

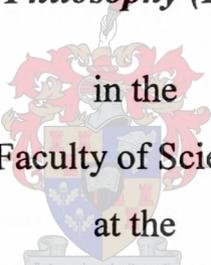
***Investigation of malt factors that influence beer  
production and quality***

by

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M.Sc. (Biochemistry)

Dissertation approved for the degree  
***Doctor of Philosophy (Biochemistry)***

in the  
Faculty of Science  
at the

The crest of the University of Stellenbosch, featuring a shield with a book, a quill, and a scale, surrounded by a red and white border. The motto 'Pietate roboretur cultus recte' is visible at the bottom of the crest.

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## Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Sandra van Nierop

Date

## Summary

A number of relevant brewing industry issues associated with malt quality were examined. These included beer foam quality, premature flocculation of yeast during fermentation and antimicrobial factors in malt.

The cause of poor foam at a brewery relative to other similar breweries was identified as being related to the boiling temperature during wort preparation and the associated conformational changes of the abundant foam protein lipid transfer protein 1 (LTP1). The temperature range of 96 to 102°C was revealed to be critical. At the higher temperature the denaturation of LTP1 was more extensive and its effectiveness as a foam protein was reduced. In addition, it was shown that the prominent role of LTP1 with respect to foam was as a lipid binding protein, forming a lipid sink and protecting foam from lipid damage.

The occurrence of malt associated premature yeast flocculation (PYF) during fermentation was induced in malt by the addition of extra-cellular fungal enzymes to the malt husk or by micro-malting barley in the presence of fungi. In addition, treating malt husk with commercial xylanase or adding commercial arabinoxylan to the fermentation also impacted on yeast flocculation. It was proposed that a range of molecular weight arabinoxylans formed by the enzymatic breakdown of the major barley husk component (arabinoxylan) resulted in PYF.

Antimicrobial activity against brewing yeast (*Saccharomyces cerevisiae*), other fungi and bacteria was found in barley, malt and malt derived wort trub. Wort trub is the non-specific precipitate of protein, polyphenols and lipids formed during wort boiling and which is, to some extent, carried over in the wort to the fermentation. Antimicrobial activity appeared to increase during malting. The growth of brewery collected yeast was inhibited in the presence of brewery production wort when compared to the same wort filtered to remove the trub. Brewery yeast was found to be more sensitive to inhibition than laboratory propagated yeast of the same strain. Different strains of *S. cerevisiae* were also found to differ in their sensitivity to inhibition. Investigation revealed that the activity originated from the inside of the barley grain and impacted on yeast sugar uptake. However, there was no direct correlation detected between levels of antimicrobial activity in malt and fermentation performance. At

high concentrations the factors were microcidal causing cell lysis. Partial characterisation of an antimicrobial extract from malt revealed the presence of a factor between 5 and 14 kDa, containing a cationic peptide component. The optimum pH stability was  $\pm 5$  when it was also most cationic. The factor easily and irreversibly lost activity at extreme pH and when exposed to certain reagents but was heat resistant in accordance with its survival in wort trub. Preliminary results showed the presence of LTP1 associated with other peptides in the active cationic fraction from the one malt tested.

The occurrence of malt related PYF and malt antimicrobial factors are associated with microbial contamination of the grain. The fungi generating the PYF factors from the barley husk while the barley's defence mechanism generates antimicrobial factors to cope with the pathogenic effect of the fungi. In addition there is a potential link between the foam protein LTP1 and malt antimicrobial activity as LTP1 or LTP1 in association with another component(s) is potentially antimicrobial.

# Opsomming

'n Aantal problematiese areas in die brouedryf, wat met mout geassosieer word, is ondersoek, naamlik bierskuimkwaliteit, voortydige flokkulering van gis tydens fermentasie en die invloed van antimikrobiële faktore in mout.

Die oorsaak van swak bierskuim by 'n spesifieke brouery relatief tot ander soortgelyke brouerye was geïdentifiseer as die moutekstrakkookpunt tydens moutekstrakbereiding. Tydens hierdie proses ondergaan dieskuimproteïen, lipiedoordrag proteïen 1 (lipid transfer protein 1, LTP1), 'n konformasieverandering. Die temperatuur tussen 96 to 102°C was kritiek t.o.v. ideale konformasieverandering vir skuimaktiwiteit. Denaturering van LTP1 het by hoër temperatuur plaasgevind wat die skuimproteïen se aktiwiteit verminder het. Daar is ook bewys dat LTP1 'n verdere rol in bierskuim speel aangesien dit 'n lipiedbindingsproteïen is wat die skuimnegatiewe lipiede verwyder.

Die voorkoms van moutgeassosieerde voortydige flokkulering van gis (PYF) tydens fermentasie is op twee maniere in mout geïnduseer, naamlik:

- deur die toevoeging van ekstrasellulêre swamensieme tot die moutdop
- deur mikrovermouting van gars in die teenwoordigheid van swamme.

Die behandeling van die moutdop met kommersiële xylanase of die toevoeging van kommersiële arabinoxilaan by fermentasies het ook die flokkulering van gis beïnvloed. Die hipotese was dat PYF veroorsaak is deur 'n reeks arabinoxilane met verskillende molekulêre massas wat gevorm het tydens die ensimatisiese afbraakproses van die primêre moutdopkomponent (arabinoxilaan).

Antimikrobiële aktiwiteit teenoor brouersgis (*Saccharomyces cerevisiae*), ander swamme en bakterië was teenwoordig in gars, mout en moutekstrakpresipitaat. Die presipitaat bestaan uit nie-spesifieke presipitate van proteïen, polifenole en lipiede wat gedeeltelik in die gekookte moutekstrak agterbly. Daar is gevind dat antimikrobiële aktiwiteit tydens vermouting toegeneem het. Die groeiproses van brouersgis, gekollekteer by 'n brouery, was geïnhibeer deur die teenwoordigheid van brouery-geproduseerde moutekstrak in vergelyking met dieselfde moutekstrak wat gefiltreer was om die presipitaat te verwyder. Die brouersgis was

meer sensitief heens inhibisie in vergeleke met dieselfde gisstam wat opgegroeï is in die laboratorium. Verskillende *S. cerevisiae* stamme het ook verskille in sensitiwiteit getoon t.o.v. die antimikrobiese komponente in die moutekstrakte. 'n Verdere ondersoek het getoon dat die oorsprong van die inhiberende aktiwiteit die interne dele van die gars is, asook dat dit die gissuikeropname beïnvloed. Daar was egter geen direkte verband tussen antimikrobiese aktiwiteit in mout en fermentasie effektiwiteit, soos gemeet onder laboratorium toestande, nie. Hoë konsentrasies van die faktore het egter gelei tot seldood weens sellise. 'n Kationiese peptiedbevattende fraksie tussen 5 en 14 kDa en 'n optimale pH stabiliteit van 5 is gevind deur gedeeltelike karakterisering van 'n antimikrobiese moutekstrak. Die aktiewe fraksie se aktiwiteit is onomkeerbaar vernietig by ekstreme pH en blootstelling aan sekere reagentie. Die aktiewe verbinding(s) is egter hittebestand en resultate het getoon dat hierdie aktiwiteit die brouproses oorleef as deel van die moutekstrakpresipitaat. Voorlopige resultate van die een mout wat getoets is het die teenwoordigheid van LTP1 getoon, asook die moontlike assosiasie met ander peptiede of kleiner komponente in die aktiewe kationiese fraksie.

Die voorkoms van moutgeassosieerde PYF en antimikrobiese faktore in mout word met die mikrobiologiese kontaminasie van gars verbind. Swamme produseer die PYF faktore vanuit die moutdopkomponente, terwyl die plant weer antimikrobiese faktore produseer as deel van 'n beskermingsmeganisme teen die patogene effek van die swamme. Daar is ook 'n potensieële verwantskap tussen bierskuimproteïen LTP1 en antimikrobiese faktore in mout, aangesien LTP1 of LTP1 tesame met 'n ander verbinding(s) moontlik antimikrobies is.

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# Table of Contents

<b>Opsomming .....</b>	<b>iii</b>
<b>Summary .....</b>	<b>v</b>
<b>Acknowledgements .....</b>	<b>viii</b>
<b>Table of contents .....</b>	<b>ix</b>
<b>List of abbreviations and acronyms .....</b>	<b>xv</b>
<b>Glossary .....</b>	<b>xviii</b>
<b>Preface .....</b>	<b>xxi</b>
<b><u>CHAPTER 1: BEER BREWING: PROCESS AND PRODUCT</u> .....</b>	<b>1-1</b>
<u>THE BEER BREWING PROCESS</u> .....	1-1
<u>BARLEY AND MALT</u> .....	1-3
<u>Structure of barley</u> .....	1-3
<u>Microbial infection of barley</u> .....	1-4
<u>Malting of barley and malt quality</u> .....	1-5
<u>Wort</u> .....	1-7
<u>YEAST AND FERMENTATION</u> .....	1-7
<u>Brewing yeast</u> .....	1-7
<u>Fermentation</u> .....	1-9
<u>Flocculation</u> .....	1-10
<u>BEER QUALITY</u> .....	1-11
<u>Factors in beer quality</u> .....	1-11
<u>Quality and microbial infections</u> .....	1-12
<u>Quality and beer foam</u> .....	1-13
<u>The mechanics of beer foam</u> .....	1-13
<u>Foam-positive factors</u> .....	1-15
<u>Foam-negative factors</u> .....	1-17
<u>Impact of raw materials on foam</u> .....	1-17
<u>Impact of process on foam</u> .....	1-18
<u>Foam assessment</u> .....	1-19
<u>REFERENCES</u> .....	1-20

**CHAPTER 2: ANTIMICROBIAL FACTORS IN BEER BREWING .....2-1**

ORIGIN OF ANTIMICROBIAL FACTORS .....2-1

PLANT DEFENCE AND ANTIMICROBIAL FACTORS .....2-1

*Proteins* .....2-3

*Peptides* .....2-3

        Thionins .....2-3

        Plant defensins .....2-4

        Lipid transfer proteins (LTP) .....2-5

        Hevein- and knottin-type antimicrobial peptides .....2-7

ANTIMICROBIAL FACTORS FROM MICRO-ORGANISMS .....2-7

*Bacteria* .....2-8

*Fungi* .....2-9

INFLUENCE OF ANTIMICROBIAL FACTORS ON BEER BREWING .....2-10

REFERENCES .....2-11

**CHAPTER 3 IMPACT OF DIFFERENT WORT BOILING TEMPERATURES ON THE BEER FOAM STABILISING PROPERTIES OF LIPID TRANSFER PROTEIN 1 .....3-1**

FOREWORD .....3-1

ACKNOWLEDGMENT .....3-1

PROCEEDINGS INSTITUTE GUILD OF BREWING, ASIA PACIFIC SECTION, ADELAIDE, 2002, PP 6 .....3-2

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY, 2004, 52, 3120-3129 .....3-7

**CHAPTER 4: DEVELOPMENT OF A SMALL SCALE PERMATURE YEAST FLOCCULATION ASSAY TO EVALUATE DIFFERETN BARLEY MALTS .....4-1**

ABSTRACT .....4-1

INTRODUCTION .....4-1

MATERIALS AND METHODS .....4-3

*Small scale PYF assay development* .....4-3

        Yeast propagation .....4-3

        Wort preparation .....4-4

        Malts and pre-fermentation analyses .....4-4

        Fermentations .....4-4

        Post-flocculation analyses .....4-5

        2L EBC tall tube fermentations .....4-5

*Preparation of PYF positive control malt* .....4-6

*Fusarium* preparation .....4-7

RESULTS AND DISCUSSION .....4-7

*Small scale PYF assay development* .....4-7

        Choice of fermentation vessel .....4-7

        Yeast propagation .....4-8

        Yeast pitching rate and wort gravity .....4-9

<u>Oxygenation</u> .....	4-9
<u>Wort analyses</u> .....	4-9
<u>Testing of the small scale PYF assay</u> .....	4-11
<u>Sensitivity of the assay</u> .....	4-11
<u>Robustness of the small-scale PYF assay</u> .....	4-14
<u>Artificial production of PYF in malt</u> .....	4-15
CONCLUSIONS .....	4-17
REFERENCES .....	4-18
<b><u>CHAPTER 5 ENZYMATIC GENERATION OF FACTORS FROM MALT RESPONSIBLE FOR PREMATURE YEAST FLOCCULATION</u></b> .....	<b>5-1</b>
FOREWORD .....	5-1
PUBLICATION J. AM. SOC. BREW. CHEM. 2004, 62, 108-116 .....	5-2
<b><u>CHAPTER 6: OPTIMISATION OF A MICROTITRE PLATE YEAST ASSAY TO SCREEN ANTIMICROBIAL ACTIVITY</u></b> .....	<b>6-1</b>
ABSTRACT .....	6-1
INTRODUCTION .....	6-1
MATERIALS AND METHODS .....	6-3
<u>Optimisation of antiyeast assay</u> .....	6-3
<u>Optimised antiyeast assay and gramicidin S dose response</u> .....	6-4
<u>Data analysis</u> .....	6-5
RESULTS AND DISCUSSION .....	6-6
<u>Microtitre plate lay out</u> .....	6-6
<u>Dilution solution for the antiyeast assay</u> .....	6-8
<u>Propagation of log phase yeast</u> .....	6-8
<u>Laboratory propagated log phase versus cryogenically stored yeast</u> .....	6-9
<u>Cell counts</u> .....	6-10
<u>Determining dose-response of yeast towards gramicidin S</u> .....	6-11
CONCLUSIONS .....	6-12
REFERENCES .....	6-13
<b><u>CHAPTER 7: SCREENING OF BARLEY, MALT AND WORT EXTRACTS FOR ANTIYEAST ACTIVITY</u></b> .....	<b>7-1</b>
ABSTRACT .....	7-1
INTRODUCTION .....	7-1
MATERIALS AND METHODS .....	7-3
<u>Malt extract preparation</u> .....	7-3
<u>Barley extract preparation</u> .....	7-4
<u>Mash extraction</u> .....	7-4
<u>Wort extraction</u> .....	7-5
<u>Assay Method</u> .....	7-5

<u>100 mL fermentations</u> .....	7-5
<u>2L EBC tall tube fermentations</u> .....	7-6
<u>Laboratory filtered and unfiltered wort as growth medium</u> .....	7-6
<u>Brewery filtered and unfiltered wort as growth medium</u> .....	7-7
<b>RESULTS AND DISCUSSION</b> .....	7-7
<u>Malt extract preparation for the antiyeast assay</u> .....	7-7
<u>Acceptable number of extracts and antiyeast analyses</u> .....	7-8
<u>Cell counts</u> .....	7-8
<u>Barley extract preparation for the antiyeast assay</u> .....	7-9
<u>Screening of malt extracts</u> .....	7-12
<u>Wort extract preparation for the antiyeast assay</u> .....	7-14
<b>CONCLUSIONS</b> .....	7-20
<b>REFERENCES</b> .....	7-21

**CHAPTER 8: SCREENING OF BREWERY YEAST STRAINS AGAINST MALT EXTRACTS TO ASSESS YEAST QUALITY .....** 8-1

<u>ABSTRACT</u> .....	8-1
<u>INTRODUCTION</u> .....	8-1
<u>EXPERIMENTAL</u> .....	8-3
<u>Antiyeast analysis</u> .....	8-3
<u>EBC 2L fermentations</u> .....	8-3
<b>RESULTS AND DISCUSSION</b> .....	8-3
<b>CONCLUSIONS</b> .....	8-7
<b>REFERENCES</b> .....	8-8

**CHAPTER 9: PARTIAL CHARACTERISATION OF ANTIMICROBIAL FACTORS IN BARLEY MALT .....** 9-1

<u>ABSTRACT</u> .....	9-1
<u>INTRODUCTION</u> .....	9-1
<u>MATERIALS AND METHODS</u> .....	9-4
<u><i>Samples for antiyeast analysis</i></u> .....	9-4
<u>Whole malt extraction</u> .....	9-4
<u>Washed malt extraction</u> .....	9-4
<u>Biomass extraction</u> .....	9-4
<u>Boiled malt extracts</u> .....	9-4
<u><i>Characterisation</i></u> .....	9-4
<u>Protein degradation</u> .....	9-4
<u>Arabinoxylan degradation</u> .....	9-5
<u>pH stability</u> .....	9-5
<u>Reagent sensitivity</u> .....	9-5
<u>Size partitioning</u> .....	9-5
<u>Lipid transfer protein 1 analysis</u> .....	9-6

<i>Antimicrobial analysis</i> .....	9-6
<u>Microflora from barley</u> .....	9-6
<u>Antibacterial assay</u> .....	9-6
<u>Antiyeast assay</u> .....	9-6
<u>Yeast lysis</u> .....	9-7
<i>Purification procedures</i> .....	9-7
<u>Gel filtration</u> .....	9-7
<u>Thin layer chromatography</u> .....	9-7
<u>Cation exchange</u> .....	9-7
<u>Liquid chromatography electrospray mass spectrometry</u> .....	9-8
<b>RESULTS AND DISCUSSION</b> .....	9-8
<i>Origin of antimicrobial activity</i> .....	9-9
<u>Antiyeast activity</u> .....	9-9
<i>Characterisation of activity</i> .....	9-10
<u>Specificity</u> .....	9-10
<u>Type of activity</u> .....	9-11
<i>Nature of antimicrobial factor(s)</i> .....	9-12
<u>Temperature stability</u> .....	9-12
<u>Enzyme stability</u> .....	9-14
<u>pH stability</u> .....	9-16
<u>Reagent sensitivity</u> .....	9-16
<u>Size of factor(s)</u> .....	9-17
<i>Partial purification</i> .....	9-18
<i>Identification</i> .....	9-21
<u>ELISA identification of LTP in extracts</u> .....	9-21
<u>Preliminary identification of factors with LC-ESMS</u> .....	9-22
<b>CONCLUSIONS</b> .....	9-24
<b>REFERENCES</b> .....	9-26
<b>APPENDIX A</b> .....	9-32
<b><u>CHAPTER 10: SUMMATION, CONCLUSIONS AND FUTURE STUDIES</u></b> .....	<b>10-1</b>
<u>INTRODUCTION</u> .....	10-1
<u>SUMMARY OF WORK</u> .....	10-1
<u>TRENDS</u> .....	10-3
<u>FUTURE WORK</u> .....	10-5
<u>CLOSING REMARKS</u> .....	10-6
<u>REFERENCES</u> .....	10-6

## List of Abbreviations and Acronyms

°C	Degrees Celsius
°P	Degrees Plato, a measure of gravity
2D	Two or three dimensional
3D	Three dimensional
AP	Antimicrobial peptide
CD	Circular dichroism
Da	Dalton
DTT	Dithiotreitol
E	Enzyme
EBC	European Brewing Congress
ELISA	Enzyme-linked immunosorbant assay
ESMS	Electrospray mass spectrometry
FAN	Free amino nitrogen (small peptides and ammonia ions)
FFA	Free fatty acids
Fig.	Figure
GC	Gas chromatography
HMW	High molecular weight
HMWP	High molecular weight polysaccharide
HPLC	High performance liquid chromatography
IC <sub>50</sub>	Inhibitory concentration for 50% inhibition of growth
kDa	kilo Dalton
L	Litre, capital used for clarity although l is the international unit
LC-ESMS	Liquid chromatography- electrospray mass spectrometry
LMW	Low molecular weight
LTP	Lipid transfer protein
M	Molarity, mols/L
Mab	Anti-foam LTP1 monoclonal antibody
MaxEnt	Algorithm in ESMS software used to calculate mass of macro molecules
mL	Millilitre

<i>m/z</i>	Mass over charge ratio
<i>M. luteus</i>	<i>Micrococcus luteus</i> , gram-positive bacterium
$M_r$	Relative molecular mass
MRS	Bacterial growth medium
MYGP	Yeast growth medium
<i>n</i>	number of samples
NIBEM	Measurement of foam expressed in seconds and represents the time it takes for foam to collapse a fixed distance (30 mm)
ns-LTP	Non-specific lipid transfer protein
OD	Optical density
P	Statistical value, probability of x falling within a 95% confidence interval
Pab	Anti-native barley LTP1 polyclonal antibody
PBS	Phosphate buffered saline
PCB	Plate count broth
Protein Z4	Abundant foam protein from barley
PYF	Premature yeast flocculation
PYF <sup>+</sup>	PYF positive
PYF <sup>-</sup>	PYF negative
R <sup>2</sup>	Correlation coefficient
RE	Residual extract
RIY	C-terminal arginine, isoleucine, tyrosine tripeptide
RP-HPLC	Reverse phase high performance liquid chromatography
rpm	revolutions per minute
rT	Temperature gradient
SAB	South African Breweries (Ltd., within South Africa)
SABMiller	SAB Plc. worldwide
SAM	Southern Associated Maltsters
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SEM	Standard error of the mean

<b>T<sub>final</sub></b>	Optical density (600nm) at the end of the antiyeast assay (Time final)
<b>TLC</b>	Thin layer chromatography
<b>Tris-HCl</b>	2-amino-2-(hydroxymethyl)-1,3-propandiol-hydrochloride
<b>T<sub>zero</sub></b>	Optical density (600nm) at the start of the antiyeast assay (Time zero)
<b>UV</b>	ultraviolet

<b>Antiyeast</b>	Antimicrobial activity against yeast
<b>Antimicrobial</b>	Ability to inhibit growth of microorganisms
<b>Beading</b>	Bubbles forming and rising to the surface of wort
<b>Brewhouse</b>	The collective term for equipment and processes used in brewing
<b>Cropping</b>	Removal of yeast, harvesting or collection of yeast from a fermentation vessel
<b>Flocculation</b>	Aggregation of yeast cells during fermentation
<b>Flocc</b>	Aggregates of yeast cells formed during fermentation
<b>Gravity</b>	Grams of solids (sucrose) per 100 g liquid wort. Fermentable sugars convert, depending on yeast strain, to ethanol and CO <sub>2</sub>
<b>Green wort</b>	Wort before it is boiled, also referred to as unstrained wort
<b>Gushing</b>	Excessive foaming or carbon dioxide release from the neck of a beer bottle causing beer to gush out
<b>High gravity</b>	Wort that has a gravity greater than 11°P
<b>Husk</b>	Outer boundary of the barley kernel, consisting of two overlapping, papery like layers rich in cellulose
<b>Internal boiler</b>	Kettle with internal heating element
<b>Kettle</b>	The vessel in which green wort is boiled
<b>Lautering</b>	Separation of sweet wort or unboiled wort from spent grain

## Glossary

Adjunct	Substances added during wort production which provide fermentable sugars in addition to those from malt (e.g. maize grits or maltose syrup)
Antiyeast	Antimicrobial activity against yeast tested using yeast as target organism
Antimicrobial	Inhibits growth of microorganism
Beading	Bubbles forming and rising to the surface of beer
Brewhouse	The collective term for equipment and processes from malt to wort
Cropping	-of yeast, harvesting or collection of sedimented/flocculated yeast from the fermentation vessel
Flocculation	Aggregation of yeast cells during fermentation resulting in flocs that sediment
Flocs	Aggregates of yeast cells formed during fermentation
Gravity	Grams of solids (sucrose) per 100 g liquid, equated to extract or fermentable sugar content, expressed in degrees Plato or °P
Green wort	Wort before it is boiled, also referred to as unboiled wort or sweet wort
Gushing	Excessive foaming or carbon dioxide release from beer on opening the bottle causing beer to gush out
High gravity	Wort that has a gravity greater than 11°P
Husk	Outer boundary of the barley kernel, consisting of two overlapping leaf-like layers rich in cellulose
Internal boiler	Kettle with internal heating element
Kettle	The vessel in which green wort is boiled
Lautering	Separation of sweet wort or unboiled wort from spent grain

Lautertun	The vessel in which lautering occurs
Log phase	The logarithmic growth phase
Malt	Barley that is malted and used as raw material for brewing
Malting	Process of germinating barley to allow synthesis of degradative enzymes and partial degradation of proteins and starch, followed by kilning to preserve and dry the grain now called malt
Mashing	In the mashtun, milled malt and water is exposed to a number of temperature stands and continually mixed to facilitate the enzymatic degradation, mainly of starch and dextrins to produce fermentable sugars
Maturation	Secondary fermentation or conditioning; little yeast is left in the beer, oxygen is minimised to avoid further yeast growth, the objective is to remove green beer flavours by enzymatic reduction in yeast
Micromalting	Small scale malting set up in the laboratory
Pitching	The addition of yeast cells to wort for fermentation
Pitching rate	The amount of yeast cells added to wort for fermentation (cells/mL)
Pronase	Cocktail of proteolytic enzymes produced by Roche Diagnostics GmbH, Mannheim, Germany
Serial re-pitching	Cropping and re-using flocculated yeast in subsequent fermentations
Steep conditioned	Pre-treatment of malt prior to milling with water to hydrate the husk milling

<b>Steeping</b>	The addition of water to barley to hydrate the grain for malting and to start germination
<b>Steep water</b>	The water added to barley during steeping, the first stage of malting
<b>Trub</b>	Precipitate formed during wort preparation (namely boiling and cooling) rich in lipids, polyphenols and metal ions
<b>Wort</b>	Boiled malt extract, dependant on the recipe may have hops and adjunct added during the boil
<b>Yeast generation</b>	The number of times yeast has been through a fermentation cycle

## Preface

The oldest proven record of the brewing of beer dates back 6000 years by the Sumarians. It was most likely discovered when bread accidentally became wet and began to ferment resulting in an inebriating pulp. The method of preparing beer by crumbling bread into water was adopted and the “divine drink” that resulted was recorded to make people feel “exhilarated, wonderful and blissful”(according to historic texts quoted by Corran, 1975).

