The Sensitivity of Brewing Micro-organisms to Silver

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Abstract

With respect to microbiological food safety, beer is thought to be very safe. This is due to the inability of pathogenic organisms to survive in the harsh environment that beer presents, due to low pH, alcohol content and hop acids. However, there are some organisms which have adapted to brewery conditions and can cause off-flavours, hazes or low ethanol yield. The effects of spoilage and subsequent product recall can result in massive economic losses for brewing companies affected. Silver nano-particle coatings for pipes and vessels have been suggested as a means of eliminating or reducing contaminants in the brewery. In this study the sensitivity of several brewery contaminants to silver has been investigated. *Pichia membranaefaciens, Brettanomyces anomalous, Candida krusei, Hansenula saturnus, Kloeckera apiculata, Rhodotorula mucilaginosa, Saccharomyces ellipsoids* var. *diastaticus*, *Lactobacillus brevis, Pediococcus damnosus,* were all tested against a range of silver nitrate concentrations (0-1 mM) in YPD, wort and beer. It was found that sensitivity to silver varied between organisms, but no tolerance exceeded 0.55 mM. It was also found that for the majority of organisms, tolerance to silver decreased under simulated brewery conditions i.e. wort, beer and microaerophillic conditions. In the investigation of potential silver tolerance mechanisms, gene microarrays of *Saccharomyces ellipsoids* var. *diastaticus* in wort and beer in the presence and absence of silver found that genes most up-regulated during silver stress were those with transmembrane transporter functions. Silver tolerance testing with gene deletion strains of selected potential silver tolerance genes demonstrated

reduced silver tolerance for the deletion strains of the *HIS1*, *COX17* and *CUP1* genes. All three of these have known functions in copper tolerance.

The data collected in this study would suggest that silver (particularily in nanoparticle form) is an effective means of microbial brewery contamination control especially under brewery conditions. However, further study is needed into the effect of silver antimicrobial surfaces on brewery microbial contaminants, silver concentrations needed in antimicrobial surfaces and silver leaching etc.

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Abbreviations

AAB – Acetic acid Bacteria
ABC – ATP-binding cassette
ABV – Alcohol by volume
ADP – Adenosine diphosphate
ANOVA – Analysis of variance
ATNCs – Apparent total N-nitroso compounds
ATP – Adenosine triphosphate
Bp – Base pair
CCP – Critical control points
cDNA – Complementary deoxyribonucleic acid
Cfu – Colony forming units
CIP – Cleaning in place
cm – Centimeter
COP – Cleaning out of place
DEFT – Direct epifluorescent filter technique
DEPEC – Diethylpyrocarbonate
Dm – Decimetre
DMS – Dimethyl sulphide
DMSO – Dimethyl sulfoxide
DNA – Deoxyribonucleic acid
EBC – European brewery convention
EDTA – Ethylenediaminetetraacetic acid
ELISA – Enzyme linked immunosorbent assay
g – Gram
g – Gravity
HACCP – Hazard analysis critical control points
ICP-MS – Inductively coupled plasma mass spectrometry
ITS – Internally transcribed spacer region
Kb - Kilobase
KEGG – Kyoto encyclopedia of genes and genomes
L – Liter
LAB – Lactic acid bacteria
LB – Lysogeny broth (medium)
M – Mol
MALDI-TOF – Matrix assisted laser desorption ionization - time of flight
mg – Milligram
MIC – Minimum inhibitory concentration
Min – Minutes
ml – Milliliter
mm – Millimeter
mM – Millimolar
mmol – Millimols
Mol – Molar
mRNA – Messenger ribonucleic acid
MRS – de Man, Rogosa and Sharpe (medium)
mtDNA – Mitochondrial deoxyribonucleic acid
MYPG – Malt yeast peptone glucose (medium)

NAD – Nicotinamide adenine dinucleotide (oxidised)
NADH – Nicotinamide adenine dinucleotide (reduced)
NADP – Nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH – Nicotinamide adenine dinucleotide phosphate (reduced)
NASC – Nottingham arabidopsis stock centre
NB – Nutrient broth (medium)
NBB – Nocive brewers bacteria (medium)
NCBI – National center for biotechnology information
ng – Nanogram
nm – Nanometer
OD – Optical Density
ORF – Open reading frame
PAA – Peracetic acid
PCR – Polymerase chain reaction
POF – Phenolic off-flavour
ppm – Parts per million
psi – Pounds per square inch
QAS – Quaternary ammonium salts
rDNA – Ribosomal deoxyribonucleic acid
RFLP – Restriction fragment length polymorphism
RNA – Ribonucleic acid
RO – Reverse osmosis (water)
ROS – Reactive oxygen species
rpm – Revolutions per minute
rRNA – Ribosomal ribonucleic acid
SDS – Sodium dodecyl sulfate
Sec – Seconds
SGD – *Saccharomyces* genome data base
TCA – Tricarboxylic acid
TEM – Transmission electron microscopy
UBA – Universal beer agar
v/v – Volume/volume
WLN – Wallerstein laboratory nutrient
w/v – Weight/volume
YPD - Yeast extract peptone dextrose (medium)
µg – Microgram
µm – Micrometer
µM – Micromolar
µl – Microlitre

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Chapter 1 Literature Review

1.1 Introduction

In general beer is considered to be microbiologically safe for human consumption. This is due to its low carbohydrate content, a low pH (approximately 3.9–4.4), alcohol content (up to 10% v/v, but typically 3.5–5.0% v/v), its redox potential (defined by the reducing capacities of the hop iso-α-acids, polyphenols, sulfhydryls and nitrogen compounds etc. present in the beer. Wort: 20-30 rH, beer at bottling 16 rH, if exposed to light 8-12 rH) and the presence of hop iso-α-acids (approximately 17–55 ppm iso-alpha-acids) which have antimicrobial activity. All of these factors make beer an innately inhospitable environment for most microorganisms. However, certain microorganisms have adapted to brewery conditions, having found specific niches within brewery processes. Therefore, to understand the danger of contamination of beer, the entire brewing process must be scrutinised (Eßlinger, 2009; Joslyn, 2012; Vriesekoop *et al*., 2013). Fortunately, none of the microbial contaminants which are able to survive within the brewing process are human pathogens. Their threat stems from the danger they pose to product flavour, quality and yield (Hough *et al*., 2006). It has been estimated that the German brewing industry alone incurs losses of €250 million each year through microbial contamination (Tamachkiarow and Schramm, 2003).

In recent years the interest in silver as an antimicrobial has increased due to the increasing rise in antibiotic resistant microorganisms (Chopra, 2007). In addition to medical uses in dressings and indwelling devices, this interest has led to research into silver nano-particles and antimicrobial surfaces made from

these particles. The results of these investigations appear promising with a variety of organisms showing sensitivity, albeit to different degrees. This project is concerned with determining the sensitivity of brewery microorganisms to silver. If silver proves to be effective at eliminating brewery contaminants, the next progression would be the manufacture of silver nano-particle surface coatings. These coatings would have to be examined for their antimicrobial effect against brewery contaminants under brewery conditions. If proven effective this coating may be applied to brewing vessel surfaces, to eliminate any contaminants as they pass through the vessels. If functional, this coating should reduce, if not remove, the need for other contaminant control measures, such as pasteurisation, potentially reducing costs for breweries and preserving flavour. Any antimicrobial surface, silver or otherwise, would have to be installed pre and post the fermentation vessel in the brewing process. This would prevent/reduce contamination in the process, but not inhibit the brewery yeast during fermentation. As a result other sanitation methods would still be necessary for the fermentation vessel.

1.2 The Brewing Process

Although every brewery will have its own recipes and methodologies specific to their beer type and brand, there are several key stages, most of which will be common to the majority of European brewing processes. These stages are Malting, Milling, Mashing, Wort Separation, Boiling, Fermentation, Conditioning and Packaging. The key ingredients: water, malt, hops, yeast and adjuncts, where necessary, will be processed through these stages (Boulton and Quain, 2006).

The Malting process is concerned with the production of malt. For this purpose a cereal, most commonly barley although other cereals such as wheat or rye can be used, is hydrated by steeping in water. Air may be passed through the steeping cereal. This promotes germination of the seeds, which are then left to rest in a cool moist atmosphere, with intermittent turning and mixing (Douglas, 1984). The germination triggers the biosynthesis of enzymes (amylases and glucanases), accumulation of sugars and other soluble materials, in addition to partially breaking down the granule cell walls of the grain (Flannigan *et al.*, 1982). When the starch in the grain is partially hydrolysed by amylases, the process is halted by heating the grain in a kiln, producing malt (Follstad and Christensen, 1962).

This malt is then milled to produce grist. At the milling stage mash tun adjuncts (cereal preparations such as flaked maize or rice) may be added, providing an additional sugar source and possibly altering the character of the beer (Hopkins and Krause, 1947). This is illegal in some countries such as Germany (Briggs *et al.*, 2004).

During the mashing process the grist is mixed with liquor (brewing quality water) at a controlled rate in a mashing vessel. This ‘mash’ is held at a specific temperature for a period of time. This allows soluble substances to dissolve from the grist into the mash e.g. carbohydrates from starch, as well as hydrolysing previously insoluble materials. The temperature and timing of the mashing process is dependent on the type of mashing used (Rübsam *et al*., 2013). In Britain one of the most common types is infusion mashing. Grist is mixed with hot liquor in a controled manner to achieve a final temperature of 65°C. The time period for which this temperature is held can vary between

30 min. and several hours. Sweet wort (liquor infused with the sugars and malt compounds from the mash) is removed from the mash in a mash tun (vessel with sieve like bottom). Any remaining sweet wort is removed from spent grain by spraying the mash with hot liquor (79°C), normally using revolving sparge arms (Briggs *et al*., 1981). The main type of mashing process used for lager in mainland Europe is the decoction system. This system requires finely ground grist made from unmodified malt. The mash produced is of low viscosity allowing it to be pumped and stirred. This method removes a fraction of the mash, which is boiled prior to addition to the main mash, thus raising its temperature (Montanari *et al.*, 2005). In Lager there are three vessels required, a mash mixing vessel, a mash cooker and a wort separation device. In the mash mixing vessel the grist is mashed-in at 40˚C, and subsequently left to stand. Then the first decoction is performed, where approximately a third of the mash is transferred to the mash cooker and heated to boiling. This is then returned to the mixing vessel increasing the temperature to 50˚C. A second decoction raises the temperature to 60-65˚C and a third to 70-75˚C. The sweet-wort is then separated from the mash in the wort separation vessel, which is normally either a mash filter or a lauter turn (Hopkins and Krause, 1947).

The sweet wort is transferred to a boiling vessel, traditionally made of copper but now more commonly made of stainless steel. Here the wort is boiled with hops or hop extracts. The exact boiling temperature and time can vary between 106°C-140°C and 3-15 min. depending on the brewery and methodology i.e. use of heat exchanger or pressure resistant kettle (Lea and Piggott, 2003). The hops added at this stage can be whole, ground or pellets

made from powder. Syrups prepared from enzymatically or acid degraded barley, maize or wheat, may also be added to increase the concentration of fermentable carbohydrates in the wort. The boiling stage has several effects on the wort's properties, it causes the coagulation of proteins into 'trub', promotes infusion of bitterness and hop aromas, as well as fulfilling an antimicrobial function due to high temperature (MacWilliam, 1971; Simpson, 1993). In addition to this, undesirable flavour and volatile aroma compounds are evaporated. The boil also effects flavour changes as well as evenly mixing the dissolved sugars, malt extracts and syrups in the wort. At the end of the boiling step the trub and hop remnants have to be removed from the wort either by a filter or in a whirlpool tank (Kühbeck *et al*., 2006). In addition to the boiling, the presence of hop iso-alpha acids also has an antimicrobial effect. These acids have an antimicrobial effect on Gram-positive bacteria, but not on most Gram-negative bacteria or yeasts (Schmalreck *et al*., 1975; Bhattacharya *et al*., 2003). Hop acids are weak acids, which act as proton ionophores in the cell membrane. This damages the cells by lowering the internal pH by transporting protons across the cell membrane and causing oxidative stress (Behr and Vogel, 2009; 2010). Additionally, there will be leakage through the membrane and respiration and the synthesis of DNA, RNA and proteins will be inhibited (Teuber and Smalreck, 1973).

Fermentation begins when the cooled wort is pitched (inoculated) by the selected brewery yeast. This yeast is usually *Saccharomyces cerevisiae,* although the exact strain used varies between breweries, altering the character and flavour of the beer, as does the type of fermentation vessel chosen e.g. open or closed top (Bolton and Quain, 2001). The yeast ferments

the substrates dissolved in the wort and replicates between 3-5 times. The major by-products of this fermentation are ethanol and carbon dioxide. Some of the yeast may be retained for future use. The excess is discarded or sold to manufacturers of yeast extract products (O'Connor-Cox, 1997). The type of yeast used and the temperature of pitching and fermentation are dictated by the type of beer. Ales require top-fermenting yeasts, which float to the top of the beer. Ales are usually pitched at 15-18˚C and fermented at 18-25˚C (optimally 20˚C) for 2-3 days. Lagers on the other hand, utilise bottom-fermenting yeasts which sink to the bottom of the fermenter. Lager yeasts are pitched and fermented at lower temperatures than ales, pitching takes place at 7-8˚C and fermentation at 12-15˚C (Knudsen, 1999).

Once the primary fermentation is complete the immature beer is processed or 'conditioned.' The immature beer is stored for a time to mature, undergoing secondary fermentation. To aid this, primary sugars or wort may be added (Boulton and Quain, 2001). In traditional lager production the beer would be stored at 0-12˚C for long periods of time. This slow process allows precipitation of tannin protein complexes and yeast to settle to the bottom of the fermentation vessel. Additionally carbon dioxide is allowed to saturate the beer and hop aroma from dry hops is allowed to develop. Post conditioning, the majority of beers are cooled and either filtered or centrifuged to remove the yeast. The 'bright' beer is then carbonated and filled into final containers such as cans, bottles or kegs, through filler lines (Rennie and Wilson, 1977). Nitrogen may be added to the containers to ensure that as much air as possible is excluded. To avoid flavour damage by microbial contaminants the beer may be passed through a sterile filter prior to filling into the container or

the beer may be pasteurised once in the container by a highly regulated heat treatment regime (specific to the brewery) in which the vessel is passed through a tunnel pasteuriser. This process subjects the vessel to increasing temperature until a top temperature (approx. 60°C) is reached, which is held for 10-20 min. The vessels are then cooled to 10-15°C (Dymond, 1992; Hoff *et al*., 2013). Alternatively flash (or plate) pasteurisation heats the vessels to 70°C for 20-30 sec. and then rapidly cools them to process temperature. In total this should take 120 sec. (Dymond, 1992).

1.3 Brewery Contaminants and Where They Occur

Despite all the antimicrobial properties beer possesses, certain spoilage organisms have adapted to specific niches of the brewing process (Fig. 1.1). These spoilage organisms can be subdivided into moulds, Gram-negative bacteria, Gram-positive bacteria and wild yeasts (Boulton and Quain, 2006). Primary contamination is introduced through contaminated raw materials or the brewing vessels, whereas secondary contamination occurs during filling into final pack, such as bottles (Storgårds, 1997). Raw materials including malt, hops and adjuncts possess their own microbiota and water must be sterilised prior to use. The brewhouse vessels may also be a source of contamination if not correctly cleaned and maintained (Vaughan, 2005). Contamination of unsealed bottles by air borne contaminants may occur before or during filling prior to sealing, possibly during transport to the filler (Storgårds, 2000).

1.3.1 Microbial Contamination of Barley and Malt

Barley and malt can be affected by moulds. Even though moulds such as *Alternaria, Epicoccum* and *Fusarium* do not directly come into contact with beer, they can damage the quality of malt, wort and beer (Lawrence, 1988; Flannigan, 1996). The degree of contamination by moulds is dependent on the condition of the fields and post-harvest storage (Flannigan, 1996). An important plant fungus genus is *Fusarium*, which can cause head blight or scab resulting in great yield loss in barley (Chelkowski, 1989). Additionally, contamination of barley by *Fusarium* is known to induce premature yeast flocculation (PYF), a condition in which yeasts flocculate before fermentation of all fermentable sugars is complete (Sarlin *et al*., 2005). The *Fusarium* strains *F. graminearum* and *F. culmorum* are able to secret mycotoxins which, if produced, will enter the final beer product (Flannigan *et al.*, 1985). As well as producing mycotoxins, *Fusarium* is also able to induce gushing (eruptive over-foaming) from bottles, cans or kegs by releasing fungal-specific hydrophobins and plant typical non-specific lipid transfer proteins (ns-LTPs) (Laitila *et al.*, 2002; Christian *et al*., 2011). Contamination of malt by moulds results in off-flavours ranging from burnt molasses to winey and harsh. In addition to moulds there is a wide range of bacteria included in the microbial load of the barley grain, however, during malting lactic acid bacteria (LAB) numbers increase drastically in comparison to other organisms (O'Sullivan *et al.*, 1999). Whilst uncontrolled growth of LAB produces off flavours, a low level presence has an antimicrobial effect on other bacteria due to production of compounds such as lactic acid and bacteriocins (O'Mahony, 2000).

1.3.2 Microbial Contamination of Mashing and Wort preparation

Due to the high temperatures and the antimicrobial hop compounds the mashing and wort preparation stages tend to reduce microbial contamination. However, if the temperature falls in the mash or unhopped wort then proliferation of microbial contaminants may occur. The majority of LAB are sensitive to hop compounds, however, if they are allowed to grow in unhopped wort then off-flavours may occur due to diacetyl production (Hough *et al.*, 1982). *Bacillus* strains can be found in these stages. These strains enter the process via malt or cereal adjuncts. They are able to survive the boiling stage as spores, but are not able to germinate due to the presence of hop compounds and the low pH of wort and beer. Contamination of wort by coliforms, such as *Pantoea agglomerans* which produces diacetyl and dimethyl sulphide (DMS) in wort, may occur if the water supply is contaminated or fluid leaks from piping (Van Vuuren *et al.*, 1980).

1.3.3 Microbial Contamination during Fermentation

Post boiling, wort is clarified, cooled and aerated to optimise yeast growth and fermentation. Unfortunately, this also optimises the growth conditions for contaminant organisms which may be inoculated during the aeration process: for example, due to insufficient cleaning and disinfection of the air lines (Vaughan, 2005). Wild yeasts are most commonly described as “any yeast not deliberately used and under full control” (Gilland, 1971). Wild yeasts can be found throughout the brewing process and particularly the fermentation stage, and are likely to be airborne, or contaminants within the fabric of the brewery or contaminants of the pitching yeast (as can other contaminating organisms). Contamination by wild yeasts during fermentation can result in turbidity, off-

flavours and aromas caused by volatile compounds such as phenol for example, produced by *Bacillus anomalus* (Hough, 1985; Cocolin *et al.*, 2004). Wild yeasts may also slow fermentation or cause superattenuation (maintaining fermentation via utilization of substrates, such as dextrins, not used by brewing strains after usual fermentable substrates are exhausted, (Andrews and Gilliland, 1952) leading to low gravity beer, with an extremely high alcohol content (Lawrence, 1988). As the definition of wild yeasts includes such a wide range of different organisms, they have been sub-categorised into *Saccharomyces* and Non-*Saccharomyces* strains (Van der Aa Kühle and Jespersen, 1998). The Non-*Saccharomyces* include genera such as *Brettanomyces, Candida, Hanseniaspora, Kluyveromyces, Pichia* and many others (Ingledew and Casey, 1982). Bacterial strains are also known to contaminate the fermentation stage of brewing. Of the LAB, *Pediococcus* strains, in particular *P. damnosus* have been found during fermentation. Contamination by these organisms results in slow fermentation times and high levels of diacetyl (McCaig and Weaver, 1983). *Obesumbacterium proteus* and *Pantoea agglomerans* (formerly *Enterobacter)* can also be found during fermentation. The most common route of contamination is inocculation into the fermentation mixed with the pitching yeast. Contamination results in low fermentation and a high pH (Priest *et al.*, 1974).

1.3.4 Microbial Contamination during Conditioning and Packaging

There are two ways in which brewers pasteurise their beer, tunnel pasteurisation and flash pasteurisation. Tunnel pasteurisation is the most common method of sterilising bottles and cans. Flash pasteurisation is usually used for keg filling. Any failure in hygiene or pasteurisation may result in the

survival of contaminants (Back, 1994; 1997). Acetic acid bacteria are well documented beer spoilage organisms; however, as the oxygen levels have decreased in brewery processes over the years the amount of spoilage has decreased as they are obligate aerobes (Ryan *et al.,* 1996). Unfortunately spoilage by organisms such as *Pectinatus* and *Megasphaera* has increased. *Pectinatus* is thought to be involved in 20-30% of bacterial beer spoilage (Back, 1994). The species *Megasphaera cerevisiae* and *Pectinatus cerevisiiphilus* are relatively newly recognized brewery contaminants, however, they are considered to be hazardous to the brewing process (Helander *et al.,* 2004). *M. cerevisiae* is an obligate anaerobic coccus, 1-1.2 µm in diameter, which is catalase-negative. Occurrence of these organisms is limited to low oxygen environments such as small pack beer due to their oxygen sensitivity. Contamination by these organisms results in off-flavours (described as foul, putrid and faecal) which are caused by hydrogen sulphide, butyric and other short-chain fatty acids (Engelmann and Weiss, 1985; Haikara and Lounatmaa, 1987). *Pectinatus cerevisiiphilus* is an obligate anaerobic curved rod, 0.8 µm x 2-3 µm in size. As with *M. cerevisiae* the oxygen sensitivity limits the species to areas with low oxygen concentrations such as canned beer. The off-flavours produced by *P. cerevisiiphilus* include sulphur compounds, acetaldehyde and propionic acid (Haikara *et al*., 1981 and Schleifer *et al*., 1990). Should there be a flaw in the pasteurisation process, bacterial contaminants may occur in the final pack. The majority of bacterial contaminants are LAB. Of the LAB the genera *Lactobacillus* and *Pediococcus* are known to be the most hazardous to the brewing process (Suzuki, 2011).

1.3.5 Microbial Contamination of Final Product

As stated previously, the most common bacterial contaminations come from the LAB (Back, 1994). Contamination by either *L. brevis* or *P. damnosus* results in hazy, low alcohol beer (Satakari *et al.*, 1999). Gram-negative bacterial contaminants of beer include acetic acid bacteria which thrive in alcohol rich environments, oxidising the ethanol to acetic acid, and have been long recognized as problematical to wine and beer producers. Acetic acid bacteria tend to be resistant to hop compounds, acid and ethanol. Contamination may occur in the headspace of bottles or cans if the packaging process is faulty (vessel sealing or pasteurisation) (Van Vuuren and Priest, 2003).

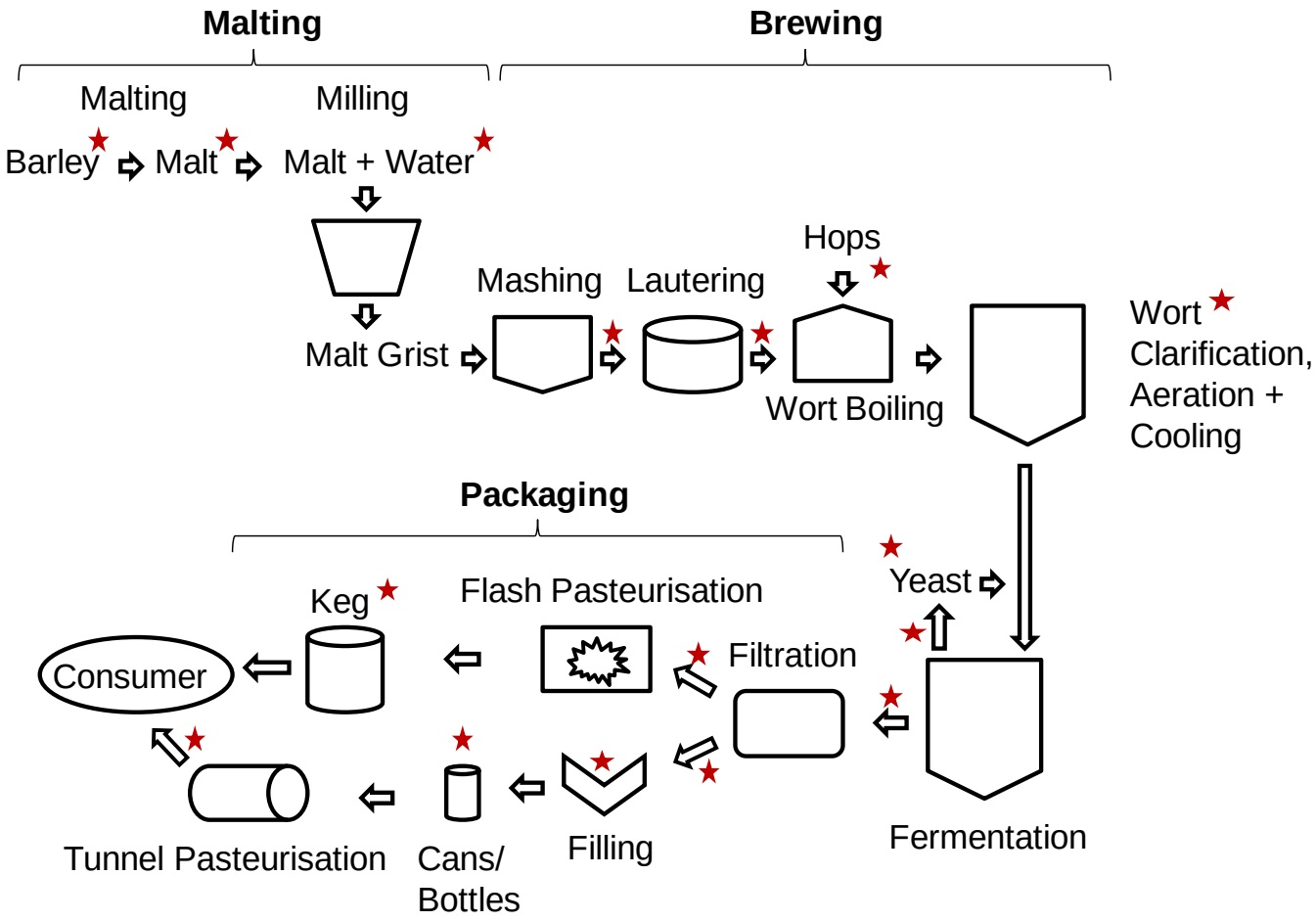


Fig. 1.1) Flow chart of the brewing process and the potential entry routes of microbial contaminants ★. The Wort boiling and Pasteurisation stages exhibit low risk of contamination if functioning correctly. The main areas at risk of of contamination are in the wort pre boiling and post fermentation. Modified from Vaughn *et al*. (2005).

1.3.6 Major Contaminant Organisms of the Brewing Industry

1.3.6.1 Gram-positive Bacterial Spoilage Organisms

The LAB are found throughout the environment, including on plant matter such as barley and malt, and dispersal through malt dust, aerosols and brewing equipment is not unusual. Examples of LAB isolated from breweries include *Pediococcus inopinatus*, *Pediococcus dextrinicus*, *Pediococcus pentosaceous*, *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus plantarum* (Jespersen and Jakobsen, 1996; Bokulich and Bamforth, 2013). However, overall the most damaging spoilage organisms to beer are *L. brevis* and *P. damnosus* as they are the most commonly reported spoilage contaminants. This is most likely due to their ethanol tolerance and resistance to hops (Pittet *et al*., 2011). Other than LAB very few Gram-positive bacteria have been reported as beer contaminants, although *Bacillus* spp. organisms have been found during wort production (Back, 1982; Smith and Smith, 1992).

1.3.6.2 Gram-negative Bacterial Spoilage Organisms

Acetic acid bacteria (AAB), although not as prevalent as they once were, are still a threat to barrel-conditioned beer (Bokulich and Bamforth, 2012). The most common AAB are *Acetobacter aceti*, *Acetobacter pasteurianus*, and *Gluconobacter oxydans*. These organisms spoil beer via the oxidation of ethanol to acetate, effectively turning beer into vinegar (Bokulich and Bamforth, 2013).

Due to the reduction of dissolved oxygen in beer, brought on by modern techniques, there has been an increase in obligate anaerobic *Veillonellaceae* organisms, such as *Pectinatus*, *Megasphaera*, *Selenomonas*, and *Zymophilus*. These mentioned organisms have only been reported in beer

and cause spoilage through haze formation, production of propionic acid, acetic acid, hydrogen sulfide and mercaptans, and inhibiting of yeast growth and alcohol production (Chowdhury *et al*., 1997). Contamination by these organisms may occur through pitching yeast, in particular repitched yeast. They are found more frequently in nonpasteurised and bottled beer (Jespersen and Jakobsen, 1996; Juvonen and Suihko, 2006).

1.3.6.3 Wild Yeast Spoilage Organisms

The most common type of wild yeast spoilage organism are variants of *Saccharomyces cerevisiae* (van der Aa Kuhle and Jespersen, 1998). Spoilage by these organisms may induce ester or phenolic off-flavour production (POF), haze or sediment formation and superattenuation (Meaden and Taylor, 1991).

Of the non *Saccharomyces* yeasts, *Brettanomyces* yeasts (teleomorph *Dekkera*) such as *Brettanomyces bruxellensis*, *Brettanomyces custersii*, and *Brettanomyces anomalus* are common spoilage organisms. Despite this *Brettanomyces* strains are actively required for the production of certain beers, such as lambics (Aguilar-Uscanga *et al*., 2003). Other non *Saccharomyces* yeasts are able to grow in beer and during beer fermentation, but spoilage is limited due to competition with *Saccharomyces cerevisiae* and the environment beer presents i.e. low dissolved oxygen, ethanol toxicity and the storage conditions. Examples of wild yeasts isolated from beer include: *Pichia anomala*, *Pichia fermentans*, *Pichia membranifaciens*, *Pichia guilliermondii*, *Candida tropicalis*, *Candida boidinii*, *Candida sake*, *Candida parapsilosis*, *Candida guilliermondii*, *Candida glabrata*, *Candida valida*, *Saccharomyces unisporus*, *Torulaspora delbrueckii*, and *Issatchenkia*

orientalis, *Kluyveromyces marxianus*, *Debaryomyces hansenii*, *Zygosaccharomyces bailii*, *Zygosaccharomyces bisporus*, *Schizosaccharomyces pombe* and *Kloeckera apiculata*. The majority of these will spoil beer by producing off-flavours, haze, sediment or surface films. These yeasts are common throughout breweries, especially in unwashed sampling ports and on other surfaces contacting beer. They are opportunistic contaminants commonly found in barrel-fermenting beer (van der Aa Kuhle and Jespersen, 1998; Timke *et al*., 2008; Bokulich and Bamforth, 2013).

1.3.7 Known Physiology of the Test Strains

In this study 7 wild yeast and 7 bacterial brewery contaminant organisms were the subject of investigation regarding their tolerance to silver. The organisms are *Lactobacillus brevis*, *Pediococcus damnosus*, *Shimwellia pseudoproteus*, *Bacillus coagulans*, *Pantoea agglomerans*, *Gluconobacter oxydans*, *Acetobacter aceti*, *Pichia membranaefaciens*, *Brettanomyces anomalus*, *Candida krusei*, *Hansenula saturnus*, *Kloeckera apiculata*, *Rhodotorula mucilaginosa* and *Saccharomyces ellipsoids* var. *diastaticus*.

1.3.7.1 Bacteria

1.3.7.1.1 *Lactobacillus brevis*

This species is part of the lactic acid bacteria (LAB) family. It is a Gram-positive, anaerobe that is catalase-negative and aerotolerant. *L. brevis* cells are thin non-motile rods that cannot form spores (Whittenbury, 1963). Strains of *L. brevis* may exhibit one of two forms of metabolism, they are facultatively heterofermentative, producing lactic acid, acetic acid, ethanol and carbon dioxide, but are also capable of homofermentative metabolism, which

produces lactic acid (Saier *et al*., 1996; Garde *et al*., 2002). Contamination results in sour off-flavours, turbidity and the formation of extracellular polysaccharide ropes. *L. brevis* prefers a pH of 4-5 and is mostly found in conditioning or final pack products (Suzuki, 2011). The presence of these organisms also increases fermentation time and leads to super-attenuation due to their ability to ferment starch and dextrins (Jespersen and Jacobsen, 1996). *L. brevis* is the most reported brewery contaminant LAB and is able to survive all brewing stages and can tolerate hop iso-alpha acids (Sakamoto *et al*., 2001; Back, 2005). However, *L. brevis* strains are able to produce bacteriocins, which have an antimicrobial effect on other spoilage organisms (Basanta *et al.*, 2008). *L. brevis* SB27 for example, produces the bacteriocin Brevicin 27, which is primarily active against other lactobacillus organisms (Benoit *et al*., 1997).

1.3.7.1.2 *Pediococcus damnosus*

This species is also part of the lactic acid bacteria family. It is a Gram-positive anaerobe that is catalase negative, but can still survive in oxygen. *P. damnosus* is a non-motile coccus which may appear singularly, in pairs or as tetrad squares (Garvie, 1974). The strain is tolerant of hop resins as well as ethanol (Haakensen *et al*., 2009). Contamination may occur in beer during fermentation and during maturation, resulting in hazes, ropiness, acidity and buttery off-flavours due to high levels of diacetyl (Back, 2005). *P. damnosus* may also bind to brewing yeast resulting in premature flocculation and reduction of fermentation (Suzuki, 2011).

1.3.7.1.3 *Shimwellia pseudoproteus*

Formerly known as *Obesumbacterium proteus* this species has been reclassified repeatedly (Priest and Barker, 2010). The cells are short, rod shaped, non-spore forming, non-motile and are Gram-negative. The species is catalase-positive and is able to survive in ethanol. The organism is most commonly found in the pitching yeast (pitching yeast have been reported as containing between 0.1%-10% *S. pseudoproteus* according to Ault (1965)), as a result of *S. pseudoproteus* contamination fermentation is inhibited so the attenuation rates will drop (yeasts do not utilize all the fermentable sugars) and the pH will not decrease as expected during fermentation. The off-flavours produced are described as fruity or parsnip. The organism can also be found in yeast heads and slurry, consequently there is a risk of breweries recycling the contaminant into the next fermentation. *S. pseudoproteus* is not found in the final product as it is intolerant of low pH (Bishop, 1942; Ault, 1965). It can grow in wort where it's presence results in the formation of diacetyl, dimethyl sulphide, acetoin, lactic acid, propanol, isobutanol and 2,3-butanediol (Thomas *et al*., 1972; Priest and Hough, 1974). Nitrates or nitrite may be reduced, forming carcinogenic nitrosamines (ATNCs, apparent total N-nitroso compounds) (Prest *et al*., 1997). Currently *S. pseudoproteus* is assigned to the *Enterobacteriaceae*. However, there are two biotypes of the species. According to phenotypic and DNA/DNA hybridisation studies, Biotype 1 is similar to *Hafnia alvei*, whilst biotype 2 is closely related to *Escherichia blattae*. Usually strains isolated from breweries are biotype 2 (Brenner, 1991; Maugueret and Walker, 2002; Koivula *et al*., 2005; Priest and Barker, 2010). However, Type 1 strains have been isolated in larger breweries (Koivula *et al.*,

2005). *Hafnia alvei* has also been isolated from animals, soil, and sewage and is the only species in the genus *Hafnia* (Sakazaki and Tamura 1991). *Escherichia blattae* has previously been isolated from the hind-gut of cockroaches (Burgess *et al*., 1973).

1.3.7.1.4 *Bacillus coagulans*

B. coagulans cells are large, motile, Gram-positive, endospore forming rods. The organisms are catalase positive, aerobic and thermoduric and thermophillic. However, they are intolerant to hop resins and do not survive a pH lower than 5. The spores can survive boiling and out-grow in sweet wort, where they produce lactic acid. They can survive boiling due to their ability to form endospores and are known to be able to grow across a large range of temperatures (55–70°C) (Calderbank and Hammond, 1989; Smith and Smith, 1992; Priest, 1999; Vaughn *et al*., 2005). In addition to this, they can grow at pH levels ranging from 5-10.5. *B. coagulans* cells also exhibit antimicrobial abilities. These abilities have been attributed to the production of lactic acid and bacteriocins such as coagulin, which are effective against coliforms and enterococci, but not lactobacilli. However, a bacteriocin which functions against Gram –positive and -negative bacteria as well as fungi has been characterised (Ripamonti *et al.*, 2009).

1.3.7.1.5 *Pantoea agglomerans*

The cells are short, fat, Gram-negative, rods, which can be motile (Vriesekoop *et al*., 2012). The species can ferment glucose, to produce acetic acid (Lategan *et al*., 1980). The organism is known to contaminate pitching yeasts and fermenting wort, however as the organism is sensitive to ethanol (>2%

ABV) it is more commonly found in top cropping ale fermentations. Contamination in wort results in the production of diacetyl, acetaldehyde, methyl acetate and dimethyl sulphide (van Vuuren *et al.*, 1980). *Enterobacter agglomerans* was reclassified as *Pantoea agglomerans* on the basis of DNA hybridization experiments (Gavini *et al*., 1989; Cruz *et al.*, 2007). The organism is motile at 25°C, but not at 37°C. *P. agglomerans* contamination in the wort during fermentation, results in increased final specific gravity due to retardation of fermentation (van Vuuren *et al*., 1980).

1.3.7.1.6 *Gluconobacter oxydans* and *Acetobacter aceti*

Both *G. oxydans* and *A. aceti* are primarily known for producing acetic acid from ethanol and are both rod shaped, Gram-negative cells approximately 4µm long. They are both pleomorphic and can form pairs or chains. The organisms are obligate aerobes and catalase-positive. *A. aceti* is a motile organism able to oxidise ethanol to carbon dioxide and water, producing acetic acid. *G. oxidans* oxidises ethanol to acetic acid. Contamination by these organisms results in the production of acetic acid and acetate, as well as the formation of hazes or pellicles (van Vuuren, 1999). *G. oxidans* can grow in a temperature range of 25-30°C and no more than 37°C. The optimal pH range is 5.5–6.0 (Mishra *et al.*, 2008). *A. aceti* has adapted to a brewery environment by becoming tolerant of heat from fermentation, ethanol is oxidised on the cell's surface to generate energy which generates acetic acid as a by-product (Okamoto-Kainuma *et al.*, 2004).

1.3.7.2 Yeasts

1.3.7.2.1 *Pichia membranaefaciens*

Pichia species such as *P. membranaefaciens* only ferment glucose and are also found in the early phase of fermentation and in draught beer. Contamination results in hazes, surface films and a sauerkraut off-flavour. The surface films and haze occur due to the formation of mycelia or pseudomycelia. The species is able to form spores that are either round or Saturn shaped, that are generally liberated (Kurtzman and Fell, 1998). *P. membranaefaciens* is known to contaminate pitching yeast, which is a possible source of process contamination (Brady, 1958). Additionally, *P. membranaefaciens* is a "killer" yeast as it has the ability to produce a zymocidal toxin. The toxin is known to bind to yeast cell surfaces via an interaction with (1-6)-β-D-glucan. *S. cerevisiae* is sensitive to this toxin, so the toxin's presence would inhibit fermentation (Barandica *et al*., 1999; Santos and Marquina, 2004).

1.3.7.2.2 *Brettanomyces anomalus*

Oxygen stimulates fermentation by *Brettanomyces anomalus*. It is able to ferment glucose and in some cases maltose, but not sucrose. The organism is most commonly found in bottle conditioned beers. The species produces acetic acid and reduces nitrate (Gilliland, 1961; Smith and van Grinsven, 1984). The organism produces volatile phenolic compounds, such as phenol, syringol and other ethylphenols, primarily causing off flavours, and can form hazes in the final product. The off-flavours produced could be described as animal or burnt plastic. This organism is difficult to control due to resistance to the usual brewery cleaning/sanitising agents such as sulphur dioxide (Cocolin

et al., 2004). *Brettanomyces anomalus* is a teleomorph (sporing form) of *Dekkera anaomalus* (Smith and Van Grinsven, 1984). Although usually considered a contaminant in beer production, small quantities of *B. anomalus* are required for the production of specific beer styles such as lambic beers. This may be due to the esterase activity exhibited by the organism (Spaepen and Verachtert, 1982; Verachtert and De Mot, 1989).

1.3.7.2.3 *Candida krusei*

Candida krusei can only metabolise glucose and requires oxygen (Samaranayak and Samaranayak, 1994). As a result this species is restricted to the early aerobic phase of fermentation process or unpasteurised draught beer. However, there are some reports of minimal anaerobic growth. The effects of contamination may include ester off-flavours and the formation of turbidity and surface pellicles which may break up into flaky particles or deposits (Kurtzman and Fell, 1998). *C. krusei* is resistant to high levels of alcohol and is tolerant to many other stresses such as acetic acid (Wei *et al.*, 2008). *C. krusei* is the anamorph (non-sporing form) of *Issatchenkia orientalis* (Middelhoven, 2002).

1.3.7.2.4 *Hansenula saturnus (Williopsis saturnus)*

H. saturnus is similar to *Pichia* strains in that it normally only metabolises glucose and is restricted to the aerobic stage of fermentation within the brewing process. Contamination may occur in unpasteurised draught beer, resulting in hazes and surface films in the beer (Boidin *et al*., 1965). In addition to this, a by-product of fermentation by *Hansenula* is the production of acetic acid. The species is also able to utilise nitrate as an N source and

form liberated Saturn shaped spores (Barnett *et al*., 2000; Yilmaztekin *et al*., 2008). Some strains of *H. saturnus* are able to produce antifungal agents which are effective against a wide range of fungi, including *Saccharomyces* (Goretti *et al.*, 2009).

1.3.7.2.5 *Kloeckera apiculata*

K. apiculata is the anamorph of *Hanseniaspora valbyensis*. The species is able to ferment wort. Contamination results in turbidity and off-flavours. The species is able to form spores which are round or Saturn shaped and are usually liberated. Vegetative growth occurs through polar budding (Barnett *et al*., 2000; Priest and Campbell, 2003). On liquid agave medium it was found that *K. apiculata* is able to produce very high levels of ethyl acetate in comparison to *S. cerevisiae* strains, but only approximately half the quantity of higher alcohols such as 1-propanol, amyl alcohols and isobutanol (Arellano *et al.*, 2008). In addition to *K. apiculata* being a synonym for *H. valbyensis,* it is a teleomorph of *Hanseniaspora guilliermondii* (Lavie, 1954).

1.3.7.2.6 *Rhodotorula mucilaginosa*

R. mucilaginosa is able to absorb and metabolise, but not ferment, a large range of sugars. *Rhodotorula* are unusual in that they have a red pigmentation. They are water borne organisms that can be found with pitching yeasts. Whilst they do not directly spoil beer they compete with the brewery yeast for sugars. Cells are oval or round and replicate by multilateral budding. They may occur singly, in pairs or as small clusters (Harrison, 1928; Libkind *et al*., 2004).

1.3.7.2.7 *Saccharomyces ellipsoides* var. diastaticus

Saccharomyces wild yeasts are considered to be more problematic than non-*Saccharomyces* yeasts. This is due to the facultatively anaerobic nature of the *Saccharomyces* strains. Due to taxonomic changes, *S. ellipsoides* is now *S. cerevisae* var. *diastaticus.* As the trend of reducing the dissolved oxygen content in beer production continues due to the unfavourable effect of oxygen on flavour and shelf life, organisms such as *S. cerevisae* var. *diastaticus* become primary spoilage yeasts as they show significant growth in the absence of oxygen. Contamination with a diastatic strain of *S. cerevisae* can occur in the fermenter or downstream (Howard and Mawer, 1977; Boulton and Quain, 2006). The spores are oval (ellipsoidal); (Lodder and Kreger-van Rij, 1952). The cells ferment vigorously, but do not form a pellicle (Kurtzman and Fell, 1998). The strain also often flocculates (Put and de Jong, 1980). The yeast is able to ferment wort oligosaccharides (dextrins) through expression of glucoamylase, making it a 'diastatic' yeast. This leads to turbidity, superattenuation (low final gravity) and phenolic off-flavour (Gilliland, 1971).

1.4 Current Means of Detection and Identification of Microbial Contamination

Although beer is an inhospitable environment for microbial growth, due to its inherent properties and the brewing process itself, as already discussed, contamination and growth in wort or beer can still occur. As a result, it is still necessary to have quality assurance methods to detect, identify and quantify any microbial contaminants in the beer or within the process. There are several methodologies available to breweries to satisfy this need, including

classical microbiology, optical techniques, molecular methods and indirect methods (Bamforth, 2006).

1.4.1 Classical Microbiology

The classical detection tests for microbial brewery contaminants consist of forcing and plating tests. The forcing test is the simplest means of detection. Beer or wort is incubated at increased temperatures and is inspected every day for haze and CO_2 production. The process may be accelerated by adding appropriate nutrients, although this will decrease selectivity of spoilage organisms. The disadvantage of this test is that, although it detects the presence of any microorganisms, there is no quantification. Additionally, many of the contaminants are extremely slow growing. The main two forcing test methods are The European Brewery Convention 1963 method, 60°C for 7 days then cooled to 0°C for 24 hours and the Harp method, 4 weeks at 37°C followed by 8 hours at 0°C (Bamforth, 2011). The plating tests consist of inoculating a beer or wort sample on to a solid nutrient medium and incubating. The number of colonies formed allows the quantification of spoilage organisms in colony forming units (cfu) per ml. A variation to this method is to filter the beer instead of applying it straight to the medium. Any organisms will be retained by the filter, which can then be applied to the medium. As this increases sample size, lower levels of contamination can be detected. Again the amount of organisms can only be approximately quantified as cfu per ml as, due to the filtration, cells may have clumped together, giving an estimate rather than true count. In addition to quantification, the large range of selective media allows the identification of some organisms down to the species level (Bamforth, 2006). There are many

different media used for the plating tests, but three of the most common are Wallerstein Laboratory Nutrient (WLN) agar, MYPG-copper agar (Malt Yeast Peptone Glucose medium), and Raka Ray agar. WLN agar allows growth of both bacteria and yeast and is made selective through the addition of 15 mg/l of cyclohexamide, allowing the detection of aerobic bacteria, such as acetic acid bacteria and wild yeasts (Quain, 1995; Hammond, 1996;, Simpson, 1996). MYGP-copper medium is utilised for the selection of wild yeasts. In this medium the selective agent is copper sulphate (usually 200 mg/l) and it has been shown that this medium positively selects for wild yeasts including *Saccharomyces*, *Pichia* and *Candida* strains (van der Aa Kuhle and Jesperson, 1996). Racka Ray agar is one of the major selective media for LAB organisms although there are others such as de Man, Rogosa and Sharpe medium (MRS) and Nocive Brewers Bacteria (NBB) medium (De Man *et al.,* 1960; Saha *et al.,* 1974; Kindraka 1987). WLN and MYPG-copper will be incubated aerobically and Racka Ray anaerobically due to the nature of the organisms being selected for (Avis, 1990). Of the classical microbiological methods the first identification methods to be used are the phenotypic characterisation methods. These begin with simple tests such as Gram staining, catalase and oxidase tests and morphological differentiation. The results of such tests allow the grouping of contaminants; breweries will have flow charts of tests, the results of which will indicate the most likely organisms. However, this is not sufficient for identification of the contaminant. Therefore, other identification methods are necessary, such as the appropriate API biochemical test system (bioMerieux) where the organism is loaded into wells containing a variety of substrates and the reaction pattern identifies the

organism (Gutteridge and Priest, 1996). For example the API 20E Microtube system has been proposed as a rapid identification method of Gram-negative brewing contaminant bacteria (Ingledew *et al.,* 1980). The main shortcoming of these methods is that they are very slow, resulting in distribution of the product before the spoilage can be detected (Funahashi *et al.*, 1998).

1.4.2 Optical Microscopic Techniques

Optical techniques can include a variety of methodologies, the simplest of which are observing the morphology of the organism under a microscope or performing a Gram stain (EBC Analytica Microbiologica, 2005). A more complete identification system is laser scanning cytometry (www.chemunex.com/literature/biblio.htm#laser).

Fluorescent markers and solid-phase laser scanning cytometry allow the quantification of organisms adhering to a filter, down to a single cell. The markers bound to the cells fluoresce as they are illuminated by the laser and the signal is interpreted by the instrument's computer. This process takes between 2-4 hours, according to which marker is used. A total viable count is possible, utilising a fluorogenic dye or fungus-specific marker, as the system is able to differentiate labelled microorganisms from autofluorescent particles (Joux and Lebaron, 2000). Specific identification would be possible using organism-specific fluorescent markers or DNA probes, if these were developed for specific spoilage organisms. The labelling takes 1.5 - 3 hours and the scanning <3 minutes. However, when evaluated by TEPRAL (Beer Research Centre for Danone) it was found to have potential, but to be too expensive (Bamforth, 2006). This system is similar to an earlier method called direct epifluorescent filter technique (DEFT). In this method organisms

adhering to a membrane filter were stained with a fluorochrome, such as acridine orange. These stained cells are visualised and counted via an epifluorescent microscope fitted with a camera linked to a computerised image analyser (Pettipher and Rodrigues, 1983; Pettipher *et al*., 1992).

Another method of identification is to use immunofluorescent immunoassays. This is founded on the interaction of a fluorescently labelled antibody with specific antigens on the surface of specific cells. These labelled cells are visible and can be quantified using microscopy, flow cytometry or laser-scanning cytometry. As antibodies are specific and can be produced on a large scale, they can also be used to identify the organism in question. Immunoassays have been produced for *Pediococcus* and *Lactobacillus* species (Whiting *et al.,* 1992; 1999). The most common method used to observe and quantify the labelled organisms is flow cytometry (Eger *et al.,* 1995). However, alternative methods have also been proposed such as the use of a CCD camera (March *et al.*, 2005).

1.4.3 Molecular Techniques

Molecular methods of identification and detection are becoming more popular. They are mostly based on genetic methods centred on analysis of the nucleotide sequence of DNA or RNA. Examples of molecular methods include hybridisation methods. Hybridisation kits have been produced for brewery spoilage organisms such as *Lactobacillus* and *Pediococcus.* Hybridisation is the formation of double stranded nucleic acids from complementary single stranded nucleic acids. In these kits magnetic beads coated in fluorescently labelled DNA probes specific to target organisms are hybridised to complementary target 16S rRNA molecules in the target organisms. The

amount of hybridisation is measured through spectroscopy in a 96 well plate by measuring fluorescence. The assay takes 24-30 hours and has a detection limit of 10^3-10^4 cells per ml (Wetmur and Fresco, 1991 and Bau *et al.*, 2005). This method detects presence and quantity of target organisms, but does not differentiate between live and dead cells.

One of the classic molecular methods still in widespread use for detection and identification of microorganisms is PCR. PCR also forms the basis of many more advanced molecular methods. Primers and DNA polymerase are used in a three step thermo-cycling process to exponentially synthesise copies of the DNA target. Within one to two hours the target DNA can be amplified up to 10^{12} times (Mullis *et al.*, 1986). Traditionally the PCR products would be electrophoresed on a gel once the end-point is reached. However, this method is awkward to automate for high throughput and quantification of the sample is difficult. This has led to the development of PCR variant methods such as Real-time PCR and PCR-ELISA.

Real-time PCR enables the synchronized amplification, detection and enumeration of the nucleotide target sequence. Each cycle of amplification is monitored and logged by detecting fluorescently labelled DNA probes or dyes bound to the amplicons using a thermo-cycler which detects fluorescence. In the exponential phase of PCR the fluorescence signal is inversely proportional to the quantity of the original target sequence. This assay takes place in a sealed tube and requires 0.5-2 hours to run (McKillip and Drake, 2004). Two examples of Real-time PCR methods are the use of dual-labelled oligonucleotide fluorogenic probes and the TaqMan® probe. Both probes consist of a non-extendable (does not act as primer) oligonucleotides,

containing a reporter fluorescent dye at the 5’ end and a quencher dye at the 3’ end. When bound to the amplicon the quencher dye reduces fluorescence. PCR amplification via DNA polymerase cleaves the probe releasing the fluorescent reporter dye (Arya, 2005).

