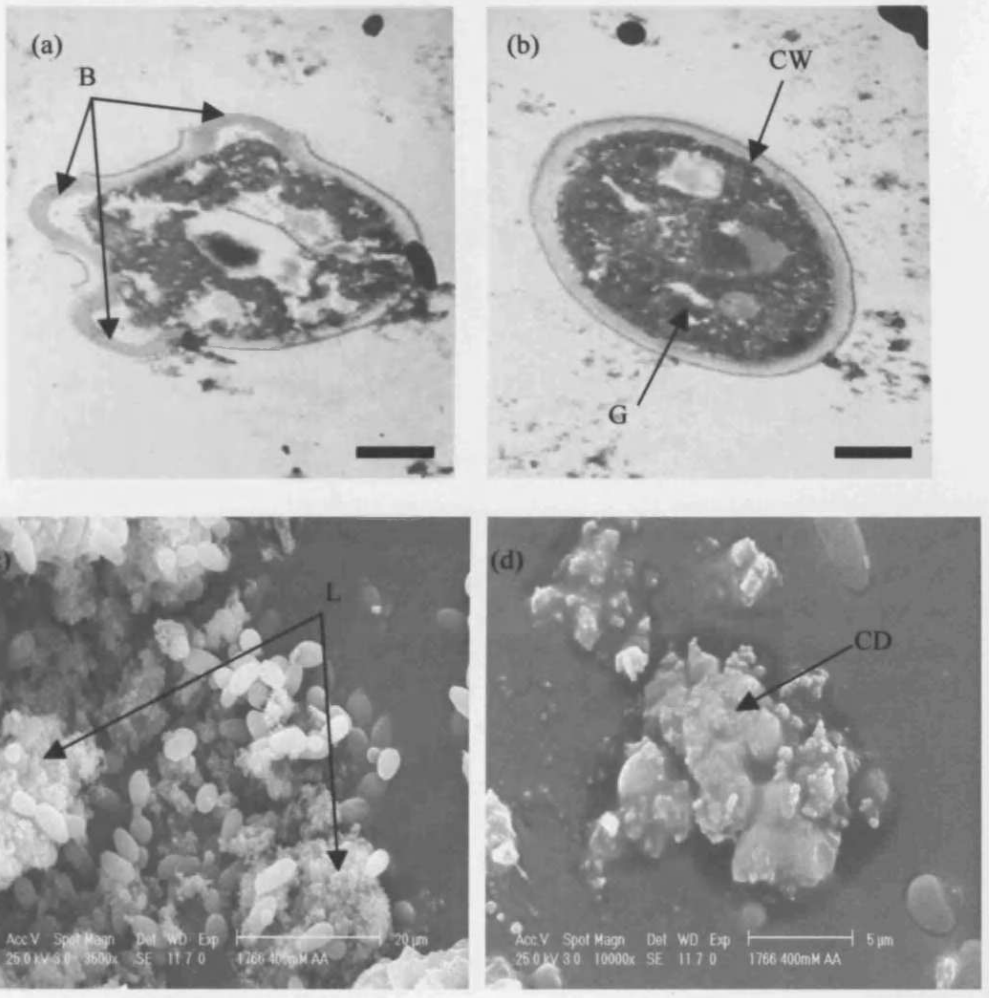
Figures 5.6-5.10 show the effects of acetic acid on yeast cell structure. The TEM images for 100 mM acetic acid treatment (Figure 5.6) illustrate that considerable structural changes are evident for *Z. bailii* NCYC 1416. The images (c-d) show that the cytoplasmic contents have become granular and detached from the periplasm. The mitochondria (M) when present appeared granular with leakage of cytoplasmic contents evident throughout the sections. The *S. cerevisiae* images (g-h) show cell debris and evidence of a granular cytoplasm. *Z. kombuchaensis* (e-f) appears to have sustained relatively little cellular damage with several cytoplasmic organelles evident including mitochondria (M) and the nucleus (N). The only difference in *Z. kombuchaensis* is that the plasma membrane appears more darkly stained than in controls. This additional staining could be indicative of some structural damage. Images (a-b) show that *Z. bailii* NCYC 1766 appeared as control cells. The SEM images obtained in Figure 5.7 supports the TEM images by confirming considerable cell debris sustained for all yeasts except *Z. bailii* NCYC 1766, which shows a similar level of debris as control. The SEM images also illustrate that the topology of the cells remain largely unchanged. At 300 mM acetic acid all yeasts show extensive cellular damage (Figure 5.8). All images are granular with no cytoplasmic organelles. The SEM images (Figure 5.9) show extensive cell debris. *Z. bailii* NCYC 1766 (a) appears to have become elongated in a similar manner to a pseudohyphal or hyphal cell in the presence of high concentrations of acetic acid. No other yeast examined showed evidence of morphological change in the presence of acetic acid. *Z. bailii* NCYC 1766 was the only yeast able to grow in acetic acid concentrations of 400 mM and the EM images obtained are shown in Figure 5.10.


(a)
CW
(b)
M
(c)
(d)
L
G
(e)
N
(f)
CM
(g)
L
(h)
V


(a)
L
Acc.V Spot Magn Det WD Exp 20 µm
25.0 kV 3.0 3500x SE 11.7 0 1766 100mM AA
(b)
Acc.V Spot Magn Det WD Exp 5 µm
25.0 kV 3.0 10000x SE 11.7 0 1766 100mM AA
(c)
Acc.V Spot Magn Det WD Exp 20 µm
25.0 kV 3.0 3500x SE 11.7 0 1416 100mM AA
(d)
L
Acc.V Spot Magn Det WD Exp 5 µm
25.0 kV 3.0 10000x SE 11.7 0 1416 100mM AA
(e)
L
20 µm
(f)
Acc.V Spot Magn Det WD Exp 5 µm
25.0 kV 3.0 10000x SE 12.0 4 200 100mM AA
(g)
L
Det WD Exp 20 µm
SE 12.0 4 957 100mM AA
(h)
Acc.V Spot Magn Det WD Exp 5 µm
20.0 kV 3.0 10000x SE 11.2 1 957 + 100mm AA


(a)
L
(b)
G
CW
(c)
G
(d)
V
PP
(e)
M
V
(f)
G
(g)
L
(h)
G
CW


(a)
PH
L
(b)
BS
(c)
L
(d)
(e)
(f)
L
(g)
L
(h)
CD


(a)
B
(b)
CW
G
(c)
L
(d)
CD

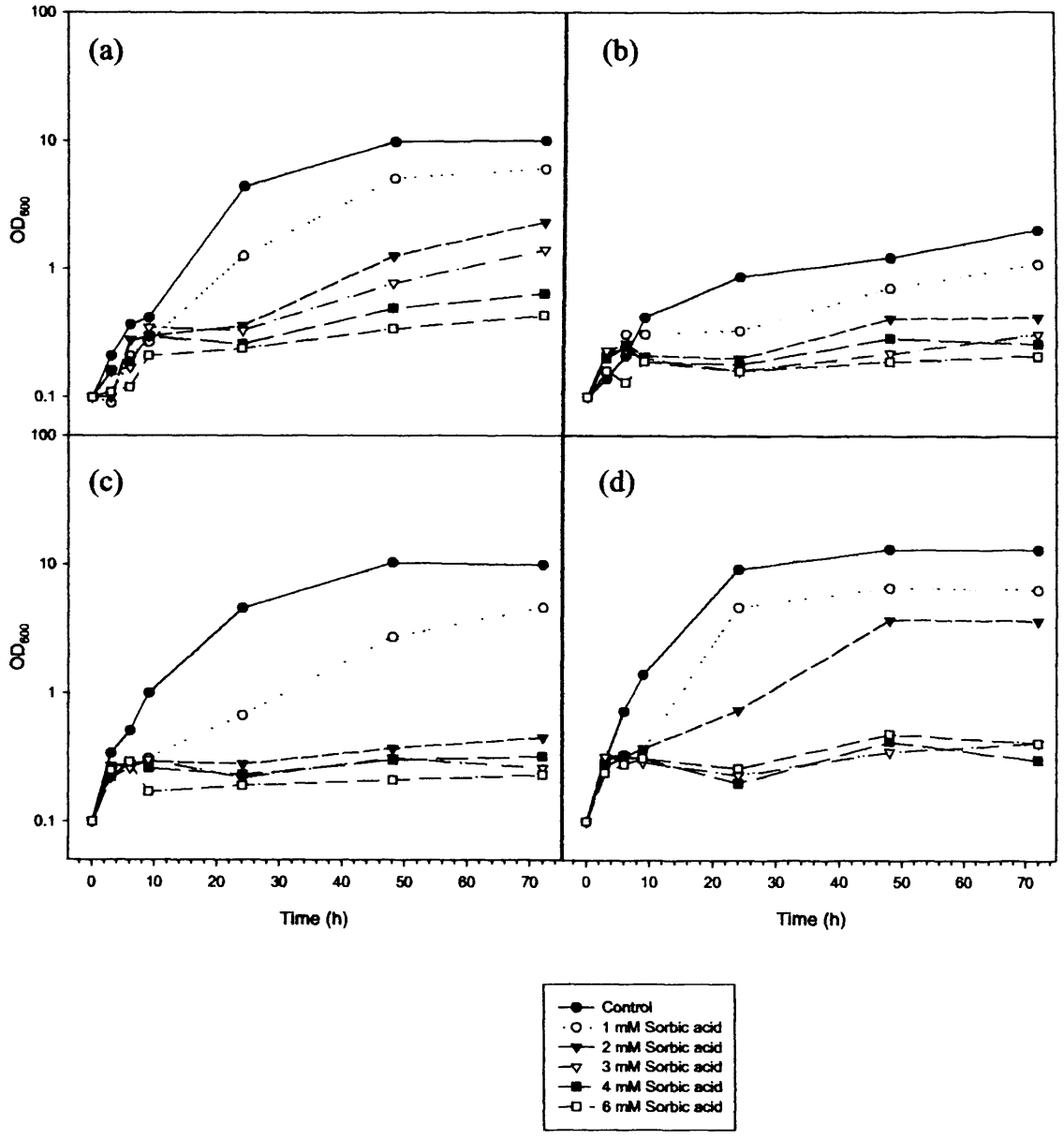
The granular image as obtained with 300 mM acetic acid treatment is still evident, with the cell wall appearing undamaged. The SEM images for *Z. bailii* NCYC 1766 show increased cell debris and cell lysis as for *S. cerevisiae* at 300 mM acetic acid.

5.2.3.2 Sorbic acid

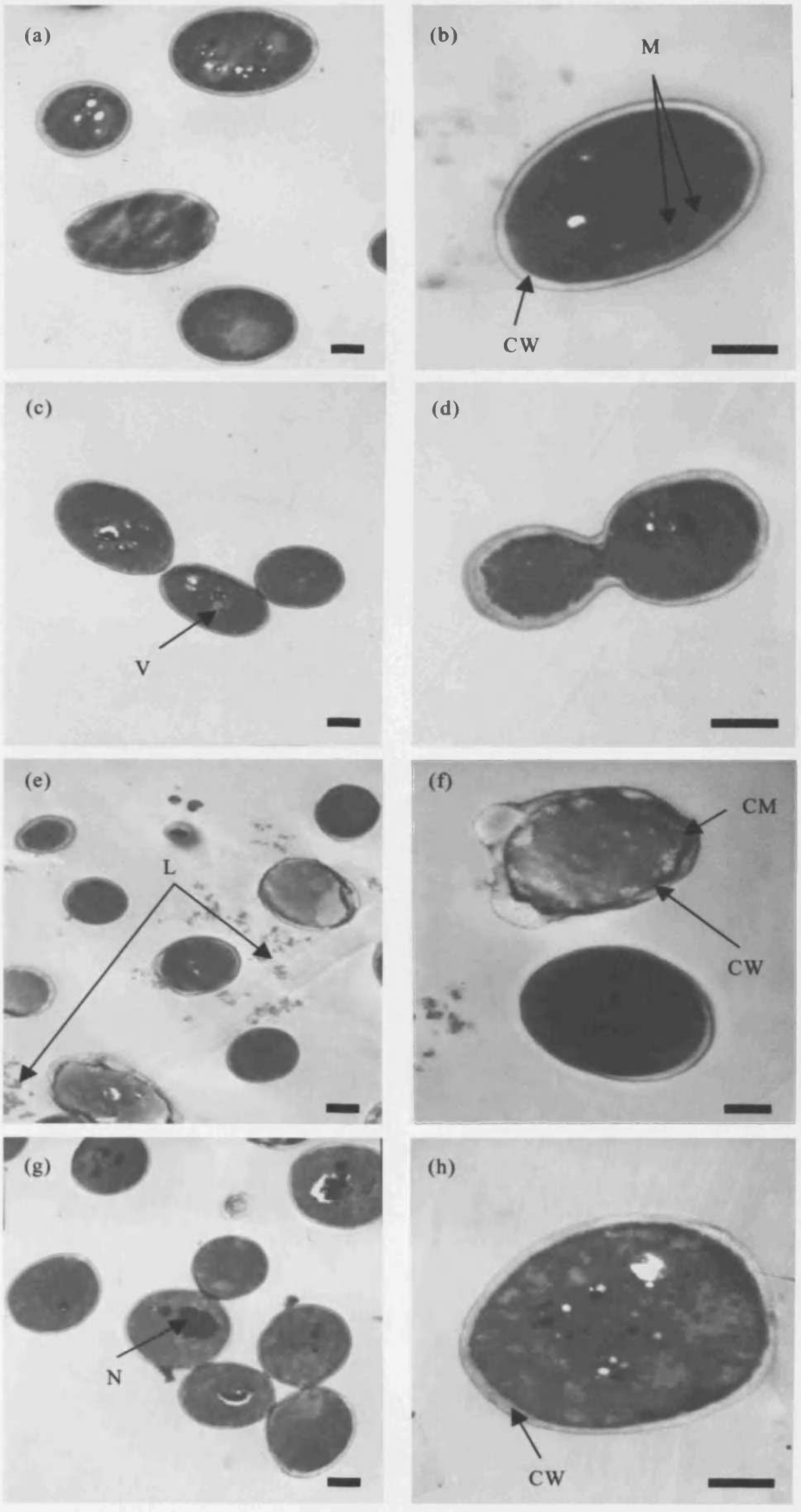
The growth curves obtained for *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* in the presence of sorbic acid are shown in Figure 5.11. *Z. bailii* NCYC 1416, *Z. kombuchaensis* and *S. cerevisiae* were only able to grow in a maximum sorbic acid concentration of 2 mM, while *Z. bailii* NCYC 1766 could grow in concentrations of 4 mM. The effects of 1 mM and 2 mM sorbic acid on yeast cell structure were analysed via EM for all the yeasts. *Z. bailii* NCYC 1766 was also analysed in concentrations of 3 mM and 4 mM.

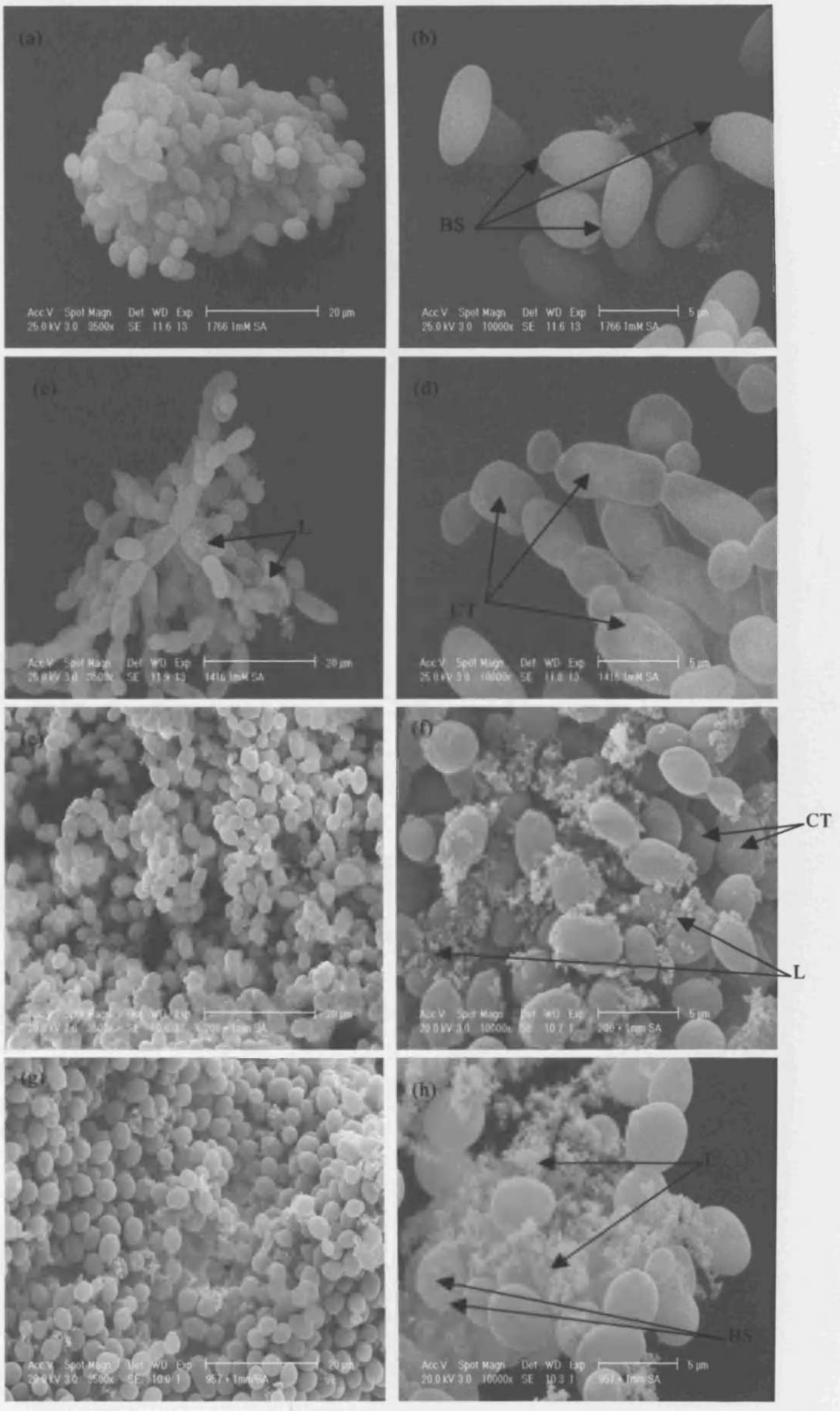
The results of the TEM and SEM analyses for the effects of sorbic acid on yeast cell structure are shown in Figures 5.12-5.16. Figure 5.12 shows the images obtained for 1 mM sorbic acid treatment. These images show that *Z. kombuchaensis* (e-f) sustained extensive structural alteration. The structural effects include cell debris plus a cytoplasmic compartment lacking visible organelles and appearing darkly stained. The biggest change in structure is that the plasma membrane and cell wall have become altered in shape, with folds being evident. Sections of the plasma membrane appear to be darkly stained and could be indicative of damage. The *Z. kombuchaensis* cells also exhibit an outgrowth from the cell wall, which appears similar to a bud scar but could be an aberration to the cell wall as a result of sorbic acid treatment. The *Z. bailii* and *S. cerevisiae* yeast exhibit fewer structural alterations than *Z. kombuchaensis*. *Z. bailii* and *S. cerevisiae,* however, contain a distinct lack of organelles and exhibit some evidence of increased staining around the area of the plasma membrane. The SEM analysis

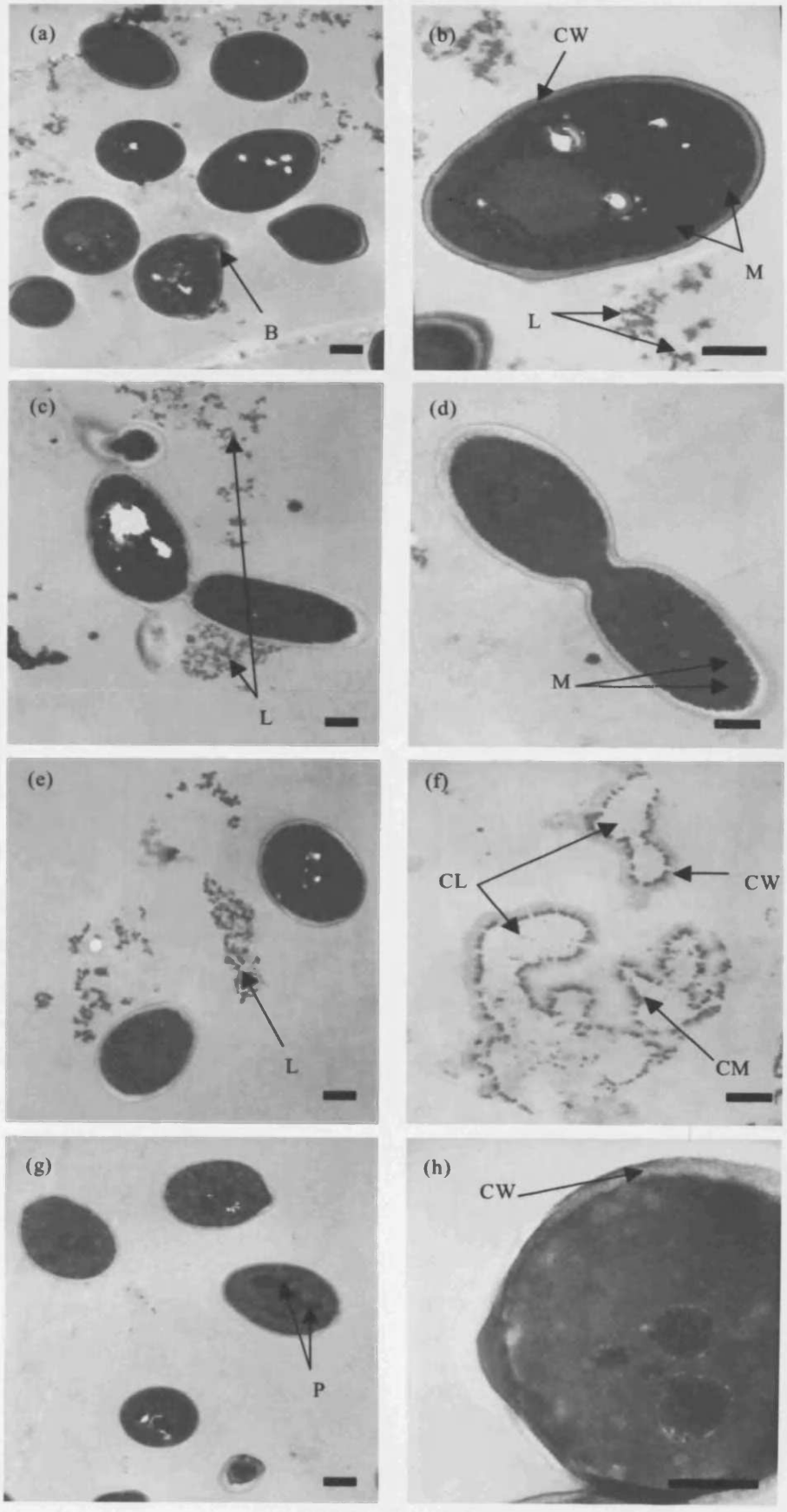
Figure 5.11 Growth curves for (a) *Z. bailii* NCYC 1766 (b) *Z. bailii* NCYC 1416 (c) *Z. kombuchaensis* NRRL YB4811 (d) *S. cerevisiae* NCYC 957 grown in YPD pH 4.0 at 25°C without shaking with concentrations of sorbic acid as indicated in the legend.

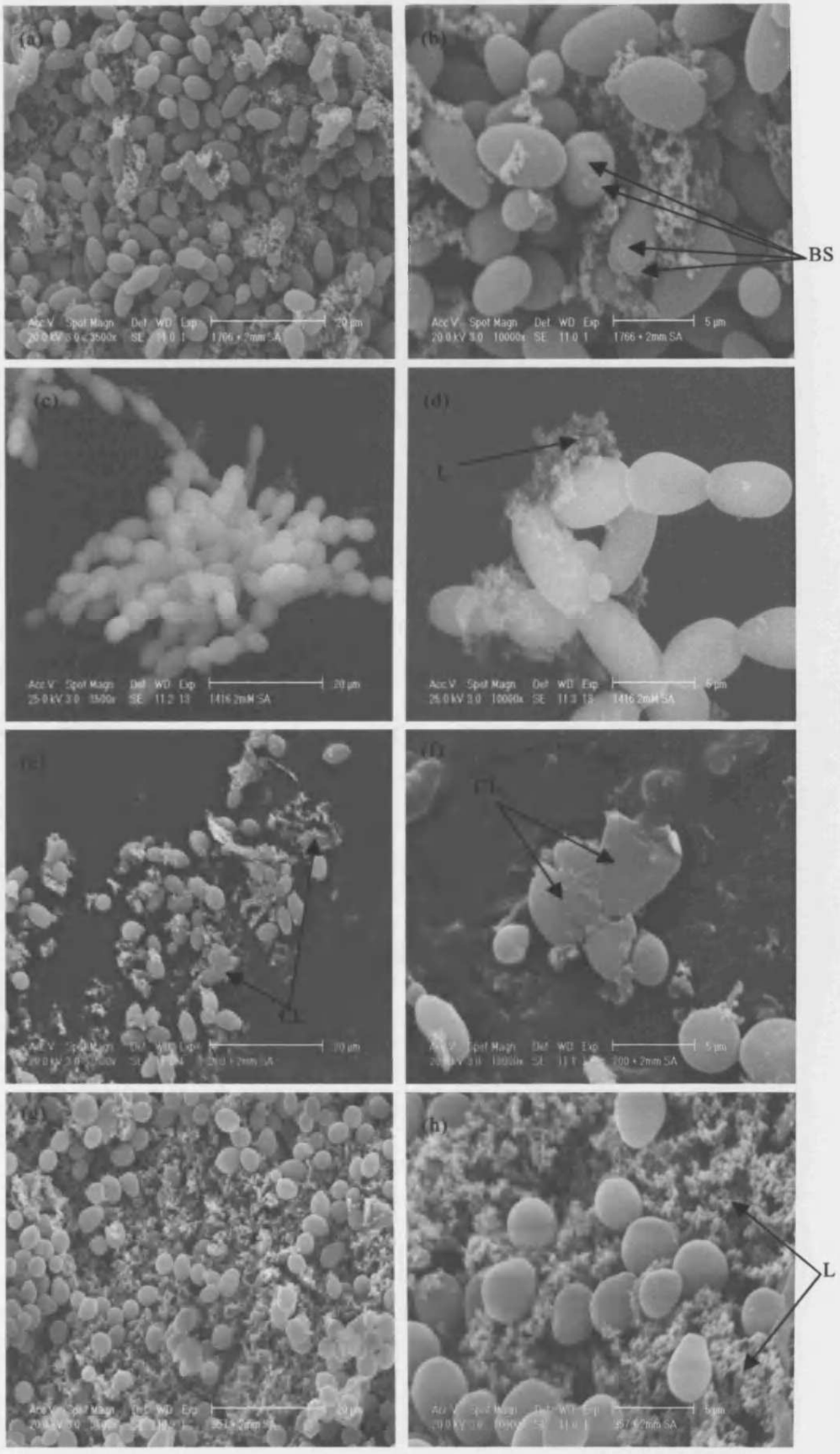


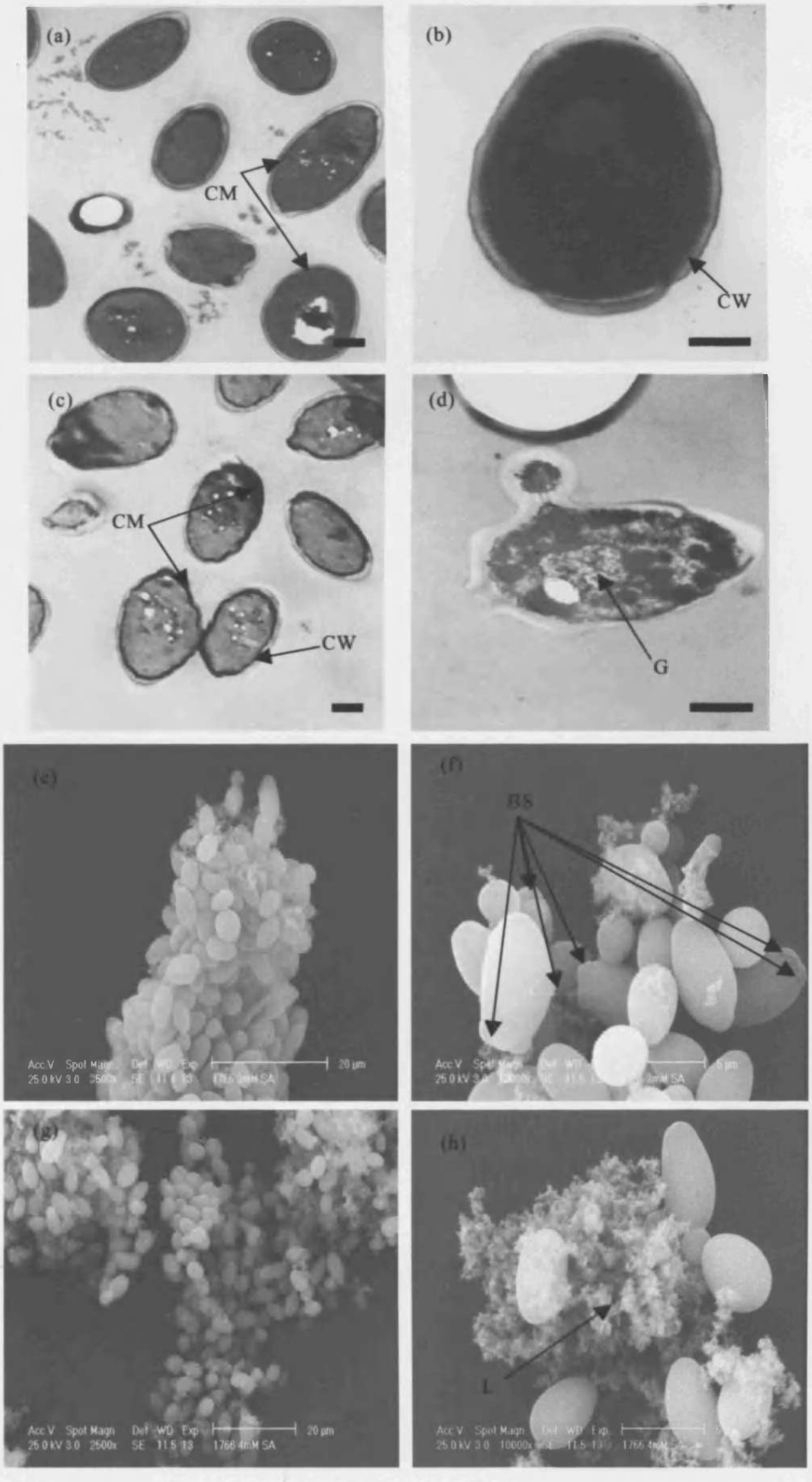
Results are representative of two independent experiments (four replicates) with standard errors <8%.


(a)
(b)
M
CW
(c)
V
(d)
(e)
L
(f)
CM
CW
(g)
N
(h)
CW


(a)
(b)
BS
(c)
L
(d)
CT
(e)
(f)
CT
L
(g)
(h)
L
BS


(a)
B
(b)
CW
M
L
(c)
L
(d)
M
(e)
L
(f)
CL
CW
CM
(g)
P
(h)
CW


(a)
(b)
BS
(c)
(d)
L
(e)
(f)
(g)
(h)
L

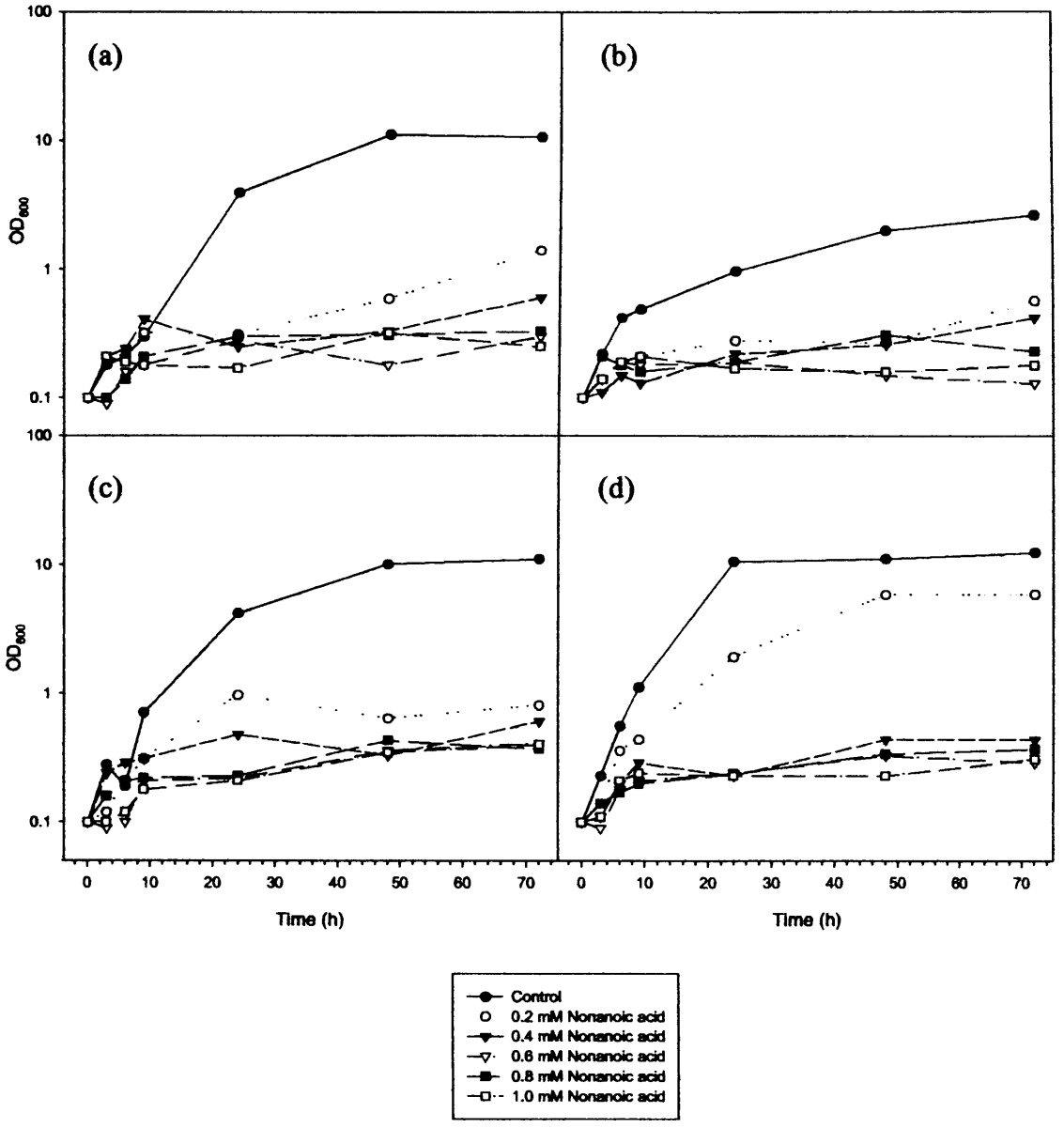

(a)
CM
(b)
CW
(c)
CM
CW
(d)
G
(e)
(f)
BS
(g)
(h)
L

(Figure 5.13) shows that 1 mM sorbic acid treatment does not result in considerable cell leakage for *Z. bailii*. *S. cerevisiae* cells exhibit some cell leakage, but at a lower level than *Z. kombuchaensis*. The *Z. kombuchaensis* images (e-f) show that cells contain alterations to the smooth outer wall. Figure 5.14 shows the effects of 2 mM sorbic acid on yeast cell structure. *Z. bailii* NCYC 1766 (a-b) contains relatively little damage as with 1 mM sorbic acid. *Z. bailii* NCYC 1416 shows signs of separation from the periplasm. *S. cerevisiae* (g-h) contains evidence of the cytoplasm becoming granular, which is not exhibited by the other yeast. *S. cerevisiae* also contains some evidence of plasma membrane folding. The effects of 2 mM sorbic acid on *Z. kombuchaensis* resulted in cell lysis (f) with extensive cell debris visible. The damage appears to be consistent with rupture of the cell membrane or cell wall. The SEM images for *Z. bailii* and *S. cerevisiae* at 2 mM sorbic acid show an increase in cell leakage from that visible at 1 mM sorbic acid treatment, but not evidence of cell lysis. Exposure of *Z. bailii* NCYC 1766 to 3 mM and 4 mM sorbic acid (Figure 5.16) results in an increase in plasma membrane staining with increased evidence of membrane and cell wall damage

5.2.3.3 Nonanoic acid

Figure 5.17 shows the growth curves obtained for *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* in the presence of YPD pH 4.0 containing 0.2-1.0 mM nonanoic acid. The results showed that the four yeasts only exhibited growth in YPD control and cultures containing 0.2 mM and 0.4 mM nonanoic acid. On the basis of OD values *S. cerevisiae* was inhibited to the smallest degree by 0.2 mM nonanoic acid. The OD values reached for all yeasts in 0.4 mM nonanoic acid were very low and only just enough cells were harvested for EM fixation.

Figure 5.17 Growth curves for (a) *Z. bailii* NCYC 1766 (b) *Z. bailii* NCYC 1416 (c) *Z. kombuchaensis* NRRL YB4811 (d) *S. cerevisiae* NCYC 957 grown in YPD pH 4.0 at 25°C without shaking with concentrations of nonanoic acid as indicated in legend.

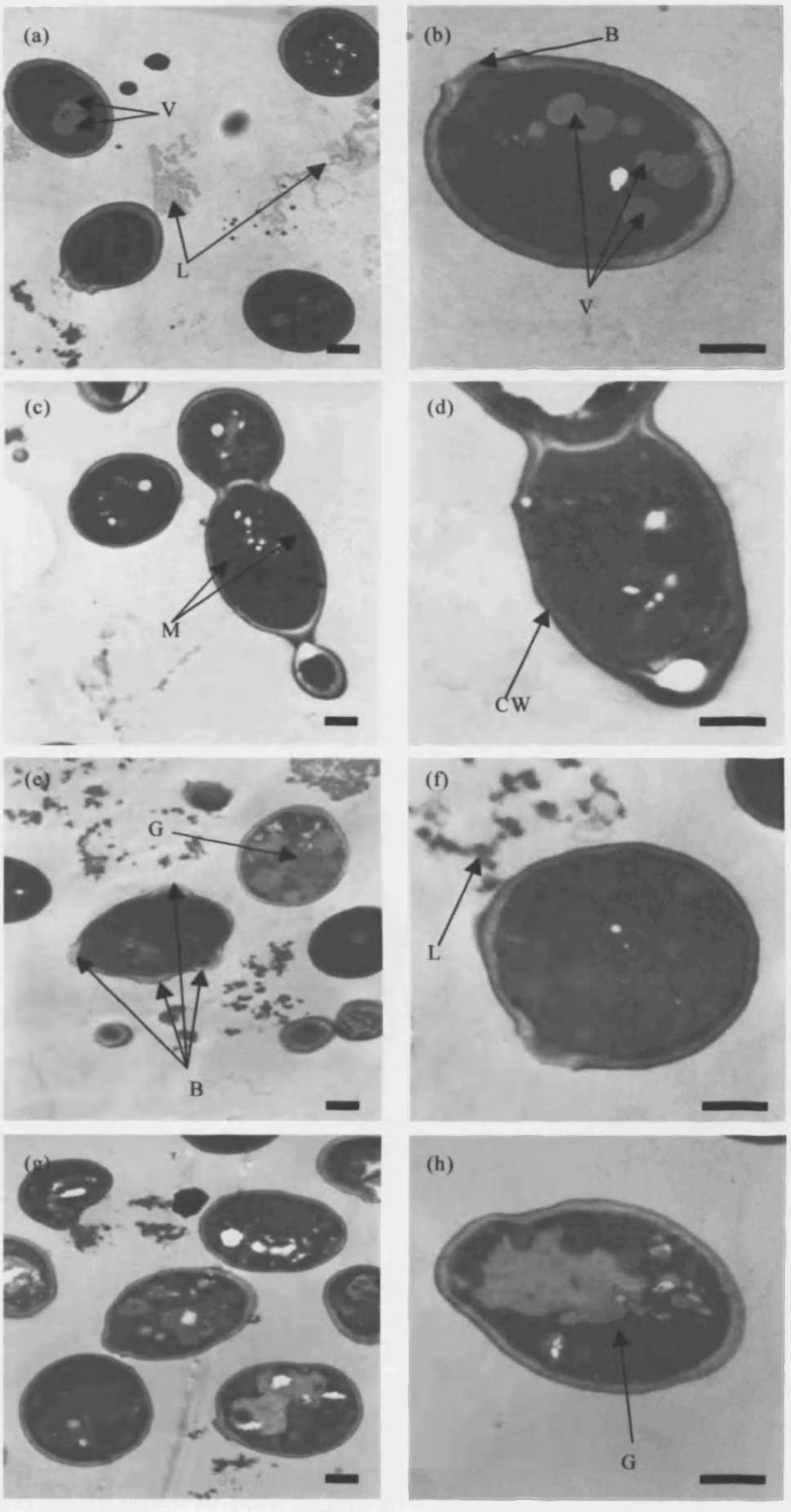


Results are representative of two independent experiments (four replicates) with standard errors <8%.