This early beer was cloudy and unfiltered and preparation was hit and miss as the fermentation relied on the yeast from the environment. A range of cereals were used to prepare beer, but once the brewing of beer came to the northern regions of Europe (around 800 B.C.), the cooler climate suited the cultivation of barley, the main cereal used today. It is not clear when the malting of grain was started, but it was certainly in use in Europe by the ninth century. It was also during this time that the flavouring of beer with a mixture of herbs was replaced with hops. The use of hops contributed to the clear character of beer and by this stage the beer made resembles what we know as beer today (Monckton, 1966).

It was only in 1876 when Louis Pasteur revealed the existence of micro-organisms associated with the production of beer and later Christian Hansen who successfully isolated a single yeast cell and induced it to reproduce on media that the process of fermentation was understood. As a result yeast propagation was used for fermentation and the taste of beer became more repeatable (Corran, 1975 and Monckton, 1966).

Although the principles of brewing are understood, both yeast and barley are complex biological entities impacted on by numerous factors not yet fully elucidated. Yeast and barley are never exactly the same and some of these variations impact negatively on the brewing process. Variations in barley quality and some of the negative impacts on both the brewing process and the quality of the final product are the focus of this work.

*In short, the aims of this Ph.D. project were to*

- *Investigate the possible impact of malt, in association with its processing in the brewery, on observed differences in beer foam quality at different breweries.* Foam is a physical attribute of beer quality and poor foam in the final product leads to indirect financial losses related to customer dissatisfaction. Perplexing differences in beer foam quality from different breweries within South African Breweries Ltd. led to an initiative to determine the cause of the observed trends starting from the raw materials including malt. Malt quality has been associated with beer foam as has the occurrence of microbial contamination of grain which can lead to uncontrolled foaming or gushing (Results in Chapter 3).
- *Elucidate the mechanism by which malt related premature flocculation (sedimentation) of yeast during the fermentation stage of beer production arises in malt.* Premature yeast flocculation (PYF) is a recurring seasonal issue observed not only in SABMiller but across the brewing industry. The effects are incomplete fermentations and inefficient utilisation of extract with associated financial losses and compromised final beer flavour and quality. This well studied issue has been associated with microbial infections of the barley in the field and their possible continued growth during malting. The mechanism as to how it arises in malt has not as yet been elucidated (Results in Chapters 4 and 5)
- *Establish the presence and potential effect of malt associated antimicrobial factors on the production of beer.* Malt has been associated with slow/incomplete fermentations observed at breweries within South African Breweries Ltd. The yeast was believed to be inhibited in its ability to take up sugars retarding yeast growth and fermentation. This was sometimes, but not necessarily, linked to premature flocculation of yeast and causes similar losses and impact on quality. The presence of antimicrobial factors in malt, be they of plant or microbial origin, and their possible impact on beer production and quality has not been examined within the brewing industry (Results in Chapters 6 to 9).

To achieve this the following objectives were set:

- Monitor the abundant foam protein, lipid transfer protein 1 (LTP1), as a marker for foam quality during brewery and laboratory trials to compare the brewing process from raw materials to product at two different breweries where one has consistently good foam quality and the other has unexplained poor foam quality with the aim of determining the cause of the poor foam.
- Attempt to induce PYF artificially in malt to help elucidate the mechanism by which malt becomes PYF positive and monitoring the occurrence of PYF by a suitably developed PYF assay.
- Develop an assay to measure the presence of antimicrobial factors in malt and investigate the impact of yeast status on antimicrobial activity.
- Characterise the antimicrobial factors in malt, using the optimised assay, and understand their origin, significance and fate in the brewing process.

Many of the chapters in this dissertation were written in article format in order to facilitate publication and this will inevitably introduce a degree of repetition but repetition was kept to a minimum. The brewing process was summarised to facilitate understanding and introduce brewing terminology (Chapter 1). Relevant literature for this thesis on the brewing process from barley to beer, malt quality, plant antimicrobial factors and antimicrobial factors of microbial origin was reviewed (Chapters 1 and 2). Work on brewery trials and elucidation of differences in beer foam with special attention to the beer foam protein LTP1 has been included as a publication and a conference proceeding (Chapter 3). Development of an assay to measure PYF in malt was described (Chapter 4) and a publication on the elucidation of the mechanism by which malt becomes PYF positive (Chapter 5) has been included. The optimisation of an assay to measure antimicrobial activity against brewing yeast (Chapter 6), the measurement of such activity in barley and malt and the fate of these antimicrobial factors in the brewing/fermentation process (Chapter 7), the application of the assay as a method to assess yeast quality (Chapter 8) and the characterisation of the antimicrobial factors isolated from malt (Chapter 9) were all presented. In closing, a summation of the work done and proposed future work was given (Chapter 10).

Note: Throughout the thesis details of the breweries mentioned, barley and malt samples used and yeast strains are not disclosed for confidentiality reasons, although a common code for breweries, malts and different yeasts was used throughout the entire thesis, e.g. malt A in Chapter 7 will correspond to the malt A referred to in Chapter 9.

## References

Corran, H.S. (1975) In: *A history of brewing*. London: David and Charles.

Monckton, H.A. (1966) In: *A history of English ale and beer*. London: Bradley Head.

# Chapter 1

## *Beer brewing: Process and product*

### **The beer brewing process**

Although the basics of the brewing process are always similar, aspects may differ for different types of beers and different brewing traditions. This overview encompasses the brewing process as used for the work presented based on South African Breweries (SAB Ltd.) with more detail added where relevant.

The major raw materials for the production of beer are barley (*Hordeum vulgare L.*) malt, water, sugar syrup adjunct, hops and brewing yeast (*Saccharomyces cerevisiae*). There are three major processes from raw materials to beer production, namely malting, brewing and fermentation.

Barley malt is produced during the malting process, when the barley is first germinated then dried for storage. During germination most hydrolytic enzymes are produced in the grain (Briggs *et al.*, 1981a). These enzymes, together with those already present in the grain, begin to break down structural polysaccharides found in the cell walls. This makes the grain more friable and easy to mill but the actual breakdown of the storage polysaccharides (starch) during germination is very limited. After germination the grain is dried by kilning. This process halts further breakdown of the grain whilst preserving the activity of hydrolytic enzymes required for breakdown of complex storage nutrients during the brewing process. At this stage the grain is called barley malt, or simply malt, and can be stored (Briggs *et al.*, 1981a).

During brewing the malt is milled and combined with water to prepare an extract. The extraction process uses a sequence of temperature stands that allows various protein and starch degrading enzymes to break down these complex nutrients to amino acids and more simple sugars which yeast can assimilate. The breakdown of the starch not only facilitates the extraction of carbohydrates, but also of protein, amino acids, lipids and polyphenols. This process is referred to as mashing. Mashing is followed by lautering, a filtration stage that

removes the solids from the liquid extract. The extract is called green or unboiled wort. The green wort is transferred and boiled in a kettle. The functions of boiling are multiple and include the following: stripping off volatiles, formation of a precipitate comprising many polyphenols, proteins and lipids, called a “hot break” or “trub”, as well as denaturation of proteins, sterilisation of the wort and reduction of volume by evaporation.

Hops, which impart the bitter flavour to beer, are added during the boil to allow for proper mixing and extraction of hop components. Sugar syrup adjunct, such as maltose syrup from maize, may also be added depending on the type of beer and the recipe used, this will increase the specific gravity of the wort. The term “gravity”, used in the brewing industry indicates the concentration of solids and is expressed in degrees Plato ( $^{\circ}\text{P}$ ), with one degree Plato defined as one gram sucrose per 100 gram solution.

After the boil, the hot break is removed and the wort is cooled. If required the wort is diluted with water to achieve a gravity of 15.5  $^{\circ}\text{P}$ . Fermentation of wort with gravity higher than 11  $^{\circ}\text{P}$  is termed “high gravity brewing” and due to the additional sugars present, results in a higher than required alcohol concentration at the end of fermentation, which is then corrected by dilution. High gravity brewing is used because it increases the production capacity of a brewery (Pfisterer and Stewart, 1976).

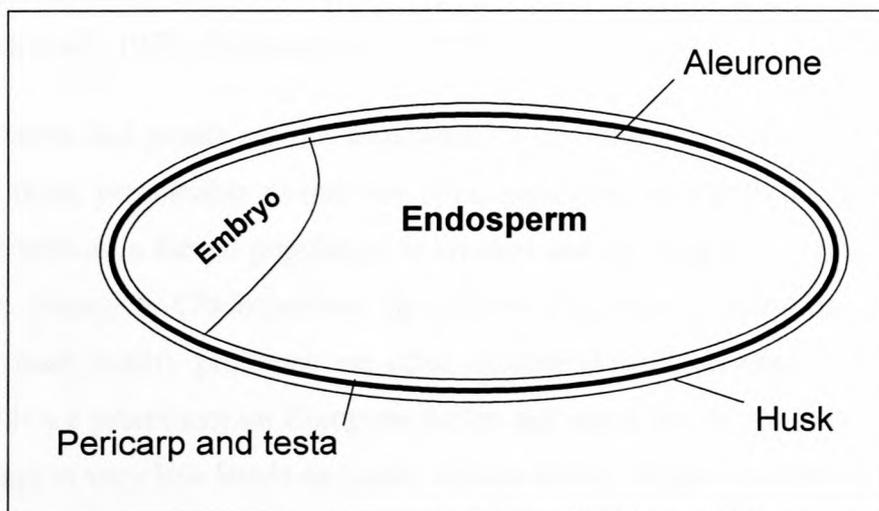
Wort is essentially the yeast growth and fermentation media. During fermentation yeast collected from a previous fermentation is added to the wort. The addition of yeast to wort is referred to as “pitching”, and the amount of yeast added per volume of wort is called the pitching rate. At the start of fermentation process there is a short lag phase while the yeast adapts to its environment. Biomass is increased with exponential yeast growth, sugars (predominantly glucose, sucrose, maltose and maltotriose (Wainwright, 1997)), free amino nitrogen (mono-, di-, tri- and sometimes also tetra-peptides as well as ammonia ions (Walker, 1998)) and lipids are predominantly utilised, decreasing their concentrations significantly. Oxygen is present in wort, but this is largely utilised during the yeast growth phase. True fermentation is anaerobic, thus the energy required for growth and other yeast metabolic functions is provided by glycolysis followed by ethanol production. Nearing the end of fermentation, which is maintained at 11 $^{\circ}\text{C}$ , the yeast flocculates (aggregates and forms flocculation complexes called “flocs”, which in the case of lager yeast sink to the bottom of the vessel) as nutrients run low and alcohol increases. The yeast is removed from the bottom of the vessel for re-pitching into the next brew. The fermented wort with a final gravity of 1 to

2°P and alcohol of 7% (v/v) is chilled to 0°C for the maturation process during which the secondary fermentation takes place by the remaining yeast. This is critical for the flavour profile of the beer and no further drop in gravity is expected. The beer is filtered, diluted with water to obtain the correct alcohol concentration (usually around 4.5% v/v) and carbonated to specification before packaging and pasteurisation (Briggs *et al.*, 1981b).

## Barley and malt

### Structure of barley

The outer layers surrounding the endosperm are the aleurone, pericarp, testa and husk where the husk forms the outermost layer. The endosperm cells are filled with starch granules embedded in protein. A cell wall consisting mainly of hemicellulose consisting of  $\beta$ -glucan ( $\beta$ -1 $\rightarrow$ 4 and  $\beta$ -1 $\rightarrow$ 3 linked glucose) and pentosans (polymers of pentoses together with some protein surround the endosperm cells). The aleurone is about three cells thick and this is where enzymes are synthesised during germination. The pericarp and testa form a waxy, semi-permeable double barrier that prevents the leakage of substances like sugars, enzymes and amino acids from the grain. The husk consists of two overlapping leaf like layers, previously part of the flower, and acts as a relatively water and insect-proof barrier (Briggs *et al.*, 1981a and Wainwright, 1997) (Fig. 1).



*Figure 1* Diagrammatic representation of a barley kernel indicating the aspects referred to in the text.

The major components of the residual cell wall that make up the husk are cellulose (linear  $\beta$ -1 $\rightarrow$ 4-glucans, 22.6%) and pentosans (20%) (Briggs *et al.*, 1981a). The predominant barley husk pentosan is arabinoxylan and the husk pentosan fraction is made up of 75% xylan, 13% arabinose and 12% uronic acid (Salo and Kotilainen, 1970). The arabinoxylans found in the endosperm are, however, different to those found in the husk. Both consist of a  $\beta$ -(1 $\rightarrow$ 4)-D-xylopyranose residues, variously substituted with  $\alpha$ -L-arabinofuranose. The backbone of the husk arabinoxylan also contains D-glucuronic acid moieties (Briggs *et al.*, 1981a and Wainwright, 1997).

### **Microbial infection of barley**

Most of the micro-flora on barley in the field consists of bacteria, wild yeast and filamentous fungi originating from the air and soil. Bacteria are the most predominant with the wild yeasts and filamentous fungi making up approximately 1 and 0.1% of the total respectively (Priest and Campell, 1987, Briggs, 1978 and Beck *et al.*, 1991). Microbial infections of the grain are generally restricted to the outer layers, namely on the husk and between the husk and pericarp, but penetration into the endosperm does occur (Schwarz *et al.*, 2002). The type and extent of infestation varies according to growing region, variety susceptibility and climate (Etchevers *et al.*, 1977) of which climate is believed to play by far the biggest role (Beck *et al.*, 1991). The microbial contamination of barley has repeatedly been linked to poor quality malt (Gjertsen *et al.*, 1965, Haikara, 1983, Noots *et al.*, 1998, Prentice, and Sloey, 1960, Schwarz *et al.*, 2001, Gyllang *et al.*, 1977, Etchevers *et al.*, 1977).

Although bacteria and yeasts are not considered to be unimportant, it is the fungi that have been studied most, presumably as they are often associated with malt quality issues (Briggs, 1978). Grain without a fungal population is unusual and the fungal genera commonly found on barley are *Altenaria*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Aspergillus* and *Penicillium*. Interestingly malt quality problems are often associated with elevated levels of *Fusarium* species, which are prominent on European barley and some North American barley, but are generally found at very low levels on South African barley. Higher overall rainfall or rainfall during harvest can cause the amount of microbial infestation to increase dramatically (especially *Fusarium*) and the increased microbial load corresponds to malt quality decline. In South Africa, where the climate is generally drier, the microbial load is not always higher, as in Europe, when there is a seasonal malt quality issue. However, the composition of the microbial population may vary due to climactic differences/stress (Ackerman A., 1998 and

Rabi and Lubben, 1993). Most of the fungi on the grain secrete enzymes including endo-xylanases,  $\beta$ -glucanases and proteinases (Hoy *et al.*, 1981, Kanauchi and Bamforth, 2002 and Schwarz *et al.*, 2002). These enzymes facilitate the breakdown of the fungal substrate, in this case the outer layers of the grain, generating nutrients that can be assimilated by the fungi.

Bacterial species most predominant on pre-harvest barley are *Erwinia herbicla* and *Xanthomonas campestris* (Flannigan *et al.*, 1996). Aside from these, other prevalent bacterial species that occur on dry stored barley include *Alcaligenes sp.*, *Arthrobacter globiformis*, *Calvibacter iranicum*, *Lactobacillus spp.* and *Pseudomonas fluorescens* (Petters *et al.*, 1988). Lactic acid bacteria are considered to be spoilage organisms in the brewing process. However, it was found that on the dry stored barley (pre-malting) the level of lactic acid bacteria were relatively low, with Gram-negative coliforms and pseudomonads more predominant (O'Sullivan *et al.*, 1999). Low levels of actinomycetes also occur on barley, mainly of the *Streptomyces* species (*S. griseus* and *S. albus*) (Hill and Lacey, 1983). The most frequent yeasts are *Candida calenulate* and *C. vini*, *Debaryomyces hansenii*, *Hansenula polymorpha*, *Kloeceera apiculata*, *Rhodotorula muciliginosa*, *Sporobolomyces roseus* and *Trichosporon beigelii* species (Petters *et al.*, 1988). Aside from the antimicrobial factors produced by some of the bacteria (see Chapter 2) many of these microorganisms also secrete enzymes, toxins, hormones and acids that may influence the grain which they are infecting (Noots *et al.*, 1998).

### **Malting of barley and malt quality**

Apart from growth of microorganisms on the grain in the field and on the dry stored barley (pre-malting), growth occurs during malting and during malt storage (Anderson *et al.*, 1967 and Prentice and Sloey, 1960). The conditions during malting are well suited for further microbial growth on barley with regard to temperatures, moisture and airflow. The population of some microorganisms increase substantially during steeping and germination (Kotheimer and Christensen, 1961), but many of these are killed off during the kilning process when the malt is dried. The total microbial load on malt is comparable to that in barley although the composition is not (Petters *et al.*, 1988).

Despite that fact that many bacteria are washed off during steeping (less so than fungi), the bacterial load can increase up to 600 times during malting. The load on malt is reported to be anything from 20 times higher (Haikara *et al.*, 1977) to less than half the original level in barley (Douglas and Flannigan, 1988). Quantifying fungal population size or load is not as simple, but a similar increase can be expected. The increase in bacterial counts is most

dramatic during steeping (although dominated by pseudomonas, the lactic acid bacteria also increase substantially), decreasing slightly at the end of germination and reduced by >98% during kilning. Some bacteria certainly remain on the malt as seen by their presence in mashing, generally lactobacilli and pediococci (which include some of the beer spoilage organisms) dominate during the early stages of mashing decreasing steadily during the mash program (O'Sullivan *et al.*, 1999 and Booysen *et al.*, 2002).

Studies have shown that certain fungi are more resistant to heat (e.g. *Aspergillus*, *Cladosporium* and *Penicillium* species) and continue to grow on the malt (Gyllang *et al.*, 1977), others even reproduce rapidly at the early kilning temperatures (e.g. *Rhizopus* and *Mucor* species) (Douglas and Flannigan, 1988 and Haikara *et al.*, 1977). Once kilned the moisture content of the stored malt is critical, up to 4-6% (w/w) moisture little change occurs in the microbial population on malt over an 18 month period. At 14% (w/w) moisture, however, a progressive increase in fungi such as *Aspergillus* species occurs. Above 20% (w/w) moisture *Penicillium* species predominate. Storage temperature and carbon dioxide levels are also critical, the optimal growth temperature for fungi is 25 to 35°C and more than 10% v/v carbon dioxide greatly reduces the fungal count (Axcell *et al.*, 1986).

Microbe-contaminated barley usually has increased post-harvest seed dormancy, a decreased capacity to germinate and increased malt yield loss during malting. The starch in the malt tends to be more modified (broken down) than normal. The increased modification is proposed to be due to the presence of additional microbial sugar hydrolases (including  $\alpha$ -amylase as observed by Gjertsen *et al.*, 1965) and proteinases (Schwarz *et al.*, 2002, Noots *et al.*, 2003 and Pekkarinen, 2003). Damage by contaminants leads to off-odour and discolouration of the malt as well as the risk of mycotoxins (Spicher, 1989).

To further support the microbial influence in these malt quality effects the addition of microorganisms such as *Fusarium* species to clean barley during malting was shown to cause detrimental changes to the malt similar to those seen when field infected barley was malted (Prentice and Sloey, 1960). Also, it has been observed that barley from a particular harvest resulted in poor quality malt from only one of two malting plants where it was used. The design of the effected malting plant was discovered to encourage more microbial growth during malting than the other plant due to the more efficient carbon dioxide extraction system.

If the carbon dioxide concentration was more than 10% (v/v), a detectable decrease in fungal growth was seen. It was speculated that the microbial population on the grain was predisposed to causing malt quality issues and was aggravated during malting (Axcell *et al.*, 1986).

## **Wort**

Malt quality directly impacts on wort, either the production thereof or the actual wort constituents. For instance, inconsistent brewhouse performance, including inefficient removal of cold break (precipitation formed during the cooling of wort after boiling), and subsequent wort clarity problems are associated with variations in the organic acid profile of batches of malt resulting from both microbial and barley metabolism during malting (Stars *et al.*, 1993). Slower mash filtration in the production of wort have also been attributed to infected barley (Prentice and Sloey, 1960). High wort colours and nitrogen has been associated with the microbially induced uncontrolled liberation of nitrogenous substances from barley during malting (Bol *et al.*, 1985). A decrease in  $\beta$ -glucan concentration as well as increased extract (sugar concentration), total soluble nitrogen and free amino nitrogen concentrations are observed in wort due to the increased starch modification during malting attributed to microbial infection. The decrease in  $\beta$ -glucan results in lower wort viscosity which has been negatively correlated to beer foam quality (Evans *et al.*, 1999a). The increased extract, total soluble nitrogen and free amino nitrogen are believed to explain the increased degree of fermentation observed by Haikara (1983).

## **Yeast and Fermentation**

### ***Brewing yeast***

There are about 700 known species of yeast, of which only a few are harmful to man. One genus of particular interest and quite different to the rest is *Saccharomyces*, which not only produces alcohol, but is able to ferment maltose and maltotriose as well as flocculate at the end of fermentation. The yeast species of interest in the case of lager beers is *S. cerevisiae*. Yeasts are fungi, typically 0.7  $\mu\text{m}$  in diameter and roughly spherical in shape. The genome has been completely sequenced with 6000 or so genes identified (Hammond, 1999). The required role of yeast in brewing is to produce alcohol and carbon dioxide with corresponding decrease in sugar levels and pH as well as producing a range of flavour active compounds. It is within this range of metabolic by-products that one beer is distinguished from another

(Lentini *et al.*, 2003). Yeast requirements are fermentable carbohydrates, assimilable nitrogen, molecular oxygen, the vitamin biotin, sources of phosphorus and sulphur, calcium and magnesium ions and trace elements such as copper and zinc ions (Walker and Birch, 1998). These are supplied mainly as maltose, amino acids, B vitamins from malt, trace elements from malt and mash (brewing) water. Yeast is a facultative anaerobic organism requiring oxygen during the initial growth phase. During fermentation sugars are metabolised to provide energy (ATP) producing alcohol, heat and carbon dioxide as by products (Lewis and Young, 1998). Due to the "Crabtree effect", glucose sensitive yeast such as brewing yeast or *S. cerevisiae* is able to ferment even in the presence of oxygen (Walker, 1998).