The PCR-ELISA (Enzyme Linked Immunosorbent Assay) test is an economical, high-throughput means of detecting and identifying many target organisms. The PCR products are targeted by species-specific monoclonal antibody probes attached to microtitre plates. Targeted DNA fragments are hybridised to these probes. Species-specific secondary antibodies carrying fluorochromes (e.g. fluorescein) bind to their bound target DNA fragment. Bound amplicons are detected through an enzymatic colour reaction enabling identification to the species level (Walker *et al.*, 2003; Gomes *et al*., 2010).

All of these methods are for the rapid detection of contaminants in product, however some methods require that the isolates to be identified have been cultured to purity e.g. Ribotyping. Ribotyping is a molecular method which is employed to define the restriction fragment length polymorphism (RFLP) of the genome using ribosomal DNA probes. The total chromosomal DNA is restricted with endonucleases and the fragments are electrophoresed on a gel. These fragments are probed with 16S and 23S rRNA genes. With automation, identification of organisms is straightforward and fast, between1-8 strains can be analysed in 8 hours (Suihko and Stackebrandt, 2003). This is dependent on the species being logged in a database for comparison. The most accurate method of identifying an organism is sequencing, followed by a database search of known sequences. As sequencing the complete genome is too expensive, time consuming and impractical, specific regions such as

genes e.g. *horA* gene (responsible for hop resistance in lactobacilli), the 16S rDNA region (a region of ribosomal DNA existing in most bacterial organisms either as a multi gene family or operons, large enough for informatics purposes and highly conserved between organisms of the same genus and species, but differerent from organisms of other genera and species) or ribosomal spacer sequences (e.g. Internally Transcribed Spacer region (ITS) has been used for yeast identification) may be used for species identification (Sami *et al.,* 1997; Bischoff *et al.,* 2001; Kurtzman and Robnett, 2003; Woo *et al*., 2008). Sequencing will determine the identity of a contaminant, but will not quantify the level of contamination, as only the sequence of the selected target region is determined, not its quantity.

1.4.4 Indirect Methods of Microbial Detection

Indirect methods include those assays which do not directly assay the growth of the contaminants, but measure microbial growth through a secondary factor. An example of an indirect method is to use ATP bioluminescence. ATP is a high-energy molecule found in viable cells, which can be used to indirectly assess biomass. The system works by detecting ATP through the luciferin-luciferase enzyme system derived from fireflies. On contact with ATP a bioluminescence reaction occurs in which energy is released as yellow-green light with a peak emission wavelength of 560 nm (Kyriakides and Patel, 1994). The minimal cell number required for detection is 1-100 for yeasts and 10^3-10^4 for bacteria (Simpson *et al.*, 1989). The main disadvantage of this system is that only the total ATP is measured, regardless of source, as a result the system can only detect the presence of microorganisms, but not identify them.

However, this method is used in numerous breweries as a hygiene control system, assessing the effectiveness of cleaning processes (Quain, 1999).

Another example of indirect methods are impedimetric techniques. Impedance functions by detecting the fluctuation in electrical charge which occurs when nutrient macromolecules are digested by microorganisms into smaller higher charged molecules. This method requires a concentration of 10^5-10^6 cfu/ml for the variation in electrical charge to be detected (Fung, 1994). In some cases impedance has been used for the assessment of viable pitching yeast and bottled beer for bacterial contamination (Bamforth, 2006). This is a non-organism specific method that merely detects the presence of contamination and does not identify it.

A third method of indirect analysis is to chemically analyse the growth medium (beer/wort). In its simplest form this may come in the shape of organoleptic assays or pH assessments. More complex chemical analysis can even indicate the cause of spoilage (Dainty, 1996). *Pectinatus* spp. are indicated by high levels of propionic acid and hydrogen sulphide in beer, just as *Megasphaera cerevisiae* is indicated by butyric, valeric and caproic acids in beer. Chemical analysis can be a very valuable tool for the identification of dead or injured organisms which are not able to be identified using culture methods (Bamforth, 2006).

1.5 Prevention and Control

Due to legal requirements in most countries, breweries have set up Hazard Analysis Critical Control Points (HACCP) management systems, striving to ensure spoilage prevention and food safety. HACCP principles apply throughout the entire process from production to sale. Prior to HACCP there

are several prerequisites which must be in place. These include measures such as a food safety policy, high risk assessments and health and safety assessments. The HACCP itself includes the formation of a HACCP team or teams depending on the size of brewery, which identify the sites of possible hazards and suitable means of hazard management. Furthermore, this team must identify steps in the procedure where control is necessary to ensure that any danger is removed or minimized to a tolerable level. These steps are referred to as Critical Control Points (CCP). In terms of microbiology the following areas could be considered CCPs and microbial sampling should occur at these points: raw materials, pitching yeast, water supply, gas supply, fermentation vessels and filler lines. Critical limits must be set for each CCP, for example at the pasteurisation stage the critical limits would be the time and temperature required to render the product microbiologically safe. Following this, monitoring procedures must be put in place to ensure that the critical limits for each CCP are adhered to. Continuing with the example of pasteurisation, this may take the form of permanent logs and proof of equipment calibration. Procedures must be put in place for corrective action should it occur that a CCP is outside its critical limits, such as the removal of inadequately pasteurised beer. As the majority of brewery contaminants are non-pathogenic, the major threat will be to product quality. Nevertheless, there are some microorganisms which can occur that do pose a health hazard such as *Salmonella*, *Escherichia coli*, *Cryptosporidium* and *Shimwellia pseudoproteus*. These organisms would be assessed under HACCP, even though they should not occur in final product. The HACCP system and plan

(flow diagram of action) should be regularly revised, logged and audited (Briggs *et al.*, 2004; Bamforth, 2006).

In large commercial breweries manual cleaning has become impractical, so all new brewery machinery has been designed so that it can be cleaned by fixed devices without disassembly. This form of cleaning is known as Cleaning In Place (CIP) and all modern breweries have all of their machinery linked into an automated CIP circuit, so that manual cleaning is unnecessary for most of the brewery. A CIP circuit will include five different vessels, as well as a pump to move the fluids around the circuit and a heating station to heat the fluid to the correct temperature. Each of the vessels contains one of the following: fresh water, stored water, disinfectant solution, acid solution or a caustic solution. The cleaning cycle consists of each of the liquids being pumped through the circuit in a specific order. The first rinse is with stored water (3-5 min.), which is then drained off (1-3 min.). The second rinse is with a 1-2% alkaline solution at 70˚C (30-50 min.). The third rinse uses 1-2% nitric acid (10-15 min.). The fourth rinse is with a disinfectant solution (1-3 min.). In between the chemical rinses, the circuit is washed out with stored water and is drained. The final rinse is with fresh water (3-5 min.). The complete cleaning cycle takes one to two hours. The insides of vessels are sanitised by both high and low pressure jets of the cleaning agents. High pressure jets are generally used for mechanical cleaning and can be up to 60 bars in pressure. Low pressure sprays of up to 6 bars permit the cleaning fluid to run down the sides of the vessels, providing a greater contact time. Spray heads and jet cleaners have to be situated so that all of the vessel can be sanitised, even deep in the corners (Kunze, 2004). Cleaning out of place (COP) is a more

elaborate means of cleaning than CIP. The machinery to be cleaned has to be disassembled into its constituent parts and manually cleaned. Even machinery that is generally cleaned with CIP should be cleaned through COP regularly. This is because it removes residues missed by CIP cleaning, even though it is more time consuming and exposes staff to sanitising agents (Bamforth, 2006). Examples of machinery parts which have to be cleaned by COP include pipe connectors, connection fittings, valves and hoses. The hoses are decontaminated in long troughs called hose baths. The preferred disinfectant solutions include quaternary ammonium compounds, halogen-based agents such as sodium hypochlorite and oxidants such as hydrogen peroxide. The halogen-based solutions form hypochlorous acids which release hypochlorous ions (OCl-). These ions have an antimicrobial effect on bacteria by inhibiting enzyme functions through oxidation of sulphydryl groups (Estrela *et al*., 2002). Hydrogen peroxide is applied in 0.01 and 0.1% solutions and also is an oxidising agent. It acts by disrupting the cell membrane and inhibiting cell functions such as respiration and protein synthesis by oxidising oxygen scavengers and thiol groups. Hydrogen peroxide is very user friendly in that it leaves no residues that have to be removed, although care must be taken whilst handling it (Finnegan *et al*., 2010). Peracetic acid (PAA) is a common brewery sanitiser, which is formed by mixing acetic acid with hydrogen peroxide in an aqueous solution, possibly using a sulphuric acid catalyst. It has a high oxidation potential, facilitating a large range of antimicrobial activity, including bacteriophage and spores (Oliver, 2011). Some of the advantages of PAA are that when decomposed no toxic residues are produced, it can be used in hard water, it can tolerate a wide temperature

spectrum (0 to 40ºC) and is active over a wide range of pH: 3.0 to 7.5 (Kunigk and Almeida, 2001). Additionally, PAA does not produce foam (important for CIP) and proteins do not affect efficiency in the absence of catalase. Concentrations as low as 40 mg/L have been found to have antimicrobial effects, however concentrations commonly vary between 50 and 750 mg/L. PAA is compatible with stainless steel, glass, silicon and some types of rubber, but not with alkalis, rust, iron, copper and nickel. A disadvantage of PAA is that it is less stable than hydrogen peroxide. It was found that at room temperature, high concentration solutions (40%) of peracetic acid lost between 1-2% of their active ingredient per month. Weak concentration solutions (1%) lost 50% of their sanitation power within 6 days. Shelf life can be prolonged by storing in original containers, under 30°C (Kunigk *et al*., 2001). Quaternary ammonium compounds are cationic compounds which are highly bactericidal. The cations bind to the negatively-charged bacterial cell membranes causing them to lose fluidity and sequestering stabilising metal ions. Unfortunately, these chemicals are also very hard to remove and often leave residues (Gilbert and Moore, 2005). The hot caustic cleaning step utilises an alkaline solution such as caustic soda (NaOH). This has a highly bactericidal effect. Unfortunately, NaOH binds to carbon dioxide forming the insoluble compound sodium hydrogen carbonate which, through precipitation, reduces the caustic agents' efficiency. To prevent this the carbon dioxide has to be expelled from the vessel. Each brewery has its own means of achieving this, either by blowing out the vessel with air, pumping the carbon dioxide out or replacing it with nitrogen (rare). The acid rinse is usually performed with nitric or phosphoric acid and is more easily performed as it does not react with

carbon dioxide as the caustic agents do. Whilst it is not sufficient as a sole cleaning agent, in combination with disinfectants and caustic agents it results in a thorough sanitation programme (Kunze, 2004).

1.6 Anti-Microbial Surfaces: Examples and Actions

Recently there has been an increase in interest in antimicrobial surfaces, mainly from the medical or dental sector in an attempt to reduce microbial loads. A commercial antimicrobial is Microban (triclosan). It has been shown to inhibit growth of Gram-positive and negative bacteria as well as fungi. The manufacturer states that this is due to an electrochemical penetration and disruption of the cell membrane, resulting in leakage of essential metabolites impairing critical cell functions (Lefebvre *et al.*, 2001). It has also been shown that triclosan binds to and inhibits FabI, the enoyl reductase enzyme from the type II fatty acid biosynthesis pathway (needed for cell membrane maintenance) in *Escherichia coli* (Sivaraman *et al.,* 2004). Microban has been used as an additive to create antimicrobial surfaces on many products e.g. cutting boards or socks. The antimicrobial surface is achieved by mixing Microban pellets with the basic materials of the final product, such as plastic for a cutting board. The Microban molecules move into the amorphous phase of the polymer, staying within the interstitial spaces. Even though it functions by disrupting microbial cell membranes, it cannot damage the cell membranes of humans or insects as they do not have the enoyl reductase enzyme that microban binds to. It has been shown to be absorbed by humans through the gastrointestinal tract but it does not accumulate and is excreted. However, Microban is not designed to create a sterile surface, but to reduce the microbial population (Medlin, 1997; Bagley and Lin, 2000; Dayan, 2007). This

might explain the results of a study concerning the effect of adding Microban to a soft denture liner on the growth of *C. albicans* which showed it did not significantly alter the cytotoxicity of the denture liner or decrease adhesion of *C. albicans* (Lefebvre *et al.*, 2001). Another example of an antimicrobial surface is a two-level antimicrobial surface investigated by Li *et al.* (2009). Glass slides were coated in a mixture of $ZnCl_2$ and encapsulated soluble ClO_2. This coating was challenged with *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*. The $ZnCl_2$ acts as a contact killing agent, inhibiting metabolism, whilst the ClO_2 diffuses out and acts as a release killing agent through oxidation of biomolecules. After a contact time of 10 min., it was found that the bacterial population (cfu/cm^2) had reduced by 5 logs. The coating was also found to be more resistant to fouling by biofilms as the ClO_2 diffuses out and disrupts its formation. Muranyi *et al.* (2009) investigated the antimicrobial effect of a titanium dioxide coating on the test strains *Aspergillus niger*, *Bacillus atrophaeus* (spores) and *Kocuria rhizophila* (spores). Glass slides were coated with TiO_2 using the sol-gel method, which was repeated fifteen times to produce a multilayered coating. As TiO_2 is a photocatalyst the slides had to be exposed to UV-light (<338 nm) to enable redox reactions to occur which produce antimicrobial products such as superoxide radicals and hydrogen peroxide. It was found that after 4 hours there was a 3.5 log reduction in the vegetative *Aspergillus niger* cells, followed by another 1 log reduction after 24 hours. The *Bacillus atrophaeus* and *Kocuria rhizophila* spores were not affected by the coating. It was further found that the antimicrobial effect could be increased by increasing the humidity. Majumdar *et al.* (2009) investigated polysiloxane polymer bound quaternary ammonium

salts (QAS) as antimicrobial surface coatings over aluminium plates. The effectiveness of the surface coating was tested against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. The bactericidal effect of QAS is based on a sequence of actions. The first is the adsorption of the QAS to the cell surface via electrostatic attraction between the positively charged QAS and the negatively charged cell surface. Post-adsorption, the QAS moves through the cell wall and disrupts the cytoplasmic membrane resulting in ion leakage and cell death. It was found that the coating had a greater antimicrobial effect on Gram-positive organisms, than Gram-negative. This was thought to be due to the Gram-negative cells having two cell membranes consisting of a phospholipid layer and a lipopolysaccharide layer either side of a peptidoglycan layer, in comparison with the Gram-positive single cell membrane and of peptidoglycan layer. It was also found that QAS with short alkyl chains had a greater antimicrobial effect than those with long chains.

1.7 Historical use of Silver as an Antimicrobial

Silver may be one of the oldest antimicrobial agents in use. Records show that silver was used for drinking water purification from 1000 B.C. onwards (Castellano *et al.*, 2007). As early as 1700, silver nitrate was used to medicate diseases such as venereal disease, fistulae and abscesses (Landsdown, 2002). In the 19th century silver nitrate was used on granulation tissue to enable epithelisation and induce crust formation over wounds. (Klasen, 2000; Castellano *et al*., 2007). Carl Crede developed silver nitrate eye drops for the treatment of opthalmia neonatorum in 1881. His son developed silver impregnated skin graft dressings (Klasen, 2000). During the 1960s 0.5% silver nitrate was used by Moyer and co-workers as a treatment

for burns which was claimed did not interfere with the healing process and had antimicrobial properties (Bellinger and Conway, 1970). Then in 1968 silver sulfadiazine cream was formulated from silver and sulphonamide, acting as a carrier for the silver. This cream acted as a broad spectrum antimicrobial for use on burns. It was found to have a great antimicrobial effect against organisms such as *E. coli, S. aureus, Klebsiella sp., Pseudomonas sp.*, as well as against fungi and viruses (Fox and Modak, 1974). When penicillin was introduced during the 1940s silver, as an antimicrobial, became less popular, but was still in use for burn treatments for example. However, with the current rise in antibiotic-resistant organisms the interest in silver as an alternative antimicrobial has returned (Chopra, 2007).

1.8 Uses of Silver compounds and Coatings

Metallic silver was used as an early antiseptic for the treatment of wounds. The strength of the antimicrobial effect generated is dependent on the concentration present and the rate of diffusion. Even though metallic silver is inert, when it is placed in contact with fluid from the wound it will become ionised (Ip *et al*., 2005). This ionised silver (Ag^+) is very reactive and when it binds to the cell surface it disrupts the cell wall and/or membrane. In addition it also prevents cell, DNA and RNA replication by binding to DNA and RNA (Furr *et al.,* 1994; Rai *et al.*, 2009).

Silver zeolite is produced by complexing alkaline earth metals with crystal aluminosilicate, that is then exchanged with silver ions through ion exchange. This complex is very common in Japan, where it is used to coat ceramics to impart antimicrobial properties to medical materials and food storage vessels (Matsumura *et al*., 2003). Silver sulfadazine, as previously mentioned, is a

formulation of silver and sulphonamide and acts as a broad-spectrum antimicrobial. It forms a reservoir from which silver ions are released into the wound. Poulter *et al.* (2009) described an antimicrobial coating for medical equipment made from a plasma deposited phosphine-stabilised silver maleimide complex. The research into novel silver coatings was carried out as traditional silver polymer coatings are expensive. The complex was formulated by reacting silver nitrate with sodium maleimide. The coating was applied by plasma deposition inside a plasma reactor on to gold-plated glass, glass microscope slides, plastic petri-dishes and polypropylene non-woven material. This method of coating is interesting due to its ability to coat tubes and fabrics. The antimicrobial films were challenged with *Pseudomonas aeruginosa*. The calculated MIC for this coating, 0.8×10^{-6} mol dm^{-3}, is much greater than that of silver sulfadiazine, 125×10^{-6} mol dm^{-3}. The antimicrobial effect seems to be based on contact killing as little leaching of silver was detected. Thati *et al.* (2007) assessed the antimicrobial abilities of a variety of silver (I) coumarin complexes against *Candida albicans*. The effect of the complexes was assessed by culturing the fungus in 96-well microplates and exposing it to the complexes. The MIC_{80}s calculated ranged between 4.6 and 332 µM. However, the highest antimicrobial effects came from 7-hydroxycoumarin-3-carboxylatosilver (I), 6-hydroxycoumarin-3-carboxylato-silver (I) and 4-oxy-3-nitrocoumarinbis (1, 10-phenanthroline) silver (I), which had MIC_{80}s of 69.3, 34.1 and 4.6 µM, respectively. It was found that the complexes reduce the amount of oxygen that the fungus utilises. This may be due to the complexes disrupting the electron transport chain by reducing the amount of cytochrome via inhibition of the cytochrome synthesis pathway.

Additionally, the presence of the complexes induced permeability of the plasma membrane, causing amino acids and other small molecules to leak from the cell. When in contact with the complexes it was found that non-specific cleavage of the fungal DNA occurred.

1.9 Silver Nano-Particles and Nano-Particle Coatings

More recently there has been an increased interest in the use of silver nano-particles as antimicrobials. Areas in which the use of these nano-particles has been considered include medical devices such as catheters or burn dressings, as well as impregnated fabrics (Liu *et al*., 2011). As with metallic silver, the exact action of antimicrobial activity is not completely understood. However, it has been suggested that silver nano-particles may attach to and pass through cell membranes more easily than other compounds. Once inside the cell the particles bind to sulphur-containing proteins in the membrane and phosphorus in the DNA. The particles preferentially inhibit the respiratory chain and cell division resulting in cell death. The antimicrobial effect is increased by the particles releasing silver ions within the cell (Liu *et al*., 2011). Other methods of cell destruction which have been suggested include the formation of pits in the cell wall, possibly through metal depletion, resulting in leakage of lipopolysaccharides and membrane proteins (Kim *et al.*, 2007). The size and shape of the nano-particles are thought to play an important part in determining the antimicrobial effect. Nano-particles have a larger percentage of surface area for interaction with microorganisms than larger particles. It has been found that silver nano-particles 2.67 nm, 6.63 nm, and 21.11 nm exhibited greater antimicrobial activity against *E. coli* when compared to silver nanoparticles of 220 nm (Duran *et al.,* 2010). The shape of

the particles can also have a great effect on antimicrobial activity. A different study indicated that spherical particles have a greater effect than rod-shaped particles and that triangular particles were the most reactive of all (Rai *et al.*, 2009). In a study by Pal *et al.* (2007) truncated triangular particles were found to exhibit almost 100% antimicrobial activity with a silver content of 1 µg per 100 ml of NB growth medium, whilst spherical particles required at least 50 µg. Rod-shaped particles still did not achieve 100% lethality with 100 µg of silver.

The ability of different silver nano-particles to act as antimicrobials has been investigated in many different ways. Kim *et al.* (2007) produced silver nano-particles by reacting silver nitrate with sodium borohydride. The antimicrobial activity was determined by testing against *E. coli* O157:H7, *S. aureus* and yeasts (unspecified) isolated from a case of bovine mastitis, using a modified agar disk diffusion method. The concentrations of nano-particles tested ranged from 0.2 to 33 nm. For the yeasts the MIC was determined to be between 6.6 nm and 13.2 nm. *E. coli* was the most sensitive with an MIC in the range of 3.3 nm to 6.6 nm. The *S. aureus* strain was shown to be the most resistant organism with an MIC of above 33 nm. Lok *et al.* (2006) produced nano-particles in a very similar manner to Kim *et al.* (2007). However, the method of analysis was based heavily on proteomics instead of classical plating techniques. The *E. coli* strain K-12 MG1655 was subjected to antibacterial levels of silver nitrate in liquid culture for 30 min. Post lysis, the samples were subjected to 2-D electrophoresis, MALDI-TOF, immuno-blotting and membrane analysis. It was found that a 0.4 nM concentration of silver nano-particles was required for growth inhibition, whereas 6 µM of silver

nitrate was required for the same result. Electrophoresis and MALDI-TOF identified several proteins that were up-regulated in response to silver nano-particles. These included methionine-binding lipoprotein MetQ, inclusion body binding proteins IbpA and IbpB and outer membrane proteins OmpA, OmpC and OmpF. It was found that the particles destabilized the cell membrane by binding to phosphates, in addition to depleting the cells of ATP.

As the antimicrobial effect of silver nano-particles has been established, there has been a move towards the utilization of these particles to make antimicrobial surfaces by binding them to polymers. Saulou *et al.* (2010) tested a silver nano-particle coating against a strain of *Saccharomyces cerevisiae* (BY4741). Silver nano-particles were embedded in a silicone-matrix on stainless steel plates through plasma deposition. These plates were then placed in test tubes containing a yeast suspension ($2x10^7$ cfu/ml). The tubes were then left to incubate at 30°C for 24 hours. The number of viable cells in suspension was calculated by plating onto YPD agar and performing a viable count. It was found that exposure to the coating resulted in a 1.4 $\log_{10}$ decrease in viable cells. TEM analysis of the cells showed silver particles attached to the cell surfaces causing punctures, resulting in leakage and the cell membrane dissociating from the cell wall. Another example of a silver nano-particle coating is the design of a silver nano-fibre coating by Kong and Jang (2008). Silver-rhodamine complexes were produced through the reaction of silver nitrate with rhodamine monomers. These complexes were formed into polymer-fibres through oxidation polymerisation. These fibres were pelleted and coated over glass slides for testing against *E. coli*, *S. aureus* and *C. albicans*. The glass slides were tested by placing in microbial suspensions

(10^6-10^7 cfu/ml). After 60 min. there was no detectable microbial growth in the suspension. The nano-fibres were also compared to silver sulfadiazine through the Kirby-Bauer method. Pellets of fibre (13 mm) were placed on agar plate lawns of the organisms and incubated for 24 hours. The zones of inhibition were measured and compared to those of silver sulfadiazine for each organism. For *E. coli* and *S. aureus* the zones of growth inhibition for the fibres were twice the size of the silver sulfadiazine control and the nano-fibre zone of growth inhibition for *C. albicans* was 50% larger than the control.

1.10 Proposed Mechanisms of Antimicrobial Action of Silver

The exact mechanism of antimicrobial effect of silver is not fully understood, although many effects have been proposed such as inhibition of protein function and production, respiration, cell wall and DNA synthesis. Silver is also purported to inhibit biofilm formation in a variety of ways (described below), in particular via the interaction with thiol groups (Rosenblatt, 2009). In the case of bacteria, silver binds to the cell wall and membrane preventing respiration, as well as inducing membrane permeability, resulting in proton leakage (Dibrov *et al*., 2002). The presence of silver ions also induces DNA to turn into its condensed form, in which it is not available for DNA replication (Rai *et al.*, 2009). Silver ions also interact with thiol groups in cysteines which inhibits cell respiration and protein function (Nies, 2003; Chen and Schluesener, 2008). One of the most damaging interactions is with the enzymes in the respiratory chain such as NADH dehydrogenase and succinate dehydrogenase, in effect shutting down electron transfer (Xu and Imlay, 2012). Another manner in which silver ions are thought to damage microorganisms is through the generation of reactive oxygen species (ROS) and inhibition of glutathione

synthesis. Glutathione is an antioxidant scavenger involved in oxidation-reduction homeostasis that protects cells from oxidative stress by binding to and reducing ROS. Silver ions prevent the synthesis of glutathione by inhibiting its synthesising enzymes GCLC and GSS (Piao *et al*., 2011). ROS are thought to be generated through the reduction of dissolved oxygen by silver ions, especially during aerobic metabolism (Xu *et al*., 2012). Additionally, the destruction of [4Fe-4S] clusters of proteins by silver and the consequent release of Fenton-active Fe into the cytoplasm, may result in additional ROS formation (Xu and Imlay, 2012; Ninganagouda *et al*., 2014). These ROS include singlet oxygen, superoxide-radicals, hydroxyl radical and hydrogen peroxide. When formed inside a cell they cause oxidative stress, potentially leading to protein denaturation, DNA denaturation and lipid peroxidation (Carlson *et al*., 2008; Liu and Hurt, 2010). Of the ROS mentioned, the singlet oxygen is possibly the most damaging due to its reactions with amine acids, vitamins, unsaturated fatty acids, proteins and steroids. The hydroxyl radical is also highly reactive and may non-selectively oxidise many types of macromolecules which include carbohydrates, nucleic acids, lipids and amino acids. The superoxide-radical itself is not a powerful oxidative agent, however dismutation reactions of superoxide-radicals produce hydrogen peroxide which may be transformed into singlet oxygen and hydroxyl radicals (Zhang *et al*., 2013).

1.11 Silver Tolerance Mechanisms

The first recorded isolation and identification of silver tolerant bacteria was from a burn wound treated with silver nitrate (Jelenko, 1969). There have been several reported cases of silver tolerant organisms in both clinical and

natural environments (Mijnendonckx *et al*., 2013). The most characterised bacterial silver tolerance mechanism was found in the pMG101 plasmid of *Salmonella enterica* serovar Typhimurium. The *silCFBA*(ORF105aa)*PRSE* region responsible for silver tolerance codes for 9 genes (Gupta *et al*., 1999; Silver, 2003). *silP* is a P-type ATPase efflux pump which is known to transport silver ions from the cell cytoplasm to the periplasm. *silF* codes for a periplasmic protein which acts as chaperone, transports Ag+ from *silP* to the *silCBA* complex. The *silCBA* complex is a cation/proton antiporter efflux pump in the cell membrane. It consists of the efflux pump *silA*, the outer membrane protein *silC* and a membrane fusion protein *silB*. This complex removes silver ions from the cell (Silver, 2003; Franke, 2007). The genes *silS* and *silR* are involved in regulating the response to silver and *silE* molecules are known to bind silver ions (Silver *et al*., 1999).

Homologues of the *sil* genes have been found on the chromosomes of *E. coli* strains. This *cusCFBARS* gene cluster contains an efflux system similar to *silCBA*. *cusF* codes for a periplasmic protein that binds copper and silver ions (Munson *et al*., 2000). *cusA* effluxes silver and copper ions from the cytoplasm and periplasm via methionine clusters (Long *et al*., 2010). It was found that *cusB* and *cusF* are constitutively expressed in silver tolerant *E. coli* and that silver tolerance is lost if *cusF* is deleted (Lok *et al*., 2008).

In terms of yeast silver tolerance, silver tolerant strains have been isolated from industrial sites, for example a strain of *Candida argentea* isolated from a disused metal mine in Wales. It was found that this strain possessed a high degree of tolerance for silver and copper (Holland *et al*., 2011). It has also been noted that the overlap between copper and silver in the use of

transporter proteins such as *Ctr1* extends to silver tolerance mechanisms including binding and efflux proteins (Bertinato *et al*., 2008; Rubino *et al*., 2010). It was found that exposure to silver up-regulated the expression of *Cup1* metallothionines which bind copper via cysteines in *S. cerevisiae* (Niazi *et al*., 2011). The deletion of the copper (Cu)-transporting P-type ATPase *CTPA*, resulted in increased sensitivity to silver in *Penicillum janthinellum* (Lai *et al*., 2009). Additionally, the copper superoxide dismutase *SOD1*, one of the *S. cerevisiae* copper tolerance mechanisms, also binds to and confers silver resistance (Ciriolo *et al*., 1994).

Aims 1.12

The aim of this project was to establish the degree of sensitivity of microbial contaminants found in breweries to silver. In particular, over the course of this study the ranges of silver tolerance exhibited by microbial brewing contaminants, including both yeasts and bacteria, were assessed in varying brewery conditions. Furthermore, the possible mechanisms by which yeast cells may exhibit resistance was investigated in a variety of brewery conditions. This may lead to the determination of whether silver or silver nano-particles could be used to combat these contaminants.

Chapter 2 - Materials and Methods

2.1. Microbial Strains

All strains (Table 2.1) were supplied by SAB Miller and unless otherwise stated, were brewery isolates, from the SAB Miller South African brewery (South African Breweries, 65 Parklane, Sandown, Sandton, Johannesburg, Republic of South Africa). The exceptions were the two *E. coli* K-12 strains J53 and J53 pMG101), originally from Professor Simon Silver (University of Illinois, Chicago, USA), which were kindly provided by Dr. Jon Hobman, University of Nottingham and the *S. cerevisiae* strain BY4741, which was acquired from the European *Saccharomyces cerevisiae* Archive for Functional Analysis (Euroscarf, Institute for Molecular Biosciences, University of Frankfurt).

Table 2.1) Test Strains

Strain	Strain no.	Isolation	Reference
Pediococcus damnosus	Davies Diagnostics ATCC® 29358™	Lager beer yeast	Barros *et al.*, 2001
Lactobacillus brevis	SAB Miller	QA culture from Coors Ltd, Burton on Trent, UK	-
Obesumbacterium proteus	SAB Miller	Unavailable from Source	-
Bacillus coagulans	SAB Miller	Unavailable from Source	-
Enterobacter agglomerans	SAB Miller	Unavailable from Source	-
Gluconobacter oxydans	SAB Miller	Unavailable from Source	-
Acetobacter aceti	Davies Diagnostics ATCC® 15973™	Alcohol turned to vinegar	Cirigliano, 1982
Pichia membranaefaciens	SAB Miller (ATCC® 26288™)	SAB Miller Reference Strain (Isolated from Elm Exudate)	Hansen, 1888
Brettanomyces anomalus	Cara Technologies	Bottle conditioned beer	Edlin *et al.*, 1998
Candida krusei	Davies Diagnostics ATCC® 14243™	Clinical Isolate (bronchomycosis)	Zeng *et al.*, 1996
Hansenula saturnus	SAB Miller	Unavailable from Source	-
Kloeckera apiculata	Davies Diagnostics ATCC® 32857™	Bee trachea (Acarapis woodi)	Hata *et al.*, 2007
Rhodotorula mucilaginosa	Davies Diagnostics ATCC® 62691™	Grapes	Thuret *et al.*, 2005
Saccharomyces ellipsoides var. diastaticus	SAB Miller	Unavailable from Source	-
Escherichia coli K-12 J53	NCTC 501050	Mutant of stool sample isolate generated for Laboratory use	Gupta *et al.*, 1998.
Escherichia coli K-12 J53 pMG101	NCTC 50110	*E. coli* K-12 J53 transformed with plasmid pMG101 from *Salmonella typhimurium* isolated in hospital burns ward	Gupta *et al.*, 1998.
S. cerevisiae BY4741	Euroscarf Y00000	ATCC	Brachmann *et al.*, 1998.

2.2. Microbial Strain Maintenance and Growth

2.2.1. Maintenance and Growth on YPD

All strains were supplied on YPD agar (§2.3.1) slopes. Representative colonies were transferred to glass universal bottles containing 20 ml of YPD broth. These bottles were incubated in a stationary incubator at 30°C, for 3 days and used to prepare cryogenic stock cultures (§2.2.3) and to inoculate YPD slopes. YPD slopes were incubated as above, stored at 4^{o}C and used to inoculate appropriate media for use in subsequent experiments. For identification experiments the strains were grown on YPD agar plates, for silver sensitivity testing the strains were cultured in YPD broth and incubated as described.

2.2.2. Maintenance and Growth of *L. brevis* and *P. damnosus* on MRS and Raka Ray

L. brevis and *P. damnosus* strains were sub-cultured from the supplied YPD slopes into MRS broth (Sharpe *et al*., 1966), at 25°C for five days. Anaerobic conditions were achieved in a 7.0 litre AnaeroPack rectangular jar (Mitsubishi Gas Chemical Company Inc.), made anaerobic by the addition of two 3.5 litre AnaeroGen anaerobic packs (Oxoid, Basingstoke, Hampshire, UK). Anaerobic conditions were monitored using anaerobic indicator paper (BR0055B, Oxoid, Basingstoke, Hampshire, UK). MRS medium (§2.3.2) was supplied by Oxoid (Basingstoke, Hampshire, UK) and made up to product specification, by adding 52 g of the formulation to 1 litre of water. The medium was sterilised by autoclaving at 121°C and 15 psi for 15 min. After incubation, MRS broth cultures were used to prepare cryogenic stock cultures (§2.2.3) and to inoculate Raka-Ray agar (§2.3.3) (Saha *et al.,* 1974) slopes and

incubated anaerobically at 25°C for five days. Raka-Ray agar was supplied by Oxoid (Basingstoke, Hampshire, UK), the preparation was dissolved in water 77.1 g/L according to the manufacturer's specification. Tween 80 (Fisher) was added to a final concentration of 1% (v/v) to the medium. The medium was then autoclaved at 121°C and 15 psi for 15 min. After incubation, slopes were stored at 4^{o}C and used to inoculate appropriate media for use in subsequent experiments. For identification experiments the strains were grown on YPD agar plates, for silver sensitivity testing the strains were cultured in YPD broth and incubated as described previously.

2.2.3. Cryogenic Storage

Cryogenic stock cultures were made by mixing 500 µl of the 3 or 5 day liquid cultures with 500 µl of sterile 50% glycerol solution in 1.2 ml cryovials (Nunc, Nalgene Nunc International, Hereford, UK). Four vials were prepared for each strain; these stocks were slow frozen and stored at -80°C.

2.3. Experimental Growth Media Composition

All water used for media production was deionised RO water.

2.3.1 YPD Medium (Cell Maintenance and Silver tolerance experiments)

YPD contained 1% (w/v) yeast extract, 2% (w/v) neutralised bacteriological peptone and 2% (w/v) glucose dissolved in water. For the production of solid medium, 2% (w/v) Technical Agar No.3 (Oxoid) was added to the broth prior to autoclaving. The medium was sterilised after preparation by autoclaving at 121°C and 15 psi for 15 min. (This medium contains 0.032% (w/v) of NaCl from the peptone content. There may be additional salt from the yeast extract, however, the manufacturer does not list this information.)

2.3.2 MRS Medium (*L. brevis* and *P. damnosus* recovery)

The Oxoid MRS Broth medium contains: peptone (1%), Lab-Lemco powder (0.8%), yeast extract (0.4%), glucose (2%), sorbitan mono-oleate (0.1%), $KHPO_4$ (0.2%), $CH_3COONa.3H_2O$ (0.5%), $(NH_4)_3C_6H_5O_7$ (0.2%), $MgSO_4.7H_2O$ (0.02%) and $MnSO_4.4H_2O$ (0.005%). For the production of solid medium, 2% (w/v) Technical Agar No.3 was added to the broth prior to autoclaving. The medium was sterilised after preparation by autoclaving at 121°C and 15 psi for 15 min.

2.3.3 Racka-ray Agar (*L. brevis* and *P. damnosus* cell maintenance)

The Oxoid Racka-Ray Broth medium contains: yeast extract (0.5%), tryptone (2%), liver concentrate (0.1%), maltose (1%), fructose (0.5%), glucose (0.5%), betaine hcl (0.2%), $C_6H_5O_7(NH_4)_2H$ (0.2%), $HOOCCH(NH_2)CH_2COOH$ (0.25%), $HOOC\text{-}CH(NH_2)\text{-}(CH_2)_2\text{-}COOH$ (0.25%), $MgSO_4$ 7H2O (0.2%), $MnSO_4$ $_{-4}$H2O (0.066%), K_3PO_4 (0.2%), $C_8H_{15}NO_6$ (0.05%) and Agar (1.7%). The medium was sterilised after preparation by autoclaving at 121°C and 15 psi for 15 min.

2.3.4 Lysine Medium (Larger/Non-Larger yeast testing)

The Oxoid Lysine medium contains: Glucose (4.45%), KH_2PO_4 (0.178%), $MgSO_4$ (0.089%), $CaICl_2$ (0.0178%), NaCl (0.0089%), adenine (0.000178%), DL-methionine (0.0000891%), L-histidine (0.0000891%), DL-tryptophane (0.0000891%), H_3BO_3 (0.00000089%), $ZnSO_4$ (0.00000356%), $H_8MoN_2O_4$ (0.00000178%), $MnSO_4$ (0.00000356%), $FeSO_4$ (0.00002225%), lysine (0.1%), inositol (0.002%), $C_{18}H_{32}CaN_2O_{10}$ (0.0002%), aneurine (0.00004%), pyrodoxin (0.00004%), $H_2NC_6H_4CO_2H$ (0.00002%), $C_6H_5NO_2$ (0.00004%),

riboflavin (0.00002%), biotin (0.0000002%), $C_{19}H_{19}N_7O_6$ (0.0000001%) and agar (1.78%). The medium was sterilised after preparation by autoclaving at 121°C and 15 psi for 15 min.

2.3.5 MYPG medium (Brewing/Wild yeast strain testing, *Cup1* knockout confirmation, Effect of silver on copper tolerance)

The MYPG medium (Briggs *et al.*, 2004) was made up of 0.3% (w/v) malt extract, 0.3% (w/v) yeast extract, 1% (w/v) glucose and 0.5% (w/v) peptone. The medium was sterilised after preparation by autoclaving at 121°C and 15 psi for 15 min.

2.3.6 Wort Agar (Silver tolerance testing in wort)

Wort agar was made by dissolving 20 g Technical Agar No.3 (Oxoid) in 500 ml wort (Peroni, Specific Gravity of 1.0567) (SAB Miller, Miller Brands, Miller House, Surrey) mixed with 500 ml deionised water, made up to 1 litre. The medium was sterilised after preparation by autoclaving at 121°C and 15 psi for 15 min.

2.3.7 Beer Agar (Silver tolerance testing in beer)

Beer agar was made by dissolving 20 g of Technical Agar No.3 (Oxoid) in 50% 500 ml beer (Peroni, 5.1% ABV) (SAB Miller, Miller Brands, Miller House, Surrey) diluted using deionised water, made up to 1 litre. The medium was sterilised after preparation by autoclaving at 121°C and 15 psi for 15 min.

2.3.8 LB Agar (Silver tolerance testing of *E. coli* strains and effect of salt)

LB Agar was prepared containing 0.5% (w/v) yeast extract, 1% (w/v) tryptone and 2% (w/v) Technical Agar No.3. The medium was sterilised after

preparation by autoclaving at 121°C and 15 psi for 15 min. (This medium contains 0.004% (w/v) of NaCl from the tryptone content. There may be additional salt from the yeast extract, however, the manufacturer does not list this information.)

2.3.8 Commercial Wort Agar (Comparison of Oxoid and Actual Wort Medium)

The Oxoid Lysine medium contains: 1.5% (w/v) malt extract, 0.078% (w/v) peptone, 1.275% (w/v) maltose, 0.275% (w/v) dextrin, 0.235% (w/v) glycerol, 0.1% (w/v) K_2HPO_4, 0.1% (w/v) NH_4Cl and 1.5% (w/v) agar. The medium was sterilised after preparation by autoclaving at 121°C and 15 psi for 15 min.

2.3.9 Commercial Beer Agar (Comparison of Oxoid and Actual Beer Medium)

The Oxoid Lysine medium contains: 1.5% (w/v) peptonised milk, 0.61% (w/v) yeast extract, 1.61% (w/v) glucose, 1.22% (w/v) tomato supplement, 0.031% (w/v) K_2HPO_4, 0.031% (w/v) KH_2PO_4, 0.0006% (w/v) NaCl, 0.0006% (w/v) $FeSO_4$, 0.012% (w/v) $MnSO_4$, 1.2% (w/v) agar and 25% (v/v) beer (Peroni). The medium was sterilised after preparation (without beer) by autoclaving at 121°C and 15 psi for 15 min. Beer was filter sterilised (0.45 µm pore filter) and added post autoclaving.

2.4. Identification of Strains

2.4.1. Microscopy

To determine the cell morphology of the organisms bright field microscopy was used. The strains were observed at x 1000 magnification on an Olympus BH-2 microscope. Representative colonies were taken from YPD plates,

resuspended in sterile water, and spread across the surface of a microscope slide prior to observation. In addition, the organisms were observed under a Nikon Optiphot microscope to which a Nikon XM1200 camera was attached allowing photographs of stained cells to be taken. The photographs were visualised using an ATC-1 computer programme (Excel Technologies).

2.4.2. Gram Staining of Bacteria

Gram staining (Smibert and Krieg, 1994) was performed on cells taken from YPD plates. Cells were heat fixed by resuspending in sterile water, spreading the cells across a microscope slide, and once the suspension was air dried, the stain was heat fixed by passing through a Bunsen burner flame several times. Cells were stained with crystal violet for 30 sec. and Gram's iodine for 1 min., de-stained with ethanol for 30 sec. and counterstained with carbol fuchsin for 1 min. Between each staining step the slides were washed by immersion in RO water. All the staining reactions occurred at room temperature. The reagents were supplied by Pro-Lab Diagnostics (Neston, South Wirral, Cheshire) as 10x solutions and were diluted to 1x solutions with deionised water. The Gram staining of the samples was observed through an Olympus BH-2 microscope at x1000 total magnification.

2.4.3. Catalase Test on Bacteria

A catalase test (Chen *et al.*, 2007) was performed by mixing representative colonies from YPD plates for each bacterial sample with a drop of 3% (w/v) hydrogen peroxide on a glass slide and observing the presence or absence of bubbles.

2.4.4. Genotypic Identification of Bacterial Strains

The DNA of the bacterial strains was extracted by placing colonies from the stock slopes into 1.5 ml Eppendorf tubes containing 50 µl of TE buffer (10 mM Tric-HCl, 1 mM EDTA) and heating them at 95˚C for 10 min. using a heating block. This DNA was amplified using a PCR reaction as described by Obodai and Dodd (2006) using the primer pair V3F and V3R (V3F, 5′-CCTACGGGAGGCAGCAG and V3R, 5′- ATTACCGCGGCTGCTGG), which amplify the V3 region of the 16S ribosomal DNA. The PCR conditions were as follows: 94˚C for 5 min., followed by 10 cycles of 94˚C for 1 min. and 66˚C for 1 min. decreasing by 1˚C each cycle until 56˚C (Techne-512). This was followed by 20 cycles of 94˚C for 1 min., 56˚C for 1 min. and 72˚C for 3 min. The sequence finished with 72˚C for 5 min. The products were held at 4˚C for 5 min. The PCR products were mixed with 3 µl of loading dye (Promega Blue/Orange) per 10 µl of product and loaded onto a 1.5% (w/v) agarose (in TAE buffer: 40 mM Tris, 20 mM glacial acetic acid, and 1 mM EDTA) gel. This gel was electrophoresed at 70 volts for 45 min. The bands were visualised on a Biorad gel transilluminator. The bands were excised using a sterile scalpel and purified using Promega Wizard SV Gel and PCR Clean-Up kit (Promega, Southampton) as per instructions (§2.4.4.1). The purified products were sent to MWG Eurofins for sequencing. The sequences were then submitted to the NCBI Blast data base for identification. Blast is the Basic Local Alignment Tool, which allows the comparison of DNA and protein sequences to those already recorded in public databases.

(http://www.ncbi.nlm.nih.gov/About/tools/restable_seq.html).

E-values of matches between the test sequence and a database sequence depend on the alignment, the length of the test sequence, and the total length of the database sequence. The E-value cut off used to determine significance was E-80. First the raw score (*S*) for the alignment must be calculated and then normalised, (*S'*). *S* is calculated through $E=Kmn\ e^{-\lambda S}$ where m and n describe the sequence lengths and K and λ characterise the high-scoring segment pairs (local alignments of equal length from both sequences being compared whose scores cannot be improved by trimming or extending). The normalised score (*S'*) is calculated by $S' = (\lambda S - \ln K) / \ln 2$ where K and λ are parameters characterising the distribution of (*S*) for the bit score. This normalised score allows the calculation of an E-value. $E = m\ n\ 2^{S'}$ where m is the length of the database sequence, n is the length of the query sequence and is the normalised score. (www.ncbi.nlm.nih.gov/BLAST/tutorial/ and www.clarkfrancis.com/blast/Blast_what_and_how.html).

2.4.4.1 PCR Product Purification

All reagents and columns were provided with the Wizard SV Gel and PCR Clean-Up kit (Promega, Southampton, UK). Sterilely excised PCR product bands were placed into 1.5 ml Eppendorf tubes. Per 10 mg of gel 10 µl of membrane binding solution (10 mM potassium acetate, 80% ethanol (v/v) and 16.7 µM EDTA) were added to each sample, which were then incubated at 65°C in a heating block until the slice was dissolved. The gel mixture was placed into SV minicolumn which had been inserted into collection tubes. The column was left to incubate at room temperature for 1 min. before centrifugation at 16,000 x g for 1 min. Flow through was discarded and the column reinserted into the column. 700 µl of wash solution (4.5 M guanidine

isothiocyanate and 0.5 M potassium acetate) was added to the column, which was centrifuged at 16,000 x g for 1 min. Flow through was discarded, 500 µl of wash solution was added and the column was centrifuged at 16,000 x g for 5 min. Flow through was discarded, the column reinserted into the collection tube and centrifuged empty for 1 min. at 16,000 x g. The collection tube was discarded and the column inserted into a clean 1.5 ml Eppendorf tube. 50 µl of nuclease free water was added to the column and left to incubate at room temperature for 1 min. before centrifuging at 16,000 x g for 1 min.

2.4.5. Growth of Yeasts on Lysine Plates

Representative yeast colonies taken from YPD plates were streaked onto Lysine (Beuchat, 1993) plates and incubated at 30°C for three days. Lysine agar (Fowell, 1965) was obtained from Oxoid (Basingstoke, Hampshire, UK). The medium (2.3.4) was prepared by dissolving 66 g of the powdered medium in 1 litre of sterile deionised water. To this, 1 ml of potassium lactate (50%) was added per 100 ml of water and brought to the boil on an electric heated stirrer. 10% Lactic acid solution (Fisher) was filter sterilised (0.45 µm pore filter) and added to the medium after boiling to make up a final concentration of 0.1% (w/v).

2.4.6. Growth of Yeasts on MYGP- Copper Plates

Representative yeast colonies taken from YPD Agar slopes were streaked onto MYGP-Copper (100 ppm or 200 ppm) plates (Briggs *et al.*, 2004). The copper solution was made up by dissolving 16 g of $CuSO_4 . 5H_2O$ in 100 ml of sterile water and sterilised by passing through a 0.45 µm pore filter. The copper solution was added to the MYGP (2.3.5) after autoclaving. To each

200 ml of MYPG were added 0.5 ml of 16% (w/v) filter sterilised copper sulphate solution to make 100 ppm MYPG-Copper and 1 ml to make 200 ppm. The plates were incubated at 30°C for three days.

2.4.7. Genotypic Identification of Yeast strains

The seven yeasts were cultured in 100 ml of YPD by incubating in an orbital incubator (120 rpm, Sartorius, Certromat BS-1) at 30˚C for 3 days. The mitochondrial DNA was extracted from these cultures (§2.4.7.1) using the method of Defontaine *et al.* (1991) modified as in Nugyen *et al.* (2000). The extracted mtDNA was used as the basis of PCR amplification of the ITS region of mitochondrial DNA as previously described by Arias *et al.* (2002) using the primer pair ITS 1 and ITS4. (ITS 1: 5' TCCGTAGGTGAACCTGCGG 3', and ITS 4: 5' TCCTCCGCTTATTGATATGC 3'). The PCR conditions were as follows: 98˚C for 30 sec., followed by 35 cycles of 98˚C for 10 sec., 55˚C for 30 sec. and 72˚C for 30 sec., the programme finished with 72˚C for 5 min. (Techne-512). The products were held at 4˚C for 5 min. The PCR products were mixed with 3 µl of loading dye (Promega Blue/Orange) per 10 µl of product and loaded onto a 1.5% (w/v) agarose TAE gel. This gel was electrophoresed at 70 volts for 1 hour. The bands were visualised on a UV gel imager, excised using a sterile scalpel and purified using Promega Wizard SV Gel and PCR Clean-Up kit (Promega, Southampton) as per manufacturers' instructions (§2.4.4.1). The purified products were sent to MWG Eurofins for sequencing. The sequences were then submitted to the NCBI data base for identification.

2.4.7.1 Mitochondrial DNA Extraction of Yeasts

Cells were pelleted by centrifuging 30 ml of yeast culture at 16,000 x g for 10 min. The supernatant was discarded and the pellets washed in 4 ml of ice cold 50 mM EDTA, prior to centrifugation at 5,000 x g for 5 min. After removing the supernatant, pellets were resuspended in RB solution (1.2 M Sorbitol, 50 mM EDTA) in addition to 2% mercaptoethanol (Sigma). This was left to incubate at 37°C for 10 min. before centrifuging at 5,000 x g for 5 min. The supernatant was removed and the cells resuspended in 5ml of solution A (0.5 M Sorbitol, 10 mM EDTA, 50 mM Tris-HCl) in addition to 20 µg/ml of zymolase to digest the yeast cell wall (Sigma). This was left to incubate at 37°C for 1 hour. Samples were sonicated at level 5 for 10 sec. before being centrifuged at 5,000 x g for 10 min. The supernatants were separated into 4 Eppendorf tubes and centrifuged at 16,000 x g for 10 min. The pellets of the four tubes were combined into one Eppendorf tube by resuspension in 0.4 ml solution A. This tube was then centrifuged at 16,000 x g for 10 min. The supernatant was removed and the pellet resuspended in 0.5 ml solution A, 2.5 µl of 1 M MgCl and 10 units of DNasa. This was left to incubate at room temperature for 10 min. 1 ml of solution A was added and the sample centrifuged at 16,000 x g for 10 min. The supernatant was removed and 0.5 µl of RNAse (10 mg/ml), 0,6 ml of lysis buffer (100 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl, 1% Sarkosyl) and 0.5 ml of phenol-chloroform (Sigma) were mixed with the pellet by pipetting. The samples were centrifuged at 16,000 x g for 5 min. The supernatant was transferred to a new Eppendorf tube and 0.6 ml of chloroform was added prior to centrifugation at 16,000 x g for 5 min. The aqueous phase was transferred to a new Eppendorf tube. To this 25 µl of 5 M

NaCl and 1 volume of isopropanol (Fisher) were added and incubated at room temperature for 30 min. before centrifugation at 16,000 x g for 30 min. The supernatant was discarded and 0.6 ml of 75% ethanol (Fisher) added to the pellet. The sample was centrifuged at 16,000 x g for 10 min. The supernatant was discarded and the pellet air dried. The pellet was resuspended in 50 µl nuclease free water (Fisher). Samples were stored at -20°C.