Figures 5.18-5.21 shows the results of TEM and SEM analysis for the effects of nonanoic acid on yeast cell structure. Nonanoic acid at a concentration of 0.2 mM resulted in cell leakage for all four yeasts, being most pronounced in *Z. kombuchaensis* as confirmed by TEM and SEM (Figures 5.18-5.19). The SEM images for *Z. kombuchaensis* (e-f) also showed some modulation of cell shape as a consequence of nonanoic acid exposure. TEM analysis reveals a granular image for all yeasts with evidence of increased vacuole formation. Mitochondria (M) are evident for some cells of each yeast but lack clear cristae. There is some evidence of cell wall (CW) modulation for both *Z. bailii* NCYC 1416 and *Z. kombuchaensis*. The effects of 0.4 mM nonanoic acid on *Z. bailii* NCYC 1766 and *Z. kombuchaensis* are similar to those of 0.2 mM nonanoic acid with a slight increase in cell leakage and debris (Figures 5.20-5.21). *Z. bailii* NCYC 1416 is more granular in the presence of 0.4 mM nonanoic acid, with no evidence of cell organelles. *S. cerevisiae* also contains a very granular cytoplasm with evidence of the cytoplasm separating from the periplasm. *Z. bailii* NCYC 1416 showed increased levels of cell leakage with 0.4 mM nonanoic acid (Figure 5.21).

5.2.4 Differences in sensitivity to cell wall/membrane disrupting agents between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

In *S. cerevisiae* the application of a number of chemicals or drugs has been applied to identify cell wall mutants. The results presented in Figure 5.22 show the differences in sensitivity between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* to several of these common chemicals. The aim being to identify possible differences in the cell wall composition between these yeasts. The control plate (a) shows that *Z. bailii* NCYC 1766, *Z. kombuchaensis* and *S. cerevisiae* showed good growth at all four dilutions. *Z. bailii* NCYC 1416, an aggregating yeast showed good growth for the first two dilutions but poor growth at the final two dilutions. Calcofluor white has been used to identify all


(a)
V
L
(b)
B
V
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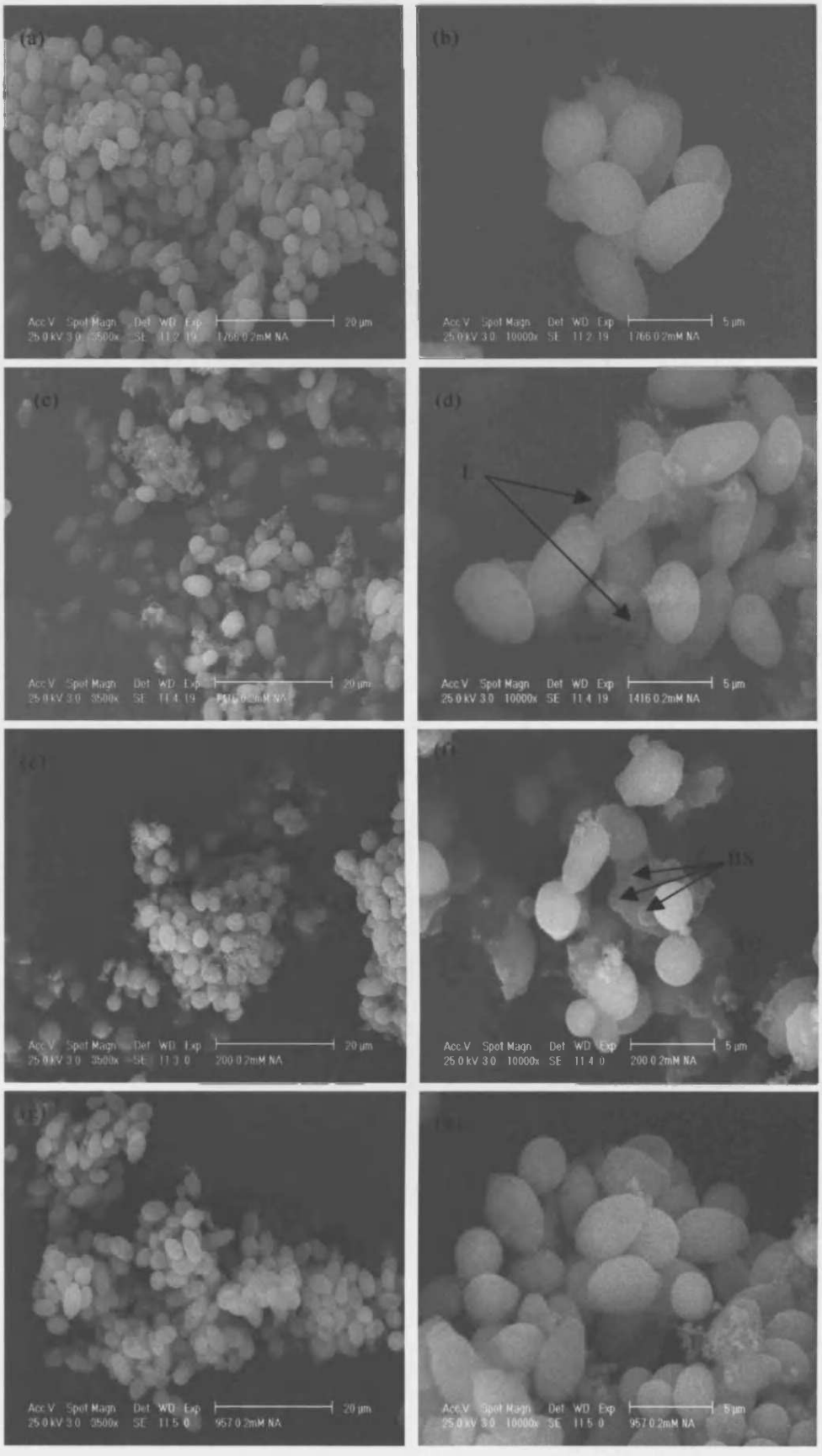
M
(d)
CW
(e)
G
B
(f)
L
(g)
(h)
G


(a)
(b)
(c)
(d)
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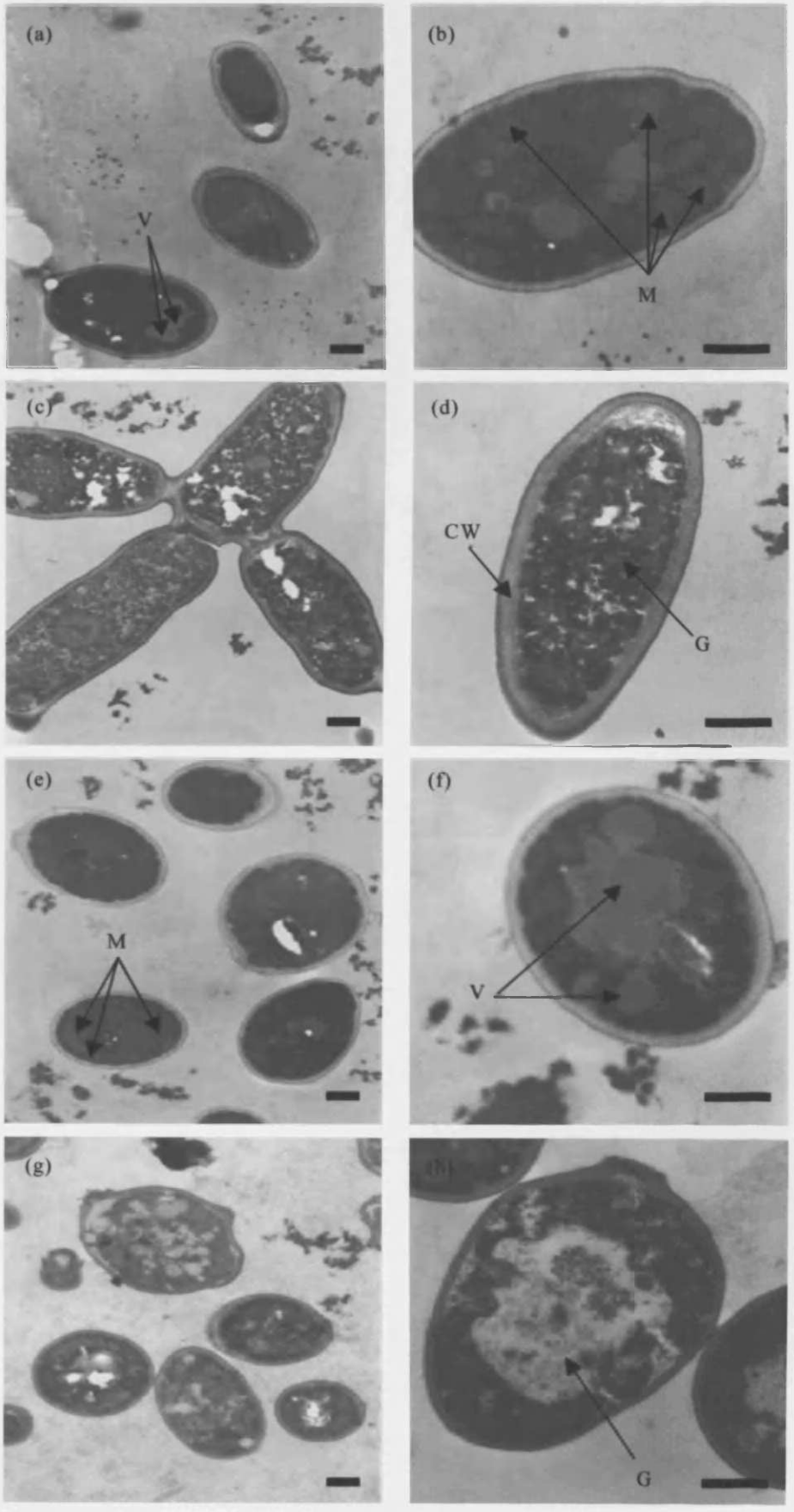
(h)


(a)
V
(b)
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(c)
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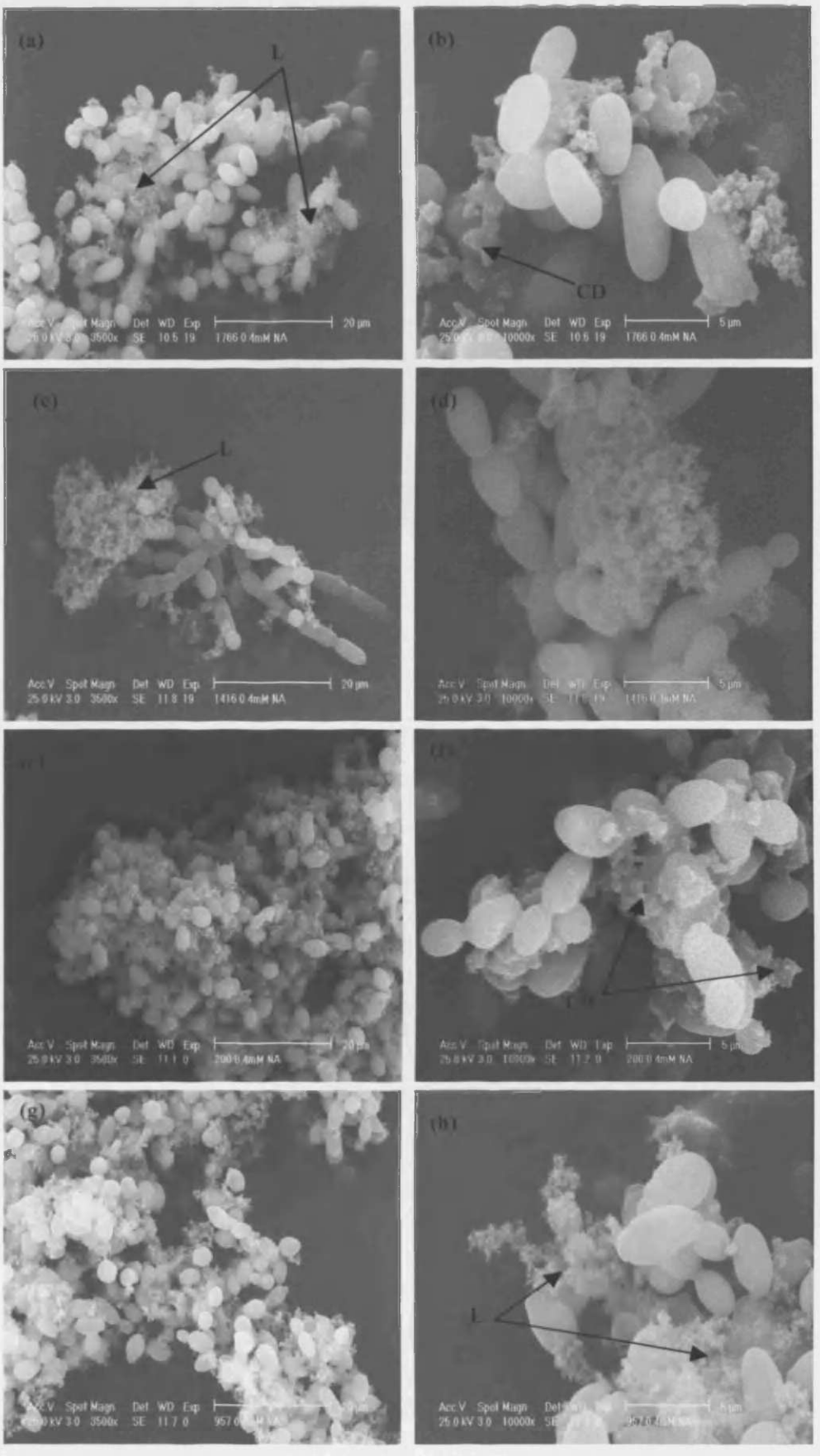
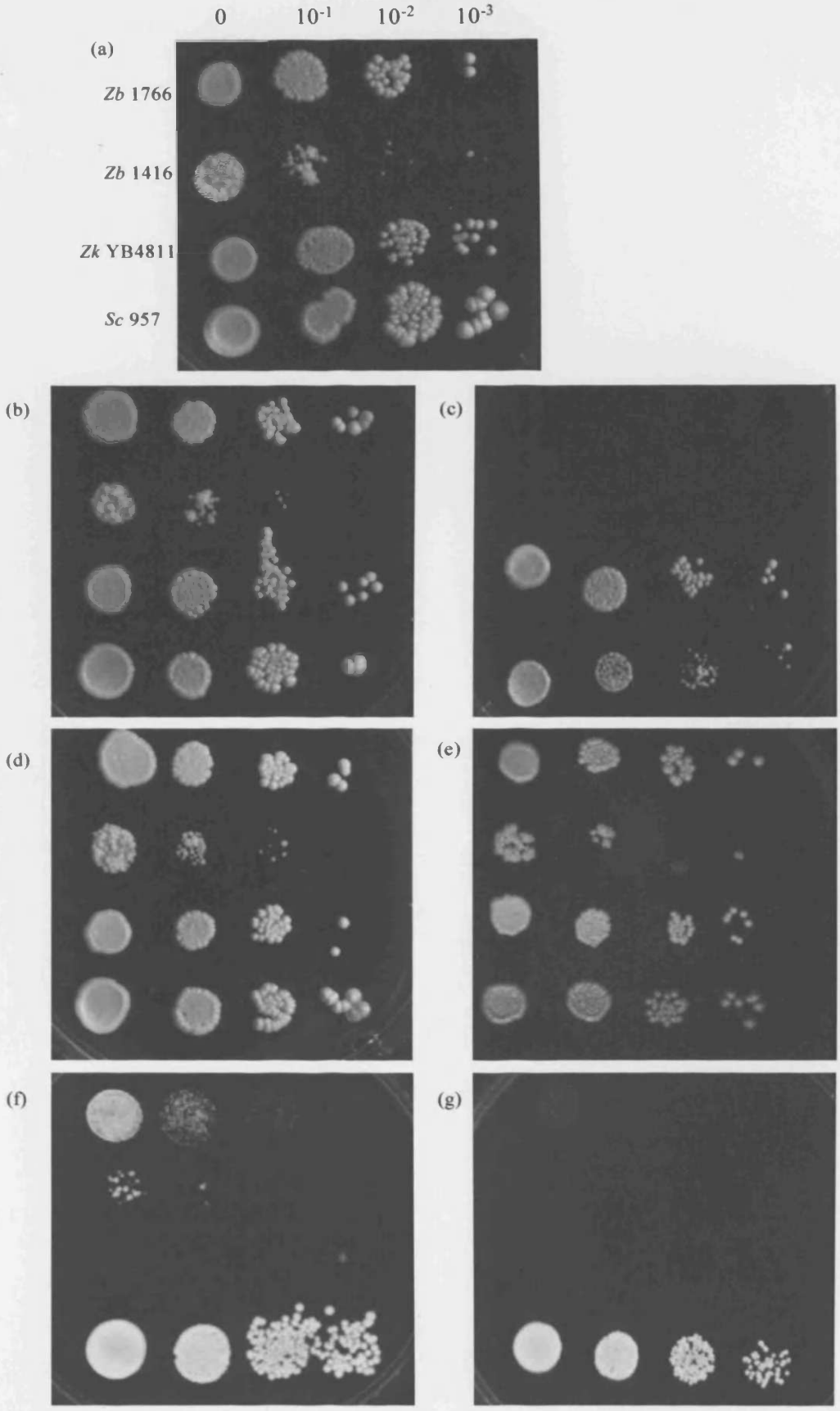
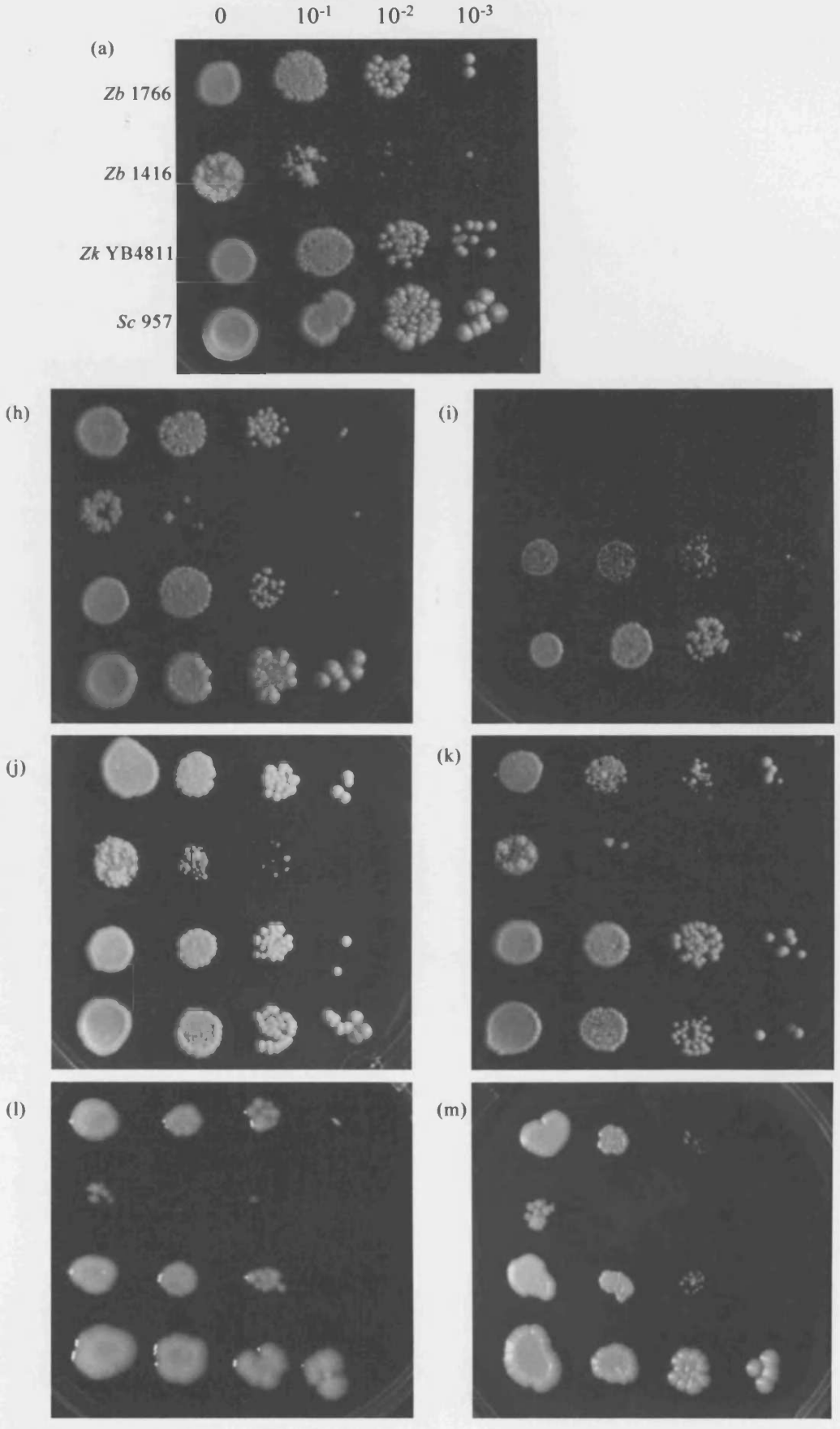
CW
G
(e)
M
(f)
V
(g)
(h)
G


(a)
L
(b)
CD
(c)
L
(d)
(e)
(f)
(g)
(h)
L

types of cell wall mutants except for those deficient in chitin in *S. cerevisiae* (Ram *et al.,* 1994). The results showed that both *Z. bailii* strains were more sensitive to Calcofluor white (b-c). Congo red is used to detect cell wall mutants defected in β 1, 3-glucan synthesis. No difference in sensitivity between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* were found at congo red concentrations of 0.025 and 0.25 mg/ml (d-e). SDS is used for the identification of cell wall mutants with possible defections in cell wall permeability. The results (f-g) show that *S. cerevisiae* was able to grow at both 0.005% and 0.05% SDS concentrations with *Z. bailii* NCYC 1766 exhibiting partial growth and *Z. bailii* NCYC 1416 growth at zero dilution on 0.005% SDS. *Z. kombuchaensis* proved the most sensitive to SDS showing no growth at either SDS concentrations. Sensitivity to caffeine is indicative of potential differences in cell wall assembly through the cell integrity pathway *PKC1*-controlled MAP kinase pathway (Costigan *et al.,* 1992). Both strains of *Z. bailii* proved caffeine sensitive showing diminished growth at 1.0 mg/ml and no growth at a caffeine concentration of 2.0 mg/ml. *Z. kombuchaensis* also showed sensitivity to caffeine but to a smaller extent than *Z. bailii. S. cerevisiae* showed growth at both concentrations of caffeine. The caffeine sensitive phenotype exhibited by *Z. bailii* and *Z. kombuchaensis* was partially rescued by 2 M sorbitol (data not shown). Sodium orthovanadate has been used in the identification of possible mutants defected in glycocosylation (Dean, 1999). The four yeasts used in this study showed no differences in sensitivity to the two concentrations of sodium orthovanadate used (j-k). Hypersensitivity to calcium, which is related to morphogenetic events, has been applied as a means of identifying cell wall mutants (Ruiz *et al.,* 1999). *Z. bailii* NCYC 1416 was the most sensitive to calcium chloride with *Z. bailii* NCYC 1766 and *Z. kombuchaensis* proving slightly less sensitive. *S. cerevisiae* was the most resistant to both concentrations of calcium chloride. The *S. cerevisiae* strain used (which does not contain a Kanamycin insertion cassette) was also more resistant to the aminoglycolyside


0
10-1
10-2
10-3
(a)
Zb 1766
Zb 1416
Zk YB4811
Sc 957
(b)
(c)
(d)
(e)
(f)
(g)


0
10-1
10-2
10-3
(a)
Zb 1766
Zb 1416
Zk YB4811
Sc 957
(h)
(i)
(j)
(k)
(l)
(m)

G418 (data not shown). The results collectively point to potential differences in the cell wall and possibly membrane structure of *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*.

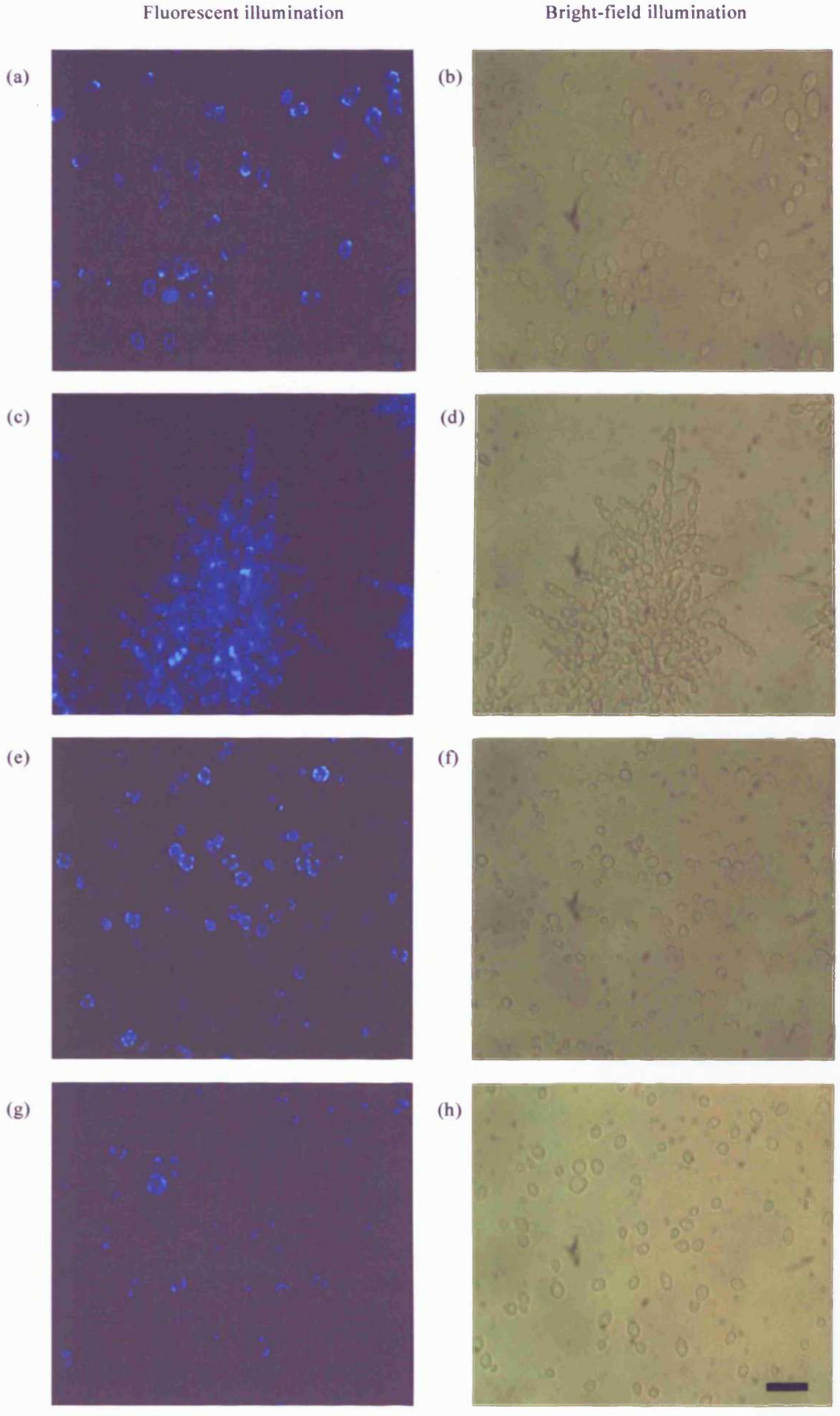
5.2.5 Chitin analysis in *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

5.2.5.1 Chitin distribution

Calcofluor white is a fluorophore used for the staining of bud scars and other chitin rich areas within yeast cells (Pringle *et al.,* 1991). Calcofluor white was applied to *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* to look for differences in chitin distribution (Figure 5.23). *Z. bailii* NCYC 1416 gave the most fluorescent cells followed by *Z. bailii* NCYC 1766, *Z. kombucahensis* and *S. cerevisiae*. The distribution of chitin on the basis of fluorescence appeared to be all over the cell for *Z. bailii* (both strains) and *Z. kombuchaensis* with fluorescence being restricted to bud scars for *S. cerevisiae*. Therefore, on the basis of Calcofluor white fluorescence, the distribution of chitin appears to differ between the *Zygosaccharomyces* yeasts and the *S. cerevisiae* strain examined.

5.2.5.2 Chitin content

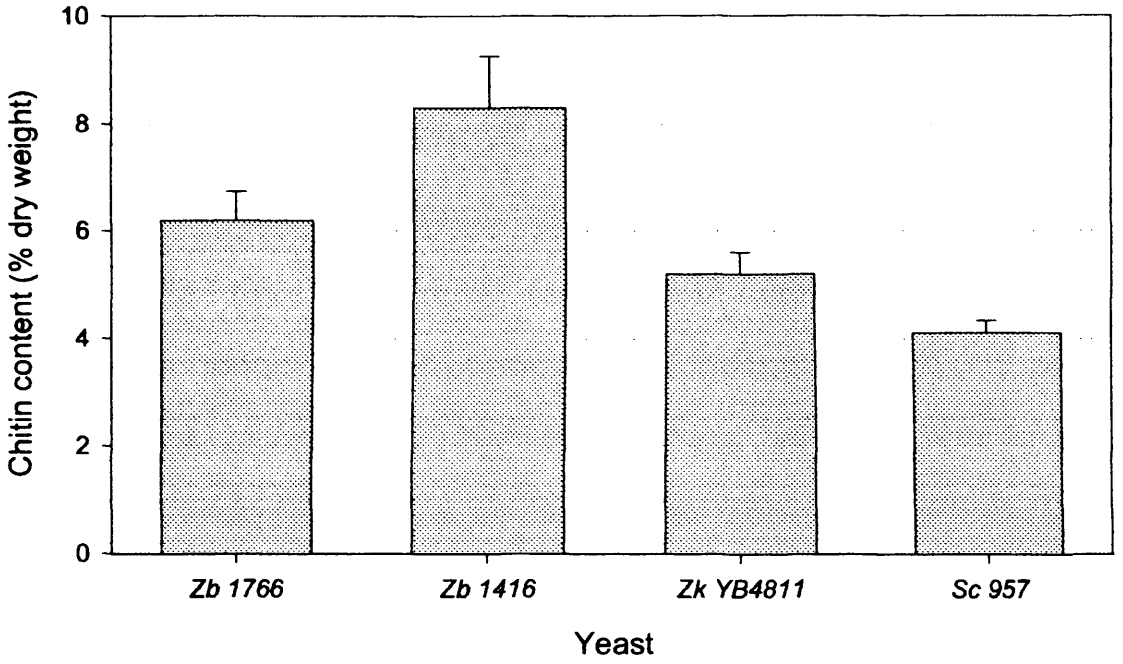
In a bid to confirm the chitin distribution results an analysis of chitin content was performed on the four yeast species growing in YPD pH 4.0, using a method based on that of Ride and Drysdale (1972). Figure 5.24 shows the results obtained for *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*. The two *Z. bailii* strains both contained the highest levels of chitin, with *Z. bailii* NCYC 1416 having the highest level. *S. cerevisiae* contained the least amount of chitin expressed as a percentage of dry weight, with *Z. kombuchaensis* showing an intermediate level. The chitin content results support the quantitative differences seen in chitin distribution between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*.


Fluorescent illumination
Bright-field illumination
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)

5.2.6 *S. cerevisiae* cell wall mutants and organic acid resistance

A lack of cell wall mutants for *Z. bailii* and *Z. kombuchaensis,* meant that *S. cerevisiae* cell wall mutants were used for elucidating whether cell wall composition can influence yeast organic acid resistance. A selection of mutants (EUROSCARF) all defective in a particular aspect of cell wall construction were selected and treated to short, medium and longer chain organic acids as described in section 2.4. The genes are involved in the following aspects of cell wall construction; *FKS1* is involved in β 1, 3-glucan synthesis; *KRE6* β 1, 6-glucan synthesis; *MNN9* mannoprotein construction and *CHS3* chitin biosynthesis. Table 5.2 shows the MICs obtained for each of the cell wall mutants and the wild-type BY4741. The MICs obtained for medium (sorbic and benozoic) acids and the longer chain (nonanoic) acid showed little difference between wild-type and mutants. The MICs obtained for the two short chain (acetic and propionic) organic acids showed differences between wild-type and mutants. *fks1* gave MICs similar to those of the wild-type, exhibiting only a slightly higher MIC for acetic acid. The other cell wall mutants, *kre6, mnn9* and *chs3* gave MICs greater than those for the wild-type by 10-20 mM (two to four culture differences). Both wild-type and mutants exhibited good growth at sub-inhibitory organic acid concentrations (data not shown). The results show that differences in cell wall composition can influence yeast organic acid resistance.

Figure 5.24 Differences in chitin content between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* grown in YPD pH 4.0 at 25°C without shaking and examined at 48 h incubation.



Results are the means of three independent cultures each performed in duplicate and converted to % chitin per mg dry weight, ± the standard error.

Table 5.2 Differences in organic acid resistance (mM) between *S. cerevisiae* cell wall mutants

	BY4741	***fks1***	***kre6***	***mnn9***	***chs3***
Acetic acid	100 ± 4.08	110 ± 7.07	130 ± 4.08	130 ± 4.08	130 ± 5.77
Propionic acid	60 ± 5.77	60 ± 4.08	70 ± 0.00	70 ± 0.00	80 ± 7.07
Sorbic acid	3.5 ± 0.20	3.5 ± 0.46	4.0 ± 0.91	3.5 ± 0.91	4.0 ± 0.20
Benzoic acid	4.0 ± 0.00	4.0 ± 0.00	4.0 ± 0.20	4.0 ± 0.46	4.0 ± 0.46
Nonanoic acid	0.5 ± 0.04	0.5 ± 0.00	0.5 ± 0.04	0.6 ± 0.09	0.6 ± 0.04

Values are mean MICs measured from at least two independent experiments (four replicates) following 14 days incubation at 25°C, ± the standard error.

5.3 Discussion

In this study, the effects of acetic, sorbic and nonanoic acid on yeast cell structure were shown to differ. The effects of acetic acid on yeast cell structure included the cytoplasmic contents becoming very granular with poor definition of organelles. The cell wall remained largely unaffected at all concentrations of acetic acid. SEM analysis at for *S. cerevisiae* and *Z. bailii* NCYC 1766 showed evidence of cell lysis. The ability of acetic acid to induce programmed cell death has recently been reported for both *S. cerevisiae* (Ludovico *et al.,* 2001) and *Z. bailii* (Ludovico *et al.,* 2002). The morphological change induced by acetic acid to *Z. bailii* NCYC 1766 is to the best of my knowledge, the first time that morphological change has been reported for *Z. bailii*. Sorbic acid treatment caused some structural changes different to those caused by acetic acid. *Z. kombuchaensis* was the most sensitive of the yeasts to sorbic acid. The effects of sorbic acid appeared to be concentrated on the cell wall and plasma membrane with alterations to both evident. The action of sorbic acid has been questioned in recent years with speculation that it does not act as a 'classic weak-acid preservative' but acts more on membrane disruption (Stratford and Anslow, 1996b, 1998). The data provided from this study is believed to be the first direct evidence for sorbic acid actually acting primarily as a membrane active substance. The work presented in this section for longer chain organic acids focussed on the less studied nonanoic acid. Nonanoic acid caused the cytoplasm of all four yeasts to become granular with little definition of organelles. The shape of the cells also became slightly altered with evidence of cell leakage. The images obtained for nonanoic acid are therefore in agreement with those reported for octanoic and decanoic acid with membrane alteration being the site of action (Viegas *et al.,* 1989; Viegas & Sá-Correira, 1995; Alexandre *et al.,* 1996; Stratford and Anslow, 1996; Cabral *et al.,* 2001). The mitochondria appear to be severely disrupted by the actions of all organic acids, as previously reported (Cole, 1987c).

Previous studies on yeast organic acid resistance involving *Z. bailii* and *S. cerevisiae* have not focused on differences in cell structure; even though differences in structure could relate to their ability to reduce acid influx, which has recently been identified as a potentially crucial factor in the ability of yeasts to tolerate high levels of organic acids (Piper *et al.,* 2001). In this study, differences were shown relating to cell size, shape, cell wall thickness and cell wall composition of *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*. The *Zygosaccharomyces* yeasts contained cells with a thicker and more electron dense cell wall than *S. cerevisiae*. Despite recent innovations in the methods available to study the cell wall including the application of various analytical techniques: HPLC and GC-MS (Hong *et al.,* 1994; Mislovicova *et al.,* 2000; Magnelli *et al.,* 2001) the cell wall is still very difficult to analyze. In this study I examined the cell wall in terms of chitin distribution and content. The results showed that staining of chitin by Calcofluor white produced fluorescence all over the cell for the three *Zygosaccharomyces* yeast but appeared only at the bud scars for *S. cerevisiae*. These results were supported by analysis of chitin content in which *Z. bailii* NCYC 1416 had the highest chitin content and *S. cerevisiae* the lowest. Chitin is known to increase for *S. cerevisiae* on exposure to cell wall stress (Dallies *et al.,* 1998). The higher chitin levels as indicated from this study for *Z. bailii, Z. kombuchaensis* may contribute to differences in withstanding organic acid induced cell stress. The phenotypic plate assays showed potential differences in the cell walls of *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*. Sensitivity to Calcofluor white and SDS are indicative of differences in chitin and permeability properties, while caffeine and calcium chloride sensitivity indicate possible differences in cell wall assembly and morphogentic events. A recent study has looked at the effects of growth conditions on yeast cell wall composition and structure, in *S. cerevisiae,* concluding that growth conditions can alter cell wall

composition (Aguilar-Uscanga and Francois, 2003). A similar study for *Z. bailii* and *Z. kombuchaensis* may prove particularly fruitful for the food industry.

In a bid to determine whether modulation of the cell wall could alter organic acid resistance, a selection of *S. cerevisiae* cell wall mutants were exposed to various organic acids and their MICs determined. The mutants selected *fks1, kre6, mnn9* and *chs3* represent different aspects of cell wall construction and have recently been used in a study on genome wide analysis of cell wall mutations in *S. cerevisiae* (Lagorce *et al.,* 2003). The results show that the mutants overall were equally resistant to medium and longer chain organic acids as the wild-type. In the case of the short chain organic acids (acetic and propionic acid), mutants were generally more resistant. This provides direct evidence that in *S. cerevisiae,* differences in the cell wall composition can lead to differences in organic acid resistance.

Any differences in cell wall composition between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* could also lead to the development of new preservation strategies. This study has shown both strains of *Z. bailii* be more sensitive to Calcofluor white than *S. cerevisiae.* It is therefore possible that chitin synthesis could be a target to prevent *Z. bailii* food spoilage. Nikkomycin Z, Fluconazole and Itraconazole are all drugs that target chitin synthesis and have been shown to work against the human pathogen *C. albicans* (Kim *et al.,* 2002; Li and Rinaldi, 1999). The fact that these are effective against *C. albicans* makes their inclusion in food unlikely but the principle remains. Chitosan, a deacetylated form of chitin, has been shown to have antifungal activity of its own (Rhoades and Roller, 2000) and increases the antimicrobial action of sodium benzoate on spoilage yeasts (Sagoo *et al.,* 2002). Indeed, Bom *et al.,* (2001) targeted the

cell wall of *Z. bailii* by interfering with GPI-CWPs; unfortunately, due to the lack of information on the cell wall of *Z. bailii* this study is the only one of its kind thus far.