Areas of interest concerning yeast quality are the aging process of yeast, yeast stress factors and yeast handling in the brewery. Yeast cells, like mammalian cells, have a limited replicative lifespan. Yeast cells generally replicate 10-30 times (species and strain dependent) before cell death occurs by senescence. During this aging process the cell undergoes physiological and morphological changes accompanied by changes in gene expression effecting sugar uptake, alcohol production, formation of flavour active components and flocculation behaviour. Cell death can also occur by a process of necrosis as a result of damage to intracellular components compromising cell integrity. Typically this is due to exposure to excess stress or repeated exposure to low-level stress (Powell *et al.*, 2000 and 2003 and Smart, 1999 and 2003). Generally aging yeast was found to be more susceptible to the effects of these stresses (Rodgers *et al.*, 1999). Yeast stress factors (Heggart *et al.*, 1999) include osmotic pressure, low pH, high ethanol concentration (White *et al.*, 2003), high temperatures, high carbon dioxide levels (Hammond, 1999, Wackerbauer *et al.*, 1997 and Kruger *et al.*, 1992), mechanical stress or shear (Stafford, 2003), high physical pressure and oxidative stress (Martin *et al.*, 2003). These stressors impact the physiological status of the yeast decreasing yeast viability (dead and alive) and vitality (health), inhibiting growth, changing cell membrane stabilisation and functioning, decreased genetic stability (Stewart *et al.*, 1999 and Casey and Ingledew, 1983) and cumulating in the activation of stress related genes (Brosnan *et al.*, 2000) and protective mechanisms. Overall stress is detrimental to fermentation performance, increasing fermentation times and impacting on beer quality (Lentini *et al.*, 2003 and Smart 2001).

After yeast flocculation, during fermentation, the yeast is cropped (collected from the fermentation vessel) to be re-used or re-pitched in subsequent fermentations in a process known as serial re-pitching. Serial re-pitching and yeast handling can result in reversible and irreversible physiological deterioration of the yeast, particularly during the cropping, storage and treatments such as acid washing used to remove contaminant bacteria (Jenkins *et al.*, 2001 and 2003a and b). An increase in surface charge with re-pitching changes the flocculation behaviour of yeast resulting in gradual increased flocculation (Jenkins *et al.*, 2002 and Rhymes and Smart, 1996 and 2001). Due to the additional stress of high gravity brewing, the yeast is being used for fewer generations than in standard gravity brewing (Cholerton, 2003). Besides the obvious role of yeast in fermentation it has been shown that improved yeast vitality results in significantly better beer quality, specifically aspects of flavour (Guido *et al.*, 2004) and beer foam (Pratt-Marshall *et al.*, 2002 and Bamforth, 2003).

### ***Fermentation***

Cooled and aerated wort is fermented by yeast in the fermentation vessel to immature or green beer by the process of primary fermentation during which the yeast also reproduces. This is followed by much slower secondary fermentation or maturation with far less yeast (post-flocculation) during which appreciable flavour development takes place and the beer is matured. The quality of the mature beer is highly dependent on the yeast strain and condition as well as on wort composition (Lewis and Young, 1998). Fermentation with high gravity wort leads to increased yeast stress due to increased osmotic pressure aggravated by the higher levels of alcohol produced and higher carbon dioxide concentrations (Heggart *et al.*, 1999).

In a good fermentation oxygen is taken up by the yeast (aiding flavour stability of the final product), yeast reproduces rapidly after only a short lag phase, sugars are utilised and ethanol is produced efficiently and the correct flavour profiles are generated. When the fermentable sugars have been utilised the yeast flocculates out at the right time and to the correct degree for cropping and maturation respectively. Good temperature control avoids any added stress on the yeast

Anomalous fermentations associated with infected malt include slower fermentation rates (based on observations at SAB, although contrary to Haikara's (1983) observations), premature flocculation of yeast leading to incomplete or hanging fermentations where the yeast will not ferment and remains in suspension (observations at SAB, Kruger *et al.*, 1982).

Premature yeast flocculation (PYF) is a recurring problem in the brewing industry and has received a lot of attention over the years (Fujii and Horie, 1975, Fujino and Yoshida, 1976, Hererra and Axcell, 1991a, Morimoto *et al.*, 1975, and Nakamura *et al.*, 1997). Although associated with infected barley the link between the infection and occurrence of PYF has not yet been explained. Yeast flocculation and PYF are discussed in more detail below.

## **Flocculation**

Yeast flocculation (Smit *et al.*, 1992, Speers, *et al.*, 1992, Stewart and Russel, 1986, Stratford, 1992, Stratford and Carter, 1993) is an integral part of the fermentation process making it possible to collect the yeast from the bottom of the fermentation vessel at the end of primary fermentation, the yeast is then re-used for subsequent fermentation. Yeast flocculation occurs when the  $\alpha$ -mannan residues of mannoproteins interact with lectin-like proteins (often glycoproteins) on adjacent cells, forming large aggregates or flocs. Although the mannan residues are always present on the yeast cell surface (Stratford and Carter, 1993) the lectin-like proteins, which specifically bind sugars, are synthesised by yeast during fermentation and positioned on the outside of the yeast cell in preparation for flocculation (Stratford and Carter, 1993). When sugars are present flocculation does not occur, probably because the sugars bind the lectin-like proteins and prevent floc formation (Verstrepen *et al.*, 2003). Once the sugars are utilised during fermentation and their concentration drops flocculation occurs (Smit *et al.*, 1992). As yeast cells age flocculation patterns become stronger. This is associated with increased cell surface hydrophobicity shown to correlate with flocculation (Powell *et al.*, 2003). Increased flocculation capacity resulting from serial re-pitching may be due to one or more of the following factors, viz., the observed increase in yeast cell surface charge associated with flocculation (Jenkins *et al.*, 2003b), the accumulation of calcium and magnesium ions (known to play a role in flocculation) from repeated exposure to wort (Jenkins *et al.*, 2003b) and the selection of more flocculent yeast during cropping from the heterogeneous yeast in the fermentation vessel cone (Powell *et al.*, 2002 and 2004).

## Premature yeast flocculation (PYF)

PYF occurs when flocculent yeast (i.e. yeast with lectin-like proteins in place on the cell surface) flocculates prematurely during yeast fermentation in the presence of high sugar concentrations (Stratford, 1992). This results in un-attenuated wort with high residual extract and low end-of-ferment cell counts, all of which impact on the beer quality (Inagaki *et al.*, 1994). The premature removal of yeast may be a purely physical event associated with one or more factors that aggregate the yeast. Alternatively the yeast could perceive nutrient starvation as a result of a factor or factors that interact with the yeast membrane and inhibits sugar uptake (Grenier *et al.*, 1993, Okada and Yoshizumi, 1970 and 1973 and Okada *et al.*, 1970), thus triggering the flocculation mechanism prematurely.

The PYF factor(s) was found to be from barley, more specifically barley husk (Axcell *et al.*, 1986 and Herrera and Axcell, 1991a), which is predominantly composed of arabinoxylan (hemicellulose or pentosan) and cellulose (90% by weight) (Briggs *et al.*, 1981, Hrmova *et al.*, 1997). The factor(s) appears to be a high molecular weight (HMW) polysaccharide(s), rich in arabinose and xylose (Axcell *et al.*, 1986 and Herrera and Axcell, 1991b), acidic in nature and containing some nitrogenous material (Fuijino and Yoshida, 1976). The latter was found to be essential for the PYF activity (Fujii and Horie, 1975 and Morimoto *et al.*, 1975). Axcell *et al.* (1986) proposed the nitrogenous component to be comprised of basic peptides produced by barley in response to microbial infections with antimicrobial properties (see Chapter 2 for more information on plant response to infections). More recently Koizumi *et al.* (2004) presented work where they claim to have identified the PYF factor as a complex non-protein containing polysaccharide (predominantly arabinose and xylose) with an average molecular weight of 40 kDa. Enzymatic digestion showed that the important region was only 5 kDa. A similar, but non-active factor was found in non-PYF malt.

## Beer quality

### *Factors in beer quality*

Beer quality can be defined in terms of physical and sensory attributes. Physical aspects such as clarity, foam, lacing and colour are very important and give the first impression of the product. The sensory characteristics include shelf life or flavour stability, consistency of flavour profile and aroma for the specific beer. In addition the beer must not contain anything

harmful such as mycotoxins (from fungi on malt) which are neither visible nor impact on the flavour. Different brands and different style beers have diverse quality requirements, for instance an off flavour in one type of beer will be a required flavour in another and some require a large head of foam while in others it is of little importance. Poor quality malt, often associated with infection of barley can, however, impact on many aspects of beer quality be it positive or negative.

### **Quality and microbial infections**

The presence of fungi on barley is associated with the presence of mycotoxins such as deoxynivalenol (Flannigan *et al.*, 1985, Schwarz *et al.*, 1997) which are secondary fungal metabolites produced under stress conditions such as an imbalance of nutrients. These mycotoxins are carried through to the beer and can have a health impact on the consumer. The concentration of mycotoxins in barley or beer does not correlate to the microbial load on the grain suggesting that the type of fungi and the environmental conditions (stress on fungi) are more important. Mycotoxins are generally heterocyclic compounds and not of a protein or carbohydrate nature (Scott, 1984 and Fischbach and Rodricks, 1973).

A decrease in flavour stability of beer and the presence of several off-flavours has been linked to the use of infected malt (Etchevers *et al.*, 1977). Infected malt can also lead to haze (fine particulate matter in the beer) in the final beer (Etchevers *et al.*, 1977)

Gushing of beer, associated predominantly, but not exclusively, with *Fusarium* species infection of barley, is the uncontrolled release of carbon dioxide occurring when a beer is opened and beer/foam gushes out (Casey, 1996, Gardner, 1973 and Gjertsen 1967). It is believed to be a nucleation process requiring solid hydrophobic particles or gas residues adsorbed onto a solid support (Gardner, 1973). Beading (the gentle release of small carbon dioxide bubbles from the body of the beer) also requires nucleation sites and helps to maintain beer foam (Shafer and Zare 1991 and Ryder *et al.*, 1991). Factors in beer, which cause beading to increase and which so far have not been determined, may be connected with gushing factors.

Fungal polypeptides are associated with the phenomenon of gushing (Amaha *et al.*, 1973, Haikara, 1980, Kitabatake and Amaha, 1974 and 1977). Hippeli and Elstner (2002) suggest that the factor isolated by Kitabatake and Amaha (1974 and 1977) is a fungal hydrophobin (16.5kDa hydrophobic peptides with eight disulphide bonds) based on the chemical and

molecular information. Hydrophobins are strongly surface active proteins produced and secreted by fungi (Wessels *et al.*, 1991, Kershaw and Talbot, 1998 and Wösten and Vocht, 2000), and are involved in the formation of infection structures, amongst other things (Wessels, 1997). This role may explain why Gjertsen *et al.* (1965) observed that the interaction of the fungi with the substrate (in this case barley) was required for gushing to occur. Hippeli and Elstner (2002) further suggest that the gushing factor isolated by Kitabatake (1978) and the factor isolated by Weidender (1992) are one and the same and again based on chemical and molecular information identified as members of the non-specific lipid binding protein (ns-LTP) multigenic family of proteins (see Chapter 2). This family of proteins includes ns-LTP1, which is an abundant protein in the aleurone layer of barley grain endosperm (Yamada, 1992 and Kader, 1996) is very surface-active, being amphipathic and extremely important in the brewing industry as a foam protein.

The factors associated with gushing are hydrophobic or amphipathic and can thus form the nucleation sites required for the release of carbon dioxide. Both gushing and beading are nucleation processes and one of the possible gushing factors (ns-LTP1) is linked to foam as is beading. This suggests there may be a commonality between gushing, beading and foam.

### **Quality and beer foam**

Beer foam is complex and multifaceted. Being an important quality parameter of the final product it has been much studied. However, the requirements from raw materials and the brewing process needed to ensure good beer foam is still not fully understood. Clearly a number of factors impact on beer foam and very likely the combination thereof is important. Beer foam will be discussed according to what foam is and how it works, the positive and negative factors impacting on foam, how foam is evaluated and the link between foam and malt quality.

#### *The mechanics of beer foam*

When beer, supersaturated with gas (usually carbon dioxide), is poured, foam forms on top of the beer when the air and the gas in the beer are encapsulated into bubbles. The bubble walls or lamellae are predominantly made up of positively charged surface active malt derived proteins/polypeptides and glycoproteins interacting with each other (Bamforth, 1995, Asano and Hashimoto, 1980). Ionic interactions with negatively charged iso- $\alpha$ -acids from hops stabilise these protein-protein interactions (Ryder *et al.*, 1991, Simpson and Hughes, 1994,

Asano and Hashimoto, 1976). Furthermore, the presence of ions (notably divalent cations) (Rudin and Hudson, 1958) stabilise these ionic interactions between the hops acids and the proteins (Rudin, 1958). Initially the foam is very wet and rapid drainage of liquid away from the foam and into the beer will occur. The rate of drainage is dependant partly on the viscosity of the beer, beer with higher viscosity results in slower drainage and more stable foam (Lusk *et al.*, 2001a). Viscosity of beer is associated with barley gums or non-starch polysaccharides (including arabinoxylan and  $\beta$ -glucans) and dextrans (residual starch, remaining after starch breakdown, not metabolised by yeast) (Comrie, 1959) and glycoproteins (Ryder *et al.*, 1991). The latter actually forms part of the foam structure and as such tends to cause a localised increase in viscosity at the foam surface, retarding foam drainage. As the foam drains and becomes drier, spherical and fairly evenly sized bubbles become polyhedral and unequally sized. The lamellae become thinner and more fragile and bubbles near the air interface start to rupture (Ryder *et al.*, 1991). Within the foam bubble coalescence occurs, where two similarly sized adjacent bubbles become one bigger bubble as the lamellae in between become thinner due to drainage and ruptures (Ronteltap *et al.*, 1991 and Bamforth, 1985). The gas pressure in smaller bubbles is greater than in the larger bubbles (Ronteltap, 1991 and Comrie, 1959), the tendency is to reduce and equilibrate the pressure, this occurs by a process of disproportionation where a smaller bubble disappear into larger bubbles, reducing the overall pressure. Both coalescence and disproportionation lead to the breakdown of foam as bubbles become larger and foam becomes coarser. The gas inside the bubbles will pass through the lamellae. The rate at which this occurs depends on the solubility of the gas in the beer. The more soluble the gas the faster disproportionation occurs and the less stable the foam. Carbon dioxide for instance is more soluble than nitrogen gas and beer with nitrogen gas typically has more creamy and stable foam (Comrie, 1959, Fisher *et al.*, 1999 and Prins and van Marle, 1999).

As the foam drains and bubbles move through the foam layer, becoming larger and finally disappearing, the foam is replenished from underneath by a fresh supply of small bubbles generated by beading within the beer. Beading and bubble formation require the presence of nucleation sites and supersaturation of the beer with gas. Increased gas and nucleation to create beading will enhance foam (Parish, 1997).

### *Foam-positive factors*

Many of the foam-positive factors have been mentioned already, such as the surface-active proteins, iso- $\alpha$ -acids, metal ions, gas composition and components that increase beer viscosity (e.g. gums, dextrans, glycoproteins). In addition, melanoidins formed during kilning of malt and derived from monosaccharide and amino acids are also foam-positive, apparently stabilising foam through ionic interactions with proteins (Jackson and Wainwright, 1980). Anderson (1966) reported the presence of a foam stabilising substance comprising 70% proteins and 30% carbohydrate, presumed to be a glycoprotein. Interestingly, the carbohydrate portion was rich in xylose and arabinose, the sugars prominent in malt husk material.

Foam proteins are of particular interest in this work and these will be reviewed in more detail. Foam active proteins from malt are generally surface active (Ryder *et al.*, 1991), of high molecular weight (Melm *et al.*, 1995) and hydrophobic in nature (Slack and Bamforth, 1983). Some of the most abundant foam proteins in beer are LTP1 (Hejgaard, 1977, Jegou *et al.*, 2000 and 2001), proteins Z4 and Z7 (Hejgaard and Kaersgaard, 1993) and members of the hordein storage protein family (Asano and Hashimoto, 1980).

LTP1 is a basic protein with a molecular weight of 9696 Da (Bernhard and Sommerville, 1989), is made up of 91 amino acids and has a basic isoelectric point between 8 and 9 (Jones, 1995). It has been sequenced and the tertiary structure has been elucidated (Heinemann *et al.*, 1996). Its tertiary structure consists of four  $\alpha$ -helixes linked by flexible loops and a hydrophobic cavity that can accept one fatty acyl chain (Shin *et al.*, 1995) (Fig. 2). Due to the lack of substrate specificity with respect to the lipids they bind, they are referred to as non-specific LTPs or ns-LTPs (Rueckert and Schimdt, 1990 and Kader, 1990).

Protein Z4 is a 40 kDa protein associated with foam stability (Hejgaard and Kaersgaard 1983 and Lewis and Young, 1998) with very high surface viscosity and elasticity properties (Maeda *et al.*, 1991, Yokio *et al.*, 1989 and Douma *et al.*, 1997). It accounts for 10-25% of non-dialysable proteins in beer, approximately one third of which is glycosylated (Hejgaard and Kaersgaard, 1983) and in which form it is considered to be even more foam-active (Curioni *et al.*, 1995). Protein Z7 is another isoform of protein Z but Z4 is by far the most prominent in the majority of varieties, making up approximately 80% of all protein Z (Evans and Hejgaard, 1999).

Hordeins consist of a complex polymorphic mixture of proteins covering a wide range of molecular weights often identified by their high levels of proline and glutamine (Sheehan *et al.*, 2000 and Sheehan and Skerritt, 1997). A number of different hordeins have been identified in beer foam (Evans and Sheehan, 2002).

#### *Foam-negative factors*

Foam-negative factors include lipids, detergents (Evans and Sheehan, 2002), basic amino acids (Furukubo *et al.*, 1993) and ethanol. Lipids (from malt or yeast) and detergents (residual from cleaning tanks) both disrupt the protein-protein interactions in the lamellae surrounding the bubbles, increasing the rate of disproportionation and coalescence (Wilde *et al.*, 2003, Dickie *et al.*, 2001, Roberts *et al.*, 1978 and Coke *et al.*, 1990). Although low concentrations of ethanol (<1%(v/v)) enhance foam, at the levels found in beer it is detrimental to foam stability. This is believed to be due to the impact of ethanol on surface tension and carbon dioxide solubility (Bumbullis and Schugerl, 1979 and Pierce, 1978) although Brierley *et al.* (1996) report reduced rigidity of the adsorbed protein layer and accelerated drainage as the cause.

#### *Impact of raw materials on foam*

The main raw materials in beer include water, malt, hops and sugar adjunct. Water and malt are a source of the metal ions considered to be foam positive. Malt and hops are a source of the foam negative lipids. The major foam components, foam positive proteins, are from the malt as are the gums and dextrans, which contribute to wort viscosity. The use of adjunct dilutes the foam material from malt as well as the negatives but generally it is detrimental to foam to use a lower percentage malt.

The contribution of malt to foam outweighs that from the other raw materials. Malting will impact on the protein population of the malt but the barley itself may contribute in a number of aspects. Differences in malt quality were shown to impact on foam stability by as much as 24%. Malt protein Z4, wort  $\beta$ -glucans and wort viscosity all correlated positively to beer foam stability while the index of malt modification (ratio of total soluble nitrogen in wort and total malt nitrogen) and malt free amino nitrogen correlated negatively. Ns-LTP1 did not correlate to foam stability, which was not surprising as LTP1 is important for foam formation, an aspect not measured by the foam method used in this case (Evan *et al.*, 1999c). In Australia, barley that tended to have higher levels of ns-LTP1 originated from the wetter growing areas regardless of variety (personal communication Dr. E. Evans of University of Tasmania), although varietal variations also exist (Evans *et al.*, 1999b). This may have been due to microbial contamination, however this has not been examined. Microbial infection of barley has a direct impact on malt quality, as mentioned earlier starch breakdown increases, and this impacts on foam through decreased wort viscosity. Higher extract impacts on fermentation, which certainly impacts on the final beer quality, including foam (Haikara, 1983).

#### *Impact of process on foam*

Foam proteins increase during malting (Evans and Hejgaard, 1999 1999a and Bamforth, 2000), but over-modification (enzymatic breakdown of starch and protein during malting) of malt will result in smaller proteins and poorer foam as will residual protease activity in the mashing process (Whitair, 1978, Krauss, 1970 and Hudson, 1971). During wort boiling a precipitate (cold break or trub) is formed due to the interaction between polyphenols and proteins, which is removed. This process removes proteins including foam active proteins. Therefore, less polyphenols would result in less proteins being removed (Pierce, 1978). Excessive kilning reduces the level of polyphenols in the malt. Trub formation during the boil also removes foam-damaging lipids (Slack and Bamforth, 1983 and Bamforth, 1985). Reduced wort boiling was seen to enhance foam presumably due to less denaturation of the nitrogenous components of foam and less protein loss due to precipitation (Comrie, 1959 and Hudson and Birthwistle, 1966).

High gravity brewing has been shown to be detrimental to foam. Less efficient extraction of proteins from malt in the thicker mash (Cooper *et al.*, 1998) and a greater loss of proteins by precipitation during the wort boil has been shown to contribute to this negative effect. In

addition high gravity brewing places additional stress on the yeast due to increased osmotic pressure in response to which yeast tends to release more proteinase A, breaking down proteins associated with foam (Bryce *et al.*, 1997, Comrie, 1959 and Bamforth, 2000). Any foaming that occurs during the brewing process is detrimental to foam due to the loss of foam material, this occurs especially during fermentation with the formation of carbon dioxide (Hudson, 1971) and is exacerbated with the higher carbon dioxide levels associated with high gravity brewing. Good yeast vitality is essential for foam quality, yeast coping better with stress and avoiding unnecessary yeast lysis which release foam negative lipids amongst other things (Roberts, 1977).

Excessive filtration of beer prior to packaging is foam negative presumably due to the removal of nucleation sites for beading. Pasteurisation is foam positive. This effect has been attributed to inactivation of any residual protease activity in the beer (Bamforth, 2000).

#### *Foam assessment*

To be able to improve and study foam, an acceptable measurement of foam quality and stability is required. None of the current methods are universally accepted. Discrete assessments of individual aspects of foam exist, measuring for instance foam stability, formation or cling (adhesion to the glass) in isolation (Bamforth, 1999 and Evans and Sheehan, 2002). Methods generate foam naturally using a pouring mechanism or artificially using gas or shearing. The former is not reproducible and the latter does not represent the foam consumers' see (Constant, 1992). One of the more widely used and accepted methods is NIBEM (Klopper, 1977), foam is generated using shearing where the beer is forced through a small aperture on route from the bottle to the glass. The collapse of the foam in the glass is followed by a probe and the time it takes for the foam to collapse a fixed distance is noted. Alternatively, instead of measuring physical aspects of the foam, the foam positive proteins are quantified and related to beer foam. Bamforth (1995) fractionated beer according to hydrophobicity and quantified the protein in the hydrophobic fraction. The measurement of specific foam protein such as protein Z4 or ns-LTP1 with antibodies and enzyme-linked immunosorbant assay (ELISA) is also widely used (Lusk *et al.*, 2001b, Ishibashi *et al.*, 1996 and 1997, Kakui *et al.*, 1998, Evans *et al.*, 1999c and Evans and Hejgaard, 1999). ELISAs for LTP measurement were also used in this study as reported in Chapter 3.