2.4.8. Confirmation of *S. ellipsoids var. diastaticus* as a diastatic yeast.

The organisms *S. ellipsoids var. diastaticus* and *S. cerevisiae* BY4741 were cultured in 10 ml of YPD broth at 30˚C for 3 days. Spot plate experiments as outlined in Section 2.5.2. were performed on standard YPD agar, YPD agar with glucose at 0.5% (w/v), YPD agar in which glucose had been replaced with 2% (w/v) Dextrin (Acros Organics) and YPD agar in which glucose was replaced with 0.5% (w/v) starch. Diastatic characteristics were confirmed by comparison of the organisms' ability to utilise starch and dextrin.

2.5. Preliminary Silver Sensitivity Testing

2.5.1. YPD Silver Nitrate Streak Plates

A 10 mM silver nitrate stock solution was prepared by adding 0.16987 g of silver nitrate (Sigma-Aldrich Company, Ltd, Dorset) to 100 ml of deionised water and filter sterilising (0.45 µm pore filter) the solution. This solution was stored at 4°C, in a glass tin-foil-wrapped bottle to exclude light. YPD agar plates were made up containing a range of silver nitrate concentrations: 0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mM $AgNO_3$. The silver nitrate concentrations were produced by adding the appropriate volume of 10 mM stock silver nitrate to the molten YPD, post autoclaving, prior to

pouring the plates. Representative colonies for each strain were taken from previously prepared YPD plates and streaked onto the silver nitrate plates using a sterile metal loop. Silver nitrate plates were wrapped in tin foil to exclude light. Aerobic strains were incubated at 30˚C for 3 days. Anaerobic strains *P. damnosus* and *L. brevis* were incubated at 25˚C in an anaerobic jar, for 3 days. After incubation the plates were photographed, using a Gel Doc XR gel imager (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) and the sensitivity of the organisms to silver nitrate evaluated.

2.5.2 Spot Plate Method

The organisms were cultured in 10 ml of YPD broth as previously, with the exception of the two *E. coli* strains which were incubated at 37˚C. The optical densities of the cultures were measured using a spectrophotometer at 600 nm (CE 2021 Spectrophotometer, CECIL, Polystyrene 10 x 4 x 45 mm Cuvettes, Sarstedt). The cultures were then diluted using sterile YPD broth until the $OD_{600\ nm}$ measured 1. Four 10-fold dilutions were made so that these dilutions represented an $OD_{600\ nm}$ of 0.1, 0.01, 0.001 and 0.0001. For each organism 5 µl of each of the 4 dilutions was spotted onto the agar plates in duplicate. The plates were photographed subsequently, using a Bio-Rad gel imager.

2.5.2.1 YPD, Wort and Beer Silver Nitrate Spot Plates

YPD agar plates containing a range of silver nitrate concentrations were made up as in Section 2.5.1 and poured into square 120 x 120 mm petri dishes (Fisher). Organisms were spotted onto the silver nitrate plates in duplicate using the spot plate method (§2.5.2). These plates were wrapped in tin foil to exclude light and incubated as described in Section 2.5.1 to prevent

decomposition of the light sensitive silver nitrate via reduction to less toxic silver species (Ag^0) which results in decreased antimicrobial activity (Kierans *et al.*, 1991; Egger *et al.*, 2009). The Minimum Inhibitory Concentration (MIC) was recorded as the lowest concentration at which no growth occurred.

Sodium nitrate plates were used as a control containing a range of concentrations: 0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.5, 0.6, 0.8 and 1 mM $NaNO_3$. A 100 mM sodium nitrate stock solution was prepared, by adding 0.849947 g of sodium nitrate (Sigma-Aldrich) to 100 ml of deionised water and sterilised by filtration (0.45 µm pore filter).

This experiment was repeated substituting the YPD medium for wort (§2.3.6) and beer agar (§2.3.7). All spot plate experiments were performed in sets of five replicates. Each spot was simply scored as growth or no growth. Results tables of the number of spots (dilutions) that grew on the various media and concentrations (silver nitrate/sodium nitrate) were created by stating the highest values of the repeats at which growth occured.

2.6. Examination of Wort and beer composition

2.6.1. ICP-MS of Wort and Beer

Multi-element analysis of wort and beer samples diluted (10 and 20%, Peroni) and digested in 1% nitric acid was performed via ICP-MS (Thermo-Fisher Scientific X-Series^{II}) employing a 'hexapole collision cell' (7% hydrogen in helium) to remove polyatomic interferences. Samples were introduced from an autosampler (Cetac ASX-520 with 4 x 60-place sample racks) through a concentric glass venturi nebuliser (Thermo-Fisher Scientific; 1 ml/min). Internal standards were introduced to the sample stream via a T-piece and

included Sc (100 µg/L), Rh (20 µg/L), Ge (20 µg/L) and Ir (10 µg/L) in 2% trace analysis grade (Fisher Scientific, UK) HNO_3. External multi-element calibration standards (Claritas-PPT grade CLMS-2 from Certiprep/Fisher, UK) included Ag, Al, As, Ba, Bi, Cd, Co, Cr, Cs, Cu, Fe, Mn, Mo, Ni, Pb, Rb, Se, Sr, U, V, and Zn, all in the range 0 – 100 µg/L (0, 20, 40, 100 µg/L). A bespoke external multi-element calibration solution (PlasmaCAL, SCP Science, France) was used to create Ca, Mg, Na and K standards in the range 0-30 mg/L. Sample processing was undertaken using Plasmalab software (version 2.5.4.; Thermo-Fisher Scientific) set to employ separate calibration blocks and internal cross-calibration where required.

2.7. Silver Nitrate Sensitivity in Liquid Media

2.7.1. Cell Metabolic Activity Measurements

For the Biolog Omnilog system assay media were prepared by mixing wort or beer with an equal volume of proprietary IFY-0 buffer and addition of the proprietary Biolog redox dye D at double the manufacturer's recommended concentration in final solution (Technopath, Limerick, Ireland). 95 µl of each wort or beer mixture was aliquotted to each well and silver or sodium nitrate was added to achieve final concentrations in the range 0 mM to 1 mM. The experiment was performed in triplicate for each concentration tested.

Organisms were cultured on YPD agar at 30°C for 3 days. Colonies were resuspended in sterile deionised water, until 62% transmittance was reached (Biolog Turbidimeter, Technopath). Cell suspensions (3 µl) were inoculated into wells containing 95 µl of assay medium. Each phenotype microarray assay was carried out in triplicate. Plates were incubated in the Biolog Omnilog at 25°C for 72 hours and metabolic activity was monitored at 15 min

intervals. Metabolic activity is detected as precipitation of the dye, which is observed via a camera and image analysis software. Metabolic activity measured in arbitrary Biolog units were plotted against time and the area under the curve (Fig. 2.1) after 72 hours incubation was determined and used to represent metabolic activity.

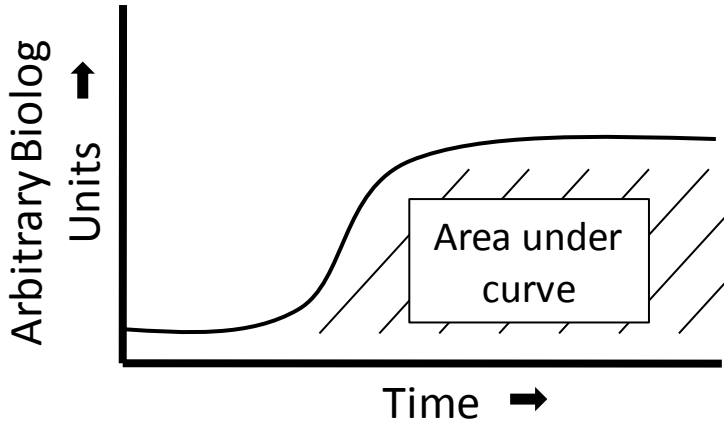


Figure 2.1) Representative metabolic activity determined by Biolog Omnilog system. Metabolic activity of test strains was recorded as arbitrary Biolog units over time. When plotted against each other this allowed the calculation of the "area under the curve" value, which all metabolic activity results were presented as.

2.7.2. Cell Growth Measurements

Growth of organisms was monitored spectrophotometrically at OD 600 nm in a 96 well plate format using a Tecan Infinity Pro 200 (Tecan Ltd, Reading, UK). Assay media containing either silver nitrate or sodium nitrate were prepared as described previously for the Biolog assays (except that the Biolog dye was omitted). Plates were incubated at 25°C for 72 hours. Readings were taken at 3 hour intervals unless otherwise stated. Optical density values obtained were used to plot growth curves for the yeasts at all silver and sodium nitrate concentrations tested. The final optical density value for each

organism was plotted against silver nitrate concentration to examine the effect of increasing silver nitrate concentration on organism growth.

2.7.3. Comparison of Cell Metabolic Activity Measurements under Aerobic and Anaerobic Conditions.

Metabolic activity experiments were performed as described in Section 2.7.1, however, the microplates were placed into sterile plastic sleeves (Technopath). These sleeves were vacuum heat sealed and the phenotype assay performed as previously. The array data of yeasts in anaerobic environments were compared to those in aerobic environments.

2.8. Gene Response to Silver Stress

2.8.1. Microarray Analysis of Gene Responses to Silver Stress

2.8.1.1 RNA Extraction

The strains *S. cerevisiae* BY4741 (Euroscarf) and *S. ellipsoids var. diastaticus* (SABMiller) were cultured in wort and beer, with and without silver nitate (0.1 mM) at 30°C for 20 hours. RNA from both strains was extracted using the Ambion RiboPure^{TM} Yeast RNA extraction kit (Life Technologies Ltd., Paisley, UK) as per manufacturer's instructions. All reagents were provided with the kit unless otherwise stated. 1 ml of pre-grown culture was centrifuged at 16,000 x g for 5 min for each sample and the supernatant discarded. To each pellet 480 µl of Lysis buffer, 10% SDS and Phenol:Chloroform:Isoamyl Alcohol were added. This mixture was vortexed for 10 sec. The mixtures were transferred to 1.5 ml screw capped tubes containing 750 µl ice cold Zirconia beads. The tubes were attached horizontally to a vortex adapter with the screw caps facing the centre. Samples were vortexed for 10 min. prior to centrifugation at 16,000 x g for 5 min. to separate the aqueous phase which was removed to a

15 ml tube. To the aqueous phase 1.9 ml of Binding Buffer and 1.25 ml of 100% Ethanol (Fisher) were added, vortexing after each addition. Filter tubes provided by the kit were inserted into collection tubes. 700 µl of sample mixture were placed into the cartridges and centrifuged at 11,000 x g for 1 min. The flow-through was discarded and the process repeated until all of the sample mixture had passed through the filter. 700 µl of wash Solution 1 was added to the cartridge and centrifuged at 11,000 x g for 1 min. 500 µl of Wash Solution 2/3 were added to the cartridge, which was centrifuged at 11,000 x g for 1 min. before discarding the flow-through. This step was repeated. Excess wash was removed by centrifugation at 11,000 x g for 1 min. After transferring the cartridge to a fresh collection tube 50 µl of Elution solution (95°C) was added to the cartridge and centrifuged at 11,000 x g for 1 min. The eluted RNA sample underwent DNase digestion by adding 5 µl of DNase Buffer and 4 µl of DNase. This mixture was left to incubate at 37°C for 30 min. Digestion was halted by addition of 6 µl of DNase inactivation reagent. After vortexing the samples were left at room temperature for 5 min. The samples were centrifuged at 16,000 x g for 3 min. before removing the supernatant RNA to RNase-free Eppendorf tubes.

2.8.1.2 Quantification of RNA

RNA quantity and purity quality control was carried out on the NanoVue (GE Healthcare UK Limited, U.K.). The NanoVue calculated RNA quantity as ng/µl. The purity was assessed via the A_{260}:A_{280} ratio. RNA samples with a ratio between 1.7-2.2 were diluted to 200 ng/µl in 15 µl total volume using DEPEC water (Fisher). These samples were used for Microarray analysis.

2.8.1.3 Microarray Analysis

The extracted RNA was submitted to NASC (Nottingham Arabidopsis Stock Centre) for MicroArray analysis on a GeneChip® Yeast Genome 2.0 Array. Sections 2.8.1.3.1 to 2.8.1.3.7 were performed by NASC. The RNA submitted to NASC was prepared for chip hybridisation using the GeneChip 3' IVT express kit (Affymetrix, U.K.). All required reagents were included in the kit unless otherwise stated.

2.8.1.3.1 Preparation of Total RNA/Poly A Control

Poly-A control stock was diluted with Poly-A control dilution buffer in a dilution series of 1:20, 1:50, 1:50 and 1:10, optimal for an RNA quantity of 100 ng. Mixtures were vortexed and centrifuged, to collect samples at the bottom of the tubes, between dilutions. 2 µl of this mixture were added to 100 ng of the RNA sample and brought to 5 µl using nuclease free water.

2.8.1.3.2 cDNA Synthesis

To assemble the first strand master mix, the reagents were thawed, placed on ice and mixed on ice in a nuclease free tube at a ratio of 4 µl First Strand Buffer mix and 1 µl First Strand Enzyme Mix, per reaction (GeneChip 3' IVT express kit, §2.8.1.3). The mix was briefly vortexed and collected at the bottom of the tube by centrifugation. A nuclease-free PCR plate was placed on ice and 5 µl of First Strand Master Mix were added per well. 5 µl of the RNA/Poly A Control mixture (§2.7.1.3.1) were added to each well. After placing a lid on the plate it was vortexed and centrifuged as previously and

placed on ice. Incubation took place at 42°C in a thermal cycler over 2 hours. The plate was centrifuged briefly and put on ice.

To assemble the second strand master mix, the reagents were mixed on ice in a nucleasefree tube in a ratio of 13 µl Nuclease free water, 5 µl Second Strand Buffer mix and 2 µl Second Strand Enzyme Mix, per reaction. The mix was briefly vortexed and collected at the bottom of the tube by centrifugation. To each 10 µl First Strand cDNA sample 20 µl of Second strand Master Mix was added. After placing a lid on the plate it was vortexed and centrifuged as previously and placed on ice. Incubation took place at 16°C for 1 hour, followed by 65°C for 10 min. in a thermal cycler. The plate was centrifuged briefly and put on ice.

2.8.1.3.3 In Vitro Synthesis and Purification of Labelled aRNA

To assemble the IVT master mix (GeneChip 3' IVT express kit, §2.8.1.3), the reagents were mixed at room temperature in a nuclease-free tube, in a ratio of 4 µl Biotin IVT label, 20 µl IVT labelling Buffer and 6 µl IVT Enzyme Mix, per reaction. The mix was vortexed and centrifuged briefly and placed on ice. To each 30 µl double stranded cDNA sample (§2.8.1.3.2), 30 µl of IVT Master Mix was added. The plate was vortexed and centrifuged as previously and placed on ice. Incubation took place at 40°C for 16 hours in a thermal cycler. Plates were placed on ice post incubation.

To assemble the aRNA binding mix, the reagents mixed at room temperature in a nuclease-free tube, in a ratio of 10 µl RNA Binding Beads and 50 µl aRNA Binding Buffer Concentrate, per reaction. To each cDNA sample, 60 µl of aRNA Binding Mix were added prior to transfer to U-bottomed well plates. To

each well 120 µl of 100% ethanol were added prior to shaking at 500 rpm for 3 min.

2.8.1.3.4 Removal of Magnetic Beads

The U-bottomed well plate was placed on a magnetic stand (96-well ring-stand, Applied Biosystems, U.K.) until the mixture became transparent. When this occurs the beads have formed pellicles against the magnets in the stand. The supernatant was aspirated, not disturbing the pellet, and discarded, after which the plate was removed from the magnetic plate. To each sample 100 µl of aRNA wash solution were added prior to shaking at 700-900 rpm for 1 min. The plate was again placed on the magnetic stand and the beads were captured as previously. The supernatant was removed without disturbing the pellet and discarded. This process was repeated with another 100 µl of aRNA wash solution. Once the supernatant was removed the plate was shaken for 1 min. at 1,000-1,200 rpm. Purified aRNA was eluted by adding 50 µl of aRNA elution solution (50° to 60°C) to the samples prior to shaking at 1,000-1,200 rpm for 3 min. After placing the plate on the magnetic stand the beads were captured by the magnets and the eluted aRNA was transferred to a nuclease-free tube and stored at -20°C.

2.8.1.3.5 aRNA Fragmentation

To assemble the aRNA fragmentation mix, the reagents were mixed at room temperature in a nuclease-free tube, in a ratio of 7.5 µg aRNA, 4 µl 5x Array Fragmentation Buffer and made up to 20 µl with nuclease-free water, per

reaction. Incubation of samples took place at 94°C for 35 min. in a thermal cycler. Samples were then placed on ice. Sample fragment sizes were determined using a Nanodrop ND-1000 and an Agilent bioanalyzer using an Agilent RNA 6000 Nano Kit.

2.8.1.3.6 Hybridisation

The samples were hybridised to the microarray chip using the GeneChip® Hybridization, Wash, and Stain Kit (Affymetrix). All reagents unless otherwise stated were provided in the kit. To assemble the Hybridisation mix, the reagents were mixed at room temperature in a nuclease-free tube, in a ratio of 5 µg fragmented and labeled aRNA, 1.7 µl Oligonucleotide B2 (3 nM), 5 µl 20 X hybridization controls (bioB, bioC, bioD and cre), 50 µl 2 x hybridization mix, 10 µl DMSO and made up to 100 µl with nuclease-free water, per single probe array. The GeneChip® Yeast Genome 2.0 Array Chip (Affymetrix) was equilibrated at room temperature prior to wetting the array with 80 µl of pre-hybridization mix. Incubation of the chip took place in a GeneChip®Hybridisation oven 640 for 10 min. at 45°C. The Hybridisation mix was incubated twice, once at 99°C for 5 min. and then at 45°C for 5 min. The hybridization mix was centrifuged at maximum speed in a microcentrifuge, for 5 min to remove insoluble materials. The pre-hybridisation mix was removed from the chip using a micropipette and discarded. The array was refilled with 80 µl of Hybridisation mix and left to hybridise in a GeneChip®Hybridisation oven 640 at 45°C for 16 hours.

2.8.1.3.7 Data Processing

Processing of the array chip occurred in a GeneChip® Fluidics station 450. The chips were analysed in an Affymetrix GeneChip scanner 3000 (Affymetrix).

2.8.1.3.8 Data Analysis

The MicroArray data, which were received as non-scaled RNA files (.cel) from the GeneChip command console (AGCC, Affymetrix Inc., USA), were interpreted using Partek Genomics Suite software. As all samples were taken in triplicate for each sample condition, the validity of fold changes in gene regulation between batches could be analysed via ANOVA. This occurred within the Partek Genomics Suite and only fold changes with a p-value of 0.05 or less were viewed as significant. To calculate P-values ANOVA first calculates an F- value. F is calculated by F= MS (within batch) / MS (between batches). MS (Mean Square) for each group (within or between) is calculated by MS=SS/DF, where SS is the sum of squared deviations from the mean (within or between) and DF is the corresponding degrees of freedom. SS (within) is calculated from SS= (value of sample - mean of group)^2. SS (between) is calculated as SS= (value of sample – total mean)^2. P values are calculated by the software as areas under the F statistic value (observed value) on the F value distribution graphs, with their corresponding degrees of freedom (Seltmann, 2009). To reduce the False Discovery Rate (proportion of false positives) the P-values determined via ANOVA are ranked in ascending order. In the calculation n represents the number of P-values which pass the constraint and m is the rank of P-value. The cut off P-value for significance in

the experiments is given as 0.05. Results which did not meet this requirement in the equation of 0.05*(n/m) were regarded as insignificant (Benjamini and Hochberg, 1995). Significant results were ranked in order of number of fold changes in both up and down-regulated genes. Only results exhibiting a 2 fold or greater change in gene expression were classified as significant. Comparisons of gene regulation fold changes were made between *S. ellipsoides var. diastaticus* in wort and wort with silver, *S. ellipsoides var. diastaticus* in beer and beer with silver, *S. ellipsoides var. diastaticus* in wort and beer and the differences between *S. ellipsoides var. diastaticus* and *S. cerevisiae* BY4741. Genes exhibiting a response to silver stress were classified into functional groups using the *Saccharomyces* Genome data base (SGD) Gene Ontology Mapper and the Kyoto Encyclopedia of Genes and Genomes (KEGG).

2.8.2. Effect of exposure to Silver Nitrate on Copper Tolerance

The organisms *S. ellipsoids var. diastaticus* and *S. cerevisiae* BY4741 were cultured in 10 ml of YPD broth and in 10 ml of YPD broth containing 150 µM silver nitrate, at 30˚C for 3 days. Spot plate experiments as outlined in Section 2.5.2 were performed on standard MYGP agar, MYGP-Copper 100 ppm and MYGP-Copper 200 ppm. These plates were incubated at 30°C for 3 days. Images were taken using a UVP Geldoc-IT Imaging System.

2.8.3. Gene Knockout Strains

The following gene knockout strains (Table 2.2) were obtained from Euroscarf, (see §2.1) as a result of microarray data indicating genes with potential silver tolerance functions. All Euroscarf knockouts were produced by inserting the kanmx4 deletion cassette into the target genes on

Saccharomyces cerevisiae BY4741 via PCR induced homologous recombination (Wach *et al.*, 1994). The exception to this was the deletion of CUP1 in *Saccharomyces cerevisiae* DTY3, to create the knockout strain DTY4. CUP 1 mutants were provided by Dr. Dennis J. Thiele (Duke University, Medical Center, Department of Pharmacology & Cancer Biology, Durham, North Carolina, USA) . Eight of the knockouts were sourced directly from Euroscarf, the remainder were provided by Dr. Trevor Phister (University of Nottingham), who originally sourced them from Euroscarf.

Table 2.2) Gene deletion mutants of *S. cerevisiae* BY4741.

Euroscarf ID No	Gene Code	Gene Name	Gene Function	Source
Y00000	Wildtype	-	-	Euroscarf
YBR296C	*HSP26*	Heat Shock Protein26	Heat shock protein with chaperone activity (DNA damage response).	Euroscarf
YCL035C	*PHO89*	Phosphate permease	Plasma membrane Na+/Pi cotransporter.	Euroscarf
YDR171W	*GRX1*	Glutaredoxin 1	Glutathione-dependent disulfide oxidoreductase (oxidative stress/DNA damage response)	Euroscarf
YDR270W	*HSP42*	Heat Shock Protein 42	Heat shock protein with chaperone activity (DNA damage response).	Euroscarf
YER103W	*CCC2*	Cross-Complements Ca(2+) phenotype of csg1	Cu(+2)-transporting P-type ATPase.	Euroscarf
YFL014W	*SSA4*	Stress-Seventy subfamily A	Heat shock protein that is highly induced upon stress; plays a role in SRP-dependent cotranslational protein-membrane targeting	Euroscarf
YBR072W	*HSP12*	Heat Shock Protein 12	Plasma membrane protein involved in membrane maintenance.	Euroscarf
YER055C	*HIS1*	Histidine 1	ATP phosphoribosyltransferase; catalyzes the first step in histidine biosynthesis; mutations cause histidine auxotrophy and sensitivity to Cu, Co, and Ni salts.	Trevor Phister's Lab (Euroscarf)
YLL009C	*COX17*	Cytochrome c oxidase	Copper metallochaperone	Trevor Phister's Lab (Euroscarf)
-	*CUP1*	-	Metallothionein; binds copper and mediates resistance to high concentrations of copper and cadmium	Dennis J. Thiele

2.8.4 Gene Knockout Confirmation of *CUP1* Gene via Copper Tolerance

The *CUP1* knockout strain DTY4 and the wildtype strain DTY3 (§2.8.3) were spot plated (§2.5.2) onto MYPG and MYPG-Copper 100 and 200 ppm plates (§2.3.6). Plates were incubated at 30°C for three days. Images were taken of the spot plates as previously described.

2.8.5. Gene Knockout Confirmation via PCR

The gene knockouts and the wildtype strain were cultured in YPD for 20 hours at 30°C. DNA was extracted using a Promega Wizard Genomic DNA Extraction kit (Promega, Southampton, UK) as per instructions (§2.8.8.1). Euroscarf quality control primers (Table 2.3) targeted at flanking regions of knockout sites, 200-400 bp from the start/stop codons of the target gene, were obtained from sigma.

(http://wwwsequence.stanford.edu/group/yeast_deletion_project/project_desc.html#delconfirm, Fig. 2.2). The PCR conditions were as follows for all reactions: 94˚C for 30 sec., followed by 35 cycles of 94˚C for 30 sec., 63˚C for 30 sec. and 72˚C for 2 min., the programme finished with 72˚C for 10 min. (Techne-512). The products were held at 4˚C for 5 min. The PCR products were mixed with 3 µl of loading dye per 10 µl of product and loaded onto a 1% (w/v) agarose gel. This gel was electrophoresed at 70 volts for 1.5 hours. Gel images were taken using a UVP Geldoc-IT Imaging System.

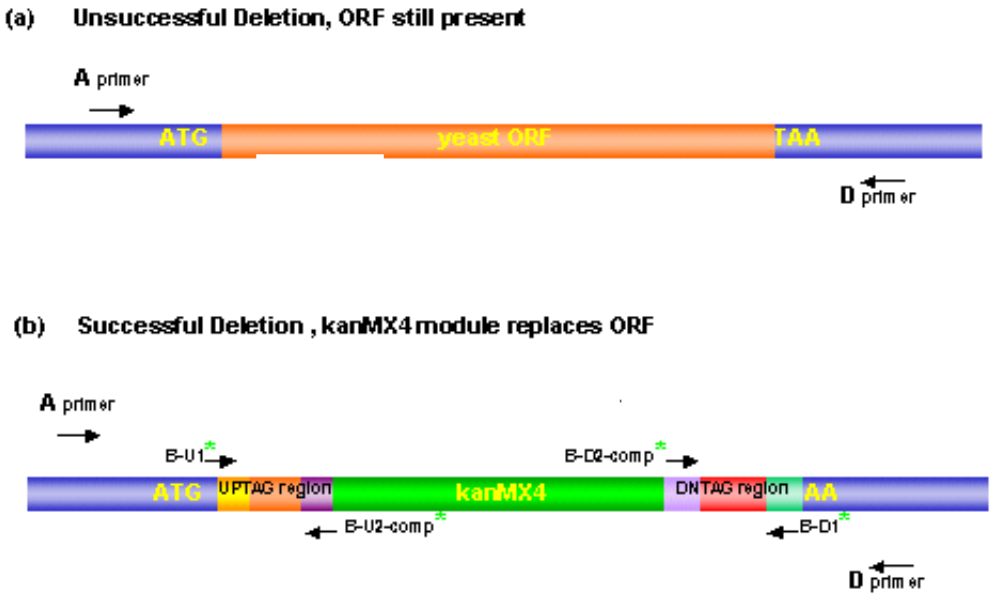


Figure 2.2) The insertion site of the kanMX4 deletion cassette and the Primer attachment sites for gene deletion confirmation primers. In this study sites A and D were used. (http://www-sequence.stanford.edu/group/yeast_deletion_project/project_desc.html)

2.8.4.1 DNA Extraction of Gene Knockout Strains

All reagents were supplied in the Promega Wizard DNA extraction kit (Promega, Southampton, UK) unless otherwise indicated. Cells were pelleted in 1.5 ml Eppendorf tubes by centrifuging 1 ml of culture at 16,000 x g for 2 min. Pellets were resuspended in 293 µl of 50 mM EDTA (Sigma). To this 7.5 µl of 20 mg/ml lyticase (Sigma) were added and left to incubate for 30 min. at 37°C. Samples were centrifuged at 16,000 x g for 2 min. Supernatant was discarded and the pellet resuspended in 300 µl of lysis solution (proprietary, contains 2-Amino-2-(hydroxymethyl)-1,3-propanediol), to which 100 µl of protein precipitation solution (proprietary) was added. Samples were left to incubate on ice for 5 min. prior to centrifugation at 16,000 x g for 3 min. Supernatants were transferred to clean Eppendorf tubes containing 300 µl of isopropanol (Fisher). Samples were mixed by inversion and centrifuged at

16,000 x g for 2 min. The supernatants were decanted and 300 µl of 70% ethanol (v/v) (Fisher) was added prior to centrifugation at 16,000 x g for 2 min. The ethanol was aspirated and the pellet allowed to air dry. 50 µl of DNA rehydration solution (10 mM Tris-HCl, 1mM EDTA) and 1.5 µl of Rnase were added to the pellet and incubated at 37°C for 15 min. DNA was rehydrated overnight at 4°C.

Table 2.3) Geneknockout Quality Control Primers

Euroscarf ID No	Gene Name	Forward Primer 5'-3'	Reverse Primer 3'-5'
YBR296C	HSP26	CTCGTAACAGTAAGGTATTCGCACT	ACATCCATAGAGATACCTCCAACAG
YCL035C	PHO89	GCACGTGGGAGACAAATAGTAATAA	AGATTTAGTCCAGGTACTGCTGCTA
YDR171W	GRX1	AGTGAGCTGTCTACAGATAACGAGC	TCTTAAAGTAATGGGCCAAGTAAAA
YDR270W	HSP42	TATATAGGTGTGTTGCCGGAGTAAT	ATCTTAGCATGTACCTCTTTTGGTG
YER103W	CCC2	GGTGCAGTTATTTGTAGTAGCGATT	AGCAGTAATTAACACCACCAGACTC
YFL014W	SSA4	GTTGTTACTTTTTCATTCGAGCAAT	TCCGAGCTAAACTCTTTGACTGTAT
YBR072W	HSP12	GTATACGCAAGCATTAATACAACCC	AGTGAAATAGAACAATACGCACACA
YER055C	HIS1	GGCATTTTACTCCTATTAACGGTTT	ACGGTAGTAAAGCTGACAAATATGC
YLL009C	COX17	CTAGAATTGGAACATCGTCTTCACT	TGAAAGAGTATTGTGAGCAGTATGG

Chapter 3 Organism Identification and Preliminary Silver Tolerance Testing

Introduction

The rationale of this research was to determine the sensitivity of bacterial and yeast brewery/beer contaminants to silver ions. Furthermore, although laboratory strains (*E. coli* J53 and J53 pMG101 containing the *sil* operon) were used as controls for benchmarking purposes, it was critical that silver sensitivity tests were conducted on microbial isolates from the brewery. To give a preliminary evaluation of the silver tolerance of the contaminant test strains, under brewery conditions, they were plated onto a standard medium for brewery organisms YPD, which contained a range of silver nitrate concentrations. Prior to commencing experimentation on the brewery contaminant organisms provided by SABMiller, their identity had to be confirmed, as they had been handled, stored and cultured by another laboratory. In the case of the organisms isolated by SABMiller both the source of isolation and original method of identification were unavailable from source. Both traditional differentiation and genotypic identification methods were employed to confirm each organism's identity. Genetic identity confirmation consisted of the sequencing of PCR amplified hypervariable regions of the test organism's 16S rDNA (bacteria) or ITS mtDNA (Internally Transcribed Spacer Region, Yeast) which was amplified using primers designed to flanking known conserved sequences. The sequences of the amplicons were then compared to the NCBI Blast database for identification.

3.1 Confirmation of Identity of Brewery Bacterial Contaminants

The isolates obtained had been previously identified, and named to species level. In order to confirm isolate identity, phenotype and genotype analysis was conducted as described below for bacterial (§ 3.1.1) and yeast (§ 3.1.2) strains.

3.1.1 Bacterial Strain Isolate Identification

Bacterial brewery contaminants were supplied on YPD slopes (§ 2.2.1). With the exception of *P. damnosus* and *A. aceti*, which are known beer spoilage organisms, all other strains had been isolated from breweries. These strains were cultured as described in the Materials and Methods and subjected to phenotypic (§ 3.1.1.1) and genotypic (§ 3.1.1.2) analysis.

3.1.1.1 Phenotypic Analysis

Phenotype analysis was conducted using Gram staining (§ 2.4.2), catalase tests (§ 2.4.3) and morphological analysis (§ 2.4.1). In regard to the morphological observation, Gram stain and catalase test, all the results were consistent with the known characteristics of the organisms (Table 3.1). The only deviation from the expected characteristics was the presumptive *B. coagulans* strain, which as a thermophile was expected to grow at 55°C, but would only grow at 30°-37°C. However, even though the results are consistent with the known characteristics of the bacterial species, these tests only establish that the test organisms have the expected characteristics, but do not definitively identify the strains. For a positive identification of the strains further identification methods were required.

Bacterial Strain	Morphology	Gram Stain	Catalase
L. brevis	Rod	+	-
P. damnosus	Cocci, tetrad formation	+	-
B. coagulans	Rod	+	+
O. proteus	Short Rod	-	+
P. agglomrans	Rod	-	+
G. oxydans	Rod	-	+
A. aceti	Rod	-	+

Table 3.1) Bacterial Phenotypes. The results from the traditional identification tests for the seven bacterial strains. The cell morphology was determined by microscopy, as was the Gram stain post Gram reaction. Whether the organisms were catalase positive (+) or negative (-) was determined by mixing colonies in 3% hydrogen peroxide.

3.1.1.2 Genotypic Identification of the Bacterial Strains

The genotypic identification of the bacteria (§ 2.4.4) is necessary as the standard brewery identification methods utilised do not definitively identify the organisms. The V3 primer set amplified a region of the 16S rDNA in each of the bacterial strains. Observation of the gel (Fig. 3.1), on a Bio-Rad gel imager, showed that the PCR produced bands of approximately 200 bp for all the bacterial strains, as expected. The positive control (previously confirmed *E. coli* K-12 DNA) also produced a band of 200 bp, indicating a successful PCR reaction and the negative control (reagents, no DNA) produced no bands indicating no contamination in the reagents. After purification via the Promega Wizard SV Gel and PCR Clean-Up kit (Promega, Southampton), the 16S rDNA bands were sent for sequencing. These sequences when submitted to the NCBI database confirmed the identities of *L. brevis* and *P. damnosus* with low E-value (probability of matches occurring at random) matches and exceeding the 95% identity (nucleotide sequence) match cut off needed for positive identification (Table 3.2). No direct matches were found for the other strains in the database. The presumptive *B. coagulans* strain was matched at the genus level to *Bacillus* spp., however no further identification was possible. The other strains could not even be identified at the genus level. For example the presumptive strain *S. pseudoproteous* found matches at 94% identity and an E-value of 8E-68 for, among others, uncultured bacterium clone 16S rRNA, *Citrobacter freundii*, *Enterobacter* sp. and *Cronobacter turicensis*.

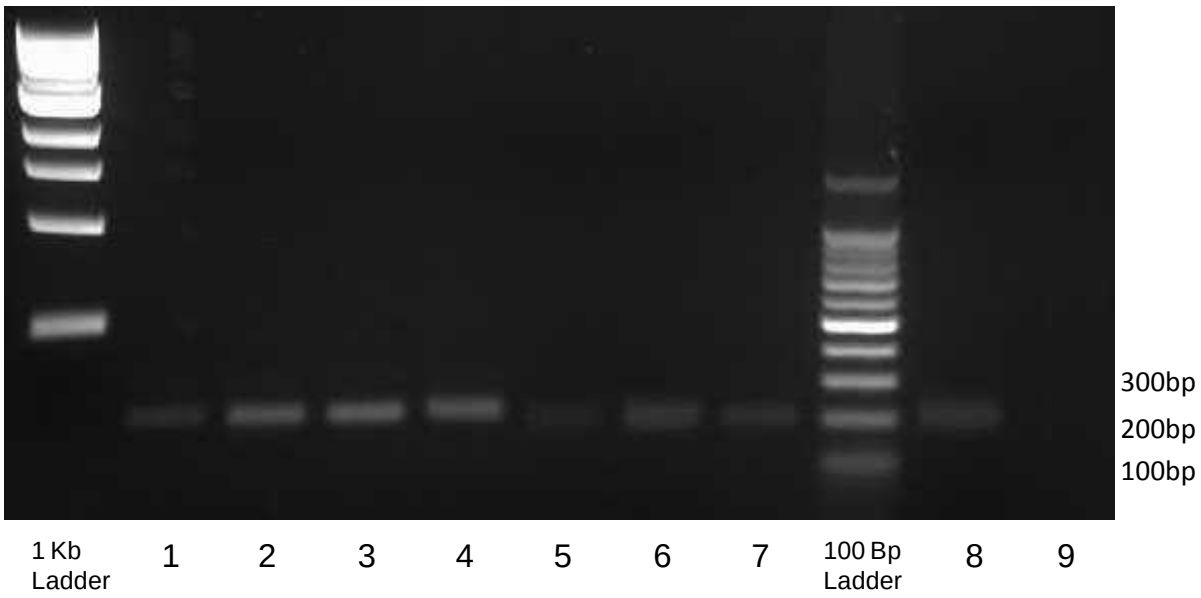


Figure 3.1) Identification of Bacterial Isolates via 16S PCR. Agarose gel (1.5%) containing 16SrDNA region PCR products gained using the V3F and V3R primer pair. The Buffer used was TAE. Bands produced are approximately 200 bp in size. The gel was electrophoresed at 70 V for 45 min. 1*: L. brevis*, 2: *P. damnosus*, 3: *B. coagulans*, 4: *O. proteus*, 5: *E. agglomerans*, 6: *G. oxydans*, 7: *A. aceti*, 8: *E. coli* K-12 (+ Control), 9: Reagents (- Control).

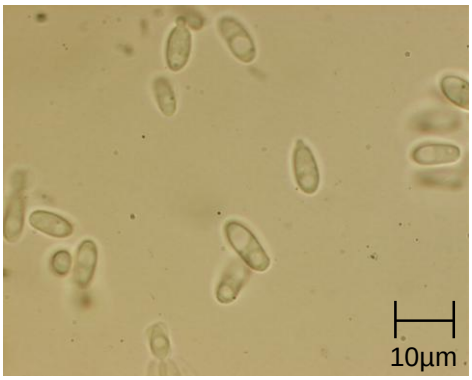
Presumptive Organism	Sequencing Result	E-Value	Max Identity
L. brevis	***L. brevis***	**2E-149**	**99%**
P. damnosus	***P. damnosus***	**8E-149**	**98%**
B. coagulans	***Bacillus* sp. 16S rRNA**	**1E-150**	**99%**

Table 3.2) Positively Identified Bacteria via Sequencing. The NCBI BLAST search results for the organisms *Lactobacillus brevis, Pediococcus damnosus* and *B. coagulans*. The table shows the organism's presumptive identity, the identity indicated by the database search, the percentage identity match and the E-value (probability of matches occurring at random).

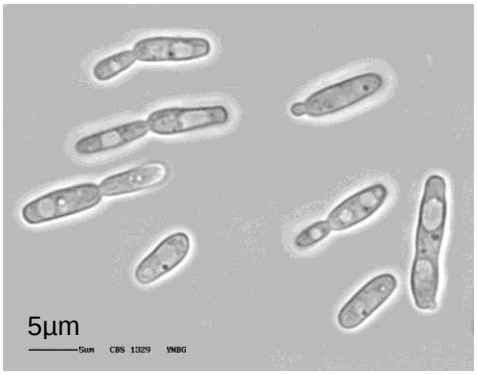
3.2 Confirmation of Identity of Brewery Yeast Contaminants

3.2.1 Physiological Differentiation

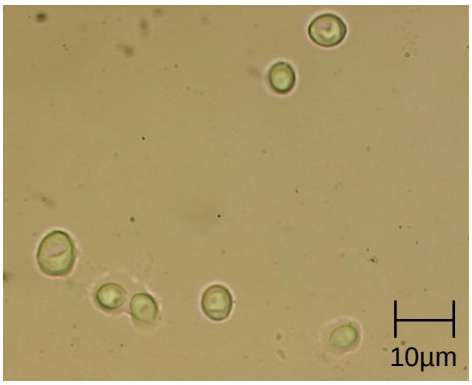
Microscopic observation (§ 2.4.1) confirmed that all of the putative yeasts possessed the correct shape and size for yeast cells (Fig. 3.2). The lysine plates for the yeast identification were used for the differentiation of wild yeasts, as brewing yeasts cannot utilise lysine as a sole nitrogen source and so cannot grow (Beuchat, 1993). All of the yeast strains grew on the lysine plates (§ 2.3.5), which is consistent with wild yeasts (Table 3.3). The greatest growth on lysine plates was exhibited by *B. anomalous* and *P. membranaefaciens* and the least growth by *K. apiculata*, which exhibited less than half the growth of *B. anomalous* and only marginally more than *S. ellipsoides* var. *diastaticus*. MYGP-Copper (§ 2.3.6) plates select for non-lager strains, as lager strains are sensitive to the copper supplement and, therefore, cannot grow on it. Non-lager *Saccharomyces* strains are able to grow on these plates as well as non-*Saccharomyces* strains (Kühle and Jespersen, 1998). All of the strains grew (Table 3.4) indicating that they are non-brewing yeasts. *C. krusei* exhibited the most growth. *B. anomalous*, *H. saturnus* and *K. apiculata* exhibited only half as much growth as C*. krusei* and only marginally more than *S. ellipsoides* var. *diastaticus*.



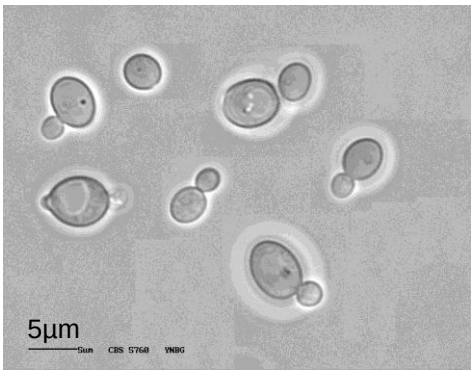
Pichia membranaefaciens



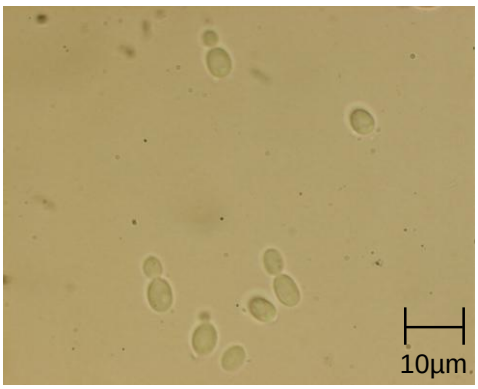
Pichia membranaefaciens (Literature)



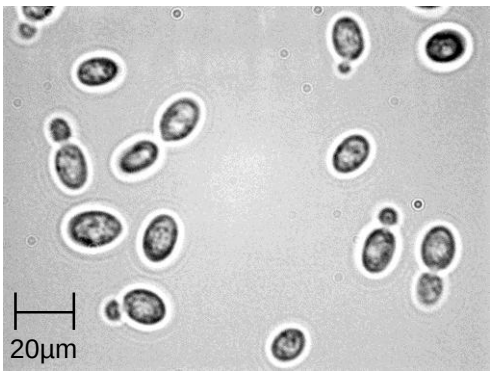
Hansenula saturnus



Hansenula saturnus (Literature)



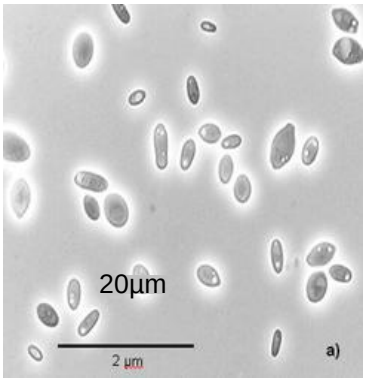
Rhodotorula mucilaginosa

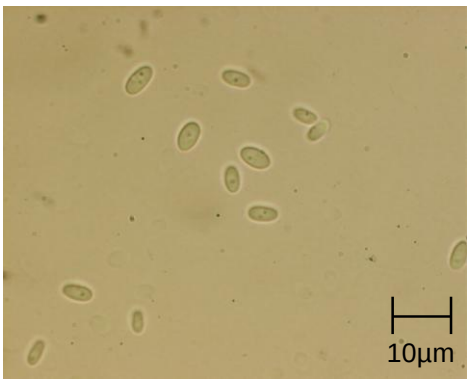


Rhodotorula mucilaginosa (Literature)

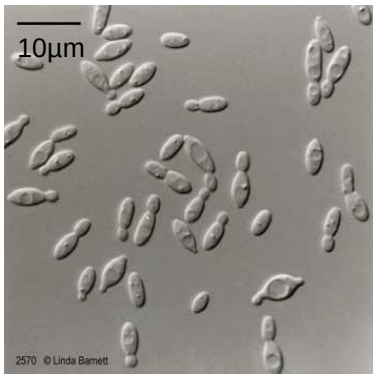
Figure 3.2a) Photographs taken of the yeast cultures under phase contrast using a Nikon Optiphot microscope at 400x magnification, with a Nikon DXM1200 camera attached. Comparative images were taken from literature. *P. membranaefaciens* and *H. saturnus* (cbs-knaw Fungal Biodiversity Center database), *R. mucilaginosa* (http://enologyaccess.org/).

Brettanomyces anomalous

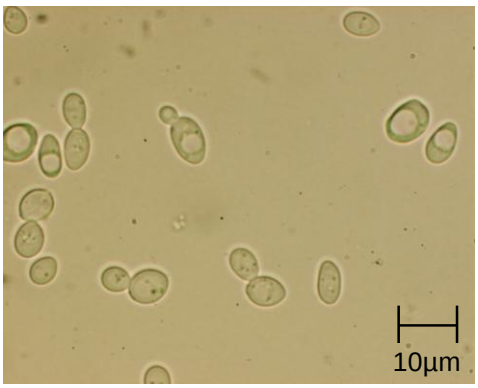




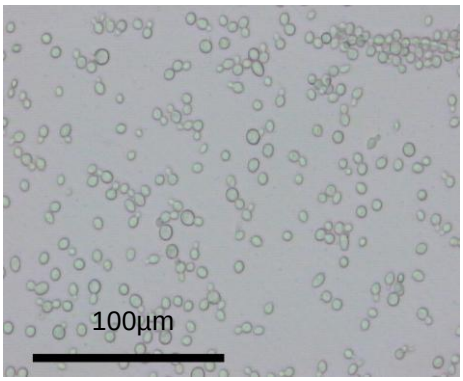
Kloeckera apiculata



Kloeckera apiculata (Literature)



Saccharomyces ellipsoides var. diastaticus



Saccharomyces ellipsoideus var. diastaticus (Literature)

Figure 3.2b) Photographs taken of the yeast cultures under phase contrast using a Nikon Optiphot microscope at 400x magnification, with a Nikon DXM1200 camera attached. Comparative images were taken from literature. *B. anomalous* (http://enologyaccess.org/), *K. apiculata* (Enology and Enotecia – Course 2013) and *Saccharomyces ellipsoideus* var. *diastaticus* (MINIATLAS MIKROORGANISMŮ).

Yeast Strain	Day 1	Day 2	Day 3
P. membranaefaciens	3	4	4
B. anomalous	3	4	4
C. krusei	2	3	3
H. saturnus	3	3	3
K. apiculata	1	1.5	2
R. mucilaginosa	4	4	4
S. ellipsoides var. diastaticus	1	1.5	1.5

Table 3.3) Differentiation of wild yeasts via lysine utilisation. Contaminant Yeasts were streaked across Lysine agar. 1= Negligible Growth, 2= Minimal Growth, 3= Moderate, 4= Good Growth. All growth was scored in relation to the growth of *P. membranaefaciens,* as it exhibited the most growth.

Yeast Strain	Day 1	Day 2	Day 2
	100 ppm	200 ppm	100 ppm
P. membranaefaciens	3	3	3
B. anomalous	3	2	3
C. krusei	4	3	4
H. saturnus	3	2	3
K. apiculata	2	2	2
R. mucilaginosa	3	3	3
S. ellipsoides var. diastaticus	1	1	1.5

Table 3.4) Differentiation of Non-Lager Saccharomyces and Non-*Saccharomyces* yeasts through copper tolerance. Contaminant Yeasts were streaked across MYPG Agar plates containing either 100 ppm or 200 ppm of copper. These plates were incubated at 30°C and observed over three days. 1= Negligible Growth, 2= Minimal Growth, 3= Moderate, 4= Good Growth All growth was scored in relation to the growth of *C. krusei,* as it exhibited the most growth.

3.2.2 Genotypic Identification of Yeast Strains

The genotypic identification of the yeasts (§ 2.4.7) is necessary as the standard brewery identification methods utilised do not definitively identify the organisms. The ITS region PCR, via the ITS1 and ITS4 primer pair, was successful and the PCR products were visualised on a Biorad gel imager (Fig. 3.3). The bands varied in size between 0.5-1 kb as expected, indicating the variable nature of the mtDNA region for which it was chosen as a means of differentiation. The bands were excised using a sterile scalpel and purified using Promega Wizard SV Gel and PCR Clean-Up kit (Promega, Southampton, UK). The purified products were sequenced by MWG Eurofins (Wolverhampton, UK). The resulting sequences when submitted to the NCBI database confirmed the identities of the yeasts with a high percentage of base - pair matches and E-values (Table 3.5) of zero (meaning the probability of the sequence matching at random is so low the database cannot calculate an E-value). The exception to this is *B. anomalous* which had a lower percentage identity (ID) match and a higher E-value. This is most likely due to the database match being *Dekkera anomala* the telomorph of *B. anomalous. S. ellipsoides* var. *diastaticus* was matched to *Saccharomyces cerevisiae* in the database. This is unsurprising as *S. ellipsoides* var. *diastaticus* is also known as *Saccharomyces cerevisiae* var. *diastaticus*. However, further confirmation that the strain is diastatic was required.

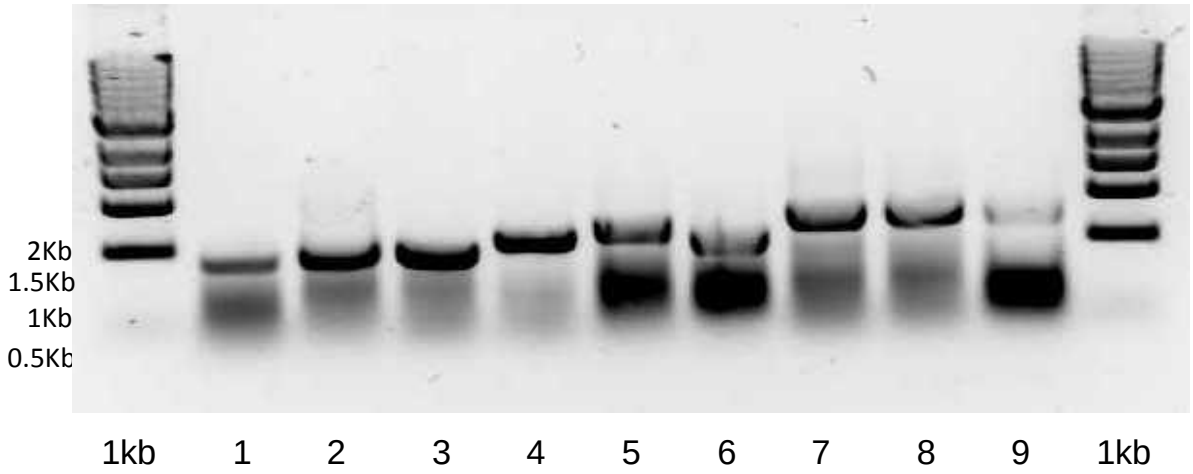


Figure 3.3) Identification of Wild Yeast Isolates via ITS PCR Agarose gel (1.5%) containing ITS region PCR products gained using the ITS1and ITS4 primer pair. The buffer system used was TAE. The gel was electrophoresed at 70 V for 1 hour. Bands produced varied in size between 0.5-1 kb. 1) *P. membranaefaciens*, 2) *B. anomalous*, 3) *C. krusei*, 4) *H. saturnus*, 5) *K. apiculata*, 6) *R. mucilaginosa*, 7) *S. elipsoides* var. *diastaticus*, 8) W34 (Control), 9) NCYC 2593N (Control).