In summary, the actions of short, medium and longer chain organic acids on yeast cell structure have been examined with the first direct evidence for the actions of sorbic acid being illustrated. The cell wall has been highlighted as an area of particular interest with a possible role in organic acid resistance, while at the same time representing a possible target for new preservation strategies. A detailed biochemical analysis of the cell wall of *Z. bailii* and *Z. kombuchaensis* is now required to elucidate its role in yeast attributed food spoilage.

6. Role of mitochondria in yeast organic acid resistance: the application of protoplast fusion

6.1 Introduction

Yeasts have traditionally been divided into two categories on the basis of their ability or inability to produce respiratory deficient mutants (Bulder, 1964). Yeasts which have the ability to form either spontaneously or when induced by various stress conditions respiratory deficient mutants which give rise to smaller (petite) colonies than wild-type and who are unable to produce ATP through respiration are classed as petite-positive. *S. cerevsiae* is known to readily give rise to petites (Piškur *et al.*, 1998). Petite-positive yeasts are characterised by large deletions in their mitochondrial DNA (*rho*$^-$) or a complete loss of mitochondrial DNA (*rho*^{o}). It is also possible to have petites as a result of nuclear mutations; these are referred to as nuclear petites (*pet*). Petite phenotypes can be induced by the intercalating agent ethidium bromide, and also at low levels by heat, ethanol and the preservatives sorbic and benzoic acid (Piper, 1999). The majority of yeasts can be classed as petite-negative due to their inability to form respiratory deficient mutants (Møller *et al.*, 2001). The greater dependence of petite-negative yeasts on the integrity of their mitochondrial DNA could be the result of the one or more of the following: an inability to achieve redox balance without fully functioning mitochondria, inability to generate a membrane potential in mitochondria lacking mitochondrial DNA and the need to form an active respiratory chain to disperse reducing substances from basal metabolism (Schafer, 2003). The possibility of non-mitochondrial encoded genes required for normal cellular functioning being dependent upon the presence of fully functioning mitochondria also exists. Indeed, only recently have developments into the physiological and biochemical basis of the petite-negative phenotype been made (Møller *et al.*, 2001; Fernet *et al.*, 2002; Schafer, 2003). It is now known that in the petite-negative yeast *Debaromyces occidentalis* the mitochondrial genome can be altered or lost without lethal effect (Fernet *et al.*, 2002). Therefore, the classification of yeasts as either petite-positive or petite-negative may be insufficient. Indeed, the focus

of research into understanding the role of mitochondria in different species seems to be focused on sequencing the mitochondrial genome. A number of mitochondrial genome sequences are already available including *S. cerevisiae* (Foury *et al.,* 1998), *Candida albicans* (Kerscher *et al.,* 2001), *Schizosaccharomyces pombe* (Bullerwell *et al.,* 2003) and *Candida glabrata* (Koszul *et al.,* 2003). The conclusions form the mitochondrial genomes available so far are that they show a high degree of diversity regarding size, gene content and organization (Koszul *et al.,* 2003).

This section describes the attempts to investigate the role of mitochondria in yeast organic acid resistance. The first part of this research focuses on the application of ethidium bromide to induce petites in *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*. Previous researchers have reported *Z. bailii* to be petite negative (Mollapour and Piper, 2001b; Merico *et al.,* 2003), but these authors used slightly differing methods to induce petites and examined different strains to those used in this study. The petite status of *Z. kombuchaensis,* however, has not been previously examined. The effect of the petite mutation on organic acid resistance in *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* is examined by challenge assays and hydrogen peroxide a known inducer of oxidative stress is also included. The stimulus for the research is that weak organic acid food preservatives have recently been shown to be pro-oxidants and mutagenic towards mitochondria (Piper, 1999). Therefore, under aerobic conditions these organic acids cause oxidative stress. Mitochondrial DNA is regarded to be particularly susceptible to the effects of oxidative stress due to a lack of protective histones and an incomplete mechanism of DNA proof reading (Kowaltowski *et al.,* 1999).

The second part of this section focuses on the application of protoplast fusion as an alternative approach in studying the involvement of mitochondria in yeast organic acid

resistance. Protoplast fusion as a technique represents an alternative means by which to perform genetic analyses on non-conventional (classed as non-*Saccharomyces*) and industrial yeasts (Zimmermann and Sipicki, 1996). The principle of protoplast fusion is that the yeast cell wall is removed enzymatically yielding protoplasts. Protoplasts are then maintained in a stabilised osmotic environment, normally containing sorbitol. Protoplasts are encouraged to aggregate and then fuse via the application of fusogenic agents, such as polyethylene glycol (PEG) and calcium chloride. The fusion of protoplasts allows the exchange of cytoplasms, organelles and nuclei of different strains. Fusants can be classified as intraspecific, interspecific or intergeneric depending upon the donor strains used in the fusion event. Protoplast fusions have been reported previously for a number of yeasts (Gumpert, 1980; Spencer and Spencer, 1981; Evans and Conrad, 1987; Philipova and Venkov, 1990; Heluane *et al.,* 1993; Lucca *et al.,* 1999; 2002). The aim of the protoplast fusion attempts was to obtain intergeneric hybrids between *Z. bailii-S. cerevisiae* and *Z. kombuchaensis-S. cerevisiae.*

The final part of the section focuses on the characterization of protoplast fusants. The hybrids were characterized in terms of morphology, physiology, petite forming capabilities, organic acid resistance and organic acid utilization. The hybrids were characterized in the aforementioned parameters in a bid to compare the differences and or similarities between donor and hybrids and how they may reflect on organic acid resistance capabilities.

6.2 Results

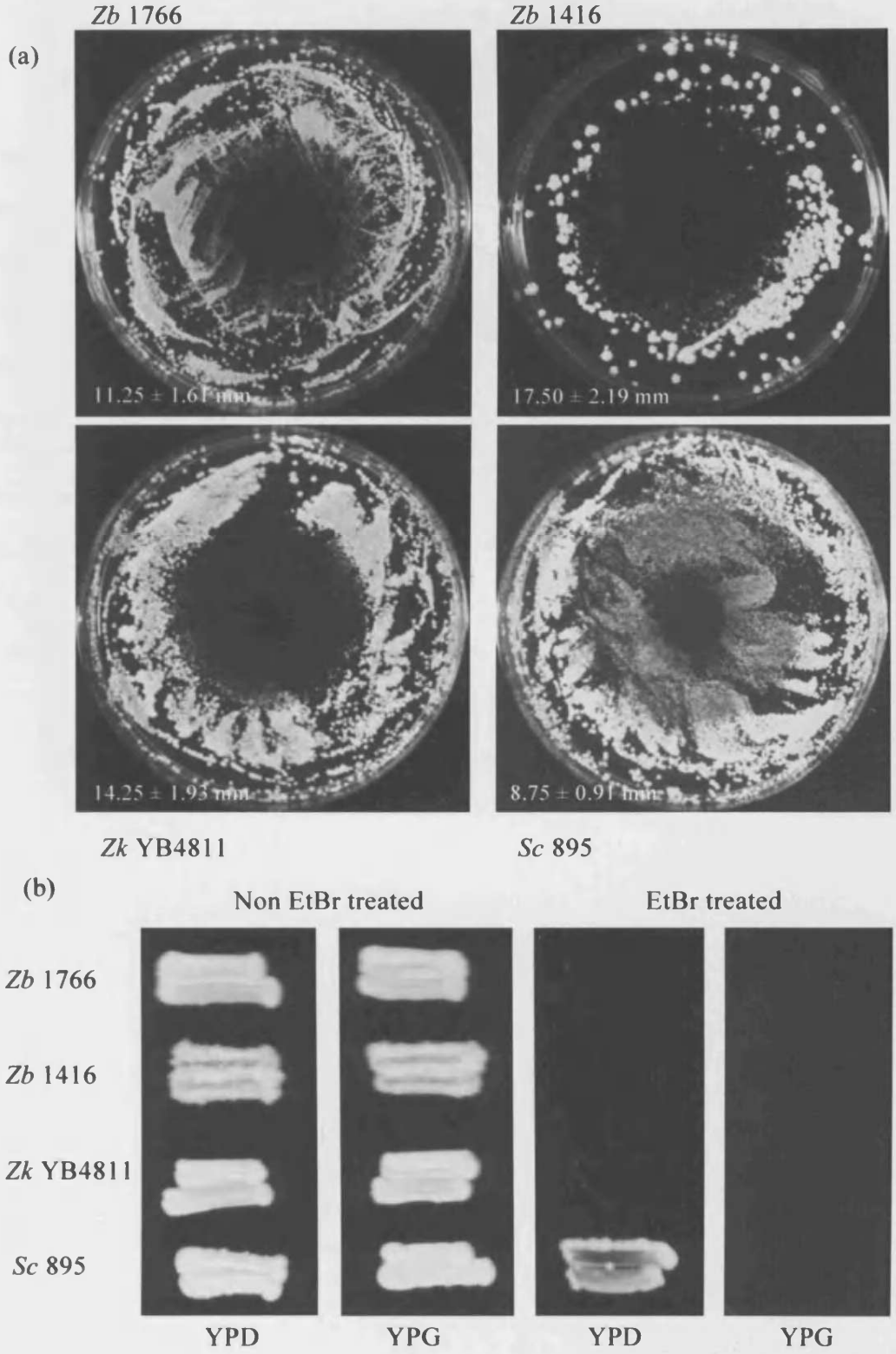
6.2.1 Differences in ethidium bromide treatment between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

Ethidium bromide was used to elucidate the petite forming capabilities of *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*. Both *Z. bailii* and *Z. kombuchaensis* were more sensitive to ethidium bromide than *S. cerevisiae* (Figure 6.1). *Z. bailii* NCYC 1416 proved the most sensitive to ethidium bromide treatment having an average zone of inhibition of 17.5 mm (radius) in comparison to 11.25 mm for *Z. bailii* NCYC 1766 and 14.25 mm for *Z. kombuchaensis* NRRL YB4811. *S. cerevisiae* had the smallest inhibition zone of 8.75 mm. Ethidium bromide treatment induced small colonies for all four yeasts. Selecting several hundred small colonies from ethidium bromide treated plates and examining for growth on non-fermentable carbon sources assessed petite forming capabilities. Figure 6.1 shows that the small colonies from ethidium bromide treated plates for both *Z. bailii* and *Z. kombuchaensis* were non-viable, exhibiting no growth on YPD plates. The small colonies selected for *S. cerevisiae* were viable on YPD but non-viable on non-fermentable carbon sources (glycerol and ethanol). *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* non-ethidium bromide treated cells exhibited poor growth on acetate plates. Acetate was therefore not examined as a non-fermentable carbon source. Several other strains from each species were also screened for petite formation, with the same results (data not shown). *Z. bailii* and *Z. kombuchaensis* were therefore classed as petite-negative and *S. cerevisiae* as petite-positive.

6.2.2 Organic acid resistance in *S. cerevisiae* grande and petite cells

The petite cells of several strains of *S. cerevisiae* were compared to grande cells for differences in organic acid resistance. The *S. cerevisiae* strains selected represent strains associated with fermentation in lager (NCYC 1324) and ale (NCYC 1119). In addition

Figure 6. 1 *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* (a) differences in sensitivity to ethidium bromide (b) petite forming capabilities.



Zones of inhibition were calculated using 10 µl of 10 mg ml^{-1} ethidium bromide in the centre of each plate and the images are representative of an experiment based on four replicates, ± the standard error.

Table 6.1 Differences in organic acid resistance (mM) between *S. cerevisiae* grande and petite cells

	NCYC 957	NCYC 1324	NCYC 1119	CDN1	JRD895
Grande					
Acetic acid	115 ± 10.20	75 ± 10.20	100 ± 10.20	100 ± 0.00	100 ± 10.20
Propionic acid	50 ± 4.08	50 ± 8.16	50 ± 4.08	50 ± 4.08	50 ± 4.08
Sorbic acid	4.0 ± 0.00	3.0 ± 0.33	4.0 ± 0.58	3.0 ± 0.33	3.0 ± 0.58
Benzoic acid	3.0 ± .041	3.0 ± 0.41	3.0 ± 0.33	3.5 ± 0.00	3.0 ± 0.00
Nonanoic acid	0.5 ± 0.00	0.5 ± 0.02	0.5 ± 0.04	0.5 ± 0.00	0.5 ± 0.00
Petite					
Acetic acid	115 ±10.20	95 ± 0.00	90 ± 10.20	105 ± 10.20	105 ± 10.20
Propionic acid	50 ± 0.00	45 ± 4.08	50 ± 8.16	50 ± 0.00	50 ± 4.08
Sorbic acid	3.5 ± 0.71	3.5 ± 0.58	3.0 ± 0.58	3.0 ± 0.00	3.5 ± 0.00
Benzoic acid	3.5 ± 0.41	3.5 ± 0.58	3.0 ± 0.00	3.0 ± 0.41	3.0 ± 0.58
Nonanoic acid	0.5 ± 0.00	0.3 ± 0.04	0.4 ± 0.02	0.4 ± 0.04	0.4 ± 0.02

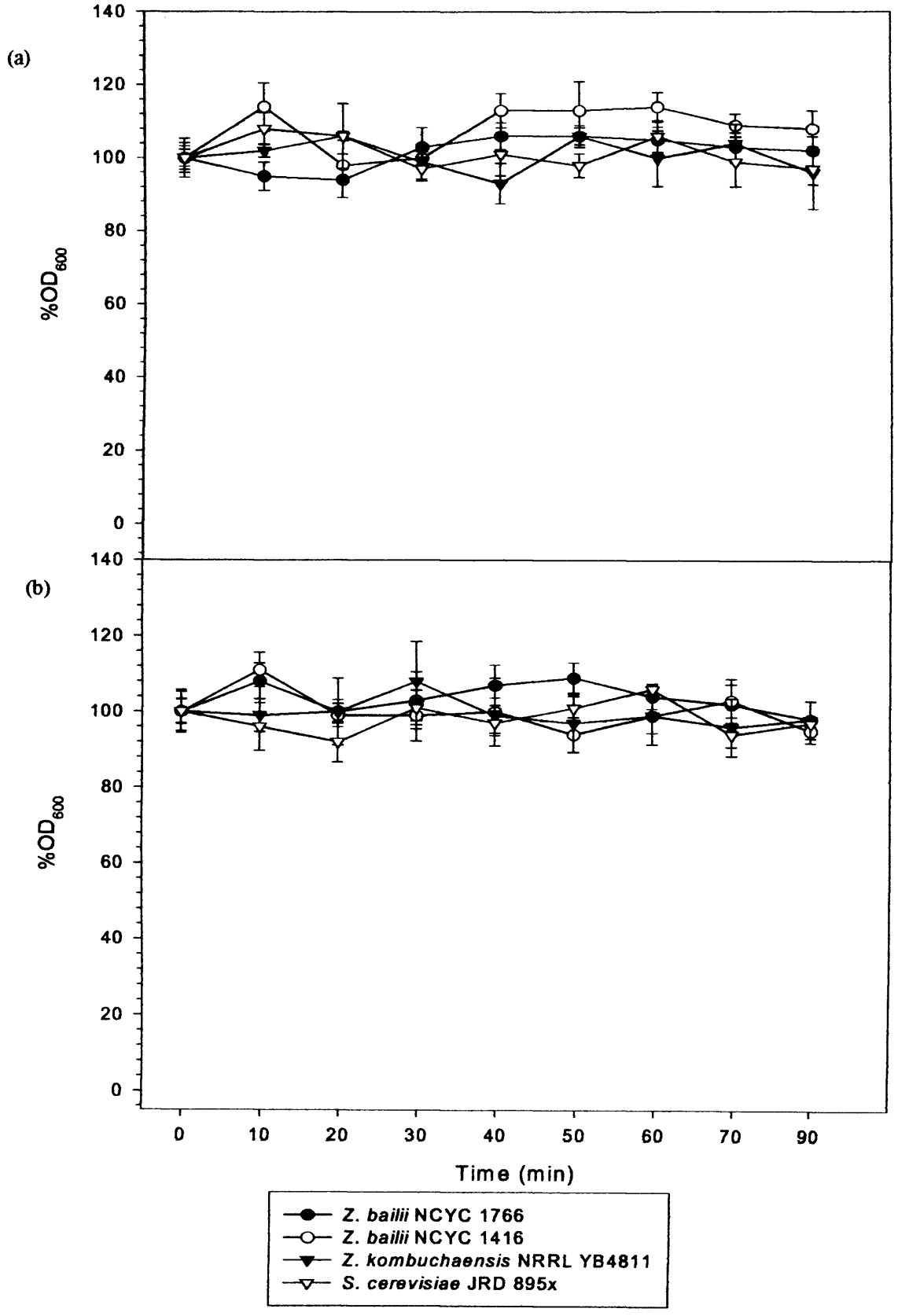
Values are mean MICs measured from at least two independent experiments (four replicates) in YPD pH 4.0 following 14 days incubation at 25°C, ± the standard error.

to a strain (CDN1) which is a diploid created from a cross between the two commonly used laboratory strains NCYC 957 and BY4741 (kindly constructed by J. R. Dickinson, Cardiff University, UK). Finally, strain 895 has been included as a strain that carries a convenient marker for protoplast fusion (section 6.2.4). Table 6.1 shows the MICs of short, medium and longer chain organic acids against both grande and petite *S. cerevisiae* cells. The MICs overall for both grande and petite cells being similar. The biggest difference in MIC between grande and petite cells is shown for strain NCYC 1324 on exposure to acetic acid. The grande MIC is 75 mM in comparison to 95 mM for the petite result. The difference in MICs for NCYC 1324 to acetic acid is representative of a difference in growth of 3-4 cultures. The MICs obtained for the other yeasts for all three classes of organic acids only show a small difference being indicative of a difference in growth of 1-2 cultures. The pattern of longer chain organic acids being the most inhibitory as shown by the lowest MICs and short chain organic acids the least inhibitory, remains constant for both grande and petite cells. In summary, *S. cerevisiae* petite cells appear to demonstrate an equivalent level of resistance to organic acid acids as grande cells.

6.2.3 Yeast protoplast formation

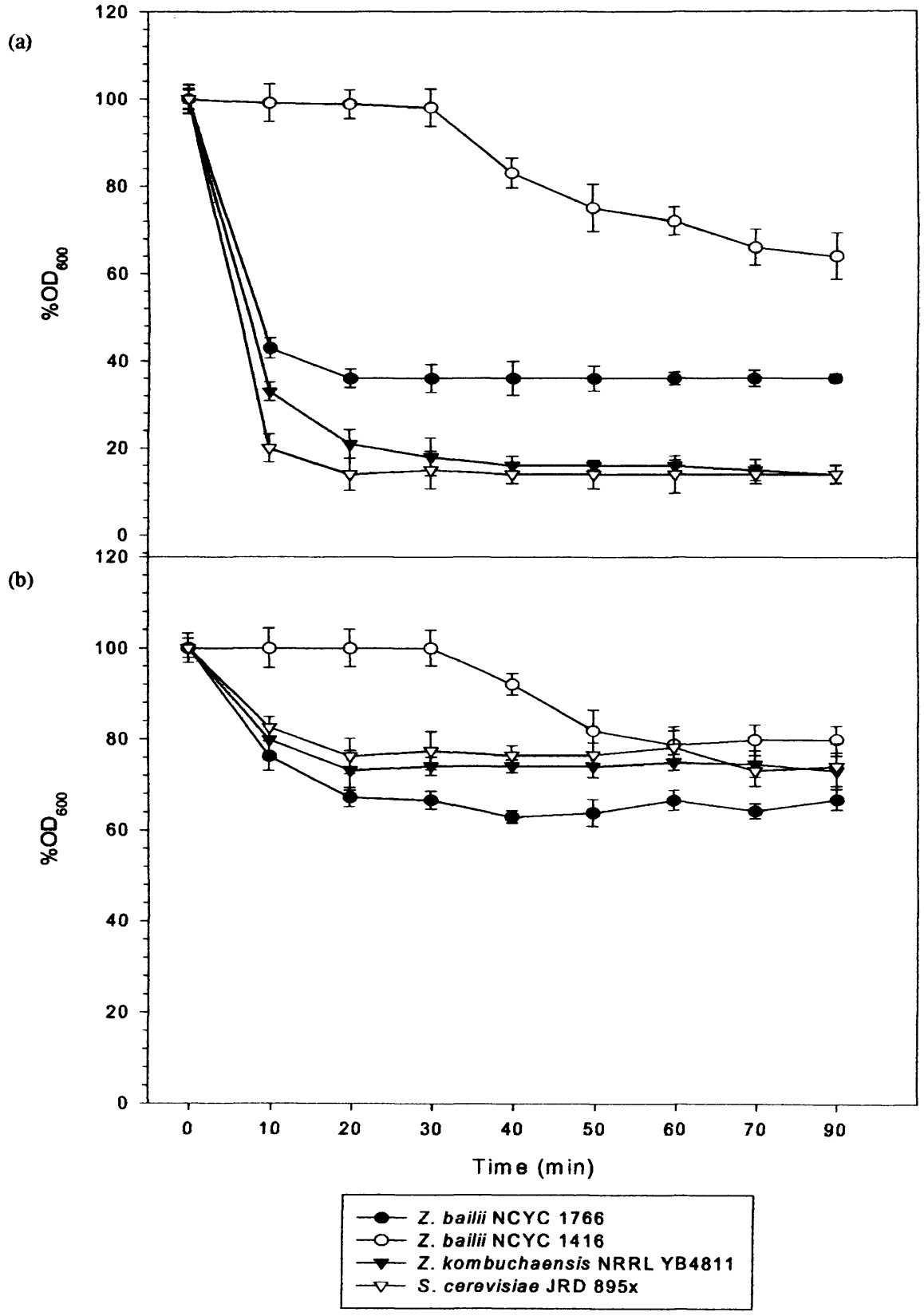
The previous results showed *S. cerevisiae* to be petite-positive with petites having a level of organic acid resistance equal to that of grande cells. The role mitochondria have if any in the petite-negative yeasts *Z. bailii* and *Z. kombuchaensis* remained unknown. Therefore, it was decided to attempt to create fused protoplasts for *Z. bailii* and *Z. kombuchaensis* with a petite *S. cerevisiae* and then attempt to create petites in any protoplast fusants. A precursor for protoplast fusion is the generation of a sufficient number of protoplasts. Attempts were therefore made to induce protoplasts in the yeasts *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*. At the concentrations used β-

Figure 6.2 Protoplast formation for *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* in (a) 100 µl ml^{-1} β-glucoronidase (b) 10 mg ml^{-1} lysozyme



Results are the mean of an experiment performed in triplicate, ± the standard error.

Figure 6.3 Protoplast formation using 1mg ml^{-1} Zymolyase (Lyticase, Sigma) for *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* in (a) distilled water (b) 2 M sorbitol



Results are the means of an experiment performed in triplicate, ± the standard error.

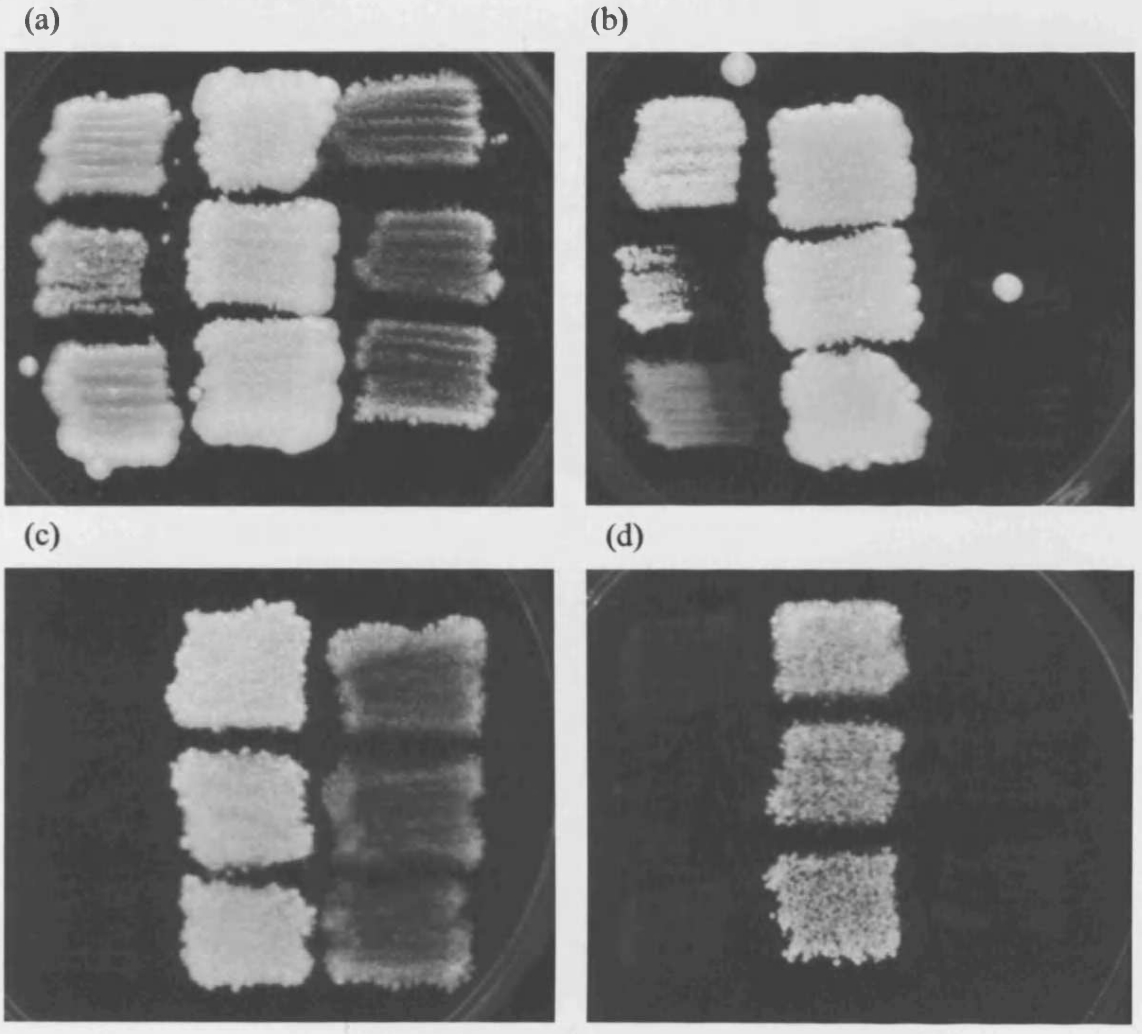
glucoronidase and lysozyme failed to induce protoplasts, as shown by the %OD which remained constant over the time frame examined (Figure 6.2). Zymolyase at the concentration indicated in Figure 6.3 induced protoplasts for *Z. bailii, Z. kombuchaensis* and *S. cerevisiae. Z. kombuchaensis* and *S. cerevisiae* were the most sensitive to the effects of Zymolyase as shown by them having the greatest fall in %OD. The two *Z. bailii* strains examined proved more resistant to the effects of Zymolyase, with NCYC 1416 showing the smallest change in %OD, therefore, being the most resistant. Even though *Z. bailii* NCYC 1416 proved the most resistant to protoplast formation, sufficient protoplasts were still generated. Protoplast formation was also confirmed microscopically for all four yeasts. Sorbitol is commonly used to prevent the lysis of protoplasts and was used in this study for that purpose. A concentration of 1 M sorbitol prevented lysis of *S. cerevisiae* protoplasts but a concentration of 1.5 M was required for *Z. kombuchaensis*. Protoplast lysis for the *Z. bailii* strains was only reduced in the presence of at least 2 M sorbitol (Figure 6.3). Zymolyase was therefore chosen for creating protoplasts in *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*.

6.2.4 Yeast protoplast fusion

Two *Z. bailii* strains were selected as donors one, which is very resistant to organic acids (NCYC 1766) and one more sensitive (NCYC 1416). The *Z. kombuchaensis* donor was NRRL YB4811. The *S. cerevisiae* donor was the petite version of 895, which contains a Kanamycin insertion cassette and therefore provides a marker for protoplast fusion in the form of resistance to the aminoglycoside G418. The basis of the fusion for both *Z. bailii* and *Z. kombuchaensis* with *S. cerevisiae* was that the *Zygosaccharomyces* yeasts would provide the ability to grow on non-fermentable carbon sources as they contain mitochondria. *S. cerevisiae* would provide the ability to grow in the presence of G418. Any protoplast fusants between *Zygosaccharomyces* and *S. cerevisiae* would,

Figure 6.4 Confirmation of protoplast fusion (a) YPD (b) YPG (c) YPD + 200 µg/ml G418 (d) YPG + 200 µg/ml G418 plates incubated for 5 days at 25°C incubation

Zb 1766	Pf 1766-895xH1	*Sc* 895x
Zb 1416	Pf 1416-895xH1	*Sc* 895x
Zk YB4811	Pf YB4811-895xH1	*Sc* 895x



therefore, be able to grow on non-fermentable carbon sources containing G418. Attempts to produce protoplast fusants for *Z. bailii* and *Z. kombuchaensis* with *S. cerevisiae* were successful as shown in Figure 6.4. A number of intergeneric protoplast fusants were produced for each attempted fusion (Table 6.2). The fusion of protoplasts between *Z. kombuchaensis* and *S. cerevisiae* generated the greatest number of fusants with *Z. bailii* NCYC 1416 and *S. cerevisiae* generating the fewest. One attempt at creating fusants using *Z. bailii* NCYC 1416 as the donor *Zygosaccharomyces* resulted in no protoplast fusants. Putative protoplast fusants were selected and taken through several rounds of marker selection to confirm stability of fusants. Finally, three fusants from each of the attempted protoplast fusions were selected for characterisation.

6.2.5 Characterisation of protoplast fusants

6.2.5.1 Morphology of protoplast fusants

The morphology of the protoplast fusants created between strains of *Z. bailii* and *Z. kombuchaensis* with that of *S. cerevisiae* was compared to that of their donor strains. Table 6.3 summarizes some of the differences in morphology between the protoplast fusants and donors. All protoplast fusants from the three different fusions showed the same morphological attributes and resemble the *S. cerevisiae* donor more than the *Zygosaccharomyces* donors. The protoplast fusants like their *S. cerevisiae* donor are round, occurring as single cells, which are non-flocculent. The protoplast fusants differ from both donor strains in that they form natural pseudohyphae or hyphae, as shown in Figures 6.5-6.6. The only donor strain capable of exhibiting natural pseudohyphae is *Z. bailii* NCYC 1416 and this is only after around 10 days incubation with YPD. The average cell sizes of the protoplast fusants are larger than those of the *S. cerevisiae* donor as a result of this natural pseudohyphal or hyphal formation. The pseudohyphal or hyphal cell morphology is clearly shown in the SEM images obtained in Figure 6.6. The

Table 6.2 Frequency of protoplast fusion between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*

Protoplast fusant	c.f.u on non-selective regeneration agar	c.f.u on selective regeneration agar	Frequency of protoplast fusion
Pf 1766-895x	1.2×10^{5}	36	3.0×10^{-4}
Pf 1416-895x	6.7×10^{6}	11	1.6×10^{-6}
Pf YB4811-895x	3.6×10^{5}	52	1.4×10^{-4}

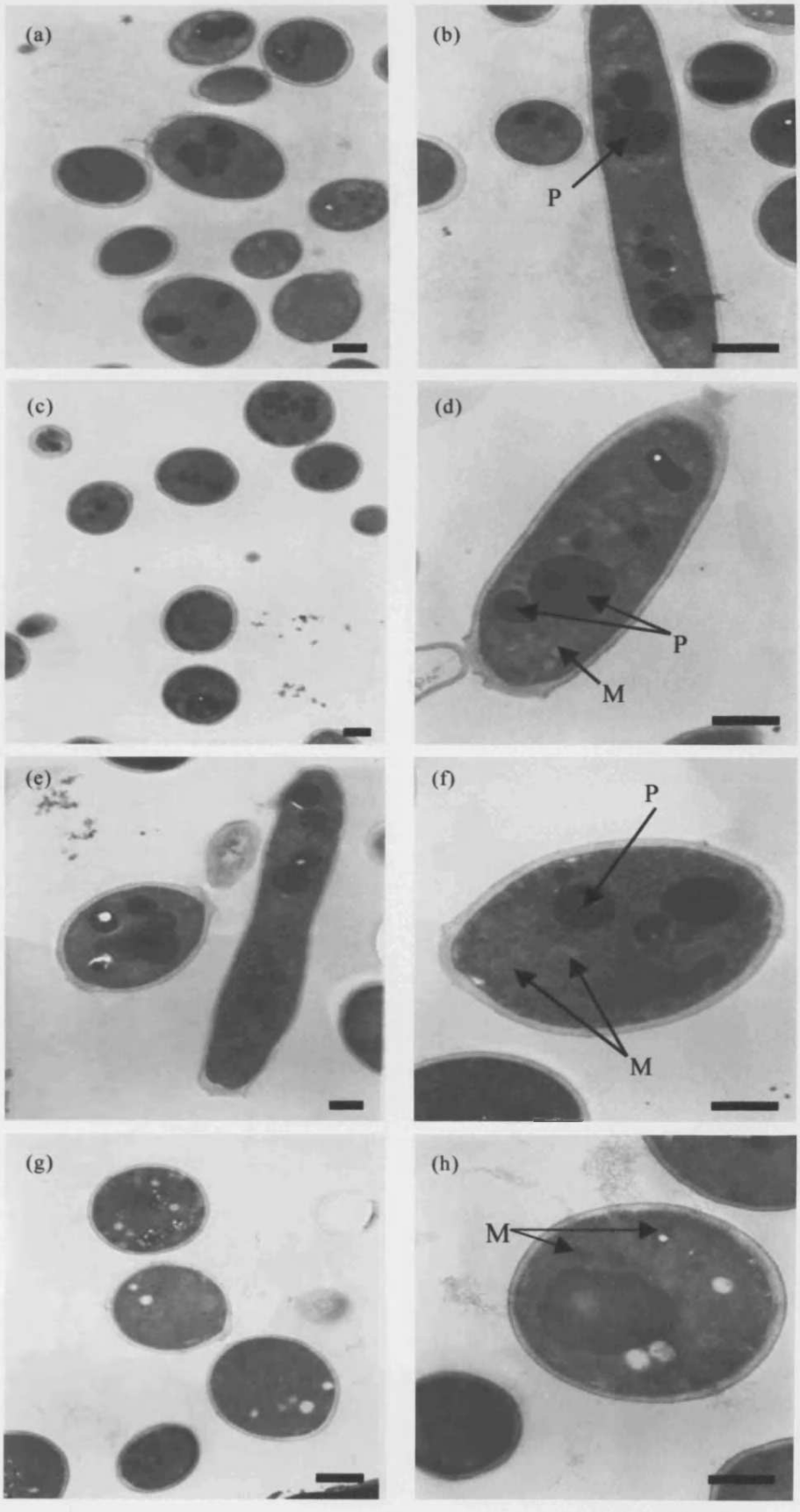
Non-selective regeneration agar is the same as selective regeneration (Table 2.3 of Methods chapter) with the absence of G418 and using glucose and not glycerol as carbon source.

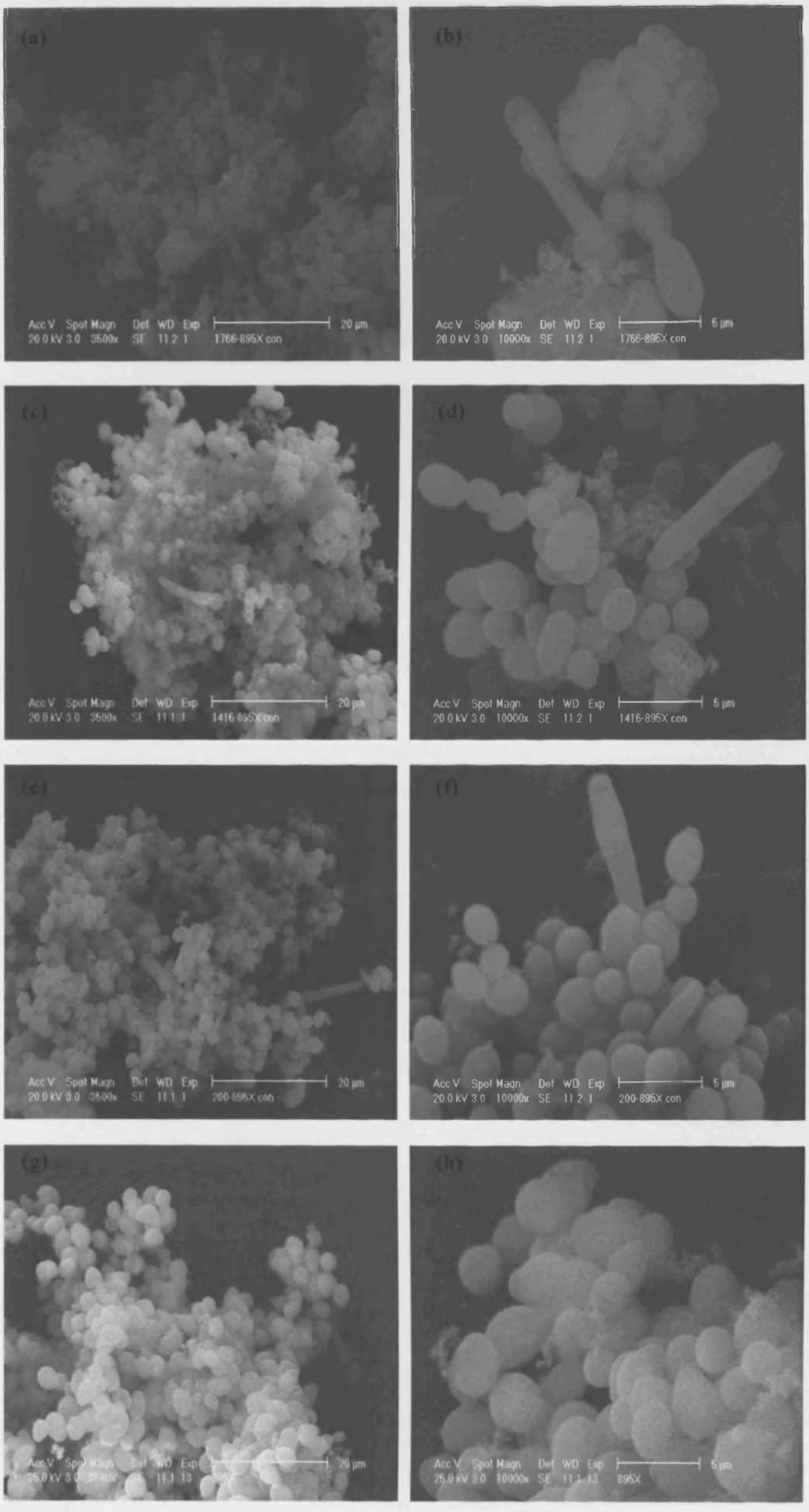
Results are the mean of two independent experiments for 1766-895x and YB4811-895x. Results for 1416-895x are based on four experiments, as one set yielded no hybrids.

Table 6.3 Physical characterisation of protoplast fusants

Physical Characteristic	*Zb* 1766	*Zb* 1416	*Zk* YB4811	*Sc* 895x	Pf 1766-895x	Pf 1416-895x	Pf YB4811-895x
Morphology:							
Shape	oval	long-oval	spherical	round	round	round	round
Arrangement	single	pair	single	single	single	single	single
Cell size (µm) ± SD	5.75 ± 0.500	6.02 ± 0.423	4.16 ± 0.322	3.23 ± 0.268	6.02 ± 1.567	5.87 ± 1.089	6.24 ± 1.023
Cell wall thickness (µm) ± SD	0.191 ± 0.023	0.196 ± 0.017	0.168 ± 0.022	0.123 ± 0.034	0.133 ± 0.026	0.139 ± 0.019	0.128 ± 0.031
Texture on agar	smooth	wrinkled	smooth	smooth	smooth	Smooth	smooth
Deposit in broth	non-flocculent	flocculent	non-flocculent	non-flocculent	non-flocculent	non-flocculent	non-flocculent
Natural pseudohyphae/hyphae	-	+	-	-	+	+	+
Growth on:							
Glucose	+	+	+	+	+	+	+
Maltose	-	-	+	+	+	+	+
Ethanol	+	+	+	-	+	+	+
Glycerol	+	+	+	-	+	+	+
Growth at:							
4°C	+	-	+	+	+	+	+
25°C	+	+	+	+	+	+	+
30°C	+	+	+	+	+	+	+
37°C	+	-	-	+	+	+	+
Sexual spores:							
Ascospores	+	-	+	-	-	-	-

Presence of physical characteristic is indicated by a + sign and its absence by a – sign. Results are based on at least two independent data sets. *Sc* 895 which contains mitochondria gave same results as *Sc* 895x which lacks mitochondria, except it gave positive growth on ethanol and glycerol. Size measurements based on 20 cells.