The role played by the microbial infection of barley on seemingly so many aspects of beer production and quality warrants reviewing the possible response of plants to microbial infestation, plant antimicrobial factors, microbial response to competition and antimicrobial factors from microorganisms (Chapter 2).

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## Chapter 2

### *Antimicrobial factors in beer brewing*

#### **Origin of antimicrobial factors**

Cereals always have microorganisms growing on them although the microbial load and composition will vary. The micro-flora on barley, consisting predominantly, in descending order, of bacteria, wild yeast and filamentous fungi (Priest and Campell, 1987, Briggs, 1978 and Beck *et al.*, 1991), has already been discussed in Chapter 1. These organisms colonise plant tissue such as the outer layers of the barley grain as a food source. This requires penetration and solubilisation achieved mainly by secretion of enzymes such as carbohydrases to break down cell walls and the use of physical mechanisms such as the formation of haustoria at the end of fungal hyphae. Not all the microorganisms found on plants are necessarily plant pathogens and pathogenicity of microorganisms will depend on the mechanisms available to the organism to invade the plant tissue and its ability to colonise it successfully (Briggs, 1978). Microbial infection of plant tissue, such as barley seeds, can elicit a number of different defence responses from the plant to protect itself from further infestation including the production of antimicrobial factors, often peptides. The microorganisms themselves can also produce antimicrobial factors as a mechanism to compete with other organisms in a heterogeneous microbial population.

#### **Plant defence and antimicrobial factors**

Plants have an innate immune system, which is either constitutive or induced to cope with sufficiently pathogenic microbial infestation. The constitutive type defence responses are in place before the plant tissue is challenged, while the induced plant defence responses are activated when plant tissue is challenged by pathogens (Carr and Klessig, 1989).

The success of the pathogenic challenge on plant tissue depends on the genetic relation between plant and pathogen – if the pathogen has the mechanism to evade the particular immune response produced by the plant, a successful infection may occur. Physical barriers such as waxes, cutin and suberin on the outside of plant organs form part of the constitutive defence response (Morrissey and Osbourn, 1999). Plant tissue at high risk of infection may

constitutively contain chemicals such as alkaloids, unsaturated lactones, sulphur compounds, saponins, phenolic compounds (Carr and Klessig, 1989), active oxygen and nitrogen species such as hydrogen peroxide and peroxyxynitrite as well as antimicrobial peptides and proteins, all of which inhibit microbial growth (Broekaert *et al.*, 1997 and García-Olmedo *et al.*, 2001). In some instances the plant synthesises inhibitors of microbial degradative enzymes to prevent the breakdown of plant tissue by these enzymes (Morrissey and Osbourn, 1999).

Gene recognition can occur between plant and pathogen, which triggers a complex signal transduction pathway in the plant cell. Dependent on the signal perceived, an appropriate plant defence response may be activated. Such induced plant defence responses include oxidative burst, lignification, hypersensitive response, an increase of defence related gene expression and an increase in antimicrobial peptide and protein synthesis. The oxidative burst response is characterised by rapid accumulation of hydrogen peroxide in the challenged plant tissue resulting in cross linking of structural proteins of the cell wall to make the plant tissue less penetrable by the pathogens (Lamb and Dixon, 1997). Lignification is closely related to the oxidative burst and is the process whereby certain compounds synthesised in the cell are polymerised in a free-radical mediated process in the presence of hydrogen peroxide and peroxidase to form an extremely strong and resistant structure (Pearce and Ride, 1980). The hypersensitive response includes mechanisms such as cell death of the infected cells inhibiting further spread of infection, production of hydrolytic enzymes, phytoalexin (low molecular weight biocides) synthesis and lignification (D'Silva *et al.*, 1998).

The presence of pathogens modifies the host cells in a number of ways, for instance, changing the ion fluxes, phosphorylation and dephosphorylation events and production of reactive oxygen species (Fritig *et al.*, 1998). These changes will trigger defence responses and lead to the production of endogenous specific defence messages such as ethylene, salicylic acid or lipid derived molecules e.g. jasmonate. These messages lead to the expression of genes and production of proteins and peptides (Creelman and Mullet, 1997).

Much of the research done on plant defence uses seed tissue, including barley seeds, suggesting that antimicrobial factors or defence mechanisms commonly occur in seeds (Osborn *et al.*, 1995). Many of the antimicrobial factors are active against a selection of bacteria and/or filamentous fungi and/or some wild yeast but only in one instance is inhibitory activity towards *Saccharomyces cerevisiae*, the yeast species used in brewing, reported (Agizzio *et al.*, 2003).

## ***Proteins***

Pathogenesis related proteins can act on microbial plasma membranes or cell walls (Van Loon and Van Strien, 1999). Little is known about the action on the plasma membrane although interaction with ion channels and osmotic pressure play a role (Batalia *et al.*, 1996). The pathogenesis related proteins include  $\beta$ -glucanases and chitinases, which degrade  $\beta$ -glucan and chitin found in the cell walls of certain fungi (Boller, 1993). Leah *et al.* (1991) characterised three barley seed proteins, a 26 kDa chitinase, a 30 kDa ribosome-inactivating protein and a 32 kDa (1-3)- $\beta$ -glucanase that synergistically inhibited the growth of fungi (tested against *Trichoderma reesei* and *Fusarium sporotrichioides*).

## ***Peptides***

Antimicrobial peptides, isolated so far from plants, all contain 4, 6 or 8 cysteine residues that form 2, 3 or 4 disulphide bonds respectively making these compounds very stable. They all act on the plasma membrane of microorganisms and occur intra- or extra-cellularly (Broekaert *et al.*, 1997). Thionins, plant defensins, non-specific lipid transfer proteins (ns-LTPs), hevein-type and knottin-type peptides are the most distinct peptide families with antimicrobial properties that have been identified so far (Florack and Stiekema, 1994 and Broekaert *et al.*, 1997).

## **Thionins**

Thionins are approximately 5 kDa in size consist of 45 to 47 amino acids (Florack and Stiekema, 1994) and all have a pronounced amphipathic character (Clare *et al.*, 1986 and 1987, Hendrickson and Teeter, 1981, Rao *et al.*, 1995 and Stec *et al.*, 1995). They have either six or eight cysteine residues corresponding to three or four disulphide bonds (Castagnero *et al.*, 1992) and apart from a couple of conserved aromatic residues the non-structural residues tend to be variable with little sequence conservation. The 3D-structure of all thionins on the contrary are very similar, characterised by an L-shape, where the long arm constitutes two anti-parallel  $\alpha$ -helices and the short arm a  $\beta$ -sheet with two anti-parallel  $\beta$ -strands.

Thionins occur in a wide variety of plant species including mono- and dicotyledons. A range of plant tissues express thionins, in barley for instance thionins have been shown to occur in the endosperm of seeds (Ponz *et al.*, 1983 and 1986), in leaves (Gausling, 1987 and Bohlmann and Apel, 1987) and roots (Steimüller *et al.*, 1986 and Castagnero *et al.*, 1992). They predominantly reside intra-cellularly in the vacuolar compartments of the cell and to a lesser

extent in the cell wall (Reinmann-Philipp *et al.*, 1989, Broekaert *et al.*, 1997, Bohlmann *et al.*, 1988, Schrader-Fisher and Apel, 1993, Castagnero *et al.*, 1992 and 1995). Thionins are released by the rupturing of vacuoles in the cells during the plant defence response, such as during the hypersensitive response (Broekaert *et al.*, 1997). These peptides have an inhibitory effect on a wide range of fungi and several Gram-positive and Gram-negative bacteria with concentrations ranging from 1 to 15  $\mu\text{g/ml}$  resulting in 50% growth inhibition ( $\text{IC}_{50}$ ) (Cammue *et al.*, 1992, Molina *et al.*, 1993(a) and Florack *et al.*, 1993).

In the presence of other cysteine rich proteins or peptides, such as storage 2S albumins and related protease inhibitors, ns-LTPs (Molina *et al.*, 1993b, Gautier *et al.*, 1994) and wheat puroindolines (Blocket *et al.*, 1993), a synergistic impact can be seen and the inhibitory activity of the thionins is greatly enhanced (Terras *et al.*, 1993a).

The interaction of thionins with phospholipids in membranes is implicated in their toxic effect (Rao *et al.*, 1995 and Stec *et al.*, 1995). They have been shown to cause leakage of potassium and phosphate ions impacting on ATP hydrolysis and causing anabolic processes to come to a halt (Guihard *et al.*, 1993). In the presence of thionins an increase in the influx of  $\text{Ca}^{2+}$  has been seen in fungal hyphae (Thevissen *et al.*, 1996) and the antifungal activity of thionins is inhibited by  $>5 \text{ mM Ca}^{2+}$  (Terras *et al.*, 1992a and Cammue *et al.*, 1995).

### **Plant defensins**

Plant defensins have a net positive charge similar to thionins. These amphiphatic peptides have 45-54 amino acids with some sequence conservation (Broekaert *et al.*, 1995) in eight cysteine residues and four other residues (Fant *et al.*, 1994). They were originally thought to be similar to thionins (Colilla *et al.*, 1990, Mendez *et al.*, 1990), but despite being of similar size (5 kDa) and having the same number of disulphide bridges (four), plant defensins are structurally unrelated to thionins (Terras *et al.*, 1992a and Buix *et al.*, 1993). These peptides comprise a triple stranded antiparallel  $\beta$ -sheet parallel to an  $\alpha$ -helix which are connected to each other by a couple of disulphide bridges (Buix *et al.*, 1993, Fant *et al.*, 1994 and 1996 and Kobayashi *et al.*, 1991).

Defensins have been isolated from a wide range of plants including barley (Molina *et al.*, 1993 and Terras *et al.*, 1992b, Broekaert *et al.*, 1995) and they are distributed in many different plant tissues, including seeds (Mendez *et al.*, 1990, Colilla *et al.*, 1990, Bloch and Richardson, 1991, Terras *et al.*, 1992a, 1993b and Osborn *et al.*, 1995). In the tissues where defensins occur they are located in the peripheral layers in accordance with their role in plant

defence. Although generally antimicrobial, these plant peptides are mostly active against fungi and play an important role in the protection of seeds and seedlings (Terras *et al.*, 1995).

There are at least two different types of plant defensins, those referred to as morphogenic reduce hyphal elongation and as a result increase hyphal branching and those referred to as non-morphogenic which only slow down hyphal elongation (Terras *et al.*, 1992b and Broekaert *et al.*, 1997). The actual mechanism of action is not known although permeabilisation of the fungal membrane does not appear to be the primary cause of inhibition. However, an increase in the influx of  $\text{Ca}^{2+}$  has been seen in the hyphae suggesting an impact on the membranes (Thevissen *et al.*, 1996 and Osborn *et al.*, 1995). Increased ionic strength inhibits the antifungal activity of plant defensins, particularly potent are the divalent cations including  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Ba}^{2+}$  (Terras *et al.*, 1993b). The  $\text{IC}_{50}$  values for plant defensins varies substantially depending on the target fungi and the ionic strength of the media, but values of 3-6  $\mu\text{g/mL}$  have been quoted for specific fungi under defined growth conditions (Osborn *et al.*, 1995).

### **Lipid transfer proteins (LTP)**

Lipid transfer proteins were originally named for their ability to facilitate the transfer of phospholipids between membranes *in vitro* (Kader *et al.*, 1984) and it was believed that their function was one of intracellular lipid transport (Aronel and Kader, 1991). It was later discovered that not all LTPs share this ability (Scofield *et al.*, 1996, Tang *et al.*, 1996, Loh *et al.*, 1995, Zhou *et al.*, 1995, Cammue *et al.*, 1995) casting some doubt on their biological role(s). It was, however, confirmed relatively recently that LTPs do bind lipids *in vivo* (Lindorff-Larsen *et al.*, 2001). In addition Lindorff-Larsen *et al.* (2001) reported that LTP bound to C17 fatty acid demonstrated putative antimicrobial activity.

LTPs are cationic polypeptides of around 9-10 kDa and are made up of 90 to 95 amino acids (Grant *et al.*, 1995). Several have been sequenced (Bouillon *et al.*, 1987, Désormeaux *et al.*, 1992 and Mundy and Rogers, 1986, Takishima *et al.*, 1986 and Yu *et al.*, 1988) including those from barley seeds (Heinemann *et al.*, 1996). Approximately 30% of the residues are conserved, tryptophan is lacking and there are eight conserved cysteine residues involved in four disulphide bonds (Yu *et al.*, 1988) and 12 conserved aromatic or hydrophobic residues. The tertiary structures are always similar and consist of four  $\alpha$ -helices linked by flexible loops and a hydrophobic cavity, which can accept one fatty acyl chain (Shin *et al.*, 1995). Due to the lack of substrate specificity of lipids they are referred to as non-specific LTPs or ns-LTPs (Rueckert and Schimdt, 1990 and Kader, 1990).

Ns-LTPs or proteins homologous to ns-LTP, have been isolated in a number of different plants, including barley and are expressed in various tissues including barley seeds (Cammue *et al.*, 1995 and Molina and García-Olmedo, 1993, Mundy and Rogers, 1989). Ns-LTPs were discovered to have antimicrobial activity and are believed to play a role in the plant defence mechanism (Terras *et al.*, 1992b, Molina *et al.*, 1993b and Segura *et al.*, 1993). Supporting evidence for this role is their extracellular location in the cell walls of the peripheral layers of plant organs and their gene expression is usually restricted to these defined peripheral cell layers (Sossountzov *et al.*, 1991 and Skiver *et al.*, 1992). In the barley seed ns-LTPs are expressed in the aleurone layer around the starchy endosperm (Mundy and Rogers, 1986). Ns-LTPs are secreted and have been detected on the outside of plant organs. Expression is also increased when plants are infected with pathogenic micro-organisms (Molina and García-Olmedo, 1993) and in response to environmental conditions such as high salt, drought and extreme temperatures, barley specifically was shown to respond to low temperatures (Kader, 1997 and García-Olmedo *et al.*, 1995). Furthermore, a large quantity of ns-LTPs is expressed in younger plant tissue (Fleming *et al.*, 1992) that is more susceptible to microbial attack.

Ns-LTPs have also been found as one of the major protein components in the outer wax layer of plant organs (Pyee *et al.*, 1994). As such it is believed ns-LTPs are involved with the transport and depositing of cutin monomers and lipophilic substances (Sterk *et al.*, 1991 and Kader, 1997). This is supported by the finding that ns-LTP expression can be induced by drought stress when enhanced cutin or wax deposition occurs as part of the adaptation process (Torres-Schumann *et al.*, 1992).

Wheat ns-LTP1e, which is very similar to barley ns-LTP1 found in the aleurone, is not antimicrobial on its own, but it is antimicrobial in the presence of  $\alpha$ -purothionins (Dubriel *et al.*, 1998). Ns-LTPs from different plants exert dissimilar antimicrobial activities (Terras *et al.*, 1992b). Some are highly active against a broad range of fungi (Cammue *et al.*, 1995) and/or bacteria (Gram positive and/or negative) (Molina *et al.*, 1993b and Segura *et al.*, 1993). The cytotoxicity of LTPs is more restricted than thionins, but they are often also more potent (Cammue *et al.*, 1995). In some cases the antimicrobial activity of ns-LTPs was affected by the ionic strength of the media, the presence of cations reducing the antimicrobial activity while in other cases the ionic strength of the media had little impact (Cammue *et al.*, 1995). Ns-LTP IC<sub>50</sub> for fungi ranges from 1 to 100  $\mu\text{g/ml}$  and for one of the bacteria it was as low as 0.6  $\mu\text{g/ml}$  (Cammue *et al.*, 1995).

## **Hevein- and knottin-type antimicrobial peptides**

Hevein is a weakly antimicrobial (Van Parijs *et al.*, 1991) chitin-binding peptide with 43 amino acid and eight cysteine residues involved in disulphide bonds (Walujono *et al.*, 1975). Structurally it is dominated by a triple stranded  $\beta$ -sheet with a short  $\alpha$ -helix turn connecting two of the strands (Andersen *et al.*, 1993). Peptides homologous to hevein have been found to be more potently antimicrobial (Broekaert *et al.*, 1992 and 1994) and constitute the hevein-type antimicrobial peptides. Two other peptides, similar to a group of proteins known as knottins, but found to be antimicrobial are referred to as the knottin-type antimicrobial peptides. They have 36 and 37 amino acids respectively with six cysteine residues (Le-Nguye *et al.*, 1990 and Chagolla-Lopez *et al.*, 1994).

Both types of antimicrobial peptides inhibit a similar range of fungi and Gram-positive bacteria (Broekaert *et al.*, 1992 and Cammue *et al.*, 1992) and they are very sensitive to divalent cations which inhibit their antimicrobial activity at  $>1$  mM (Broekaert *et al.*, 1992 and Cammue *et al.*, 1995). As mentioned throughout, some antimicrobial peptides are inhibited by cations and it may be questioned whether they actually exert their antimicrobial activity *in vivo* in the presence of physiological concentrations of inorganic cations (Roberts and Selitrennikoff, 1990, Broekaert *et al.*, 1992, Cammue *et al.*, 1992, Terras *et al.*, 1992a and 1993b).

There are a number of other types of antimicrobial peptides for instance those with four cysteine residues which are involved in two disulphide bonds, but otherwise unrelated to any of the other peptide groups (Tailor *et al.*, 1997), peptides homologues to other antimicrobial peptides such as plant defensins (Bloch and Richardson, 1991), ns-LTPs (Campas and Richardson, 1984) and knottin-type peptides (Chagolla-Lopez *et al.*, 1994), but which act by inhibiting microbial enzymes.

## **Antimicrobial factors from micro-organisms**

Both bacteria and fungi produce antimicrobial factors, which act on competing organisms. Whereas plant antimicrobial factors are generally active against a broad range of organisms, microbial antimicrobial factors mostly act on a much narrower range of organisms.

## ***Bacteria***

Most if not all bacteria are able to produce a heterogeneous array of molecules inhibitory to closely related bacteria. These molecules include toxins, bacteriolytic enzymes, bacteriophages, by-products of the primary metabolic pathways (organic acids, ammonia, hydrogen peroxide), secondary metabolites or idiolytes, antibiotic substances like gramicidin S (synthesised by multi-enzyme complexes) and finally bacteriocins or bacteriocin-like molecules (ribosomally synthesised as polypeptides or precursor polypeptides) (Sahl and Bierbaum, 1998). The term bacteriocin is applied to antibacterial peptides from bacteria. Any proteinaceous species that are analogue, but act against other microorganism (i.e. not bacteria) are referred to as bacteriocin-like.

Bacteriocins from Gram-negative bacteria are generally large (29-90 kDa) domain-structured protein toxins (e.g. colicins), with receptor-mediated narrow spectrum activity. Some form ion channels in the cytoplasmic membrane, while others exhibit nuclease activity once inside a sensitive cell (Jack *et al.*, 1995). Bacteriocins from Gram-positive bacteria are mostly peptides with similarities to plant antimicrobial peptides; 20-40 amino acids long, <10kDa, cationic, amphipathic and derived from pre-peptides. Gram-positive bacteriocins have a broader spectrum of activity with little adsorption specificity, generally active against a wide range of Gram-positive bacteria and in some cases inhibitory of Gram-negative species (Jack *et al.*, 1995). Although the majority of peptide bacteriocins are linear peptides (e.g. lactococcin, pediocins and lactacins), which may or may not contain cysteine residues and disulphide bonds, a significant number are subject to unique post-translational modifications resulting in unusual structural features. Such bacteriocins include the lantibiotics (e.g. nisin, microcin and subtilin) so named since they contain modified residues of the thioether amino acids lanthionine and methyllanthionine (Sahl and Bierbaum, 1998). The plain low molecular weight bacteriocins are generally membrane active as are the lantibiotics but their membrane insertion appears to be voltage dependent (Jack *et al.*, 1995 and Rao, 1995).

Lactic acid bacteria are widely used in the food industry since they produce a wide range of antimicrobial factors including organic acids, hydrogen peroxide, diacetyl, carbon dioxide, bacteriocins and antibiotics, which have a broad range of activity. Lactobacilli for instance produce a range of antimicrobial factors some of which are active against one or more of the following; Gram-positive bacteria (e.g. *Lactobacilli casei* and *Bacillus subtilis* and *L. delbrueckii* species), Gram-negative bacteria (e.g. *Pseudomonas putida* and *Enterobacter cloacae* species), filamentous fungi (e.g. *Aspergillus parasiticus* species and members of the

genus *Fusarium*), and yeasts (e.g. *Candida albicans* and *Saccharomyces cerevisiae* species). Activity is listed from most common to least, yeast as a target organism being rare. Many of these factors are organic acids and short chain fatty acids, but in some cases they are proteinaceous (Atanassova *et al.*, 2003 and Magnusson *et al.*, 2003). Antifungal peptides, like bacteriocins are membrane active causing cell lysis (Shai, 1995) or interference with cell wall synthesis (Debono and Gordee, 1994).

Gramicidin S, a cyclic antibiotic peptide produced by Gram-positive *Bacillus brevis*, is known to be active against numerous Gram-positive and a few Gram-negative bacteria, adsorbing to the cell membrane and preventing the functioning of the membrane. If it adsorbs to the entire membrane it can be bacteriocidal (Prenner *et al.*, 1997 and Yonezawa *et al.*, 1986). It was more recently reported to be antifungal as well, inhibiting the growth of *Spaerotheca fuliginea* (Schmitt *et al.*, 1999). Several *Bacillus subtilis* species produce small antifungal cyclic lipopeptides belonging to the group of molecules called iturins which affects membrane surface tension and causes pore formation leading to leakage of vital ions (Maget-Dana and Peypoux 1994 and Mhammedi *et al.*, 1982). Iturins have been shown to inhibit growth of the various fungi e.g. *Aspergillus niger*, *A. flavus*, *Candida albicans*, *Fusarium oxysporum* and *F. moniliforme* (Klich *et al.*, 1991) and *Saccharomyces cerevisiae* species (Besson *et al.*, 1984 and Latoud *et al.*, 1987). Some species of the *Pseudomonas syringae* produce small cyclic lipodepsipeptides known as syringomycins, which increase the plasma membrane potential in plants and yeasts (Reidle and Takemoto, 1987). They also produce another family of peptides called pseudomycins with broad-spectrum antifungal activity (Harrison *et al.*, 1991).

## ***Fungi***

Singh *et al.* (1971) reported the incidence of antibacterial compounds in fungi such as *Candida albicans*, *Rhizopus nigricans*, *Aspergillus leukensis*, *Penicillium funiculosum* and *Trichoderma virid.* Most of these compounds were found to be sterols, phenolic compounds and tannins. Some species of *Aspergillus* (*A. gigantues* and *A. clavatus*), produce ribosome inactivating proteins called gigantins and clavin- both 17 kDa, noted for their antiviral activity and anti-proliferative effect on mammalian cells, but no mention has been made of their effect on other fungi or bacteria. Another large peptide (51 amino acids) isolated from *A. gigantues* inhibited the growth of some filamentous fungi, but had no effect on bacteria or yeast (Ng, 2004).

## Influence of antimicrobial factors on beer brewing

Antimicrobial producing microorganisms and their antimicrobial products have been shown to occur naturally on barley and malt Vaughan *et al.* (2001). Members of the genus *Enterococcus* and a number of *Lactobacillus lactis* species that produce broad range bacteriocins, also active against a number of beer spoilage organisms, were isolated on barley grain and were able to produce bacteriocins when cultured in wort validating their possible application to control the growth of beer spoilage organisms in the brewing process (Hartnett *et al.*, 2002 and O'Mahony *et al.*, 2000).