Presumptive Organism	**Sequencing Result**	**E-Value**	**Max Identity**
P. membranaefaciens	*Pichia membranaefaciens*	0	95%
B. anomalous (teleomorph: *Dekkera anomala)*	*Dekkera anomala*	8E-66	78%
C. krusei (alias: *Issatchenkia orientalis)*	*Issatchenkia orientalis*	0	97%
H. saturnus	*Hansenula saturnus*	0	98%
*K. apiculata (*alias: *Hanseniaspora guilliermondii)*	*Hanseniaspora guilliermondii*	0	95%
R. mucilaginosa	*Rhodotorula mucilaginosa*	0	99%
S. ellipsoides var. diastaticus	*Saccharomyces cerevisiae*	0	97%

Table 3.5) Positively Identified Yeast via Sequencing. The NCBI BLAST search results for the yeast contaminant test strains. The table shows the organisms suspected identity, the identity indicated by the database search, the percentage identity match and the E-value (probability of matches occurring at random).

3.2.3 Confirmation of Presumptive *S. ellipsoides* var. *diastaticus* as a diastatic Strain

The presumptive *S. ellipsoides* var. *diastaticus* strain, also known as *S. cerevisiae* var. *diastaticus*, was genotypically identified as *S. cerevisiae* in Section 3.2.2. The presumptive strain was confirmed to be diastatic by spot plating (§ 2.4.8) the organism and the laboratory strain *S. cerevisiae* BY4741 (as control) onto YPD agar and YPD agar in which glucose had been replaced with dextrin or starch. Both organisms exhibited strong growth on YPD agar (Fig. 3.4). However, on the dextrin and starch media the test strain exhibited much stronger growth creating a lawn of confluent growth on each spot, whereas the control strain produced weaker spots made up of individual colonies.

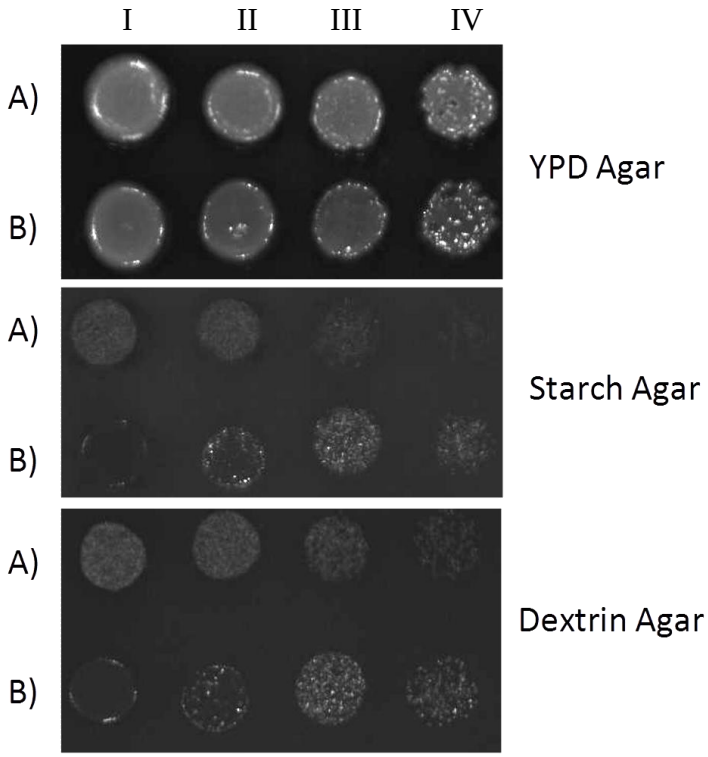


Figure 3.4) Confirmation of *S. cerevisiae* as a diastatic strain. The dilutions of the presumptive *S. ellipsoides* var. *diastaticus* (A) and the control strain *S. cerevisiae* BY4741 (B) were spot plated onto YPD agar and YPD agar in which glucose had been replaced with starch or dextrin (I: OD 0.1, II: OD 0.01, III: OD 0.001, IV: OD 0.001). Plates were incubated at 30°C for 3 days. Images were taken using a Bio-Rad gel imager.

3.3 Preliminary Tolerance of Brewing Microbes to Silver Nitrate

The brewery isolates and beer spoilage organisms were exposed to a range of silver nitrate concentrations to test their tolerance to silver ions. Silver nitrate was used as a silver stress for the organisms as it is a readily available form of soluble silver, which has been widely used in the published literature. The tolerance levels exhibited would indicate which of the organisms are sensitive to silver and which of the organisms should be investigated for possible silver resistance mechanisms. These experiments will also indicate the range of silver concentrations which should be used for future experiments. These experiments were performed only on the contaminant yeasts, as these could be positively identified and on the bacterial control strains *E. coli* J53 and *E. coli* J53 (pMG101) as their levels of silver sensitivity and resistance are known and so provide a reference point for silver tolerance testing.

3.3.1 YPD Silver Nitrate Streak Plates

The streak test on YPG plates (§ 2.5.1) was the first silver sensitivity test performed (Fig. 3.5, Table 3.6). This test was aimed at providing an estimate of where the MICs (Minimum Inhibitory Concentration) of the test strains may lie. YPD was chosen as a first medium as all the organisms are brewery contaminants and YPD is the standard medium for brewery organisms. All the organisms demonstrated good growth on YPD under optimal conditions. Silver nitrate was used to test the silver sensitivity of the strains as it was the most readily available, soluble, and practical source of silver for resistance testing. Of the yeasts, *P. membranaefaciens*, *C. krusei*, *H. saturnus* and *K. apiculata* showed no growth at 0.2 mM silver nitrate present in the YPD

medium. *P. membranaefaciens* showed good growth up to a concentration of 0.04 mM. *C. krusei* and *H. saturnus* only exhibited minimal growth up to 0.1 mM of silver nitrate. *K. apiculata* had moderate growth up to 0.08 mM and minimal growth at 0.1 mM of silver nitrate. *B. anomalus* exhibited good growth up to 0.04 mM, moderate growth up to 0.08 mM, minimal growth at 0.1 mM and negligible growth up to 0.4 mM. *S. ellipsoides* var*. diastaticus* exhibited good growth up to 0.06, moderate growth up to 0.2, minimal growth at 0.4 and negligible growth right up to 1 mM. For the purposes of grading the growth of yeasts on the streak plates, the growth of *P. membranaefaciens* was used as a standard for comparison.

The silver tolerances of the *E. coli* control strains are known. J53 (pMG101) has a known silver nitrate tolerance of 0.6 mM and J53 a known tolerance of 0.4 mM in low salt LB agar. However, the experimental silver tolerances exhibited were higher than expected. Neither of the *E. coli* strains J53 and J53 (pMG101) exhibited an MIC point. Up to 0.06 mM good growth was displayed, moderate growth at 0.08 and minimal growth up to 0.2 mM. J53 exhibited negligible growth (1-2 individual colonies) whilst J53 (pMG101) displayed minimal growth.

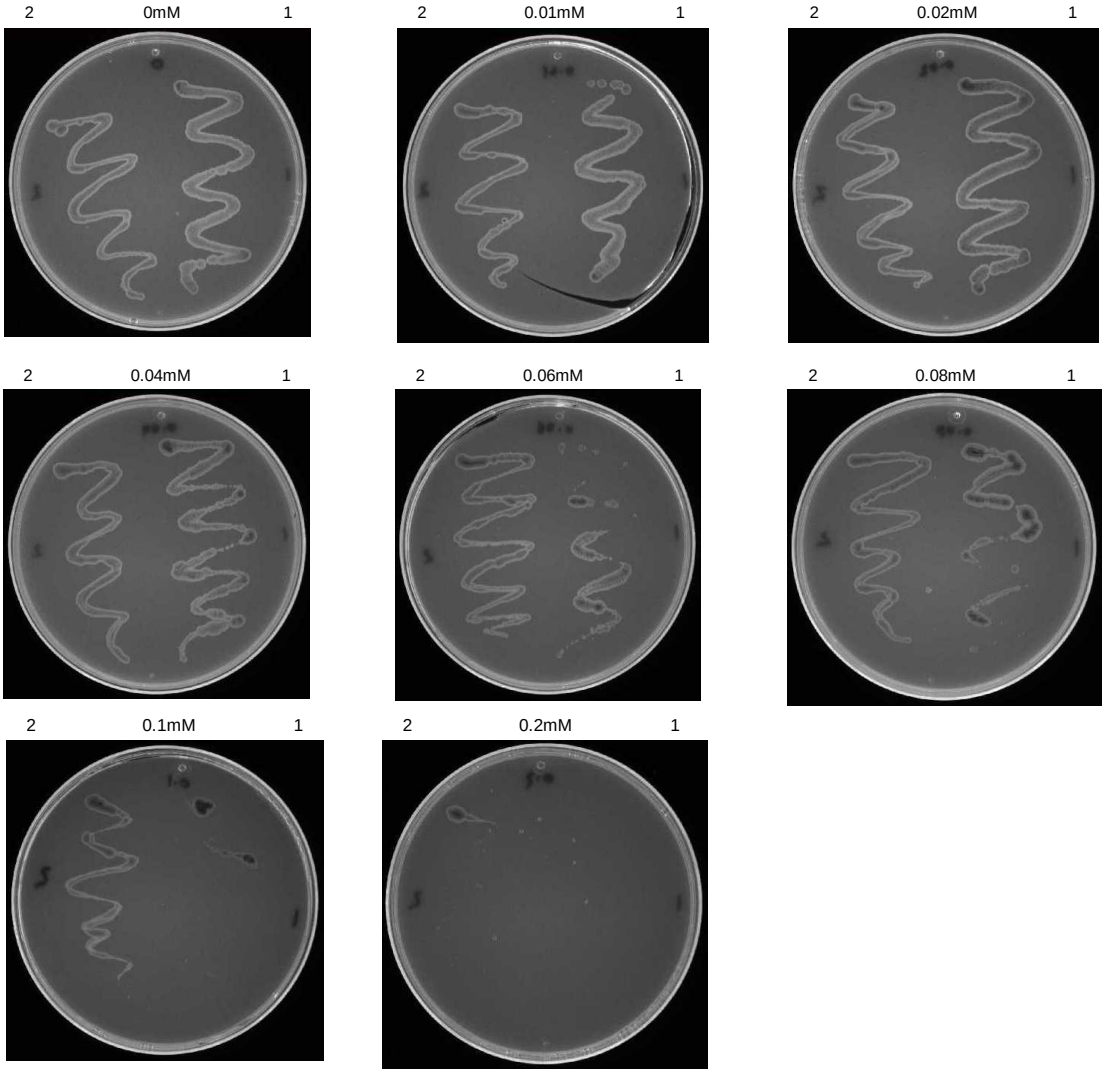


Figure 3.5) Estimation of silver nitrate MIC for contaminant isolates via streak plating. Example of Streak Plates on YPD containing a range of silver nitrate concentrations, 0-1 mM. Organisms were taken from stock slopes and streaked across the test plates. The plates were incubated at 30°C for 3 days. Images were taken using a gel transilluminator. Key to strains used in the streak tests and shown above 1: *P. membranaefaciens*, 2: *B. anomalus*. The growth of *P. membranefaciens* (1) was used as a standard to define growth in Table 6, summarising streak plate growth. Growth as on the 0-0.04 mM plates: Good Growth, 0.06 mM: Moderate Growth, 0.08 mM: Minimal Growth and 0.1 mM: Negligible growth.

Strain/ mM $AgNO_3$	0	0.01	0.02	0.04	0.06	0.08	0.1	0.2	0.4	0.6	0.8
P. membranaefaciens	4	4	4	4	3	2	1	-			
C. krusei	2	2	2	2	2	2	2	-			
H. saturnus	2	2	2	2	2	2	2	-			
K. apiculata	3	3	3	3	3	3	2	-			
B. anomalus	4	4	4	4	3	3	2	1	1	-	
S. ellipsoides var. diastaticus	4	4	4	4	4	3	3	3	2	1	1
***E. coli* J53**	4	4	4	4	4	3	2	2	1	1	1
***E. coli* J53 pMG101**	4	4	4	4	4	3	2	2	2	2	2

Table 3.6) Summary results of silver nitrate streak plate experiment. To determine the approximate levels of silver tolerance all the organisms were streaked across YPG agar plates containing a range of silver nitrate concentrations, 0-1 mM. The results for *R. mucilaginosa* were omitted as they did not produce growth even in the control.4: Good Growth, 3: Moderate Growth, 2: Minimal Growth, 1: Negligible Growth, - : No Growth. Growth categories were defined by the growth of *P. membranaefaciens* as shown in Fig.3.

3.3.2 *E. coli* Control Strain $AgNO_3$ Resistance and Growth Tests

The results of the silver nitrate sensitivity streak plate experiments performed on YPD agar (Table 3.6) all showed no MIC for either control strain. The expected levels of resistance derived from Gupta *et al.* (2001) are up to 0.1 mM for J53 and up to and above 0.6 mM for J53 (pMG101). Due to these variations the viability and silver tolerances of the control strains were examined using a modified method described by Gupta *et al.* (2001), which originaly determined the silver tolerance levels. Spot plates were performed, using these two strains, as previously described onto LB agar plates (containing a range of NaCl concentrations; 0, 5, 10, 20 and 30 g/L Fig. 3.6) containing a range of silver nitrate concentrations 0-1 mM. These plates were incubated overnight at 37°C. It was found that the highest concentration of silver nitrate that the *E. coli* strain J53 was able to tolerate was 0.2 mM at a salt concentration of 0 g/L (Table 3.7), however, at this silver concentration only the spot containing the highest cell density of the culture dilution series grew. The highest silver nitrate concentration J53 (pMG101) strain was able to tolerate was 1 mM at a salt concentration of 5 g/L (Table 3.7), producing growth on all five spots. For J53 any increase in salt from 0 g/L resulted in decreased growth. For J53 (pMG101) the increase in salt from 0 to 5 g/L resulted in increased growth on the silver nitrate plates from 0.4 mM to 1 mM of silver nitrate. Any increase in salt above 5 g/L resulted in a decrease in growth. These results confirm that the strains are still viable and indicate that there is a difference in silver tolerance between the strains. Additionally the results indicate that levels of salt present may affect the concentration needed to achieve antimicrobial levels in the medium.

NaCl g/L	Strain/ mM $AgNO_3$	0	0.01	0.02	0.04	0.05	0.06	0.08	0.1	0.15	0.2	0.4	0.5	0.55	0.6	0.7	0.8	1
0	J53	5	5	5	5	5	5	5	4	3	1							
	pMG101	5	5	5	5	5	5	5	5	5	5	1	-					
5	J53	5	5	5	5	5	5	5	5	1	-							
	pMG101	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
10	J53	5	5	5	5	5	5	5	2	-								
	pMG101	5	5	5	5	5	5	5	5	5	5	5	-					
20	J53	5	5	5	5	5	5	4	1	-								
	pMG101	5	5	5	5	5	5	5	5	4	-							
30	J53	5	5	5	5	5	4	4	1	-								
	pMG101	5	5	5	5	5	5	5	5	1	-							

Table 3.7) Summary results of *E. coli* J53 and *E. coli* J53 pM101 spot plate experiments to determine viability and maintenance of silver tolerace. *E. coli* control strain spot plates on LB agar (0, 5, 10, 20, 30 g/L NaCl) containing a range of silver nitrate concentrations. Both strains were cultured in LB broth prior to the dilutions and spotting. These spot plates demonstrate that the strains are still viable and their levels of resistance to silver nitrate.

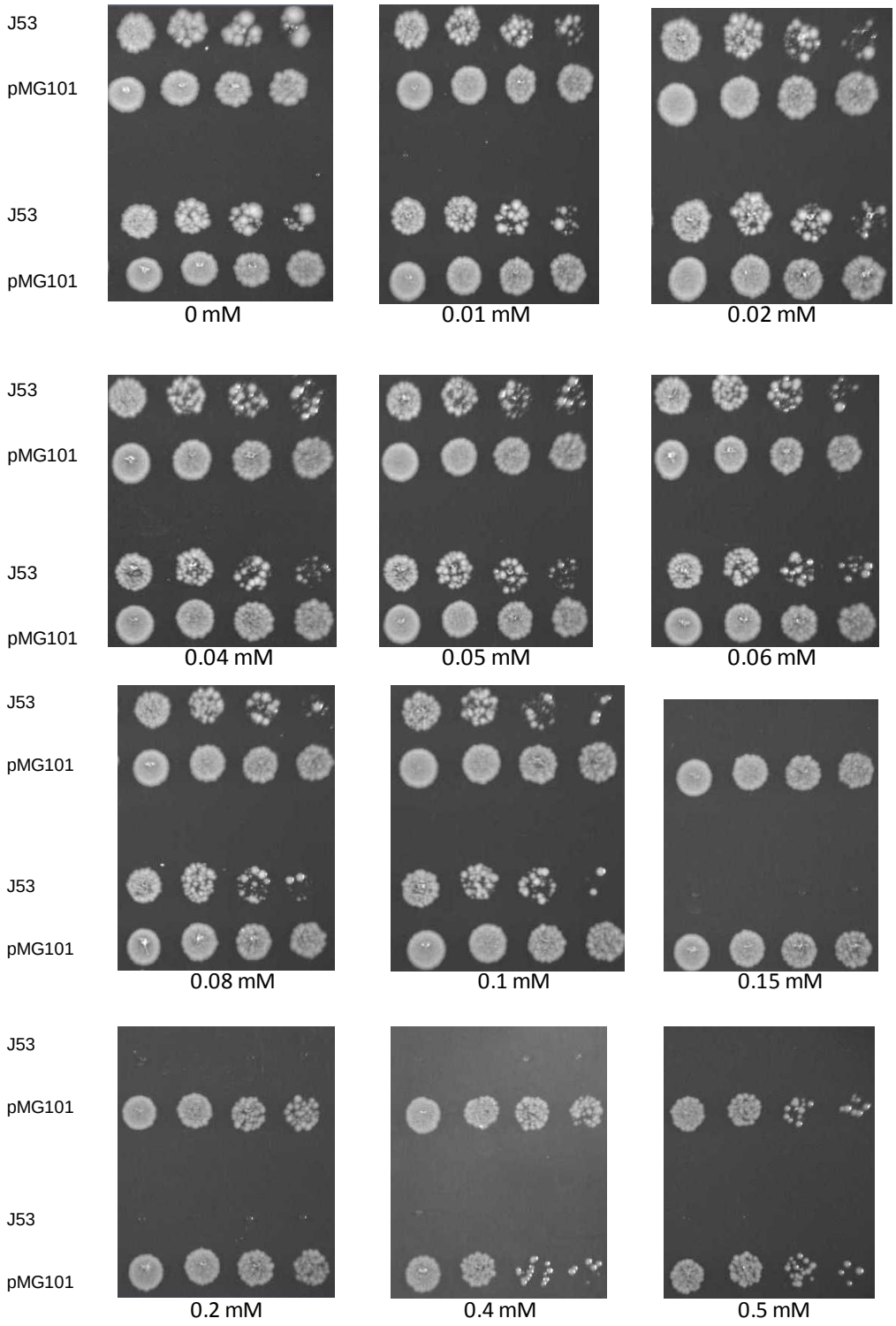


Figure 3.6a) *E. coli* J53 and *E. coli* J53 pM101 spot plate experiments to determine viability and maintenance of silver tolerace. Spot plates of the *E. coli* control strains J53 and J53 pMG101 on LB (5 g/L NaCl) agar containing a range of silver nitrate concentrations 0-1 mM. The strains were grown to an OD (600 nm) reading of 1 and then diluted in a 4 step 10 fold dilutions to produce dilutions of 0.1, 0.001, 0.0001 and 0.00001. 5 µl of each dilution were spotted in duplicate onto LB agar plates.

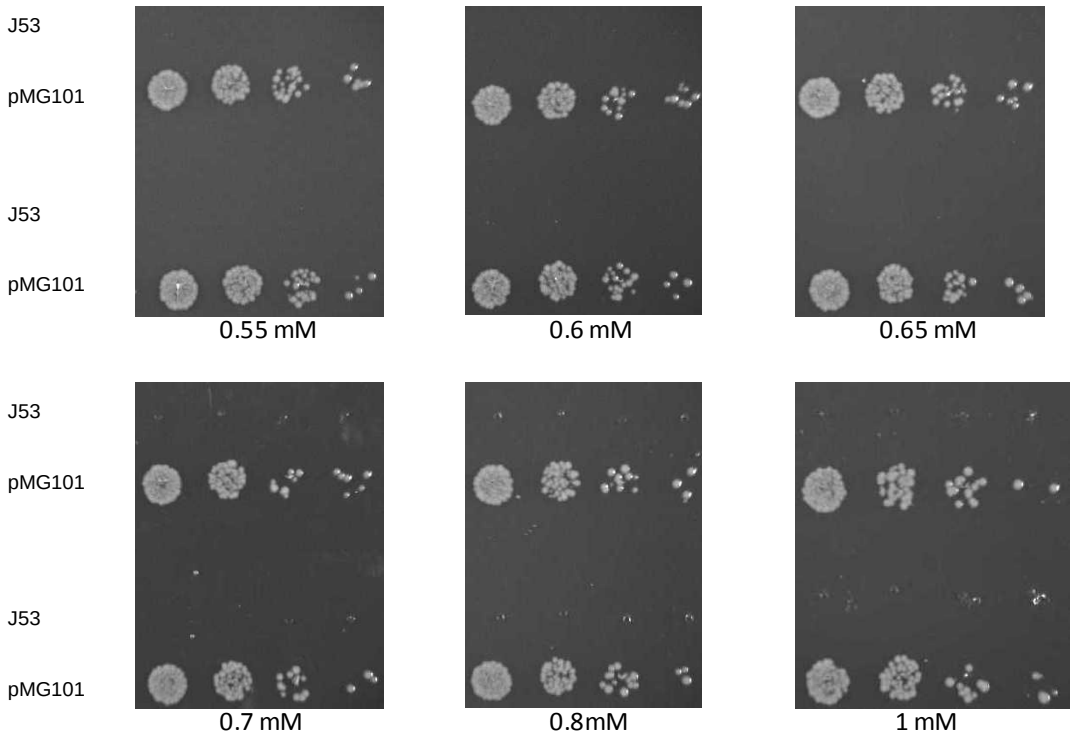


Figure 3.6b) *E. coli* J53 and *E. coli* J53 pM101 spot plate experiments to determine viability and maintenance of silver tolerace. Spot plates of the *E. coli* control strains J53 and J53 pMG101 on LB (5 g/L NaCl) agar containing a range of silver nitrate concentrations 0-1 mM. The strains were grown to an OD (600 nm) reading of 1 and then diluted in a 4 step 10 fold dilutions to produce dilutions of 0.1, 0.001, 0.0001 and 0.00001. 5 µl of each dilution were spotted in duplicate onto LB agar plates.

3.3.3 Maintenance of Silver Resistance plasmid in J53 pMG101

The results of the silver nitrate resistance streak plate test on YPD (Table 3.6) showed that both control strains exhibited growth up to a silver nitrate concentration of 0.4 mM. Therefore J53 (pMG101) was assessed, to determine whether the pMG101 plasmid responsible for the increased silver resistance, had been maintained. The plasmid also contains an ampicillin resistance gene. Both strains were streaked onto two LB agar (no salt) plates, one of which contained 100 µg/ml ampicillin (Fig.3.7). These plates were left to incubate overnight at 37°C. The J53 (pMG101) strain was able to grow whilst the J53 strain did not. This indicates that the pMG101 plasmid has been maintained in J53 (pMG101) and is not present in J53.

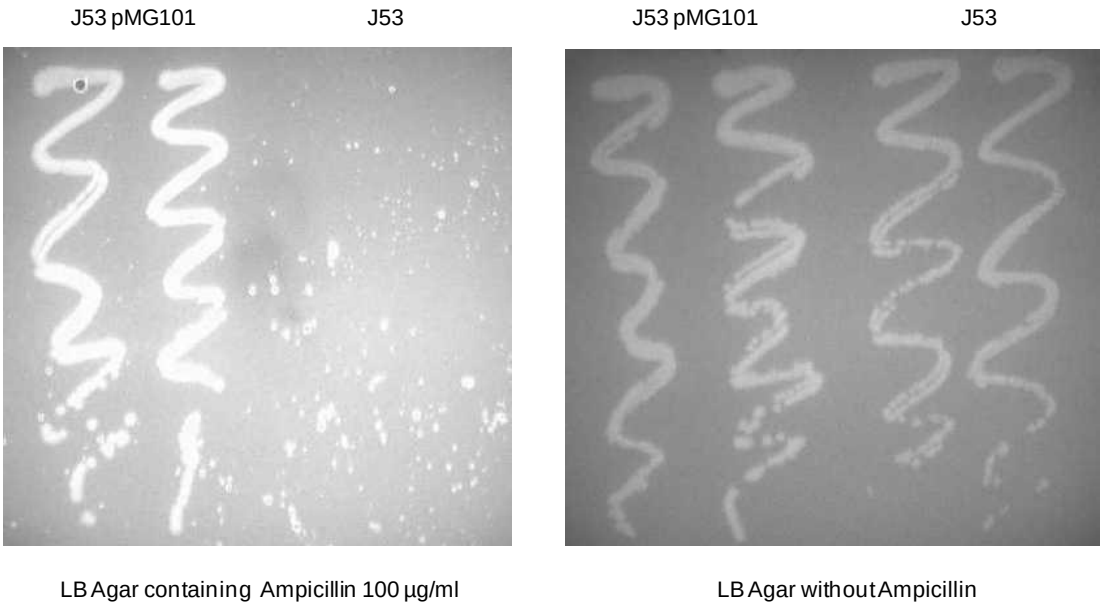


Figure 3.7) *E. coli* ampicillin streak plates to determine maintenance of pMG101 plasmid. *The E. coli* control strains J53 and J53 (pMG101), were grown overnight at 37°C. These cultures were then streaked onto two LB agar plates, one of which contained 100 µg/ml of ampicillin. The plates were left overnight to incubate at 37°C. Only the pMG101 strain was able to grow on the plate containing ampicillin.

Chapter 3 Discussion

3.4 Confirmation of Identity of Brewing Bacterial Contaminants

3.4.1 Physiological Differentiation

All of the results of the bacterial identification methods, from microscopy to Gram staining to the catalase test, were congruent with the known morphology and physiology of the organisms. One note of interest is the inability of the supposedly thermotolerant *B. coagulans* strain to grow at 55°C, seeming to have a temperature maximum of 30-37°C. This may or may not be due to an adaptation to brewery conditions or could represent a mis-identification of the species.

3.4.2 Genotypic Identification

All the bacterial 16S PCR products were approximately 200 bp in size which is the expected size of product from a 16S V3 primer pair amplicon (Obodai and Dodd, 2006). The purified bands were sent for sequencing. The sequences were used to perform a NCBI database search to confirm the identity of the organisms. Matches were only found for the presumptive organisms *L. brevis* and *P.damnosus.* The high % matches and E-values of zero would indicate that these organisms are *L. brevis* and *P.damnosus.* Although the presumptive *B. coagulans* strain could be identified to the genus, the fact that the organism is not a thermophile (§ 3.1.1.1) casts doubt on its identity as *B. coagulans*. As no matches could be found for the other bacterial organisms, their identity could not be positively confirmed. It may be that as the organisms tested are brewery conditioned contaminant organisms they are atypical examples of the genus and their 16S sequences are sufficiently different from

strains isolated in other environments that no matches could be found, or the strains may not be what they have previously been identified as.

3.5 Confirmation of Identity of Brewing Yeast Contaminants

3.5.1 Physiological Differentiation

Microscopy confirmed that all of the strains were consistent in cell size and shape with yeasts. All of the strains grew on the lysine and MYPG-Copper selective plates confirming that they were all wild yeasts. It must be noted that the strain *S. ellipsoides* var*. diastaticus* grew slowly, exhibiting minimal growth, approximately 50% of the growth of *K. apiculata* the second weakest in terms of growth. This may or may not be due to being a *Saccharomyces* (now *Saccharomyces cerevisiae diastaticus,* (§1.3.7.2.7*)* and so possibly phenotypically closer to the brewing yeasts than the other strains, as it cannot grow well on either medium. These results are consistent with the known characteristics of the organisms, however, they do not provide a definitive identification.

3.5.2 Genotypic Identification

The ITS PCR products produced in this study, were purified, sequenced and used for database searches to confirm the identity of the yeasts as noted previously**.** The high % matches and E-values of zero would indicate that these yeasts are the organisms they were suspected to be. The only organism with a comparatively lower % identity match was *Brettanomyces anomalous*. This organism was matched to *Dekkera anomalous*, which is the teleomorph (sexual reproduction stage) of *B. anomalous* (Smith and Van Grinsven, 1984). This difference in reproductive state might explain the difference in % identity

match, as teleomorphosis is the sexual stage of the fungal life cycle and the cell undergoes chromosomal crossover/recombination as part of meiosis. The ITS sequence of *S. ellipsoides* var*. diastaticus* was most homologous to *Saccharomyces cerevisiae* which the strain is also known as (§1.3.7.2.7). Further investigation confirmed the strain as diastatic (§3.5.3).

3.5.3 Confirmation of Presumptive *S. ellipsoides* var. *diastaticus* as Diastatic Strain

The strong growth that the presumptive *S. ellipsoides* var. *diastaticus* strain exhibited on medium containing dextrin or starch as sole carbon source in comparison to the laboratory strain *S. cerevisiae* BY4741 (§3.2.3), shows that the test strain is diastatic, where diastatic is defined as possessing the capability to produce glucoamylase and possess the ability to break down starch and dextrin (Kleinman *et al*., 1988).

3.6 Tolerance of Brewing Microbes to Silver Nitrate

3.6.1 YPD Silver Nitrate Streak Plates

The silver nitrate streak test on YPD agar was a preliminary test to determine the MIC, to allow more refined experimentation. Of the yeasts, *P. membranaefaciens*, *C. krusei*, *H. saturnus* and *K. apiculata* were all unable to grow at a silver nitrate concentration of 0.2 mM. *B. anomalous* could not grow at 0.6 mM and no end point could be determined for *S. ellipsoids* var. *diastaticus*. The *E.coli* control strains J53 and J53 pMG101 both exhibited growth past the maximum concentration tested. The control strain J53 pMG101 was expected to exhibit resistance to silver nitrate up to a concentration of 0.6 mM and the J53 strain a resistance up to 0.1 mM (Gupta

et al., 2001). That the growth of both *E. coli* control strains exceeded their predicted MIC values may indicate that the toxicity of silver nitrate in the medium is inhibited in some manner. It is possible that this may be caused by substances such as salt (§3.6.6) contained within the yeast extract in the YPD agar, chelating the silver, thus decreasing the overall activity of the silver content, as was suggested in section 3.3.2 for the spot plates performed on LB agar containing a range of NaCl concentrations. However, constituents in yeast extract are also known to bind toxic metals.

3.6.2 *E. coli* Control Strain $AgNO_3$ Resistance and Growth Tests

It was found that the maximum concentration of silver nitrate that *E. coli* J53 could grow on was 0.2 mM, this was achieved on the LB agar plates containing no added salt. Any increase in salt reduced the concentration of silver nitrate needed to inhibit growth. The higher the salt concentrations present the lower the MIC of silver nitrate. The same was true of the *E. coli* strain J53 (pMG101). However, the strain was able to grow at a higher concentration of silver nitrate (1mM) in the presence of a low concentration of salt (5 g/L) than on medium containing no salt (0.4 mM). Any further increase in salt concentration resulted in increased sensitivity to silver nitrate.

Gupta *et al.* (1998) suggested that the increased sensitivity to silver in increasing concentrations of NaCl is due to the salt ions increasing the cell membrane permeability to silver ions. This would increase the amount of internalised silver in the organism, so less silver is needed to achieve an antimicrobial effect.

3.6.7 Maintenance of Silver Resistance plasmid in J53 pMG101

The results of the silver nitrate spot plate tests on YPD (Table 3.7), showed both *E. coli* J53 and J53 (pMG101) exhibiting growth up to 0.4 mM of silver nitrate. The expected tolerances derived from Gupta *et al.* (1998) were 0.1 mM for J53 and 0.6 mM for J53 (pMG101) in Luria-Bertani (LB) medium. Although no plasmid map exists for the pMG101 plasmid in the *E. coli* control strain J53 pMG101, it is known to contain genes for resistance to ampicillin, sulphonamide, tetracycline, chloramphenicol, streptomycin, potassium tellurite and mercuric chloride, in addition to silver resistance genes. Therefore, to prove that the plasmid has been maintained in J53 and has not transferred to J53 both organisms were streaked onto two LB agar plates, one of which contained 100 µg/ml ampicillin. Both strains grew on the plate without ampicillin, but only J53 pMG101 could grow on the plate containing ampicillin. This infers that the plasmid is maintained and has not been transferred to J53.

3.7 Conclusion

These data would suggest that all the organisms are sensitive to silver, but that the sensitivity varies between the different organisms. The two *E. coli* strains tended to exhibit similar silver tolerances in YPD media even though J53 (pMG101) was expected to exhibit a greater degree of resistance due to the *sil* silver resistance genes included on the pMG101 plasmid (Gupta *et al.,* 2001). As these organisms were meant to provide fixed reference points of silver tolerance and silver toxicity levels this discrepancy was investigated. When examined on ampicillin plates, it was found that J53 (pMG101) had maintained its plasmid and that J53 has not acquired it. Furthermore, spot plates on LB agar containing silver indicated a marked difference between the

highly silver tolerant J53 (pMG101) and the less tolerant J53. However, it must be stated that the exact degree of tolerance was affected by the salt concentration present in the medium, as noted by Gupta *et al.* (1998). The greater the salt concentration, the lower was the tolerance of the strains to silver.

S. ellipsoides var. *diastaticus*, as the most silver tolerant organism, will have to be observed closely in future silver tolerance experiments. It would appear that in YPD there is generally little difference in sensitivity between the bacterial control strains and *S. ellipsoides* var. *diastaticus*. In chapter 4 experiments will be performed with wort and beer media to accurately investigate silver tolerance of the brewery contaminants under brewery conditions. Further experimentation is needed to establish the exact MICs of silver for the test strains, and the effects of environmental conditions on these MICs. The streak plate experiment would suggest that emphasis should be placed on investigating the 0.1-0.8 mM range. The project will be continued using the bacteria *Lactobacillus brevis* and *Pediococcus damnosus*. These are arguably the most important spoilage bacteria and the test organisms have been positively identified (Vaughan *et al.,* 2005). In addition to these two organisms, all seven of the wild yeasts will also be investigated.

Chapter 4 - The Effect of Silver Stress on Microbial Brewery Contaminants in Brewery Media

Beer has a low nutritional content, a low pH, contains alcohol, has a high redox potential and contains antimicrobial hop iso-α-acids, which make beer an innately inhospitable environment for the majority of microorganisms (Suzuki *et al.*, 2007). However, as with most environments, there are groups of microorganisms which can tolerate these conditions and have found an ecological niche within the brewing process (Ogden *et al.*, 1988). These contaminants, whilst generally non-pathogenic, may negatively impact product flavour, quality and yield, making them economically important (Hough *et al.*, 1982; Boulton and Quain, 2006). Microbiological contamination is damaging to the brewer in terms of both consumer confidence as well as product retrieval costs (Suzuki *et al*., 2006). Wild yeasts have been isolated throughout the brewing process, possibly entering the system during wort aeration and establishing themselves due to incomplete sanitisation (Vaughn *et al*., 2005). Biofilm formation on brewery surfaces poses a two-fold problem as they are both a continuing source of contamination and also provide protection for the microorganisms from cleaning agents (Storgårds *et al*., 2003).

Historically silver has been used as an antimicrobial in a number of applications including drinking water purification (Castellano *et al.,* 2007) and for disease medication (Landsdown, 2002). Low concentrations of ionic silver (0.05–0.1 ppm) have also been used as an antimicrobial agent as part of water treatment for drinking and swimming pool water (Just and Szniolis, 1936; Landeen *et al.,* 1989). Silver has also been used in the cleaning of seafood, fruit and vegetables as well as the disinfection of lettuce, removing

bacterial, yeast and mould contaminants (Gopal *et al.*, 2010). Silver is also reported to inhibit biofilm formation (Rosenblatt, 2009). Silver surface coatings are known to have antimicrobial activity and are used on surfaces ranging from medical devices such as catheters, to food storage vessels in Japan (Poulter *et al.*, 2009; Rai *et al.,* 2009). Silver antimicrobial surfaces may reduce the risk of microbial contamination and the need for contamination counter measures in breweries.

The Biolog system is a micro plate reader in which each well of the microplate contains a growth medium or a biochemical test reagent, in addition to a tetrazolium dye. The Biolog™ Omnilog uses a redox reaction based on respiration as a reporter to measure metabolic activity. A cell's active metabolism causes the reduction of the tetrazolium dye as electrons are taken from NADH in the electron transport chain; this reduction results in a purple precipitate. An increased rate of metabolism/respiration results in increased reduction of the dye, increasing the intensity of colour. The changes in intensity of colour are detected and recorded via an internal colour video camera in the Omnilog plate reader (Bochner, 2009). This system allows for the quantification of metabolic activity as well as detection of presence or absence of metabolic activity in response to varying carbon sources or potential stress factors. The Biolog system has previously been applied to the study of stress responses, in particular to metals such as cadmium, zinc, lead and copper, for example in soil microbial soil samples and *Pseudomonas pseudoalcaligenes* (Muhammad *et al.*, 2005, Liao and Xie, 2007, Tremaroli *et al.*, 2009). The system has also been applied to the phenotypic differentiation of brewery isolates of the brewing contaminant *Obesumbacterium proteus*

(Prest *et al.*, 1994). However, all of these studies relied upon premade, commercially-available Biolog plates and do not take account of the impact that the brewery environment (beer, wort and atmosphere) would impart. In this study the relative tolerances of 7 wild yeast contaminants, *P. membranaefaciens*, *B. anomalous*, *C. krusei*, *H. saturnus*, *K. apiculata*, *R. mucilaginosa*, *S. ellipsoides* var. *diastaticus,* and 2 bacterial species: *L. brevis* and *P. damnosus* to silver and the effect of brewing environments (wort and beer) on these tolerances were examined. As in Chapter 3, silver nitrate was used to determine the silver tolerance of the yeasts. The comparative silver tolerances of the yeasts in true wort and beer medium at different silver nitrate concentrations were tested by examining growth via traditional spot plates and optical density readings (Tecan), in addition to metabolic activity measurements using the Biolog phenotype microarray system. As the Biolog phenotype array measures metabolic activity, metabolising cells will be detected during silver tolerance experiments even if no growth is detected by the other assays.

4.1 Nitrate Tolerance

To determine whether the sensitivities to silver nitrate exhibited in the tolerance experiments were due to the silver or the nitrate, duplicate experiments were performed for growth assays (§2.7.2, Fig. 4.1), utilising sodium nitrate. No decrease in growth or viability was detected over the same concentration range of sodium nitrate as was used for the silver nitrate experiments. Although the all yeast cell cultures were inoculated into the wells at 62% turbidity, there is some variation in OD values beyween the yeasts. This may be due to some yeasts cultures flocculating.

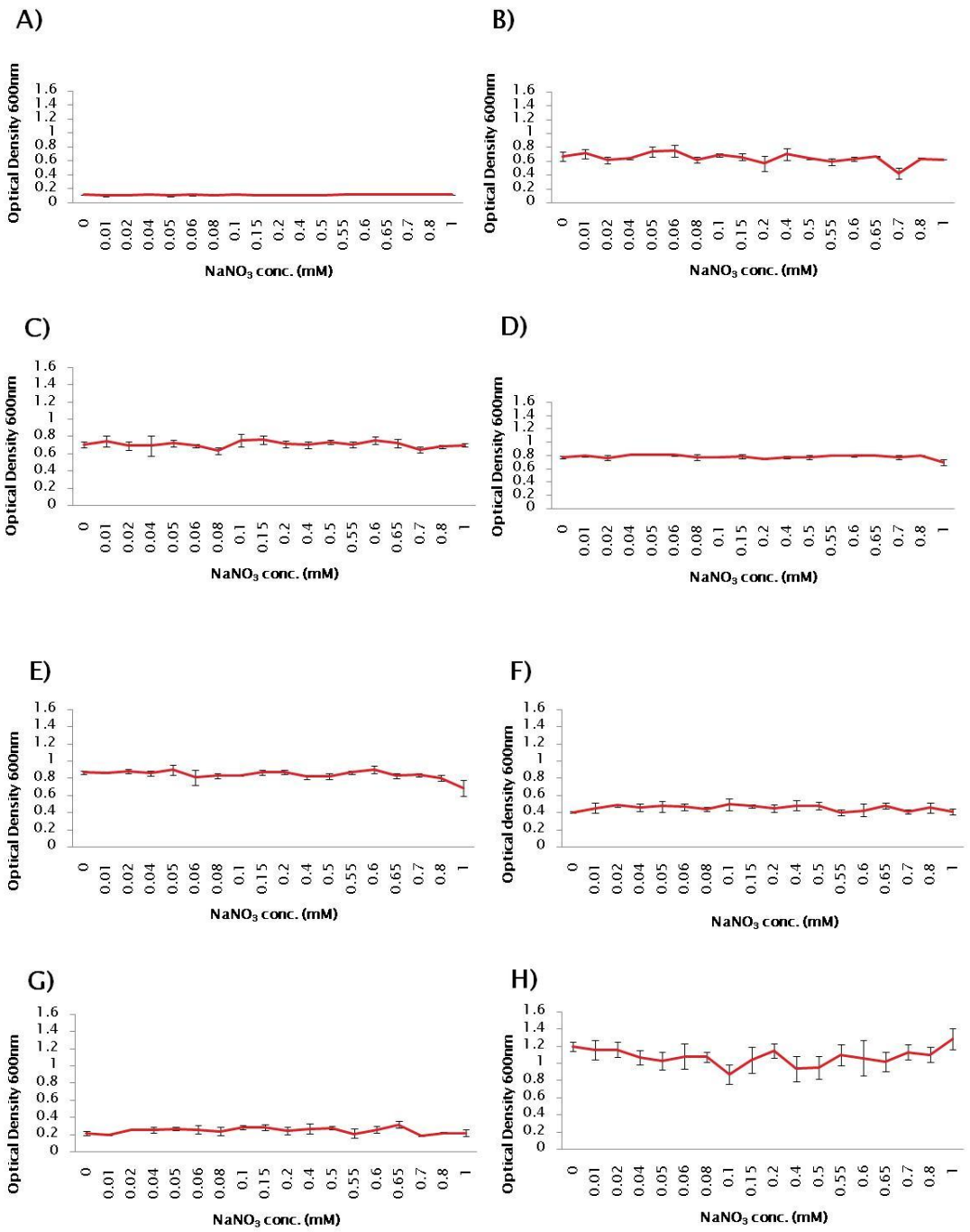


Figure 4.1) Effect of sodium nitrate on growth. Graphs illustrating growth of wild yeasts exposed to sodium nitrate. Plates were set up to contain 95 µl of 50% Wort (Hopped Peroni, S.G. 1.0567) mixed with IFY, containing the appropriate quantity of sodium nitrate. To each well 3 µl of cell culture (62% turbidity) were added prior to incubation at 25°C for 72 hours. Growth was recorded using a Tecan microplate optical density meter at 600 nm. **A)** Control, **B)** *P. membranaefaciens*, **C)** *B. anomalus*, **D)** *C. krusei*, **E)** *H. saturnus*, **F)** *K. apiculata*, **G)** *R. mucilaginosa*, **H)** *S. elipsoides var. diastaticus*.

4.2 Spot Plate Testing

Spot plate silver tolerance assays (§2.5.2.1) were performed using both yeast and bacterial test strains on YPD (Fig. 4.2, Table 4.1), beer (Table 4.2) and wort (Table 4.3) agar media. The yeast strains, exhibited a range of silver nitrate tolerances in their growth patterns on all media. On YPD agar the tolerances ranged from 0.65 mM silver nitrate for *S. ellipsoides var. diastaticus* to 0.2 mM for both *B. anomalous* and *R. mucilaginosa*. Of the bacterial strains *L. brevis* was able to tolerate up to 0.55 mM of silver nitrate. *P. damnosus* was able to tolerate up to 0.8 mM of silver nitrate, exhibiting a greater silver tolerance than *S. ellipsoides var. diastaticus* on YPD without additional stresses.

On both wort and beer agar the silver tolerances of the organisms still varied, however, they were greatly reduced. On Wort agar the highest silver tolerance exhibited by a yeast was 0.15 mM, by both *C. krusei* and *R. mucilaginosa*. The lowest tolerance of 0.06 mM was exhibited by *B. anomalous* and *K. apiculata*. Of the bacteria only *L. brevis* showed growth on wort agar with a silver tolerance of 0.4 mM.

On beer agar the bacterial strains showed no growth at any silver nitrate concentration. The yeast strains exhibited a tolerance range from 0.55 mM by *R. mucilaginosa* to 0.04 mM by *B. anomalous*.

4.2.1 Comparison of Silver Tolerance on Solid Commercial and Actual Wort and Beer Media

The silver tolerances of the brewery contaminant organisms on commercial Oxoid wort agar (§2.3.8) and actual wort agar (§2.3.6) were compared via spot plate experiments (Table 4.2). It was found that *P. damnosus* was unable

to grow on either medium. *C. krusei*, *H. saturnus*, *R.mucilaginosa* and *L. brevis* were all able to grow on actual wort agar but not on the commercial wort agar. The organisms *B. anomalous* and *K. apiculata* were able to grow on both media in the absence of silver, but only on actual wort agar in the presence of silver. The organism *P. membranaefaciens* was able to grow on both media in the presence of silver and could tolerate up to a concentration of 0.06 mM silver on actual wort agar but only 0.01 mM on commercial wort agar. *S. ellipsoides* var. *diastaticus* was also able to grow on both media, but was able to tolerate silver up to a concentration of 0.8 mM on commercial wort agar and only up to 0.1 mM on actual wort agar.

The silver tolerances of the brewery contaminant organisms on commercial Oxoid universal beer (UBA) agar (§2.3.9) and actual beer agar (§2.3.7) were compared using spot plate experiments (Table 4.3). The organisms *L. brevis* and *P. damnosus* were unable to grow on either medium. *H. saturnus* was able to grow on actual beer agar but not on commercial UBA. With the exception of *B. anomalous* all the remaining yeast were able to grow on both media and tolerate silver to some degree, however, greater tolerance was exhibited on actual beer agar than UBA. *B. anomalous* exhibited growth on both media with and without silver, but demonstrated greater silver tolerance on UBA than on actual beer agar.

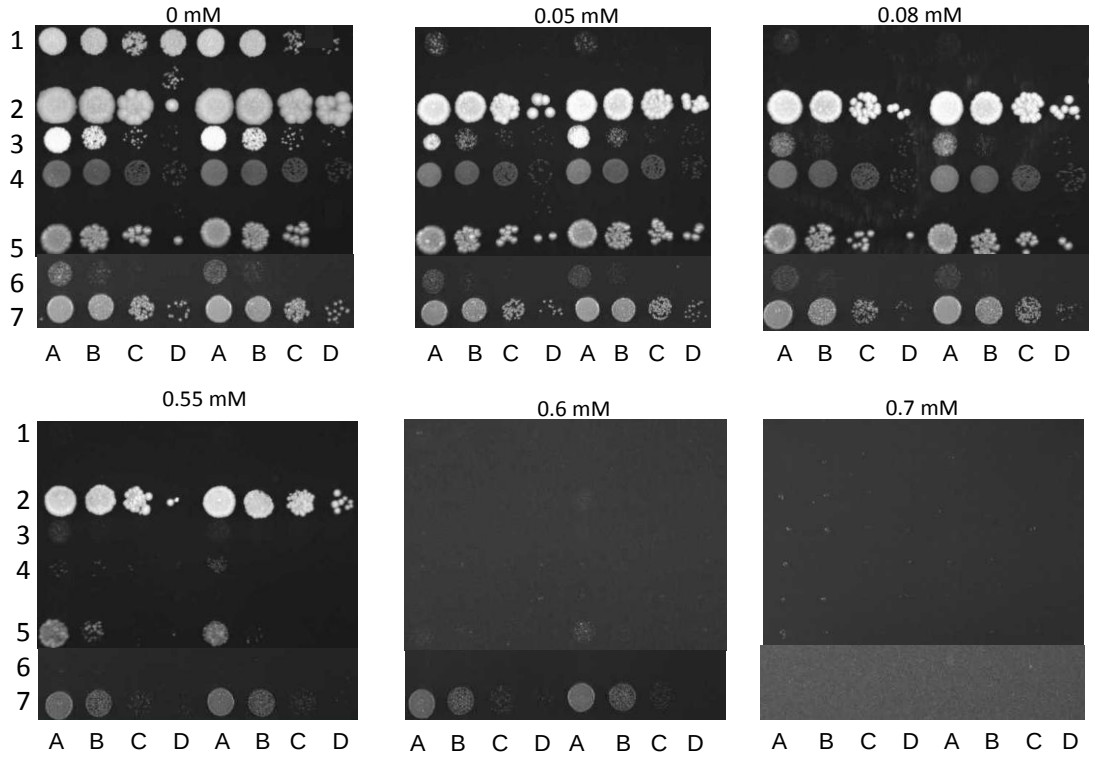


Figure 4.2) Tolerance of wild yeast brewery isolates to silver nitrate in aerobic conditions on YPD. Dilutions of each yeast (A: OD 0.1, B: OD 0.01, C: OD 0.001, D: OD 0.001) were spotted onto YPD agar containing a range of silver nitrate concentrations (0-1 mM) and incubated at 30°C for 3 days. 1: *P. membranaefaciens*, 2: *C. krusei*, 3: *H. saturnus*, 4: *K. apiculata*, 5: *S. elipsoides var. diastaticus,* 6: *B. anomalus*, 7: *R. mucilaginosa.*

YPD	Silver nitrate Concentration (mM)																
Organisms	**0**	**0.01**	**0.02**	**0.04**	**0.05**	**0.06**	**0.08**	**0.1**	**0.15**	**0.2**	**0.4**	**0.5**	**0.55**	**0.6**	**0.65**	**0.7**	**0.8**
P. membranaefaciens	4	3	3	2	1	1	1	1	1	1	-						
B. anomalous	2	2	2	2	2	2	1	1	1	-							
C. krusei	4	4	4	4	4	4	4	4	4	4	4	4	3	-			
H. saturnus	3	3	3	3	3	3	2	2	1	1	1	1	-				
K. apiculata	4	4	4	4	4	4	4	4	4	4	4	2	1	-			
R. mucilaginosa	4	4	4	4	4	4	4	4	1	-							
S. ellipsoids var. *diast.*	4	4	4	4	4	4	4	4	4	3	3	3	3	3	2	-	
L. brevis	4	4	4	4	4	4	4	4	4	4	4	3	-				
P. damnosus	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

Table 4.1) Assessment of minimum inhibitory concentration (MIC) in the presence of silver nitrate on YPD agar. MICs of the four dilutions (OD 0.1, OD 0.01, OD 0.001, OD 0.001), of each yeast spotted onto YPD agar plates containing a range (0-1 mM) of silver nitrate concentrations. Plates were incubated at 30°C for 3 days. 4: All dilutions Grow, 3: 3 Highest Cell Densities, 2: 2 Highest Cell Densities, 1: Highest Cell Density, - : No Growth

Wort	Silver nitrate Concentration (mM)																
Organisms	**0**	**0.01**	**0.02**	**0.04**	**0.05**	**0.06**	**0.08**	**0.1**	**0.15**	**0.2**	**0.4**	**0.5**	**0.55**	**0.6**	**0.65**	**0.7**	**0.8**
P. membranaefaciens	4(4)	4(2)	2(-)	1	1	1	-										
B. anomalous	2(3)	2(-)	2	1	1	-											
C. krusei	4(-)	4	4	4	2	1	1	1	-								
H. saturnus	2(-)	2	2	2	2	1	-										
K. apiculata	4(4)	4(-)	4	4	2	-											
R. mucilaginosa	4(-)	4	4	4	4	4	4	4	-								
S. ellipsoids var. *diast.*	3(4)	3(3)	3(3)	3(3)	2(3)	1(3)	1(3)	-(3)	(3)	(3)	(3)	(3)	(3)	(2)	(2)	(2)	(-)
L. brevis	2(-)	1	1	1	1	1	1	1	1	1	-						
P. damnosus	-(-)																

Table 4.2) Growth on 50% wort and commercial wort agar in the absence and presence of silver nitrate. Hopped wort at a specific gravity of 1.0567 was diluted with sterile deionised water to achieve a concentration of 50%. Where required, silver nitrate was added to achieve a concentration within the range 0 and 1 mM. Plates were incubated with 5 µl of cell suspension grown in YPD and diluted with sterile deionised water to achieve optical densities of OD 0.1, OD 0.01, OD 0.001, OD 0.001 at 600 nm. Plates were incubated at 30°C for 3 days. Values represent the relative growth detected on plate and are denoted as 4: All dilutions Grow, 3: 3 Highest Cell Densities, 2: 2 Highest Cell Densities, 1: Highest Cell Density, - : No Growth. Values given in () represent the growth of the organisms on commercial oxoid wort agar under the same conditions.