(a)
(b)
P
(c)
(d)
P
M
(e)
(f)
P
M
(g)
(h)
M

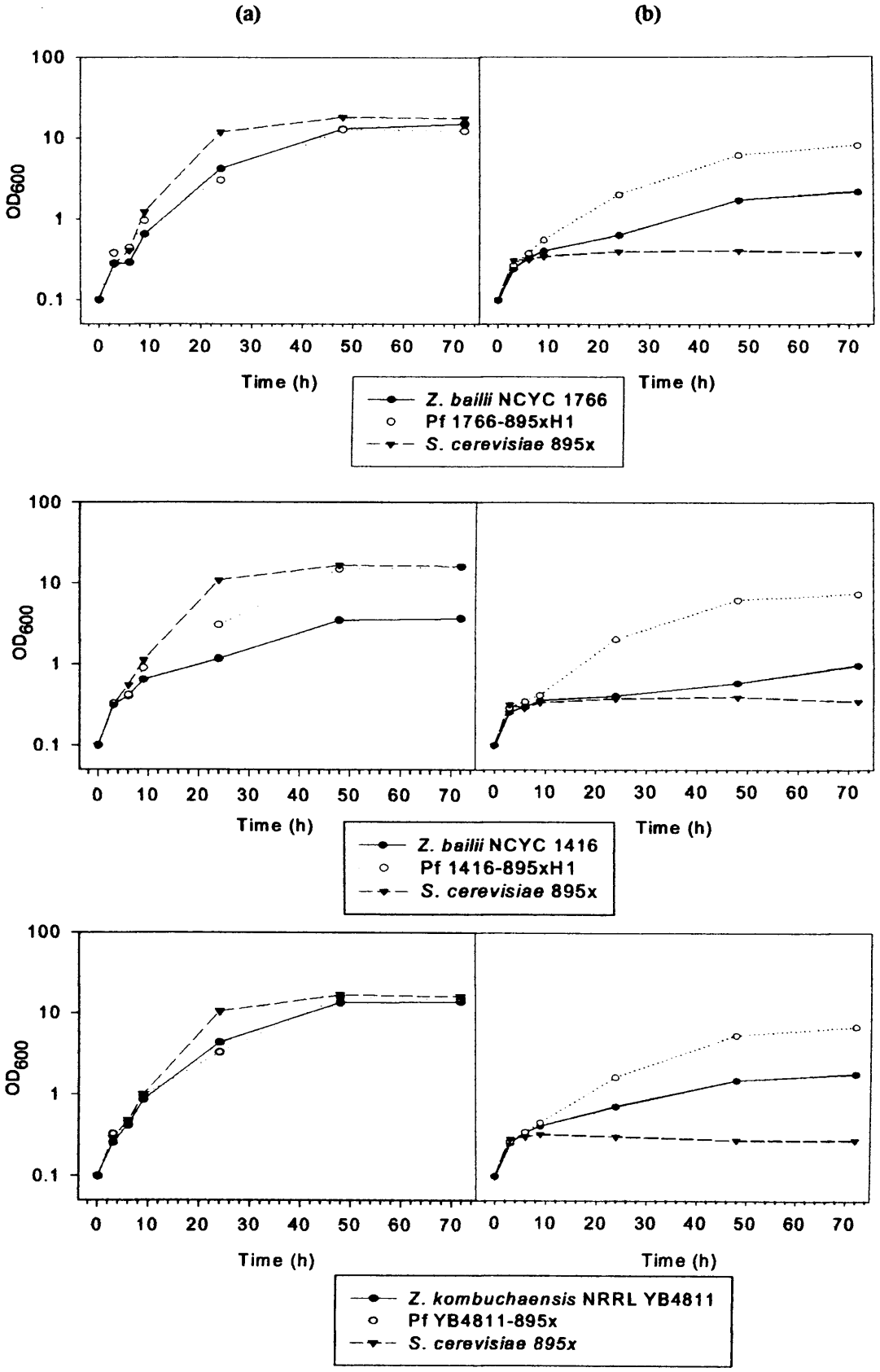

(a)
Acc.V Spot Magn Det WD Exp 20 µm
20.0 kV 3.0 3500x SE 11.2 1 1766-895X con
(b)
Acc.V Spot Magn Det WD Exp 5 µm
20.0 kV 3.0 10000x SE 11.2 1 1766-895X con
(c)
Acc.V Spot Magn Det WD Exp 20 µm
20.0 kV 3.0 3500x SE 11.1 1 1416-895X con
(d)
Acc.V Spot Magn Det WD Exp 5 µm
20.0 kV 3.0 10000x SE 11.2 1 1416-895X con
(e)
Acc.V Spot Magn Det WD Exp 20 µm
20.0 kV 3.0 3500x SE 11.1 1 200-895X con
(f)
Acc.V Spot Magn Det WD Exp 5 µm
20.0 kV 3.0 10000x SE 11.2 1 200-895X con
(g)
20 µm
(h)
Acc.V Spot Magn Det WD Exp 5 µm
25.0 kV 3.0 10000x SE 11.1 13 895X

TEM comparison of fusants shows that their cell walls are of a similar thickness to the *S. cerevisiae* donor and are thinner than those of the *Zygosaccharomyces* donors. The TEM micrographs show that each protoplast fusant contains several darkly stained bodies, which appear to be peroxisomes (P). Peroxisomes are largely absent from the *S. cerevisiae* donor appearing in only one or two cells (Figure 6.5). The *Zygosaccharomyces* donors appear devoid of peroxisomes (section 5, Figure 5.2). Mitochondria (M) are present for the fusants but lack clear distinct cristae. The morphology of the mitochondria also appears to vary in both size and shape. The *S. cerevisiae* petite donor, which lacks all or some mitochondrial DNA as a result of ethidium bromide treatment, still exhibits some remnants of mitochondria (Figure 6.5, g-h).

6.2.5.2 Growth of protoplast fusants

The ability of protoplast fusants to grow on a number of fermentable and non-fermentable carbon sources was examined. The *Z. bailii* donor strains (*Zb* 1766 and *Zb* 1416) were unable to grow with maltose as a carbon source (Table 6.3). The fact that the protoplast fusants created between these strains and *S. cerevisiae* 895 are able to grow on medium with maltose as the carbon source means that this ability has been inherited from the *S. cerevisiae* donor. The protoplast fusants were also able to use the non-fermentable carbon sources glycerol and ethanol as a result of mitochondria being inherited from the *Zygosaccharomyces* donor strains. The ability of the protoplast fusants to grow in the fermentable carbon source glucose was compared to the non-fermentable carbon source glycerol (Figure 6.7). The results show that the fusants exhibited a similar pattern of growth in glucose containing media to that of the donor strains. The exception to this is the *Z. bailii* 1416 donor, which is known to be a slow growing aggregating yeast. The growth pattern for the fusants in the presence of

Figure 6.7 Growth curves for *Z. bailii, Z. kombuchaensis, S. cerevisiae* and protoplast fusants in (a) YPD (b) YPG pH 4.0 incubated at 25°C without shaking.



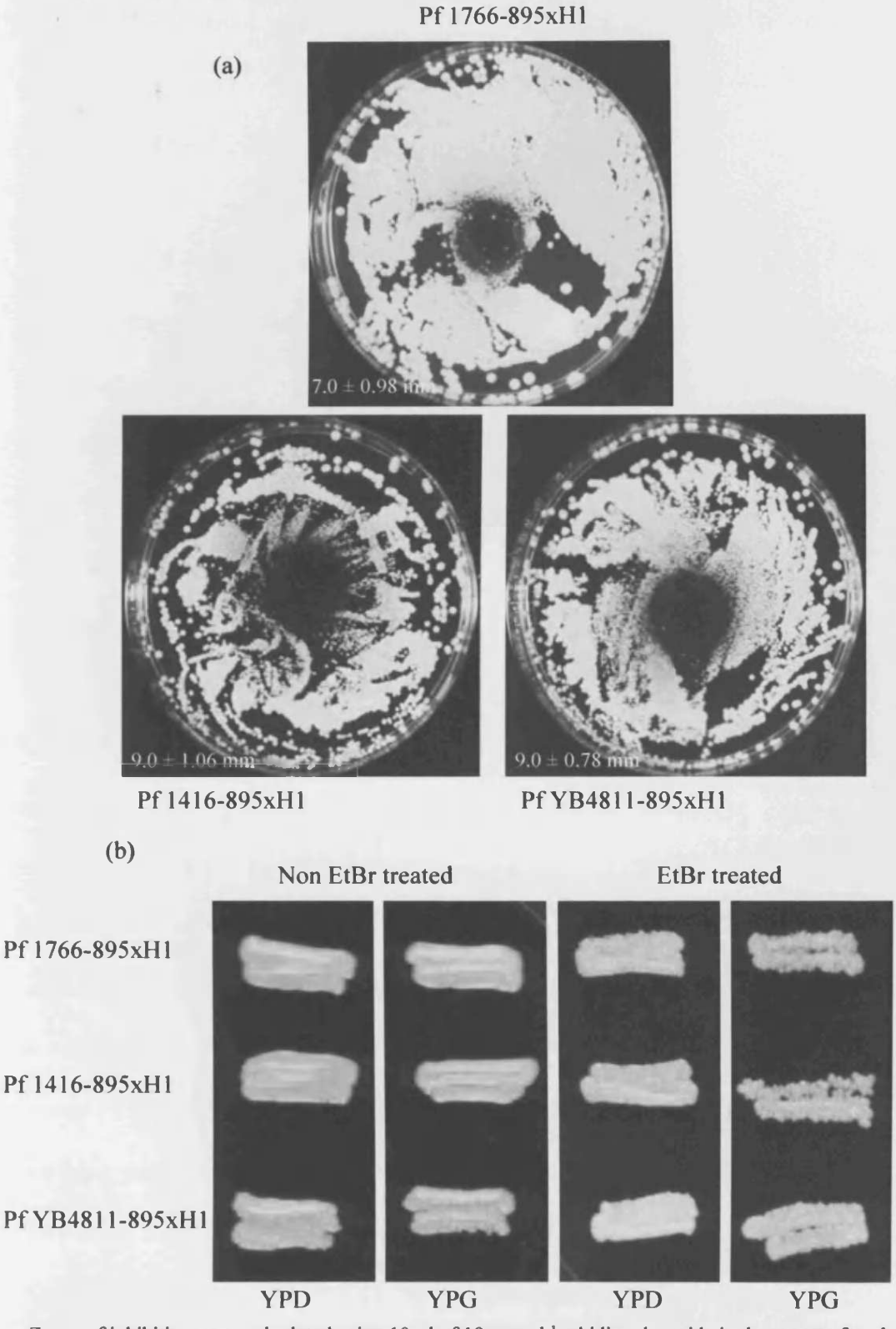
Results are the means of three experiments with standard errors <5%.

glycerol differed to that of donor strains. The fusants exhibited better growth than either of its donor strains (Figure 6.7). *S. cerevisiae* 895x with its petite status was unable to grow in the presence of glycerol, its grande equivalent; however, still exhibited poorer growth in glycerol than fusants (data not shown). It would, therefore, appear that the fusants have a greater ability to grow on the non-fermentable carbon source glycerol than their donor strains. The growth of the fusants at various temperatures was also examined (Table 6.3). The results show that the fusants were capable of growth at all four temperatures examined. The *Z. bailii* 1766 and *S. cerevisiae* 895 donor strains were also capable of growth over the same temperature range. *Z. bailii* 1416 and *Z. kombuchaensis* YB4811 exhibited no growth for at least one temperature examined. The growth of the protoplast fusants can be accounted for by their donor strains, except for the enhanced ability to grow in media containing glycerol.

6.2.5.3 Effects of ethidium bromide treatment on protoplast fusants

In a bid to elucidate the potential role of mitochondria from *Z. bailii* and *Z. kombuchaensis* in organic acid resistance, it was decided to attempt to create petites in the protoplast fusants. The mitochondria present in the fusants as confirmed by TEM analysis (Figure 6.5) and ability to grow on non-fermentable carbon sources (Table 6.3) were inherited from the *Zygosaccharomyces* donors. Ethidium bromide was used to determine the petite status of the fusants. Figure 6.8 shows that all three types of protoplast fusants were resistant to ethidium bromide treatment. The average radius of the inhibition zones being 7 mm for Pf 1766-895x and 9 mm for fusants Pf 1416-895x and YB4811-895x. Small colonies were formed for all three fusants in the presence of ethidium bromide. Several hundred small colonies for each fusant were selected and viability determined on YPD plates. All colonies selected were viable. Colonies were then replica plated onto plates containing the non-fermentable carbon sources ethanol

Figure 6.8 Protoplast fusants (a) differences in sensitivity to ethidium bromide (b) petite forming capabilities



Zones of inhibition were calculated using 10 µl of 10 mg ml^{-1} ethidium bromide in the centre of each plate and the images are representative of an experiment based on four replicates, ± the standard error.

and glycerol. Figure 6.8 shows that the ethidium bromide treated cells could not be distinguished from the non-ethidium bromide treated cells in terms of growth on glucose and glycerol media. Therefore, even though ethidium bromide treatment induced small petite like colonies in the fusants these colonies did not exhibit a respiratory deficient phenotype.

6.2.5.4 Organic acid resistance of protoplast fusants

The organic acid resistance capabilities of protoplast fusants pre- and post-ethidium bromide treatment were assessed. Table 6.4 shows the MICs obtained for protoplast fusants pre-ethidium bromide treatment on exposure to short, medium and longer chain organic acids. The MICs obtained for all three types of protoplast fusants were closer to those of the *S. cerevisiae* donor than the *Z. bailii* and *Z. kombuchaensis*. All fusants were more sensitive to both acetic and propionic acid than those of their respective donors. Differences in MICs between the *S. cerevisiae* donor and the protoplast fusants for medium (sorbic and benzoic) and longer (nonanoic) chain organic acids were representative of a difference in growth of 1-2 cultures. The fusants were also exposed to oxidative stress in the form of hydrogen peroxide. Hydrogen peroxide was included in the MICs determinations due to the differences growth on non-fermentable carbon sources shown between the fusants and donor strains (section 6.2.5.2). Fusants showed an average MIC of 14 mM to hydrogen peroxide being considerably greater than that obtained for the donor strains (Table 6.4). The only donor strain to have an MIC for hydrogen peroxide close to that of the fusants was *Z. bailii* 1766 with an average MIC of 10 mM. The organic acid resistance capabilities of fusants post-ethidium bromide treatment were also tested (Table 6.5). Ethidium bromide appeared to exert little effect on the MICs obtained for fusants with the results for pre and post-ethidium bromide treatment being similar.

Table 6.4 Organic acid resistance (mM) of (a) *Z. bailii* NCYC 1766 (b) *Z. bailii* NCYC 1416 (c) *Z. kombuchaensis* NRRL YB4811 in comparison with *S. cerevisiae* 895x and protoplast fusants

(a)

	Zb 1766	*Sc* 895x	Pf 1766-895x1	Pf 1766-895x2	Pf 1766-895x3
Acetic acid	400 ± 20.41	125 ± 10.20	100 ± 0.00	100 ± 10.20	100 ± 10.20
Propionic acid	110 ± 8.16	60 ± 4.08	50 ± 8.16	50 ± 0.00	50 ± 4.08
Sorbic acid	8.0 ± 0.71	3.0 ± 0.33	4.0 ± 0.71	4.0 ± 0.71	3.0 ± 0.33
Benzoic acid	10 ± 0.48	4.0 ± 0.48	4.0 ± 0.91	5.0 ± 0.71	5.0 ± 0.48
Nonanoic acid	0.7 ± 0.17	0.5 ± 0.04	0.6 ± 0.11	0.6 ± 0.09	0.6 ± 0.00
Hydrogen peroxide	10 ± 0.93	6 ± 0.85	14 ± 1.18	14 ± 0.85	14 ± 0.93

(b)

	Zb 1416	*Sc* 895x	Pf 1416-895x1	Pf 1416-895x2	Pf 1416-895x3
Acetic acid	300 ± 28.87	125 ± 10.20	100 ± 10.20	100 ± 10.20	100 ± 20.41
Propionic acid	80 ± 11.55	70 ± 8.16	50 ± 0.00	50 ± 4.08	50 ± 4.08
Sorbic acid	5.0 ± 0.71	4.0 ± 0.58	5.0 ± 0.00	5.0 ± 0.33	5.0 ± 0.71
Benzoic acid	5.0 ± 1.22	4.0 ± 0.91	5.0 ± 0.33	5.0 ± 0.58	5.0 ± 0.58
Nonanoic acid	0.6 ± 0.09	0.5 ± 0.04	0.6 ± 0.09	0.6 ± 0.00	0.6 ± 0.00
Hydrogen peroxide	6 ± 1.22	6 ± 0.85	14 ± 0.93	12 ± 1.22	14 ± 1.22

(c)

	Zk YB4811	*Sc* 895x	Pf 4811-895x1	Pf 4811-895x2	Pf 4811-895x3
Acetic acid	300 ± 20.41	125 ± 10.20	75 ± 20.41	100 ± 10.20	75 ± 10.20
Propionic acid	120 ± 0.00	60 ± 8.16	40 ± 0.00	40 ± 4.08	40 ± 4.08
Sorbic acid	1.2 ± 0.13	3.0 ± 0.58	3.0 ± 0.91	3.0 ± 0.71	3.0 ± 0.58
Benzoic acid	5.0 ± 0.71	4.0 ± 0.71	5.0 ± 0.00	5.0 ± 0.33	5.0 ± 0.71
Nonanoic acid	0.5 ± 0.09	0.5 ± 0.11	0.6 ± 0.00	0.6 ± 0.04	0.6 ± 0.04
Hydrogen peroxide	4 ± 0.91	6 ± 0.00	14 ± 0.00	14 ± 0.93	14 ± 1.22

Values are mean MICs measured from at least two independent experiments (four replicates) in YPD pH 4.0 following 14 days incubation at 25°C, ± the standard error.

Table 6.5 Organic acid resistance (mM) of (a) 1766-895x protoplast fusants (b) 1416-895x protoplast fusants (c) NRRL YB4811-895x protoplast fusants after ethidium bromide treatment

(a)

	Pf 1766-895x1	Pf 1766-895x2	Pf 1766-895x3
Acetic acid	100 ± 10.20	100 ± 10.20	100 ± 0.00
Propionic acid	60 ± 4.08	50 ± 4.08	50 ± 4.08
Sorbic acid	4.0 ± 0.33	3.5 ± 0.58	3.5 ± 0.58
Benzoic acid	4.0 ± 0.33	4.0 ± 0.71	4.0 ± 0.33
Nonanoic acid	0.5 ± 0.00	0.5 ± 0.00	0.6 ± 0.04
Hydrogen peroxide	14 ± 0.93	14 ± 1.22	14 ± 0.93

(b)

	Pf 1416-895x1	Pf 1416-895x2	Pf 1416-895x3
Acetic acid	110 ± 20.41	100 ± 10.20	100 ± 10.20
Propionic acid	50 ± 7.01	50 ± 0.00	60 ± 4.08
Sorbic acid	5.0 ± 0.58	4.0 ± 0.33	5.0 ± 0.71
Benzoic acid	5.0 ± 0.00	5.0 ± 0.33	5.0 ± 0.00
Nonanoic acid	0.6 ± 0.04	0.5 ± 0.00	0.6 ± 0.00
Hydrogen peroxide	14 ± 1.22	14 ± 0.93	14 ± 0.00

(c)

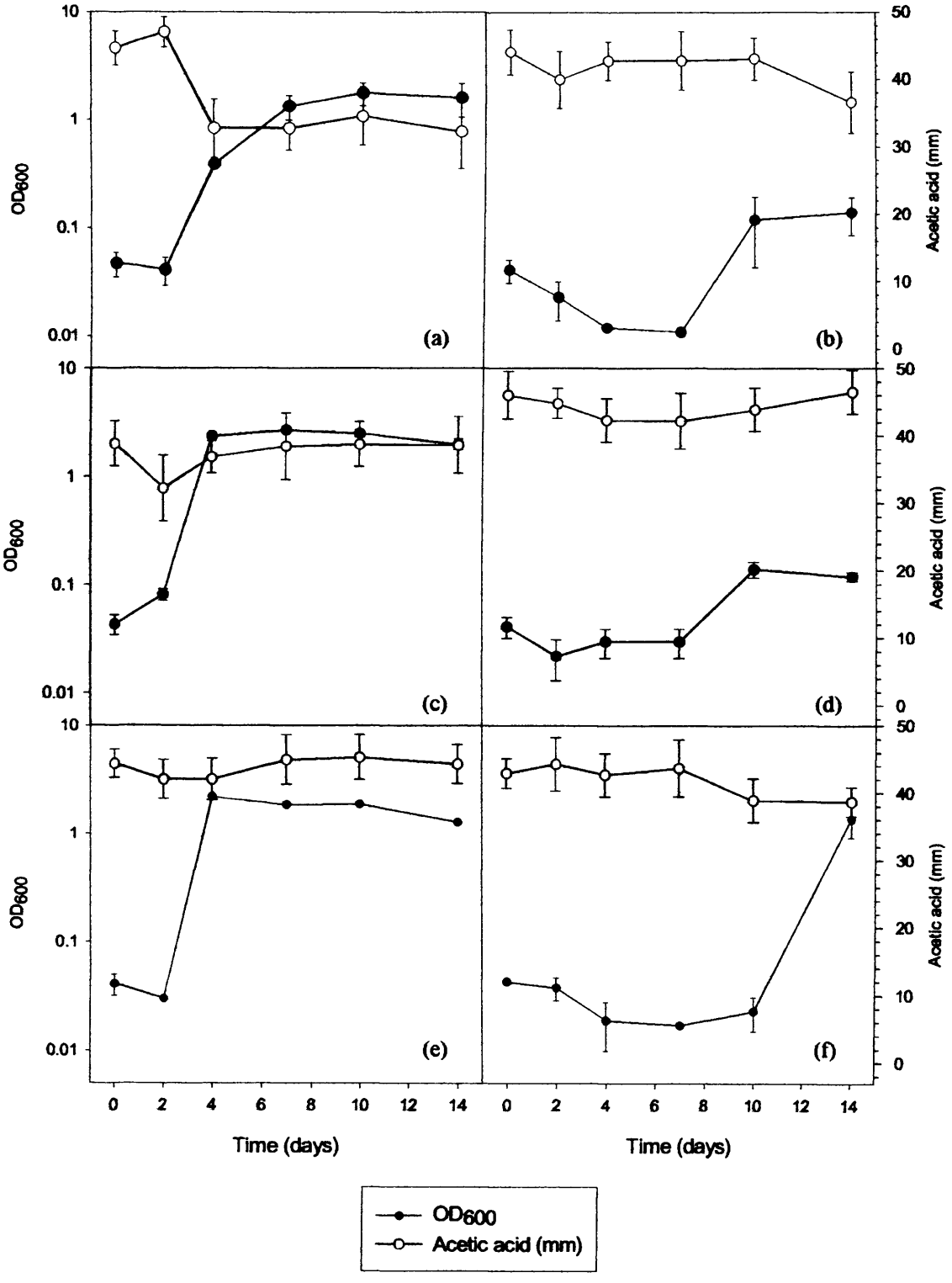
	Pf 4811-895x1	Pf 4811-895x1	Pf 4811-895x1
Acetic acid	80 ± 20.41	90 ± 20.41	80 ± 10.20
Propionic acid	40 ± 8.16	50 ± 4.08	40 ± 7.01
Sorbic acid	3.0 ± 0.33	3.5 ± 0.58	3.5 ± 0.58
Benzoic acid	5.0 ± 0.71	5.0 ± 0.00	4.0 ± 0.58
Nonanoic acid	0.5 ± 0.02	0.5 ± 0.04	0.5 ± 0.00
Hydrogen peroxide	14 ± 0.93	14 ± 0.93	14 ± 1.22

Values are mean MICs measured from at least two independent experiments (four replicates) in YPD pH 4.0 following 14 days incubation at 25°C, ± the standard error.

6.2.5.5 Organic acid utilisation by protoplast fusants

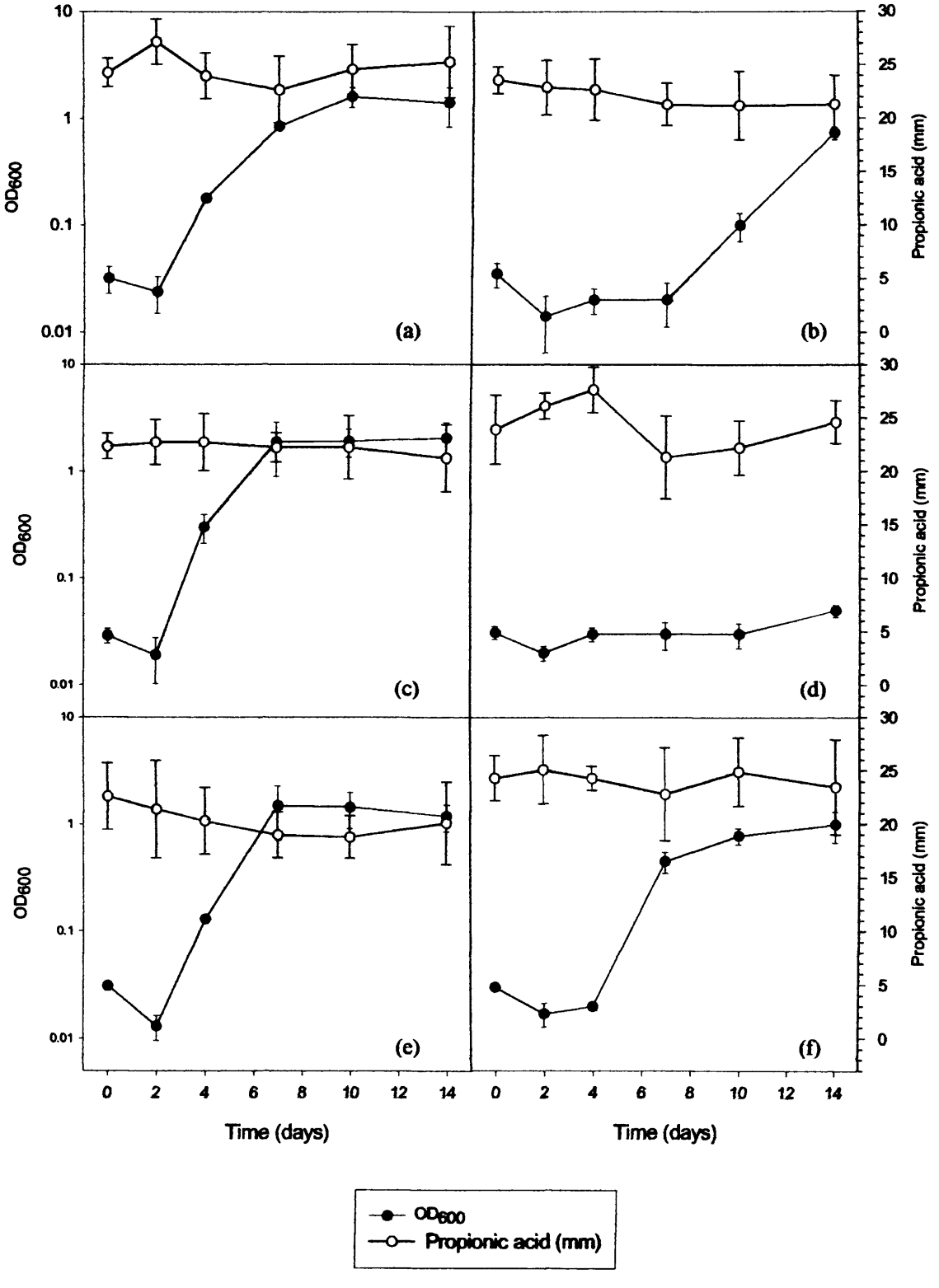
The ability of fusants to utilise acetic and propionic acid were compared to those of their donors. The ability to utilise acetic acid is shown in Figure 6.9. All six yeasts examined were able to grow in the presence of 50 mM acetic acid in minimal medium. The yeasts were grown in minimal medium as complex medium produced a complicated chromatogram with many peaks making the identification and quantification of acids difficult. *Z. bailii* 1766 was the only yeast clearly capable of using acetic acid. The acetic acid concentration for *Z. bailii* 1766 reached approximately 35 mM after 4 days incubation. Propionic acid was not utilised by any of the yeasts investigated (Figure 6.10). The results for *S. cerevisiae* grande and petite cells in terms of both growth and acetic and propionic acid utilisation are very similar. The growth for some of the yeasts for both acetic and propionic acid show a trough. The trough in growth could be the result of the acids on addition to culture generating a precipitate giving a slightly higher OD value in addition to some growth variation between cultures. The inclusion of the growth data was to show that the yeasts could grow in these concentrations of acids and in all cases the OD values are at the greatest at the end of the experiment. The significance of the OD values being at the greatest at the end of the experiment is that the cells have had sufficient time to grow and utilise the acid. The results for both acetic and propionic acid even at time zero shows that the concentration of acid detected by HPLC was lower than those originally added to the cultures. The difference in the level of acid detected and that added to the cultures could be the result of the following: the acids are volatile and this would cause some loss, some of the acid would be attached to the yeast, differences may be evident in sampling and some variation would be evident between HPLC runs.

Figure 6.9 Differences in growth and acetic acid utilisation between (a) *Z. bailii* NCYC 1766 (b) *Z. bailii* NCYC 1416 (c) *Z. kombuchaensis* NRRL YB4811 (d) Pf 1766-895x (e) *S. cerevisiae* 895 (f) *S. cerevisiae* 895x.



Results are the mean of two independent experiments (four replicates) conducted in 10 ml minimal medium pH 4.0 with 50 mM acetic acid incubated for 14 days at 25°C without shaking, ± the standard error.

Figure 6.10 Differences in growth and propionic acid utilisation between (a) *Z. bailii* NCYC 1766 (b) *Z. bailii* NCYC 1416 (c) *Z. kombuchaensis* NRRL YB4811 (d) Pf 1766-895x (e) *S. cerevisiae* 895 (f) *S. cerevisiae* 895x.



Results are the mean of two independent experiments (four replicates) conducted in 10 ml minimal medium pH 4.0 with 30 mM propionic acid incubated for 14 days at 25°C without shaking, ± the standard error.

6.3 Discussion

Ethidium bromide,a common inhibitor of mitochondrial function, was used to determine the petite status of *Z. bailii* and *Z. kombuchaensis*. (Grant *et al.*, 1997; Møller *et al.*, 2001; Pearce *et al.*, 2001b; Fernet *et al.*, 2002). *Z. bailii* and *Z. kombuchaensis* were both more sensitive to ethidium bromide treatment than *S. cerevisiae*. The small colonies produced as a result of ethidium bromide treatment were non-viable for both *Z. bailii* and *Z. kombuchaensis*. Loss or considerable damage to the mitochondria of these yeasts therefore appears to represent a lethal event. *Z. bailii* and *Z. kombuchaensis* were therefore classified as petite-negative. Previous researchers have found *Z. bailii* to be petite-negative (Mollapour and Piper, 2001b; Merico *et al.*, 2003). The petite-positive yeast *S. cerevisiae* showed no overall difference in organic acid resistance between grande and petite cells. Therefore, loss or considerable damage to the mitochondria of *S. cerevisiae* has no detrimental effect on organic acid resistance.

The possible role of mitochondria in *Z. bailii* and *Z. kombuchaensis* organic acid resistance remained unknown due to the inability to form petites. I therefore produced fusants between the respiratory competent *Z. bailii* and *Z. kombuchaensis* with that of the respiratory deficient *S. cerevisiae*. Three different enzymes were used in a bid to induce protoplasts. Lysozyme and β-glucroronidase at the concentrations examined did not yield protoplasts. Zymolyase was able to produce protoplasts to differing degrees in *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* and was used to form protoplasts. The restoration of respiratory function to a petite yeast via protoplast fusion with a respiratory competent yeast has been previously reported (Ferenczy and Maráz, 1977; Spencer and Spencer, 1981; Goodey and Bevan, 1983). To the best of my knowledge this is the first time that protoplast fusion has been used as a means of studying yeast organic acid resistance.

The fusants generally resembled their *S. cerevisiae* donor more than their *Zygosaccharomyces* donor. The shape of fusants' varied with many cells exhibiting natural pseudohyphae or hyphae. The shape and size of protoplast fusants have been shown to differ from those of their donors (Ferenczy and Maráz, 1977; Groves and Oliver, 1984). The TEM images obtained for the protoplast fusants show the presence of several mitochondria of various shapes and sizes but with no clear cristae (Figure 6.5). The mitochondria present in the hybrids are probably the result of the fusion of *S. cerevisiae* mitochondrial remnants (from ethidium bromide treatment) with those of the fully functioning *Zygosaccharomyces* mitochondria. The presence of a large number of peroxisomes in the protoplast fusants may explain several of their unusual properties. The fusants were able to grow on YPG (a non-fermentable carbon source) better than their respective donors. They also showed hydrogen peroxide resistance greater than that of their donor strains. Peroxisomes are known to contain catalase and several oxidases involved in the oxidative utilisation of specific carbon sources (Walker, 1998). Catalase is known to be important for resistance to hydrogen peroxide as it catalyses the breakdown of hydrodgen peroxide to oxygen and water (Jamieson, 1998). Therefore, if yeasts were to contain a greater number of peroxisomes it would seem logical for them to be more resistant to hydrogen peroxide.

The fusants showed a similar sensitivity to ethidium bromide treatment as *S. cerevisiae* being more resistant than the *Zygosaccharomyces* donors. The small cells selected from ethidium bromide treatment were capable of growth on fermentable and non-fermentable carbon sources. This result differs from that of either donor and one can only speculate the reason for this unusual and unexpected result. The result suggests the following possibilities; the fusants can use glycerol without mitochondria, and/or that fusants contain a means of overcoming the effects of ethidium bromide treatment.

The organic acid resistance of the fusants before and after ethidium bromide treatment was determined. Ethidium bromide treatment resulted in no overall differences in organic acid resistance. All the fusants were slightly more sensitive to short chain organic acids than the *S. cerevsiae* donor. These results are of interest in that common food preservatives many of which are organic acids including acetic acid have been reported to exert an oxidative stress (Piper, 1999). The results here show the protoplast fusants, which are considerably more resistant to hydrogen peroxide a known inducer of oxidative stress, to be the most sensitive of the yeasts examined to short chain acids. The exact nature of the oxidative stress induced by organic acids appears not to be fully elucidated.

Z. bailii unlike *S. cerevisiae* has been reported to metabolise acetic acid simultaneously with glucose (Sousa *et al.,* 1996). The only yeast clearly capable of using acetic acid under the conditions examined was *Z. bailii* NCYC 1766. None of the yeasts appeared able to utilise propionic acid. The resistance of *Z. kombuchaensis* unlike that of *Z. bailii* NCYC 1766 to acetic acid is therefore not based only the metabolism of the acid, suggesting more than one mechanism is involved in resistance to acetic acid.

In conclusion, the mitochondria of the petite-positive yeast *S. cerevisiae* were shown to have little influence on organic acid resistance. *Z. bailii* and *Z. kombuchaensis* have been shown to be petite-negative with loss or damage to mitochondria representing a lethal event. The ability of organic acids to be mutagenic towards mitochondria and generate oxidative stress warrants further investigation.

7. Effects of glycerol on isoamyl alcohol induced pseudohyphal formation: a role for the HOG pathway

7.1 Introduction

This section was stimulated by the observation of elongated *Z. bailii* cells in the presence of acetic acid (section 5). The first part of this research examines the ability of isoamyl alcohol, a known inducer of yeast pseudohyphal formation, to induce morphological change in *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*. The second part of this section investigates the nature of the morphological change induced by isoamyl alcohol, as this still remains speculative (Lorenz *et al.,* 2000; Martínez-Anaya *et al.,* 2003). The final section shows a role for the HOG pathway in isoamyl alcohol induced pseudohyphal formation.

Yeast dimorphism (in which yeasts exist in at least two morphological forms) has been reported for numerous yeasts including; *S. cerevisiae* (Gimeno *et al.,* 1992; Dickinson, 1994; 1996; Kron, 1997), *D. hansenii* (Cruz *et al.,* 2000) and *C. albicans* (Alonso-Monge *et al.,* 1999; Tzung *et al.,* 2001). The filamentous forms of *C. albicans* have been implicated in its pathogenic ability (Gow *et al.,* 1995; Alonso-Monge *et al.,* 1999). The majority of research into yeast dimorphism has been conducted in the model yeast *S. cerevisiae*. The ability of *S. cerevisiae* to grow in a filamentous form was reported over 80 years ago (Guilliermond, 1920). However, it is only since the work by Gimeno *et al.* (1992) that yeast dimorphism has become an area of intense research.

It is now known that under certain environmental conditions a variety of yeasts will differentiate into hyphal-like extensions (Black *et al.,* 1995; Dickinson, 1996) or pseudohyphae (Gimeno *et al.,* 1992). Pseudohyphae can be defined as elongated chains of cells, in which unipolar budding predominates (Dickinson, 1999). Hyphal-like extensions are elongated cells in which no bud formation takes place, resulting in an extended form of the cell, but which lacks the isotropic swelling characteristic of the

normal cell (Dickinson, 1996). In *S. cerevisiae* haploid strains form what has been termed "invasive filaments" as they penetrate the surface of the agar. The ability of diploids to form pseudohyphae and haploids to form invasive filaments is not mutually exclusive (Gancedo, 2001). There have also been some reports of *S. cerevisiae* mutants forming rod shaped cells rather than the typical ovoid (Blacketer *et al.*, 1995). The general view regarding yeast pseudohyphal formation is that it provides a means for yeast to forage for more favourable conditions when under nutrient stress (Martínez-Anaya *et al.*, 2003).

The signal for yeast to form pseudohyphae is believed to be mainly the result of nitrogen starvation (Kron *et al.*, 1994). Pseudohyphal formation in addition to being induced by nutrient limitation can be induced by fusel alcohols. Fusel alcohols are the end products of amino acid catabolism in *S. cerevisiae*. Isoamyl alcohol, isobutyl alcohol, 2-phenylethanol and tryptophol are the end products of leucine, valine, phenylalanine and tryptophan catabolism respectively (Dickinson *et al.*, 1997; 1998; 2003). The fusel alcohols known to induce filamentous growth are isoamyl alcohol (Dickinson, 1996; Martínez-Anaya *et al.*, 2003), isobutyl alcohol (Lorenz *et al.*, 2000) and butanol (Ashe *et al.*, 2001). The means by which fusel alcohols induce morphological change still remains speculative, despite recent research (Ashe *et al.*, 2001; Martínez-Anaya *et al.*, 2003). Isoamyl alcohol and butanol have been shown to cause a rapid inhibition of translation at the initiation step by targeting the translation initiation factor eIF2B (Ashe *et al.*, 2001). This however does not account for their ability to induce a morphological switch. It has been proposed that *S. cerevisiae* detects a combination of nutrient limitation and metabolic by-products to regulate pseudohyphal differentiation (Lorenz *et al.*, 2000). It is therefore possible that the production of fusel alcohols and the depletion of nitrogen source collectively result in

pseudohyphal formation. A number of other factors including oxygen (Wright *et al.*, 1993; Kuriyama & Slaughter, 1995) and carbon source (Vivier *et al.*, 1997) have been shown to be involved in pseudohyphal formation. The toxic nature of isoamyl alcohol also requires further research as this may point to the means of pseudohyphae induction.

The control of filamentous growth in *S. cerevisiae* has recently been the subject of several reviews (Pan *et al.*, 2000; Gancedo, 2001; Palecek *et al.*, 2002). In brief, the basic elements of filamentous control are as follows. In *S. cerevisiae* two signal transduction pathways are required for the induction of pseudohyphae both of which are activated by Ras2. The first pathway is a Mitogen Activated Protein (MAP) kinase pathway. The MAP kinase pathways in *S. cerevisiae* and the current known elements for each pathway are shown in Figure 7.1. There is generally accepted to be cross-talk between the various MAP kinase pathways (Levin & Errede, 1995; Pan *et al.*, 2000). The second pathway for pseudohyphal formation is the cAMP dependent pathway (Pan & Heitman, 1999). The cAMP dependent pathway consists of three main components that are required for pseudohyphal formation: G-protein, protein kinase A and cAMP. The importance of the cAMP dependent pathway is illustrated by the ability of external cAMP to stimulate pseudohyphal formation (Kübler *et al.*, 1997; Lorenz & Heitman, 1998; Jung and Stateva, 2003). Both the MAP kinase and cAMP dependent pathways converge on *FLO11*, which encodes a cell-surface flocculin necessary for pseudohyphal formation (Lo & Dranginis, 1998). The cAMP pathway has also been shown to be involved with cell wall biogenesis (Tomlin *et al.*, 2000; Jones *et al.*, 2001; 2003). The cell cycle is also known to play a role in pseudohyphal formation as such growth requires an extended G2 in addition to a switch to more extensive polarised growth (Kron *et al.*, 1994).