Although antimicrobial factors and plant defence have been studied at length, any possible relevance to the brewing industry seems largely if not entirely neglected. Antimicrobial activity of microorganisms on the barley may influence the malting process by impacting on the composition of the micro-flora. This has been exploited in the use of starter cultures in malting where less pathogenic fungi (*Rhizopus* and *Geotrichum* species) or bacteria (*Lactobacillus* species) are selected and added to the steep water to inhibit (due to the presence of antimicrobials) the less desirable organisms. Controlling the microbial population on barley in malting has led to detectable improvements in malt quality both in terms of processing and final beer (Dufait and Coppens, 2004). Members of the genus *Fusarium* associated with mycotoxin production and gushing in beer was successfully inhibited using *Lactobacillus plantarum* species (Laitila *et al.*, 2002) or *Geotrichum* species (Boivin and Malanda, 1997) as starter culture during malting. Malts prepared with a *Rhizopus* starter culture in steep were better modified (hydrolysed) with higher levels of xylanase and beta-glucanase. Proteolytic activity, wort colour and wort pH were also influenced (Noots *et al.*, 2001). Starter cultures of lactic acid bacteria have even been employed on barley in the field resulting in decreased levels of *Fusarium*, water sensitivity of the grain and gushing tendency while free amino nitrogen, alpha amylase activity, malt modification and wort filterability increased (Reinikainen *et al.*, 1999).

The brewing process uses yeast to produce alcohol amongst other products/compounds and any antimicrobial factors surviving the production of wort, be it from the microorganisms on the barley/malt or from the malt itself may pose a threat to the functioning of the yeast. Any negative impact on the yeast is detrimental to beer processing and beer quality as reviewed in Chapter 1. Interestingly early on in the research of antimicrobial peptides Okada and Yoshizuma (1973) detected barley and wheat antimicrobial peptides that at low concentrations (0.4 µg/mL) were shown to adsorb on to the cell wall and membrane of yeast

*Saccharomyces cerevisiae*, inhibiting respiration, fermentation and incorporation of sugars into the cell and causing membrane permeabilisation seen by leakage of potassium and phosphate ions as well as nucleotides and proteins out of the cells. At higher concentrations (4 µg/mL) cell death was observed. It was determined that these peptides had a molecular weight of 9.8 kDa, an  $\alpha$ -helix content of 34.5% (Okada and Yoshizuma, 1970) and their toxic effect could be removed by the presence of divalent cations such as  $\text{Ca}^{2+}$  at concentrations of >5 mM (Okada *et al.*, 1970). Moreover, the peptide could be digested with a cysteine protease like trypsin, but not chymotrypsin and carboxypeptidase. All these observations point to the identity of these peptides as ns-LTP (Douliez *et al.*, 2001). It should be noted that the antimicrobial extracts from barley and wheat were not heated at any stage, hence giving no indication of survival in a true brewing process. However, the extraction procedure was harsh suggesting the factor is quite stable.

The aim of the first two chapters was to provide the relevant literature background for the ensuing chapters of the research done with the underlying common theme of malt quality, the role of peptides and the effect of microbial contamination of barley. These subsequent chapters follow the basic sequence as the literature review; investigation of the possible impact of malt and its processing on differences in beer foam quality at a number of breweries, elucidation of the mechanism by which malt related premature yeast flocculation arises in malt and establishing the presence and potential effect of malt associated antimicrobial factors on the production of beer.

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## Chapter 3

### *Impact of different wort boiling temperatures on the beer foam stabilising properties of lipid transfer protein 1*

#### Foreword

When faced with the question as to why a particular brewery within a group of breweries had consistent poor beer foam quality without any apparent cause, an investigation was undertaken. The aim was to establish the cause and mechanism of the poor foam quality and how to improve the foam quality. The role of malt factors was examined and in particular the abundant foam protein, lipid transfer protein 1 (LTP1).

The work on beer foam and LTP1 is described in this chapter and was presented as well as published as a conference proceeding (*Proc. Inst. Guild of Brewing, Asia Pacific Section, Adelaide, 2002*, pp 6). Subsequent to this, more work was done on the LTP1 and its conformational changes during brewing, this was published as a peer reviewed article in the *Journal of Agricultural and Food Chemistry* (2004, 52, 3120-3129)<sup>1</sup>. The research reported in the conference proceedings was summarised for peer review in the publication leading to a degree of unavoidable repetition.

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The RP-HPLC reported in Figure 4 (Van Nierop *et al.*, 2004) was performed by the co-author, Dr. E. Evans. The 2D gel electrophoresis reported in Figure 5 (Van Nierop *et al.*, 2004) was done in conjunction with Dr. E. Evans. The CD spectroscopy reported in Figure 7 (Van Nierop *et al.*, 2004) was performed by the co-author, Dr. M. Rautenbach.

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<sup>1</sup> Editorial errors: Table 4, the footnote refers to figure 8, this should be figure 6. Table 5, the foam capacity at 96-100°C should be high, represented by two ticks not one and at 102-106°C the foam capacity should be less, represented by one tick, not two.

# Studies on Beer Foam Proteins in a Commercial Brewing Process

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## Abstract

A survey of in package beer foam attributes including foam proteins, LTP1 and Z4 led to a series of microbrewery and commercial brewing trials. Monitoring these proteins through the brewing process identified the temperature of wort boil as a critical stage effecting foam protein levels in beer. Boil temperature differences between sea level and higher altitude breweries caused a major difference in the survival of LTP1 through to the finished beer. The concentration of the specific foam proteins, LTP1 and Z4, in wort and in beer frequently relates to the beer foam stability. However, a lack in balance between foam positives and negatives can cause this relationship not to be evident.

**KEYWORDS:** commercial scale, in-process, wort boiling, foam, foam proteins, LTP1

## Introduction

A CONTINUING priority within South African Breweries (SAB) is to improve quality consistency of national brands produced across the seven breweries in South Africa which all differ slightly in configuration and equipment. Ongoing monitoring showed two breweries habitually had beers with the lowest and highest foams in the group in spite of the fact that raw materials and processing protocols were common.

Beer foam, both in its appearance and stability, is an important aspect of overall beer quality and establishes the taste expectation of the product for the consumer<sup>1</sup>. The basis of foam stability is the interaction of a number of parameters including hop iso- $\mu$ -acids and beer proteins/polypeptides<sup>2</sup>. More specifically, for a good foam, foam positives such as hop acids<sup>3</sup>, proteins<sup>4</sup>, metal ions<sup>5</sup> and gas composition<sup>6</sup> need to be at a maximum while foam negatives such as lipids<sup>7</sup>, basic amino acids<sup>8</sup>, ethanol<sup>9</sup>, yeast protease activity<sup>10</sup> and excessively modified proteins<sup>11</sup> need to be at a minimum to create the optimum balance.

An area of much interest in foam research is the foam positive proteins<sup>12,13</sup>. The key malt foam proteins are LTP1<sup>14,15,16</sup>, proteins Z4 and Z7<sup>17</sup> and the members of the hordein storage protein family<sup>18</sup>. LTP1 is reportedly associated with foam formation and foam stability, the latter only when in conjunction with other proteins. It has been documented to be only foam active in the heat denatured form<sup>13,17,19</sup>. Furthermore LTP1 is believed to play an additional role as a lipid binding protein enabling it to mop up foam negative lipid materials and so protect beer foam from lipid related damage<sup>20,21,22</sup>. In contrast, protein Z4 is considered to be only associated with foam stability<sup>22</sup>. Both LTP1 and Z4 are tolerant of high temperatures and resistant to proteolysis<sup>24,25,26,27</sup>, factors which contribute to their resilience during the brewing process. Monitoring specific foam proteins such as LTP1 and Z4 by enzyme-linked immunosorbant assay (ELISA) on the basis of antibody recognition is well established as a very sensitive and specific method<sup>12,17,27</sup>. Although these specific foam proteins have their own attributes, it follows that they may also reflect the behaviour of other foam positive proteins

in beer, and so, by monitoring these proteins they may act as a marker of all foam positive protein material<sup>14</sup>.

Of the items deemed negative to foam, much attention has been directed to certain lipids<sup>8,29</sup>. These lipids, derived from raw materials or yeast, are regarded as detrimental to foam as they disrupt the continuity of the polypeptide film<sup>30</sup>. Numerous other materials have been reported to exert a negative effect on foam but, by their very variety, their measurement and identification can be difficult. Some estimation of the balance of positives and negatives can be made by beer dilution foam tests<sup>2,20</sup>. In the case of lipids, their impact on foam may be determined by challenging beers with free fatty acids. This test is referred to as foam robustness<sup>2,31</sup>.

The beer dilution, foam robustness, LTP1 and protein Z4 ELISA tests have been applied in an investigation to determine the cause of the observed differences in foam. Both microbrewery and commercial brewery scale trials were undertaken. However, in contrast to earlier reported works<sup>12,14,27</sup>, it is the extension of the study to commercial brew scale that makes the conclusions more relevant and realistic.

## Experimental

### LTP1 and Z4 ELISA

LTP1 and Z4 was determined by quantitative double sandwich ELISA according to Evans and Hejgaard<sup>17</sup> using polyclonal LTP1 and Z4 antibodies and standards developed in their laboratory. Wort and beer samples were diluted 2000 times for LTP1 analysis and 2814 times for Z4 analysis. The coefficient of variance of the LTP1 and Z4 analysis was less than 10%. The absorbance at 415nm was read on a Bio-Rad (Richmond, CA) model 450 microplate reader.

### Microbrewery trials

A 40 L brewlength BAM (Bavarian Apparatus and Machinery, Freising, Germany) microbrewery was used with 32 L fermenters and a Krones (Neutraubling, Germany) single head filler packaging system.

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## Chapter 4

### ***Development of a small scale premature yeast flocculation assay to evaluate different barley malts***

#### **Abstract**

A small-scale fermentation type assay for faster detection of premature yeast flocculation (PYF) was developed and optimised as a research tool for subsequent investigative work to improve our understanding of this phenomenon. The method was validated by comparison with the well-established two litre EBC tube laboratory fermentation method, which provides more information about many fermentation parameters including flocculation, but is time consuming and labour intensive. Visual assessment of flocculated biomass, post-flocculation wort gravity (residual extract) measurement and cell counts in suspension were used as measures of flocculation relative to the controls. Results were evaluated by comparing the PYF positive and PYF negative control malts with test malts. The preparation of artificially induced PYF positive malt, as an alternative PYF positive control, is also described.

#### **Introduction**

Malt analysis has been the topic of much debate for the past few decades, because the current analyses do not satisfactorily predict the performance of the malt in the brewery (Glen *et al.*, 1998, Inagaki *et al.*, 1994, Jones *et al.*, 1976, Palmer, 1985, Silberhumer, 1990, and Wainwright and Buckee, 1977). The majority of these analyses directly or indirectly evaluated the modification of the grain during the malting process. For instance many methods quantified the extent of protein and starch breakdown while others measured the enzyme activity by which this breakdown occurs. These factors contribute to the amount of “extract” (sugars) that can be recovered from the grain, wherein lies the value of malt. It is this extract that is converted to alcohol during the fermentation on which the effectiveness of the process was evaluated (Briggs *et al.*, 1981). However, the sole aim of brewing beer is not to just produce alcohol, but also to produce a consistent quality alcoholic beverage. The malt, as the major raw material, contributes to consistency and product quality along with the actual processing. Although several processing and quality issues have been attributed to malt

(Axcell *et al.*, 2000, Herrera and Axcell, 1991, Morimoto *et al.*, 1975, Okada and Yoshizumi, 1970, Okada *et al.*, 1970, Palmer, 1985 and Sarx, 2002), these were not predicted by the existing malt analyses. The discrepancy between specific data that is required from malt analyses and the data current analyses provided, indicated a need for the development of alternative/additional malt analyses that can predict critical aspects of malt performance in the brewery.

Premature yeast flocculation (PYF) is malt related and one of the aspects not predicted by conventional malt analyses. PYF during fermentation leads to incomplete fermentation due to lack of yeast cells in suspension. This results in low alcohol concentrations and high levels of residual sugar (Smit *et al.*, 1992, Speers *et al.*, 1992, Stratford, 1992 and Verstrepen *et al.*, 2003). The end-of-ferment yeast counts observed in commercial breweries can range from  $2 \times 10^6$  to  $>20 \times 10^6$  cells/mL. These differences influence secondary fermentation and hence the beer flavour profile and stability. It is a recurring problem in the brewing industry and has significant financial implications resulting from the associated incomplete extract utilization. Predicting this phenomenon in malt would allow evasive action at the barley/malt purchasing stage or before/during the brewing process. Yeast flocculation itself and the process of PYF during fermentation have been well-studied and are mostly elucidated (Smit *et al.*, 1992, Speers *et al.*, 1992, Stratford 1992 and Verstrepen *et al.*, 2003). However, a small scale malt PYF assay with a sufficiently fast turn around time would facilitate the study of the mechanism leading to the development of PYF malt, which as yet is not fully understood.

Various methods for the measurement of PYF in malt have been reported (Baker and Kirsop, 1972, Fujino and Yoshida, 1976, Koizumi *et al.*, 2004, Mochaba *et al.*, 2001, Inagaki *et al.*, 1994 and Ishimaru *et al.*, 1967), of which some even allow for measurement a step earlier in the barley (Nakamura *et al.*, 1997). Overall there appear to be two different types of PYF assays: one that does not require fermentation using malt extracts (Koizumi and Ogawa, 2004 and Mochaba *et al.*, 2001) and the other based on small scale fermentations using wort (boiled extract of raw materials as used in the brewing process) (Inagaki *et al.*, 1994 and Ishimaru *et al.*, 1967). For the latter type of assay differences tend to be around scale, measurements/assessment, the fermentation medium used and the yeast. Many of these differences depend on the aim of the assay and the brewing conditions they are trying to emulate. Likewise for these purposes, it was necessary to develop an assay reflecting our brewing process and the sensitivity of yeast to PYF under those conditions. Not all yeast strains are sensitive to flocculation changes and a particular brewery's processing can be more

or less susceptible to PYF. None of the published PYF assays are recommended as a routine method in the brewing analysis manuals, indicating the complexity of measuring PYF reliably. The development of a small scale PYF assay is reported here and was used in further research on PYF (Chapter 5). In addition, a method to artificially induce PYF in malt was established, because PYF positive malt, which must be used as a positive control for such a PYF assay, is not always available, as the problem is intermittent.

## **Materials and methods**

### ***Small scale PYF assay development***

The assay consisted of small-scale fermentations and is based on similar existing methods (Fujino and Yoshida, 1976, Inagaki *et al.*, 1994 and Ishimaru *et al.*, 1967). Each assay was a set of up to ten duplicate fermentations, run in parallel, including a positive and a negative PYF control fermentation. The assay controls were prepared from a PYF negative malt with normal fermentability and flocculation properties and a strongly PYF positive malt. The same control malts were used throughout all the assays to enable comparison from run to run. The results were assessed relative to the controls within a run using visual assessment of the flocculation, post-fermentation cell counts remaining in suspension in the wort and post-flocculation wort gravity measurements.

### **Yeast propagation**

Yeast used for the assay was propagated in steam sterilised enriched MYGP broth (115 g maltose, 3 g malt extract, 3 g yeast extract, 27.5 g glucose and 5 g peptone (Biolab Diagnostics, Midrand, RSA) dissolved in 1 L deionised water) from cryogenically preserved lager yeast (SAB Ltd. [Sandton, South Africa] brewing yeast) in two stages.

For the first stage, the propagation medium consisted of 12 mL enriched MYGP broth diluted with 3 mL sterile deionised water in 100 mL Erlenmeyer flasks plugged with cotton wool and covered with foil. Cryogenically stored yeast was inoculated at approximately  $1 \times 10^6$  cells/mL and cultures were incubated at 25°C for 20 hours with continuous shaking at 150 rpm, by which stage the culture was in log phase. For the second stage 8.5 mL was transferred aseptically to 200 mL sterile enriched MYGP broth in 1 L Erlenmeyer flasks plugged with cotton wool and covered with foil, followed by incubation at 20°C with continuous shaking at 150 rpm. Yeast was used when in stationary phase (28 hours) for the pitching (inoculation) of

wort. Yeast cells were counted in a Hawksley–Cristallite Haemocytometer with improved Neubauer ruling (Boeco, Hamberg, Germany).

After counting, the volume of cells required to obtain a pitching rate (cell concentration) of  $20 \times 10^6$  cells/mL for 250 mL wort samples was calculated, aseptically dispensed into sterile centrifuge tubes and centrifuged at 5°C for 5 minutes at 5000xg. The supernatant was discarded and the pellet aseptically re-suspended in 250 mL of wort for fermentation.

### **Wort preparation**

The wort used for the assay was prepared by an adaptation of the method of Kruger *et al.* (1982) to give an all malt wort with a gravity of 13 degrees Plato (°P) using a R. Chaix M.E.C.A. mash bath (Nancy, France) with 500 mL beakers and overhead paddle stirrers. 1°P is defined as 1 g solids / 100 g solution and was measured using an Anton PAAR Beer Analyser (Anton PAAR, Graz, Austria). A ratio of 350 mL mash water (deionised water with 1.2 mM CaCl<sub>2</sub>.2H<sub>2</sub>O (Sigma, St. Louis, USA) and with pH adjusted to 3.0-3.1 using a dilute solution of lactic acid (Sigma, St. Louis, USA) to give a final pH of 5.2) and 130 g malt was used. The mash preparation temperature profile was: 60 minutes at 63°C, 20 minutes at 72°C and 5 minutes at 76°C, with temperature increasing at a rate of 1°C/min between temperature stands, temperatures were controlled within  $\pm 1^\circ\text{C}$ . After filtration, wort was steamed for 30 minutes in 250 mL aliquots in 1L Duran Schott bottles (BDH, Dorset, England) (sufficient wort for duplicate 100 mL fermentations) and stored at 5°C for no longer than 2 weeks.

### **Malts and pre-fermentation analyses**

For each assay, wort was prepared from PYF negative and PYF positive control malts and the test malts. Carbohydrate profiles and wort free amino nitrogen (FAN) were determined according to EBC methods 8.7 and 8.10 (European Brewing Convention, 1998) respectively to ensure differences in flocculation were not due to differences in concentrations of fermentable carbohydrates. In order for worts to be used in the same assay carbohydrate values were required not to vary by more than 15% and the FAN concentration to be equal or greater than 200 mg/mL.

### **Fermentations**

For the fermentations, the appropriate amount of harvested yeast for a pitching rate of  $20 \times 10^6$  cells/mL was added to 250 mL sterile wort in 1L Duran Schott bottles. The bottles were

capped and then shaken 35 times to aerate the wort as described by Phaweni *et al.* (1992). The wort was allowed to stand for five minutes to drain the foam formed during shaking. After swirling the wort to ensure even suspension, 100 mL was measured in duplicate and poured aseptically into two pre-sterilised 100 mL separation funnels plugged with cotton wool covered with foil.

Fermentation funnels were incubated at 12.5-13.0°C. Flocculation for PYF positive and PYF negative samples occurred between 70 and 80 hours post-pitching. Visible assessment of the flocculation of the test samples was made relative to the controls during this time. Digital images of the flocculation were acquired as a record of results.

### **Post-flocculation analyses**

Analyses were carried out 12 hours post-flocculation of the PYF negative control fermentation. Yeast count sampling was done at a standard depth (1.5 cm) below the surface of the fermentation. Yeast cells were counted as before, alternatively relative yeast concentration was determined by measuring the light dispersion or optical density (OD) of the yeast suspension at 600 nm using a dual beam UV/visible spectrophotometer (Shimatzu, UV-1600, Tokyo, Japan). The cell counts were compared to the control samples, with a positive result having a third or less cells than the negative control. The remainder of the wort was centrifuged at 5000g for 5 minutes, filtered through Whatman no.1 filter paper (Whatman International Ltd., Maidstone, England) and the gravity (also referred to as residual extract or RE) was measured and expressed in °P. The PYF positive control fermentations used in this work routinely had gravities of up to 20% higher than the PYF negative control fermentations. However, presence of PYF does not always result in higher gravities in the rapid test, thus this parameter cannot be used as a definitive indicator of PYF.

### **2L EBC tall tube fermentations**

Brewery yeast was collected aseptically from the yeast storage vessel within 6 hours of cropping (harvesting from the bottom of the fermentation vessel) and stored on ice at 5°C for no longer than 12 hours.

Wort with a gravity of 16°P was prepared with 40% dextrose syrup/60% malt, using 12 x 2 L bucket mash bath with overhead paddle stirrers as described by Kruger *et al.* (1982). The wort was steamed for 30 minutes in 2 L aliquots in 5 L round flasks with cotton wool plugs covered in foil and stored at 5°C for no longer than two weeks.

EBC 2L fermentations were done using a modification of Kruger *et al.* (1982) and Phaweni *et al.* (1992). The stand time after pitching and first wort aeration was changed from 2 to 4 hours. Fermentations were also monitored daily for yeast counts and gravity as described earlier.

### ***Preparation of PYF positive control malt***

South African grown Chariot (cv.) barley corresponding to non-PYF malt (used as a control malt for comparison) was sourced for micro-malting. Micro-malting was performed in 2 L plastic jars, covered with foil inside a standard laboratory incubator with an ultrasonic humidifier (Salton, Lake Forest, Illinois) placed inside to avoid drying out of the grain. Steep water constituted tap water boiled for one hour and cooled. During steeping enough water was added to submerge the grain entirely. The barley was malted using the following regime: two cycles of 17 hours wet (steep) followed by seven hours dry, then one final hour of wet and then germination, which took between 44 and 64 hours. Malt was turned and mixed approximately every 12 hours. Germination was terminated based on rootlet growth. The malt was dried at 45°C for 48 hours and kilned at 80°C for a further 12 hours. The malts produced were subjected to the SAB Ltd. (Sandton, South Africa) standard full malt analysis as done by SAM (South African Maltsters, Caledon, South Africa) and compared to the control malt. In addition, malts were tested for PYF activity.

Various aspects of the malting process were tested for their impact on the generation of PYF in the resultant malt. This was done by:

1. washing; rinsing of the malt with deionised water prior to the first steep (no soaking) and subsequent frequent changes of steep water;
2. aeration, using any standard fish tank pump and sinter stones, during steeping to aerate the water. Steep water was also aerated prior to use.
3. re-using steep water by retaining it for the subsequent steeps and topping up with fresh steep water as required and
4. addition of fungi isolated from South African grown barley (*Fusarium*) to the steep water.

### ***Fusarium* preparation**

Microflora from the outside of barley grain grown in South Africa was cultured on potato dextrose agar plates (Difco, Le Pont de Claix, France). *Fusarium* was selected by identification of spores (A. Ackerman, personal communication) from the culture and sub-cultured. Spores were washed from semi-pure *Fusarium* rich culture plates with 25% glycerol (BDH, Poole, England) and stored at -20°C. Approximately  $52 \times 10^6$  spores per kg of barley were used in the steep water.

## **Results and Discussion**

Previous results, using 2L EBC fermentations, indicated that PYF was more pronounced in 100% malt wort than in the adjunct type wort used in the breweries. 100% malt wort was therefore used throughout the optimisation process using known PYF (positive control) and non-PYF (negative control) malts. 13°P all malt wort was used, as this contains more malt, and therefore a higher concentration of PYF factors, than 15.5-16°P adjunct wort used in the breweries for high gravity brewing. Aeration of pitched wort to oxygen saturation was done by shaking wort in a sealed bottle immediately post pitching, which achieved an oxygen charge of approximately 8 mg/L. Brewery yeast was used as per 2L EBC fermentation protocol (Kruger *et al.*, 1982).