Beer	Silver nitrate Concentration (mM)																
Organisms	**0**	**0.01**	**0.02**	**0.04**	**0.05**	**0.06**	**0.08**	**0.1**	**0.15**	**0.2**	**0.4**	**0.5**	**0.55**	**0.6**	**0.65**	**0.7**	**0.8**
P. membranaefaciens	4(4)	4(4)	4(4)	2(1)	1(-)	1	-										
B. anomalous	3(3)	3(3)	2(3)	-(3)	(1)	(1)	(1)	(-)									
C. krusei	4(2)	4(1)	4(1)	4(1)	3(1)	2(1)	1(-)	1	1	-							
H. saturnus	2(-)	2	2	1	1	1	1	1	-								
K. apiculata	3(4)	3(4)	3(3)	2(-)	1	-											
R. mucilaginosa	2(2)	2(2)	1(1)	1(1)	1(1)	1(1)	1(-)	1	1	1	1	1	-				
S. ellipsoids var. *diast.*	2(4)	2(4)	2(4)	2(4)	1(4)	1(4)	1(4)	1(3)	1(-)	-							
L. brevis	-(-)																
P. damnosus	-(-)																

Table 4.3) Growth on 50% beer and commercial universal beer (UBA) agar in the absence and presence of silver nitrate. Lager beer (ABV 5.1%) was diluted with sterile deionised water to achieve a concentration of 50%. Where required, silver nitrate was added to achieve a concentration within the range 0 and 1 mM. Plates were incubated with 5 µl of cell suspension grown in YPD and diluted with sterile deionised water to achieve optical densities of OD 0.1, OD 0.01, OD 0.001, OD 0.001 at 600 nm. Plates were incubated at 30°C for 3 days. Values represent the relative growth detected on plate and are denoted as 4: All dilutions Grow, 3: 3 Highest Cell Densities, 2: 2 Highest Cell Densities, 1: Highest Cell Density, - : No Growth. Values given in () represent the growth of the organisms on commercial oxoid universal beer agar (UBA) under the same conditions.

4.3 Silver Tolerance Testing in Yeast

4.3.1 Metabolic Assay in Yeast

The metabolic activity assays (§2.7.1, Appendix 1) (Figs. 4.3 and 4.4) show a range of silver nitrate tolerances for the yeasts in both wort and beer. In wort the highest tolerances were exhibited by *H. saturnus* and *S. ellipsoides* var. *diastaticus,* as these strains maintained metabolic activity up to a silver nitrate concentration of 0.2 mM. In beer the highest tolerances were demonstrated by *C. krusei* and *H.saturnus*, maintaining metabolic activity up to 0.15 mM silver nitrate. The lowest tolerance was exhibited by *K. apiculata* at 0.01 mM.

4.3.1.1 Metabolic Assay Method Development

As the majority of Omnilog/Biolog users only use pre-prepared and not bespoke purpose made plates and no work using this system has been performed to assess the tolerance of microbial brewery contaminants against silver has been reported in the literature, method development and optimisation was needed.

Firstly, metabolic activity assays (§2.7.1) were performed in the absence of silver, utilising 50% beer and wort (IFY buffer as diluent) and 100% beer and wort as the growth medium for the contaminant yeast strains. It was found that the darker colour of the 100% wort and beer obscured some of the purple precipitate from the camera leading to a reduced calculated value of metabolic activity from the Omnilog system. Therefore, 50% wort and beer were used for all metabolic activity assays.

Secondly, there was concern about the effect of silver nitrate on the tetrazolium dye and about the black precipitate formed by silver nitrate when

exposed to light. Therefore, metabolic activity assays (§2.7.1) were performed in 50% beer and wort, without organisms, at silver nitrate concentrations of 0, 0.05, 0.1, 0.4, 0.6 and 1 mM. It was found that although silver nitrate did not affect the tetrazolium dye, concentrations of 0.4 mM or greater could cause an increase in calculated metabolic activity due to the formation of black precipitate. This precipitate is most likely an insoluble silver halide and so may affect the concentration of silver ions in solution. This precipitate could impact on the methodology if formed during experimentation and has to be taken into account whilst evaluating the metabolic activity data.

4.3.2 Growth Assay in Yeast

The growth assays (§2.7.2, Appendix 2) (Fig. 4.3 and 4.5) show the growth of the yeasts in beer and wort in the presence of a range of silver nitrate concentrations, measured by optical density (600 nm). In wort the organism that exhibited the highest silver tolerance was *S. ellipsoides* var. *diastaticus*, demonstrating growth up to a concentration of 0.4 mM. The lowest tolerance was exhibited by *B. anomalus* at 0.04 mM. In beer the highest tolerance was exhibited by *H. saturnus* at 0.5 mM and the lowest by *P. membranaefaciens* at 0.02 mM. The 96 well microtitre plates (plates in triplicate for each strain and medium) were incubated at 25°C for 72 hours. Data was recorded using a Tecan microplate optical density meter at 600 nm at 0, 18, 21, 24, 42, 45, 48, 66, 69, 72 hours. This was a practical necessity due to the volume of plates and the single plate capacity of the Tecan plate reader. The time gaps between readings may be the cause of the sudden jumps in OD demonstrated in Fig. 4.5. However, as the aim of the experiment was to determine the silver

MIC's exhibited by the contaminant yeasts in wort and beer, the focus is on the concentration at which there is no change in OD (so no growth) over all the time points.

4.3.3 Comparison of Silver Tolerance Assays

In Fig. 4.4 the silver MICs of the yeast brewery contaminant organisms in wort and beer, determined by spot plating (Tables 4.2-4.3), growth assays (Fig. 4.5) and metabolic activity assays (Fig. 4.4), are summarised. In wort all three assays determined very similar MIC's. The exceptions to this were *H. saturnus* where the spot plate assay determined an MIC of half the concentration determined by the other assays and *S.ellipsoides var. diastaticus* where the growth assay indicates a much higher MIC than the other two assays. In beer the assays also tended to determine similar results. In beer these exceptions were *H. saturnus* where the growth assay indicated a much higher MIC than the other two assays and *R. mucilaginosa* and *S.ellipsoides var. diastaticu* for both of which the spot plate assay determined a higher MIC than the other assays. All MIC data on Fig. 4.3 is the concentration of silver at which there is no growth/metabolic activity determined by the correlated data of replica experiments for each individual condition for each assay type. For reproducibility of data see individual assays.

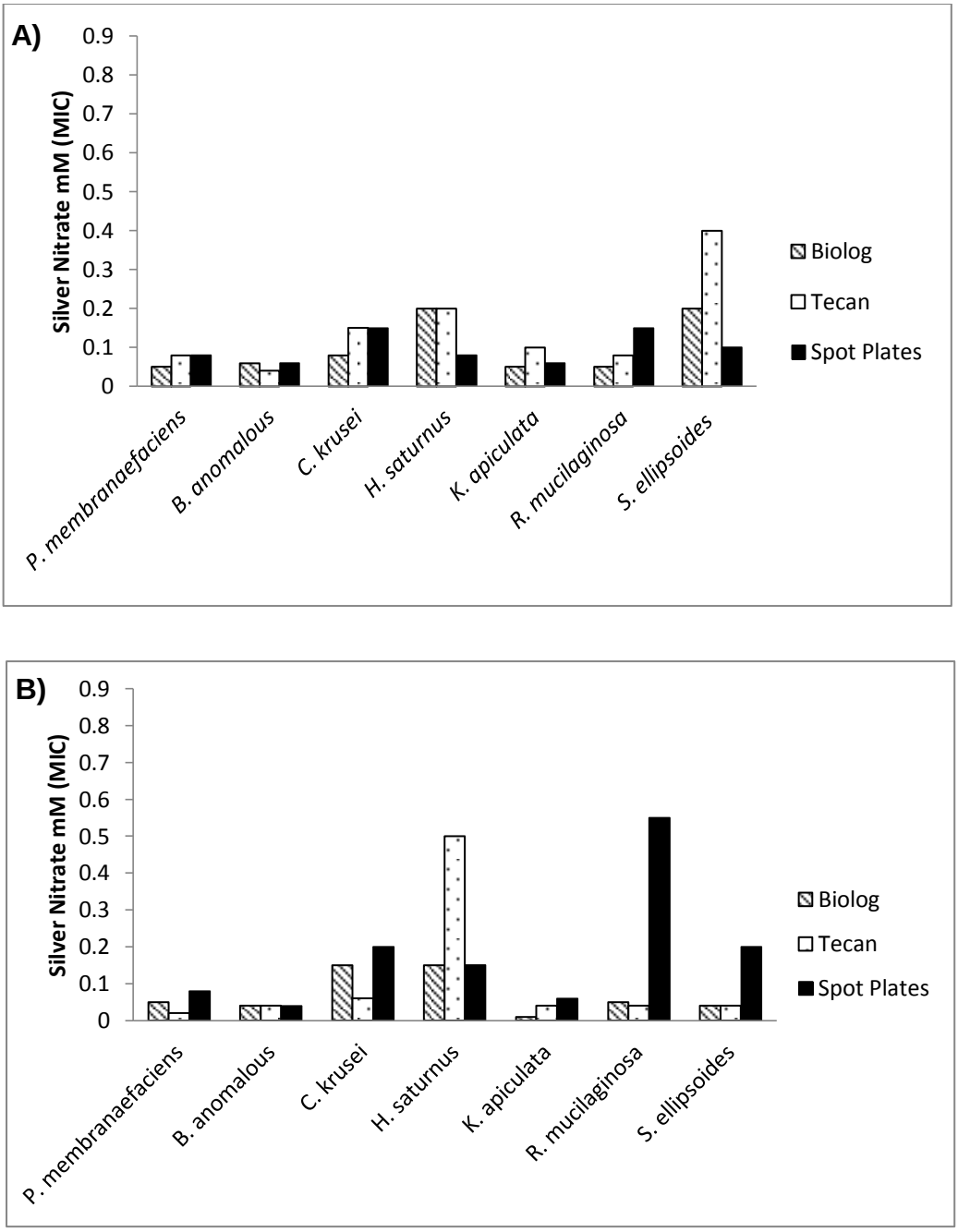


Figure 4.3) Correlated Silver tolerances of wild yeasts. A) Correlated silver nitrate minimum inhibitory concentrations (mM) of yeasts in 50% wort (Hopped Peroni, S.G. 1.0567) from Biolog (metabolic activity), Tecan (OD 600nm) and Spot plate results. **B)** Correlated silver nitrate minimum inhibitory concentrations (mM) of yeasts in 50% beer (Peroni, 5.1% ABV) from Biolog (metabolic activity, Fig. 4.4), Tecan (OD 600 nm, eg. Fig. 4.5) and Spot plate results (Tables 4.2-4.3).

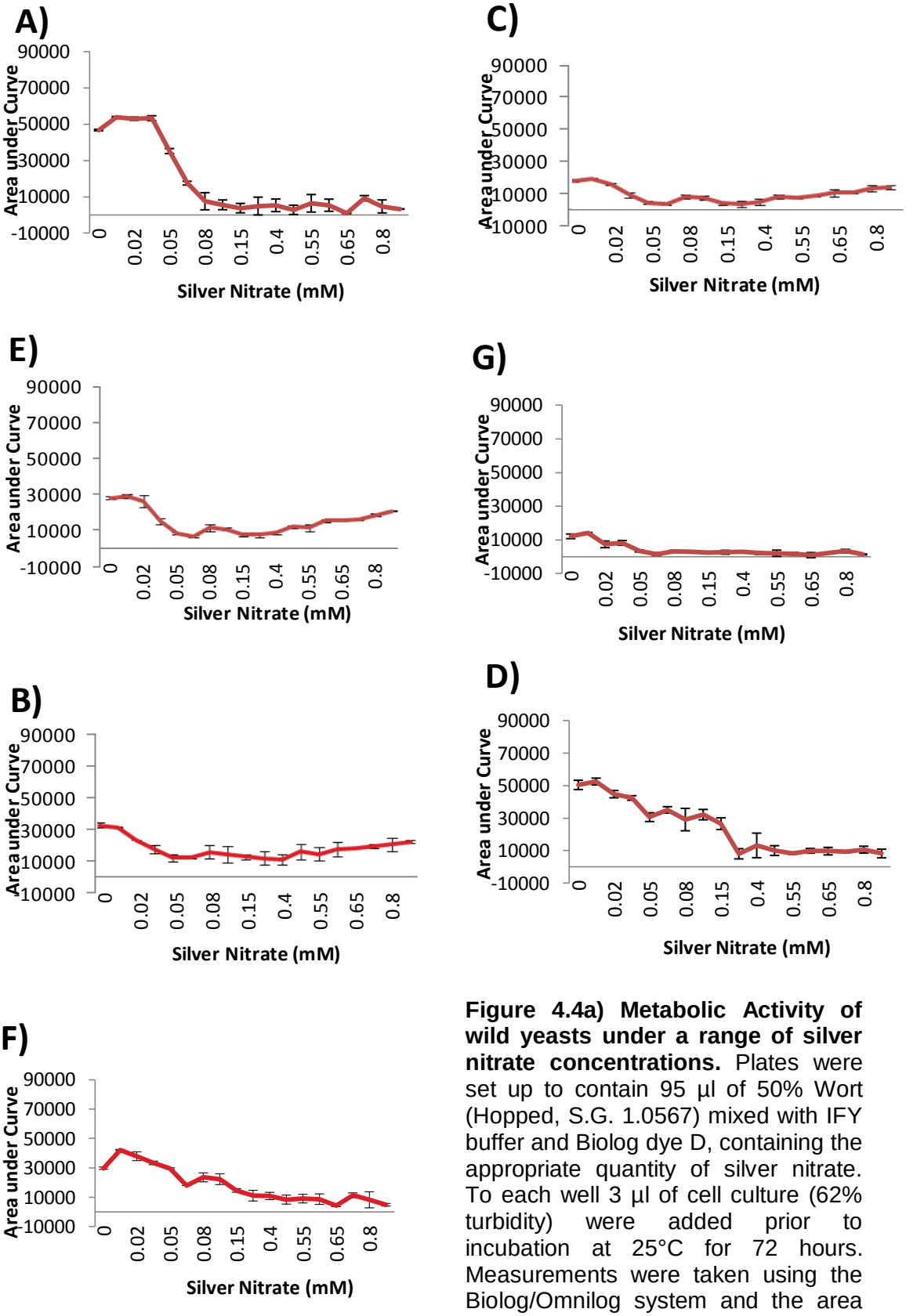


Figure 4.4a) Metabolic Activity of wild yeasts under a range of silver nitrate concentrations. Plates were set up to contain 95 µl of 50% Wort (Hopped, S.G. 1.0567) mixed with IFY buffer and Biolog dye D, containing the appropriate quantity of silver nitrate. To each well 3 µl of cell culture (62% turbidity) were added prior to incubation at 25°C for 72 hours. Measurements were taken using the Biolog/Omnilog system and the area under the curve taken as a measure of metabolic activity. A) *C. krusei*, B) *P. membranaefaciens,*C) *K. apiculata*, D) *S. ellipsoids* var. *diastaticus*, E) *B. anomalous*, F) *H. saturnus*, G) *R. mucilaginosa.*

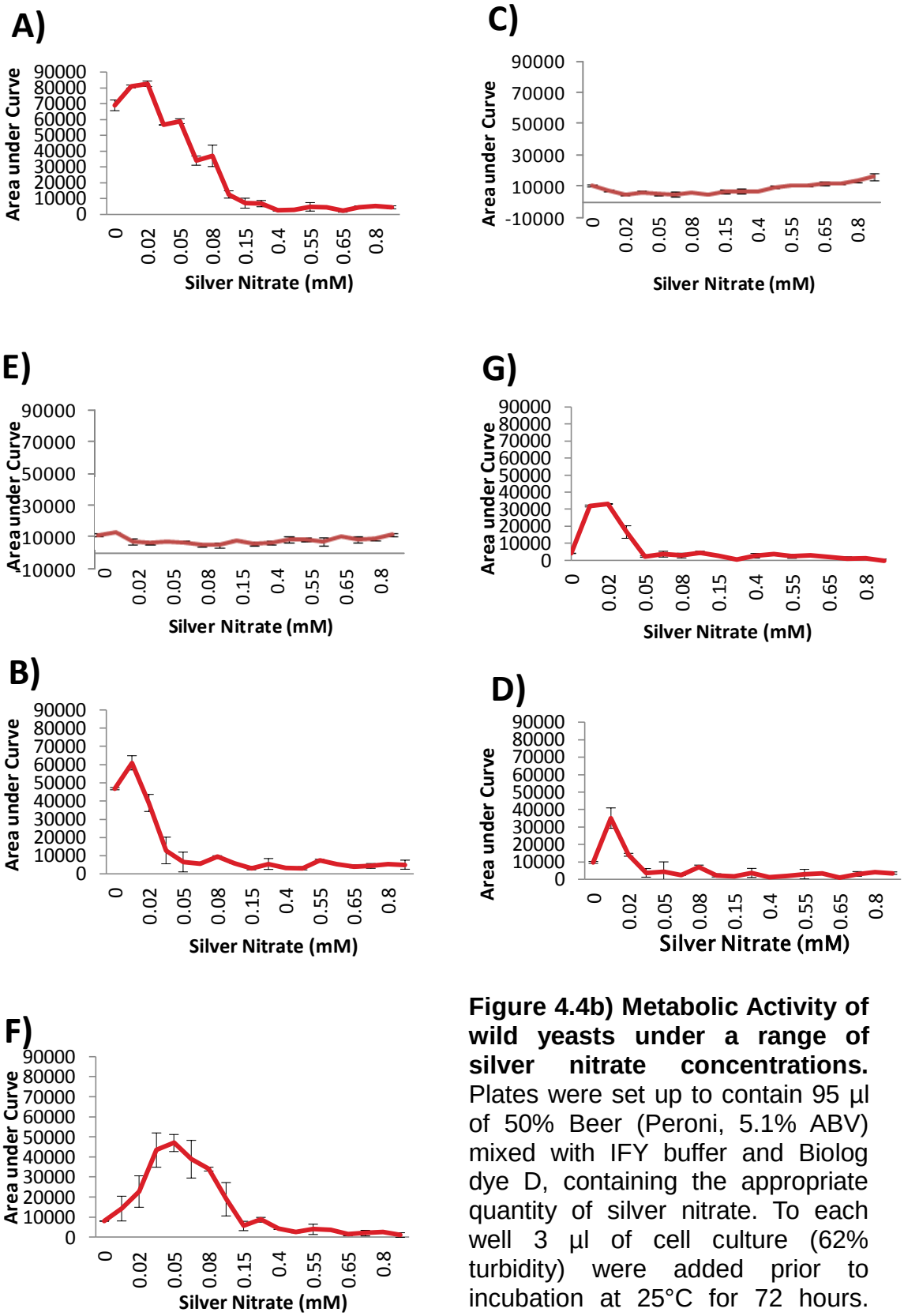


Figure 4.4b) Metabolic Activity of wild yeasts under a range of silver nitrate concentrations. Plates were set up to contain 95 µl of 50% Beer (Peroni, 5.1% ABV) mixed with IFY buffer and Biolog dye D, containing the appropriate quantity of silver nitrate. To each well 3 µl of cell culture (62% turbidity) were added prior to incubation at 25°C for 72 hours. Measurements were taken using the Biolog/Omnilog system and the area under the curve taken as a measure of metabolic activity. A) *C. krusei*, B) *P. membranaefaciens,* C) *K. apiculata*, D) *S. ellipsoids* var. *diastaticus*, E) *B. anomalous*, F) *H. saturnus*, G) *R. mucilaginosa*

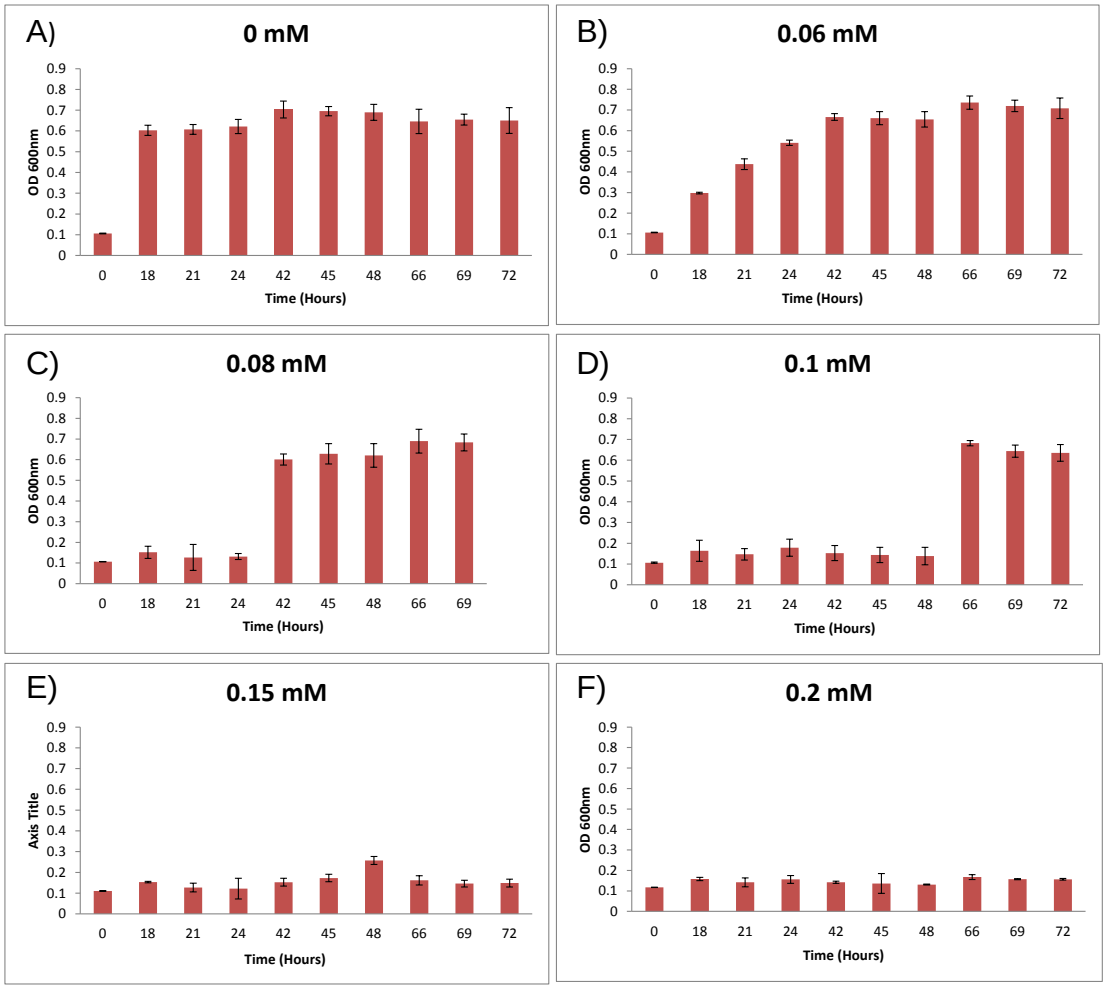


Figure 4.5) Optical Density assay to determine yeast silver nitrate tolerance. Graphs illustrating growth of the wild yeast *C. krusei*. Plates were set up to contain 95 µl of 50% Wort (Hopped Peroni, S.G. 1.0567) mixed with IFY, containing the appropriate quantity of silver nitrate. To each well 3 µl of cell culture (62% turbidity) were added prior to incubation at 25°C for 72 hours in a stationary incubator. Growth was recorded at the given time points using a Tecan microplate optical density meter at 600 nm. Representative silver nitrate test concentrations shown include **A)** 0, **B)** 0.06, **C)** 0.08, **D)** 0.1, **E)** 0.15 and **F)** 0.2 mM.

4.4 Silver Tolerance Testing in Bacteria

4.4.1 Metabolic Assay in Bacteria

The metabolic activity assays (§2.7.1) for the silver tolerance of bacterial strains in wort and beer (Fig. 4.6) indicate that in wort *L. brevis* maintained metabolic activity up to a silver nitrate concentration of 0.15 mM and *P. damnosus* up to 0.5 mM. In beer *L. brevis* was only able to maintain metabolic activity up to 0.06 mM and *P. damnosus* only up to 0.08 mM.

4.4.2 Growth Assay in Bacteria

The growth assays (§2.7.2) for the bacterial strains in wort and beer measured silver tolerance by recording growth via optical density spectrophotometry (600 nm) (Fig. 4.7). In wort both *L. brevis* and *P. damnosus* grew up to a silver nitrate concentration of 0.02 mM. Both also exhibited increased growth at 0.01 mM of silver nitrate in comparison to growth without silver. In beer neither organism exhibited growth.

4.5 Effect of Anaerobic and Aerobic Conditions on Yeast Silver Tolerance

To assess the effect of aerobic versus anaerobic conditions on silver tolerance, the yeasts were subjected to further metabolic activity assays (§2.7.3) in wort and beer in both conditions. In wort under aerobic conditions the yeasts exhibited higher or equal silver tolerances to those determined under anaerobic conditions (Fig. 4.8). The only exception to this was *K. apiculata* which exhibited a higher tolerance under anaerobic conditions. In beer the yeasts exhibited no difference in silver tolerance between aerobic and anaerobic conditions. Two exceptions to this were *K. apiculata* and *R. mucilaginosa*, both of which only exhibited growth under aerobic conditions.

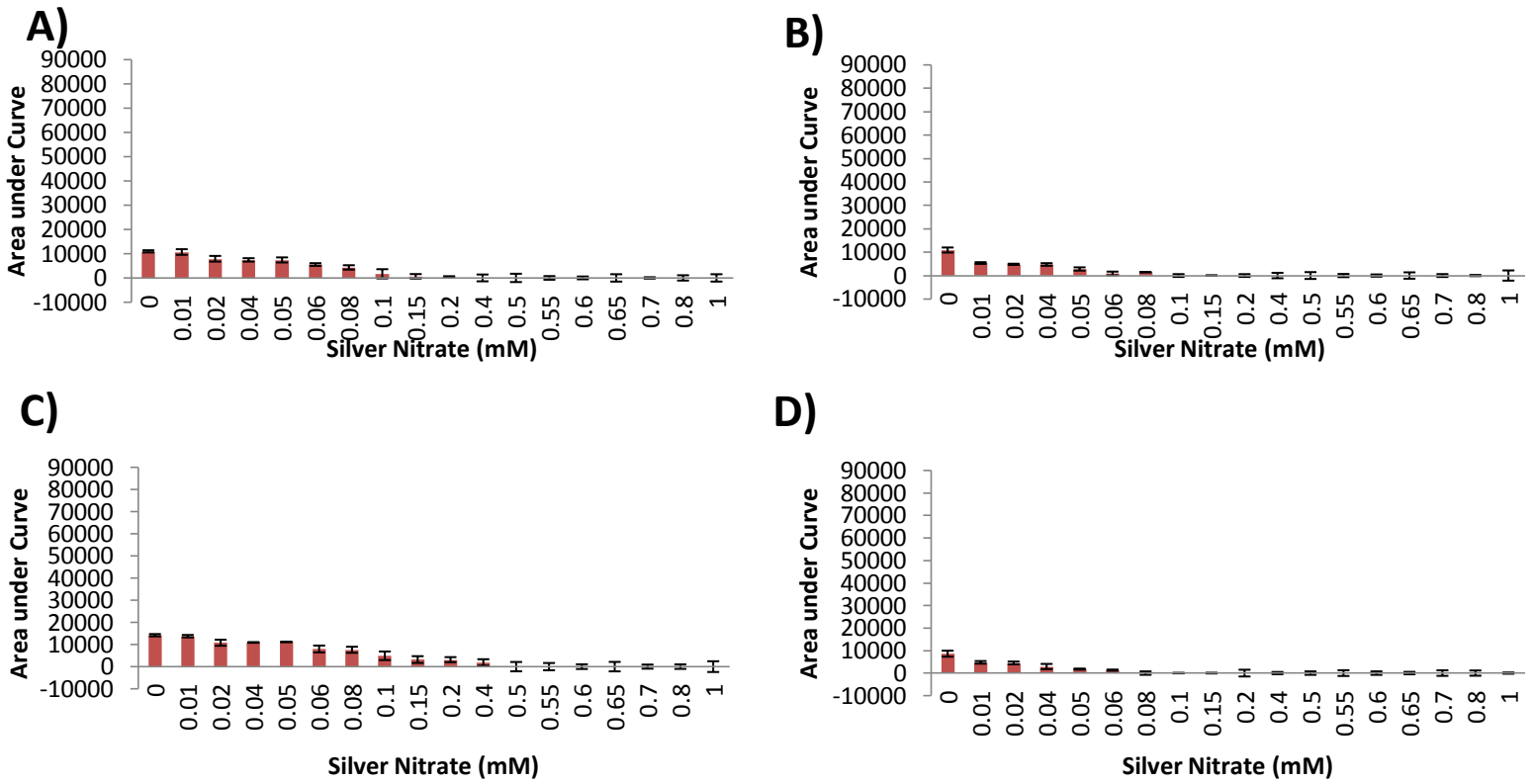


Figure 4.6) Metabolic Activity of *L. brevis* and P*. damnosus* under a range of silver nitrate concentrations. Plates were set up to contain 95 µl of **A+C)** 50% Wort (Hopped, S.G. 1.0567) and **B+D)** 50% Beer (Peroni, 5.1% ABV) mixed with IFY buffer and Biolog dye D, containing the appropriate quantity of silver nitrate. To each well 3 µl of cell culture (62% turbidity, **A** and **B**: *L. brevis*, **C** and **D**: *P. damnosus*) were added prior to incubation at 25°C for 72 hours. Measurements were taken using the Biolog/Omnilog system and the area under the curve taken as a measure of metabolic activity.

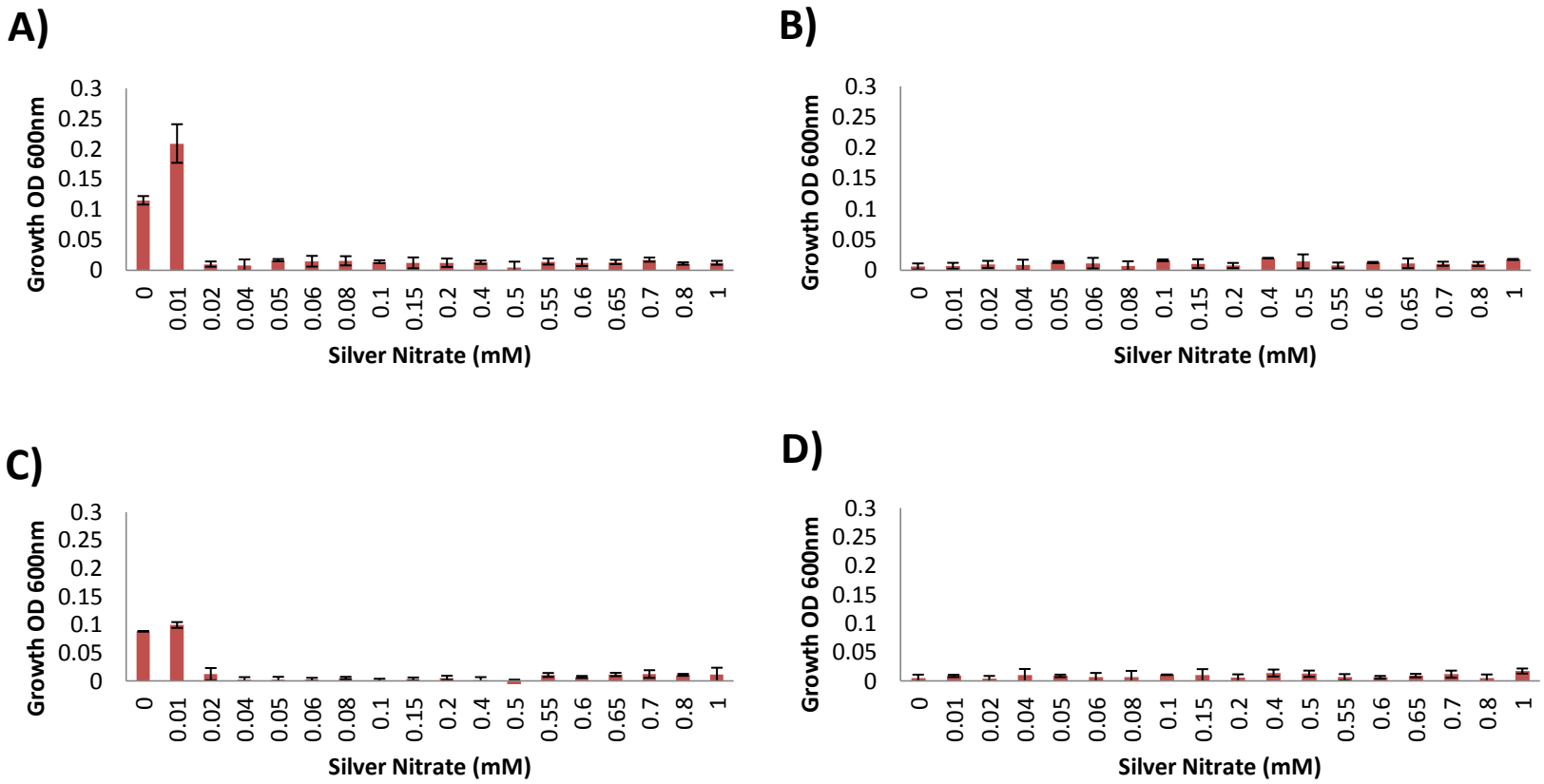


Figure 4.7) Growth assay of *L. brevis* and P*. damnosus* under a range of silver nitrate concentrations. Plates were set up to contain 95 µl of **A+C)** 50% Wort (Hopped, S.G. 1.0567) and **B+D)** 50% Beer (Peroni, 5.1% ABV) mixed with IFY buffer and Biolog dye D, containing the appropriate quantity of silver nitrate. To each well 3 µl of cell culture (62% turbidity, **A** and **B**: *L. brevis*, **C** and **D**: *P. damnosus*) were added prior to incubation at 25°C for 72 hours. Growth was recorded using a Tecan microplate optical density meter at 600 nm.

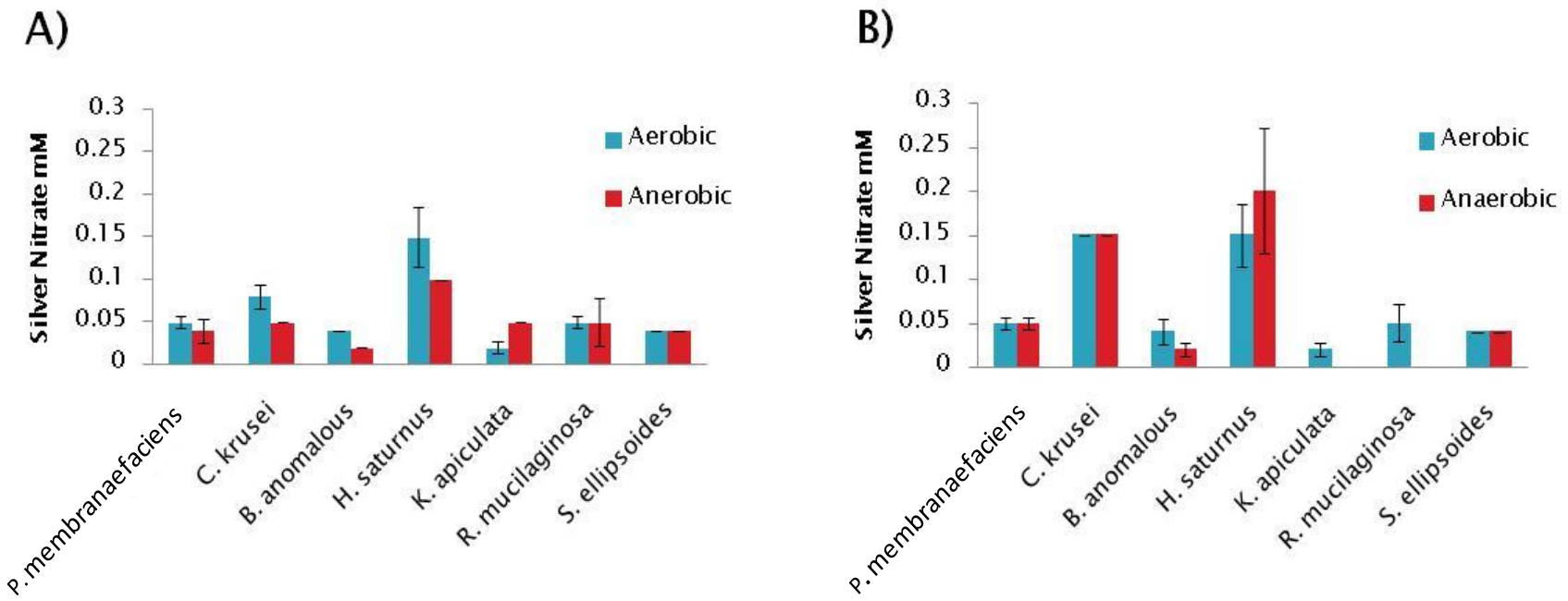


Figure 4.8) Comparison of Aerobic and Anaerobic Growth of Brewery Contaminant Strains. Graphs of comparative MICs of silver nitrate for all 7 yeasts in aerobic and anaerobic conditions, showing results from metabolic activity assays in **A)** 50% wort and **B)** beer (Peroni). Measurements were taken using the Biolog/Omnilog system and the area under the curve taken as a measure of metabolic activity. Microaerophillic conditions were achieved by vacuum sealing the Biolog plates into sterile plastic sleeves.

Chapter 4 Discussion

4.6 Nitrate Tolerance

In the current study silver nitrate was used to establish the relative sensitivity of brewery-isolated wild yeast strains to silver. In order to confirm that the nitrate itself was not toxic to the strains assessed, the relative sensitivity of strains to sodium nitrate was examined by determining the impact of $NaNO_3$ on yeast growth. Growth was assessed by colony development on spot plates (data not shown) and by monitoring optical density in liquid culture (Fig. 4.1). No decrease in growth was seen using either means of assessment for any of the strains examined in the presence of 0 mM to 1 mM sodium nitrate. This observation suggests any negative impact of silver nitrate on strain growth would not be caused by nitrate. This supports the findings of Xia *et al,* (2006), who determinined that sodium nitrate concentrations of 0.5-10 mmol/L have no antimicrobial effect on oral pathogens including yeasts such as *Candida albicans.*

4.7 Impact of Silver on Wild Yeast Strain Growth on Solid Medium

Brewery isolates typically establish a monolayer on brewery equipment surfaces as the first stage in developing a biofilm (Timke, 2004). To assess the impact of silver on the capacity of the yeast isolates to establish growth on a solid surface, 10 fold serial dilutions of the strains were inoculated onto YPD agar incorporating 0-1mM silver nitrate and incubated at 30° C for 3 days after which time they were assessed for colony formation (Fig. 4.2). Whilst not a true test of the antimicrobial effects of silver on biofilm formation, this test demonstrates the effect of silver on the ability of these brewery contaminants to grow on a solid surface that releases silver ions. All yeasts were able to

form colonies at all dilutions on YPD agar at 0mM silver nitrate demonstrating the ability to grow. The spot plates showed that as the concentration of silver nitrate increased the ability of the yeasts to form colonies was inhibited, beginning with the spots containing the greatest dilution of cell culture, until a concentration was reached where no growth occurred. The degree of silver tolerance exhibited appeared highly species dependent. The minimum inhibitory concentrations for each strain is provided in Table 1. The most silver tolerant yeast, *S. ellipsoides* var. *diastaticus* was able to grow up to a concentration of 0.65 mM, whereas the least tolerant organisms: *B. anomalous* and *R. mucilaginosa*, were unable to grow above concentrations of 0.15 mM. All of these tolerances were lower than expected as previous silver nitrate tolerance experiments based on yeasts such as *Debaryomyces hansenii*, *Candida albicans*, *Saccharomyces cerevisiae*, *Rhodotorula rubra* and *Aureobasidium pullulans* growing on solid medium indicated silver nitrate tolerances of up to 1-2 mM. This may be due to the volatile and non-volatile reducing compounds that were found to be produced by some of these organisms, which reduce Ag^{+} to the less toxic Ag^{0} (Kierans *et al*., 1991).

4.8 Traditional Spot Plate Screening of Yeast Silver Tolerance on Wort and Beer Agar

The impact of brewery media (beer and wort) on the sensitivity of wild yeasts to silver has not been previously described. Before silver can be used as part of an antimicrobial surface in breweries, the effect of these brewery media on the silver tolerance of wild yeast contaminants must be established. Minimum inhibitory concentrations for each strain on solidified wort and beer media (Tables 4.2 and 4.3 respectively) showed that each yeast exhibited similar

tolerances to silver nitrate on both brewery media. However, on all media tested the silver nitrate tolerances varied greatly between organisms. Most yeasts on both wort agar (0.06-0.15 mM) and beer agar (0.04-0.2 mM) exhibited lower tolerances than on YPD agar (0.2-0.6 mM). The test strain *R. mucilaginosa* did not follow this trend and showed the highest tolerance on beer agar (0.5 mM). This organism can be found during fermentation suggesting an adaptation to beer as a growth medium (Matei *et al.*, 2011).

4.8.1 Comparison of Silver Tolerance on Solid Commercial and Actual Wort and Beer Media

In the comparison of actual and commercial media, (excluding *P. damnosus* on wort and *L. brevis* and *P.damnosus* on beer as they grew on neither actual nor commercial agar), the general trend is that less growth and lower tolerances to silver were exhibited on the commercial agar. This trend is more apparent in the comparison of the wort agars, where many organisms were only capable of growth on actual wort agar. Of those that were able to grow on both media only *P. membranaefaciens* exhibited any silver tolerance on commercial agar, a sixth of the tolerance exhibited on actual wort agar. This is surprising as commercial wort agar is meant for the culture and enumeration of yeasts and moulds. The results may be due to the nature of the commercial wort agar. It has a pH of 4.8 meant to inhibit most bacteria and was designed and optimised for yeasts and moulds isolated from butter, not from beer or wort (Parfitt, 1933; Atlas, 2006). As a result commercial wort agar may not be an ideal medium for the culture of brewery isolates.

On beer, (keeping the exclusions mentioned above in mind), the majority of organisms could grow on both media. Greater or equal growth was exhibited

on the commercial medium in the absence of silver, however, greater silver tolerances was exhibited on actual beer agar. Greater growth was to be expected on commercial beer agar as it is designed as a recovery and culture medium for yeast and bacterial beer contaminants (Kozulis and Page, 1968). Actual beer agar presents a harsher environment than commercial beer agar. It has higher alcohol content and higher levels of dissolved CO_2. This could be an explaination as to why higher levels of silver are tolerated on actual beer agar, as the stress responses of the test organisms to manage this environment may also result in increased silver tolerance.

There were only two exceptions to the general trend, *S. ellipsoids* var. *diastaticus* on wort and *B. anomalous* on beer. Both of these organisms grew on both actual and commercial agar, however, they both exhibited greater silver tolerance on commercial agar.

4.9 Silver Tolerance in Liquid Medium

4.9.1 Effect of Silver on Yeast Metabolism

Similar to the spot plate experiments, the silver nitrate tolerances indicated by the metabolic activity assays vary between yeasts. However, the yeasts in wort exhibited slightly higher tolerances, as higher concentrations of silver nitrate were needed to completely inhibit metabolic activity than in beer (Fig. 4.3). The only exceptions to this were *C. krusei* which exhibited lower tolerance and *P. membranaefaciens* which did not exhibit any difference in silver tolerance between the media. The gradual decrease in metabolic activity in response to increasing silver nitrate concentrations may indicate inhibition of cell functions such as respiration or DNA replication as the mechanism of toxicity for silver as suggested by Rai *et al.* (2009). Biolog

experiments measured the effect of silver nitrate on the metabolic activity of the wild yeasts (Fig. 4.4), in wort and beer. With the exception of *P. membranaefaciens*, *R. mucilaginosa* and *S. ellipsoides* var. *diastaticus* in wort and *K. apiculata* in beer, the yeasts all exhibited an increase in metabolic activity at low concentrations of silver nitrate (Fig. 4.4). Increased metabolic activity was demonstrated between 0-0.01 mM silver nitrate in both wort and beer, although in beer *C. krusei* and *R. mucilaginosa* exhibited an increase in activity up to 0.02 mM and *H. saturnus* up to 0.05 mM. This may indicate that in minute quantities metals, such as silver or copper, have a stimulatory effect on metabolism, possibly as the yeasts bioaccumulate the metal as a form of tolerance (Blackwell *et al.*, 1995, Gomes *et al.*, 1999; de Silóniz, *et al*., 2002). As the silver nitrate concentration increased in both wort and beer, many yeasts exhibited a transitory increase in metabolic activity, occurring between 0.06 and 0.1 mM silver nitrate. Although this effect was observed in both media it was demonstrated most clearly in wort. This may be the result of the up-regulation of a tolerance mechanism at a silver nitrate concentration of 0.06 mM, which is overwhelmed at concentrations higher than 0.1 mM. This would be a hormetic effect as exposure to a small dose of a harmful substance results in resistance to higher doses of the same, if transient, or other substances (Leroy *et al.*, 2012). This, and the initial increase in metabolic activity mentioned previously, agrees with the findings of Niazi *et al.* (2011), in which the genetic response of *Saccharomyces cerevisiae* to silver stress was examined, revealing the up regulation of genes such as *CUP1-1*/*CUP1-2* (Copper efflux pump), *PHO89* (Na+/Pi co-transporter), *HSP12* and *HSP26* (heat shock genes). The induction of copper genes was unsurprising

as silver is known to induce the *Cup1* gene (Fürst and Hamer, 1989). Copper resistance mechanisms such as a P1-Type ATPase copper efflux pump have also been reported to confer silver tolerance (Riggle and Kumamoto, 2000). Any further increase in silver nitrate concentration led to decreasing metabolic activity.

4.9.2 Effect of Silver on Bacterial Metabolism

In both beer and wort *P. damnosus* exhibited greater silver nitrate tolerance than *L. brevis* by demonstrating metabolic activity at higher concentrations of silver nitrate (Fig. 4.6) . However, in beer this difference was only marginal. Both organisms exhibited decreased silver tolerance in beer in comparison to wort. In fact in beer metabolic activity appeared reduced across all concentrations tested, even in the absence of silver nitrate. This may be due to additional stresses found in beer including the alcohol content, pH and lack of nutrients (Bergveinson *et al.*, 2012). In comparison to the yeast metabolic activity assays, the metabolic activity of the bacteria seems reduced for both beer and wort.

4.9.3 The Effect of Silver on Yeast Growth in Brewery Media

Optical density experiments measured the effect of silver nitrate on the growth of the wild yeasts in wort and beer. The data given in Fig. 4.5 show growth of the representative yeast *C. krusei* in each silver nitrate concentration. Fig. 4.5 shows that whilst the final cell density measured is not initially affected by increasing silver nitrate concentration, the length of lag phase does increase until a concentration is reached at which there is no growth. This is the MIC value given for the Tecan results in Fig. 4.3. As in the previous experiments

individual tolerances for $AgNO_3$ varied between yeasts. For most of the yeasts $AgNO_3$ growth inhibition approximately matched $AgNO_3$ levels that inhibited metabolic activity (Fig. 4.3). The exceptions to this were *K. apiculata* and *S. ellipsoides* var. *diastaticus* in wort and *H. saturnus* in beer, where the tolerance indicated via growth (0.1 mM, 0.4 mM and 0.5 mM) was higher than that indicated by metabolic activity (0.05 mM, 0.2 mM and 0.15 mM). This discrepancy may be due to the "area under the curve" measurement used to quantify metabolic activity. A brief period of low level metabolic activity may have occurred allowing a degree of cellular replication (detected by growth assay) before metabolic activity was inhibited. Similar to the gradual decrease in metabolic activity, this would also suggest inhibition of cell functions as mechanisms of silver toxicity, rather than rupturing or distortion of cell surfaces as suggested by Saulou *et al.* (2010).

4.9.4 Effect of Silver on Bacterial Growth in Brewery Media

Optical density growth assays recorded the growth of the test bacteria in wort and beer when exposed to a range of silver nitrate concentrations. Both organisms only exhibited growth up to a silver nitrate concentration of 0.02 mM in wort and did not demonstrate any growth in beer. This is not consistent with the bacterial metabolic activity assays in which both organisms show metabolic activity at higher levels of silver nitrate than 0.02 mM in both beer and wort. This may be explained by the fact that when these organisms are present in breweries they are often found to be viable but not culturable (Suzuki *et al.,* 2007). This is an example of why both the growth assays and the metabolic assays are needed to determine silver tolerance. At a given concentration of silver nitrate, or other test condition, the metabolic activity

assay may only detect metabolic activity for an organism and thus confirm its viability, however, it may not determine whether the organism is replicating. To confirm cell replication the optical density growth assay is required. Similar to the yeast metabolic activity, assays for both bacteria demonstrated increased growth at low levels of silver nitrate (0.01 mM), which was terminated at higher concentrations of silver nitrate (0.02 mM). As for the yeasts this may be a hermitic response. The bacterial spot plates on beer agar match the results for the bacterial growth assays in beer. However, in wort *P. damnosus* exhibits no growth, whilst *L. brevis* exhibits a silver nitrate up to 0.2 mM which is similar to the metabolic activity data for *L. brevis* in wort (0.15 mM). It is, however, only the highest cell density spot of the *L. brevis* dilution series that can grow above 0.01 mM silver nitrate on wort agar.