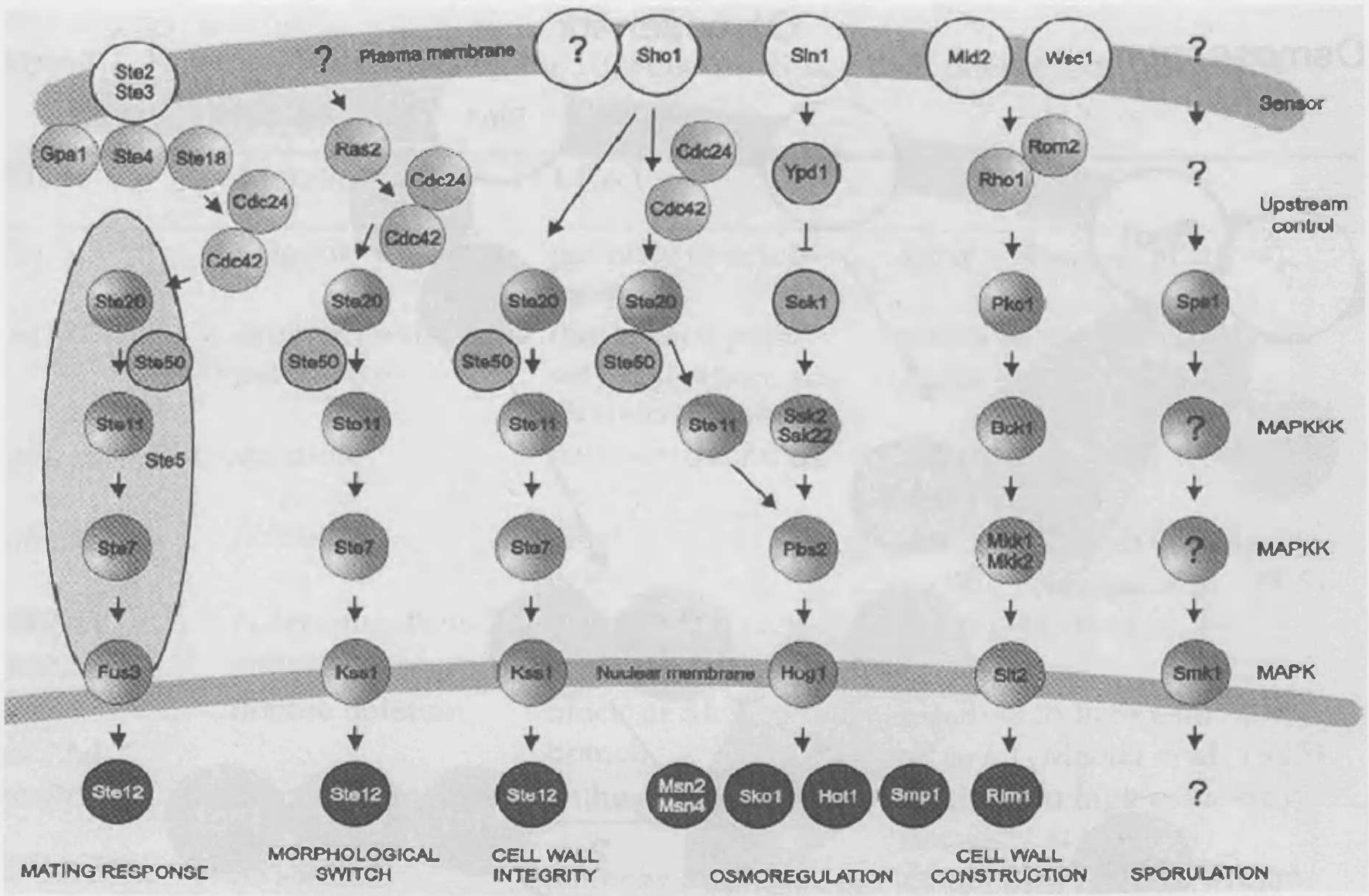


Figure 7.1 Components of the *S. cerevisiae* MAP kinase pathways. Reproduced from Tamás and Hohmann (2003)

7.2 Results

7.2.1 Effects of isoamyl alcohol on yeast growth and pseudohyphal formation

Preliminary experiments analysing the inhibitory effects of acetic acid against *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* revealed the formation of elongated cells for one strain of *Z. bailii* (NCYC 1766). These cells appeared similar to pseudohyphae in *S. cerevisiae.* The effects of isoamyl alcohol a known inducer of morphological change on growth and pseudohyphal formation have been investigated in the three aforementioned yeasts. The results in Figure 7.2 show that both *S. cerevisiae* and *Z. kombuchaensis* formed pseudohyphae in the presence of 0.5% (v/v) isoamyl alcohol (4.6 mM). *Z. bailii* showed no morphological change. In YPD without 0.5% (v/v) isoamyl alcohol neither *Z. bailii, Z. kombuchaensis* nor *S. cerevisiae* exhibited morphological change. Isoamyl alcohol caused a significant reduction in growth for all three yeasts, with *S. cerevisiae* showing the greatest suppression of growth and highest pseudohyphal count (Figure 7.3). The peak of pseudohyphal formation for *S. cerevisiae* was at 24 h and at 48 h for *Z. kombuchaensis*, with a considerable reduction evident by 72 h for both yeasts (Figure 7.3). Growth in the presence of isoamyl alcohol increased with time for all three yeasts, being at its maximum after 72 h.

7.2.2 Effects of isoamyl alcohol on yeast cell structure

7.2.2.1 Transmission electron microscopy comparison

The effects of isoamyl alcohol on yeast cell structure were analysed via TEM. The results in Figure 7.4 shows the effects of 0.5% (v/v) isoamyl alcohol on the cell structure of *Z. bailii, Z. kombuchaensis* and *S. cerevisiae.* Isoamyl alcohol at a concentration of 0.5% (v/v) caused the development of vacuoles (V) and a proliferation of mitochondria (M) for all three yeasts in comparison to control YPD cultures (Figure 7.4 g-h and section 5; Figure 5.3). Differences in cell structure on exposure to isoamyl

Figure 7.2 Morphology of (a-c) *Z. bailii* NCYC 1766 (d-f) *Z. kombuchaensis* NRRL YB4811 (g-i) *S. cerevisiae* IWD72 grown in (left to right) YPD, YPD + 0.5% (v/v) isoamyl alcohol and YPD + 0.5% (v/v) isoamyl alcohol + 10% (w/v) glycerol at 48 h incubation. Bar = 10 μm.

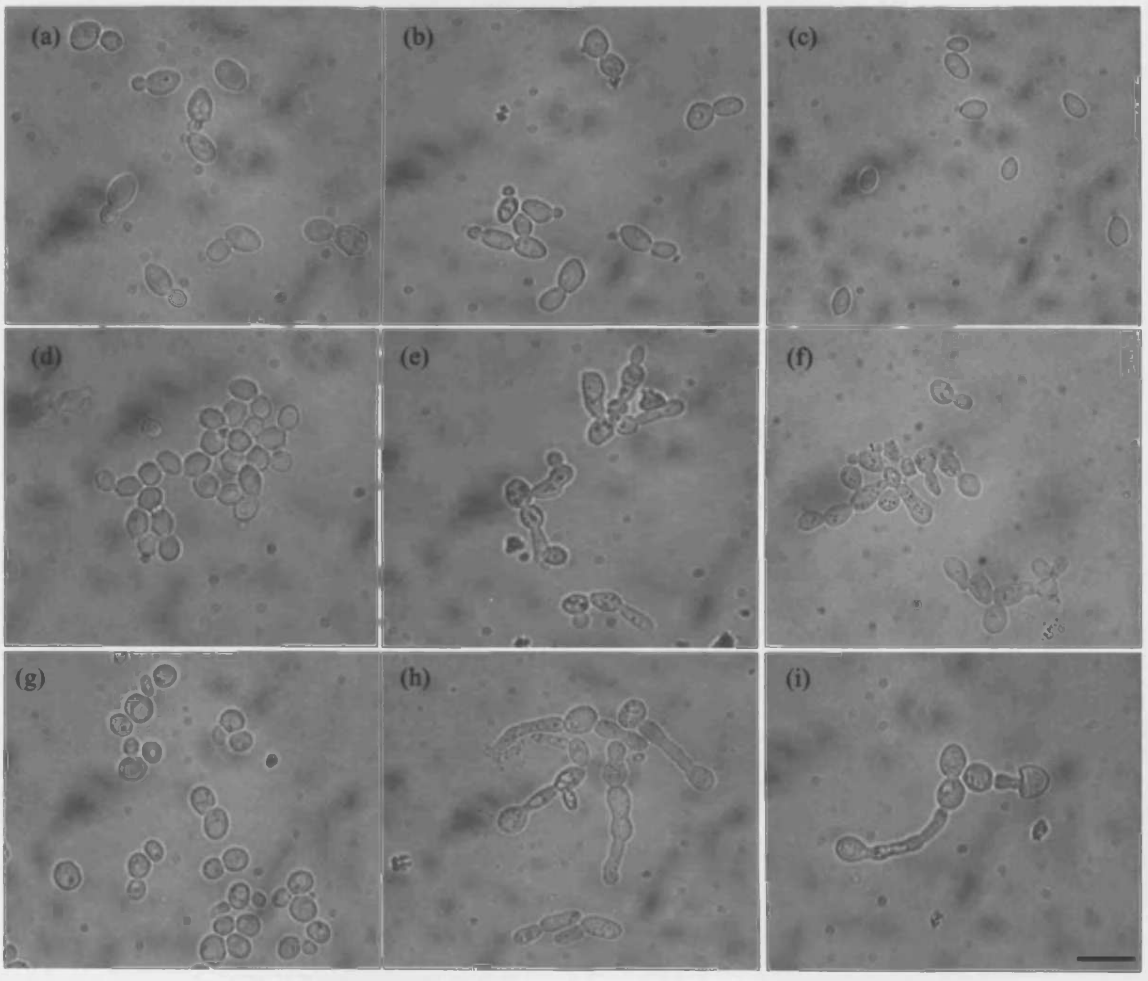
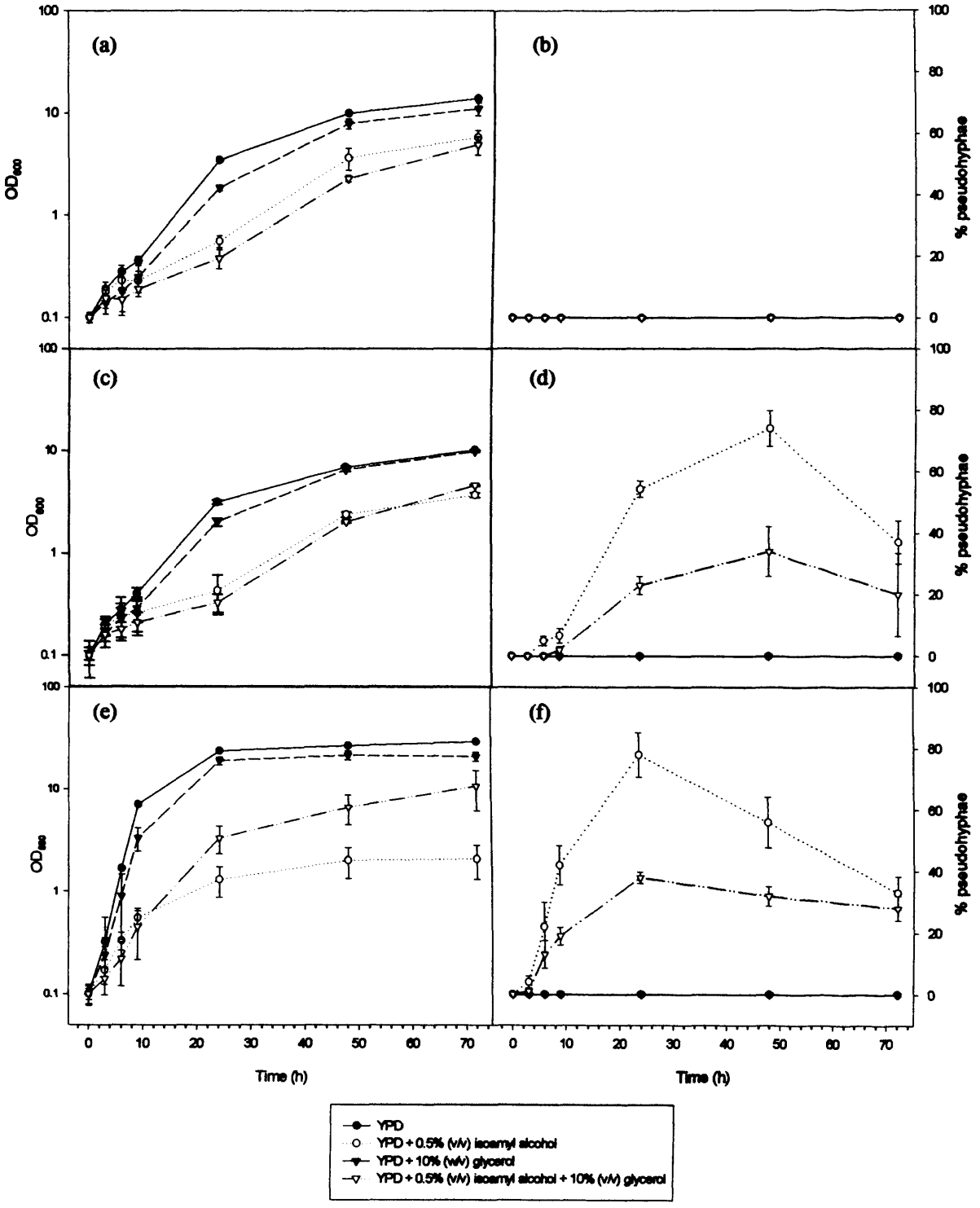


Figure 7.3 Effects of isoamyl alcohol and glycerol on yeast growth and pseudohyphal formation in (a, b) *Z. bailii* NCYC 1766 (c, d) *Z. kombuchaensis* NRRL YB4811 grown in YPD at 25°C without shaking and (e, f) *S. cerevisiae* IWD72 grown at 30°C at 160 r.p.m



Results are the means of three experiments, ± the standard error.

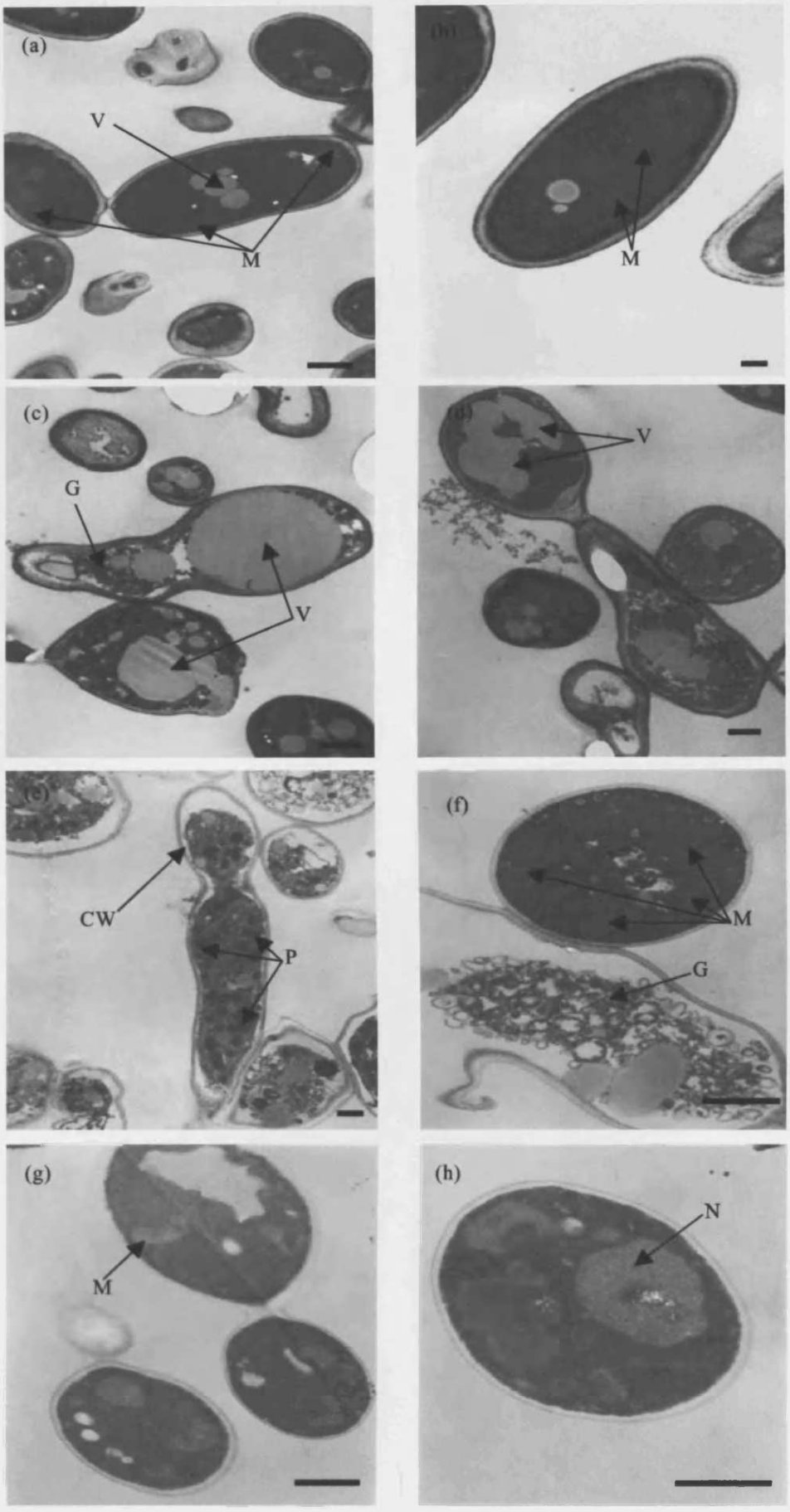
alcohol are also evident. *Z. kombuchaensis* and *S. cerevisiae* have cells exhibiting a granular (G) cytosol with extensive cell damage being evident. In such cells the cytosol appears to have detached from the cell envelope. No structural damage was evident for *Z. bailii*. *S. cerevisiae* cells also appear to contain a number of peroxisomes (P) which are absent for the *Zygosaccharomyces* yeasts. The cell wall (CW) for all three yeasts appears to have remained undamaged.

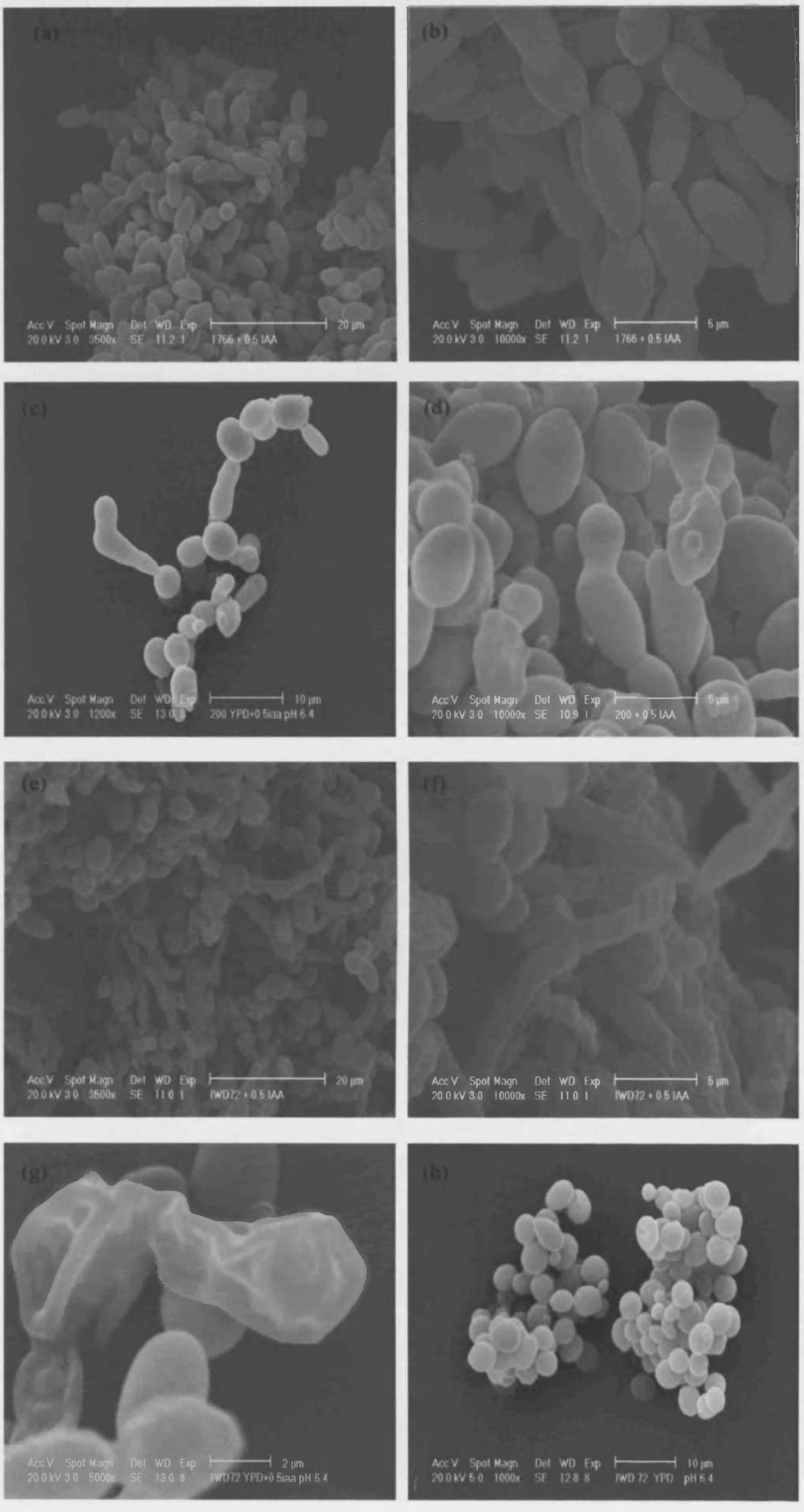
7.2.2.2 Scanning electron microscopy comparison

The scanning electron micrographs shown in Figure 7.5 show the range of shapes formed by pseudohyphal cells of *Z. kombuchaensis and S. cerevisiae* in the presence of 0.5% (v/v) isoamyl alcohol. The SEM images show no pseudohyphae for *Z. bailii* with cells remaining long oval in YPD with and without isoamyl alcohol. The *S. cerevisiae* (e, f) and *Z. kombuchaensis* (c, d) cells show variation in size with the *S. cerevisiae* pseudohyphal cells appearing the longer. The cells for all three yeasts show no evidence of cell leakage, with cells appearing intact. Bud scars are largely absent for the majority of cells for all three yeasts. Figure 7.5 (g) shows the range of contortions formed by *S. cerevisiae* cells in the presence of 0.5% (v/v) isoamyl alcohol. The contortions are reminiscent of aged 'mothers' as they reach senescence (Nestelbacher *et al.*, 1999).

7.2.3 Effects of glycerol on isoamyl alcohol induced pseudohyphal formation

The effects of glycerol were studied in the presence of 0.5% (v/v) isoamyl alcohol for *Z. bailii*, *Z. kombuchaensis* and *S. cerevisiae*. Glycerol at a concentration of 10% (w/v) resulted in at least a five-fold improvement in growth for *S. cerevisiae* IWD72 in the presence of 0.5% (v/v) isoamyl alcohol (Figure 7.3). The addition of 10% (w/v) glycerol to *Z. kombuchaensis* cultures containing 0.5% (v/v) isoamyl alcohol resulted in a marginal increase in growth but similar reduction in pseudohyphae as *S. cerevisiae*


(a)
V
M
(b)
M
(c)
G
V
(d)
V
(e)
CW
P
(f)
M
G
(g)
M
(h)
N


(a)
Acc.V Spot Magn Det WD Exp 20 µm
20.0 kV 3.0 3500x SE 11.2 1 1766 + 0.5 IAA
(b)
Acc.V Spot Magn Det WD Exp 5 µm
20.0 kV 3.0 10000x SE 11.2 1 1766 + 0.5 IAA
(c)
Acc.V Spot Magn Det WD Exp 10 µm
20.0 kV 3.0 1200x SE 13.0 1 200 YPD+0.5iaa pH 6.4
(d)
Acc.V Spot Magn Det WD Exp 5 µm
20.0 kV 3.0 10000x SE 10.9 1 200 + 0.5 IAA
(e)
Acc.V Spot Magn Det WD Exp 20 µm
20.0 kV 3.0 3500x SE 11.0 1 IWD72 + 0.5 IAA
(f)
Acc.V Spot Magn Det WD Exp 5 µm
20.0 kV 3.0 10000x SE 11.0 1 IWD72 + 0.5 IAA
(g)
Acc.V Spot Magn Det WD Exp 2 µm
20.0 kV 3.0 5000x SE 13.0 8 IWD72 YPD+0.5iaa pH 6.4
(h)
Acc.V Spot Magn Det WD Exp 10 µm
20.0 kV 5.0 1000x SE 12.8 8 IWD 72 YPD pH 6.4

(Figures 7.2 and 7.3). Glycerol (10% w/v) in the presence of 0.5% (v/v) isoamyl alcohol for both *S. cerevisiae* and *Z. kombuchaensis* caused the pseudohyphal counts to remain below 40% throughout the experiments. The peak in pseudohyphal formation of 24 and 48 h for *S. cerevisiae* and *Z. kombuchaensis* remained unchanged when glycerol was added to cultures containing 0.5% (v/v) isoamyl alcohol. The addition of glycerol to *Z. bailii* cultures containing 0.5% (v/v) isoamyl alcohol resulted in a slight reduction in growth than was evident in 0.5% (v/v) isoamyl alcohol cultures alone. The addition of 10% (w/v) glycerol to YPD cultures for all three yeasts in the absence of isoamyl alcohol resulted in slight suppression of growth in comparison to YPD alone and resulted in no morphological change (Figure 7.2 and 7.3).

7.2.4 Effects of isoamyl alcohol and glycerol on c.f.u counts

The application of methylene blue to samples withdrawn from cultures of *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* containing 0.5% (v/v) isoamyl alcohol showed many cells to be stained blue indicating a loss of viability (data not shown). In order to further study the effects of 0.5% (v/v) isoamyl alcohol on growth of *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* c.f.u counts were performed at various time intervals. Table 7.1 shows that the addition of 0.5% (v/v) isoamyl alcohol to YPD cultures caused a reduction in c.f.u counts. The biggest difference in c.f.u counts was shown for *S. cerevisiae* after 24 h incubation in which the number of c.f.u were just 0.02% of that in an equivalent culture without isoamyl alcohol. Indeed, all three yeasts only showed a maximum c.f.u count of approximately 10% that of an equivalent culture without isoamyl alcohol. Isoamyl alcohol therefore causes a significant reduction in c.f.u counts. The number of c.f.u counts increased with time for *S. cerevisiae*. The percentage of c.f.u counts for *Z. bailii* and *Z. kombuchaensis* showed a percentage trough at 48 h. All three yeasts reached a similar c.f.u count at 72 h incubation of 1-2 $\times 10^8$. The addition of 10%

Table 7.1 Differences in c.f.u counts between (a) *Z. bailii* NCYC 1766 (b) *Z. kombuchaensis* NRRL YB4811 and (c) *S. cerevisiae* IWD72 on exposure to isoamyl alcohol in the absence and presence of glycerol. Values in brackets show the % c.f.u counts in the corresponding culture without isoamyl alcohol

(a)	Time (h)	YPD	YPD + 0.5% (v/v) IAA	YPD + 10% (w/v) glycerol	YPD + 10% (w/v) glycerol + 0.5% (v/v) IAA
	24	1.93×10^7	1.10×10^6 (5.90)	1.05×10^7	4.90×10^5 (4.7)
	48	1.07×10^9	1.42×10^7 (1.30)	9.08×10^8	3.90×10^6 (0.40)
	72	5.00×10^9	1.28×10^8 (2.56)	5.20×10^8	9.07×10^7 (17.60)

(b)	Time (h)	YPD	YPD + 0.5% (v/v) IAA	YPD + 10% (w/v) glycerol	YPD + 10% (w/v) glycerol + 0.5% (v/v) IAA
	24	2.21×10^7	6.60×10^5 (2.90)	8.20×10^6	2.22×10^5 (2.7)
	48	1.06×10^9	2.70×10^6 (0.25)	1.40×10^9	1.50×10^6 (0.11)
	72	1.10×10^9	2.10×10^8 (10.00)	7.00×10^7	9.0×10^6 (12.9)

(c)	Time (h)	YPD	YPD + 0.5% (v/v) IAA	YPD + 10% (w/v) glycerol	YPD + 10% (w/v) glycerol + 0.5% (v/v) IAA
	24	2.86×10^8	6.70×10^4 (0.02)	2.23×10^8	2.42×10^5 (0.64)
	48	2.60×10^8	8.00×10^5 (0.31)	9.00×10^7	4.00×10^6 (4.40)
	72	1.20×10^9	1.30×10^8 (10.80)	1.09×10^9	1.50×10^8 (15.0)

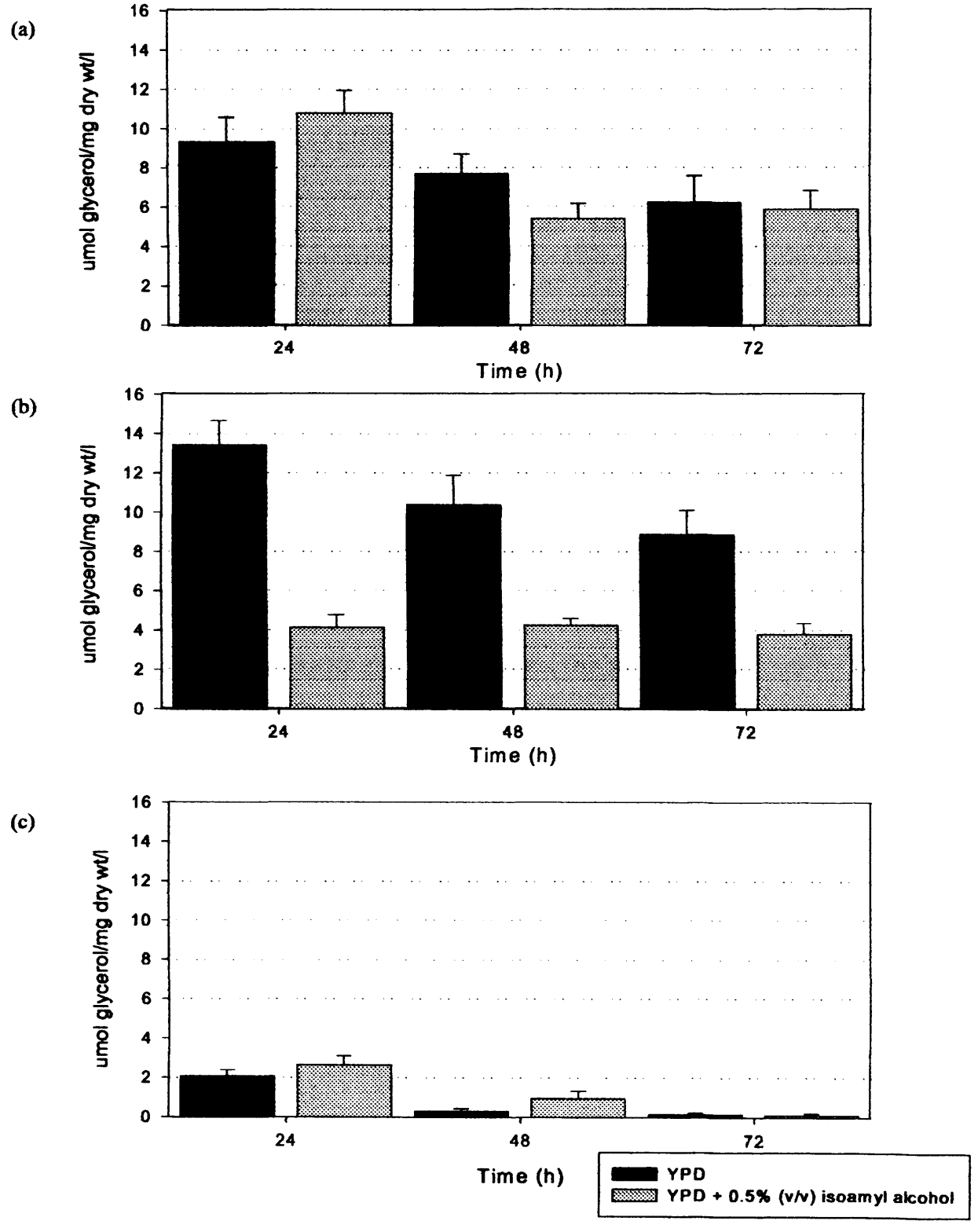
Data is representative of an experiment performed in duplicate with similar result

(w/v) glycerol to YPD cultures had no effect on the overall c.f.u counts for *S. cerevisiae* with the c.f.u counts remaining similar for both YPD and YPD with glycerol. Glycerol additions alone, however, did have a detrimental effect on the c.f.u count of both *Z. bailii* and *Z. kombuchaensis* (Table 7.1). These results are in agreement with OD results, which showed 10% (w/v) glycerol to cause slight inhibition of growth (Figure 7.3). The addition of 10% (w/v) glycerol to cultures containing 0.5% (v/v) isoamyl alcohol resulted in an increase in percentage c.f.u counts for all three yeasts, in comparison to cultures containing glycerol addition alone. Isoamyl alcohol therefore caused a reduction in the percentage c.f.u counts for all three yeasts that was counteracted by the addition of 10% (w/v) glycerol.

7.2.5 Glycerol content and isoamyl alcohol induced pseudohyphal formation

Glycerol caused a reduction in the percentage of isoamyl alcohol-induced pseudohyphal cells for *Z. kombuchaensis* and *S. cerevisiae*. *Z. bailii* did not exhibit pseudohyphal formation in the presence of 0.5% (v/v) isoamyl alcohol and is known to be osmotolerant. Since glycerol is a compatible solute synthesised in response to osmotic shock, it seemed prudent to compare the glycerol content of these three yeasts with and without isoamyl alcohol. The glycerol content of the three yeasts differed from one another and showed a different pattern in the presence of isoamyl alcohol (Figure 7.6). In the absence of isoamyl alcohol *Z. kombuchaensis* had the highest glycerol content followed by *Z. bailii* and *S. cerevisiae*. *S. cerevisiae* showed a level of glycerol at least four times lower than that of *Z. bailii* and *Z. kombuchaensis* at each time interval. Isoamyl alcohol caused an increase in total glycerol content for *S. cerevisiae* at 24 and 48 h. The glycerol levels at 72 h for *S. cerevisiae* with and without isoamyl alcohol being equivalent. In *Z. kombuchaensis* the glycerol levels in the presence of isoamyl alcohol were significantly lower than those without isoamyl alcohol. The glycerol levels

Figure 7.6 Effects of isoamyl alcohol on total glycerol content in (a) *Z. bailii* NCYC 1766 (b) *Z. kombuchaensis* NRRL YB4811 grown in YPD at 25°C incubation without shaking and (c) *S. cerevisiae* IWD72 grown at 30°C 160 r.p.m



Results are the mean of two independent experiments (four replicates), ± the standard error.

for *Z. kombuchaensis* without isoamyl alcohol decreased with time while in the presence of isoamyl alcohol they remained constant. *Z. bailii* showed the highest level of total glycerol in the presence of isoamyl alcohol being at its highest at 24 h. In the absence of isoamyl alcohol glycerol content decreased with time for all three yeasts.