### ***Small scale PYF assay development***

#### **Choice of fermentation vessel**

Several different vessels were used in the published methods ranging from 100 mL to 1.5 L (Baker and Kirsop, 1972, Fujino and Yoshida, 1976, Inagaki *et al.*, 1994, Ishimaru *et al.*, 1967 and Nakamura *et al.*, 1997). Vessels with a conical base with volumes ranging from 10 mL to 250 mL were used to ferment control worts in duplicate and flocculation was evaluated visually. Fermentations (125 mL and 250 mL) were carried out in separating funnels and 100 mL fermentations in cylindro-conical tubes. For each of these fermentation volumes, differences in flocculation between controls were observed – the PYF positive controls flocculated 48 hours after pitching, whilst the negative controls flocculated 72 hours after pitching. Differences in fermentations carried out in 10 and 50 mL conical tubes could not be accurately assessed. Further experiments were conducted with 100 mL fermentations. Based on the results and assay requirements it was found that 100 mL was small enough to enable

## **Yeast pitching rate and wort gravity**

All reported PYF assays ferment 13°P wort (Fujino and Yoshida 1976, Ishimura *et al.*, 1967 and Nakamura *et al.*, 1997,) or lower (Baker and Kirsop, 1972 and Inagaki *et al.*, 1994). High gravity (16°P) 100% malt wort and 13°P wort were pitched with cryo-preserved yeast propagated in MYGP. This was done at the normal pitching rate used for 2L EBC fermentations and in the breweries ( $20 \times 10^6$  cells/mL) and at a higher pitching rate ( $30 \times 10^6$  cells/mL). These pitching rates are 3-4 times higher than those reported in other PYF methods (Baker and Kirsop, 1972, Fujino and Yoshida, 1976 and Inagaki *et al.*, 1994) but comparable to that used in the breweries. The increased gravity and pitching rate was tested to try and accelerate the fermentation to shorten the assay duration. Differentiation was not as clear with high gravity wort and at the normal pitching rate flocculation was significantly slower. The fastest and greatest difference between PYF positive and negative reference fermentations was obtained when using 13°P wort and high pitching rate. However, the time window in which this could be observed was very short and thus easy to miss. The experiment was repeated, using 13°P wort and the normal pitching rate. Differences were consistently observed after approximately 72 hours. This ratio of gravity/pitching rate was selected as the most appropriate for this assay.

## **Oxygenation**

Only the method by Baker and Kirsop (1972) mentions oxygenation of pitched wort, but this is standard practice for 2L EBC fermentations. MYGP propagated cryo-preserved yeast was pitched into the PYF positive and negative controls worts and shaken to oxygenate as for the 2L EBC fermentations; 35 shakes post-pitching, repeated four hours later before dispensing into the fermentation vessels. This was repeated, omitting the second shaking, to shorten the time it took to set up the assay. This reduces the oxygen supplied from 14 to 8  $\mu\text{g/mL}$  (Phaweni *et al.*, 1992) which is the normal air saturation. No effect was observed on the flocculation and this was thus adopted as part of the assay.

## **Wort analyses**

All pre-fermentation worts was analysed for FAN and carbohydrate profiles to ensure differences in flocculation were not due to nutritional differences. Carbohydrate values that vary less than 15% are not significantly different and the FAN concentration must be  $>200$  mg/mL for worts to be used in the same assay. These values were determined from previous 2L EBC fermentation data. A typical wort carbohydrate and FAN profile is shown (Table 1).

Table 1 13°P all malt wort carbohydrate profile and FAN concentrations for the PYF assay control worts (average of five samples and standard deviation (SD) expressed in brackets).

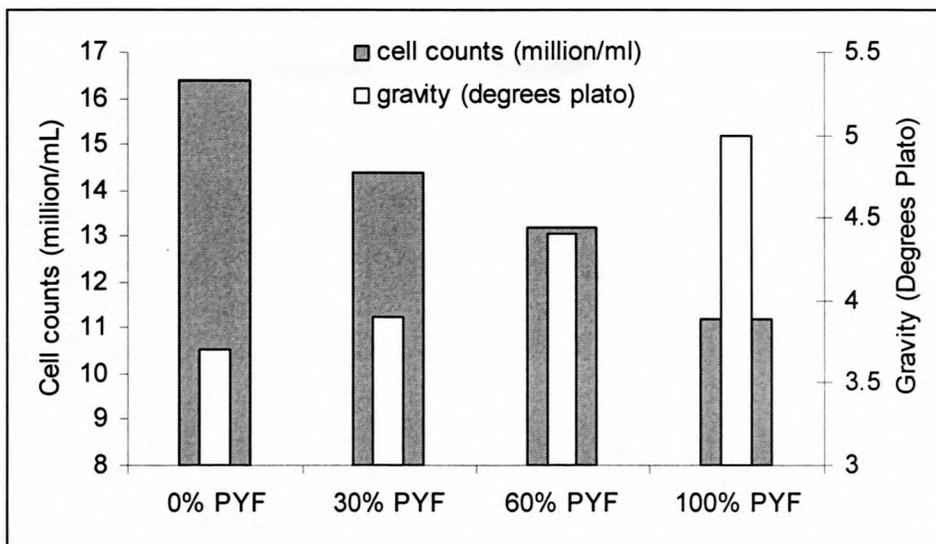
Parameters	PYF negative reference	PYF positive reference
Dextrins (g/100mL)	2.68 (0.19)	3.08 (0.40)
Maltotriose (g/100mL)	1.80 (0.24)	2.05 (0.51)
Maltose (g/100 mL)	7.13 (0.74)	7.26 (0.58)
Glucose (g/100 mL)	1.27 (0.45)	1.26 (0.11)
FAN ( $\mu\text{g/mL}$ )	245.50 (28.21)	205.22 (28.67)

Concentration of yeast cells in suspension and wort gravity was determined 12-14 hours post-flocculation. Other methods rely on visual assessment only (Baker and Kirsop, 1972), others in addition take gravity measurements at the end of fermentation on day eight (Nakamura *et al.*, 1997) or do cell counts and gravity measurements daily for the full eight days of fermentation (Fujino and Yoshida, 1976, Inagaki *et al.*, 1994 and Ishimura *et al.*, 1967). To minimise mixing and standardise sampling for the cell counts a pipette was inserted from the top of the vessel to a standard depth (1.5 cm) and 1.0 mL was withdraw for counting. The PYF reference fermentations had higher gravities and lower yeast counts than negative reference, supporting visual assessment and typical presentation of PYF fermentations (Table 2).

Table 2 Small scale PYF assay cell counts and wort gravity measurements 96 hours post-pitching (all flocculated). Values represent average of duplicate PYF assays. Variation of cell count method was 15%. Average SD for gravity measurement method is 0.04°P.

Reference sample	Yeast Counts (million/mL)*	Gravity (°P)*
PYF positive	0.2	2.54
PYF negative	4.8	2.07

\*Each value was the average of duplicate fermentations.



*Figure 3* 2L EBC fermentation end-of-ferment parameters for worts prepared with a range of PYP malt percentages. Values represent average of duplicate PYP assays, variation for the cell count method was 15% and average SD of gravity measurement method is 0.04.

Further malts were tested by both methods to determine if the PYP results corresponded. All PYP positive malts according to the 2L EBC fermentation method tested positive relative to the control using the small-scale PYP assay on a visual and post-flocculation cell count basis. It appears that for the less extreme PYP malts, the small-scale PYP assay post-flocculation gravities do not show differences. However, at the end of fermentation in the 2L EBC tubes the gravities did confirm the visual PYP assessment (Figs. 4 and 5). This discrepancy was explained by the time of sampling, post-flocculation is not the same as end-of-ferment. Extension of the small-scale PYP assay to attain true end-of-ferment resulted in similar gravities as found with the longer 2L EBC tube assay (results not shown). As a consequence, post-flocculation gravities were measured as an indication that fermentation occurred as sugars were utilised, but results were not used in the interpretation of PYP. For both the 100 mL PYP assay and the 2L EBC fermentations it was the trends, relative to the PYP positive and negative controls using the same propagated yeast, rather than the absolute values that can be compared from experiment to experiment.

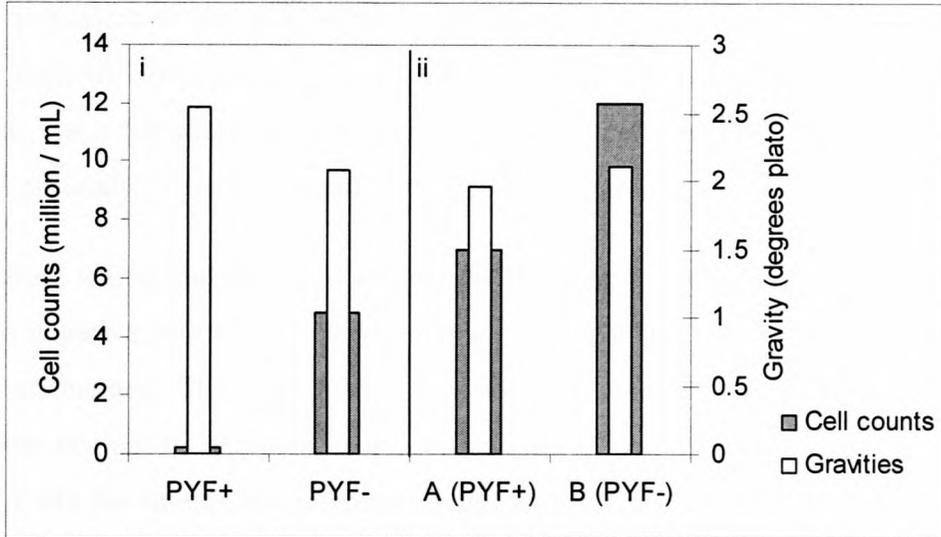


Figure 4

Small-scale PYF assay results for the original control malts, PYF+ and PYF- (i) and two other malts, A and B (ii). In the first case (i), both post-flocculation cell counts and gravities support the visual PYF assessment. In the second case (ii) the cell counts support the PYF results the gravities do not. (i) and (ii) represent two different experiments hence trends can be compared, but not the absolute values. Each value represents average of PYF assay duplicates, variation of the cell count method was 15% and average SD of gravity measurement was 0.04.

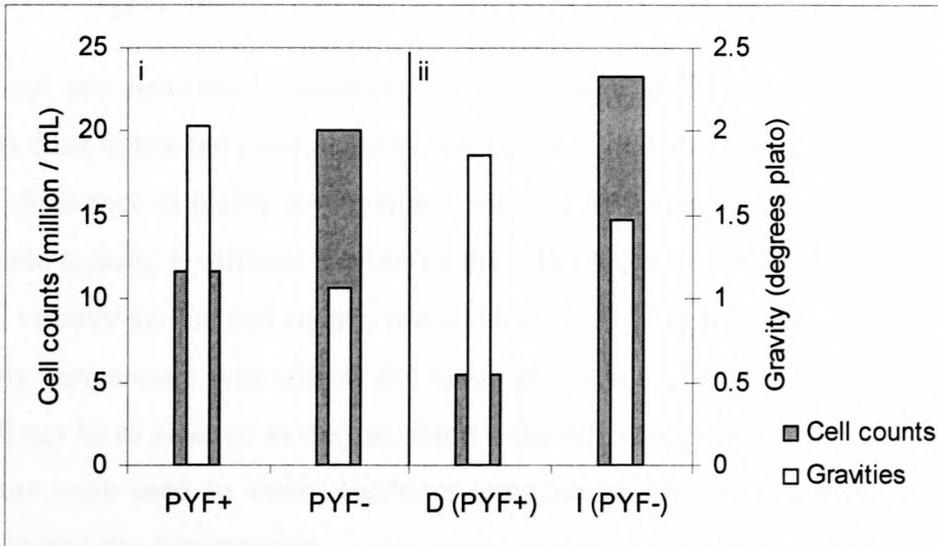


Figure 5

2L EBC fermentation results for the original control malts, PYF+ and PYF- (i) and two other malts, D and I (ii), in all cases end of ferment cell counts and gravities support the visual PYF assessment. (i) and (ii) represent two different experiments hence trends can be compared, but not the absolute values. Each value represents average of PYF assay duplicates, variation of the cell count method was 15% and average SD of gravity measurement is 0.04.

### **Robustness of the small-scale PYF assay**

Finally, the specification limits were set for the assay to determine how much variation was acceptable without influencing the results. Malt milling, mash water pH, mashing temperatures, mash filtration, fermentation temperature and yeast cell counts were examined (Table 3). Wort analysis pre-fermentation was also monitored.

The mill setting was found not to be critical, as the percentage difference between the PYF positive and negative reference values remained high and the wort carbohydrate and FAN profile was unchanged. The specification was set as  $1 \text{ mm} \pm 0.2 \text{ mm}$  (Table 3). The mash water pH was critical to attain the correct final pH in the mash, a too low pH caused a decrease in FAN for one of the reference malts and this also reflected in the carbohydrate results (results not shown). The specification was set between pH 3.0 and 3.1 to attain a final pH of 5.2-5.3 in the mash (Table 3).

To determine the sensitivity of the PYF assay to the mashing temperature used to prepare the wort, the stated temperature in the mashing method was increase/decreased by  $2^{\circ}\text{C}$ . The lower temperature had no significant impact, but at the higher temperature PYF was less pronounced (Table 3). The specification was set between the optimal temperature stated in the method and two degrees below.

Fermentations are notoriously sensitive to temperature differences. Data from the two experiments done to test temperature influence, revealed that although cell counts show a high percentage difference at higher temperatures, visually both samples flocculate within a short period of time making it difficult to observe the differences in flocculation. The overall best result, both visually and on cell counts, was as close to  $13^{\circ}\text{C}$  as possible, targeting  $12.5\text{-}13^{\circ}\text{C}$ . Undoubtedly temperature was critical and although outside of this range the assay will still work it will not be as clear or as easy to observe the differences between controls. Calibrated thermometers were used to verify incubator temperature settings and monitor temperature fluctuation during the fermentation.

Table 3 Small scale PYF assay results determining acceptable deviations of various parameters. The % difference represents the difference in post-flocculation cell counts between the controls; PYF+ versus PYF- where the PYF positive control has lower cell counts than the negative control. Variation in the method by which cell counts were determined was 15% and values represent average of PYF assay duplicates. Where applicable FAN analysis of the wort pre-fermentation was included, the variation of the FAN method is 10% and again each value is the average of duplicate PYF assays. The values in bold italic font indicate where an impact was observed.

	% difference, PYF + vs PYF -	FAN ( $\mu\text{g/mL}$ ) PYF+	FAN ( $\mu\text{g/mL}$ ) PYF-
Mill setting			
0.8mm	66	215	230
1mm	83	207	216
1.2mm	76	219	233
Mashwater pH			
pH 2.9	-	<b><i>154</i></b>	231
pH 3	83	207	216
pH 3.1	82	214	221
Mash temperature			
less 2°C	85	230	244
Specification	79	220	225
plus 2°C	36	<b><i>198</i></b>	<b><i>203</i></b>
Ferment temperature (1)		Visual assessment	
12°C	23	possible	
13°C	50	possible	
14°C	51	Too fast	
Ferment temperature (2)		Visual assessment	
12.5°C	38	possible	
13°C	71	possible	
13.5°C	160	Fast	

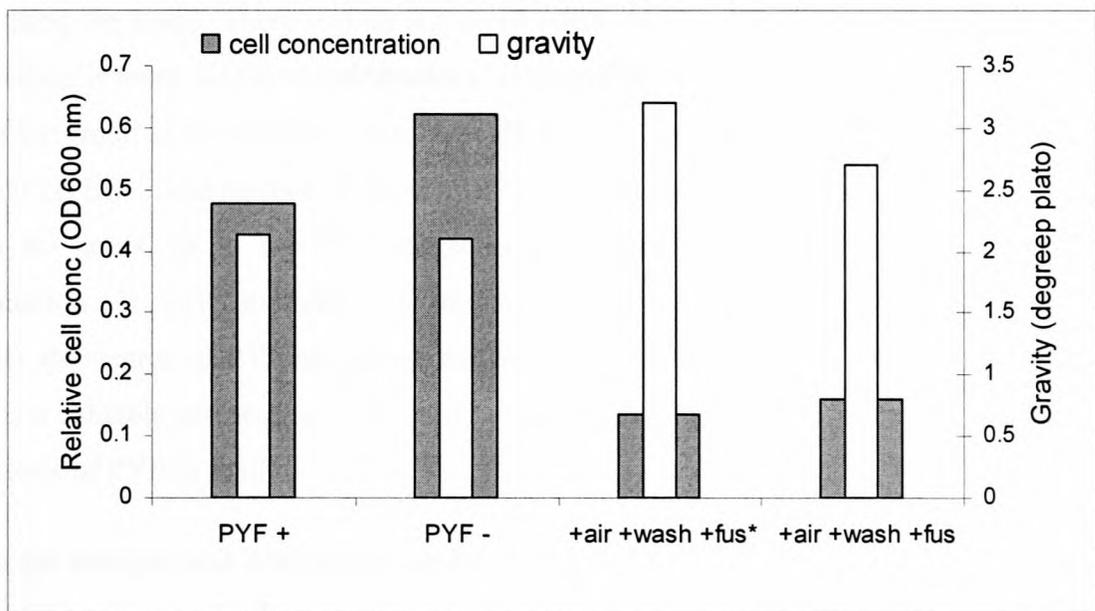
### ***Artificial production of PYF in malt***

A simple laboratory micro-malting was set up to prepare PYF positive malt from barley. Before trying to induce PYF, micro-malting conditions were adjusted to produce malt with similar in-specification malt analyses as production malt malted from the same barley (data not shown). This was done to ensure that the malts produced would make a filterable and fermentable wort with sufficient fermentable sugars and FAN for the yeast. Conditions were then varied to attempt inducing PYF.

The microbial population on a non-PYF barley may inhibit PYF, hence washing the barley prior to malting was used to remove the majority of this population with the intent of replacing it with organisms such as *Fusarium*, often associated with PYF. Contrary to expectation, in two out of three cases it was demonstrated that the washing alone was sufficient to induce PYF (results not shown). Possibly washing changes the dynamics of the microbial population and the competition within the population, allowing specific microorganisms to flourish, which in some cases may have been conducive to the induction of PYF.

To encourage this type of PYF inducing microbial growth, aeration was introduced into the steep. Washing and aeration combined again induced PYF in one out of two cases. This supports the above suggestion as to why washing may affect PYF. If the specific inducing microorganism population was present, the aeration will enhance PYF development.

These attempts at producing PYF malt by micro-malting were so far inconsistent. To ensure a better success rate, the remaining microbial population on washed barley was enriched with a likely candidate for the induction of PYF, namely *Fusarium* (Prentice and Sloey, 1960) and aerated to encourage growth. This combination always induced PYF (4 out of 4). To further ensure PYF development, the steep water containing the *Fusarium* was retained and re-used for all the steep stages, further reducing post-flocculation cell counts (Fig. 6). The PYF malts produced were extremely PYF, relative to the natural PYF positive control malt, dramatically affecting both post-flocculation cell counts (low) and gravity (high) unlike the natural PYF positive control malt used in this case which impacts on cell counts only (Fig. 6). Using this extremely PYF malt, any degree of premature flocculation can be achieved by mixing the malt with normal malt as previously demonstrated (Fig. 2), thus matching the natural PYF positive malt if required.



**Figure 6** PYF assay results of the control malts (PYF + and -) and micro-malted PYF malts prepared from washed barley (+wash) which was aerated (+air) in the steep and had *Fusarium* (+fus) added. \* Indicates the steep water was re-used. Each value represents average of duplicate PYF assays. Variation of the cell count method using optimal density (OD) was 7% and average SD for gravity measurement was 0.04.

To confirm the findings on inducing PYF in micro-malting, no aeration, no washing and no fungal addition was used and this malt was relatively less PYF, but not as non-PYF as the negative control malt. Overall all the micro-malted malts were at least marginally PYF, hence micro-malting cannot be used to prepare a PYF negative control malt, but fortunately these are more readily available. Once these conditions were established, a large batch of PYF malt was produced. This batch can be used as an alternative PYF positive control malt should natural PYF positive malt not be available.

## Conclusions

The 2L EBC tube fermentation method, which provides information about fermentation, the yeast and the raw materials, was used as a starting point to develop a simpler, faster method to measure PYF only under conditions resembling those of the brewery. The developed assay was faster, taking less time to set up with the smaller volumes (100 mL) allowing more samples to be prepared simultaneously and the fermentation stage was shorter (<80 hours) allowing for the observation of flocculation, but not requiring the extension to end of fermentation. However, it has limitations in terms of its sensitivity that need to be considered

when using the assay. There will be situations where 2L EBC fermentations are called for as this method is more sensitive and provides additional fermentation information. However, the aim of the small scale method was to provide a faster and more practicable PYF assessment than the 2L EBC tube method. In particular, it was able to provide a simple YES/NO answer within 80 hours, as to the PYF status of the malt compared to eight days for a full fermentation (2L EBC method). For this type of answer the method was not required to quantify the degree of PYF and hence the sensitivity determined was sufficient. As such the assay is a valuable research tool that will be used to investigate the mechanism leading to the occurrence of PYF in malt.

Due to the seasonal and intermittent nature of PYF, research was often halted when PYF malt was no longer available. To overcome this, PYF malt was produced artificially to be used as an alternative reference sample for the assay. The artificially treated malt was clearly extremely PYF, both in terms of low post-flocculation cell counts and high gravities, enabling continued research on PYF and allowing the standardisation of this small-scale rapid PYF assay. It is also clear from these findings that the malting process may contribute to the occurrence of PYF in malt.

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## Chapter 5

### *Enzymatic generation of factors from malt responsible for premature yeast flocculation*

#### **Foreword**

The occurrence of malt associate premature yeast flocculation (PYF) is a major concern within the brewing industry resulting in beer quality and financial losses. Although a lot of work has been done to examine the factors associated with PYF the mechanism as to how a malt becomes PYF positive has not examined.

The work described in the following Chapter, investigating the mechanism by which malt becomes a premature yeast flocculating malt, was published in the *Journal American Society of Brewing Chemists* (2004, 62, 108-116). The small scale premature yeast flocculation assay detailed in Chapter 4 was used in this investigation. The development of the assay was however not published, hence for the purpose of the publication the assay was described and this may lead to some repetition.

# Enzymatic Generation of Factors from Malt Responsible for Premature Yeast Flocculation

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## ABSTRACT

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Using a rapid, small-scale fermentation assay for testing premature yeast flocculation (PYF), it has been shown that PYF is associated with malt husk and can be induced by fungal infection during malting. Exposure of malt to extracellular fungal extracts induced PYF and exposure to commercial fungal xylanase had an impact on PYF. Arabinoxylan, the polysaccharide product of xylanase action on husk, also had an impact on PYF.

Keywords: Arabinoxylan, Barley, Fungal, Husk, Xylanase

## RESUMEN

### Generación Enzimática de Factores de Malta Responsables por la Floculación Prematura de Levadura

Usando un análisis rápido de fermentación en escala reducida para probar la floculación prematura de levadura (FPL), se ha demostrado que la FPL está asociada con la cáscara de malta y puede ser inducida por infección de hongos durante el malteo. La exposición de malta a extractos de hongos extracelulares indujo FPL y la exposición a xilanasa comercial de hongos tuvo un impacto en FPL. Arabinoxilana, el producto polisacárido de acción xylanasa en la cáscara, también tuvo un impacto en FPL.

Palabras claves: Arabinoxilana, Cáscara, Cebada, Hongo, Xilanasa

Premature yeast flocculation (PYF) is a recurring problem in the brewing industry and it has significant financial implications resulting from the associated incomplete extract utilization. Although much research has been undertaken on PYF (11,12,14-16,22,23), a definitive cause and mechanism has not been elucidated.