4.10 Aerobic vs Anerobic Growth of Yeasts under Silver Stress

The effect of aerobic and microaerophillic conditions on yeast silver tolerance in beer and wort was assessed using metabolic activity assays. Overall in both beer and wort, with the exception of *K. apiculata* in wort, the organisms exhibited greater or at the least equal tolerance to silver nitrate in aerobic conditions as in microaerophillic conditions. This may be due to the facultatively anaerobic nature of the yeasts. The fermentation occurring in microaerophillic conditions may be more susceptible to the ability of silver to inhibit ATP production than the respiration occurring in aerobic conditions. This is due to the interactions of silver with thiol groups inhibiting enzymes such as NADH dehydrogenase (Klasen, 2000; Park *et al.*, 2008). This inhibition of NAD^+ production is more damaging to fermentation than respiration, as fermentation relies exclusively on glycolysis, which requires

NAD^+. Additionaly fermentation is comparatively inefficient, producing 2 ATPs per molecule of glucose in comparison to the 36-38 produced by respiration (Solomon *et al*., 2005). So any reduction in NAD would have a greater impact on fermenting organisms than respiring organisms. Therefore, the addition of silver may lead to a greater reduction of metabolic activity in microaerophillic conditions than it does in aerobic conditions. Additionally, copper is more toxic to microbes under anaerobic conditions as more of its ions are in their Cu(I) form rather than Cu(II). The same may apply to Ag(I) ions as silver and copper are very similar (Beswick *et al*., 1976; Outten *et al.*, 2001). Under anaerobic conditions *E. coli* and yeasts have demonstrated increased copper accumulation; as silver utilises many copper transport proteins, the same may be true of silver (Outten *et al*., 2001; Strain and Culotta, 1996; Weissman *et al*., 2000; Gudipaty *et al*., 2012).

4.11 Conclusions

The data indicated that levels of silver tolerance vary greatly between individual yeasts as well as the bacteria tested; this appears true for all conditions tested. The gradual decrease in metabolic activity and the increasing lag phase during growth could suggest that inhibition of cell function is the mechanism of silver toxicity. There is evidence of low level resistance/tolerance mechanisms in the yeasts indicated by the recovery in metabolic activity around 0.06-0.1 mM silver nitrate. However, this mechanism appears to be overwhelmed by silver concentrations in excess of 0.1 mM. All wort experiments in both liquid and on solid medium exhibited similar silver tolerances and this would support the findings of Tilton and Rosenberg (1973), which indicated that agar had no effect on silver toxicity. However,

yeast cells grown on beer agar exhibited higher tolerances to silver than in the liquid growth experiments. This may be due to the smaller surface area on the agar on which the silver is in contact with the yeasts and reduced diffusion of silver ions, resulting in localised depletion of silver in the medium and additional stresses of beer (alcohol, pH etc.) cannot interact with the yeasts to the same degree as in the liquid media. Examination of metabolic activity suggested that wild yeasts exhibited slightly greater tolerance to silver in wort rather than beer, as the rate of decrease in metabolic activity was greater and the minimum inhibitory concentrations lower in beer than in wort. This may be due to the additional stress factors in beer, including lower pH, ethanol content and lower sugar content (Menz *et al.*, 2010). Conversely for the yeasts on solid media, higher silver tolerances were evident on beer than on wort medium. Of the bacterial strains only *L. brevis* was able to grow on wort and neither of the bacteria were able to grow on beer agar. These results indicate that the surrounding medium (wort/beer) can have an effect on the tolerance of the bacteria to silver. This, and the variation in silver tolerance between yeasts, would have an impact on the effectiveness of any potential antimicrobial surface, possibly requiring different silver concentrations in the antimicrobial bioactive silver surfaces in different parts of the brewery, depending on the environment e.g. wort or beer and likely contamination threats. The decreased silver nitrate tolerances exhibited by a large proportion of the yeasts under microaerophillic conditions, is of interest from a brewing perspective, as the microaerophillic conditions are more representative of the atmospheric conditions of the brewing process. The fact that silver tolerances decreased, or at the least did not increase, for the

yeasts tested in more brewery accurate conditions, are encouraging for the development of silver as an antimicrobial within the process.

The results indicate that both the metabolic assay and the optical density assay are similar in sensitivity with regard to determining MIC. However, the Biolog system detected the onset of inhibition in the form of decreased metabolic activity at lower concentrations of silver nitrate than those required by the optical density system. Additionally examining the metabolic activity of the organisms via the Biolog system provided insight into the cells' function during stress, such as an increase in metabolic activity in response to minute quantities of silver nitrate and the possibility of a resistance mechanism. The Biolog system could potentially be a useful tool for the screening of industrial media for microbial growth and responses to stress.

The data suggest that silver has potential as an antimicrobial targeted at contaminants in the brewery. However, the work carried out here uses silver in a soluble form and further investigation must be performed to determine the effectiveness of solid metallic silver and eventually silver antimicrobial surfaces, such as silver nanoparticle surfaces, as a means of preventing or reducing contamination in the brewery. The metabolic activity data shows that low doses of silver nitrate increase metabolic activity in the yeast strains tested, suggesting up regulation of silver tolerance or general stress response mechanisms, including increased metabolism. Whilst this low level tolerance is overwhelmed at higher levels of silver nitrate, potential silver tolerance mechanisms in the contaminant strains will have to be investigated.

Chapter 5 – The Genetic Response to Silver Stress in Brewing Conditions

In chapter 4 it was suggested that the brewery contaminant strains tested, in particular the yeast strains, exhibited a degree of silver tolerance to low level silver nitrate. In this chapter the genetic response of the brewery contaminant strain *S. ellipsoides var. diastaticus* to silver stress in wort and beer was investigated. Additionally, genes potentially involved in silver tolerance mechanisms were investigated. The genetic responses to silver stress in beer and wort were investigated via RNA microarray transcriptome analysis (§2.8.1) on an Affymetrix GeneChip® Yeast Genome 2.0 Array. Microarrays function on the basic premise that complimentary nucleic acids have base pair affinity and will hybridize. An Affymetrix microarray consists of a "chip", which has short oligonucleotides (25-mer) printed on it, corresponding to known genes in the target genome (11 probe pairs per sequence). The Affymetrix GeneChip® Yeast Genome 2.0 Array includes 5,744 probe sets for 5,841 of the 5,845 genes in *S. cerevisiae* and 5,021 probe sets for the 5,031 genes in *S. pombe*. Fluorescently labelled RNA (Biotin IVT label) of the test organism will hybridize to it's complimentary probe. After washing the unbound molecules from the array, hybridised probes were stained with streptavidin phycoerythrin conjugate and can be detected via laser microscopy (Affymetrix GeneChip scanner 3000). The amount of light emitted at 570 nm is proportional to the bound RNA at each location on the probe array. Comparison of gene expression levels is possible through quantifying the ratio of fluorescence for the dot corresponding to each gene (Hoopes, 2008; GeneChip® Expression Analysis Technical Manual). As a quality control of hybridisation, the array is hybridized with bioB, bioC, bioD labled RNA controls

(of known concentration) from *E. coli* and *cre* from P1 bacteriophage. These should not cross-hybridise with non-bacterial and non-viral samples. Therefore, the quantities of RNA indicated by the array should match those of the RNA spike. The quality controls for RNA labelling consist of diaminopimelic acid, lysine, phenylalanine, threonine and tryptophan polyA RNA from *Bacillus subtilis*. They are spiked in at RNA labelling and allow the validation of the target prep steps. The poly-A controls Dap, Thr, Phe and Lys should be present at a proportionally decreasing intensity, to verify that there was no bias during the retro-transcription between highly expressed genes and low expression genes. For mRNA quantity normalisation purposes GAPDH, Actin, EAF5, SRB4, TFIID, RIP1, URA3, and WBP1 were used as housekeeping/control genes (Zang *et al*., 2007). Microarrays have been previously utilised to analyse other stress responses such as the gene response of *S. cerevisiae* to copper stress and the screening of yeasts for sensitivity to potential new antifungal agents (Hodgins-Davis *et al.,* 2012; Tebbets *et al.*, 2012). Microarrays have even been used to analyse the stress response to silver and silver nano-particles previously, however, only for the laboratory strain *Saccharomyces cerevisiae* S288C grown in YPD medium (Niazi *et al.*, 2011). This study suggested copper tolerance mechanisms such as the metalloprotein *CUP1*, which binds copper ions, are highly induced under silver stress. Another study suggested that a mechanism for silver tolerance has been found in the ectomycorrhizal fungus *Amanita strobiliformis*, by the metallothionein *AsMT1*a, which has been found to sequester silver and also copper. This was still found to be the case when *AsMT1*a was inserted into *S. cerevisiae* DTY113 and DTY168 (Osobova *et*

al., 2011). It has also been found that copper induces the *PCA1* gene, leading to expression of a cadmium efflux P1B-type ATPase which also transports silver and copper ions in *Saccharomyces cerevisiae* (Adle *et al.*, 2007). Copper homeostasis is part of normal cellular function, as copper is both essential and toxic to cells (Dong *et al.*, 2013). Copper levels are maintained by induction of copper uptake mechanisms such as the plasma membrane high-affinity copper transporter *CTR1* in times of copper depletion (Dancis *et al.*, 1994; Schwartz *et al.*, 2013). In times of copper stress these uptake mechanisms are inhibited and copper chelation via metallothionienes such as *Cup1* and *CRD2* increases (Riggle and Kumamoto, 2000).

The yeast *S. ellipsoides var. diastaticus* was chosen from amongst the contaminant test strains for investigation of genetic response to silver stress, as from previous silver tolerance experimentation in chapter 4 it appears to be amongst the most tolerant of the yeasts. Therefore, this organism may exhibit a more visible silver linked stress response than other organisms. Additionally, the Affymetrix *Saccharomyces* gene chips (GeneChip® Yeast Genome 2.0 Array) are readily available and contain probe sets for 5,841 of the 5,845 genes present in *S. cerevisiae*. Once potential tolerance genes have been identified, *S. cerevisiae* BY4741based gene knockout strains are available for confirmation experiments, making this an efficient experimental system. *S. ellipsoides* var*. diastaticus* is also known as *S. cerevisiae* var.*diastaticus*, so with the exception of lacking the *STA1*, *2* and *3* genes for glucoamylase production the contaminant strain should match the gene chip closely (Pretorius *et al.*, 1991).

5.1 ICP-MS of Wort and Beer

Inductively coupled plasma mass spectrometry was performed on the wort and beer (Peroni) used for experimentation (§2.6.1). This revealed that there is no trace amount of silver in either beer or wort (Table. 5.1). However, Copper was found in both beer and wort, at 42.93 µg/L (0.676 mM) and 71.88 µg/L (1.131 mM), respectively. This would indicate that the copper levels in wort are higher than those found in beer.

Concentration of Copper (µg/L)			
Wort		Beer	
71.57		41.36	
71.68		43.71	
72.39		43.74	
Mean	71.88	Mean	42.93667
Standard Deviation	1.365516	Standard Deviation	0.445084
Mean (mM)	1.131mM	Mean (mM)	0.676

Table 5.1) Table of Copper concentrations detected in wort and beer detected via ICP-MS. Three replicates were taken for both beer (different bottles) and wort (same keg). Mean copper concentrations in both media were calculated as µl/L as well as the respective standard deviations. These copper concentrations were then converted to mM concentrations.

5.2 Microarrays

As stated in section 2.8.1.1 the strains *S. cerevisiae* BY4741 (Euroscarf) and *S. ellipsoids var. diastaticus* (SABMiller) were cultured in wort and beer, with and without silver nitrate (0.1 mM) at 30°C for 20 hours. These cells were harvested via centrifugation and supernatant discarded. All conditions were

repeated in triplicate, with the yeasts being innoculated into the replicate growth conditions from the same culture. RNA from both strains was extracted using the Ambion RiboPure^{TM} Yeast RNA extraction kit. The purity and quantity of the extracted RNA was assessed using spectroscopy (§2.8.1.2). The NanoVue calculated RNA quantity as ng/µl (Table 5.2). The purity was assessed via the A_{260}:A_{280} ratio. RNA samples with a ratio between 1.7-2.2 were diluted to 200 ng/µl in 15 µl total volume using DEPEC water (Fisher). These samples were used for Microarray analysis by NASC. The Microarray data received from NASC was analysed using the Partec Express™ software (§2.8.1). Using the software to compare gene expression in two condition batches (e.g. gene expression of *S. ellipsoids* var. *diastaticus* in wort compared to in wort with 0.1 mM silver nitrate, both performed in triplicate) produced fold change data. The significance of gene expression (fold) change between sample batches was determined via ANOVA within the software (as described in §2.8.1.3.7). The cut off P-value for significance in the experiments is given as 0.05. Results which did not meet this requirement in the equation of 0.05*(n/m) were regarded as insignificant (Benjamini and Hochberg, 1995). Significant results were ranked in order of number of fold changes in both up and down-regulated genes. Only results exhibiting a 2 fold or greater change in gene expression were classified as significant. Both the up and down-regulated genes were sorted into categories of gene function, via the Kyoto Encyclopedia of Genes and Genomes (KEGG). KEGG is a comprehensive database of biological information for the functions and processes of cells, organisms and ecosystems. The KEGG PATHWAY Mapping function allows the sorting of the genes identified via transcriptomics

into functional groups, such as biological processes, according to their pathway function by comparison to the KEGG PATHWAY database. Some genes may be involved in multiple cellular processes and so were listed in multiple functional categories. Comparisons of gene responses were made for *S. ellipsoids var. diastaticus* in wort and beer, with and without silver. Additionally the silver stress responses of the contaminant strain *S. ellipsoides var. diastaticus* were compared to the laboratory strain *S. cerevisiae* BY4741.

Wort - *Saccharomyces ellipsoides var. diastaticus*						
	Wort			**Wort (Silver)**		
	1	**2**	**3**	**1**	**2**	**3**
A260 nm	19.88	31.07	27.08	20.288	16.549	19.495
A280 nm	9.09	22.18	18.01	9.24	7.572	8.977
A260/A280	2.19	1.99	1.97	2.2	2.19	2.17
ng/µl	835	713	737	1014.4	1027.4	974.8

Wort - *Saccharomyces cerevisiae* BY4741						
	Wort			**Wort (Silver)**		
	1	**2**	**3**	**1**	**2**	**3**
A260 nm	23.15	5.01	4.79	4.649	2.690	3.042
A280 nm	12.77	3.34	3.22	2.135	1.242	1.407
A260/A280	1.9	2.037	2.047	2.18	2.17	2.16
ng/µl	214	131	123	232.5	134.5	152.1

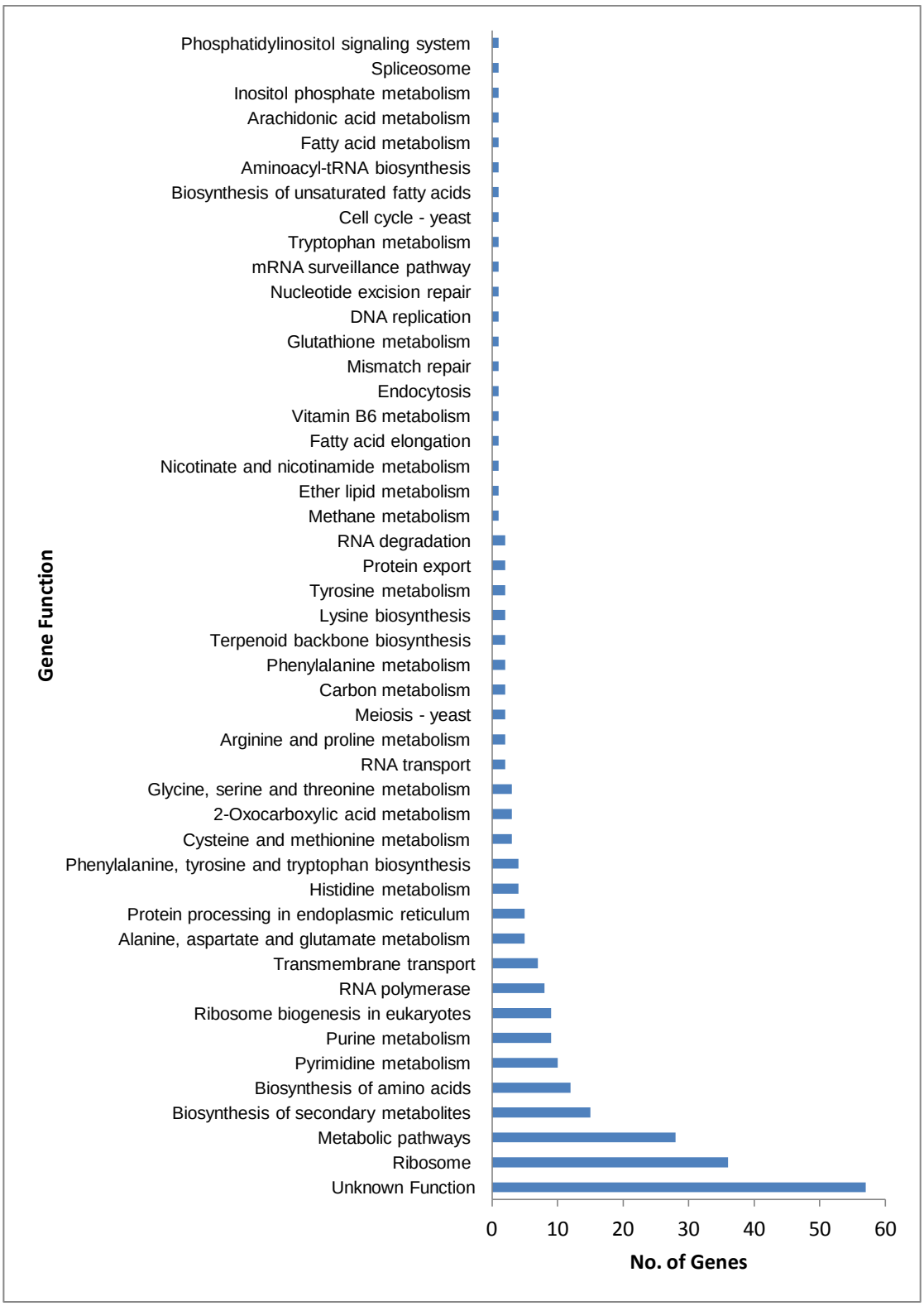
Beer - *Saccharomyces ellipsoides var. diastaticus*						
	Wort			**Wort (Silver)**		
	1	**2**	**3**	**1**	**2**	**3**
A260 nm	2.6	3.16	1.34	3.23	3.85	1.99
A280 nm	1.54	2.01	0.94	2.05	2.75	1.17
A260/A280	1.891	1.871	1.69	1.861	1.786	1.837
ng/µl	90	100	99	101.2	100	100

Table 5.2) Quantity and Purity of extracted RNA. RNA was extracted from *S. ellipsoides var. diastaticus* and *S. cerevisiae* BY4741 cultured in wort and beer, with and without silver (0.1 mM) for 20 hours at 30°C, in triplicate. Data given represents the quantity and quality results of the spectrophotometry assay. RNA quantities for each sample are given in ng/µl. RNA of sufficient purity requires a ratio of 1.7-2.2 between the values of the spectrophotometer readings at 260 nm and 280 nm.

5.2.1 Silver Stress in Wort

Excluding the genes of unknown molecular function, the majority of genes up-regulated in response to silver stress in wort by *S. ellipsoides var. diastaticus* (Fig. 5.1), are genes involved in the synthesis/maintenance of ribosome sub units, a variety of metabolic pathways and biosynthesis of amino acids. The metabolic pathways category is a total of all the metabolic pathway categories affected (e.g. pyrimidine metabolism). Other than genes with transmembrane transport (Table 5.3) and RNA and DNA synthesis functions, which make up the next largest group, the rest of the functional groups appear to encode genes involving the cell cycle and meiosis. The gene types most down-regulated (Fig. 5.2) appear to be genes involved in the metabolism of a variety of sugars followed by those involved in meiosis (HXT13, HXT17 and HXT5) which also act as transporters for hexose, fructose and glucose. Overall the genes most highly up-regulated were RPA12, an RNA polymerase sub-unit and PDR12, a plasma membrane ATP-binding cassette (ABC) transporter. The functions of the remaining ten most up-regulated genes (Table 5.4) consist of ribosome sub-units, rRNA processing, cell wall regulation and methionine, threonine and purine nucleotide biosynthesis. The most down-regulated genes were HXT13 and HXT5, both of which are Hexose transporters induced in the presence of non-fermentable carbon sources. The functions of the remaining ten most down-regulated genes (Table 5.5) concern cyclin transcriptional repression, gluconeogenesis pathway regulation, mitochondrial functions, cell wall protein and RNA binding required for sporulation.

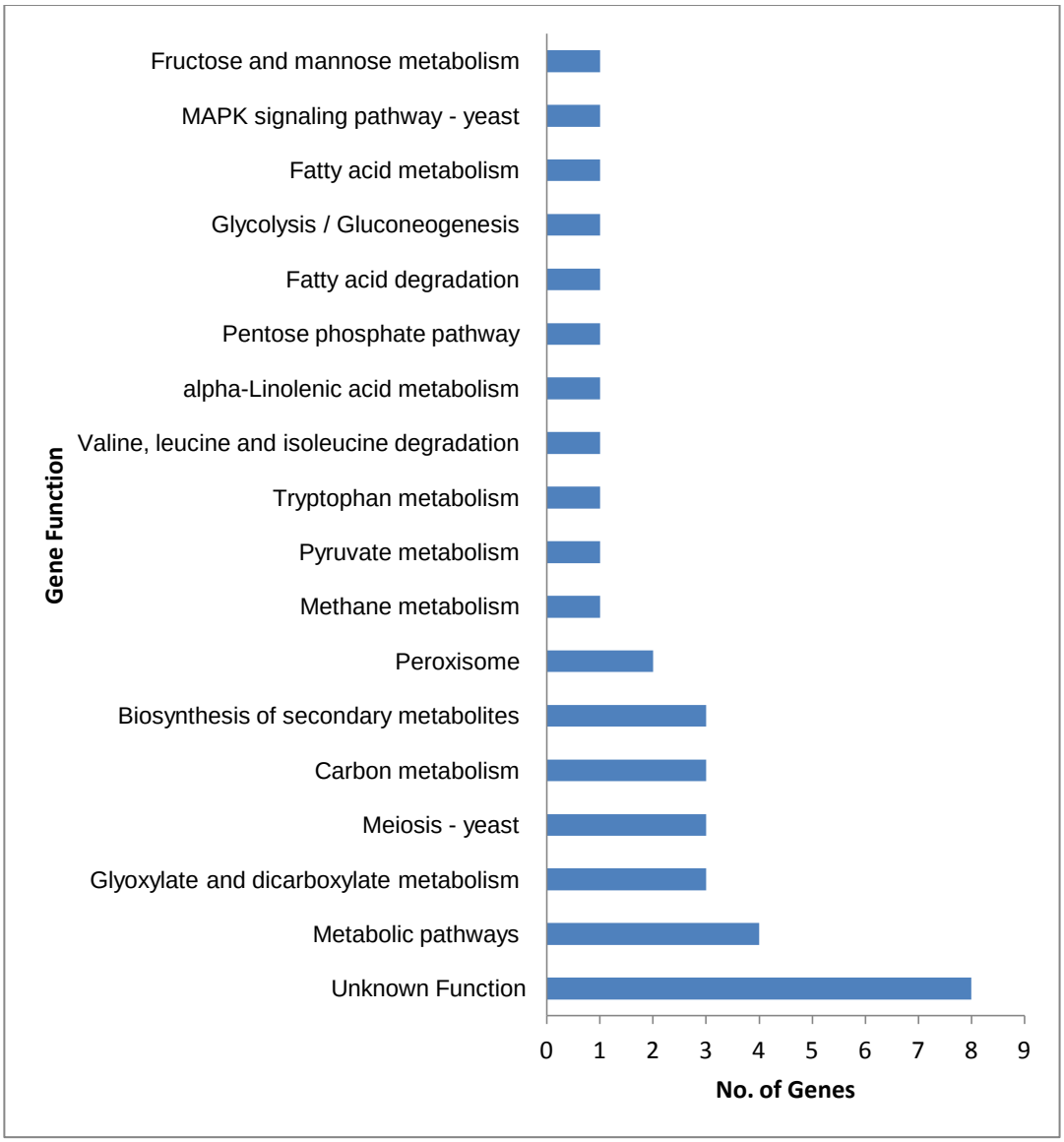
Figure 5.1) Graph of gene functions up-regulated in wort during silver stress sorted into activity categories. Data gained from transcriptome microarrays and analysed using Partec Express™ and KEGG Pathway mapper soft ware.



Transmembrane Transporter Activity		
Gene	**Fold Change**	**Function**
AQR1	2.43	Plasma membrane transporter; confers resistance to short-chain monocarboxylic acids and quinidine, relocalizes from plasma membrane to cytoplasm upon DNA replication stress
ATR1	2.32	Multidrug efflux pump; required for resistance to aminotriazole and 4-nitroquinoline-N-oxide; protein abundance increases in response to DNA replication stress
FLX1	2.12	Protein required for transport of flavin adenine dinucleotide (FAD)
HXT3	2.91	Low affinity glucose transporter
PDR12	8.77	Plasma membrane ATP-binding cassette (ABC) transporter, weak-acid-inducible multidrug transporter required for weak organic acid resistance
RIM2	2.17	Mitochondrial pyrimidine nucleotide transporter
YHM2	2.69	Carrier protein that exports citrate from and imports oxoglutarate into the mitochondrion, causing net export of NADPH reducing equivalents

Table 5.3) Transporter genes upregulated by *S. ellipsoids* var. *diastaticus* during silver stress in wort. Genes listed are from the functional activity groupTransmembrane Transporter Activity. Genes are listed next to their known function.

Figure 5.2) Graph of genes down-regulated in wort during silver stress sorted into activity categories. Data gained from transcriptome microarrays and analysed using Partec Express™ and KEGG Pathway mapper soft ware.



Up-Regulated Genes		
Gene	**Fold Change**	**Function**
RPA12	9.30	RNA polymerase I subunit A12.2; contains two zinc binding domains, and the N terminal domain is responsible for anchoring to the RNA pol I complex.
PDR12	8.77	Plasma membrane ATP-binding cassette (ABC) transporter; weak-acid-inducible multidrug transporter required for weak organic acid resistance; induced by sorbate and benzoate and regulated by War1p; mutants exhibit sorbate hypersensitivity.
CGR1	7.14	Protein involved in nucleolar integrity and processing of pre-rRNA; has a role in processing rRNA for the 60S ribosome subunit; transcript is induced in response to cytotoxic stress but not genotoxic stress; relocalizes from nucleus to nucleolus upon DNA replication stress.
RPS28B	6.75	Protein component of the small (40S) ribosomal subunit; homologous to mammalian ribosomal protein S28, no bacterial homolog.
HOM3	6.24	Aspartate kinase (L-aspartate 4-P-transferase); cytoplasmic enzyme that catalyzes the first step in the common pathway for methionine and threonine biosynthesis; expression regulated by Gcn4p and the general control of amino acid synthesis.
HLR1	6.21	Protein involved in regulation of cell wall composition and integrity; also involved in cell wall response to osmotic stress; overproduction suppresses a lysis sensitive PKC mutation; similar to Lre1p, which functions antagonistically to protein kinase A.
UTR2	5.90	Chitin transglycosylase; functions in the transfer of chitin to beta(1-6) and beta(1-3) glucans in the cell wall; similar to and functionally redundant with Crh1; glycosylphosphatidylinositol (GPI)-anchored protein localized to bud neck.
RRN11	5.89	Component of the core factor (CF) rDNA transcription factor complex; CF is required for transcription of 35S rRNA genes by RNA polymerase I and is composed of Rrn6p, Rrn7p, and Rrn11p.
ADE4	5.77	Phosphoribosylpyrophosphate amidotransferase (PRPPAT); catalyzes first step of the 'de novo' purine nucleotide biosynthetic pathway; also known as amidophosphoribosyltransferase.
RFU1	5.68	Protein that inhibits Doa4p deubiquitinating activity; contributes to ubiquitin homeostasis by regulating the conversion of free ubiquitin chains to ubiquitin monomers by Doa4p; GFP-fusion protein localizes to endosomes.

Table 5.4) Ten most up-regulated genes of *S. ellipsoids var. diastaticus* during silver stress in wort. Data gained from transcriptome microarrays and analysed using Partec Express™.

Down-Regulated Genes		
Gene	**Fold Change**	**Function**
HXT5	2.96	Hexose transporter with moderate affinity for glucose; induced in the presence of non-fermentable carbon sources, induced by a decrease in growth rate, contains an extended N-terminal domain relative to other HXTs.
HXT13	2.37	Hexose transporter; induced in the presence of non-fermentable carbon sources, induced by low levels of glucose, repressed by high levels of glucose.
XBP1	2.33	Transcriptional repressor; binds to promoter sequences of the cyclin genes, CYS3, and SMF2; expression is induced during mitosis, and late in meiosis; member of the Swi4p/Mbp1p family; potential Cdc28p substrate; relative distribution to the nucleus increases upon DNA replication stress.
HXT17	2.28	Hexose transporter; up-regulated in media containing raffinose and galactose at pH 7.7 versus pH 4.7, repressed by high levels of glucose; HXT17 has a paralog.
FBP1	2.21	Fructose-1,6-bisphosphatase; key regulatory enzyme in the gluconeogenesis pathway, required for glucose metabolism; undergoes either proteasome-mediated or autophagy-mediated degradation depending on growth conditions; glucose starvation results in redistribution to the periplasm; interacts with Vid30p.
CTT1	2.18	Cytosolic catalase T; has a role in protection from oxidative damage by hydrogen peroxide.
RIM4	2.14	Putative RNA-binding protein; required for the expression of early and middle sporulation genes .
MBR1	2.13	Protein involved in mitochondrial functions and stress response; overexpression suppresses growth defects of hap2, hap3, and hap4 mutants.
CAT2	2.12	Carnitine acetyl-CoA transferase; present in both mitochondria and peroxisomes; transfers activated acetyl groups to carnitine to form acetylcarnitine which can be shuttled across membranes.
PIR3	2.11	O-glycosylated covalently-bound cell wall protein; required for cell wall stability; expression is cell cycle regulated, peaking in M/G1 and also subject to regulation by the cell integrity pathway; coding sequence contains length polymorphisms in different strains.

Table 5.5) Ten most down-regulated genes of *S. ellipsoids var. diastaticus* during silver stress in wort. Data derived from transcriptome microarrays and analysed using Partec Express™.

5.2.2 Silver Stress in Beer

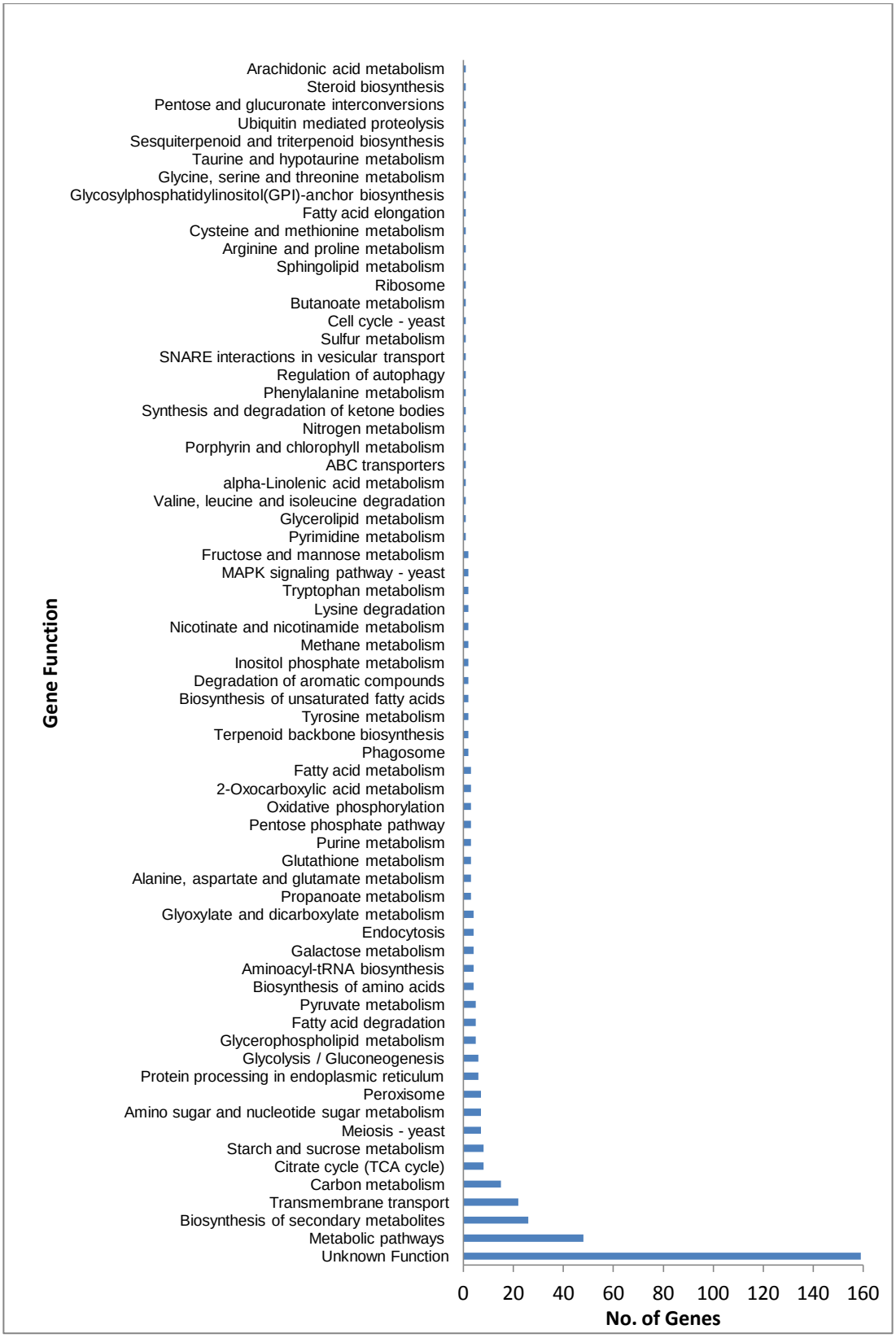
The gene categories most up-regulated in response to silver stress in beer (Fig. 5.3) are those associated withmetabolic pathways (e.g. COX17, a copper metallochaperone ion transporter in mitochondria), synthesis of secondary metabolites (e.g. ADH5, an alcohol dehydrogenase), transmembrane transport (Table 5.6) and energy production functions (carbon, starch and amino sugar metabolism, as well as TCA cycle genes). Other gene function categories which are up-regulated include both cell replication functions such as meiosis and cell cycle genes and structural functions such as ribosome sub-unit synthesis. The gene function categories down-regulated (Fig. 5.4) seem to primarily consist of metabolic pathways as well as ribosome biogenesis. RNA related functions such as RNA polymerase, degradation and transport are also down-regulated, as are mitosis genes (e.g. HXT4, a hexose, fructose, glucose and pentose transporter). The up-regulated genes with the highest fold change are CMK2, a calmodulin-dependent protein kinase and PUG1 a plasma membrane protein with roles in the uptake of protoprophyrin IX. The functions of the remaining ten most up-regulated genes (Table 5.7) consist of membrane transporter proteins, mitochondrial genes, cell wall maintenance and gluconeogenesis regulation. The down-regulated genes with the highest fold change was HXT4, a high-affinity glucose transporter. The functions of the remaining ten most down-regulated genes (Table 5.8) are comprised of plasma membrane riboflavin transporter, thiamin uptake, plasma membrane targeting dehydrogenase/reductase, ribosome biogenesis, RNA helicase, mitochondrial inner membrane ADP/ATP translocation proteins, and proteins of unknown function.

5.2.3 Comparison of Silver Stress in Wort and Beer

In the comparison of gene responses of yeast grown in wort and beer without silver (Fig. 5.5), the gene function categories most up-regulated in wort when compared to beer are those of metabolic pathways, secondary metabolite synthesis, as well as energy production functions (carbon, starch and amino sugar metabolism and TCA cycle genes). Additionally, cell replication pathway categories such as meiosis and cell cycle genes and cell maintenance categories such as protein processing/export and RNA degradation were also up-regulated. The gene categories most down-regulated in wort compared to beer are those of metabolic pathways and ribosome biogenesis and sub-unit synthesis. Additionally, cell replication pathway categories such as meiosis and cell cycle genes, and cell maintenance categories such as protein processing/export, DNA replication and RNA transport, polymerase and degradation were also down-regulated. (Fig. 5.6). When comparing transcriptional responses in wort to those in beer in the presence of silver (Fig. 5.7) there is little difference to the results seen where cells were grown without silver. Gene categories most up-regulated are those of metabolic pathway functions and energy productionfunctions. Other up-regulated categories include RNA degradation and transport and DNA synthesis. The gene categories most down-regulated (Fig. 5.8) also appear to be similar to those down-regulated without silver. The exception to this, are the functional categories of ribosome sub unit synthesis, DNA replication and RNA transport, RNA polymerase and RNA degradation. For these categories fewer genes were down-regulated in the presence of silver than in the absence. The genes by far the most up-regulated in wort compared to beer during silver

stress are DAN1 and PAU24, both of which are cell wall mannoproteins. Cell wall mannoproteins are often produced during fermentation, which would occur in wort more readily than beer, as part of the budding process (Blasco *et al.*, 2011). The functions of the remaining ten most up-regulated genes consist (Table 5.9) of additional cell wall mannoproteins, members of the seripauperin multigene family, vacuolar membrane amino acid permease and ergosterol biosynthesis regulation. The functions of the ten most down-regulated genes (Table 5.10) consist of plasma membrane permeases, transporters and proton symporters, galactose metabolism, stress induced ATPase, cell wall modulation, gluconeogenesis, glyoxylate cycle and methionine and alanine catabolism.

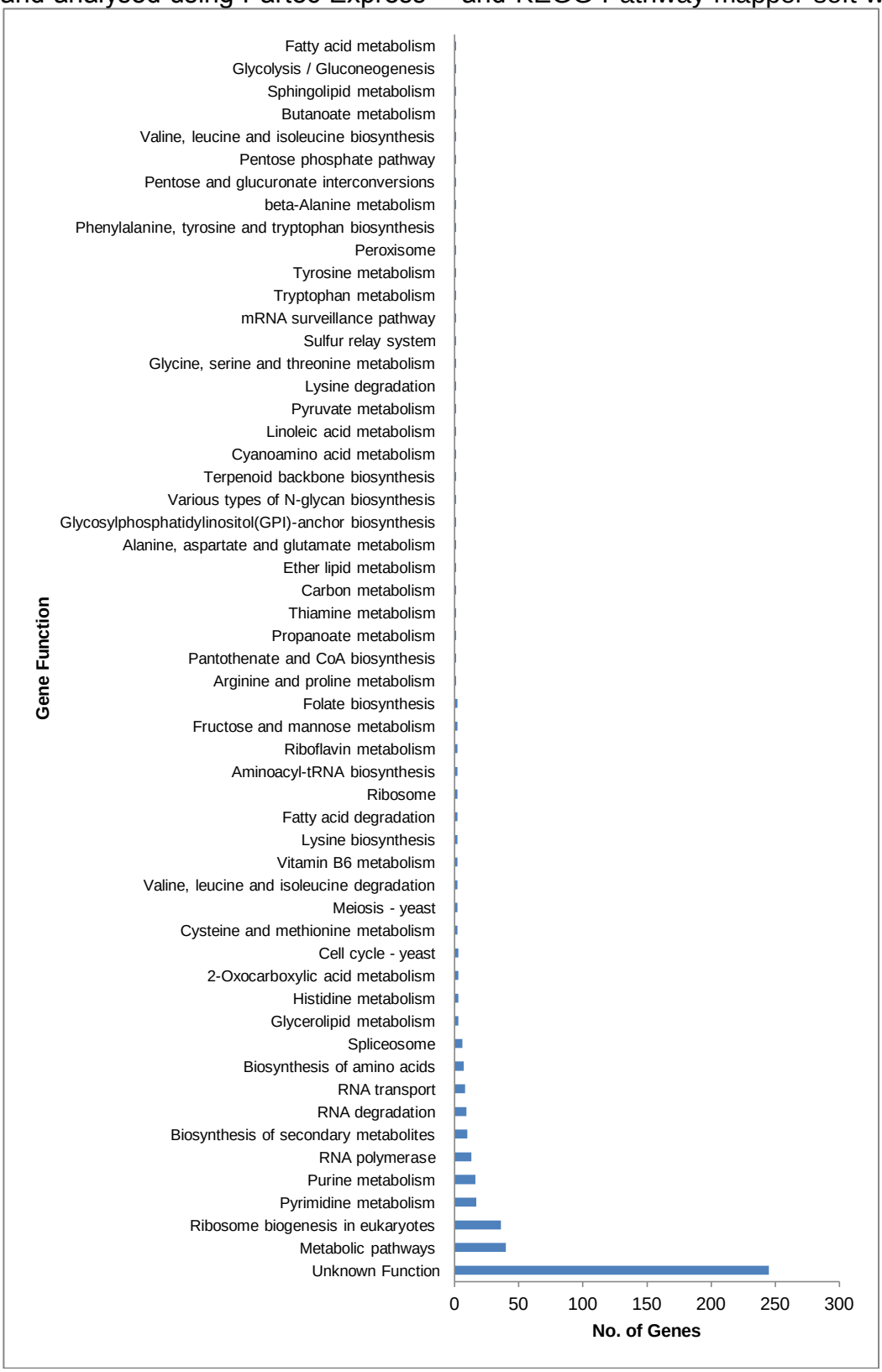
Figure 5.3) Graph of genes up-regulated in beer during silver stress sorted into activity categories. Data gained from transcriptome microarrays and analysed using Partec Express™ and KEGG Pathway mappersoft ware.



Transmembrane Transporter Activity		
Gene	**Fold Change**	**Function**
AGP2	2.8	Plasma membrane regulator of polyamine and carnitine transport
ATO2	2.75	Putative transmembrane protein involved in export of ammonia
AZR1	4.3	Plasma membrane transporter of the major facilitator superfamily; involved in resistance to azole drugs such as ketoconazole and fluconazole
CCC2	2.2	Cu(+2)-transporting P-type ATPase; required for export of copper from the cytosol into an extracytosolic compartment
DIC1	2.5	Mitochondrial dicarboxylate carrier
FMP37	2.04	Highly conserved subunit of the mitochondrial pyruvate carrier
GAL2	2.28	Galactose permease, required for utilization of galactose
GIT1	4.08	Plasma membrane permease; mediates uptake of glycerophosphoinositol and glycerophosphocholine
HXT2	5.32	High-affinity glucose transporter of the major facilitator superfamily, expression is induced by low levels of glucose
JEN1	2.58	Monocarboxylate/proton symporter of the plasma membrane; mediates high-affinity uptake of carbon sources lactate, pyuvate, and acetate
MPH3	2.51	Alpha-glucoside permease; transports maltose, maltotriose, alpha-methylglucoside, and turanose
PET8	2.93	S-adenosylmethionine transporter of the mitochondrial inner membrane
PHO89	2.2	Na+/Pi cotransporter
PMC1	6.73	Vacuolar Ca2+ ATPase involved in depleting cytosol of Ca2+ ions
PMR1	2.46	High affinity Ca2+/Mn2+ P-type ATPase required for Ca2+ and Mn2+ transport into Golgi
PXA1	2.6	Subunit of a heterodimeric peroxisomal ATP-binding cassette transporter complex (Pxa1p-Pxa2p), required for import of long-chain fatty acids
SGE1	3.21	Plasma membrane multidrug transporter, acts as an extrusion permease
SMF3	2.08	Putative divalent metal ion transporter involved in iron homeostasis
STV1	2.23	Subunit a of the vacuolar-ATPase V0 domain
SUL1	3.87	High affinity sulfate permease, mediates sulphate uptake
TPO4	8.19	Polyamine transporter
VNX1	2.12	Calcium/H+ antiporter localized to the endoplasmic reticulum membrane

Table 5.6) Transporter genes upregulated by *S. ellipsoids* var. *diastaticus* during silver stress in beer. Genes are from the Oxidoreductase Activity group

Figure 5.4) Graph of genes down-regulated in beer during silver stress sorted into activity categories. Data gained from transcriptome microarrays and analysed using Partec Express™ and KEGG Pathway mapper soft ware.



Up-Regulated Genes		
Gene	**Fold Change**	**Function**
CMK2	13.48	Calmodulin-dependent protein kinase; may play a role in stress response, many CA++/calmodulan dependent phosphorylation substrates demonstrated in vitro, amino acid sequence similar to mammalian Cam Kinase II.
PUG1	11.79	Plasma membrane protein involved in protoprophyrin and heme transport; roles in the uptake of protoprophyrin IX and the efflux of heme; expression is induced under both low-heme and low-oxygen conditions; member of the fungal lipid-translocating exporter (LTE) family of proteins.
UIP3	9.99	Putative integral membrane protein of unknown function; interacts with Ulp1p at the nuclear periphery; member of DUP240 gene family.
AIM17	9	Putative protein of unknown function; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies; null mutant displays reduced frequency of mitochondrial genome loss.
TPO4	8.19	Polyamine transporter of the major facilitator superfamily; member of the 12-spanner drug:H(+) antiporter DHA1 family; recognizes spermine, putrescine, and spermidine; localizes to the plasma membrane.
PMC1	6.73	Vacuolar Ca2+ ATPase involved in depleting cytosol of Ca2+ ions; prevents growth inhibition by activation of calcineurin in the presence of elevated concentrations of calcium.
YPS1	6.25	Aspartic protease; member of the yapsin family of proteases involved in cell wall growth and maintenance; attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor.
HEM15	5.43	Ferrochelatase; a mitochondrial inner membrane protein, catalyzes the insertion of ferrous iron into protoporphyrin IX, the eighth and final step in the heme biosynthetic pathway.
FBP1	5.41	Fructose-1,6-bisphosphatase; key regulatory enzyme in the gluconeogenesis pathway, required for glucose metabolism; undergoes either proteasome-mediated or autophagy-mediated degradation depending on growth conditions; glucose starvation results in redistribution to the periplasm.
HXT2	5.32	High-affinity glucose transporter of the major facilitator superfamily; expression is induced by low levels of glucose and repressed by high levels of glucose.

Table 5.7) Ten most up-regulated genes of *S. ellipsoids var. diastaticus* during silver stress in beer. Data gained from transcriptome microarrays and analysed using Partec Express™.

Up-Regulated Genes		
Gene	**Fold Change**	**Function**
ANS1	9.29	Putative protein of unknown function; transcription dependent upon Azf1p.
HXT4	6.77	High-affinity glucose transporter; member of the major facilitator superfamily, expression is induced by low levels of glucose and repressed by high levels of glucose.
MCH5	6.26	Plasma membrane riboflavin transporter; facilitates the uptake of vitamin B2; required for FAD-dependent processes
PHO3	5.98	Constitutively expressed acid phosphatase similar to Pho5p; brought to the cell surface by transport vesicles; hydrolyzes thiamin phosphates in the periplasmic space, increasing cellular thiamin uptake; expression is repressed by thiamine.
AST1	4.99	Lipid raft associated protein; interacts with the plasma membrane ATPase Pma1p and has a role in its targeting to the plasma membrane by influencing its incorporation into lipid rafts; sometimes classified in the medium-chain dehydrogenase/reductases (MDRs) superfamily.
RSA4	4.94	WD-repeat protein involved in ribosome biogenesis; may interact with ribosomes; required for maturation and efficient intra-nuclear transport or pre-60S ribosomal subunits, localizes to the nucleolus.
HOR2	4.84	DL-glycerol-3-phosphate phosphatase involved in glycerol biosynthesis; also known as glycerol-1-phosphatase; induced in response to hyperosmotic or oxidative stress, and during diauxic shift.
JJJ3	4.82	Protein of unknown function; contains a CSL Zn finger and a DnaJ-domain; involved in diphthamide biosynthesis.
DHR2	4.59	Predominantly nucleolar DEAH-box ATP-dependent RNA helicase; required for 18S rRNA synthesis.
AAC3	4.48	Mitochondrial inner membrane ADP/ATP translocator; exchanges cytosolic ADP for mitochondrially synthesized ATP; expressed under anaerobic conditions; similar to Aac1p; has roles in maintenance of viability and in respiration.

Table 5.8) Ten most down-regulated genes of *S. ellipsoids var. diastaticus* during silver stress in beer. Data gained from transcriptome microarrays and analysed using Partec Express™.

Figure 5.5) Graph of genes up-regulated in wort compared to beer without silver stress, sorted into activity categories. Data gained from transcriptome microarrays and analysed using Partec Express™ and KEGG Pathway mapper soft ware.

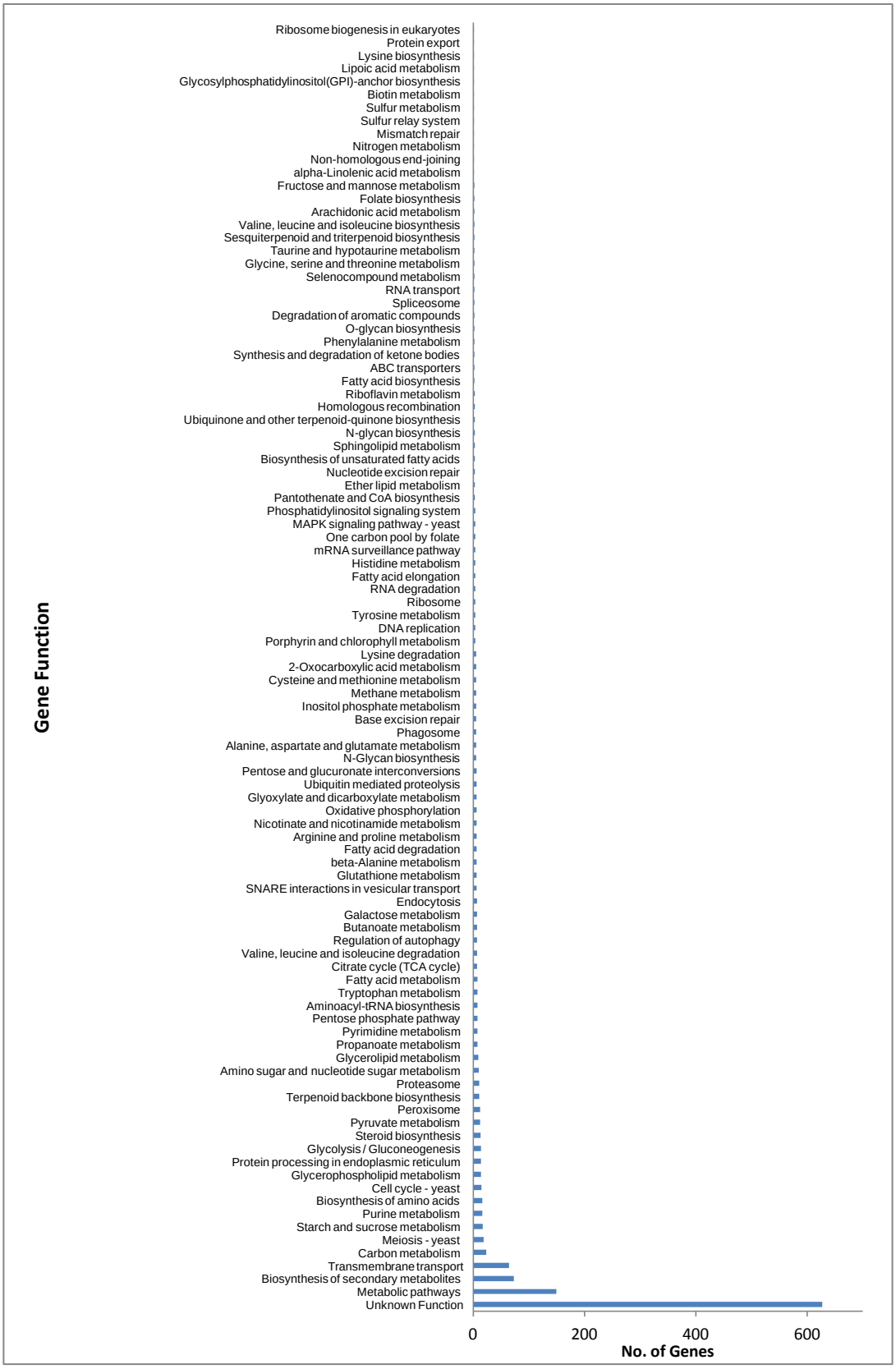


Figure 5.6) Graph of genes down-regulated in wort compared to beer without silver stress, sorted into activity categories. Data gained from transcriptome microarrays and analysed using Partec Express™ and KEGG Pathway mapper soft ware.