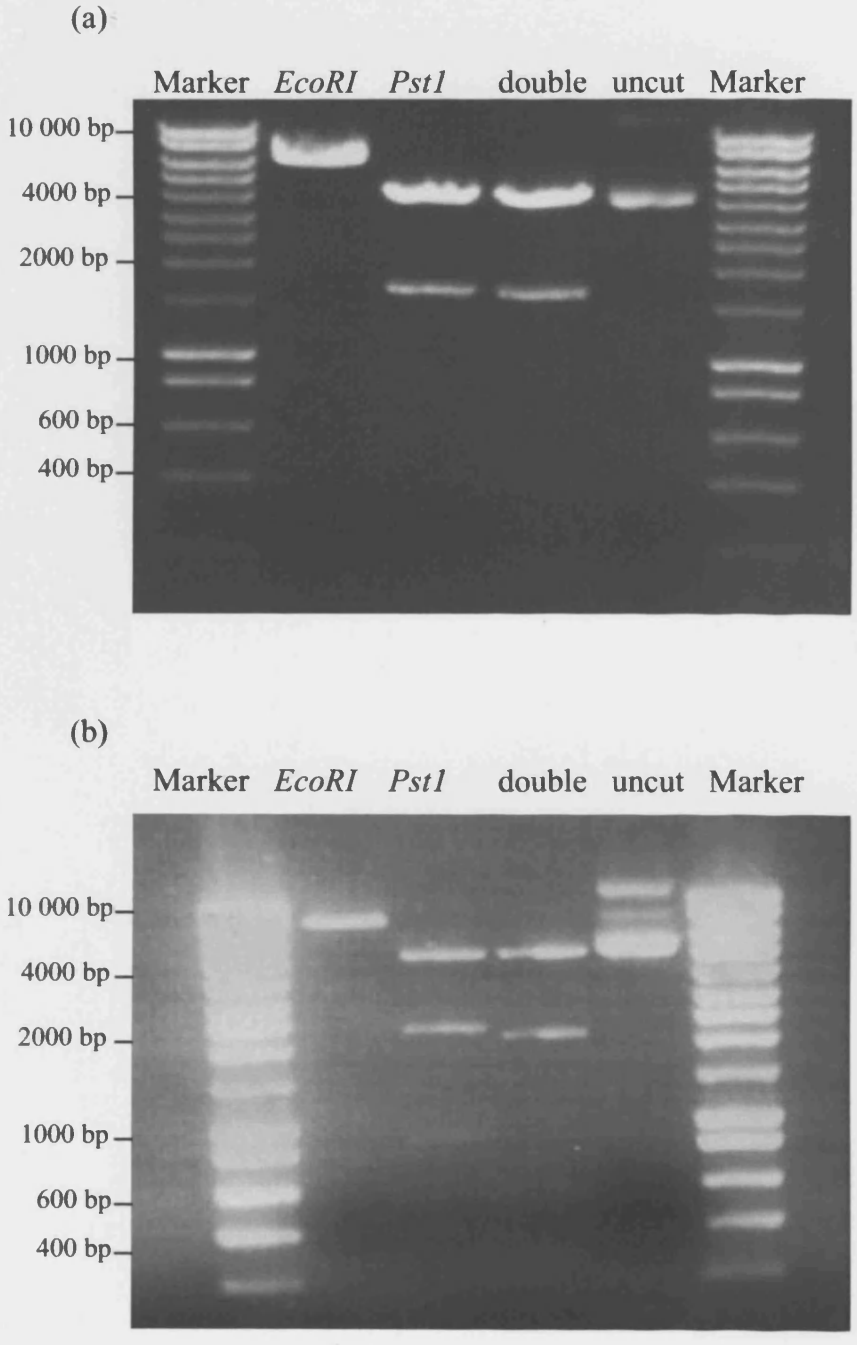
7.2.6 The HOG pathway in isoamyl alcohol induced pseudohyphal formation

7.2.6.1 Confirmation of plasmids and transformation

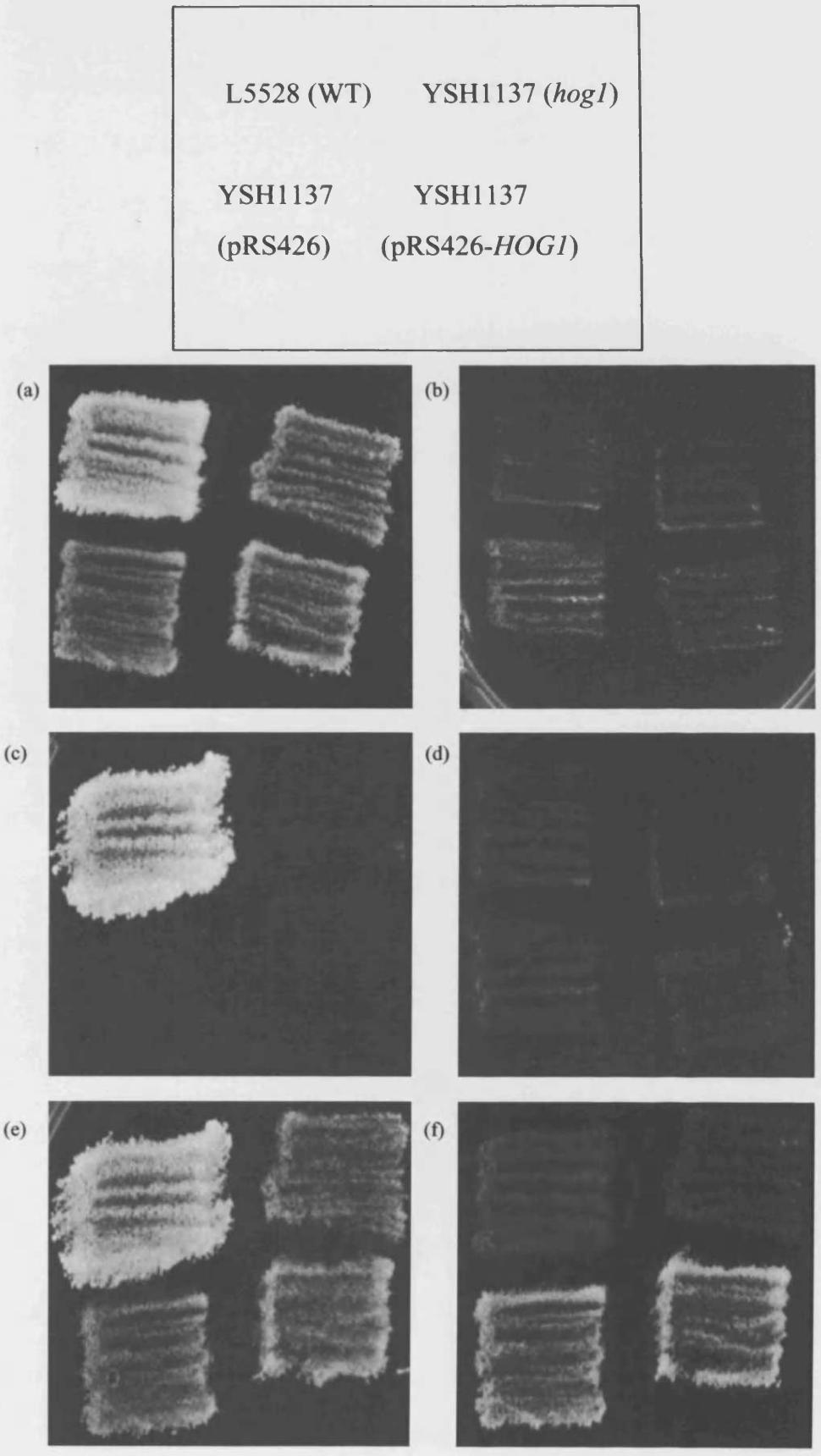
Salgado (2001) showed a *hog1* mutant to exhibit isoamyl alcohol induced pseudohyphae even in the presence of glycerol. This work extends on that by complementing the *hog1* mutant with a plasmid containing a functional *HOG1* gene (pRS426-*HOG1*). The same plasmid (pRS426) lacking the *HOG1* gene was used as an empty vector control. The plasmid identities were confirmed by *EcoR1* and *Pst1* restriction sites prior to transformation via restriction endonuclease digests. Figure 7.7 shows that the expected restriction fragments were obtained. The three bands present in the uncut pRS426-*HOG1* digest are representative of the different coiled circular forms of the plasmid, which all migrate at different rates. The pRS426 and pRS426-*HOG1* plasmids were confirmed to be approximately 6000 bp and 8000 bp respectively by the bands produced with *Eco*RI digests. A plasmid map of pRS426 and pRS426-*HOG1* can be found in appendix. The *hog1* mutant was in a different background to *S. cerevisiae* IWD72 therefore the parent of the *hog1* mutant L5528 was used as wild-type in all experiments relating to the HOG pathway. Figure 7.8 shows confirmation of *hog1* mutant with plasmids pRS426 and pRS426-*HOG1* by complementation of auxotrophic requirements. Both plasmids contain a functional *URA3* gene and transformation is confirmed by growth on minimal medium + uracil D/O. The other auxotrophic requirements of each strain are also confirmed. To confirm the presence of each plasmid colony PCR was used to amplify a section of the *HOG1* gene. The sequence of the

Figure 7.7 Plasmid confirmation by restriction endonuclease analysis

(a) pRS426 (b) pRS426-*HOG1*



Method used for restriction analysis can be found in section 2.11.3

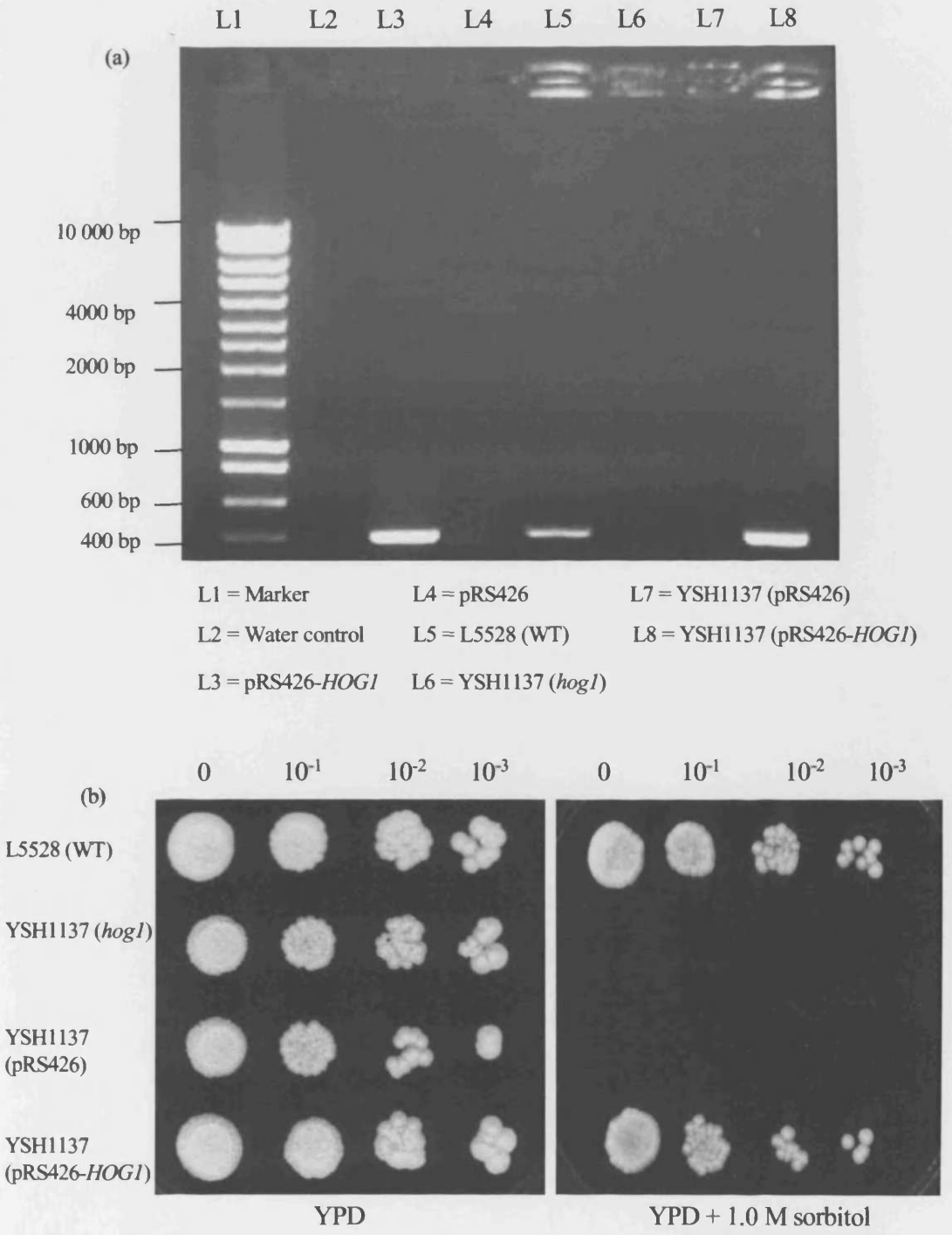

L5528 (WT)
YSH1137 (hog1)
YSH1137 (pRS426)
YSH1137 (pRS426-HOG1)
(a)
(b)
(c)
(d)
(e)
(f)

HOG gene is located in the appendix. Figure 7.9 (a) shows that only the pRS426-*HOG1* plasmid control, wild-type (L5528) and the strain transformed with pRS426-*HOG1* (YSH1137 pRS426-*HOG1*) gave positive PCR bands. The size of this fragment is in the region of 400 bp, which is the correct size for the length to be amplified by the HOG primers. The transformation of both pRS426 and pRS426-*HOG1* was also confirmed phenotypically by growth on media containing sorbitol (Figure 7.9, b). All four yeasts were capable of growth on YPD while only L5528 and YSH1137 (pRS426-*HOG1*) were capable of growth on media containing 1 M sorbitol. This confirms a functional HOG pathway for YSH1137 (pRS426-*HOG1*) and the lack of such a functional pathway in the empty vector control YSH1137 (pRS426).

7.2.6.2 Isoamyl alcohol induced pseudohyphal formation in a *S. cerevisiae hog1* mutant

Isoamyl alcohol induced pseudohyphae in the wild-type, *hog1* mutant, *hog1* mutant plus empty vector (YSH1137-pRS426) and the reconstituted *HOG1* wild-type (YSH1137-pRS426-*HOG1*) as shown in Figure 7.10. The pseudohyphal count for L5528 (WT) and YSH1137 (pRS426-*HOG1*) in the presence of isoamyl alcohol was greater than that of YSH1137 (*hog1* mutant) and the empty vector control YSH1137 (pRS426). Glycerol caused a decrease in percentage pseudohyphae for both wild-type and complemented *hog1* mutant (YSH1137-pRS426-*HOG1*). The level of pseudohyphae produced by the *hog1* mutant and empty vector control (YSH1137-pRS426) remained unchanged by the presence of glycerol (Figure 7.11). The addition of 10% (w/v) glycerol in the absence of isoamyl alcohol, however, induced morphological change in the *hog1* mutant and its empty vector control (YSH1137-pRS426). These cells in the presence of glycerol addition alone exhibited a range of morphologies. The strains used in this work were less sensitive to the effects of isoamyl alcohol than *S. cerevisiae* IWD72 as shown by

Figure 7.9 Confirmation of pRS426 and pRS426-*HOG1* transformation by (a) colony PCR (b) Growth on YPD and YPD + 1.0 M sorbitol



A suspension of 2 × 10^7 cells for each yeast was prepared and 10 fold dilutions prepared in peptone water. A 3 µl aliquot of each dilution was spotted onto each plate and incubated at 25°C for 5 days.

Figure 7.10 Morphology of (a) *S. cerevisiae* L5528 (WT) (b) *S. cerevisiae* YSH1137 (*hog1*) (c) *S. cerevisiae* YSH1137-pRS426 (d) *S. cerevisiae* YSH1137-pRS426-*HOG1* grown for 48 h incubation at 30°C 160 r.p.m in media as indicated above micrographs. Bar = 10 µm

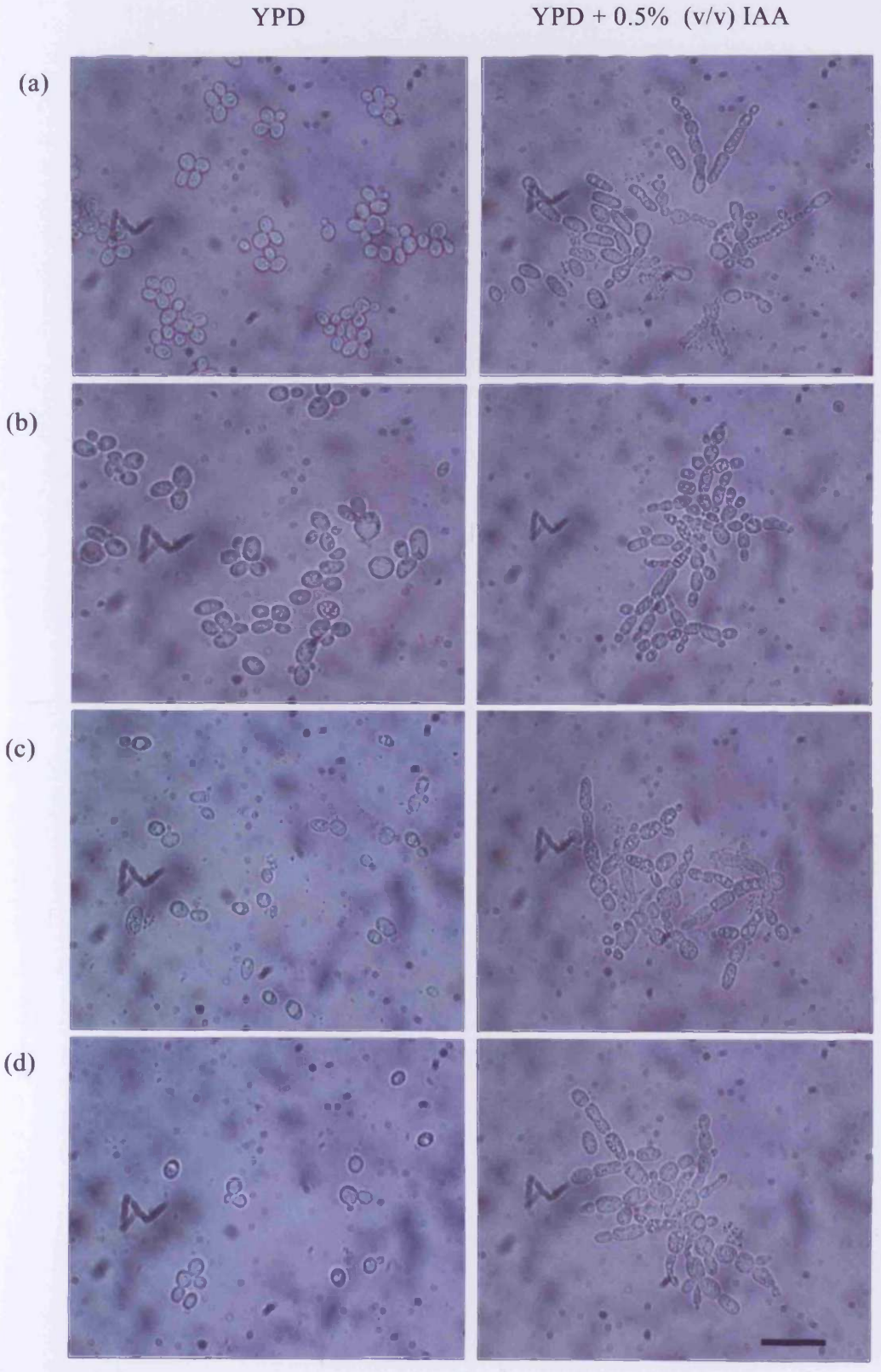


Figure 7.10 cont Morphology of (a) *S. cerevisiae* L5528 (WT) (b) *S. cerevisiae* YSH1137 (*hog1*) (c) *S. cerevisiae* YSH1137-pRS426 (d) *S. cerevisiae* YSH1137-pRS426-*HOG1* grown for 48 h incubation at 30°C 160 r.p.m in media as indicated above micrographs. Bar = 10 µm

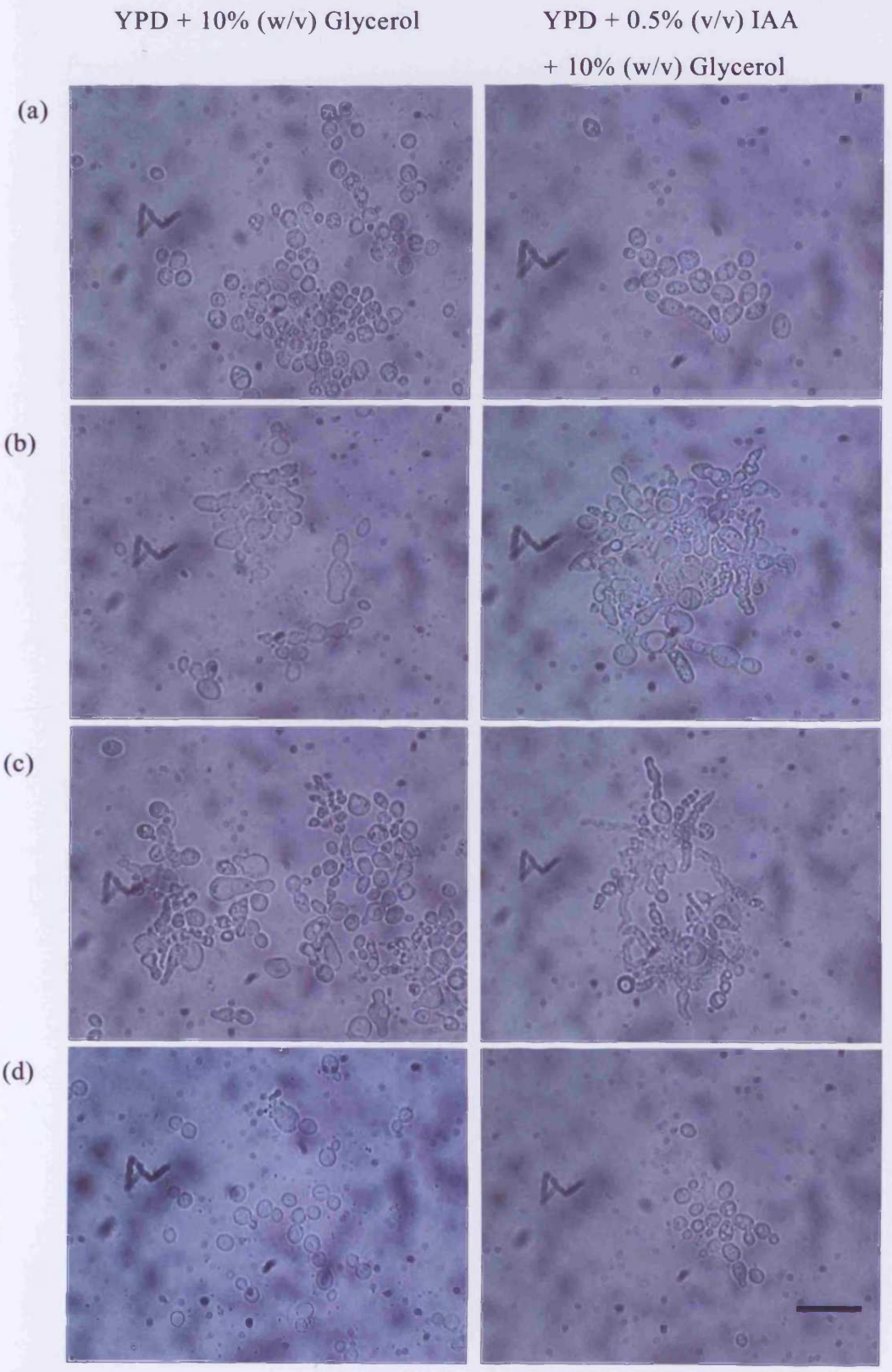
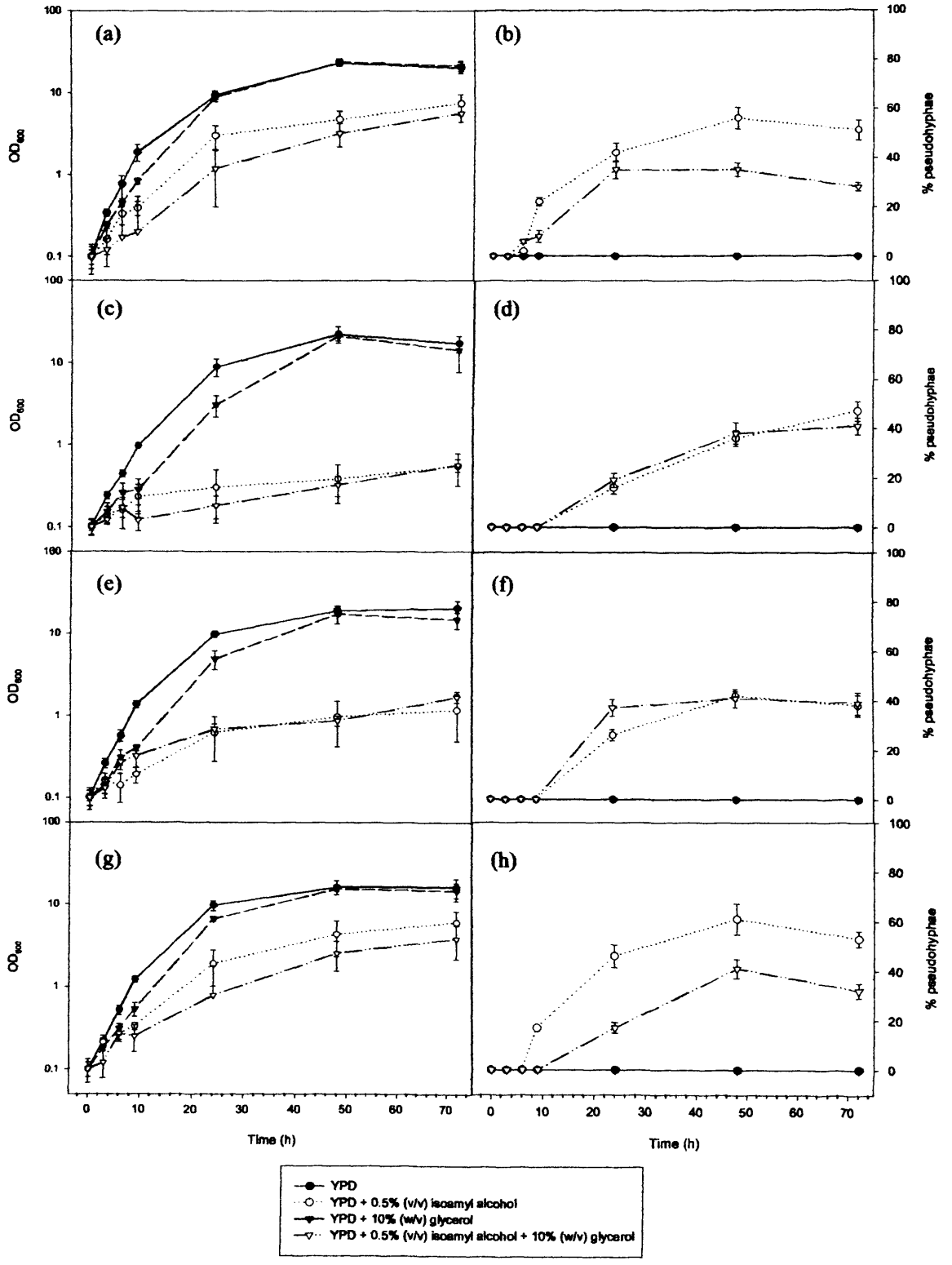


Figure 7.11 Effects of isoamyl alcohol and glycerol on yeast growth and pseudohyphal formation in *S. cerevisiae* (a, b) L5528 (WT) (c, d) YSH1137 (*hog1*) (e, f) YSH1137-pRS426 (g, h) YSH1137-pRS426-*HOG1* grown in YPD at 30°C 160 r.p.m



Results are the means of three experiments, ± the standard error.

the L5528 background strains reaching higher OD values (Figure 7.11). All the strains examined in this investigation into the role of the HOG pathway in isoamyl alcohol resistance exhibited similar levels of growth in both YPD and YPD + 10% (w/v) glycerol. The *hog1* mutant (YSH1137) and the empty vector control (YSH1137-pRS426) reached lower OD values in the presence of isoamyl alcohol with and without glycerol than the wild-type (L5528) and reconstituted *HOG1* wild-type (YSH1137-pRS426-*HOG1*). Therefore, the *hog1* mutant and empty vector control were more sensitive to the effects of isoamyl alcohol on growth.

7.2.6.3 Differences in sensitivity to cell wall/membrane disrupting agents between *hog1* mutants

The HOG pathway was shown to have an influence on isoamyl alcohol induced pseudohyphae (7.2.6.2). The nature of the involvement of the HOG pathway in isoamyl alcohol induced pseudohyphal formation remained unknown. In a bid to elucidate more information on the interaction between the HOG pathway and pseudohyphal formation it was decided to examine whether there were differences in the cell wall. Figure 7.12 shows the results obtained for sensitivity to several cell wall/membrane disrupting agents. All four strains showed growth at all dilutions examined on YPD. No differences in sensitivity were observed on exposure to congo red or caffeine over the concentrations examined. However, differences in sensitivity to Calcofluor white and SDS were observed. All strains exhibited some sensitivity to 0.25 mg/ml Calcofluor white, which was more pronounced for strains carrying the pRS426 and pRS426-HOG1 plasmids (Figure 7.12, c). SDS an indicator of differences in cell membrane permeability showed the wild-type (L5528) and the reconstituted *HOG1* wild-type (YSH1137-pRS426-*HOG1*) to be more resistant than the *hog1* mutant and empty vector control (YSH1137-pRS426) to 0.05% SDS.

Figure 7.12 Differences in cell wall/cell membrane disrupting agents between *S. cerevisiae* wildtype and HOG strains (a) YPD (b) 0.025 mg/ml Calcofluor white (c) 0.25 mg/ml Calcofluor white (d) 0.025 mg/ml congo red (e) 0.25 mg/ml congo red. Cell suspensions of 2 $\times 10^7$ cells ml in peptone water and 10 fold dilutions were prepared. A 3 µl aliquot of each dilution was spotted onto each plate and incubated for 5 days at 25°C in an inverted position.

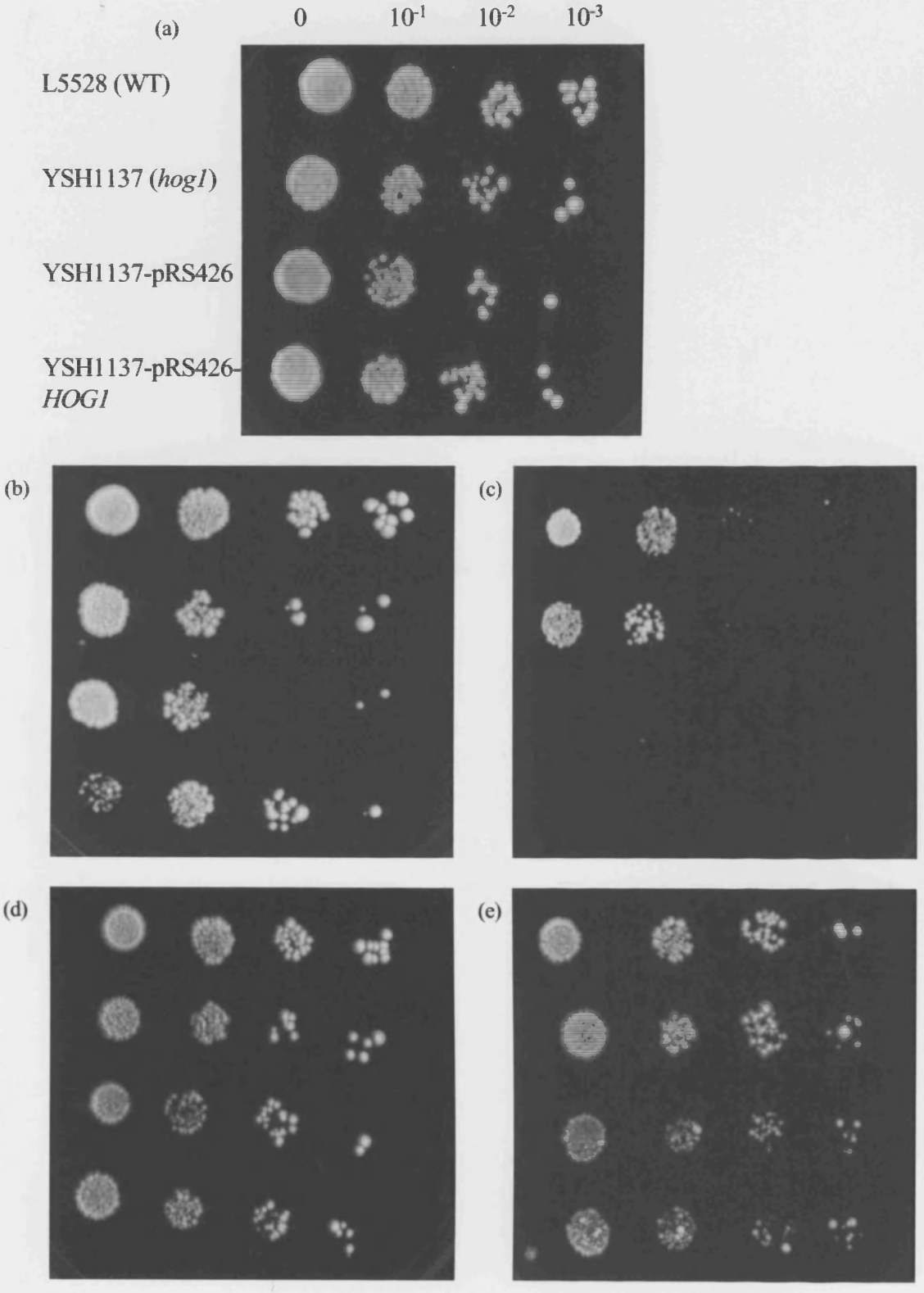
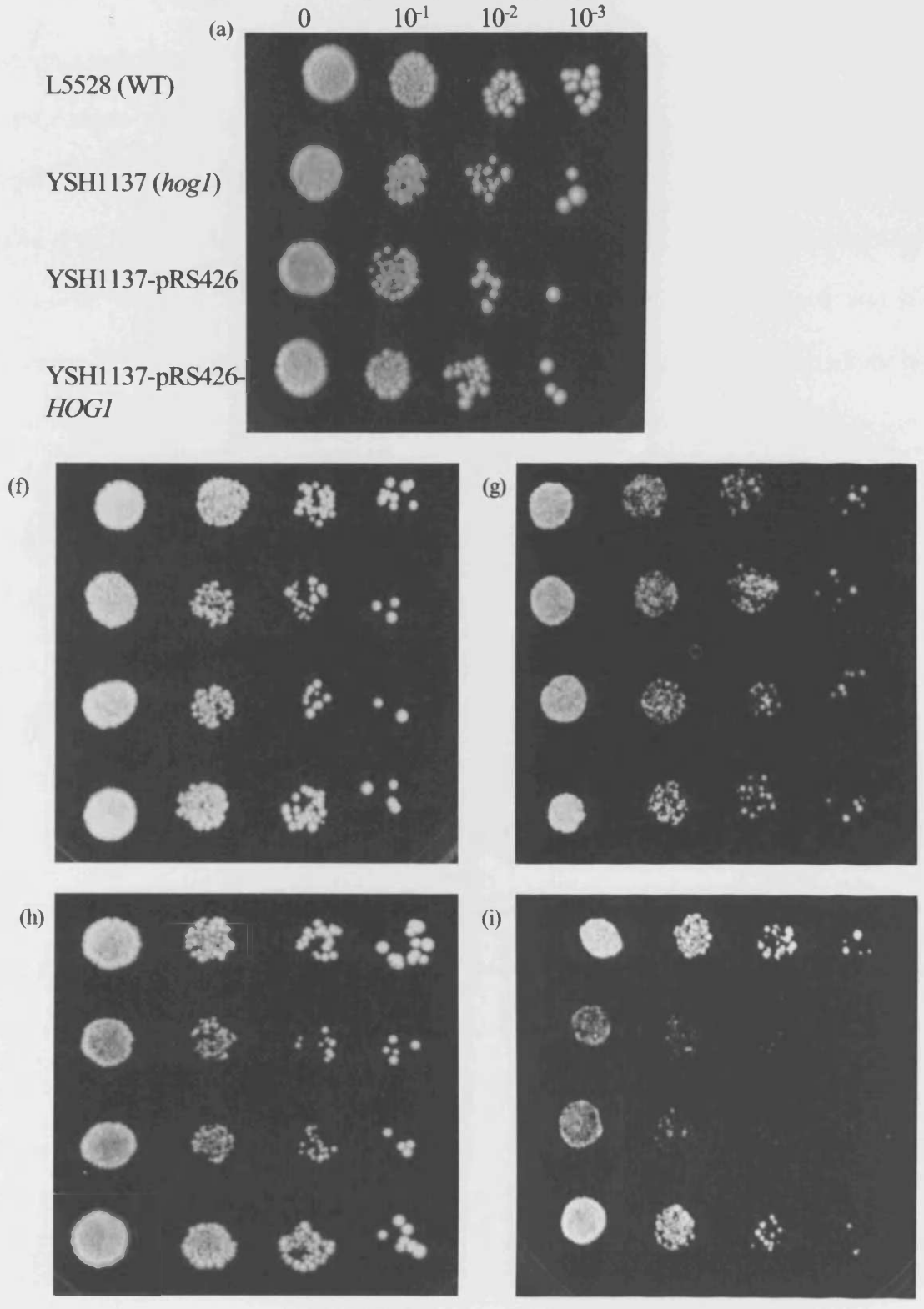


Figure 7.12 cont. Differences in cell wall/cell membrane disrupting agents between *S. cerevisiae* wildtype and HOG strains (a) YPD (f) 1.0 mg/ml caffeine (g) 2.0 mg/ml caffeine (h) 0.005% SDS (i) 0.05% SDS. Cell suspension of 2×10^7 cells ml in peptone water and 10 fold dilutions were prepared. A 3 µl aliquot of each dilution was spotted onto each plate and incubated for 5 days at 25°C in an inverted position.



7.3 Discussion

In this section, the first known study of morphological change in the *Zygosaccharomyces* yeasts, isoamyl alcohol was shown to induce pseudohyphae in *S. cerevisiae* and *Z. kombuchaensis* but not *Z. bailii*. One possible reason for this is that isoamyl alcohol causes an osmotic stress that triggers pseudohyphal formation. The osmotic stress caused by isoamyl alcohol could be insufficient to cause the development of pseudohyphae in the more osmotolerant *Z. bailii* (Thomas and Davenport, 1985; Steels *et al.*, 2000). The TEM results support the possibility of isoamyl alcohol causing an osmotic stress as many of the pseudohyphal cells for *Z. kombuchaensis* and *S. cerevisiae* show evidence of the cytosol detaching from the cell envelope, which is characteristic of hyper-osmotic stress (Tamás and Hohmann, 2003). Vacuoles are known to play an important role in plant osmoregulation by acting as a water reservoir for the cytosol (Chrispeels *et al.*, 2001). The presence of large vacuoles particularly for *Z. kombuchaensis* in the presence of isoamyl alcohol is therefore indicative of osmotic stress. Isoamyl alcohol, therefore, causes a number of changes to the cell structure that are characteristic of hyper-osmotic stress.

Salgado (2001) exposed *S. cerevisiae* to glycerol, in the presence of isoamyl alcohol on the basis that isoamyl alcohol exerted some sort of osmotic stress. Glycerol at an optimal concentration of 10% (w/v) was shown to increase growth and reduce pseudohyphal formation in the presence of isoamyl alcohol for *S. cerevisiae*. Salgado (2001) showed this response to be specific for glycerol with other compatible solutes including sorbitol, mannitol, trehalose and the di-ol 1, 3-butanediol having no effect despite being involved in osmotic stress responses (Rains & Valentine, 1980; Nevoigt & Stahl, 1997). Glycerol was also shown to reduce isoamyl alcohol induced pseudohyphal formation for *Z. kombuchaensis* (Figure 7.3). Therefore, glycerol has been shown to

specifically reduce isoamyl alcohol induced pseudohyphal formation in both *S. cerevisiae* and *Z. kombuchaensis*. The addition of 10% (w/v) glycerol in the presence of 0.5% (v/v) isoamyl alcohol resulted in an increase in percentage c.f.u for *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*. Therefore, even though isoamyl alcohol does not induce pseudohyphae in *Z. bailii* the addition of glycerol still leads to an increase in c.f.u counts.

The formation of glycerol is an integral part of osmoregulation, which in *S. cerevisiae* is closely linked to the high osmolarity glycerol (HOG) pathway (Tamás *et al.*, 2000). A number of the MAP kinase pathways have a number of areas of overlap including Cdc24, Cdc42, Ste20 and Ste11 (Tamás and Hohmann, 2003). It is therefore possible for cross-talk to occur between many of the pathways. Any cross-talk between MAP kinase pathways could account for the role of glycerol in isoamyl alcohol induced pseudohyphal formation. The HOG pathway could sense isoamyl alcohol, which leads to the initiation of pseudohyphal formation via the morphological switch MAP kinase pathway. This could then lead to an accumulation of glycerol which, when present at a given threshold, prevents the initiation of pseudohyphal formation. There is a variety of evidence in support of this hypothesis. The fact that the percentage of pseudohyphae is reduced at 72 h suggests that the cells have adapted to the effects of isoamyl alcohol. The glycerol content for *S. cerevisiae* was shown to increase at 24 and 48 h in the presence of 0.5% (v/v) isoamyl alcohol, which may be sufficient to prevent further pseudohyphal formation. The *hog1* mutant and empty vector control showed no reduction in percentage pseudohyphal formation in the presence of isoamyl alcohol and glycerol. The wild-type and the re-constituted *HOG1* wild-type, however, did show a reduction in isoamyl alcohol induced pseudohyphae in the presence of glycerol.

There are a number of observations from the experiments conducted that suggest isoamyl alcohol may not just be causing an osmotic stress. Indeed, if the effect of isoamyl alcohol on yeasts were only that of osmotic stress then one would expect any alcohol to have some morphological effect. The fact that only a few alcohols including isoamyl alcohol (Dickinson 1996; Ashe *et al.* 2001; Martínez-Anaya *et al.* 2003) induce morphological change suggests that osmotic stress is not the only factor. This is further supported by the mitigation of the effects of isoamyl alcohol being specific for glycerol and not any compatible solute. *Z. kombuchaensis* had a glycerol content of 5-10 times greater than *S. cerevisiae* both in the absence and presence of isoamyl alcohol. If the ability of isoamyl alcohol to induce pseudohyphae was only related to osmotic stress then *Z. kombuchaensis* with its higher glycerol content should not exhibit pseudohyphal formation. Indeed, isoamyl alcohol actually caused the glycerol content of *Z. kombuchaensis* to decrease rather than increase as with *S. cerevisiae*. In a bid to understand the role of glycerol in isoamyl alcohol induced pseudoyphal formation in *S. cerevisiae,* experiments in which the activity of key glycerol metabolic enzymes including glycerol-3-phosphate dehydrogenase (Gpd) and glycerol-3-phosphate phosphatase (Gpp) would prove of interest. The activity of these enzymes in the presence of isoamyl alcohol, ethanol and sorbitol would aid the understanding of glycerol in isoamyl alcohol stress.

Isoamyl alcohol has recently been reported to cause a proliferation of mitochondria for *S. cerevisiae* (Martínez-Anaya *et al.* 2003). The same proliferation was evident for *Z. kombuchaensis* and *Z. bailii*. The reason for the proliferation of mitochondria in the presence of isoamyl alcohol remains unknown. It could be the result of some form of oxidative stress. Evidence to support this theory is shown by the HOG pathway being

activated by oxidative stress in *C. albicans* (Alonso-Monge *et al.*, 2003; Navarro-Garcia *et al.*, 2003).

The *hog1* mutant and its empty vector control were shown to be more sensitive to the effects of SDS a known indicator of differences in permeability than the wild-type and complemented *hog1* mutant. This is an interesting result and means that permeability may play a role in the effects of isoamyl alcohol on yeast pseudohyphal formation. Evidence has recently been presented for the HOG pathway being stimulated by turgor pressure rather than water stress (Tamás *et al.*, 2000). It is therefore possible for isoamyl alcohol to cause an alteration in turgor rather than water stress, which is detected by the HOG pathway. The HOG pathway could then trigger the morphological switching pathway resulting in the formation of pseudohyphae and the activation of glycerol accumulation. Isoamyl alcohol is also known to target eIF2B in order to allow regulation of translation (Ashe *et al.*, 2001). The possibility of the HOG pathway altering eIF2B function in response to alcohols cannot be dismissed.