The process of yeast flocculation has been studied and is well understood (29-34,36). Yeast flocculation occurs when the  $\alpha$ -mannan residues of mannoproteins, which are always present on the yeast cell wall (33), interact with lectin-like proteins (often glycoproteins) on adjacent cells, forming large aggregates or flocs. In the case of lager yeast these flocs sediment at the bottom of the fermentation vessel. The lectin-like proteins, which specifically bind sugars, are synthesized by yeast and positioned on the outside of the yeast cell in preparation for flocculation (33). In brewer's yeast, flocculation is reversibly inhibited by mannose, as well as maltose, glucose, and sucrose (36), probably as a result of the sugars binding with the lectin-like proteins. Thus, flocculation only occurs when most of the sugars in wort have been assimilated (29).

Flocculant yeast (i.e., yeast with lectin-like proteins in place on the cell surface) can flocculate prematurely during yeast fermentation in the presence of high sugar concentrations (32). The onset of flocculation can be at the same time as normal flocculation or slightly earlier, but the rate and extent of flocculation tends to be more dramatic (*unpublished results*),

leading to a marked reduction in the number of yeast cells in suspension. This results in unattenuated wort with high levels of residual extract and low end-of-ferment cell counts, all of which impact the beer quality. The premature removal of yeast may be a purely physical event associated with a factor (factor may refer to a number of factors) that aggregates the yeast. Alternatively, the yeast could perceive nutrient starvation as a result of a factor that interacts with the yeast membrane and inhibits sugar uptake (13,24-26), thus triggering the flocculation mechanism prematurely. For purposes of this paper, PYF has been defined as the visual observation that the yeast flocculates earlier as well as the presence of high levels of residual wort extract and low yeast cell counts at the end of fermentation.

The end-of-ferment yeast counts observed in commercial breweries range from 2 to  $>20 \times 10^6$  cells per mL. These differences influence secondary fermentation, and hence, beer flavor profile and stability. When cell counts are extremely low, PYF will be clearly defined. However, the range of counts that occur suggests varying degrees of PYF may occur with corresponding variations on the effects on beer quality. PYF may be an extreme example of a condition that can be present to some degree in all wort. This is supported by the findings of Herrera and Axcell (16) in which the PYF factor was detected on PYF-negative and PYF-positive malts although the concentrations differed.

According to current dogma, the occurrence of PYF is associated with seasonal differences, such as wet conditions during harvest, to which the barley is exposed. This leads to increased microbial load, in particular fungi, on the grain (2). In other cases, the occurrence of PYF is not associated with wet harvest, neither is the fungal load detectably higher, but it is still seasonal. In this case, it is believed that some weather related stress may enable certain microflora to survive better than others, and hence, it is the population rather than the load that is important. However, the exact relationship of climatic conditions to PYF remains speculative.

Although certain bacteria have also been shown to cause flocculation of nonflocculant yeast by bridging the lectins via the carbohydrates on the membrane surface (37), this paper focuses only on fungal effects on PYF. It has also been shown that the malting process itself can influence PYF development but this may not be seasonal (3).

The PYF factor was found derived from barley, more specifically barley husk (3,15,23), which is predominantly composed of arabinoxylan and cellulose (90% by weight) (7,18). The factor appears to be a high-molecular-weight (HMW) polysaccharide rich in arabinose and xylose (3,15), acidic in nature, and containing some nitrogenous material (12), which was found to be essential for the PYF activity (11,22). Axcell et al (4) proposed the nitrogenous component to be basic peptides produced by barley in response to microbial infections with antimicrobial properties.

Investigations by a number of major international brewery groups over three decades have implicated malt factors as responsible for causing premature yeast flocculation during fermentation. The nature of these factors has remained elusive because of the confusing interactions among brewing raw materials, yeast strains, and differing brewing processes. The objective of this work was to use a novel approach and routinely induce the pro-

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pitching and first wort aeration was changed from 2 to 4 hr. As per method, fermentations were also monitored daily for yeast counts and gravity.

#### Micromalting with Fungal Stress

Approximately 5 kg of South African-grown barley (cv. Chariot) with normal fungal load according to the maltsters standard screening was rinsed by submerging the barley with 10 L of previously boiled (1 hr) tap water and pouring it off after gently hand mixing the grain and water for 1 min. The same water treatment was applied for all the water used in the micromalting. The barley was steeped by covering the grain in water in open basins (35 × 60 cm and 18 cm deep) at 15°C in a standard laboratory incubator for 6 hr, followed by 16-hr dry stand after all the water had been drained off, followed by another cycle of 7-hr steep in fresh water. Germination was under the same condition for three days during which the germinating barley was turned, moistened, and roots untangled twice a day. The green malt was kilned at 40°C in a standard laboratory-drying oven for 48 hr and finally cured at 80°C for 14 hr.

For the test, fungal flora isolated from South African malt grown on potato dextrose agar (Biolab Diagnostics) plates was washed into the first steep water, and the barley was not rinsed before starting. Although the inoculum was not quantified, it was a mixed culture and two overgrown petri dishes were used per 5 kg of malting. The steep water was retained and reused for the second steep. Germination was slower for the fungal infected malt, which took six days compared with three days required for the control to have similar rootlet development.

#### Removing Husk

Malt was milled with a Bühler Universal Disc Mill (model DLFU, 1 mm setting; (Bühler-Miag Ltd., Braunschweig, Germany). The milled malt was sifted through a 2-mm square mesh steel test screen (Star Screens, Nigel, South Africa). The husk fraction on top of the screen was discarded. The retained fraction was used to prepare wort according to the method detailed for the PYF assay above. The mash was lautered through the used filter bed of the PYF-negative control malt described for the PYF assay.

#### Washing Whole Malt

Four hundred milliliters of deionized water was added to 130 g of whole malt and stirred at 100 rpm in a 60°C water bath for 1 hr. The grain was filtered through cheesecloth and rinsed with a further 200 mL of 60°C deionized water. The filtrate was retained, and the grain was spread out onto a tray and dried in a standard laboratory-drying oven at 40°C for 24 hr.

The filtrate or wash was freeze-dried, and the solid was resuspended in 350 mL of mash water that was used in the preparation of wort as described for the PYF assay.

#### Husk Pretreatment

Malt was milled and sifted as for husk removal. The malt minus the bulk of the husk was retained, and 200 mL of deionized water was added to the predominantly husk fraction from the top of the screen in a 400-mL glass beaker. The extracellular fungal extracts or the xylanase enzymes (see below) were added to this, and the beaker was shaken on an orbital shaker at 100 rpm at room temperature for 16 hr. When mashing-in, the pretreated husk fraction was added to 150 mL of mash water and the retained milled malt minus the husk. One milliliter of 0.18M CaCl<sub>2</sub>·2H<sub>2</sub>O (Sigma) was added to the mash to compensate for the lack of calcium relative to the normal mashes using 350 mL of mash water (described for wort preparation of the PYF assay) instead of 150 mL.

#### Extracellular Fungal Extract Preparation and Analysis

Five different *Aspergillus* species (*A. niger*, *A. terreus*, *A. oryzae*, *A. aculeatus*, and *A. ficuum*) were inoculated separately at  $1 \times 10^6$  spores per mL in 150-mL minimal media (0.5% [w/v] yeast extract [Biolab Diagnostics], 0.2% [w/v] casamino acids [Difco Laboratories, Detroit, MI], and 6% [w/v] sodium nitrate [Sigma]) containing 2% [w/v] whole South African-grown barley in 500-mL Erlenmeyer flasks with nonabsorbent cotton wool plugs covered in aluminum foil and shaken at 300 rpm for seven days at 30°C. The mycelia and insoluble grain material were filtered out through cheesecloth. The release of reducing sugars by fungal enzymes, such as xylanase, endoglucanase, mannanase, and amylase, in the filtrate was determined by the 3,5-dinitrosalicylic acid method of Bailey et al (5). As described in the method, birchwood xylan, carboxymethyl cellulose, locust bean gum, and soluble starch were used as the respective substrates. All the extracts exhibited >10 units (U) of xylanase activity per liter. The *A. niger* and *A. aculeatus* also exhibited significant levels of amylase activity. The extracts were freeze-dried and stored at 4°C until used.

An additional amount of *A. oryzae* extracellular extract was prepared together with a number of controls. All the extracts were analyzed for enzyme activity. The additional extract prepared contained low levels of endoglucanase, mannanase, and amylase as well as relatively high levels of xylanase activity. The controls were as follows: 1) extract prepared with no fungus present (none of the above enzyme activity detected); 2) extract prepared with no barley present (some mannanase enzyme activity was detected) and; 3) extracellular extract prepared with fungus and barley and boiled prior to use (no enzyme activity detected).

#### Xylanase

Commercial endo-xylanase M3 from *Trichoderma longibrachiatum* (Megazyme, Bray, Ireland) was used to treat husk from 130 g of malt (used for the 350-mL PYF assay mash) at activities ranging from 10 to 1,000 U.

#### Arabinoxylan Addition to Mash

Soluble and insoluble arabinoxylan from wheat starchy endosperm (Megazyme) was added to the 350 mL of PYF assay mash in a ratio of 30% soluble and 70% insoluble at concentrations (soluble plus insoluble) ranging from 100 to 500 µg/mL.

## RESULTS AND DISCUSSION

#### A Reliable PYF Assay

Although 2-L EBC tall tube fermentations are reliable tools for fermentability and PYF measurements, they are time consuming and labor intensive in addition to requiring larger amounts of malt. Monitoring PYF was central to this work, so a rapid, small-scale fermentation type assay was previously developed and optimized (*unpublished*) to test malts for PYF within SAB Ltd. for research purposes. Parallel fermentations in EBC tall tubes demonstrated that the rapid test had a comparable ability to detect PYF (results not shown).

Two control malts were used to set the boundaries of the test, a strong PYF-positive malt that flocculated very early and a PYF-negative malt with normal fermentability and flocculation properties. The malts being tested were assessed relative to these controls and trends rather than absolute values obtained. Test samples were repeated to confirm any findings. The visual assessment of PYF was used in the original method, later the addition of postflocculation cell counts and wort gravity (residual extract) were added. In some cases, therefore, only visual assessment is reported. Similar types of methods have been published (12,19), the main differences included in the current method are the use of

standardized yeast (grown on defined media), 13°P all-malt wort without hops, and the fermentation vessel design (Fig. 1).

#### Establishing a Link Between PYF and Fungi on Barley

Six malts originating from barley reportedly having higher than normal fungal loads, according to the testing applied by the various maltsters, were collected and screened for PYF. In all cases, they were PYF positive according to the visual assessment (data not shown). The gushing assay positive control malt available from Carlsberg, Copenhagen, Denmark (intentionally infected with *Fusarium culmorum*) was also PYF positive.

To further explore the relationship between PYF and fungal infection, South African-grown barley (cv. Chariot) with a normal fungal load was micromalted in the laboratory with (test) and without (control) additional fungi obtained from South African malt added to the steep water. Forty percent dextrose adjunct worts at 16°P were prepared from the test and control malts and fermented in EBC 2-L tall tube fermentations. The fungal infected malt caused PYF relative to the control (Fig. 2), with lower cell counts and higher levels of residual extract (RE) in the wort after flocculation. Impaired sugar uptake by the yeast in the presence of wort prepared from the fungal infected malt contributed to these results (slower maltose uptake relative to the control, data not shown). The findings indicate there is a link between fungi on barley and the occurrence of PYF.

In addition, work in progress at SAB (Sandton, South Africa) on malt antimicrobial factors active against yeast led to the testing of the antiyeast activity of malt used for this work. The level of antiyeast activity was determined with a method modified from Broekaert et al (8) in which growth is measured by increase in absorbance over time relative to a control (SAB Ltd. brewing yeast was used as the test organism [to be published]). The results indicated that all the malts tested to date were either PYF negative with low levels of antiyeast activity, PYF positive with low levels of antiyeast activity, or PYF positive with high levels of antiyeast activity. Interestingly, high levels of antiyeast activity were not observed in PYF-negative malts, inferring a relationship between PYF and antiyeast activity in barley. The fact that antimicrobial factors (often peptides), which play a role in plant defense are

present at low levels in plant tissue at all times and stress, such as microbial infection, can trigger the production of more antimicrobial factors, supports this inferred relationship (9,35).

Furthermore, the PYF-positive control malt (from high fungal barley) not only caused PYF but also inhibition of yeast sugar uptake (maltose) in fermentation (Fig. 3) coinciding with the above observation and supported by previous work in which basic antimicrobial peptides from barley have been shown to inhibit sugar uptake by yeast (13,24–26).

#### Removal of Husk—Impact on PYF

On the assumption that the PYF factor was a degradation product of husk, husk was removed from PYF-positive malt and PYF characteristics of the resultant wort were measured with 2-L EBC

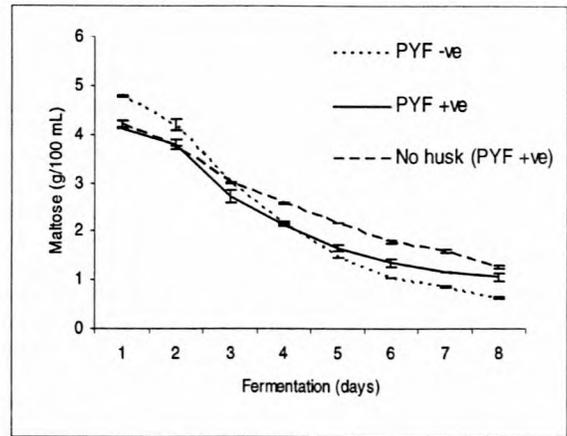


Fig. 3. Maltose concentrations during 2-L European Brewing Convention tall tube fermentations of wort prepared from premature yeast flocculation (PYF)-negative control malt, PYF- positive control malt and PYF-positive malt without husk. Bars represent min and max of duplicate fermentations. Maltotriose profiles of the same samples followed the same profiles (data not shown).

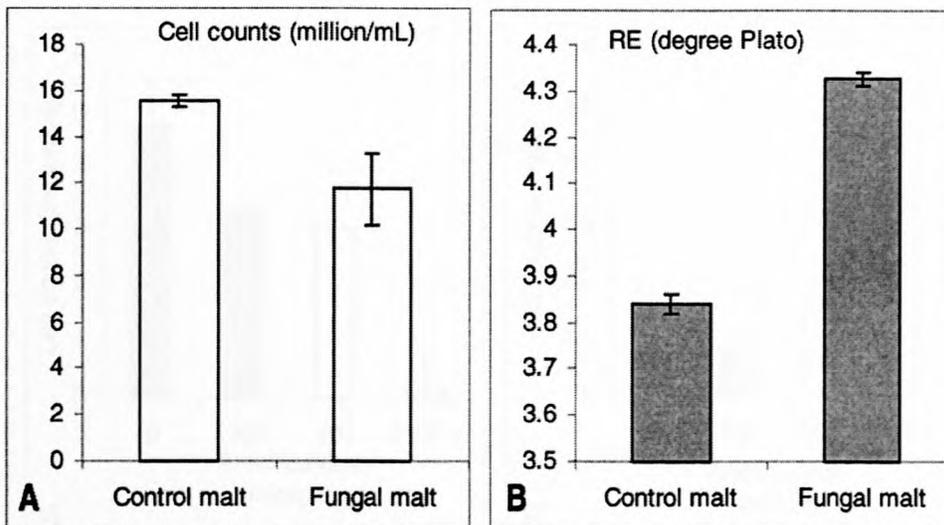


Fig. 2. Two-liter European Brewing Convention (EBC) tall tube fermentation of 40% dextrose adjunct wort at 16°Plato, prepared from malt micromalted with and without fungal addition in steep. Postflocculation wort cell counts (A) and residual extract (RE) (B) from the premature yeast flocculation (PYF) assay results are shown. Bars represent minimum and maximum for duplicate fermentations. Repeat micromalting gave similar results using PYF assay (data not shown).

arabinoxylans, which supports the finding that barley infected with *Fusarium* had more xylanases than the uninfected control (28). Additionally, *A. niger* produces and excretes large amounts of xylanase relative to any other enzymes (1). Filamentous fungi in general produce far more xylanases than either yeast or bacteria (21), supporting the suggestion that it is the fungal infection that is more important with respect to PYF.

**Extracellular Fungal Extracts.** The PYF-negative malt husk fraction was pretreated with the extracellular fungal (*Aspergillus*) extracts before mashing-in and preparation of wort for PYF analysis. In each case, PYF was induced by the pretreatment of the husk with the extracts relative to the control (presoaked husk, no extract added) (Fig. 4).

To determine the relative importance of the fungi (*Aspergillus*), the enzymes, and the barley in the extracts, three additional controls were prepared and tested for PYF-inducing activity (Table III). *Aspergillus* was essential for inducing PYF activity, since the extract where the *Aspergillus* inoculum was left out did not induce PYF. Boiled extract did not induce PYF presumably because

of the inactivation of enzymes required to generate the PYF factor. This implies that the factor must survive the brewing process (including boiling) to cause its effect in fermentation. Although nonenzymatic proteins present in the fungal extract would also be denatured, these are unlikely to be associated with the occurrence of PYF since they would not survive the brewing process. Although the presence of barley in the growth media enhanced the PYF activity of the extract, it was not essential since the extract from fungi grown on minimal media without barley still induced PYF but to a lesser extent (Table III). The latter suggests the fungal enzymes required to break down husk material are constitutively produced and secreted by the growing fungi, but the presence of material that needs to be degraded before it can be assimilated increases the amount secreted (21).

**Commercial Endo-Xylanase.** Having established the importance of the fungal extracellular enzymes in PYF and the presence of xylanase in the extracellular fungal extracts, commercially available endo-xylanase from *T. longibrachiatum* was tested (with the same method of pretreating the husk) for its PYF-inducing capability.

At 10 and 50 U of xylanase per 350 mL of mash, flocculation was not affected (Table IV). At 100 U of xylanase, PYF was induced according to the visual assessment and postflocculation yeast counts, although the residual extract remained unchanged. This observation supports the finding that some husk component is required for normal flocculation and also suggests that the required component is likely to be the same as the PYF factor. This is in agreement with the findings of Herrera and Axcell (16) who detected PYF factor at different levels in PYF-negative and -positive malts. Above 150 U, flocculation was impaired. The proposed PYF factor produced by the breakdown of the husk arabinoxylan by xylanase may have been degraded further resulting in lower-molecular-weight arabinoxylans, which no longer impacted flocculation.

The fungal endo-xylanase level of activity on the malt husk appears to create factors that influence flocculation, although no impact was detected on attenuation. There may be several reasons for this, e.g., the fungal extracellular extracts are likely to contain several different enzymes and only one was tested in isolation in

**TABLE IV**  
Flocculation Results (PYF Assay) of All-Malt Wort<sup>a</sup>

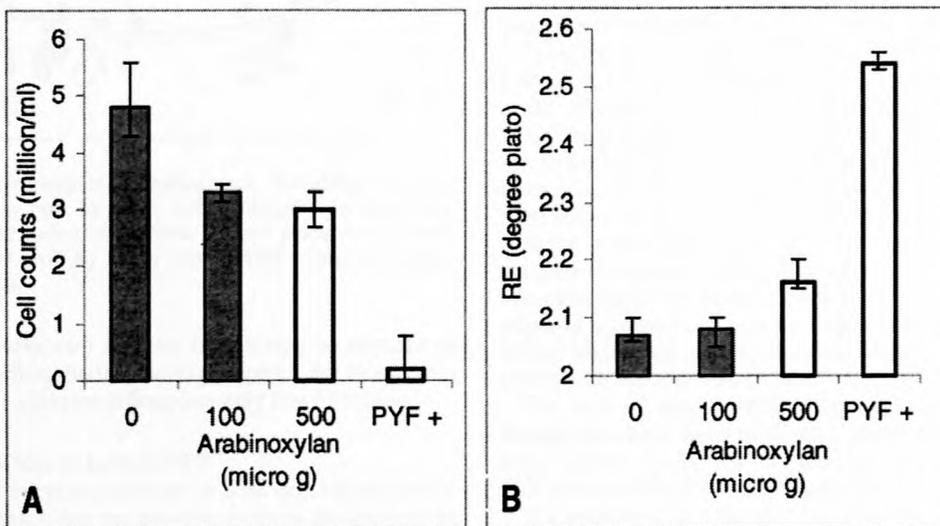
Enzyme/350 mL of Mash	Flocculation (Visual Assessment)	Postflocculation Wort Analysis	
		Yeast Counts (10 <sup>6</sup> cells/mL)	RE <sup>b</sup> (°P)
0 Units (U) (control)	Normal	26	2.04
10 U <sup>c</sup>	Normal	23.8	2.03
100 U	Early	13.3	1.9
1,000 U <sup>d</sup>	Delayed	42	2.2

<sup>a</sup> All-malt wort (13°Plato [°P]) prepared with malt where the husk was pretreated with differing concentrations of commercial xylanase. Cell counts and residual extract was measured postflocculation. Average values for duplicate fermentations are reported and repeat experiments indicated similar trends (data not shown). PYF = premature yeast flocculation.

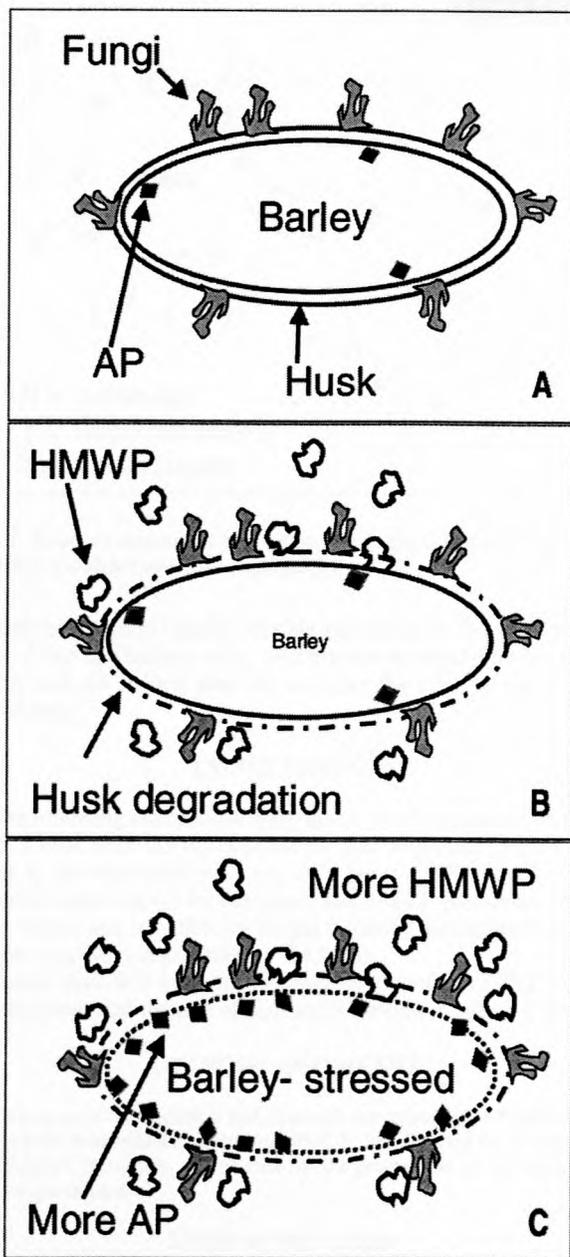
<sup>b</sup> RE = residual extract.

<sup>c</sup> 10 and 50 U of xylanase were tested and results were comparable.

<sup>d</sup> 150, 200, 500, and 1,000 U of xylanase were tested and all results were comparable.



**Fig. 5.** Postflocculation wort cell counts (A) and residual extract (B) from the premature yeast flocculation (PYF) assay results for arabinoxylan additions to the mash. Visually assessed as not PYF (shaded bar) and visually assessed as PYF (white bar). Assay was done in duplicate and bars represent min and max. The experiment and assay were repeated and gave similar results.