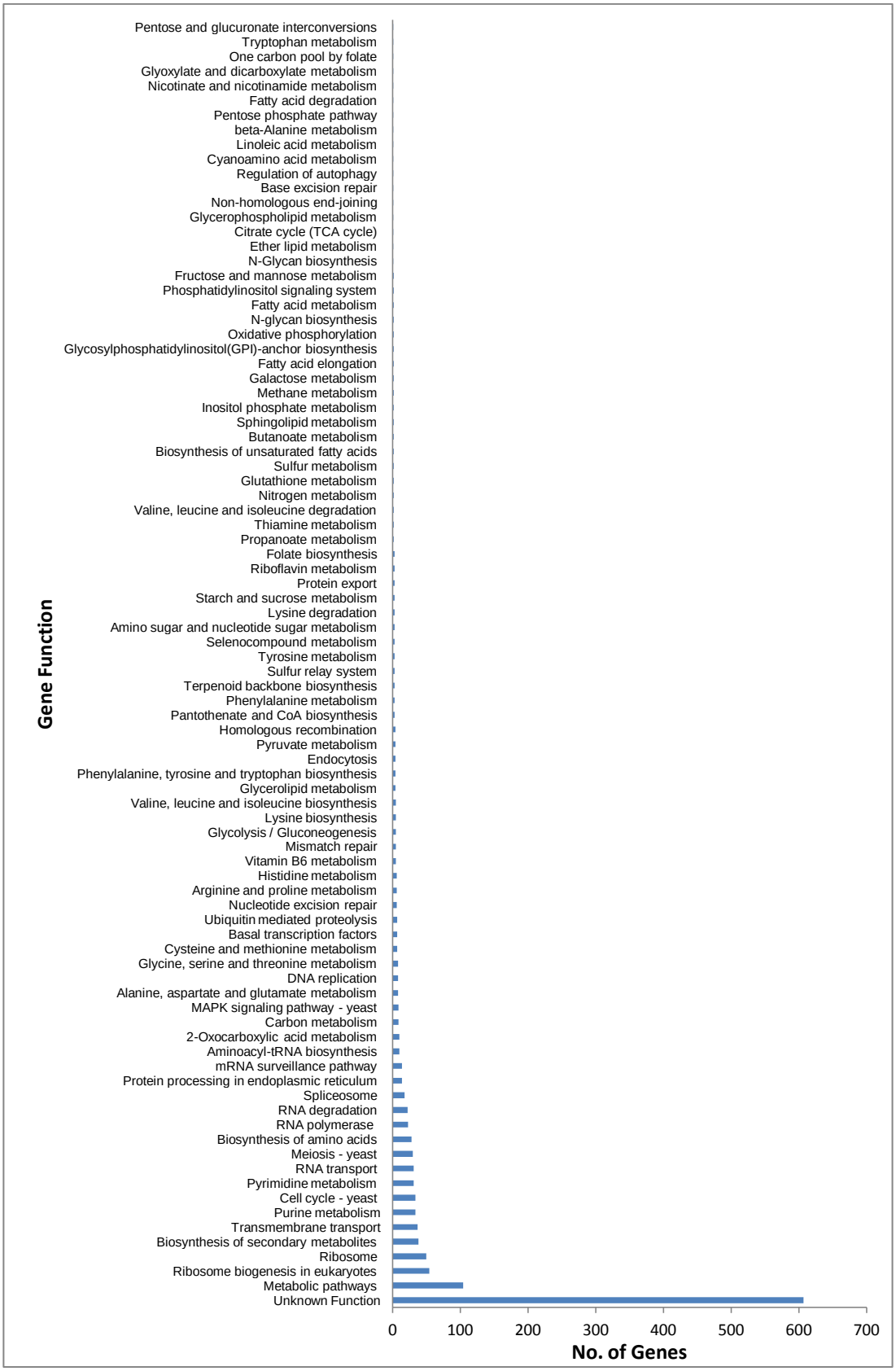


Figure 5.7) Graph of genes up-regulated in wort compared to beer during silver stress, sorted into activity categories. Data gained from transcriptome microarrays and analysed using Partec Express™ and KEGG Pathway mappersoft ware.

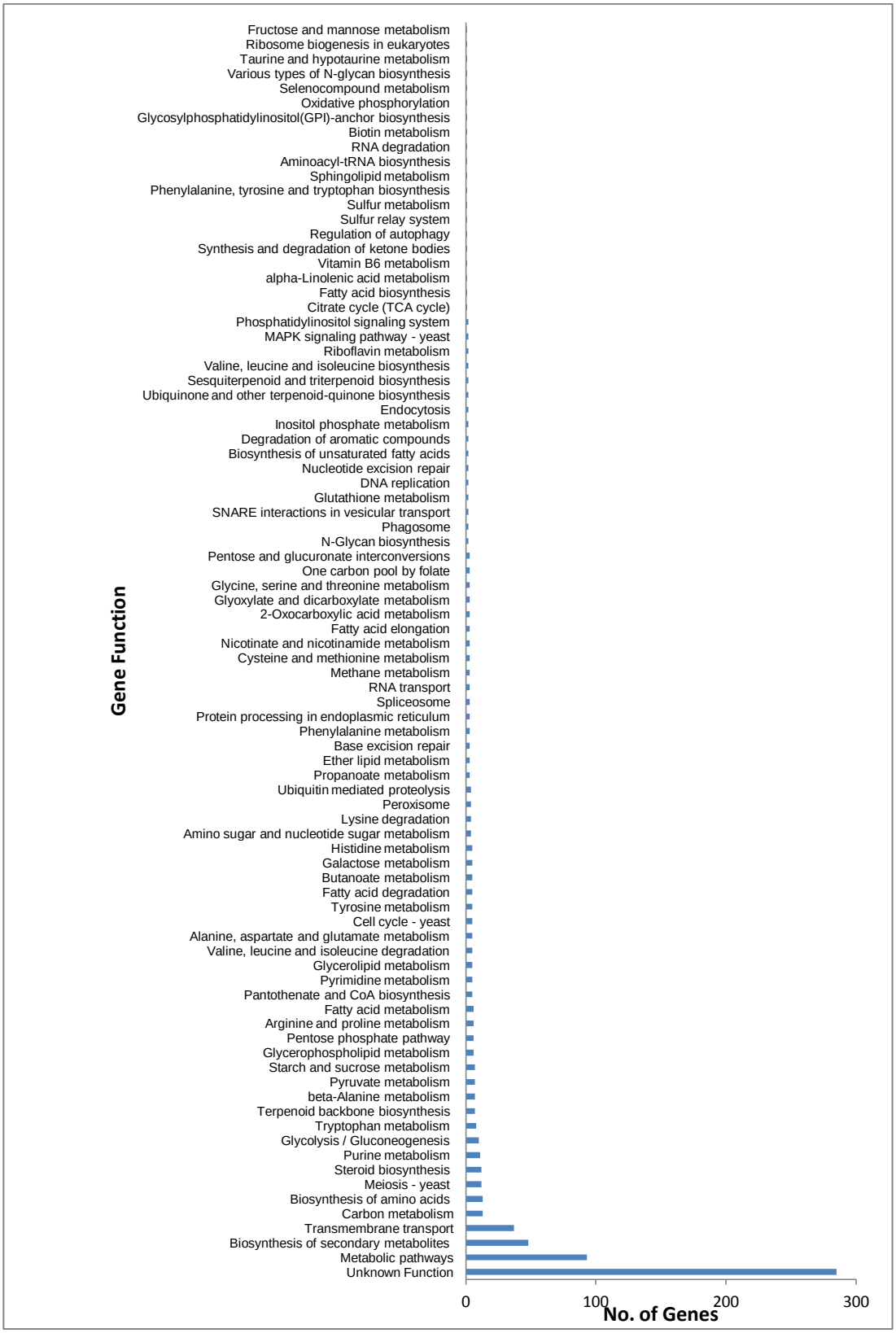
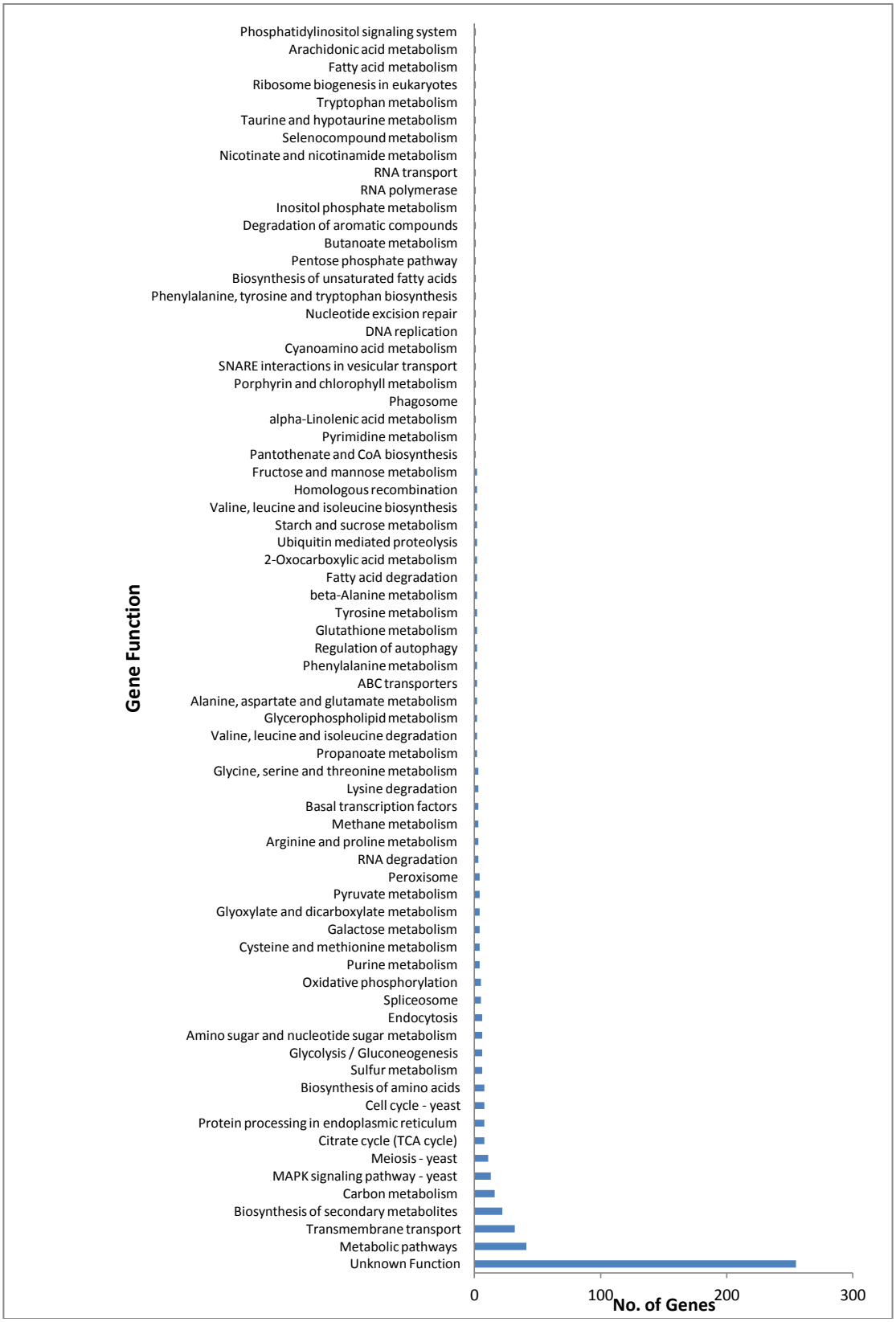


Figure 5.8) Graph of genes down-regulated in wort compared to beer during silver stress, sorted into activity categories. Data gained from transcriptome microarrays and analysed using Partec Express™ and KEGG Pathway mappersoft ware.



Up-Regulated Genes		
Gene	**Fold Change**	**Function**
FHN1	103.41	Protein of unknown function; induced by ketoconazole; promoter region contains sterol regulatory element motif, which has been identified as a Upc2p-binding site; overexpression complements function of Nce102p in NCE102 deletion strain.
DAN1	100.48	Cell wall mannoprotein; has similarity to Tir1p, Tir2p, Tir3p, and Tir4p; expressed under anaerobic conditions, completely repressed during aerobic growth.
PAU24	48.86	Cell wall mannoprotein; has similarity to Tir1p, Tir2p, Tir3p, and Tir4p; member of the seripauperin multigene family encoded mainly in subtelomeric regions; expressed under anaerobic conditions, completely repressed during aerobic growth.
VBA3	44.84	Permease of basic amino acids in the vacuolar membrane
PAU5	38.41	Member of the seripauperin multigene family; encoded mainly in subtelomeric regions; induced during alcoholic fermentation; induced by low temperature and also by anaerobic conditions; negatively regulated by oxygen and repressed by heme.
HES1	35.12	Protein implicated in the regulation of ergosterol biosynthesis; one of a seven member gene family with a common essential function and non-essential unique functions.
TIR1	34.35	Cell wall mannoprotein; expression is downregulated at acidic pH and induced by cold shock and anaerobiosis; abundance is increased in cells cultured without shaking; member of the Srp1p/Tip1p family of serine-alanine-rich proteins.
TIR3	31.15	Cell wall mannoprotein; member of Srp1p/Tip1p family of serine-alanine-rich proteins; expressed under anaerobic conditions and required for anaerobic growth.
PAU7	30.43	Member of the seripauperin multigene family; active during alcoholic fermentation, regulated by anaerobiosis, inhibited by oxygen, repressed by heme.
PAU17	25.81	Protein of unknown function, member of the seripauperin multigene family encoded mainly in subtelomeric regions.

Table 5.9) Ten most up-regulated genes of *S. ellipsoids var. diastaticus* in wort compared to beer, during silver stress. Data gained from transcriptome microarrays and analysed using Partec Express™.

Down-Regulated Genes		
Gene	**Fold Change**	**Function**
GIT1	48.04	Plasma membrane permease; mediates uptake of glycerophosphoinositol and glycerophosphocholine as sources of the nutrients inositol and phosphate; expression and transport rate are regulated by phosphate and inositol availability.
AZR1	20.29	Plasma membrane transporter of the major facilitator superfamily; involved in resistance to azole drugs such as ketoconazole and fluconazole.
SUL1	20.23	High affinity sulfate permease of the SulP anion transporter family; sulfate uptake is mediated by specific sulfate transporters Sul1p and Sul2p, which control the concentration of endogenous activated sulfate intermediates.
GAL10	12.92	UDP-glucose-4-epimerase; catalyzes the interconversion of UDP-galactose and UDP-D-glucose in galactose metabolism; also catalyzes the conversion of alpha-D-glucose or alpha-D-galactose to their beta-anomers.
HSP30	12.75	Negative regulator of the H(+)-ATPase Pma1p; stress-responsive protein; hydrophobic plasma membrane localized; induced by heat shock, ethanol treatment, weak organic acid, glucose limitation, and entry into stationary phase.
RPI1	11.03	Transcription factor, allelic differences between S288C and Sigma1278b; mediates fermentation stress tolerance by modulating cell wall integrity; overexpression suppresses heat shock sensitivity of wild-type RAS2 overexpression and also suppresses cell lysis defect of mpk1 mutation; allele from S288c can confer fMAPK pathway independent transcription of FLO11; S288C and Sigma1278b alleles differ in number of tandem repeats within ORF.
STL1	10.82	Glycerol proton symporter of the plasma membrane; subject to glucose-induced inactivation, strongly but transiently induced when cells are subjected to osmotic shock.
PCK1	10.51	Phosphoenolpyruvate carboxykinase; key enzyme in gluconeogenesis, catalyzes early reaction in carbohydrate biosynthesis, glucose represses transcription and accelerates mRNA degradation, regulated by Mcm1p and Cat8p, located in the cytosol.
MLS1	9.61	Malate synthase, enzyme of the glyoxylate cycle; involved in utilization of non-fermentable carbon sources; expression is subject to carbon catabolite repression; localizes in peroxisomes during growth on oleic acid, otherwise cytosolic; can accept butyryl-CoA as acyl-CoA donor in addition to traditional substrate acetyl-CoA.
ARO10	9.04	Phenylpyruvate decarboxylase; catalyzes decarboxylation of phenylpyruvate to phenylacetaldehyde, which is the first specific step in the Ehrlich pathway; involved in protein N-terminal Met and Ala catabolism.

Table 5.10) Ten most down-regulated genes of *S. ellipsoids var. diastaticus* in wort compared to beer, during silver stress. Data gained from transcriptome microarrays and analysed using Partec Express™.

5.2.4 Comparison of Gene Responses in Brewing Contaminant and Laboratory Strain Yeast

The comparison of gene responses of the brewery contaminant strain *S. ellipsoides var. diastaticus* and the laboratory strain *S. cerevisiae* BY4741 was only performed in wort. The gene function categories most up-regulated in the diastatic strain in comparison to the laboratory strain are metabolic pathways and genes involved in the biosynthesis of secondary metabolites and amino acids (Fig. 5.9). Other highly up-regulated groups of genes include ribosome sub-unit formation and ribosome biogenesis. Additionally, the functional categories: meiosis (sugar transporters and DNA helicases), cell cycle, DNA replication and RNA transport, RNA polymerase and RNA degradation are also up-regulated. *S. ellipsoides var. diastaticus* functional categories most down-regulated (Fig. 5.10) in comparison to the laboratory strain include metabolic pathways and biosynthesis of secondary metabolites and amino acids. Other down-regulated groups of genes include those involved in meiosis, cell cycle and TCA cycle. The most up-regulated gene function groups in the diastatic strain during silver stress (Fig. 5.11) compared to the lab strain are similar to those in the absence of silver stress. However, during silver stress more genes are expressed in many categories. The functional groups down-regulated (Fig. 5.12) are similar to those down-regulated in the absence of silver. However, during silver stress the number of down-regulated genes increased in the metabolic pathways, Biosynthesis of secondary metabolites, carbon metabolism categories and TCA cycle. Additionally, the number of down-regulated genes decreased in the categories meiosis and cell cycle. The three most up-regulated genes (highest fold increase) in the diastatic strain compared to the lab strain are URA3; an

orotidine-5'-phosphate (OMP) decarboxylases, MET17; a methionine and cysteine synthase and LEU2; a beta-isopropylmalate dehydrogenase (IMDH). The functions of the remaining ten most up-regulated genes (Table 5.11) consist of isomaltose utilization, maltose catabolism, vacuolar membrane amino acid permease, ferric reductase and sterol and lipid biosynthesis. The functions of the ten most down-regulated genes (Table 5.12) consist of a haze-protective mannoprotein, asparagine catabolism, alpha-glucosidase, enolase regulation, endosomal iron transport, mating pheromone factors and proteins and hexose transport.

Figure 5.9) Graph of genes up-regulated in *Saccharomyces ellipsoides* var. *diastaticus* compared to *Saccharomyces cerevisiae* BY4741 in wort, sorted into activity categories. Data gained from transcriptome microarrays and analysed using Partec Express™ and KEGG Pathway mapper soft ware.

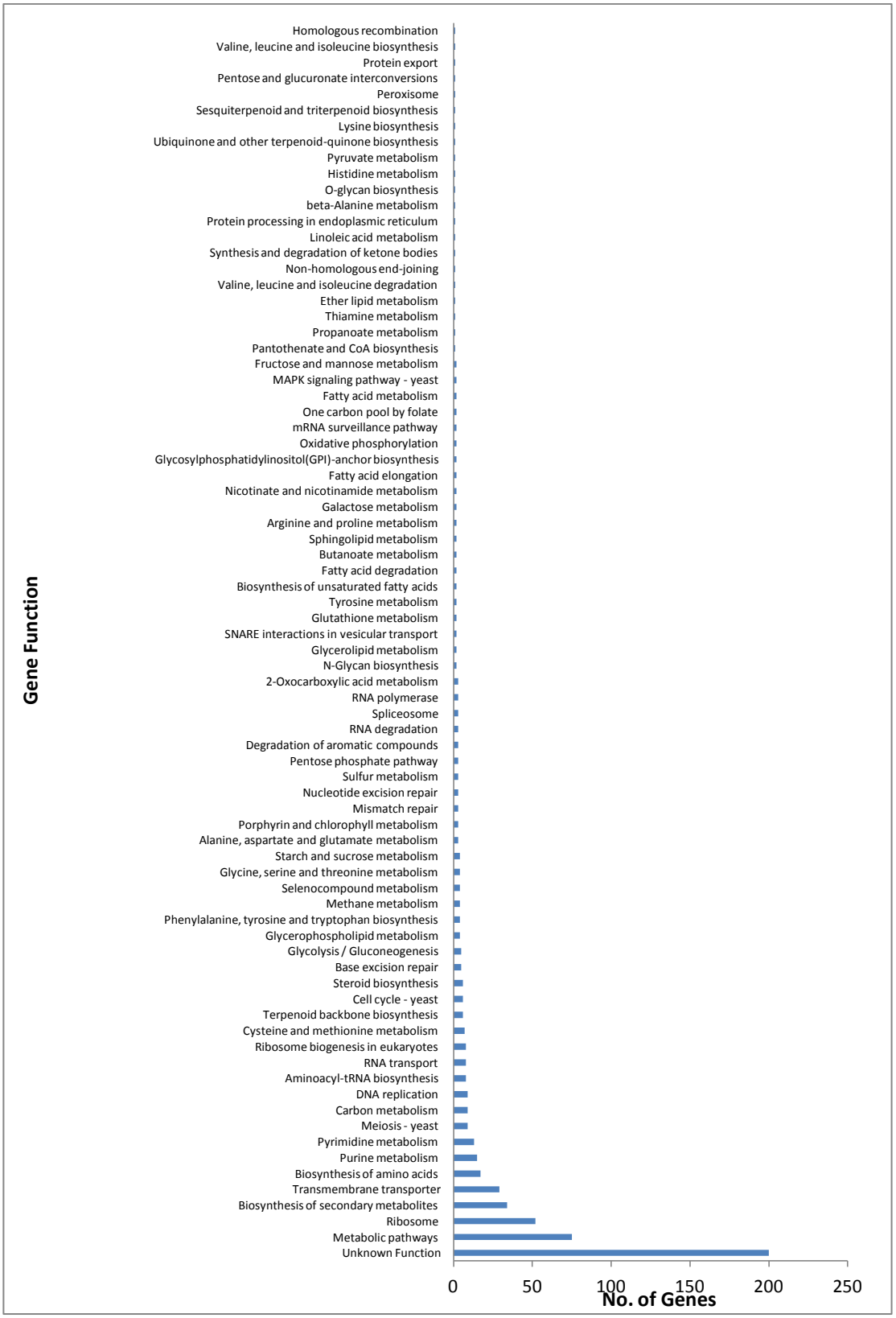


Figure 5.10) Graph of genes down-regulated in *Saccharomyces ellipsoids* var. *diastaticus* compared to *Saccharomyces cerevisiae* BY4741 in wort, sorted into activity categories. Data gained from transcriptome microarrays and analysed using Partec Express™ and KEGG Pathway mapper soft ware.

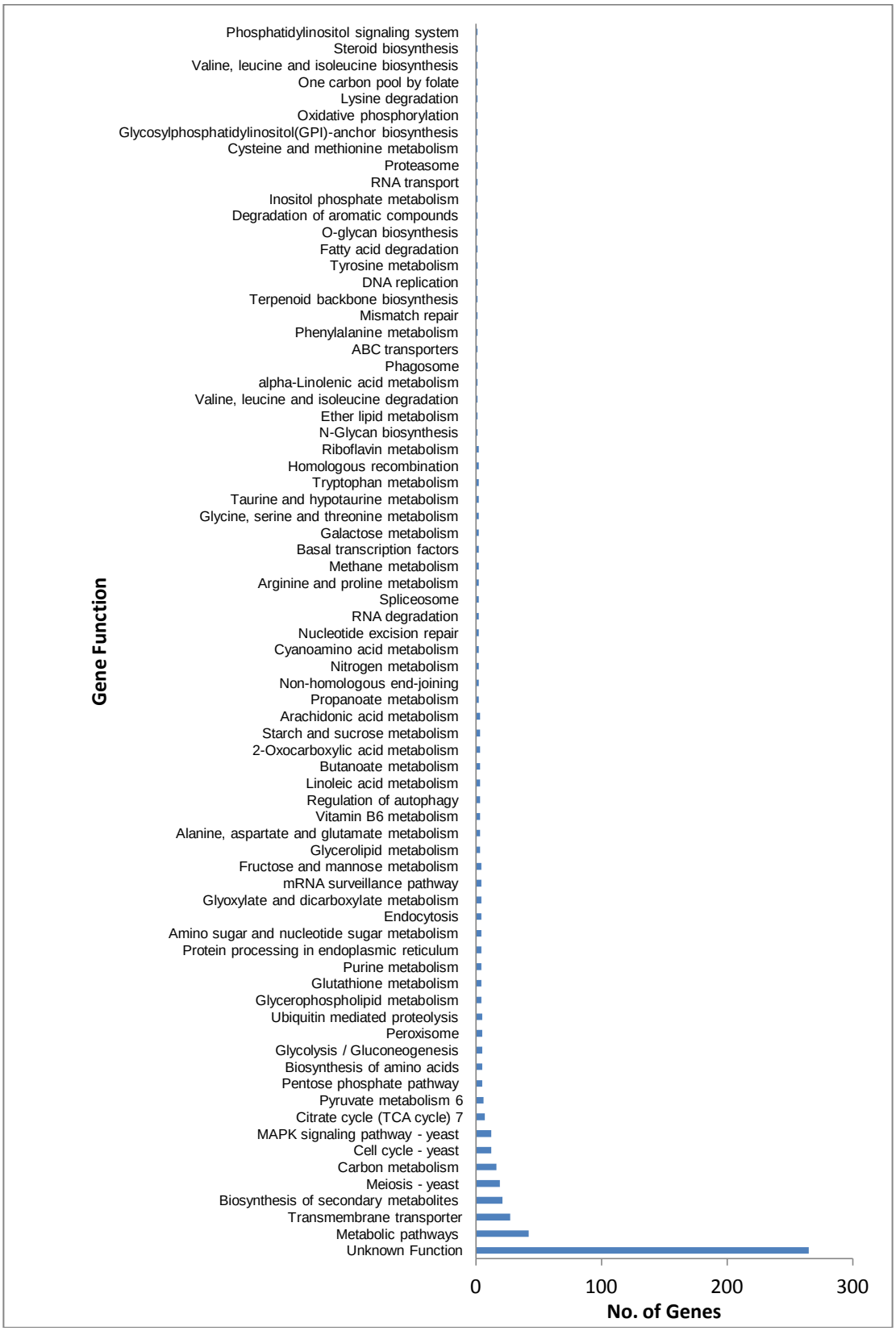


Figure 5.11) Graph of genes up-regulated in *Saccharomyces ellipsoids* var. *diastaticus* compared to *Saccharomyces cerevisiae* BY4741 in wort during silver stress, sorted into activity categories. Data gained from transcriptome microarrays and analysed using Partec Express™ and KEGG Pathway mapper soft ware.

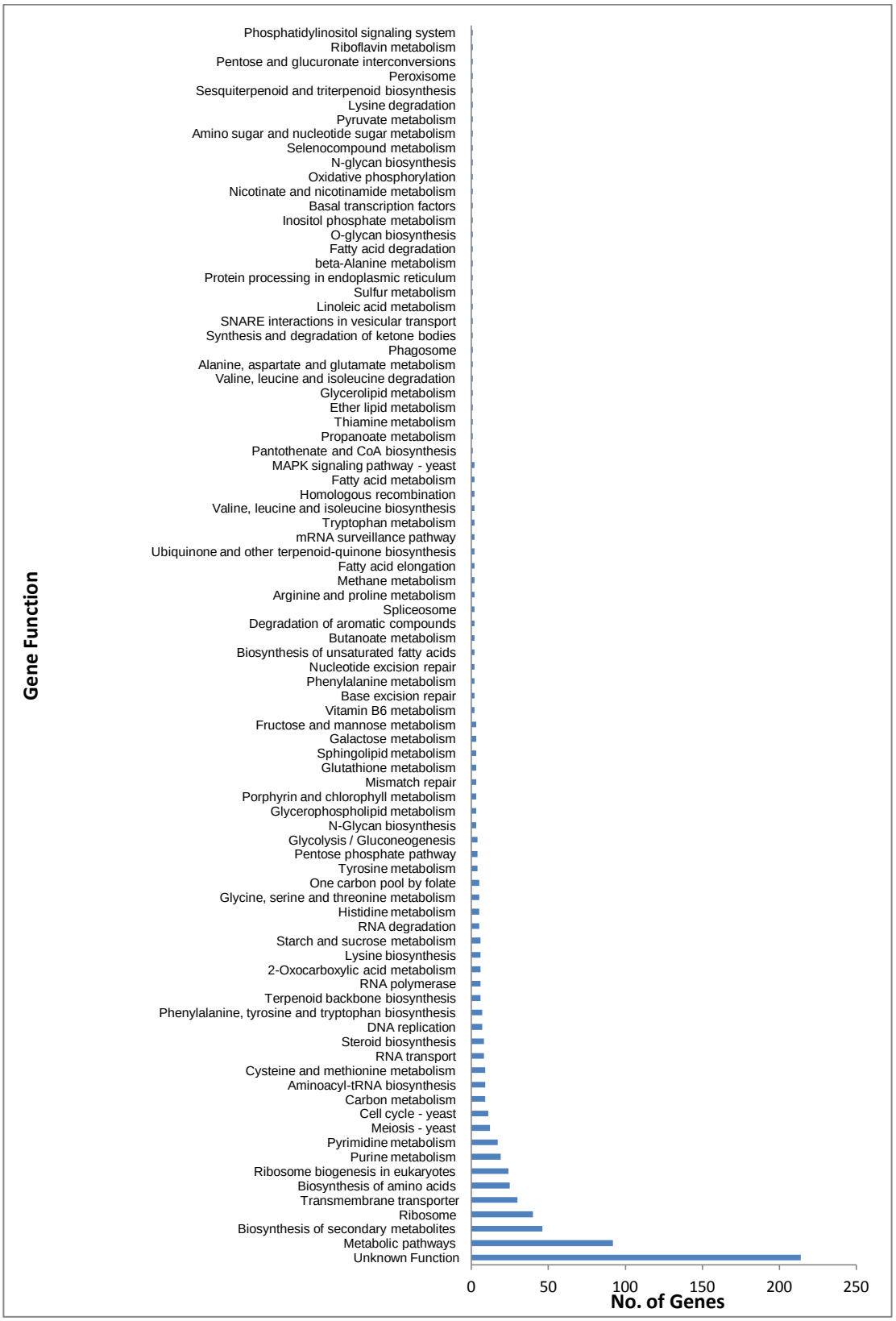
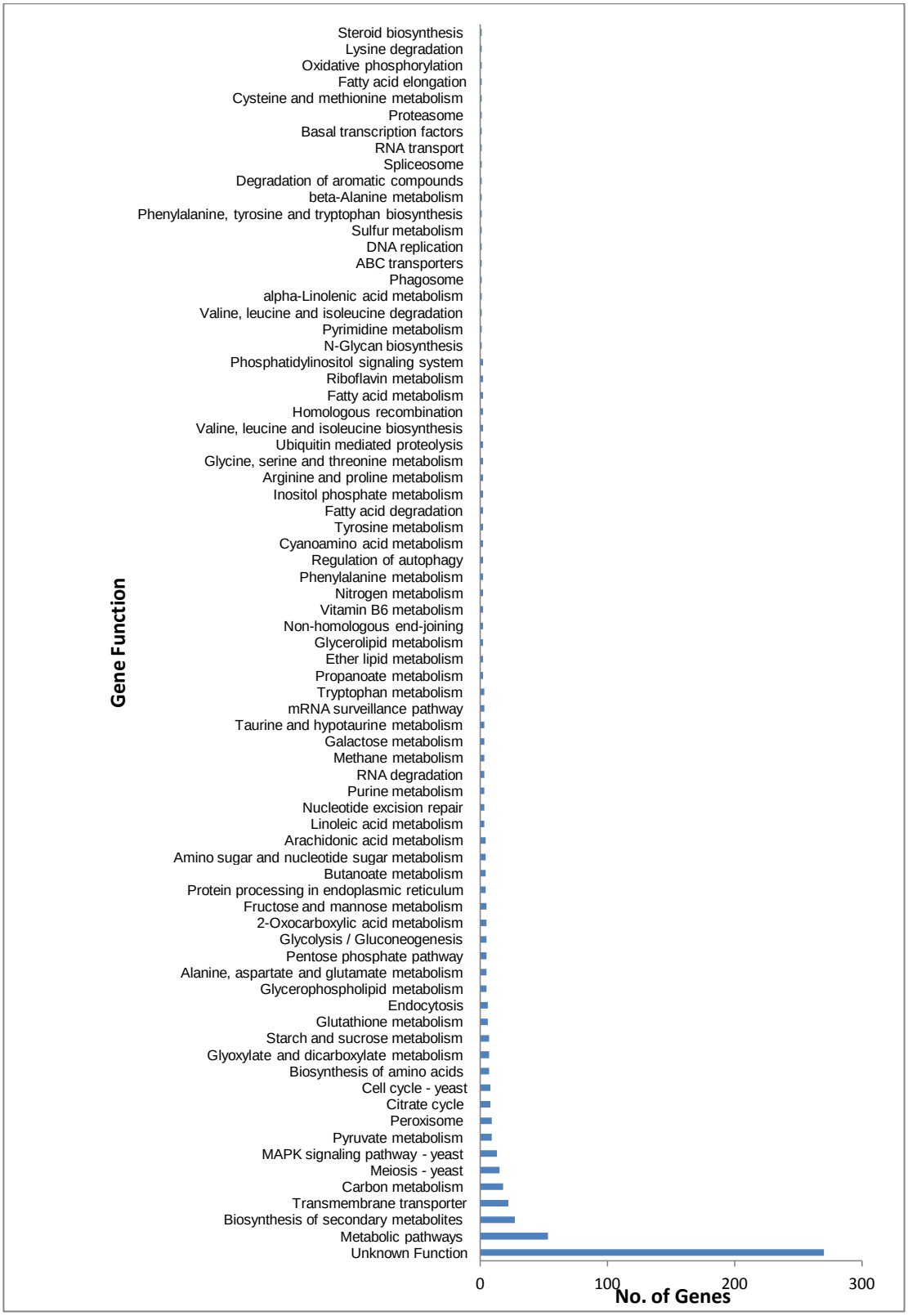


Figure 5.12) Graph of genes down-regulated in *Saccharomyces ellipsoids* var. *diastaticus* compared to *Saccharomyces cerevisiae* BY4741 in wort during silver stress, sorted into activity categories. Data gained from transcriptome microarrays and analysed using Partec Express™ and KEGG Pathway mapper soft ware.



Up-Regulated Genes		
Gene	**Fold Change**	**Function**
URA3	106.89	Orotidine-5'-phosphate (OMP) decarboxylase; catalyzes the sixth enzymatic step in the de novo biosynthesis of pyrimidines, converting OMP into uridine monophosphate (UMP); converts 5-FOA into 5-fluorouracil, a toxic compound.
MET17	93.26	O-acetyl homoserine-O-acetyl serine sulfhydrylase; required for Methionine and cysteine biosynthesis.
LEU2	85.42	Beta-isopropylmalate dehydrogenase (IMDH); catalyzes the third step in the leucine biosynthesis pathway; can additionally catalyze the conversion of β-ethylmalate into α-ketovalerate.
IMA1	39.5	Major isomaltase (alpha-1,6-glucosidase/alpha-methylglucosidase); required for isomaltose utilization; specificity for isomaltose, alpha-methylglucoside, and palatinose; member of the IMA isomaltase family.
VBA3	34.1	Permease of basic amino acids in the vacuolar membrane .
VEL1	30.36	Protein of unknown function; highly induced in zinc-depleted conditions and has increased expression in NAP1 deletion mutants.
MAL12	20.07	Maltase (alpha-D-glucosidase); inducible protein involved in maltose catabolism; encoded in the MAL1 complex locus; hydrolyzes the disaccharides maltose, turanose, maltotriose, and sucrose.
FRE4	18.24	Ferric reductase; reduces a specific subset of siderophore-bound iron prior to uptake by transporters; expression induced by low iron levels.
PAU15	17.05	Protein of unknown function; member of the seripauperin multigene family encoded mainly in subtelomeric regions.
CYB5	15.32	Cytochrome b5; involved in the sterol and lipid biosynthesis pathways; acts as an electron donor to support sterol C5-6 desaturation.

Table 5.11) Ten most up-regulated genes of *S. ellipsoids var. diastaticus* in comparison to *S. cerevisiae BY4741*, during silver stress. Data gained from transcriptome microarrays and analysed using Partec Express™.

Down-Regulated Genes		
Gene	**Fold Change**	**Function**
HPF1	448.7	Haze-protective mannoprotein; reduces the particle size of aggregated proteins in white wines.
ASP3-1	245.1	Cell-wall L-asparaginase II involved in asparagine catabolism; expression induced during nitrogen starvation; ORF contains a short non-coding RNA that enhances expression of full-length gene; likely arose in via horizontal gene transfer from the wine yeast Wickerhamomyces anomalus or a close relative; reference strain S288C has four copies of ASP3.
MFA2	68.08	Mating pheromone a-factor; made by a cells; interacts with alpha cells to induce cell cycle arrest and other responses leading to mating; biogenesis involves C-terminal modification, N-terminal proteolysis, and export; also encoded by MFA1.
IMA2	61.35	Alpha-glucosidase with specificity for isomaltase, methyl-alpha-glucoside, and palatinose.
REE1	52.25	Cytoplasmic protein involved in the regulation of enolase (ENO1); mRNA expression is induced by calcium shortage, copper deficiency (via Mac1p) and the presence of galactose (via Gal4p); mRNA expression is also regulated by the cell cycle.
COS6	49.15	Protein of unknown function; member of the DUP380 subfamily of conserved, often subtelomerically-encoded proteins.
ENB1	42.58	Endosomal ferric enterobactin transporter; expressed under conditions of iron deprivation; member of the major facilitator superfamily; expression is regulated by Rcs1p and affected by chloroquine treatment.
PRM8	35.84	Pheromone-regulated protein; contains with 2 predicted transmembrane segments and an FF sequence, a motif involved in COPII binding; forms a complex with Prp9p in the ER; member of DUP240 gene family.
MFA1	35.26	Mating pheromone a-factor; made by a cells; interacts with alpha cells to induce cell cycle arrest and other responses leading to mating; biogenesis involves C-terminal modification, N-terminal proteolysis, and export; also encoded by MFA2.
HXT5	34.83	Hexose transporter with moderate affinity for glucose; induced in the presence of non-fermentable carbon sources, induced by a decrease in growth rate, contains an extended N-terminal domain relative to other HXTs.

Table 5.12) Ten most down-regulated genes of *S. ellipsoids var. diastaticus* in comparison to *S. cerevisiae* BY4741, during silver stress. Data gained from transcriptome microarrays and analysed using Partec Express™.

5.3 Confirmation of Selected Genes involvement in Silver Tolerance via Gene-knockout Experiments

Ten genes (§2.8.3) were selected for investigation into their role in aiding silver tolerance. These were selected from both a survey of the literature and the microarray experimental results (§5.2). All gene knockout strains were purchased from Euroscarf and all knockouts are based on the wildtype strain *S. cerevisiae* BY4741. The exception to this is the deletion of CUP1 in *Saccharomyces cerevisiae* DTY3, to create the knockout strain DTY4. CUP 1 mutants were provided by Dr. Dennis J. Thiele (§2.8.3) as these were not available from Euroscarf.

5.3.1 Confirmation of Gene-knockout Identity via Traditional Plating

The Euroscarf gene knockout strains and the wildtype strain were spot plated onto YPD agar in the presence and absence of 200 µM Geneticin (Fig. 5.13, §2.5.2.1). All of the knockout strains exhibited growth on YPD agar in both the presence and absence of geneticin. This indicates that the Kanmx4 deletion cassette has been incorporated as this confers resistance to geneticin via the *KANMX4* gene. The wildtype strain only exhibited growth on the plates without Geneticin. For the *CUP1* knockout confirmation, both the *Saccharomyces cerevisiae* DTY3 wildtype strain and the DTY4 knockout strain were spot plated onto MYPG, MYPG-copper 100 ppm and MYPG-copper 200 ppm plates (Fig. 5.14, §2.8.4). The wildtype strain was able to grow at all concentrations of copper, due to the copper metalloprotein encoded by *CUP1*, whereas the knockout strain only exhibited growth at all cell density spots in the absence of copper and growth at its highest cell density spots in the presence of copper.

Figure 5.13) Confirmation of Kanmx4 cassette insertion in gene knockout strains through spot plating onto Geneticin. Euroscarf gene knockout strains and the wildtype strain *Saccharomyces cerevisiae* BY4741 spot plated on YPD agar with and without 200 µM Geneticin and incubated at 30°C for three days. Images taken with the UVP Geldoc-IT Imaging System.

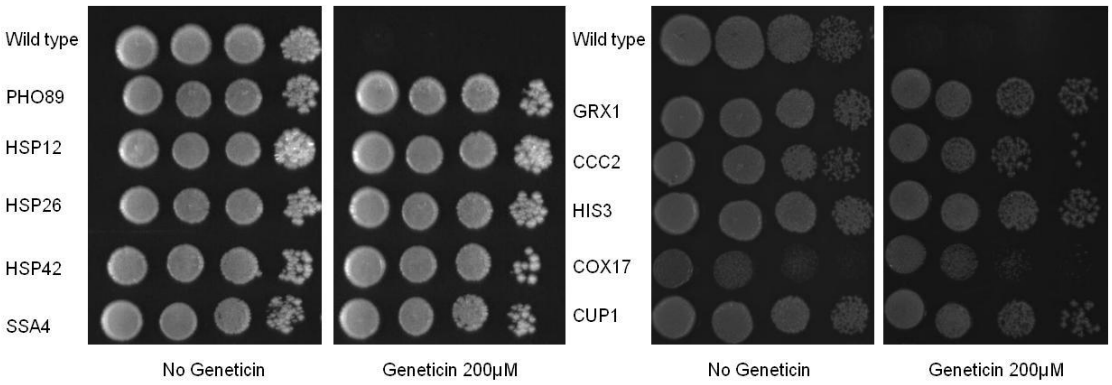
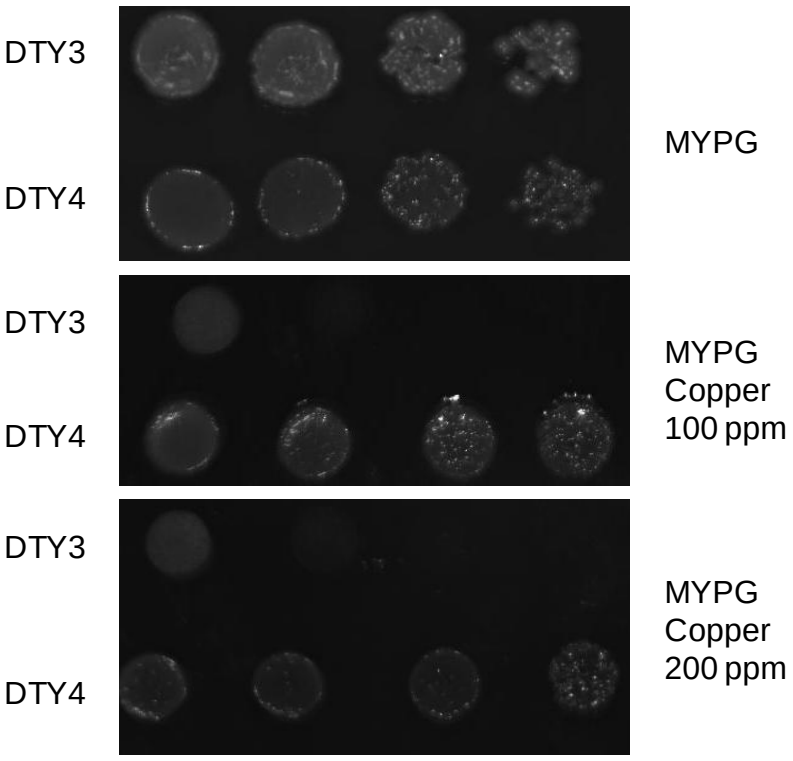


Figure 5.14) Confirmation of *CUP1* gene knockout via copper tolerance spot plating. *Cup1* deletion strain DTY4 and wildtype strain DTY3 were spot plated onto MYPG medium and MYPG medium containing copper (100 and 200 ppm). Plates were incubated at 30°C for three days. Images taken with the UVP Geldoc-IT Imaging System.



5.3.2 Confirmation of Gene-knockout Identity by PCR

PCR gene knockout confirmation experiments were performed for all the Euroscarf strains as described in section 2.8.5. The regions surrounding the target sites were amplified via PCR, using specific primers for each target gene, in both the wildtype strain and the gene knockouts. The PCR products of each gene knockout strain were electrophoresed on a 1% agarose gel (TAE), side by side with their wildtype strain PCR product counterpart (Fig. 5.15). All of the knockout strain PCR products were of a different base pair length to their wildtype counterparts and matched the expected PCR product lengths of their specific gene deletion target site with the kanmx4 deletion cassette inserted (Table 5.13).

5.3.3 Metabolic Activity Assays of Knockouts

Silver stress metabolic activity assays were performed for the selected gene knockout strains and compared to those of the wildtype strain in both wort and beer (§2.7.1). In wort (Fig. 5.16) only the gene knockout strains for HIS1, COX17 and CUP1 showed reduced silver tolerance, exhibiting metabolic activity only to silver nitrate concentrations lower than those of the wildtype. In beer (Fig. 5.17) the only gene knockout strain which differed from the wildtype strain was the SSA4 knockout, which showed no metabolic activity at all.

5.3.4 Growth Assays of Knockouts

Silver stress growth assays were performed for the selected gene knockout strain and compared to those of the wildtype strain in both wort and beer (§2.7.2). In wort (Fig. 5.16) the only knockout strains to differ from the wildtype strain were those for HIS1 and CUP1, both of which only exhibited growth at

silver nitrate concentrations lower than those of the wildtype. In beer (Fig. 5.17) the gene knockout strain for SSA4 produced no growth at any concentration of silver tested. All other knockout strains exhibited lower silver tolerances than the wildtype strain. The knockouts with the lowest tolerances were those for HIS1, COX17 and CUP1.

Figure 5.15) **Confirmation of Gene-knockout Identity via PCR.** PCR products of gene knockout regions in both the wildtype strain (A) and knockout strain (B). PCR products were electrophoresed on a 1% agarose gel.

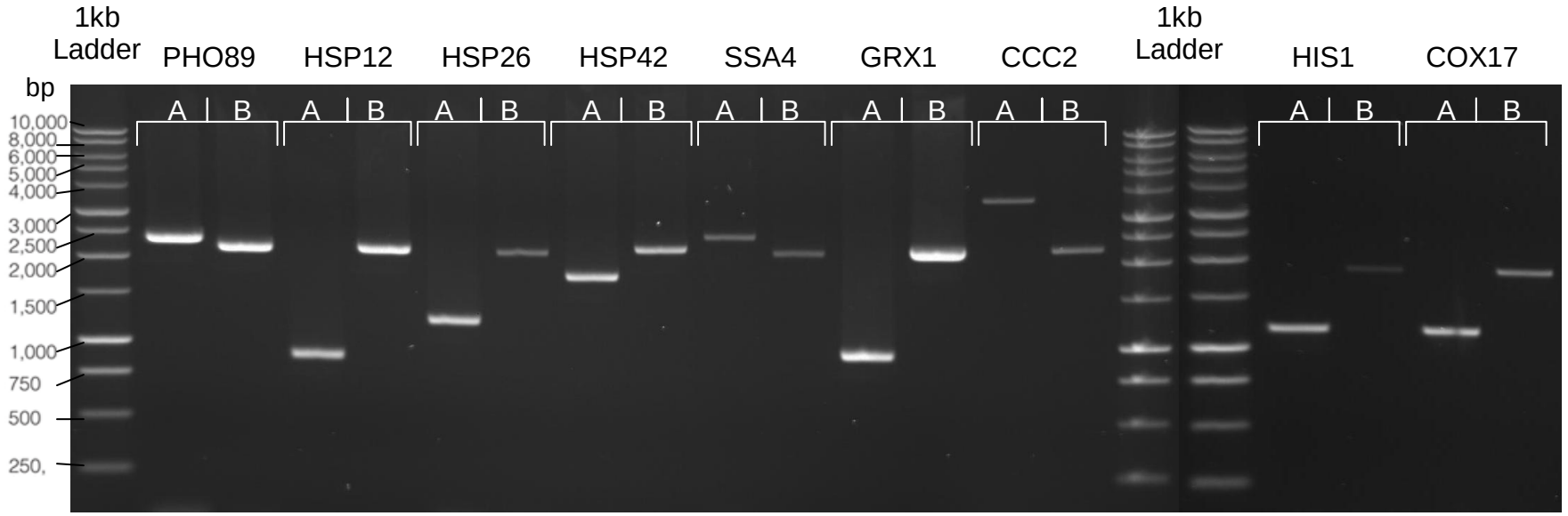


Table 5.13) Wild type and Knock out strain PCR fragment lengths. Expected sizes of PCR amplicons of the kanmx4 gene deletion target site in the wildtype strain S. cerevisiae BY4741 and the gene deletion mutant version. Expected amplicon sizes of the kanmx4 target site for each gene deletion and its wild type counter part are given in bp (base pairs).

	PHO89	**HSP12**	**HSP26**	**HSP42**	**SSA4**	**GRX1**	**CCC2**	**HIS1**	**COX17**
Wild type	2357	909	1214	1769	2500	949	3681	1183	1006
Deletion Strain	2216	2163	2153	2225	2155	2200	2250	1873	2380

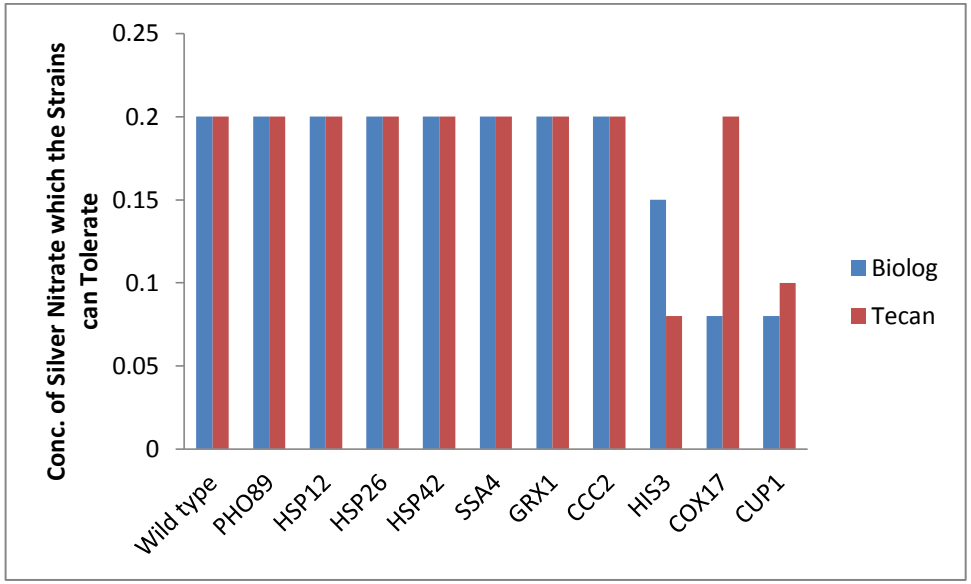


Figure 5.16) Comparison of gene knockout strain silver tolerances to that of the wildtype strain in wort. Data shown is from both metabolic activity and growth assays of the gene knockout strains. Plates were set up to contain 95 µl of 50% Wort (Hopped Peroni, S.G. 1.0567) mixed with IFY, containing the appropriate quantity of silver nitrate and also Biolog dye D for the metabolic assay. To each well 3 µl of cell culture (62% turbidity) were added prior to incubation at 25°C for 72 hours in a stationary incubator. Growth was recorded using a Tecan microplate optical density meter at 600 nm or via the Omnilog system for the metabolic assay.