Overall, *Z. bailii* unlike *Z. kombuchaensis* and *S. cerevisiae* did not from psedudohypahe in the presence of isoamyl alcohol. Isoamyl alcohol would appear to cause some degree of osmotic stress with a role for the HOG pathway being demonstrated. The involvement of oxidative stress, turgor pressure and a role for the eukaryotic initiation factor eIF2B in isoamyl alcohol induced pseudohyphae remain interesting possibilities.

8. Re-examination of *Z. bailii* classification

8.1 Introduction

Traditionally the physiological identification of *Zygosaccharomyces* species has proved difficult due to the limited ability of these yeasts to ferment or assimilate carbon sources (Kurtzman, 1990). On the basis of traditional physiological tests eight species of *Zygosaccharomyces* were identified (Yarrow, 1984). These comprised of *Z. bailii, Z. bisporus, Z. cidri, Z. fermentati, Z. florentinus, Z. microellipsoides, Z. mrakii* and *Z. rouxii*. A ninth species, *Z. mellis* was identified as a separate species from *Z. rouxii* on the basis of DNA relatedness (Kurtzman, 1990). Two additional species of *Zygosaccharomyces* have been identified: *Z. lentus* was identified on the basis of molecular and physiological differences from yeasts originally classed as *Z. bailii* (Steels *et al.*, 1999a, b). The latest species of *Zygosaccharomyces* to be identified is *Z. kombuchaensis,* which was isolated from 'kombucha tea' with four strains being currently known (Kurtzman *et al.*, 2001). The application of 26S rDNA D1/D2 sequence analysis has shown *Z. kombuchaensis* to be closely related to *Z. lentus* (Steels *et al.*, 2002). The eleven aforementioned species of *Zygosaccharomyces* based on physiological and molecular data have recently been reduced to the following six species: *Z. bailii, Z. bisporus, Z. kombuchaensis, Z. lentus, Z. mellis* and *Z. rouxii* (Kurtzman 2003). The classification of *Zygosaccharomyces* yeasts is therefore one under constant review.

In this section, differences in organic acid resistance between strains of *Z. bailii* (described in section 1) are re-examined in addition to resistance to the inhibitors ethanol, sodium chloride and hydrogen peroxide. The morphology, physiology and molecular composition of the *Z. bailii* strains are also examined. The overall aim was to determine whether the differences in *Z. bailii* were due to strain variation or represented a greater divergence within the species.

8.2 Results

8.2.1 Differences in inhibitor resistance between strains of *Z. bailii*

The organic acid resistance results for *Z. bailii* reported in section 3 were re-examined in addition to the inhibitors ethanol, sodium chloride and hydrogen peroxide. The collective results are presented in Table 8.1. The results show *Z. bailii* NCYC 1416 and NCYC 385 to have lower MICs to all inhibitors except decanoic acid and ethanol. The biggest differences in inhibitor resistance within the *Z. bailii* strains were found for medium chain organic acids (hexanoic, sorbic and benzoic acid). On the basis of the inhibitor MICs the *Z. bailii* strains examined were divided into two groups. Group A included *Z. bailii* NCYC 1766, 11, NCYC 1520, 20 and 80. The two remaining yeasts *Z. bailii* NCYC 1416 and 385 were designated as group B. Despite *Z. bailii* 11 (an isolate from an American soft drinks factory) exhibiting MICs for hydrogen peroxide, sodium chloride and sorbic acid similar to those of the more sensitive (NCYC 1416 and NCYC 385) *Z. bailii* strains it was placed into group A on the basis of the majority of MICs. The mean MICs for organic acids (including standard errors) for the two *Z. bailii* groups were compared to those of *Z. kombuchaensis* and *S. cerevisiae*. The results are presented in Table 8.2. The small standard errors for *Z. kombuchaensis* and *S. cerevisiae* for all organic acids except acetic acid are indicative of little strain variation. *Z. bailii* group B also had small standard errors for the MICs to all organic acids. *Z. bailii* group A, generally, shows the most strain variation as shown by the standard errors. The placing of all *Z. bailii* strains into one group resulted in considerably larger standard errors for all organic acids except decanoic acid (data not shown). The two groups of *Z. bailii* are distinct from one another on the basis of MICs except for formic acid in which the standard errors are indicative of a small overlap of MICs. *Z. bailii* group B shows a similar level of resistance to medium and longer chain organic acids as *Z. kombuchaensis* and *S. cerevisiae* (except for the known extreme sorbic acid

Table 8.1 Differences in inhibitor resistance between strains of *Z. bailii*

Inhibitor	NCYC 1766	NCYC 1416	11	NCYC 385	NCYC 1520	20	80
Formic acid (mM)	110 ± 8.16	90 ± 14.14	90 ± 8.16	70 ± 9.57	90 ± 8.16	90 ± 9.57	90 ± 8.16
Acetic acid (mM)	400 ± 20.41	300 ± 28.87	350 ± 20.41	300 ± 20.41	400 ± 20.41	350 ± 28.87	400 ± 0.00
Propionic acid (mM)	100 ± 8.16	80 ± 11.55	100 ± 0.00	80 ± 11.54	100 ± 8.16	100 ± 0.00	100 ± 8.16
Hexanoic acid (mM)	8 ± 0.41	4 ± 0.58	6 ± 0.00	3 ± 0.41	8 ± 0.91	6 ± 0.00	8 ± 0.91
Sorbic acid (mM)	8 ± 0.71	4 ± 0.58	5 ± 0.71	3 ± 0.91	8 ± 0.33	8 ± 0.58	8 ± 0.33
Benzoic acid (mM)	9 ± 0.48	5 ± 1.22	9 ± 1.08	5 ± 0.71	9 ± 0.48	8 ± 0.00	9 ± 0.71
Octanoic acid (mM)	2.1 ± 0.17	1.5 ± 0.21	1.8 ± 0.00	0.6 ± 0.00	2.1 ± 0.00	1.8 ± 0.00	2.1 ± 0.17
Nonanoic acid (mM)	0.6 ± 0.00	0.5 ± 0.11	0.6 ± 0.04	0.4 ± 0.11	0.7 ± 0.09	0.7 ± 0.04	0.8 ± 0.00
Decanoic acid (mM)	0.2 ± 0.00	0.2 ± 0.04	0.2 ± 0.00	0.2 ± 0.04	0.2 ± 0.00	0.2 ± 0.00	0.2 ± 0.02
Ethanol (M)	1.8 ± 0.12	1.8 ± 0.17	1.8 ± 0.17	2.3 ± 0.24	1.8 ± 0.12	1.8 ± 0.12	1.8 ± 0.17
Sodium Chloride (M)	2.4 ± 0.00	2.0 ± 0.18	2.0 ± 0.08	2.0 ± 0.18	2.4 ± 0.08	2.4 ± 0.00	2.4 ± 0.14
Hydrogen peroxide (mM)	10 ± 0.41	6 ± 0.91	6 ± 0.00	6 ± 0.41	10 ± 0.41	8 ± 0.71	8 ± 0.00

Values are mean MICs measured from at least two independent experiments (four replicates) in YPD pH 4.0 following 14 days incubation at 25°C, ± the standard error.

Table 8.2 Mean MIC (mM) ± SE of organic acids on *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*.

	Z. bailii group A	*Z. bailii* group B	*Z. kombuchaensis*	*S. cerevisiae*
Short chain acids				
Formic acid	94 ± 4.00	80 ± 10.00	70 ± 0.00	50 ± 0.00
Acetic acid	380 ± 14.43	300 ± 0.00	313 ± 12.5	100 ± 10.21
Propionic acid	100 ± 0.00	80 ± 0.00	120 ± 0.00	50 ± 0.00
Medium chain acids				
Hexanoic acid	7.20 ± 0.49	3.50 ± 0.50	4.50 ± 0.29	3.00 ± 0.00
Sorbic acid	7.40 ± 0.60	3.50 ± 0.50	1.25 ± 0.08	3.50 ± 0.29
Benzoic acid	8.80 ± 0.20	5.00 ± 0.00	6.00 ± 0.00	3.25 ± 0.08
Longer chain acids				
Octanoic acid	1.98 ± 0.07	1.05 ± 0.45	1.05 ± 0.09	1.05 ± 0.09
Nonanoic acid	0.68 ± 0.04	0.45 ± 0.05	0.40 ± 0.00	0.48 ± 0.03
Decanoic acid	0.20 ± 0.00	0.20 ± 0.00	0.15 ± 0.00	0.15 ± 0.00

Results are based on at least duplicate experiments in YPD pH 4.0 following 14 days incubation at 25°C.
Z. bailii group A comprises NCYC 1766, 11, NCYC 1520, 20 & 80. *Z. bailii* group B comprises NCYC 1416 & NCYC 385.
Z. kombuchaensis comprises NRRL YB4810, NRRL Y27163, NRRL Y27162 & NRRL YB4811.
S. cerevisiae comprises NCYC 957, NCYC 1324, NCYC 1119 & BY4741.

sensitivity of *Z. kombuchaensis*) but is clearly more resistant than *S. cerevisiae* to short chain organic acids. Overall, the differences in organic acid resistance between *Z. bailii* strains allow the formation of two groups: one that is resistant and the other more sensitive to inhibitors.

8.2.2 Morphological differences between strains of *Z. bailii*

During the investigations into the differences in inhibitor resistance between *Z. bailii, Z. kombuchanesis* and *S. cerevsiae,* two strains of *Z. bailii* were shown to exhibit a number of morphological differences. The morphological differences between strains of *Z. bailii* are summarised in Table 8.3. *Z. bailii* strains NCYC 1416 and NCYC 385 even though differing in shape with the former being long-oval and the latter round, showed a number of morphological properties distinct from the other *Z. bailii* strains. The two strains were shown to form pairs and to aggregate, the latter property being classed as flocculation. These two strains were also shown to form rough colonies on agar while all the other strains represented by *Z. bailii* NCYC 1766 and *Z. bailii* 11 were shown to form smooth colonies (Figure 8.1). The ability of *Z. bailii* strains to form natural pseudohyphae was assessed. *Z. bailii* NCYC 1416 and NCYC 385 were the only strains to show natural pseudohyphae on prolonged culture to YPD (Figure 8.2). In summary, the two *Z. bailii* strains, which were shown to differ in inhibitor resistance, could also be distinguished from the other *Z. bailii* strains by morphological differences.

8.2.3 Physiological differences between strains of *Z. bailii*

The inhibitor resistance and morphological differences reported between strains of *Z. bailii* were extended to look for physiological differences. The two strains of *Z. bailii* NCYC 1416 and NCYC 385 were again shown to differ from the other *Z. bailii* strains examined (Table 8.3). Testing for growth on media with different carbon sources

Table 8.3 Morphological and physiological differences between strains of *Z. bailii*

Physical Characteristic	NCYC 1766	NCYC 1416	11	NCYC 385	NCYC 1520	20	80
Morphology:							
Shape	oval	Long-oval	oval	round	oval	oval	oval
Arrangement	single	pair	single	pair	single	single	single
Texture on agar	smooth	wrinkled	smooth	wrinkled	smooth	smooth	smooth
Deposit in broth	non-flocculent	flocculent	non-flocculent	flocculent	non-flocculent	non-flocculent	non-flocculent
pseudohyphae	-	+	-	+	-	-	-
Growth on:							
Glucose	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Sorbose	-	+	+	+	-	-	-
Mannose	+	+	+	+	+	+	+
Maltose	-	-	-	-	-	-	-
Galactose	+	-	+	-	+	+	+
Growth at:							
4°C	+	-	+	-	+	+	+
25°C	+	+	+	+	+	+	+
30°C	+	+	+	+	+	+	+
37°C	+	-	-	-	+	+	+

Presence of physical characteristic is indicated by a + sign and its absence by a – sign. Results are based on at least two independent data sets.

Figure 8.1 Colony morphology after 48 h incubation at 25°C on YPD plates (a) *Z. bailii* NCYC 1766 (b) *Z. bailii* NCYC 1416 (c) *Z. bailii* 11 and (d) *Z. bailii* NCYC 385

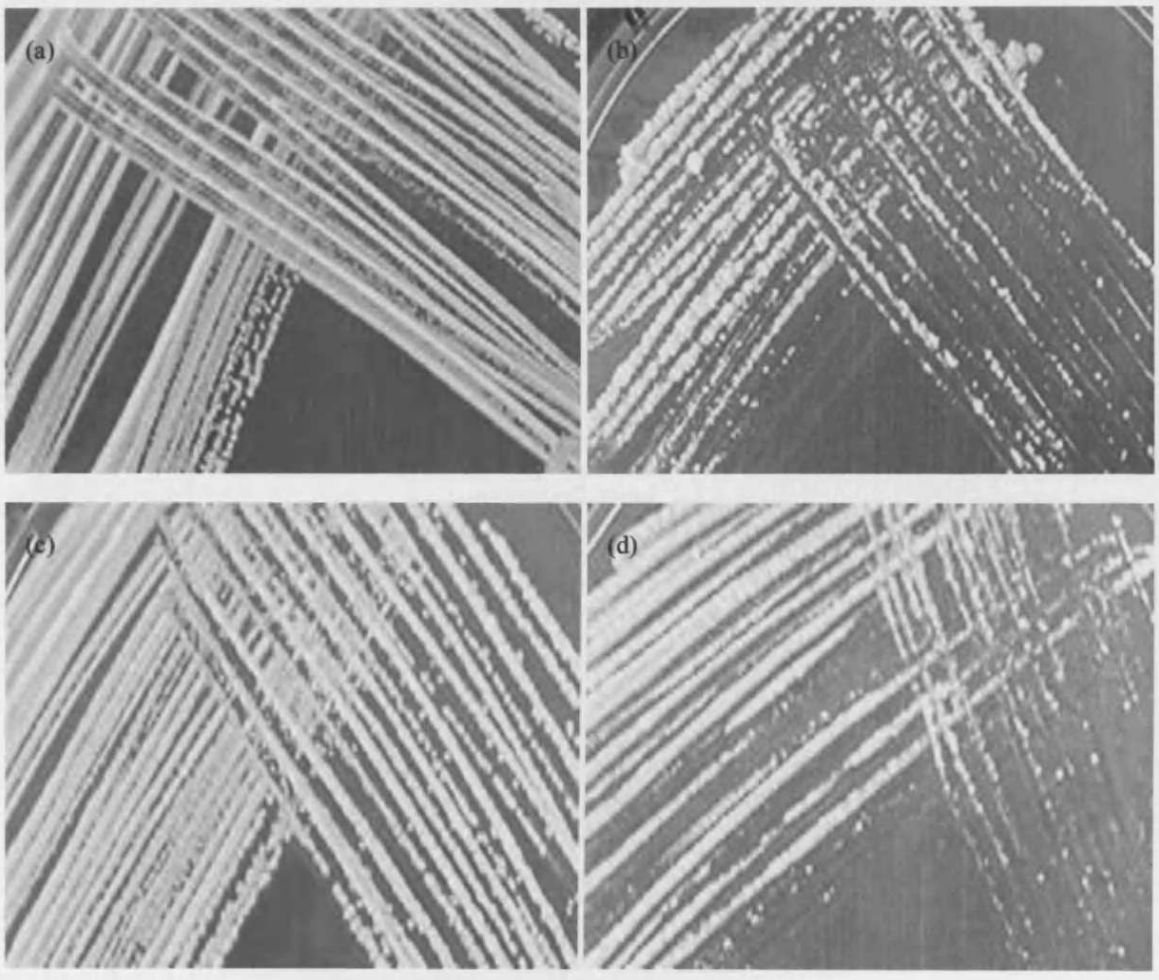
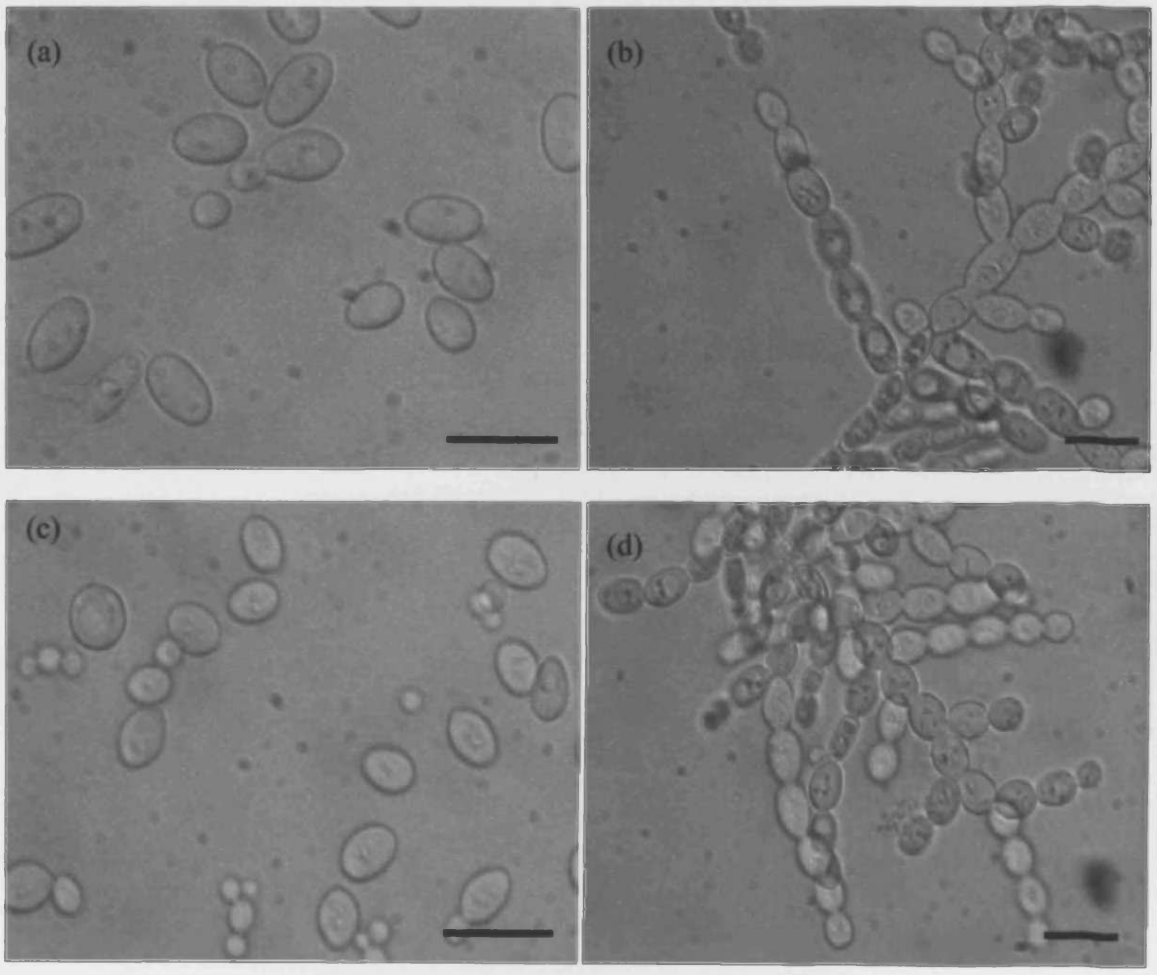
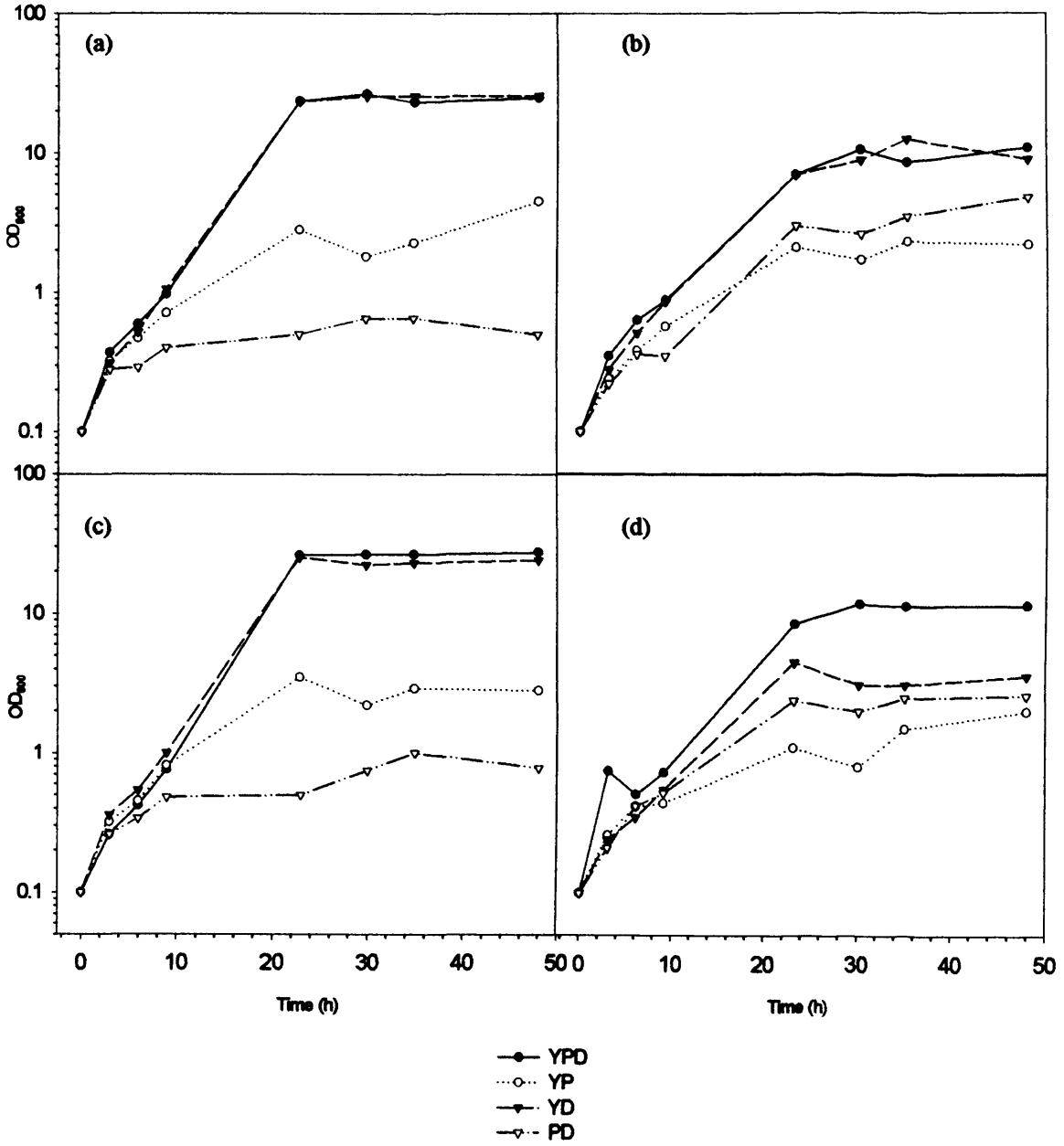


Figure 8.2 Cell morphology after 10 days incubation at 25°C on YPD plates (a) *Z. bailii* NCYC 1766 (b) *Z. bailii* NCYC 1416 (c) *Z. bailii* 11 and (d) *Z. bailii* NCYC 385. Bar = 10 μm.



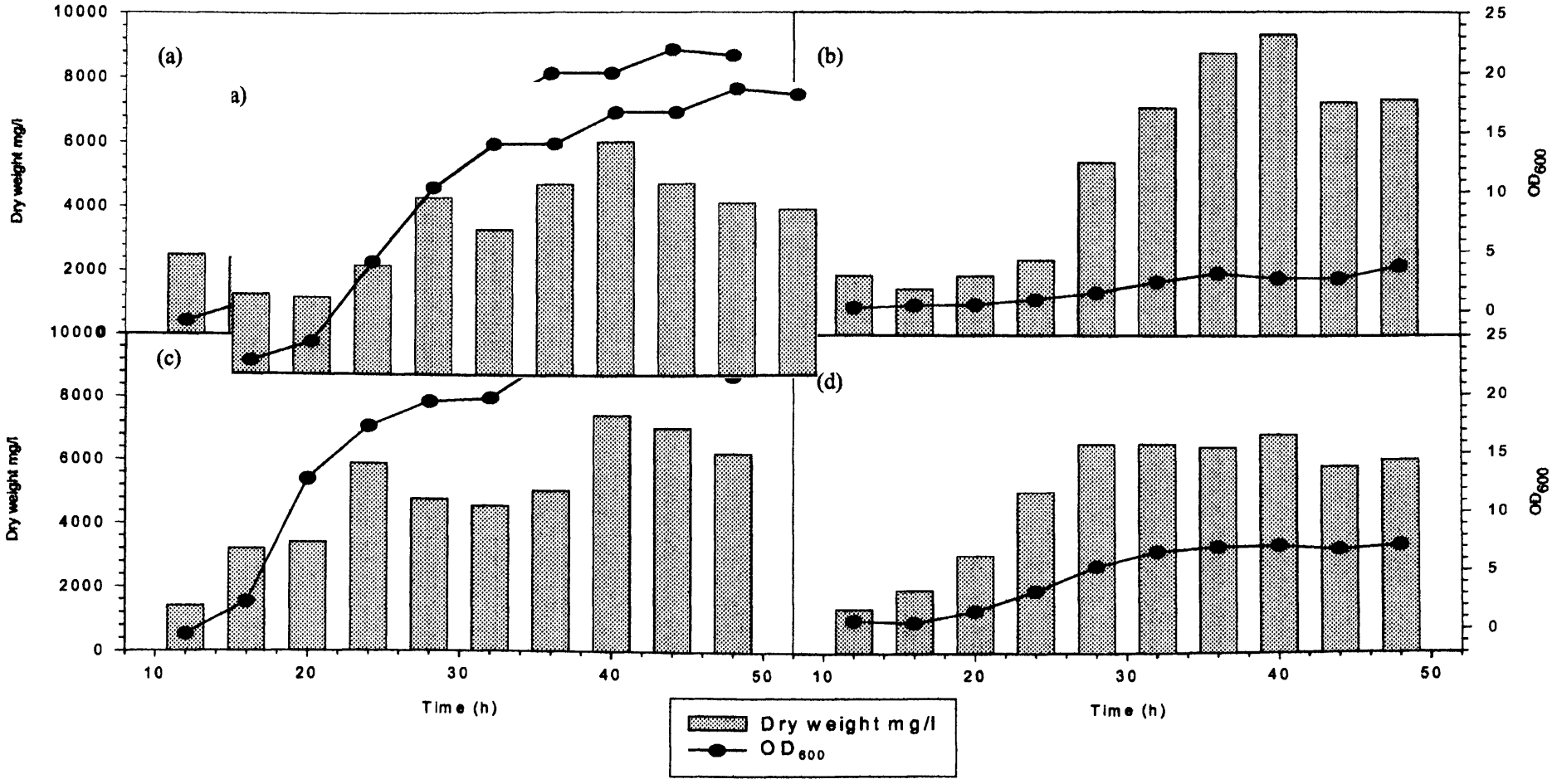
showed the two aforementioned yeasts to be the only *Z. bailii* strains examined to be unable to grow on galactose. *Z. bailii* 11, NCYC 1416 and NCYC 385, however, were the only *Z. bailii* strains capable of growth on sorbose. The ability of *Z. bailii* strains to grow at different temperatures was also examined. Strains NCYC 1416 and NCYC 385 could not grow at 4°C after prolonged incubation (six weeks). Growth at 37°C was also absent for strains NCYC 1416 and NCYC 385 in addition to *Z. bailii* 11. All the remaining *Z. bailii* strains showed growth at all four temperatures examined. The composition of complex medium was shown to have an influence on yeast organic acid resistance (section 4). Therefore, the effects of complex medium on the growth of *Z. bailii* strains were examined (Figure 8.3). The results show that the *Z. bailii* strains examined could be divided into two groups; one group comprising NCYC 1416 and 385 which showed the absence of dextrose to cause the greatest reduction in growth. The second group consisting of all the remaining *Z. bailii* strains showed growth most affected by the absence of yeast extract. The OD values reached by *Z. bailii* NCYC 1416 and NCYC 385 even in complete complex medium (YPD) were considerably lower than those of the remaining strains. The time taken to reach stationary phase by the two aforementioned strains was also longer than those of the other *Z. bailii* strains. On the basis of differences in OD values corresponding to stationary phase between *Z. bailii* NCYC 1416 and NCYC 385 with the remaining *Z. bailii* strains, dry weights were compared. The dry weight results are shown in Figure 8.4. The dry weight determinations show that despite strains NCYC 1416 and NCYC 385 reaching lower OD values, their dry weights were at least equivalent to those of the other *Z. bailii* strains examined. *Z. bailii* NCYC 1416 reached the lowest OD values but showed the highest dry weight. The results collectively point to a number of physiological differences between the proposed *Z. bailii* groups.

Figure 8.3 Differences in growth in complex medium between strains of *Z. bailii* (a) NCYC 1766 (b) NCYC 1416 (c) 11 (d) NCYC 385 grown at 25°C at 160 r.p.m



Results are the means of two independent experiments (four replicates) with standard errors of <5%

Figure 8.4 Dry weight comparison of *Z. bailii* strains (a) NCYC 1766 (b) NCYC 1416 (c) 11 (d) NCYC 385 grown in YPD pH 4.0 at 25°C without shaking



Results are representative of an experiment performed in triplicate with standard errors of <10%.

8.2.4 Molecular differences between strains of *Z. bailii*

To extend upon the differences in inhibitor resistance, morphology and physiology reported for *Z. bailii* potential differences in their molecular composition were examined. An analysis of the 26S rDNA D1/D2 sequences of the *Z. bailii* strains used throughout this investigation was kindly conducted by S. A. James (NCYC, Norwich, UK) and is presented with permission in Figures 8.5 and 8.6. An analysis of the two atypical *Z. bailii* strains NCYC 1416 and NCYC 385 revealed 100% sequence identity for the 26S rDNA D1/D2 sequences (data not shown). The 26S rDNA D1/D2 sequences of *Z. bailii* NCYC 1416/NCYC385 were compared to the other *Z. bailii* strains. The sequence alignment showed that three subgroups of *Z. bailii* could be identified with a number of sequence differences (indicated by *) being evident (Figure 8.5). *Z. baiilii* NCYC 1416 and 385 formed one distinct group with NCYC 1520 and 11 another and the final group comprising NCYC 1766 and 80. *Z. bailii* 20 was not included into a group due to variation in the end sequence, despite several sequence attempts. The sequence alignments of the three *Z. bailii* groups were compared as a phylogenetic tree to the type strains of several closely related members of *Z. bailii* (Figure 8.6). *Z. bailii* is, thus far, the only one of these closely related species to exhibit subgroups in its 26S rDNA D1/D2 sequences. In summary, molecular examination shows differences in the 26S rDNA D1/D2 sequences of *Z. bailii* with the atypical strains NCYC 1416 and NCYC 385 compromising of a distinct subgroup.

Figure 8.5 26S rDNA D1/D2 sequence alignment of *Z. bailii* strains

```
NCYC_385/1416                                                GCGGCAAAA GCTCAAATTT
Uni_20                                    TTAGTAA CGGCGAGTGA AGCGGCAAAA GCTCAAATTT
NCYC_1766          AACCGGGATT GCCTTAGTAA CGGCGAGTGA AGCGGCAAAA GCTCAAATTT
Uni_80                 GGGATT GCCTTAGTAA CGGCGAGTGA AGCGGCAAAA GCTCAAATTT
NCYC_1520                                CGGCGAGTGA AGCGGCAAAA GCTCAAATTT
Uni_11                        GCCTTAGTAA CGGCGAGTGA AGCGGCAAAA GCTCAAATTT

NCYC385/1416       GAAATCTGGT ACCTTCGGTG CCCGAGTTGT AATTTGTAGA AGGCGACTCT
Uni_20             GAAATCTGGT ACCTTCGGTG CCCGAGTTGT AATTTGTAGA AGGCGACTCT
NCYC_1766          GAAATCTGGT ACCTTCGGTG CCCGAGTTGT AATTTGTAGA AGGCGACTCT
Uni_80             GAAATCTGGT ACCTTCGGTG CCCGAGTTGT AATTTGTAGA AGGCGACTCT
NCYC_1520          GAAATCTGGT ACCTTCGGTG CCCGAGTTGT AATTTGTAGA AGGCGACTCT
Uni_11             GAAATCTGGT ACCTTCGGTG CCCGAGTTGT AATTTGTAGA AGGCGACTCT

                      *
NCYC_385           GGGGCTGGTC CTTGTCTATG TTCCTTGGAA CAGGACGTCA TGGAGGGTGA
Uni_20             GGGGCTGGTC CTTGTCTATG TTCCTTGGAA CAGGACGTCA TGGAGGGTGA
NCYC_1766          GGGACTGGTC CTTGTCTATG TTCCTTGGAA CAGGACGTCA TGGAGGGTGA
Uni_80             GGGACTGGTC CTTGTCTATG TTCCTTGGAA CAGGACGTCA TGGAGGGTGA
NCYC_1520          GGGGCTGGTC CTTGTCTATG TTCCTTGGAA CAGGACGTCA TGGAGGGTGA
Uni_11             GGGGCTGGTC CTTGTCTATG TTCCTTGGAA CAGGACGTCA TGGAGGGTGA

NCYC_385/1416      GAATCCCGTA TGGCGAGGAT CCCAGTTCTT TGTAGAGTGC CTTCGAAGAG
Uni_20             GAATCCCGTA TGGCGAGGAT CCCAGTTCTT TGTAGAGTGC CTTCGAAGAG
NCYC_1766          GAATCCCGTA TGGCGAGGAT CCCAGTTCTT TGTAGAGTGC CTTCGAAGAG
Uni_80             GAATCCCGTA TGGCGAGGAT CCCAGTTCTT TGTAGAGTGC CTTCGAAGAG
NCYC_1520          GAATCCCGTA TGGCGAGGAT CCCAGTTCTT TGTAGAGTGC CTTCGAAGAG
Uni_11             GAATCCCGTA TGGCGAGGAT CCCAGTTCTT TGTAGAGTGC CTTCGAAGAG

NCYC_385/1416      TCGAGTTGTT TGGGAATGCA GCTCTAAGTG GGTGGTAAAT TCCATCTAAA
Uni_20             TCGAGTTGTT TGGGAATGCA GCTCTAAGTG GGTGGTAAAT TCCATCTAAA
NCYC_1766          TCGAGTTGTT TGGGAATGCA GCTCTAAGTG GGTGGTAAAT TCCATCTAAA
Uni_80             TCGAGTTGTT TGGGAATGCA GCTCTAAGTG GGTGGTAAAT TCCATCTAAA
NCYC_1520          TCGAGTTGTT TGGGAATGCA GCTCTAAGTG GGTGGTAAAT TCCATCTAAA
Uni_11             TCGAGTTGTT TGGGAATGCA GCTCTAAGTG GGTGGTAAAT TCCATCTAAA

NCYC_385/1416      GCTAAATATT GGCGAGAGAC CGATAGCGAA CAAGTACAGT GATGGAAAGA
Uni_20             GCTAAATATT GGCGAGAGAC CGATAGCGAA CAAGTACAGT GATGGAAAGA
NCYC_1766          GCTAAATATT GGCGAGAGAC CGATAGCGAA CAAGTACAGT GATGGAAAGA
Uni_80             GCTAAATATT GGCGAGAGAC CGATAGCGAA CAAGTACAGT GATGGAAAGA
NCYC_1520          GCTAAATATT GGCGAGAGAC CGATAGCGAA CAAGTACAGT GATGGAAAGA
Uni_11             GCTAAATATT GGCGAGAGAC CGATAGCGAA CAAGTACAGT GATGGAAAGA

NCYC_385           TGAAAAGAAC TTTGAAAAGA GAGTGAAAAA GTACGTGAAA TTGTTGAAAG
Uni_20             TGAAAAGAAC TTTGAAAAGA GAGTGAAAAA GTACGTGAAA TTGTTGAAAG
NCYC_1766          TGAAAAGAAC TTTGAAAAGA GAGTGAAAAA GTACGTGAAA TTGTTGAAAG
Uni_80             TGAAAAGAAC TTTGAAAAGA GAGTGAAAAA GTACGTGAAA TTGTTGAAAG
NCYC_1520          TGAAAAGAAC TTTGAAAAGA GAGTGAAAAA GTACGTGAAA TTGTTGAAAG
Uni_11             TGAAAAGAAC TTTGAAAAGA GAGTGAAAAA GTACGTGAAA TTGTTGAAAG
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```
NCYC_385/1416   GGAAGGGCAT TTGATCAGAC ATGGTGTTTT GCGCCCCTCG CCTCTCGTGG
Uni_20          GGAAGGGCAT TTGATCAGAC ATGGTGTTTT GCGCCCCTCG CCTCTCGTGG
NCYC_1766       GGAAGGGCAT TTGATCAGAC ATGGTGTTTT GCGCCCCTCG CCTCTCGTGG
Uni_80          GGAAGGGCAT TTGATCAGAC ATGGTGTTTT GCGCCCCTCG CCTCTCGTGG
NCYC_1520       GGAAGGGCAT TTGATCAGAC ATGGTGTTTT GCGCCCCTCG CCTCTCGTGG
Uni_11          GGAAGGGCAT TTGATCAGAC ATGGTGTTTT GCGCCCCTCG CCTCTCGTGG

                                    *
NCYC_385/1416   GTGGGGGAAT CTCGCAGTTC ACTGGGCCAG CATCAGTTTT GGCGGCAGGA
Uni_20          GTGGGGGAAT CTCGCAGTTC ACTGGGCCAG CATCAGTTTT GGCGGCAGGA
NCYC_1766       GTGGGGGAAT CTCGCAGCTC ACTGGGCCAG CATCAGTTTT GGCGGCAGGA
Uni_80          GTGGGGGAAT CTCGCAGCTC ACTGGGCCAG CATCAGTTTT GGCGGCAGGA
NCYC_1520       GTGGGGGAAT CTCGCAGCTC ACTGGGCCAG CATCAGTTTT GGCGGCAGGA
Uni_11          GTGGGGGAAT CTCGCAGCTC ACTGGGCCAG CATCAGTTTT GGCGGCAGGA

NCYC_385/1416   TAAATCCCTG GGAATGTAGC TCTACCACTT CGTGGCGGAC GAACTTATAG
Uni_20          TAAATCCCTG GGAATGTAGC TCTACCACTT CGTGGCGGAC GAACTTATAG
NCYC_1766       TAAATCCCTG GGAATGTAGC TCTACCACTT CGTGGCGGAC GAACTTATAG
Uni_80          TAAATCCCTG GGAATGTAGC TCTACCACTT CGTGGCGGAC GAACTTATAG
NCYC_1520       TAAATCCCTG GGAATGTAGC TCTACCACTT CGTGGCGGAC GAACTTATAG
Uni_11          TAAATCCCTG GGAATGTAGC TCTACCACTT CGTGGCGGAC GAACTTATAG

                                                            **
NCYC_385/1416   TCCAGGGGAA TACTGCCAGC TGGGACTGAG GAATGCGACT TTT--AGTCA
Uni_20          TCCAGGGGAA TACTGCCAGC TGGGACTGAG GAATGCGACT TTT--ATACA
NCYC_1766       TCCAGGGGAA TACTGCCAGC TGGGACTGAG GAATGCGACT TTTTTAGTCA
Uni_80          TCCAGGGGAA TACTGCCAGC TGGGACTGAG GAATGCGACT TTTTTAGTCA
NCYC_1520       TCCAGGGGAA TACTGCCAGC TGGGACTGAG GAATGCGACT TTTTTAGTCA
Uni_11          TCCAGGGGAA TACTGCCAGC TGGGACTGAG GAATGCGACT TTTTTAGTCA

NCYC_385/1416   AGGATGCTGG CATAATGGTT ATATGCCGCC CGTCTTGAAA CACGG
Uni_20          CAG------- ---------- ---------- ---------- -----
NCYC_1766       AGGATGCTGG CATAATGGTT ATATGCCGCC CGTCTTGAAA CACGG
Uni_80          AGGATGCTGG CATAATGGTT ATATGCCGCC CGTCTTGAAA CACGG
NCYC_1520       AGGATGCTGG CATAATGGTT ATATGCCGCC CGTCTTGAAA CACGG
Uni_11          AGGATGCTGG CATAATGGTT ATATGCCGCC CGTCTTGAAA CACGG
```

Sequence-based subgroups

Group 1: NCYC 385, NCYC 1416^T
Group 2: NCYC 1520, (Unilever) 11
Group 3: NCYC 1766, (Unilever) 80

Sequence for *Z. bailii* NCYC 1416 and NCYC 385 are 100% identical.