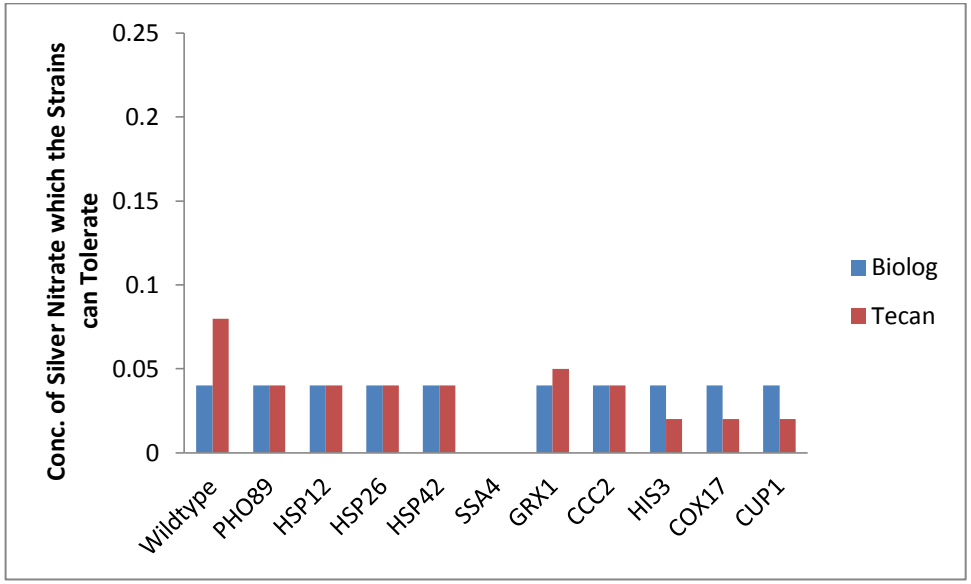


Figure 5.17) Comparison of gene knockout strain silver tolerances to that of the wildtype strain in beer. Data shown is from both metabolic activity and growth assays of the gene knockout strains. Plates were set up to contain 95 µl of 50% Beer (Hopped Peroni) mixed with IFY, containing the appropriate quantity of silver nitrate and also Biolog dye D for the metabolic assay. To each well 3 µl of cell culture (62% turbidity) were added prior to incubation at 25°C for 72 hours in a stationary incubator. Growth was recorded using a Tecan microplate optical density meter at 600 nm or via the Omnilog system for the metabolic assay.

5.4 Effect of Silver on Copper Tolerance

The organisms *S. ellipsoides var. diastaticus* and *S. cerevisiae* BY4741 were cultured in YPD broth with and without 150 µM silver nitrate. These cultures were spot plated onto MYPG, MYGP-Copper 100 ppm and MYGP-Copper 200 ppm agar (§2.8.2). On MYPG without copper both strains grew at all cell densities (Fig. 5.18). However, in the presence of 100 and 200 ppm copper only the strains pre-exposed to silver grew at all cell densities. The strains without the pre-exposure to silver only grew at their highest cell densities, which confirms the previous experimental data (§3.2.1).

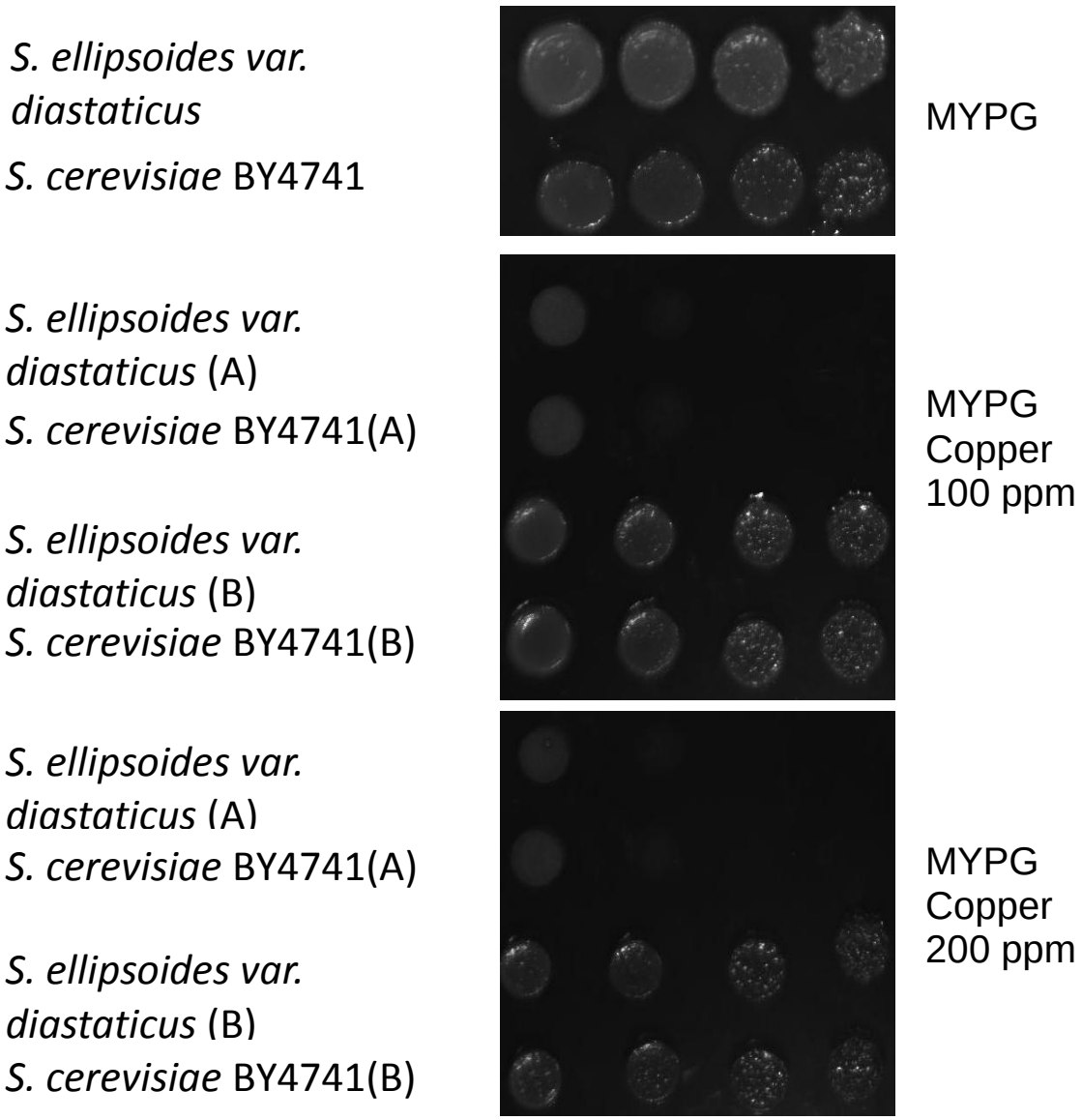


Figure 5.18) Effect of exposure to silver nitrate on copper tolerance. Spot plate growth of *S. ellipsoides var. diastaticus* and *S. cerevisiae* BY4741 on MYPG medium and MYPG medium with copper (100 and 200 ppm). Growth of strains cultured in YPD without silver (A) on copper plates was contrasted with that of strains grown in YPD containing 150 µM silver nitrate (B). Culture in YPD and on MYPG plates took place at 30°C over three days.

Chapter 5 Discussion

5.5 ICP-MS of Wort and Beer

The concentrations of copper found in wort and beer are equal to some of the concentrations of silver nitrate tested. The levels of copper present in the media used for experimentation must be taken into account when analysing the experimental data, due to the similarities between copper and silver. For example it is known that copper and silver induce some of the same tolerance mechanisms, such as the cadmium efflux P1B-type ATPase (Adle *et al.*, 2007). Therefore, it may be that the presence of copper is masking certain gene responses to silver stress in the microarray experiments, by up or down regulating genes induced or suppressed by both copper and silver in the samples without silver. In the comparison of gene responses in samples with and without silver this would lead to a decrease in fold changes in gene expression measured, or even no fold change at all. However, when comparing the silver stress responses of *S. ellipsoides var. diastaticus* in wort and beer found in this study, to those of *S. cerevisiae* in YPD (identified by Niazi *et al.*, 2011), the number of gene responses in beer was more than twice that in YPD. In wort (151) the number of genes up-regulated was similar to that in YPD (161), however the number of down-regulated genes in wort (17) was much lower than in YPD (73).

These data would suggest that higher concentrations of copper are present in wort than in beer. Heavy metals such as copper may be introduced to the brewing process via water, cereals, hops, yeast and containers/vessels, from leaching of metal from pipework or vessels, or in the case of the ingredients by metal contamination from fertilizers, pesticides or industrial processing

(Nascentes *et al*., 2005). The decrease in copper concentrations from wort to beer may be due to biosorbtion (attachment to cell surface) and/or bioaccumulation (internalisation) of copper by brewery yeasts such as *S. cerevisiae* (Brady *et al*., 1994; Han *et al*., 2006; Wang and Chen, 2006; Li *et al*., 2014).

5.6 Gene Regulation

5.6.1 Gene Response During Silver Stress

In both beer and wort a large proportion of genes both up- and down-regulated were of unknown function. It is therefore possible that gene responses directly linked to silver stress are taking place that remain unidentified. In wort the gene response types most up-regulated are those linked to the synthesis of ribosomal sub-units. The next most commonly up-regulated gene types, for wort and beer, are those genes associated with metabolic pathways and biosynthesis of secondary metabolites, transmembrane transport and DNA/Protein maintenance activities. In wort the gene responses seem targeted at the synthesis and processing of DNA, RNA, proteins and their components such as purine, pyrimidine and amino acids. In addition to promoting cellular replication via genes involved in the cell cycle and meiosis. This is most likely a response to the disruption or inhibition of DNA, RNA, proteins, cell membrane and arrest of the cell cycle caused by silver toxicity (Wang *et al*., 2013; Kim *et al*., 2009). In beer, although these functional groups are also present, a large proportion of the pathways up-regulated appear to be involved in the acquisition or utilisation of carbon sources e.g. carbon metabolism, starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism and gluconeogenesis. This may be a

response to a combination of the nutrient depleted media that beer presents and the ability of silver ions to disrupt the electron transport chain leading to an energy deficit (Bragg and Rainnie, 1974). In both beer and wort transmembrane transport functions are up-regulated. These include genes such as *ATR1* (multidrug efflux pump), *PDR12* (Plasma membrane ATP-binding cassette (ABC) transporter) and several others. These are most likely induced as part of a generalised attempt to remove toxic materials from the cell. *PRD12* specifically, is a plasma membrane ATP-binding cassette transporter, required for weak acid resistance, and so may play an additional role in hop acid resistance (Piper *et al.*, 1998). Niazi *et al.* (2011) found that when exposed to silver ions in YPD, *Saccharomyces cerevisiae* up-regulated 73 genes, the majority of which are transport and homeostasis related. For instance silver and copper ions induce the efflux of potassium (Vagabov *et al.*, 2008). Although gene functional groups down-regulated during silver stress include transporter molecules (meiosis; HXT4, HXT5, HXT13 and HXT17 sugar transporters) and DNA, RNA and protein synthesis, in wort these are down-regulated (18 genes) in much smaller numbers than were up-regulated (153 genes). However, in beer more genes are down-regulated (359 genes) than up-regulated (243 genes), which is in keeping with the gene responses (73 up-regulated and 161 down-regulated genes) to silver stress of *S. cerevisiae* in YPD (Niazi *et al.*, 2011).

As stated in section 5.2.1 the most up-regulated genes in wort during silver stress are RPA12 and PDR12, an RNA polymerase subunit and a plasma membrane ATP-binding cassette (ABC) transporter respectively. The remaining ten most up-regulated genes consist of ribosomal subunits

(RPS28B), rRNA processing (CGR1, RRN11), cell wall regulation (HLR1, UTR2) and methionine, threonine (HOM3) and purine (ADE4) nucleotide biosynthesis. The RNA genes RPA12, CGR1 and RRN11 and the ribosome sub-unit encoded by RPS28B, may be up-regulated in order to increase transcription of proteins as a response to stress. CGR1 in particular is known to be induced by cytotoxic stress (Caba *et al*., 2005). As a weak-acid-induced multidrug transporter responsible for acid efflux, PDR12 may be up-regulated in response to the acidic conditions caused by the addition of Ag^{+} ions, as well as the hop isoalpha acids. Multi drug transporter pathways, such as HOG1, are known to be up regulated in *Candida glabrata* in response to heavy metal ions and weak acids (Cannon *et al*., 2007). This is also paralleled in bacteria, for example the multi drug efflux operon *mdt*ABC is induced by and confers resistance to copper and zinc in *Lactococcus lactis, Escherichia coli and Salmonella* (Maynaud *et al*., 2013). The cell wall genes HLR1 and UTR2 are involved in cell wall regulation and integrity and therefore may be up-regulated in response to cell wall damage that silver stress is known to cause (Alonso-Monge *et al*., 2001; Cabib, 2009; Xia *et al*., 2014). ADE4 is involved in the biosynthesis of purine, which is a component of DNA, RNA and other biomolocules (Kowalski *et al*., 2008). Therefore, ADE4 may be up-regulated during silver stress as either a generalised stress response or due to the inhibition of DNA and RNA synthesis caused by silver ions (Hwang *et al*., 2012). Similarily HOM3 is involved in the biosynthesis of the amino acids methionine and threonine (Mountain *et al*., 1991). HOM3 may be up-regulated as amino acids are needed for synthesis of proteins and silver ions are known to inhibit protein synthesis and denature existing proteins (Wang *et al*., 2013).

In particular, methionine is needed as it is coded by the initiation codon AUG which determines the mRNA's coding region where translation to protein begins (Meinnel *et al*., 1993).

As stated in section 5.2.1 the most down-regulated genes in wort during silver stress are HXT13 and HXT5, both of which are Hexose transporters induced in the presence of non-fermentable carbon sources (Greatrix and van Vuuren, 2006). That these genes in addition to HXT17, another hexose transporter and FBP1 a gluconeogenesis pathway regulator, are down-regulated would suggest an abundance of fermentable sugars available in wort (Rintala *et al*., 2008; Alibhoy *et al*., 2012). A sufficient level of fermentable sugars may also explain why RIM4 is down-regulated, as it is involved in RNA binding during sporulation, which it induces if expressed in rich media (Deng and Saunders, 2001). The gene XBP1 is involved in the transcriptional repression of the cyclin genes CYS3 and SMF2 (Mai and Breeden, 2000). CYS3 codes for cystathionine gamma-lyase which is involved in the transsulphuration pathway that produces cysteine from homocysteine (Hiraishi *et al*., 2008). This protein is known to increase in abundance during DNA replication stress, which silver ions are known to cause (Tkach *et al*., 2012). SMF2 codes for a divalent metal ion transporter involved in manganese homeostasis, which has broad specificity for divalent and trivalent metals including cobalt, iron and copper ions (Culotta *et al*., 2005). This may explain why XBP1 is down-regulated during silver stress. The expression of the cell wall protein gene PIR3 is known to be cell cycle related, peaking in M/G1 (Porter *et al*., 2002). Sampling may have occurred in a down-regulated phase and silver ions may arrest the cell cycle (Kim *et al*., 2009). The remaining down-regulated genes MBR1 and

CAT2 concern mitochondrial functions (Daignan-Fornier *et al*., 1994). CAT2 is responsible for the transport of acetyl-CoA into the mitochondria needed for carbon metabolism. This is only needed in the absence of glucose in the growth media, as in the presence of glucose acetyl-CoA is synthesised inside the mitochondria (Strijbis *et al*., 2010). This may be why in wort CAT2 is down-regulated. It must be stated that even the most down-regulated gene HXT5 was only down-regulated by a 2.96 fold factor.

The most up-regulated genes in beer during silver stress are CMK2 and PUG1, a calmodulin-dependent protein kinase and a plasma membrane protein with roles in the uptake of protoporphyrin IX and haem efflux respectively (Dudgeon et al., 2008; Manente et al., 2009). CMK2 has been suggested as a link in stress activated signalling pathways, catalysation of protein phosphoryliastion, leading to oxidative stress responses (Ding *et al*., 2014). PUG1 may be up-regulated in an attempt to remove the Fe^{2+} ions contained in haem (Protchenko *et al*., 2008). The functions of the remaining ten most up-regulated genes consist of membrane transporter proteins (TPO4, HXT2, PMC1), mitochondrial genes (AIM17, HEM15), cell wall maintenance (YPS1) and gluconeogenesis regulation (FBP1). Of the transporter genes TPO4 acts as a polyamine transporter allowing the efflux of polyamines such as spermine, putrescine and spermidine, which are toxic at high levels (Albertsen *et al*., 2003). PMC1 encodes a vaccuolar Ca2+ ATPase efflux mechanism aiding in the removal of Ca2+ before growth inhibition occurs (Folkina *et al*., 2012). HXT2 is a hexose transporter induced by low glucose levels, which are found in beer as glucose has been depleted. Induction results in import of hexose, fructose, pentose and mannose

alternative carbon sources (Reifenberger *et al*., 1995). Of the mitochondrial genes, HEM15 catalyses the insertion of ferrous iron into protoporphyrin IX imported by PUG1 (Lesuisse *et al*., 2003). AIM17 encodes a protein of unknown function found in mitochondria, which is repressed in the presence of glucose (Hess *et al*., 2009; Kim *et al*., 2013). YPS1 is involved with cell wall maintenance and so may be up-regulated in response to damage caused by silver stress (Miyazaki *et al*., 2011). FBP1 catalyses gluconeogenesis, the formation of glucose from noncarbohydrate precursors such as pyruvate, amino acids and glycerol (Hung *et al*., 2004). This is most likely a response to the depletion of fermentable carbon sources in beer.

The most down-regulated gene of known function in beer during silver stress is HXT4, a high-affinity glucose transporter (Ozcan and Johnston, 1999). The functions of the remaining ten most down-regulated genes comprise of Plasma membrane riboflavin transporter (MCH5), thiamin uptake (PHO3), plasma membrane targeting dehydrogenase/reductase (AST1), ribosome biogenesis (RSA4), glycerol biosynthesis (HOR2), RNA helicase (DHR2), mitochondrial inner membrane ADP/ATP translocation (AAC3) and proteins of unknown function (ANS1, JJJ3). The the down-regulated genes HXT4, MCH5, PHO3, AST1 and AAC3 are all involved in some form of trans plasma membrane import, which may have been inhibited as part of a generalised stress response (Chang and Fink, 1995; Ozcan and Johnston, 1999; Nosaka *et al*., 2005; Spitzner *et al*., 2008; Palmieri, 2013). HXT2 may be up-reguated whilst HXT4 is down-regulated, due to a higher number of induction regulators (12) compared to HXT4 (3).

5.6.2 Effect of Wort and Beer on Gene Responses

Quantitatively, more transmembrane transporter genes are up-regulated in wort than in beer. This may be due to the comparative abundance of carbon sources and nutrients found in wort. This would correlate to the genes involved in carbon metabolism, starch and sucrose metabolism exhibiting comparative up-regulation in wort. However, the gene groups for structural molecules such as purine, pyrimidine and amino acids, ribosome maintenance and biogenesis, and DNA/RNA synthesis and processing i.e. cell maintenance processes, are more up-regulated in beer (still present in wort). This may be due to the ethanol, pH and nutrient starvation stresses exerted by beer (Suzuki *et al.,* 2007). This is in addition to the stress caused by the presence of antimicrobial hop acids in wort and beer, which act as ionophores dissipating the pH gradient of the cell membrane and lowering the internal pH inhibiting enzyme activity (Behr *et al.*, 2006). Whilst this is antimicrobial for most bacteria, yeasts appear tolerant of the hop acids (Srinivasan *et al.*, 2004). Possibly due to stress management responses such as those mentioned. In wort this pattern continues in the presence of silver. In beer however, the focus in pathway up-regulation appears to shift from cell maintenance (e.g. DNA synthesis) to alternative carbon source utilisation, meiosis, TCA and cell cycle with the addition of silver.

The most up-regulated gene in wort compared to beer is FHN1. FHN1 is a protein of unknown function putatively involved in sterol regulation in the cell membrane and protein localisation to the plasma membrane (Loibl *et al*., 2010). Of the ten most up-regulated genes the fourth is VBA3 a permease of basic amino acids in the vacuolar membrane, involved with the transport of

basic amino acids across the membrane (Dias and Sá-Correia, 2013). Most of the other ten most up-regulated genes are cell wall mannoproteins (DAN1, PAU24, TIR1, TIR3) and members of the seripauperin multigene family (PAU5, PAU7, PAU17). The seripauperin multigene family genes are induced by alcoholic fermentation, which occurs in wort (Luo and Vuuren, 2008; Luo and Vuuren, 2009). The cell wall mannoprotein genes are induced by anaerobic conditions such as during fermentation in wort and are repressed by acidic pH as are found in beer (Sertil *et al*., 2007; Luo and Vuuren, 2009). Of the top ten most down-regulated genes in wort compared to beer plasma membrane permeases (GIT1, AZR1), transporters (SUL1) and proton symporters (STL1), galactose metabolism (GAL10), stress induced ATPase (HSP30), cell wall modulation (RPI1), gluconeogenesis (PCK1), glyoxylate cycle (MLS1) and methionine and alanine catabolism (ARO10). These are all mechanisms to cope with stresses such as lack of fermentable carbon sources, acidic pH and ethanol content found in beer (Suzuki *et al.,* 2007).

5.6.3 Differences in Gene Responses between Brewery Contaminant and Laboratory Strain Yeast

The transcriptional responses of the brewery contaminant strain *S. ellipsoides var. diastaticus* and the laboratory strain *S. cerevisiae* BY4741 were first compared without silver. It was found that although the following gene types were also expressed in the laboratory strain, in terms of the number of genes in each category, the gene functions for structural activities such as, structural molecule synthesis (purine, pyrimidine), synthesis/maintenance of ribosomes and DNA/RNA synthesis/ processing were more highly expressed in the diastatic strain. Similarly, although also expressed in the diastatic strain,

numerically, more genes were expressed with meiosis, cell cycle and TCA cycle functions in the laboratory strain than in *S. ellipsoides* var*. diastaticus*. Transmembrane transporter genes were expressed by both strains in approximately equal numbers. However, when silver stress was introduced it was found that whilst still expressed in the laboratory strain the gene groups for transmembrane transporter and cell cycle functions were more highly expressed in the contaminant strain as well as the previous categories. Additionally, during silver stress meiosis genes are expressed in approximately equal quantities in both organisms. This indicates that the diastatic strain may be more adapted to metal or oxidative stress than the laboratory strain. The most up-regulated genes in the diastatic strain in comparison to the laboratory strain during silver stress are URA3; an orotidine-5'-phosphate (OMP) decarboxylase (involved in pyrimidine biosynthesis), MET17; (Alias: Met15) a methionine and cysteine synthase and LEU2; a beta-isopropylmalate dehydrogenase (IMDH) (Ko *et al*., 2008; Branduardi *et al*., 2013; Goryanova *et al*., 2013; Sadhu *et al*., 2014). The functions of the remaining ten most up-regulated genes consist of isomaltose utilization (IMA1), maltose catabolism (MAL12), vacuolar membrane amino acid permease (VBA3), ferric reductase (FRE4) and sterol and lipid biosynthesis (CYB5) (Brown *et al*., 2010; Gutiérrez *et al*., 2013; Plitzko *et al*., 2013; Deng *et al*., 2014; Saikia *et al*., 2014). This would suggest that the wild type diastatic strain possesses increased sugar utilisation, amino acid synthesis/transport and electron transport (CYB5 and FRE4) capabilities in comparison to the laboratory strain. The functions of the ten most down-regulated genes consist of an haze-protective mannoprotein (HPF1),

asparagine catabolism (ASP3-1), alpha-glucosidase (IMA2), enolase regulation (REE1), endosomal iron transport (ENB1), mating pheromone factors and proteins (MFA2, PRM8, MFA1) and hexose transport (HXT5) (Sandmann *et al*., 2003; Brown *et al*.,2007; Froissard *et al*., 2007; Choi *et al*., 2008; Naumoff and Naumov, 2010; League *et al*., 2012; Rodgers *et al*., 2012; Bermejo *et al*., 2013). This would suggest that the laboratory strain up regulates stress management systems such as alternative carbon source utilisation and copper, iron, calcium and nitrogen deficiency management systems.

5.7 Gene Knockout Experiments

5.7.1 Confirmation of Knockout Identity

The transcriptomics data from this study and that of Niazi *et al.* (2011) identified the genes HSP12 (7.5 fold), HSP26 (3.2), HSP42 (5.7), PHO89 (5.7), GRX1 (2.8), CCC2 (4.6), SSA4 (6.5), HIS1 (5), COX17 (2.7) and CUP1 (22.6) as induced when 0.1 mM silver was added to the growth medium. To assess the importance of these genes to silver tolerance gene knockout mutants in the reference strain *S. cerevisiae* BY4741 were purchased (§2.7.3). The identity of the gene knockouts was confirmed through a combination of traditional plating and molecular genetic techniques. The traditional plating technique (§5.3.1) confirmed that all the knockout strains have taken up the kanmx4 deletion cassette (containing the geneticin resistance gene *KANMX4*) as this confers tolerance to 200 µM Geneticin, which all the strains except the wildtype exhibited (Jauert *et al.*, 2005). The genetic technique (§5.3.2) confirmed that the kanmx4 cassettes have deleted

the correct gene in each case, as the primers specifically targeted at the genes of interest produced different size PCR products for the wildtype and knockout strains. This is due to the cassette replacing the gene via homologous recombination in the knockouts, having a different nucleotide length to the target gene (Kastenmyer *et al.*, 2013). The gene deletion amplicons produced were the expected lenth of the specific gene deletion target site with the kanmx cassette inserted.

5.7.2 Effect of Gene Knockouts on Silver Tolerance in Wort and Beer.

Generally speaking the tolerances exhibited in the metabolic activity assays (§5.3.3) matched those of the growth assays (§5.3.4). The tolerances recorded by both assay types for all strains in beer were approximately half of their corresponding values in wort. In wort the knockout strains for *HIS1* and *CUP1* showed reduced silver tolerance in comparison to the wildtype strain in both assay types. *COX17* only exhibited reduced tolerance through the metabolic activity assay. In beer the only decrease in silver tolerance was demonstrated by *HIS1*, *COX17* and *CUP1* and only in the growth assays. That deletion of *HIS1*, *COX17* and *CUP1* reduces silver tolerance may be due to their function. *HIS1* codes for an ATP phosphoribosyltransferase, which catalyses the first step in histidine biosynthesis. Mutations in this gene have been known to cause histidine auxotrophy and sensitivity to Cu salts. It has been suggested that intracellular histidine, possibly in the vaccuole, reduces pH-dependant copper toxicity in *S. cerevisiae* (Baganz *et al.*, 1998; Pearce and Sherman, 1999). This may also be the case for silver. *COX17* codes for a copper metallochaperone which aids in the transfer of copper to cytochrome c oxidase (Horng *et al.*, 2004). *CUP1* codes for a metallothionein which binds

copper to promote tolerance to high levels of copper and is known to be induced by silver ions (Jensen *et al.*, 1996 and Tohoyama *et al.*, 2001). The function of COX17 and CUP1 in silver tolerance, as metallochaperone and metallothionine respectively, is most likely the binding of silver ions to their cysteine pairs as they would copper (Silar *et al.*, 1991; Banci *et al.*, 2008). All three of the genes which may play a role in silver tolerance also appear to be linked to the tolerance of copper. This would seem logical as copper and silver are isoelectronic, bind similar molecules and appear to utilise the same transmembrane transporters etc. (Nies, 1999; Osobova *et al.*, 2011).

5.8 Effect of Silver on Copper Tolerance

The data would suggest that pre-exposure to a non-lethal dose of silver increases copper tolerance, as both strains, when exposed to silver prior to plating were able to tolerate higher concentrations of copper than without exposure to silver. This implies a link between copper and silver tolerance. This agrees with the microarray data, which showed that one of the effects of exposure to silver for the test strains is the up regulation of copper tolerance mechanisms.

Chapter 5 Conclusion

In the comparison of gene responses under all the conditions tested it must be taken into account that the microarray chip utilised for the experiments, GeneChip® Yeast Genome 2.0, was designed for *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Therefore, there may be genes other than the *STA1*, *2* and *3* glucoamylase genes missing from the array which are present in brewery contaminant strain *S. ellipsoides* var*. diastaticus*

(*S. cerevisiae* var. *diastaticus*). Additionally, some of the silver stress gene responses that are recorded by the array may be masked by the copper present in the test media. This, and the fact that a sizable portion of the gene responses (both up and down-regulated) were of unknown function, means that some gene responses directly linked to silver stress may not have been identified. It must also be taken into account that the microarray experiments are only performed in *S. cerevisiae* BY4741 and *S. ellipsoides var. diastaticus*, so the other contaminant strains, including the bacterial strains, may have other potential tolerance mechanisms which are not recorded.

When comparing the gene responses to silver stress of *S. ellipsoides var. diastaticus* in wort and beer, it was found that in wort the responses appeared to be centred on detoxification i.e. transmembrane transporters, whilst in beer they seemed centred on cell structural maintenance and carbon source utilisation. The overall gene response to silver stress in both beer and wort appears to be an up-regulation of transmembrane transporters. This is most likely an attempt to remove toxic elements from the cell and repair damage caused by silver ions. Other up-regulated potential silver tolerance mechanisms include Ferrodoxin (*YAH1*), required for the assembly of iron-sulphate proteins, which are known to be inhibited by silver ions (Lange *et al*., 2000; Xu and Imlay, 2012). Additionally, the up-regulation of a variety of DNA, RNA and protein activities, for example SSA2 which codes for an ATP-binding protein involved in protein folding and vacuolar import of proteins, may be in response to silver ions inhibiting DNA and Protein functions or it may be part of a general stress response (Unno *et al.*, 1997). This would correlate to the ten most up-regulated genes in wort and beer during silver

stress. The most up-regulated genes in wort during silver stress are involved in RNA polymerase sub-unit synthesis, a plasma membrane ATP-binding cassette (ABC) transporter, rRNA processing, cell wall regulation, and methionine, threonine and purine nucleotide biosynthesis. The most up-regulated genes in beer during silver stress are involved in a calmodulin-dependent protein kinase, a plasma membrane protein, membrane transporter proteins, mitochondrial genes, cell wall maintenance and gluconeogenesis regulation.

In the comparison of the contaminant strain *S. ellipsoides* var. *diastaticus* and the laboratory strain *S. cerevisiae* BY4741 it was found that without silver, cell maintenance activities, such as DNA, RNA, purine, pyrimidine and ribosome sub-unit synthesis, were more highly expressed in the contaminant strain, and meiosis, cell cycle and TCA cycle functions in the laboratory strain. Detoxification functions such as transmembrane transporters were equally expressed in the absence of silver, but more highly expressed in the contaminant strain during silver stress. The most up-regulated genes in the contaminant strain in comparison to the laboratory strain during silver stress are an orotidine-5'-phosphate (OMP) decarboxylase (involved in pyrimidine biosynthesis), a methionine and cysteine synthase, a beta-isopropylmalate dehydrogenase (IMDH), isomaltose utilization, maltose catabolism, a vacuolar membrane amino acid permease, a ferric reductase and sterol and lipid biosynthesis. The functions of the most down-regulated genes in the contaminant strain in comparison to the laboratory strain during silver stress consist of a haze-protective mannoprotein, asparagine catabolism, an alpha-

glucosidase, enolase regulation, endosomal iron transport, mating pheromone factors and proteins and hexose transport.

Of the ten gene knockout strains selected due to the up regulation of their genes during silver stress, either in literature or current study, only three exhibited a decrease in silver tolerance in comparison to the wild type. These genes: *HIS1*, *COX17* and *CUP1*, although diverse in function, are all involved in copper tolerance mechanisms. This, and the finding that pre-exposure to silver increases copper tolerance by pre-up-regulating copper tolerance mechanisms, would suggest that copper and silver share some of the same tolerance mechanisms. This would include the three genes discussed, as well as others potentially. However, not all copper tolerance mechanisms appear linked to silver tolerance as shown by *CCC2*, a Cu(+2)-transporting P-type ATPase, which exports copper from the cytosol into an extracytosolic copper oxidase (Huffman and O'Halloran, 2000). No decrease in silver tolerance was detected for the deletion mutant of this gene.

Chapter 6 – Conclusion

The aim of this project was to establish the degree of sensitivity of microbial contaminants found in breweries to silver. In particular, over the course of this project the ranges of silver tolerance exhibited by microbial brewing contaminants, including both yeasts and bacteria, were assessed in simulated brewery growth conditions. Furthermore, the possible mechanisms by which the microorganisms may exhibit tolerance/resistance was investigated in beer and wort. This was done with the aim of establishing a foundation of knowledge upon which to base a determination of whether silver or silver nano-particles could be used to combat these contaminants within breweries, in the form of an antimicrobial surface. In Chapter 3 it was determined that the bacterial and yeast contaminant test organisms exhibited physiological and morphological characteristics which were consistent with their documented characteristics. The exception to this was the presumptive *B. coagulans* strain, which as a thermophillic strain should have been able to grow at 55-70°C, but only grew at temperatures under 37°C. As some of the test strains were brewery isolates and all the strains were received as YPD streak plates cultured by a secondary party, they had to undergo genotypic identification. The genotypic identification only yielded positive identification for *Pichia membranaefaciens*, *Brettanomyces anomalous*, *Candida krusei*, *Hansenula saturnus*, *Kloeckera apiculata*, *Rhodotorula mucilaginosa*, *Saccharomyces ellipsoides* var. *diastaticus, Lactobacillus brevis* and *Pediococcus damnosus*. As these represent some of the most common brewery contaminant strains and their identity was positively confirmed, experimentation was confined to

these organisms. The presumptive strain *B. coagulans* could only be identified to the genus level *Bacillus* spp.. This, in combination with the lack of thermophillic growth exhibited, casts doubt on the identity as *B. coagulans*. The remaining test strains, *Shimwellia pseudoproteus*, *Bacillus coagulans*, *Pantoea agglomerans*, *Gluconobacter oxydans*, *Acetobacter aceti* could not be identified even to the genus level. The presumptive identities were not on the list of potential matches found in the database and all of the matches that were found had low % identity matches, well below the cut off, with high E-values. This would suggest that either these organisms are not what they had previously been identified as or that due to their specialisation as brewery contaminants their 16S sequences are sufficiently different from strains isolated in other environments that no matches could be found. Phylogenetic analyses would need to be undertalken to conclusively identify these strains.

However, to fully understand the effectiveness of silver as an antimicrobial against brewery contaminant microorganisms, further study is needed on bacterial contaminants as the contaminant organisms investigated for silver sensitivity in this study comprised primarily yeast. These bacterial studies should include the bacteria excluded from this study, due to inconclusive identification, if their identity is verified. Additionally, *Pectinatus* sp. and *Megasphaera* sp. organisms should be investigated as they are known beer spoilage organisms (Iijima *et al*., 2008).

Chapter 4 was concerned with the effect of silver stress on these microbial brewery contaminants in simulated brewery conditions. The examination of the effect of silver stress on colony formation via spot plate analysis revealed that silver tolerances were decreased in brewery media in comparison to YPD

and that in brewery media no growth on solid surfaces was exhibited at silver nitrate concentrations higher than 0.55 mM. The comparison of metabolic activity and growth during silver stress in wort and beer indicated that although silver tolerance was highly organism dependant, no tolerance exceeded 0.5 mM silver nitrate in liquid medium. The silver tolerances exhibited, even that of *L. brevis* (0.2 mM in wort), exceed the known tolerances of bacterial organisms such as *E.coli* J53, but not those of known silver tolerant organisms such as *E.coli* J53 (pMG101) (Gupta *et al.,* 2001). Decreased silver nitrate tolerances were exhibited by all the yeasts under microaerophillic conditions during metabolic activity assays in wort with the exception of *S. ellipsoids* var. *diastaticus* and *K. apiculata.* In beer a decrease in silver tolerance was detected in *B. anomalus*, *K. apiculata* and *R. mucilaginosa* during microaerophillic conditions. The other yeasts exhibited no difference in silver tolerance between aerobic and microaerophillic conditions. This is of interest from a brewing perspective, as the microaerophillic conditions are more representative of the internal conditions of the brewing process. The fact that silver tolerances decrease, or at the least do not increase for the yeasts tested in more brewery-like conditions, are encouraging for the development of silver as an antimicrobial within the process. The data gathered would suggest that silver has potential as an antimicrobial against the range of brewery contaminants selected for this study. However, this study focused on silver in soluble form. Therefore, further investigation of the sensitivity of brewery contaminants to solid metallic silver and eventually, silver nano-particles is needed before silver-based antimicrobial surfaces can be developed. Particular focus should be placed on

fixed silver antimicrobial surfaces such as silver nano-particle surfaces and their effect on biofilms. Various studies have investigated the antimicrobial effects of both free and fixed silver nano-particles. The study of Inbakandan *et al*. (2013) focused on the effect of unbound silver nano-particles (spherical shape, 15-34 nm) on marine bacterial biofilms of organisms including *Micrococcus luteus* and *Bacillus pumilus*. In this study biofilms were allowed to form inside microtubes in Zobell marine broth and treated with silver nano-particles. The degree of biofilm inhibition was assessed via staining with crystal violet and optical density spectroscopy at 540 nm. A study similar to this showed the inhibition of *Candida glabrata* and *Candida albicans* biofilm formation on acrylic due to silver nano-particle exposure (Silva *et al*., 2013). As in the previous work, biofilm formation was assessed through crystal violet staining as well as viable counts. An example of an investigation into the effect of surface-fixed silver nano-particles on biofilms was the study by Prahbhakar *et al*. (2011). In this study silver nano-particles (average 22 nm) generated from silver nitrate, using sodium citrate as a stabilising agent, were bound to medical grade polyurethane slides. These slides were placed in conical flasks containing nutrient broth inoculated with *Bacillus subtilis*. After 24 hours of incubation at 37°C in a shaking incubator (100 rpm) it was found that polyurethane slides coated in silver nano-particles exhibited 50.5% less attached cells (cfu) than the uncoated control slide. Similar experiments could be performed for brewery contaminant bacteria. This should be done in both wort and beer as growth medium and both aerobically and anaerobically to simulate brewery environments. In addition to this viable cell counts should be

performed on the media to assess the effect of surface fixed silver nano-particles on organisms not in direct contact with the surface.

In Chapter 5 the brewery contaminant strain *Saccharomyces ellipsoides* var. *diastaticus* was examined for gene responses to and potential tolerance mechanisms against silver stress using microarrays. Gene responses to silver stress were analysed in both wort and beer. In the comparison of gene responses in wort and beer, it was found that in wort the responses appeared to be centred on stress factor removal i.e. transmembrane transporters and cell maintenance and repair (RNA and DNA synthesis, cell cycle and meiosis). In beer the responses were centred on cell structural maintenance as well as transmembrane transporters and carbon source utilisation. The main gene responses to silver stress in both media appear to be the up regulation transmembrane transporters. This is most likely an attempt to remove toxic elements from the cell, in terms of both efflux and reduction of the ion or damage reduction from oxidation by silver. The up regulation of several DNA and protein activities may be a response to silver ions inhibiting DNA and protein functions or it may be part of a general stress response. From previous published work on copper/silver response in yeast (Niazi *et al.* 2001) and the transcriptional responses to silver stress in Chapter 5, ten genes potentially linked to silver tolerance were selected. Gene knockout strains were acquired for these genes and silver stress metabolic activity and growth assays performed. Of the ten genes only *HIS1*, *COX17* and *CUP1* exhibited a link to silver tolerance. All of these are linked to copper tolerance. *HIS1* codes for an ATP phosphoribosyltransferase, which catalyses the first step in histidine biosynthesis. Mutations in this gene have been known to cause

histidine auxotrophy and sensitivity to Cu salts. It has been suggested that intracellular histidine, possibly in the vaccuole reduces pH-dependant copper toxicity in *S. cerevisiae* (Baganz *et al.*, 1998; Pearce and Sherman, 1999). *COX17* codes for a copper metallochaperone which aids in the transfer of copper to cytochrome c oxidase (Horng *et al.*, 2004). *CUP1* codes for a metallothionein which binds copper to promote tolerance to high levels of copper and is known to be induced by silver ions (Jensen *et al.*, 1996; Tohoyama *et al.*, 2001). When these copper tolerance genes are knocked out (§5.7), silver tolerance decreases and that pre- exposure to low levels of silver increases copper tolerance (§5.8) indicates that copper and silver tolerances are closely linked if not identical. This establishes that of the contaminant strains at least *S. ellipsoides* var. *diastaticus* has silver management mechanisms. However, despite these mechanisms silver nitrate concentrations of 0.55 mM on solid and 0.5 mM in liquid brewery medium still inhibit growth and metabolic activity. Although three components of the silver tolerance mechanisms of *S. ellipsoides* var. *diastaticus* have been identified, there were other genes up-regulated in response to silver stress such as PDR12, a plasma membrane ATP-binding cassette (ABC) transporter and PMC1, a vacuolar Ca^{2+} ATPase involved in depleting cytosol of Ca^{2+} ions (Holyoak *et al*., 2000; Luo *et al*., 2004). Particular attention should be paid to genes involved with copper tolerance or homeostasis mechanisms. In addition to this, potential tolerance mechanisms must be investigated in other organisms such as *Lactobacillus brevis* and *Candida krusei,* as they are important brewery contaminants. Similar methodologies as for *S. ellipsoides* var. *diastaticus* could be used as microarray gene chips are available for

Lactobacillus and *Candida* strains (Azcarate-Peril *et al*., 2005; Leimena *et al*., 2012; Marotta *et al*., 2013; Tamakawa *et al*., 2013).

If silver nano-particles prove to be effective at eliminating brewery contaminants, the next progression would be the manufacture of silver nano-particle surface coatings and their effects on beer. These coatings would have to be examined for their antimicrobial effect against brewery contaminants under brewery conditions. If proven effective, this coating may be applied to brewing vessel surfaces, pre and post fermentation, to eliminate any contaminants as they pass through the vessels. If functional, this coating should reduce, if not remove, the need for other contaminant control measures, such as pasteurisation, potentially reducing costs for breweries, particularly in terms of energy and water. In addition to antimicrobial efficiency any potential surface coating must also be examined in terms of leaching of silver into the product, durability of the coating, effect on beer flavour due to the oxidising nature of silver and the effect on pure brewing yeast cultures. The effect on pure brewing yeast cultures must be studied, as due to their use within breweries there is a risk of surface colonisation by these yeasts. In the majority of beers the presence of pure brewing yeast culture down stream of fermentation is undesirable, therefore, a preventative measure such as the silver antimicrobial surface would be advantageous if effective.

The potential leaching of the silver coating must be investigated for several reasons. In terms of the brewing process, if leaching into the product occurs upstream of fermentation then the silver antimicrobial may be introduced to the fermentation vessel leading to potential inhibition of fermentation. If leaching into product occurs downstream of fermentation, then the silver may

be introduced right down into final pack. In this case the prolonged exposure of beer to silver may have negative effects on flavour due to oxidation. This would be especially true for cask or bottle conditioned beers, due to inhibition of the yeasts. If leaching occurs during the sanitation stages then, depending on the breweries' recycling processes/policy of waste water, then the leached silver may have an environmental effect if disposed of in brewery waste water effluent.

In terms of the effect of leached silver in final product on human health, there is some debate about the toxicity of silver. Generally speaking silver does not have an adverse effect on humans. However, long term exposure to moderate concentrations of silver, particularly in colloidal form or silver containing medicine or silver containing solid material implants into skin/body, has been known to cause argyria. Argyria is the irreversible bluish-gray or ash gray pigmentation of the skin and/or the eyes (argyrosis), caused by silver granules or silver sulphide precipitates in the dermis. Fortunately, this condition is rare and only has a cosmetic effect (Silver, 2003; Drake and Hazelwood, 2005). However, as there is even a slight possibility of negative effects on humans, leching must be investigated. Additionally there may be a concern that continued exposure of oral and gut microflora to low silver levels, could lead to silver resistance developing in oral and enteric flora including enteric pathognes and could establish further antimicrobial resistance in bacterial populations.

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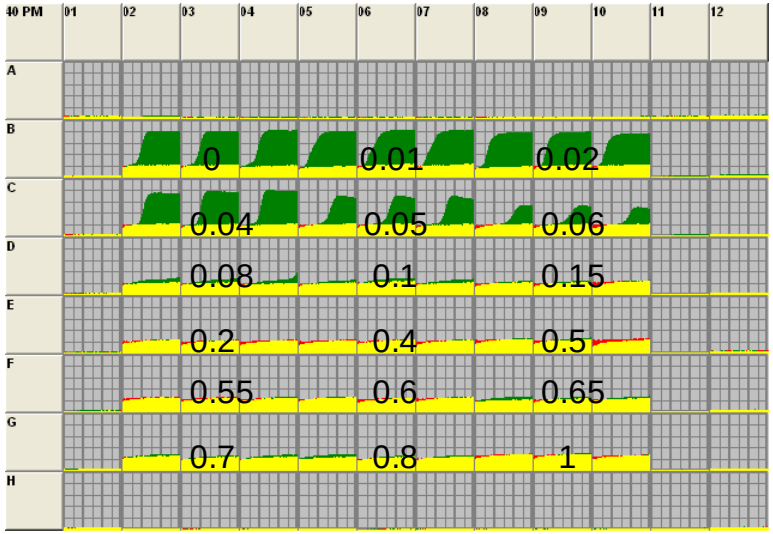
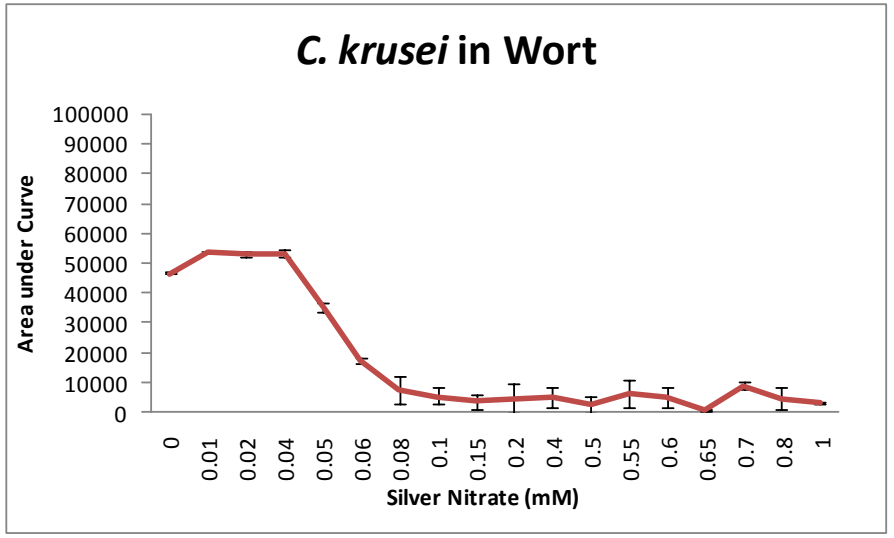
Appendix 1) Example of Biolog Metabolic Activity Assay in Wort

A)



C)



B)

91349	93223	95575	96134	97535	94558	95860	92920
85763	85962	62530	63226	65028	46275	47672	45480
36391	35732	33397	39137	35426	31449	31582	32778
31198	30838	30934	32677	32179	35465	35568	31130
34577	35757	36767	33508	36050	36458	35250	36628
35543	37725	39729	35221	36424	40102	41469	37925

Appendix 1) Example Biolog curves (Biolog units over time, A) of C. krusei in 50% wort, exposed to a range of silver nitrate concentration (0-1mM) over 72hours at 25°C. The experiment was performed in triplicate for each concentration. Image A) shows the graphs (green) and indicates the background readings (yellow) of the medium and silver nitrate without organisms. B) Area under the curve values were calculated for each graph and the control readings subtracted. This allowed the plotting of metabolic activity against silver nitrate concentration (C).

Appendix 2) Example of Optical Density Growth Assay in Wort

		Silver Nitrate Concentration (mM)								
		0	0.01	0.02	0.04	0.05	0.06	0.08	0.1	0.15
Time (Hrs)	0	0.1059	0.106767	0.107	0.104367	0.106067	0.105767	0.106633	0.106267	0.110367
	18	0.6032	0.583333	0.5445	0.5642	0.297633	0.449267	0.152067	0.163533	0.153333
	21	0.607233	0.6176	0.597267	0.590033	0.437367	0.536033	0.126867	0.146767	0.126667
	24	0.6211	0.622367	0.6312	0.608167	0.5409	0.5793	0.131433	0.178467	0.1218
	42	0.703433	0.74915	0.7596	0.717167	0.6657	0.661367	0.601067	0.152633	0.152767
	45	0.695433	0.724833	0.755	0.697267	0.660333	0.652033	0.628433	0.1436	0.172767
	48	0.689567	0.721433	0.7484	0.700067	0.654467	0.649	0.620467	0.1385	0.257433
	66	0.645767	0.75975	0.745633	0.8148	0.7356	0.682633	0.689833	0.682633	0.161667
	69	0.6546	0.7206	0.722733	0.7823	0.719633	0.66055	0.6835	0.643867	0.145867

		Silver Nitrate Concentration (mM)								
		0.2	0.4	0.5	0.55	0.6	0.65	0.7	0.8	1
Time (Hrs)	0	0.1173	0.119333	0.121033	0.119867	0.128067	0.1233	0.135533	0.104667	0.110367
	18	0.172633	0.155933	0.167567	0.1768	0.156867	0.1514	0.194033	0.564267	0.153333
	21	0.149	0.154433	0.1527	0.165433	0.155467	0.142933	0.193567	0.6008	0.126667
	24	0.198933	0.148967	0.127767	0.158233	0.147833	0.1226	0.183967	0.5837	0.1218
	42	0.17	0.1711	0.1392	0.1697	0.171967	0.137433	0.203233	0.691233	0.152767
	45	0.162	0.169167	0.131633	0.1604	0.16	0.137533	0.193367	0.6747	0.172767
	48	0.159733	0.1604	0.1263	0.1578	0.153967	0.1299	0.184067	0.672867	0.257433
	66	0.142467	0.1872	0.187967	0.1457	0.1829	0.179	0.142367	0.199867	0.161667
	69	0.135667	0.16145	0.174867	0.129767	0.163167	0.15945	0.133867	0.1879	0.145867

Appendix 2) Example of optical density growth results, Tables show mean results for *C. krusei* in 50% wort, exposed to a range of silver nitrate concentrations (0-1 mM) over 72 hours at 25°C. From this growth was plotted against time in a range of silver concentrations and silver MICs determined (4.3.2).