Z. bailii 20 could not be placed into any of the subgroups due to slightly differing 26S rDNA D1/D2 sequences.

* Differences in the 26S rDNA D1/D2 sequence.
- No sequence obtained.
_ Variable sequence obtained.

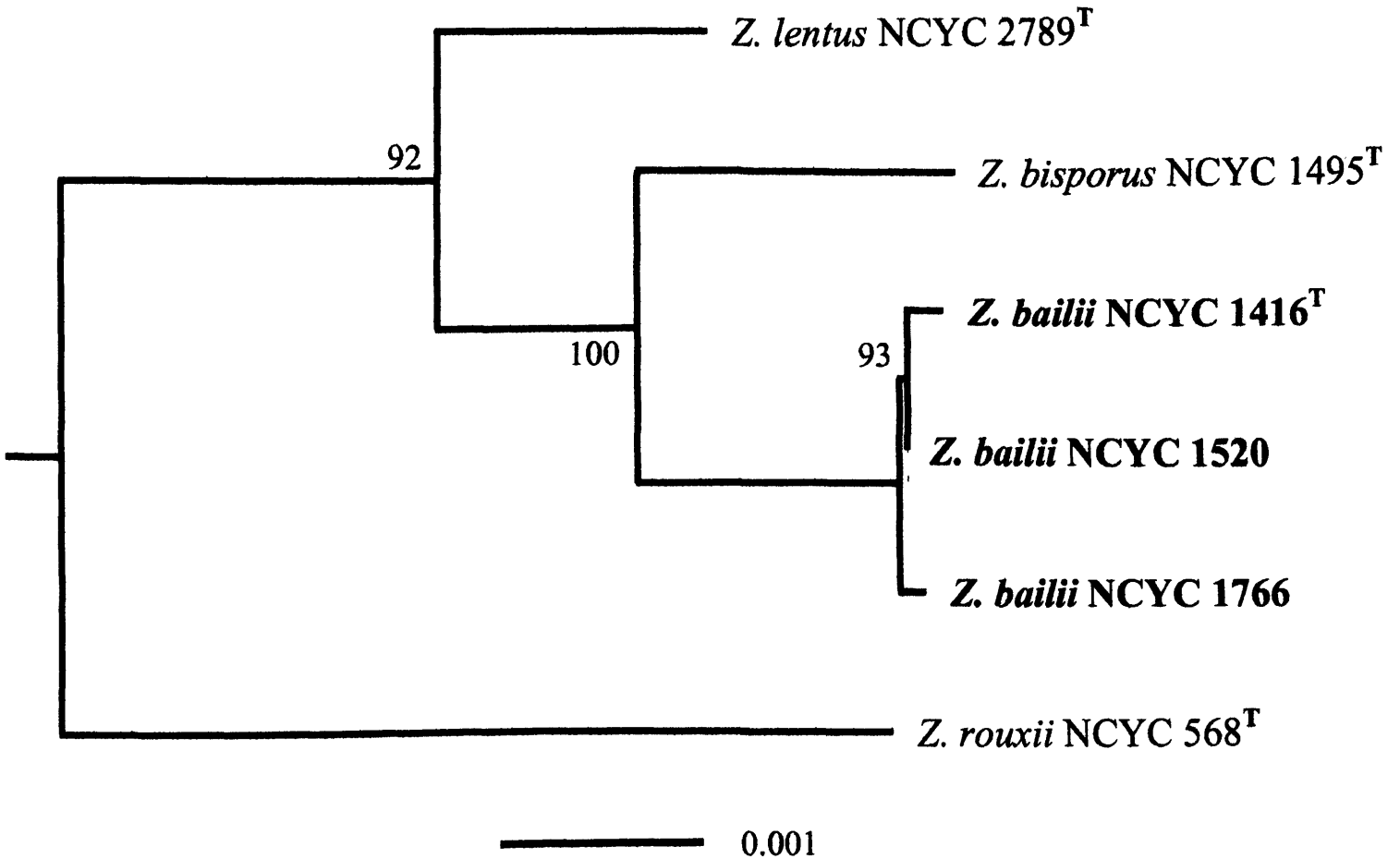

Figure 8.6 Phylogenetic tree of *Z. bailii* 26S rDNA D1/D2 sequences with other closely related *Zygosaccharomyces* species.

Bar is representative of one substitution per 1000 nucleotide position. Bootstrap values >50% are given. Sequence alignment and phylogenetic tree were constructed using DNAMAN version 5.1.5 (Lynnon BioSoft). [T] Type strain. Figure constructed by S. A. James NCYC, Norwich, UK.

8.3 Discussion

Z. bailii has been shown to contain a subgroup which shows differences relating to inhibitor resistance, morphology, physiology and molecular classification compared with the other *Z. bailii* strains. The subgroup consists of NCYC 1416 and NCYC 385 with the main group consisting of NCYC 1766, 11, NCYC 1520, 20 and 80. The former being the more sensitive to all inhibitors examined except for decanoic acid and ethanol. The mean MICs of these two groups allowed clear distinction of their organic acid resistance. The exception to this was formic acid in which the standard errors were indicative of a small overlap of the mean MIC. The biggest difference in mean MICs between the two proposed groups of *Z. bailii* were shown for medium chain organic acids; comprising hexanoic, sorbic and benzoic acid. The mean MICs obtained for all three of these medium chain acids in the resistant group were almost double that of the mean MICs obtained for the more sensitive group. There are therefore some strains of *Z. bailii* with a mechanism of resistance to medium chain organic acids that is absent in other strains.

Morphological differences between the two aforementioned subgroups included: texture on agar, aggregation or flocculation capabilities and ability to form natural pseudohyphae. Physiological differences in the form of carbon source utilisation, range of growth temperatures, growth in complex medium and dry weight analysis also allowed the distinction of *Z. bailii* NCYC 1416 and NCYC 385 from the other *Z. bailii* strains. The type strain of *Z. bailii* (NCYC 1416) has recently been shown to have a lower UV resistance and smaller DNA content than a highly resistant weak carboxylic acid *Z. bailii* strain (Rodrigues *et al.*, 2003). This is further supportive of differences between the strains of *Z. bailii*.

The 26S rDNA D1/D2 sequence alignments proposed the existence of three subgroups in *Z. bailii*. *Z. bailii* NCYC 1416 and NCYC 385 comprised of one subgroup, NCYC 1520 and 11 another with the final subgroup comprising NCYC 1766 and 80. *Z. bailii* 20 (isolated from an orange concentrate) could not be placed into any one of the three subgroups due to slightly differing 26S rDNA D1/D2 sequences. This phenomenon has recently been reported for a number of *Clavispora lusitaniae* strains (Lachance *et al.*, 2003). The level of divergence between the subgroups proposed by 26S rDNA D1/D2 classification, however, was less than the 1% value generally used for the identification of a new species (Kurtzman & Blanz, 1998). The level of divergence for 26S rDNA D1/D2 sequences is indicative of a species currently diverging (S. A. James, personal communication). The divergence in *Z. bailii* is somewhat akin to that reported for the *Saccharomyces* sensu stricto complex (Edwards *et al.*, 2003; Fernandez-espinar *et al.*, 2003; Kurtzman and Robnett, 2003, Kurtzman, 2003). A recent study examining the molecular characterisation of the genus *Zygosaccharomyces* highlighted *Z. bailii* as showing considerable variability supporting the results in this thesis (Esteve-Zarzoso *et al.*, 2003).

In summary, evidence has been presented for a subgroup in *Z. bailii* molecular classification extended upon this by proposing the existence of three subgroups in *Z. bailii*. The relevance to the food industry is that for the first time *Z. bailii* has been shown to contain a subgroup of organic acid sensitive strains, and by studying the differences between resistance and sensitive groups the mechanisms of extreme organic acid resistance utilized by some *Z. bailii* strains could be revealed.

9. General Discussion

The lack of research into yeast attributed food spoilage, in comparison to that of bacterial spoilage, is now beginning to be re-addressed. The primary reason for this is that food spoilage, as a result of yeast contamination, is becoming increasingly well reported. The results presented in this thesis add to our understanding of yeast attributed food spoilage by examining differences in organic acid resistance and the basis of these differences between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*. The following considers the results of this research, future work and the relevance of this research to the food industry.

This is the first known study to compare organic acid resistance in *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*. The data showed that organic acid resistance for the three aforementioned yeast species differed. In general, *S. cerevisiae* was the most sensitive to organic acids followed by *Z. kombuchaensis*. *Z. bailii* was shown to be a diverse species containing strains both resistant and sensitive to organic acids. The information on organic acid resistance gathered from this research can be applied directly to the food industry providing a detailed account of the capabilities of these yeasts to grow in the presence of organic acid food preservatives.

The method used in determining MICs was challenge assays in universal bottles (Steels *et al.*, 1999b; 2000; 2002; Stratford *et al.*, 2002; Fitzgerald *et al.*, 2003). This method has many advantages including the fact that many inhibitors can be assessed simultaneously, a number of yeasts can be examined, it is of relatively low cost, low skill and relatively quick. The biggest advantage is that this method simulates many of the conditions in which yeast cause spoilage including growth in complex media (with an excess of nutrients), under static conditions and with limited oxygen. The potential problems with this method are that it is very labour intensive, it requires the use of large

incubators and the recording of MICs can be considered subjective. The subjectivity of the method, which is based on the accurate recording of MICs by visual inspection of each culture, was not considered to be problematic in these investigations as the MICs obtained from duplicate experiments were found to differ by only 1-2 cultures. Alternative methods to record MICs included solid media, micro-titre plates and large flasks.

Experiments examining the effects of growth conditions on yeast organic acid resistance produced a number of interesting results. *Z. bailii* and *Z. kombuchaensis* had lower MICs in complex media in which yeast extract was omitted. As yeast extract contains a complex series of nutrients (appendix), experiments in which individual components of yeast extract are omitted from the growth media would allow for the elucidation of the dependency of yeasts on specific nutrients. Discovering the effects of nutrients on growth particularly for *S. cerevisiae* would be of interest to brewing and biotechnology industries. The inability of some *Z. bailii* strains to grow in the presence of maltose and for some strains galactose, could lead to the development of new food products with a reduced risk of spoilage, by *Z. bailii*. In fact, there is currently, a high energy sports drink available called '*G-PUSH*' (source of information http://www.gpush.com). '*G-PUSH*' contains galactose instead of glucose as the main carbon source. Therefore, in addition to this product appealing to a specific area of the market, it also has a reduced risk of spoilage. A similar situation exists for *Z. kombuchaensis* in which organic acid resistance varied with nitrogen source. Collectively, the data provides a greater understanding of the factors affecting yeast growth in foods and beverages.

Z. bailii and *Z. kombuchaensis* at extreme concentrations of organic acids produced low OD values being indicative of growth of only a few cells. *S. cerevisiae* did not show a

similar pattern with growth ending abruptly at the MIC. The poor growth of *Z. bailii* and *Z. kombuchaensis* at extreme organic acid concentrations could be the result of "super" cells as, previously, described for *Z. bailii* in the presence of sorbic acid (Steels *et al.*, 2000). The presence of "super" cells could account for the ability of *Z. bailii* to cause spoilage from as little as one cell per litre (Thomas and Davenport, 1985). Recently, a review on phenotypic heterogeneity, defined as 'non-genetic variation that exists between individual cells within an isogenic population' in *S. cerevisiae* was published (Sumner and Avery, 2002). It is possible that the cells which are able to grow at extreme concentrations of organic acids for *Z. bailii* and *Z. kombuchaensis* are representative of phenotypic heterogeneity. If *Z. bailii* were shown to contain a higher natural pool of "super" cells then this could be crucial regarding their proliferation in high concentration of organic acids. The EM analyses showed that the *Zygosaccharomyces* yeasts under organic acid stress form multiple buds. The formation of multiple buds may be a means of surviving cell stress and could contribute to the phenomenon of phenotypic heterogeneity. If this is the case then the application of flow-cytometry, which can differentiate at the single-cell level (Attfield *et al.*, 2001) and has been previously applied to *Z. bailii* (Prudêncio *et al.*, 1999), could prove extremely useful. Elucidating more about the ability of *Zygosaccharomyces* yeasts to form "super" cells could aid research into yeast organic acid resistance and the general phenomenon of phenotypic heterogeneity.

The application of electron microscopy in this study has added to our knowledge on the modes of inhibition caused by organic acids. For example, the actions of sorbic acid have been found to be concentrated on the plasma membrane and/or cell wall, where it results in cell lysis for the sorbic acid sensitive *Z. kombuchaensis*. This is believed to be the first direct microscopic evidence for sorbic acid acting as a membrane active

substance supporting the theory of Stratford and Anslow (1996b, 1998) that sorbic acid does not act as a 'classic weak-acid preservative'. The 'weak-acid preservative theory' is based upon undissociated acid molecules entering the cell via simple diffusion and dissociating releasing protons and anions. The anions and protons accumulate within the cytoplasm resulting in intracellular acidification. If the 'weak-acid preservative theory' holds then acids with similar pK_a values should inhibit yeast at similar concentrations. Differences in the MIC of acetic and sorbic acid both of which have similar pK_a values have been discussed previously in relation to the 'weak-acid preservative theory' (Stratford and Ueckert, submitted). In this study, possible further discrepancies in the 'weak-acid preservative theory' have been highlighted. Propionic acid and hexanoic acid both have a pK_a of 4.87, but show a 15-30 fold difference in MICs. Therefore, the 'weak-acid preservative theory' does not account for differences in inhibitory concentrations between organic acids with similar pK_a values. On the basis of these findings it may be appropriate to revise the 'classic weak-acid preservative theory'. Any such review would have to take into account the importance of intracellular acidification and the relevance of other mechanisms utilised by organic acids to inhibit yeast growth.

A large part of the research presented in this thesis was conducted to build upon the limited information regarding the morphology and physiology of *Zygosaccharomyces* spoilage yeasts. In this thesis, three main questions relating to morphology and physiology of *Zygosaccharomyces* yeasts were addressed. Each question will be considered individually.

Are there differences in morphology and physiology between *Z. bailii*, *Z. kombuchaensis* and *S. cerevisiae*?

A number of morphological and physiological differences have been highlighted between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae*. Differences relating to the cell structure in terms of cell size, cell wall thickness, sensitivity to cell wall enzymes, cell wall composition and pseudohyphal formation were found. A detailed analysis of the cell wall of *Z. bailii* and *Z. kombuchaensis* to build upon the data presented in this thesis represents one of the more immediate aspects of future work. *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* were also shown to differ in sensitivity to ethidium bromide and isoamyl alcohol, in petite forming capabilities and glycerol production in addition to a number of physiological growth tests (e.g. carbon source utilisation).

Do any differences relate to organic acid resistance?

A number of the differences in morphology and physiology reported in this thesis for *Z. bailii, Z. kombuchaensis* and *S. cerevisae* relate to organic acid resistance. The cell wall was shown to be involved in yeast organic acid resistance as cell wall mutants of *S. cerevisiae* exhibited differences in organic acid resistance to the wild-type. *Z. bailii* contains a higher chitin level than *S. cerevisiae* with chitin being distributed throughout the cell, and not restricted to the bud scars. This is correlated with the higher resistance of *Z. bailii* to Zymolyase (an enzyme known to breakdown β-1, 3 glucans) and indicates that *Z. bailii* is likely to contain more chitin and β-1, 3 glucan complexes. The increased number of these complexes could add additional mechanical strength and change permeability properties. An even simpler way in which the cell wall could contribute to the differences in organic acid resistance between *Zygosaccharomyces* yeasts and *S. cerevisiae,* could be that the thicker cell wall of the *Zygosaccharomyces* yeast allows the cells more time to adapt to the stress. *Z. bailii* and *Z. kombuchaensis* were also shown to have considerably higher glycerol levels than *S. cerevisiae.* The addition of compatible solutes in the form of glycerol, sorbitol and mannitol resulted in a slight increase in

organic acid resistance for all three yeast species. Therefore, organic acids may exert some osmotic stress, which the *Zygosaccharomyces* yeasts with their higher glycerol levels are better able to tolerate. Mitochondria may also have a role to play in yeast organic acid resistance, as organic acids have recently been shown to cause an oxidative stress (Piper, 1999). The following supports this: firstly, *Z. bailii* and *Z. kombuchaensis* are more dependent upon mitochondria than *S. cerevisiae* as cells in which mitochondrial DNA had been damaged or lost were non-viable. Secondly, *ZbYME2* a gene isolated from *Z. bailii* allows the utilisation of sorbate and benzoate when heterologously expressed in *S. cerevisiae*. Expression of *ZbYME2* as a functional fusion to green fluorescent protein (GFP) in *S. cerevisiae* on benzoate was largely localised to the mitochondria (Mollapour and Piper, 2001 b).

Do any differences present alternative targets for food preservation?

Z. bailii was shown to be more sensitive to Calcofluor white which in yeast preferentially binds to chitin. The application of a compound to target chitin would be one possible strategy to target food preservation. Both the *Zygosaccharomyces* yeasts were also shown to be more sensitive to SDS, caffeine, calcium chloride and the aminoglycoside G418 all of which have been used in *S. cerevisiae* as indicators of differences in cell wall composition. The cell wall would, therefore, appear to be a potential target in the prevention of *Z. bailii* and *Z. kombuchaensis* attributed spoilage. This hypothesis is strengthened by the fact that the cell wall in *Z. bailii* and *S. cerevisiae* has been targeted as a means of inhibiting growth during the course of conducting this research (Bom *et al.*, 2001). The mitochondria of *Zygosaccharomyces* appear to play a role in yeast organic acid resistance as mentioned previously, but the fact that these yeasts are non-viable when the mitochondria are damaged also makes it a potential target for food preservation. *Z. kombuchaensis* and *S. cerevisiae* formed pseudohyphae

in the presence of isoamyl alcohol. Pseudohyphal cells are induced in response to stress with the rationale being that they provide a means of foraging for more favourable conditions (Kron *et al.*, 1994). The viability of pseduohyphal cells and ability to switch back into yeast form, however, remains to be fully elucidated. If pseudohyphal cells showed a reduction in cell viability then the addition of a substance to induce pseduohyphae in the case of yeast contamination, would present a possible strategy to prevent food spoilage. Isoamyl alcohol has also been shown to be inhibitory to *Z. bailii, Z. kombuchaensis* and *S. cerevisiae.* The application of fusel alcohols and their esters to the preservation of food is also a possibility. Isoamyl acetate for example has a banana like smell and would, therefore, also add a unique taste to the product.

Collectively, the information relating to morphological and physiological differences between *Z. bailii, Z. kombuchaensis* and *S. cerevisiae* should give new insights into the mechanisms used by yeasts to high levels of organic acids, and provide alternative targets for food preservation. The cell wall would appear to be an area of particular interest by contributing to organic acid resistance, while at the same time presenting a possible target for preventing food spoilage.

This thesis has reported the first known successful protoplast fusions between *Z. bailii* and *Z. kombuchaensis* with *S. cerevisiae.* The hybrids formed were shown to be stable and to be sensitive to organic acids but resistance to oxidative stress in the form of hydrogen peroxide. The ability of hybrids to grow on YPG post-ethidium bromide treatment could mean that they are insensitive to the effects of ethidium bromide on their mitochondria or that they can use glycerol via some unknown metabolic pathway. The possible alternative pathway could be the Salicylhydroxamic acid (SHAM) sensitive pathway, which uses an alternative oxidase to accept electrons and reduce

oxygen. The SHAM sensitive pathway could allow for growth on glycerol and other non-fermentable carbon sources and such a pathway is evident for the petite-negative yeast *Debaromyces (Schwanniomyces) occidentalis* (Fernet *et al.*, 2002). The hybrids were also shown to contain a high number of peroxisomes. In humans, Zellweger syndrome is a congenital cerebro-hepato-renal disorder characterised by an absence or deficiency in peroxisomes (Waterham and Cregg, 1997). The use of the hybrids created by this research with molecular biology techniques could aid studying such a disorder. Hybrids between *Z. bailii* and *Z. kombuchaensis* could be produced by employing a method based on that of Lucca *et al.* (1999). *Z. bailii* is unable to grow on maltose where as *Z. kombuchaensis* is capable of growth on maltose. Therefore, *Z. kombuchaensis* would be heat killed and protoplasts mixed with those of *Z. bailii* and the hybrids selected as those viable and capable of growth on maltose containing media. The creation of such hybrids may add to our understanding of the unusual attributes of these yeasts.

Evidence has been presented in this thesis for the existence of a subgroup in *Z. bailii* (consisting of NCYC 1416 and NCYC 385) based upon differences in organic acid resistance, morphology and physiology. Molecular examination in the form of 26S rDNA D1/D2 sequences showed the two *Z. bailii* strains in the subgroup (NCYC 1416 and NCYC 385) to be 100% identical. Further examination of the 26S rDNA D1/D2 sequences for the remaining *Z. bailii* strains used in this study revealed the existence of three subgroups for *Z. bailii*. *Z. bailii* NCYC 1416 and NCYC 385 comprised of one subgroup, NCYC 1520 and 11 another with the final subgroup comprising NCYC 1766 and 80. An extension to the 26S rDNA study would be to examine the divergence of ITS1 and ITS2 sequences. *Z. bailii* has been previously shown to comprise three ITS subgroups with strains NCYC 1416 and NCYC 1766 being in different groups (James *et al,* 1994). Differences in ITS sequences would be further supportive of the existence of

subgroups in *Z. bailii*. An interesting series of observations can be made relating to *Z. bailii* 11. This strain produced a few results characteristic of the NCYC 1416 and NCYC 385 subgroup. These results included slightly increased sensitivity to sorbic acid, hydrogen peroxide and sodium chloride, the ability to use sorbose and no growth at 37°C. Interestingly, the 26S rDNA D1/D2 sequences showed this strain to be in a group, which does not contain the most organic acid sensitive strain (NCYC 385) or the most resistant strain (NCYC 1766). *Z. bailii* 11 therefore indicates further divisions within the species of *Z. bailii*. On analysis of the NCYC database there are a number of *Z. bailii* strains, which may prove of interest in terms of the subgroups proposed in this thesis. These include NCYC 417 and NCYC 1427 both of which share a number of similar properties to the NCYC 1416 and NCYC 385 subgroup. Comparing the subgroups within *Z. bailii* could make further insights into the mechanisms of yeast organic acid resistance.

Modern molecular tools are increasingly being applied to *Z. bailii* including: targeted gene deletion (Mollapour and Piper, 2001a), the construction of genomic libraries (Rodrigues *et al.*, 2001 b) and the isolation, cloning and sequencing of genes (Merico *et al.*, 2001; Rodrigues *et al.*, 2001b; Branduardi, 2002). Indeed, *Z. bailii* has recently been developed as a host for heterologous protein production, secretion and metabolic engineering applications (Branduardi *et al.*, 2004). These molecular tools are collectively allowing us to exploit the unusual attributes of this yeast while at the same time elucidating more about these attributes.

Generally, it is hoped that the work presented in this thesis will not only be of interest to the food industry, but that it will also be of interest to researchers working within the fields of biotechnology, brewing, systematics and applied biology.

9.1 Future Work

Future work that would be a continuation of the work presented in this thesis is as follows:

- The application of flow cytometry to investigate the existence of highly resistant "super" cells as reported for *Z. bailii* to sorbic acid by Steels *et al.* (2000) would be of particular interest. The results in this thesis show that for the spoilage yeasts *Z. bailii* and *Z. kombuchaensis* at very high concentrations of organic acids only a few cells are capable of growth. The cells capable of growth at extreme organic acid concentrations could be representative of phenotypic heterogeneity and may be a key factor in the ability of these yeasts to cause food spoilage.

- Investigations into the effects of growth conditions on yeast organic acid resistance should be extended. The inclusion of more strains coupled with a larger range of inhibitors and an examination of extrinsic in addition to the intrinsic factors examined in section 4, would provide a more complete overview of the abilities of spoilage yeast to spoil foodstuffs and aid spoilage risk assessments.

- Biochemical investigations into the composition of the cell wall of the spoilage yeasts *Z. bailii* and *Z. kombuchaensis* represents an area of more immediate work. The analyses from this thesis have shown that differences in the cell wall can affect yeast organic acid resistance in *S. cerevisiae*. The possibility of the cell wall contributing to the organic acid resistance of spoilage yeasts, while also presenting a possible target for preservation strategies, should be of considerable interest to the food industry.

- Identification of the role of the HOG pathway in isomayl alcohol induced pseudohyphal formation. The results in this thesis show some role for the HOG pathway in yeast pseudohyphal formation but the identity of that role remains unknown. There are a number of possibilities to how the HOG pathway may respond to isoamyl alcohol, which now need pursuing in order to identify the stress response triggered by isomayl alcohol. The result of such studies may provide new insights into the mechanisms of yeast morphological change, which are of particular importance for the human pathogen *C. albicans.*

- An investigation into the classification of all currently known *Z. bailii* strains would prove of interest to researchers in the food industry and to those involved in yeast systematics. This thesis has shown for the first time the existence of at least one subgroup in *Z. bailii* with an increased sensitivity to organic acids, while the possibility of other subgroups has been shown by 26S rDNA comparisons. The application of multigene sequence analyses (Kurtzman and Robnett, 2003; Kurtzman, 2003), would aid the elucidation of the classification of *Z. bailii*. Molecular investigations into the classification of *Z. bailii* may aid the development of molecular techniques to distinguish particular strains, while comparisons of organic acid resistant and sensitive strains may prove invaluable in identifying the mechanisms of organic acid resistance in *Z. bailii*, which have been speculated to differ from those of *S. cerevisiae* (Piper *et al.*, 2001).

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11. Appendix

Yeast Extract

Typical analysis
Source of information: Becton-Dickinson Microbiology, Oxford, UK

Physical Characteristics (%)	
Ash	11.2
Clarity, 1% solution (N10)	1.6
Filterability (g/cm^2)	2.7
Loss on Drying	3.1
pH, 1% solution	6.7
Carbohydrate (%)	
Total	17.5
Nitrogen content (%)	
Total Nitrogen	10.9
Amino Nitrogen	6.0
Amino Nitrogen/Total Nitrogen	20.7
Amino Acids (%)	
Alanine	5.36
Arginine	3.92
Aspartic Acid	6.59
Cystine	0.74
Glutamic Acid	14.20
Glycine	3.25
Histidine	1.20
Isoleucine	3.23
Leucine	4.69
Lysine	5.15
Methionine	1.03
Phenylalanine	2.63
Proline	2.80
Serine	2.84
Threonine	2.95
Tryptophan	1.36
Tyrosine	1.20
Valine	3.79
Inorganics (%)	
Calcium	0.012
Chloride	0.380
Cobalt	<0.001
Copper	<0.001
Iron	<0.001
Lead	<0.001
Magnesium	0.075
Manganese	<0.001
Phosphate	3.275
Potassium	3.195

Sodium	1.490
Sulphate	0.091
Sulphur	0.634
Tin	<0.001
Zinc	0.011

Vitamins (µg/g)

Biotin	3.3
Choline (as Choline chloride)	300.0
Cyanocobalamin	<0.1
Folic Acid	1.5
Inositol	1400.0
Nicotinic Acid	597.9
PABA	763.0
Panthothenic Acid	273.7
Pyridoxine	43.2
Riboflavin	116.5
Thiamine	529.9
Thymidine	217.5

Bacto Peptone

Typical analysis
Source of information: Becton-Dickinson Microbiology, Oxford, UK

Physical Characteristics (%)	
Ash	4.4
Clarity, 1% solution (N10)	0.5
Filterability (g/cm^2)	0.5
Loss on Drying	3.0
pH, 1% solution	7.0
Carbohydrate (%)	
Total	6.9
Nitrogen content (%)	
Total Nitrogen	15.6
Amino Nitrogen	3.1
Amino Nitrogen/Total Nitrogen	20.0
Amino Acids (%)	
Alanine	8.67
Arginine	6.76
Aspartic Acid	6.80
Cystine	0.20
Glutamic Acid	10.21
Glycine	15.69
Histidine	0.58
Isoleucine	1.45
Leucine	3.01
Lysine	3.42
Methionine	1.19
Phenylalanine	1.81
Proline	8.60
Serine	2.67
Threonine	1.81
Tryptophan	0.36
Tyrosine	0.64
Valine	2.35
Inorganics (%)	
Calcium	0.008
Chloride	1.086
Cobalt	<0.001
Copper	<0.001
Iron	0.004
Lead	<0.001
Magnesium	0.007
Manganese	<0.001
Phosphate	0.445
Potassium	0.303

Sodium	1.759
Sulphate	0.244
Sulphur	0.410
Tin	<0.001
Zinc	0.001

Vitamins (µg/g)

Biotin	0.2
Choline (as Choline chloride)	2000.0
Cyanocobalamin	<0.1
Folic Acid	0.3
Inositol	2400.0
Nicotinic Acid	21.9
PABA	<0.5
Panthothenic Acid	5.9
Pyridoxine	1.7
Riboflavin	3.9
Thiamine	<0.1
Thymidine	413.0

Sequence alignment for *S. cerevisiae* YLR113W/HOG1

YLR113W/HOG1 on chromosome XII from coordinates 371621 to 372928

Chromosome XII Sequence.
Johnston *et al.* (1997). The nucleotide sequence of *Saccharomyces cerevisiae* chromosome XII. *Nature* **387**, 87-90

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   1 ATGACCACTAACGAGGAATTCATTAGGACACAGATATTCGGTACAGTTTT
  51 CGAGATCACAAATAGATACAATGATTTAAACCCCGTTGGGATGGGGGCAT
 101 TTGGGTTGGTTTGCTCAGCCACGGACACTTTGACATCTCAGCCAGTTGCC
 151 ATTAAGAAAATCATGAAACCTTTTTCCACTGCAGTGCTGGCCAAAAGGAC
 201 ATATCGTGAACTAAAACTACTAAAACATCTAAGACACGAGAACTTGATTT
 251 GCCTTCAGGACATATTTCTTTCTCCATTGGAAGATATATATTTTGTCACG
 301 GAATTACAAGGAACAGATTTACATAGACTCTTGCAAACAAGACCCTTGGA
 351 AAAGCAATTTGTTCAGTATTTCCTATACCAAATTCTAAGGGGTTTAAAAT
 401 ACGTTCACTCCGCGGGCGTCATTCATAGAGATTTGAAACCGAGCAACATT
 451 CTGATTAATGAAAACTGTGATTTGAAGATTTGCGATTTCGGTCTAGCAAG
 501 AATTCAAGACCCTCAAATGACAGGCTATGTTTCCACTAGATACTACAGGG
 551 CACCTGAAATCATGCTAACGTGGCAAAAATATGACGTCGAGGTCGACATT
 601 TGGTCCGCTGGTTGTATTTTTGCCGAAATGATTGAAGGTAAGCCTTTGTT
 651 CCCTGGGAAAGATCATGTTCACCAATTTTCGATCATCACTGACTTGTTGG
 701 GATCTCCGCCAAAGGATGTGATAAATACTATTTGTTCCGAAAATACTCTA
 751 AAATTTGTTACTTCGTTACCACACAGAGATCCAATTCCATTTTCTGAAAG
 801 ATTTAAAACAGTCGAACCTGATGCCGTAGACCTTTTGGAAAAAATGCTGG
 851 TTTTTGATCCTAAGAAGAGAATCACTGCGGCGGATGCCTTGGCTCATCCT
 901 TATTCGGCTCCTTACCACGATCCAACGGATGAACCAGTAGCCGATGCCAA
 951 GTTCGATTGGCACTTTAATGACGCTGATCTGCCTGTCGATACCTGGCGTG
1001 TTATGATGTACTCAGAAATCCTAGACTTCCATAAGATTGGTGGCAGTGAT
1051 GGACAGATTGATATATCTGCCACGTTTGATGACCAAGTTGCTGCAGCCAC
1101 CGCTGCCGCGGCGCAGGCACAGGCTCAGGCTCAGGCTCAAGTTCAGTTAA
1151 ACATGGCTGCGCATTCGCATAATGGCGCTGGCACTACTGGAAATGATCAC
1201 TCAGATATAGCTGGTGGAAACAAAGTCAGCGATCATGTAGCTGCAAATGA
1251 CACCATTACGGACTACGGTAACCAGGCCATACAGTACGCTAATGAGTTCC
1301 AACAGTAA
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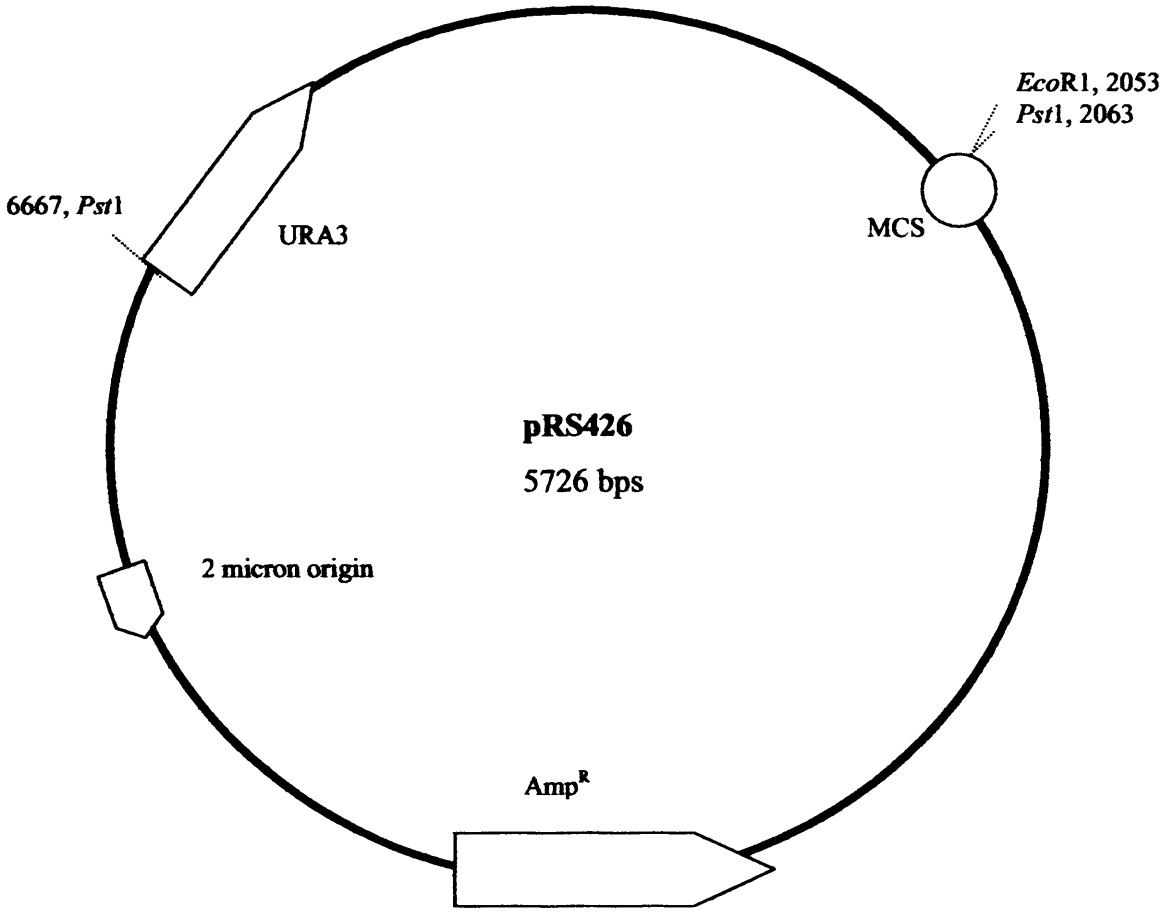
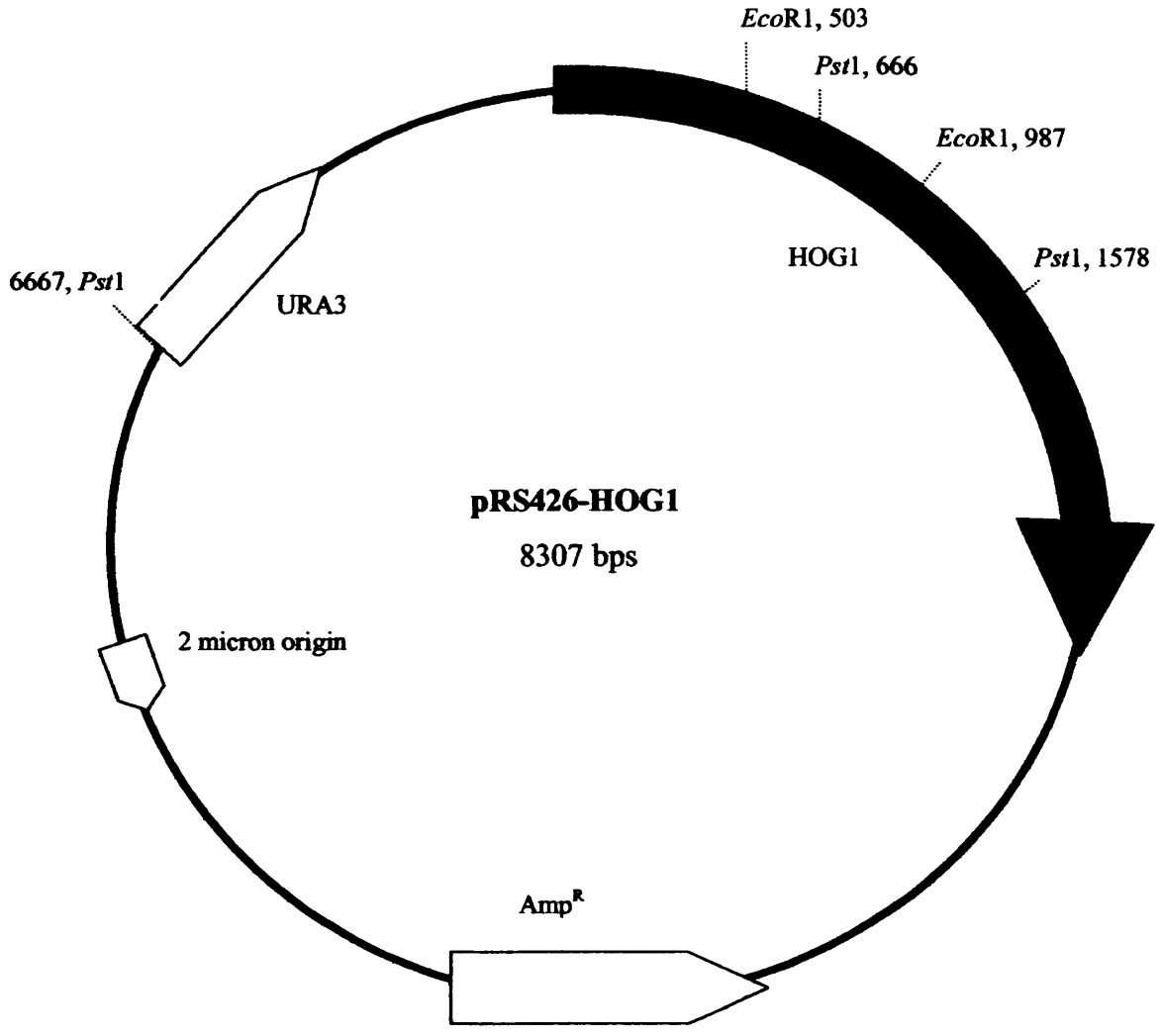
Underlined and bold sequences were used in the design of primers P1 and P2 as detailed in 2.11.5. Sequences retrieved from the *Saccharomyces* genome database (SGD).

pRS426 vector map and restriction sites



pRS426 series of vectors are based on pBluescript II SK+ (Christianson *et al.*, 1992). The pRS426 vector was obtained from S. Oliver (Manchester University, UK). Only restriction sites used in plasmid digests are shown.

pRS426-HOG1 vector map and restriction sites.



The *HOG1* gene with its own promoter was cloned into the pRS426 vector at the *Cla*1/*Bam*H1restriction sites within the MCS. The pRS426-HOG1 vector and map was obtained from S. Hohmann (Göteborg University, Sweden) and originally constructed by M. Gustin (Rice University, USA). Only restriction sites used in plasmid digests are shown.