**Fig. 6.** Proposed mechanism of premature yeast flocculation factor(s) generation from barley husk by fungi. Initial infestation by fungi (A), fungal enzymatic degradation of the husk (B) and production of more antimicrobial peptides (AP) by barley (C). HMWP = high-molecular-weight polysaccharides.

this case. Alternatively, two separate factors may be required to induce an effect on flocculation and sugar uptake, the factor from husk degradation of xylanase influencing only floc formation.

#### HMW Polysaccharides to Induce PYF

Since the fungal enzymes involved in husk breakdown induce PYF, it stands to reason that the enzyme products are involved in PYF. Xylanase activity specifically was shown to impact flocculation, and its products from the degradation of husk would be a range of molecular weight arabinoxylans. Arabinoxylan is commercially available from wheat starchy endosperm, which only

differs from barley husk arabinoxylan in that it does not contain glucuronic acid residues. These glucuronic acid residues make the husk arabinoxylan acidic or negatively charged (7), possibly accounting for the previous finding that the PYF factor is an acidic polysaccharide (12). The importance of the acidic character of the PYF factor is not known hence the wheat endosperm arabinoxylan used may not be as effective as barley husk arabinoxylan.

Arabinoxylan is available in soluble and insoluble forms. The latter does not dissolve in water but hydrates and forms a gel. In most cereals, the ratio of soluble to insoluble arabinoxylan is 30:70% (18). This ratio was used when arabinoxylan was added to the mash at various concentrations. Since the PYF factor can be washed off the grain, it suggests that the factor is soluble. Morimoto et al (22) also reported this. Results indicate that arabinoxylan did induce PYF at higher concentrations (Fig. 5).

#### Proposed Mechanisms of PYF

*From the Barley Perspective* (Fig. 6A, B, and C). Seasonal conditions impact the barley microbial load (2). Fungi occur on the surface or husk of the grain (6,17) (Fig. 6A). To generate nutrients that the fungi can assimilate, enzymes are secreted (mainly xylanases and glucanases) that degrade the husk (1,28) (Fig. 6B), which is made up predominantly of arabinoxylan and cellulose (18). The products of xylanase degradation from barley husk (7) are acidic arabinoxylans with a range of molecular weights. PYF is associated with acidic HMW polysaccharides (12), which we suggest are the acidic arabinoxylan products of husk degradation by xylanase. Cellulose is not associated with PYF and is also resistant to enzymatic degradation (22).

These HMW polysaccharides (possibly arabinoxylans) remain on the surface of the grain. Since PYF can be removed by washing grain, the causative agent (arabinoxylans) must be soluble, and indeed it has been established that xylanase degradation products, albeit of wheat starch endosperm, consist of soluble and insoluble components (1).

Should the fungal infection of the barley be heavy, or such that the integrity of the grain is compromised as a result of severe degradation, the plant may respond by synthesizing more antimicrobial factors (likely to be basic peptides) (Fig. 6C). These are present constitutively at low levels and the up-regulation is a common mechanism found in plants (9,35). The observation that high antiyeast activity is only associated with malts that are also PYF but that not all PYF malts have high antiyeast activity supports this proposed mechanism.

*From the Yeast Perspective* (Fig. 7A and B). Once the yeast is flocculent, but the sugar concentration in the wort is still high, the acidic arabinoxylans (which we postulate to be PYF factors generated by fungal enzymatic degradation of barley husk) cross-link the lectin-like proteins on the yeast cell surface forming yeast flocs (Fig. 7A). The polysaccharides associated with PYF have been shown to have a greater affinity for the lectin-like proteins than the simple sugars (32), enabling the polysaccharides to out-compete the simple sugars. This would result in normal fermentation rates up to the onset of early flocculation when the yeast is removed by a physical process because of floc formation leaving behind high levels of residual extract in the wort; a scenario coinciding with brewery observations.

The role of antimicrobial polypeptides remains speculative, though they have been implicated in the ability to impair yeast sugar uptake (13,24–26) and there is an apparent link between PYF and antimicrobial activity against yeast.

It is possible that if the malt antimicrobial factors are present at higher than constitutive levels, in addition to associating with the HMW polysaccharides PYF factor, they could interact with the yeast cell membrane and curtail the sugar uptake capabilities of the yeast (Fig. 7B).

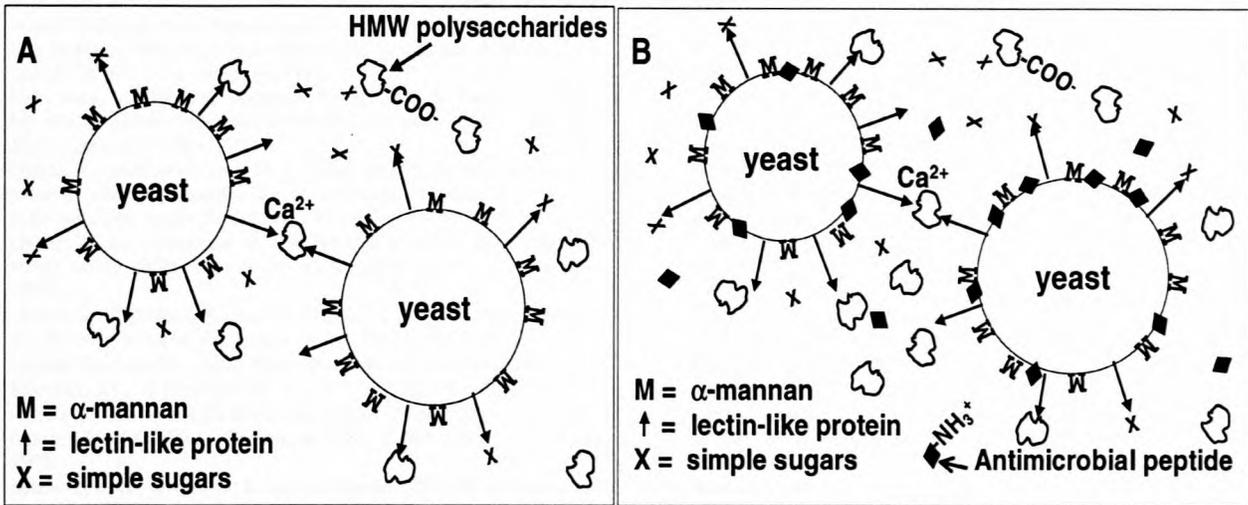


Fig. 7. Proposed mechanism of premature yeast flocculation by high-molecular-weight (HMW) polysaccharides only (A) and HMW polysaccharides in association with antimicrobial peptides (B).

Although several equally feasible variations on the smaller details of this mechanism exist, they are not included here for simplicity and we believe they do not alter the main theme of the hypothesis.

**CONCLUSIONS**

The following conclusions were made: 1) A mechanism on how PYF occurs from the onset in barley was proposed; 2) PYF appears to be associated with not only heavy PYF but also with impaired sugar uptake by the yeast; and 3) PYF was shown to be husk related and inducible by fungal infection during malting and fungal enzymatic degradation of the husk.

Future work will examine the possible reduction of PYF at the malting stage and the role of malt antimicrobial factors in PYF.

**ACKNOWLEDGMENTS**

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## Chapter 6

### *Optimisation of a microtitre plate yeast assay to screen antimicrobial activity*

#### **Abstract**

The optimisation of an antiyeast assay, developed from a number of existing assays, using South African Breweries Ltd. (SAB Ltd.) production lager yeast strain (*Saccharomyces cerevisiae*) as the test organism, is described. Throughout the optimisation a known antimicrobial peptide from *Bacillus brevis*, gramicidin S, was used as a model antibiotic and control for the assay. The assay was performed in a 96 well microtitre plate of which the layout was optimised for consistent and reproducible yeast growth. The preparation of the yeast prior to the assay and the cell concentration used in the assay was optimised to attain good differentiation or sensitivity and robustness. Growth in the presence or absence of gramicidin S was measured over time by the relative increase in light dispersion (optical density). A doubling dilution series was used to generate a dose response curve. The software package Prism<sup>®</sup> 3.0 (Graphpad Software Inc.) was used to analyse data expressed as the concentration required for 50% microbial growth inhibition (IC<sub>50</sub>) and to compare dose response growth inhibition curves. Gramicidin S was found to have an IC<sub>50</sub> value of 12.3±1.1 µg/mL. The antiyeast assay was optimised with the view of screening barley malt derived extracts to investigate the possible impact of yeast inhibitory factors in malt on the brewing process.

#### **Introduction**

The presence of antimicrobial factors in grain and in particular barley is well established (Broekaert *et al.*, 1995, Leah *et al.*, 1991, Molina *et al.*, 1993 and Molina and García-Olmedo, 1993, Mundy and Rogers, 1986 and Ponz *et al.*, 1983 and 1986). However, in the brewing industry it has not been investigated if the presence of such factors in barley or the malt prepared from barley (the major raw material for beer brewing) impacts on the brewing process. The impact, if any, may be direct or indirect. The direct impact would be the inhibition of yeast metabolism during fermentation by the antimicrobial factors from the malt. The indirect impact may be much more complex, whereby the occurrence of microbial

contamination on grain and the defence/stress response to that is indicative of a range of other malt quality aspects that may not necessarily affect yeast (e.g. flavour stability and haze in beer (Etchevers *et al.*, 1977)). In order to examine this, a reliable and sufficiently sensitive antimicrobial assay using brewing yeast as an indicator organism was required.

Various methods for the evaluation of antimicrobial activity already exist. Some focus on the assessment of the microbial membrane permeabilisation, e.g. measuring leakage of ions such as potassium out of the microorganism (Terras *et al.*, 1993 and Thevissen *et al.*, 1996) or the uptake of a dye, such as SYTOX Green, into the organism (Thevissen *et al.*, 1999). Others assess membrane lysis, signifying a cidal rather than a static effect on organisms, measured by the detection of intracellular enzymes such as  $\alpha$ -glucosidase extracellularly (Jewell *et al.*, 2002). Changes in membrane function can also be detected by monitoring the uptake of glucose by the microorganism (Wetter *et al.*, 2001).

Other methods focus on microbial growth itself, which is by far the most commonly used and least cumbersome. Initially the inhibition of growth was assessed using culture plates e.g. disk-plate diffusion where disks soaked in test sample were placed on a developing lawn of microbial growth, resulting in inhibition zones. This was only semi-quantitative since the concentration of the test sample in the disk was difficult to assess and inhibition zones were scored by arbitrary ratings (Hadacek and Greger, 2000). Alternative growth assays related growth to increase in turbidity enabling quantification of growth inhibition but these required large volumes of the test samples (Amsterdam, 1996 and Boeira *et al.*, 1999). This type of growth inhibition assay was adapted to microtitre plates (Broekaert *et al.*, 1990, Hancock, 1997 and Steinberg and Lehrer, 1997) which required far less test samples and allowed for simultaneous testing of many different samples or samples over a range of concentrations by micro-dilution. In the same microtitre plate format Yamashoji *et al.*, (2004) used spectroscopy and chemiluminescence to relate viable cells to active oxygen species, although this was not applied to testing antimicrobials.

According to Hadacek and Greger (2000), who compared different types of antimicrobial assays, the microtitre plate micro-dilution type method offered the greatest potential of all the bioassays. Many of the growth inhibition assays where growth was assessed spectrophotometrically used fungi as test organism (e.g. *Fusarium culmorum* (Thevissen *et al.*, 1997), *Botrytis cinerea* (Broekaert *et al.*, 1992) and *Neurospora crassa* (Thevissen *et al.*, 1996)), some used bacteria (*Micrococcus luteus* (Du Toit and Rautenbach, 2000)) and others

used yeast (*Saccharomyces cerevisiae* strains) (Boeira *et al.*, 1999, Kubo *et al.*, 2003, Kubo and Himejima, 1992, Okada *et al.*, 1970, Thevissen *et al.*, 2000 and Wetter *et al.*, 2001). Most of these organisms were used to test plant derived antimicrobial factors. Yeast, however, was used to test factors such as mycotoxins (Boeira *et al.*, 1999), antimicrobial factors from bacteria (Thevissen *et al.*, 2000), various chemicals including alcohols (Ken-ichi and Kubo, 2002 and Kubo and Himejima, 1992) and using brewing yeast specifically, barley derived antimicrobial factors (Okada *et al.*, 1970).

Known antimicrobial compounds were sometimes used as positive control in antimicrobial assays. These compounds are mostly known antimicrobial peptides from bacteria such as the antifungal Nystatin from *Streptomyces noursei* (Broekaert *et al.*, 1990) and Nikkomycin Z from *Streptomyces tendae* (Broekaert *et al.*, 1992) and an antibacterial peptide from *Bacillus brevis*, namely gramicidin S (Du Toit and Rautenbach, 2000). The latter is a known antibacterial that prevents the functioning of the cell membrane by disrupting the integrity of its phospholipid bilayer (Yonezawa *et al.*, 1986).

The microtitre plate growth inhibition type assays, summarised here, were used as a basis to develop a suitable antiyeast assay to determine the impact of antimicrobial factors on yeast with the aim of applying this method to the investigation of malt antimicrobial factors and their possible impact on the brewing process. The following aspects were optimised for the assay: the microtitre plate set up and incubation conditions, the impact of sample solvent or dilution solution used for the assay, the yeast preparation and the yeast cell concentration.

## **Materials and methods**

### **Optimisation of antiyeast assay**

Growth medium and yeast were added to all 96 wells of the microtitre plates (flat bottomed non-binding, Bibby Sterilin, Staffordshire, England) used for the assay. The impact of incubation temperature and airflow as well as the location of the well was examined by monitoring yeast growth in each well. Each well contained 100  $\mu$ L MYGP broth (3 g each of malt and yeast extract, 5 g peptone and 10 g glucose (Biolab Diagnostics, Midrand, South Africa) dissolved in 1 L water and autoclaved 15 minutes under pressure to attain 121°C) and  $3.6 \pm 0.5 \times 10^2$  cells of SAB Ltd. lager brewing yeast (*Saccharomyces cerevisiae*). Yeast counts were determined by using a Hawksley–Cristallite haemocytometer with improved Neubauer ruling (Boeco, Hamburg, Germany). Yeast growth was monitored by measuring the increase

in light dispersion at 595 nm using a microtitre plate reader (Bio-Rad model 450 microtitre plate reader, Richmond, CA, USA).

The sample solvent or dilution solution (25% acetonitrile (HiPerSolv™, BDH, Poole, England)) used for the assay and its impact on the yeast growth was established by monitoring yeast growth in the presence of increasing concentrations of acetonitrile. To each well 80 µL MYGP containing  $3.6 \pm 0.05 \times 10^5$  cells of yeast/mL and 20 µL sample solvent was added.

The optimal yeast preparation for the assay was determined by comparing cryogenically stored yeast to propagated log phase yeast. *Saccharomyces cerevisiae* (SAB Ltd. lager brewing yeast) was propagated by inoculating 15 mL MYGP with cryogenically stored yeast at  $1 \times 10^6$  cells/mL in a 100 mL Erlenmeyer flask. The yeast was allowed to grow for between 7.5 and 10 hours (25°C) while shaking (150 rpm) to attain log phase yeast. The yeast was then diluted in MYGP to the required cell concentration for the assay. Cell counts were optimised for the assay by testing and comparing cell counts ranging from 0.22 to  $3.6 \times 10^6$  cells/mL.

#### **Optimised antiyeast assay and gramicidin S dose response**

Only the inner block of wells, eight across and four down, of the 96 well microtitre plates were used and analysed for the assay itself (Fig. 2), although yeast in liquid medium was added to the remaining wells. Dilution solution (20 µL 25% acetonitrile) and 80 µL liquid growth medium with or without yeast was added to each well of the entire plate. A positive control (100 µg/mL gramicidin S from *Bacillus brevis* (Sigma, St. Louis, USA) in 25% acetonitrile), a negative control (25% acetonitrile, taken as 100% growth) and a contamination control (25% acetonitrile and media without yeast) were included in each run (consisting of up to 20 plates). Dilution solution (20 µL) was dispensed into all the wells of the entire plate except the first well of the rows within the central block where dilution series were required. Gramicidin S (40 µL of 100 µg/mL) was dispensed into the first well of a row in the central assay block for the start of the dilution series. The gramicidin S was diluted two-fold across the rows (eight wells) within the central assay block by transferring 20 µL of the 40 µL to the next well and discarding the final 20 µL. MYGP broth without yeast (80 µL) was added to the wells designated as the contamination control and MYGP with yeast (80 µL) was added to the remaining wells on the plate already containing sample or dilution solution.

Cryogenically stored yeast was propagated as described above and was diluted in MYGP to a final concentration of between  $3.6$  and  $8.3 \times 10^5$  cells/mL.

Each gramicidin S concentration was tested in duplicate using duplicate log phase yeast grown from two different batches of cryogenically preserved yeast. The content of the wells were mixed by holding the plate on a vibrating vortex mixer for several seconds, set to avoid spillage and frothing. The light dispersion or optical density (OD) at 600 nm was determined using a microtitre plate reader at time zero (at the start of the assay,  $T_{zero}$ ) and after between 22 and 24 hours (when the controls had a light dispersion of between 0.2-0.3,  $T_{final}$ ). The plates are wrapped in foil and left to incubate at room temperature ( $23 \pm 1^\circ\text{C}$ ) or in an incubator. An assay was regarded as contamination free when there was no significant increase in absorbance for the blank wells ( $< +0.006$ ) over the growth period.

### Data analysis

The data analysis protocol was adapted from Du Toit and Rautenbach (2000). The change in absorbance for each well was calculated and expressed as a percent growth relative to the negative reagent control. All the reagent control values were averaged (Average  $\delta T$ ).

$$\left( \frac{(T_{final} - T_{initial})}{Average \delta T} \right) \times 100 = \% \text{ growth}$$

The percent growth was corrected such that the percent growth at the lowest concentration of the test sample represented 100% growth and the rest of the dilution series was expressed relative to this.

$$\left( \frac{\% \text{ growth}}{\% \text{ growth}_{\text{lowest concentration}}} \right) \times 100 = \text{corrected \% growth}$$

These values were entered into Graphpad Prism version 3.0 for Windows (Graphpad Software, San Diego, CA, USA ([www.graphpad.com](http://www.graphpad.com))) to calculate the inhibitory concentration at which 50% growth inhibition ( $IC_{50}$ ) occurred by performing nonlinear regression on the dose response data and fitting a sigmoidal curve with variable slope to the data as described by Du Toit and Rautenbach (2000). The correlation coefficient ( $R^2$ ) of the data to the selected curve was also considered and only data sets with  $R^2 > 0.99$  were selected. Wherever relevant the standard error of the mean (SEM) was used to establish any significant differences.

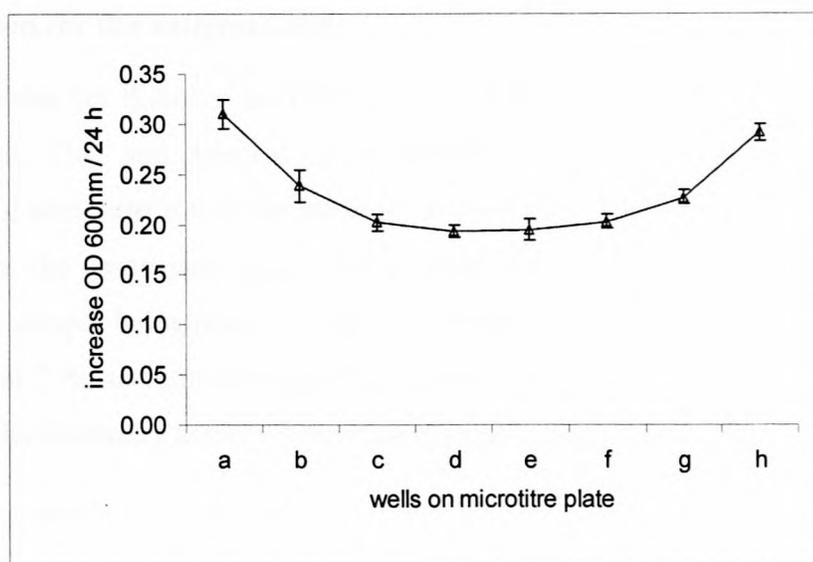
$$\frac{SD}{\sqrt{n}} = SEM$$

Where  $SD$  is standard deviation and  $n$  is number of values or samples.

## **Results and Discussion**

### **Microtitre plate lay out**

Growth in the wells across the microtitre plate was found to be inconsistent. On closer examination it was found that growth was greater in the wells near the edges than in the centre causing the negative control to “smile” across the plate (Fig. 1). It was proposed that this may be attributed to the airflow inside the incubator effecting the outer wells more than the inner wells and/or differences in temperature between the wells as a result of heat generated by the yeast. Temperature was proposed to be the critical parameter as any attempts to seal the plates to minimise the airflow had not impact at all. The problem was resolved by only using the inner eight by four wells for the assay and the outer two rows of wells were inoculated with yeast as for the negative control but not regarded in the data set. The growth in these wells probably generate the correct temperature environment for the inner wells. This format was used as four rows each consisting of eight wells, samples were added to the first well in each row and diluted two fold across the row, i.e. seven two-fold dilution steps per sample, per row (Fig. 2).



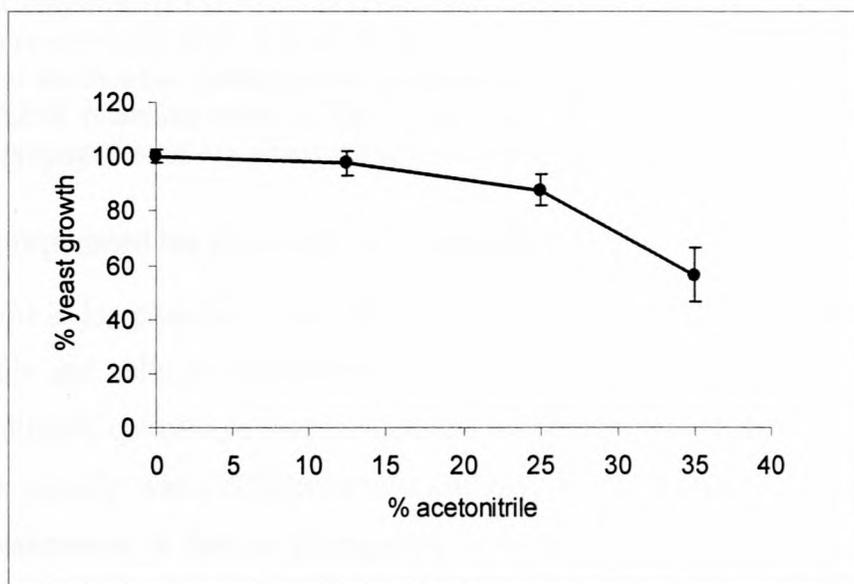
*Figure 1* Example of the “Smiling” effect of yeast growth (measured by change in optical density at 600nm / 24 hours) observed down a column in this case but also seen across rows on a microtitre plate. Error bars represent SD (standard deviation) of 7 sets of data from 7 different experiments (n = 7).

1A	2	3	4	5	6	7	8	9	10	11	12
1B											
1C		1.000w	0.500w	0.250w	0.125w	0.063w	0.031w	0.016w	0.008w		
1D		1.000x	0.500x	0.250x	0.125x	0.063x	0.031x	0.016x	0.008x		
1E		1.000y	0.500y	0.250y	0.125y	0.063y	0.031y	0.016y	0.008y		
1F		1.000z	0.500z	0.250za	0.125z	0.063z	0.031z	0.016z	0.008z		
1G											
1H											

*Figure 2* Diagrammatic representation of the 96 well microtitre plate lay out used for the antiyeast assay. Only the central block of 8 by 4 clear wells were used for the assay. Rows C to F were used for different samples or controls; w to z. Doubling dilution was made across each row indicated by the numbers and starting with an arbitrary concentration of 1.000.

### Dilution solution for the antiyeast assay

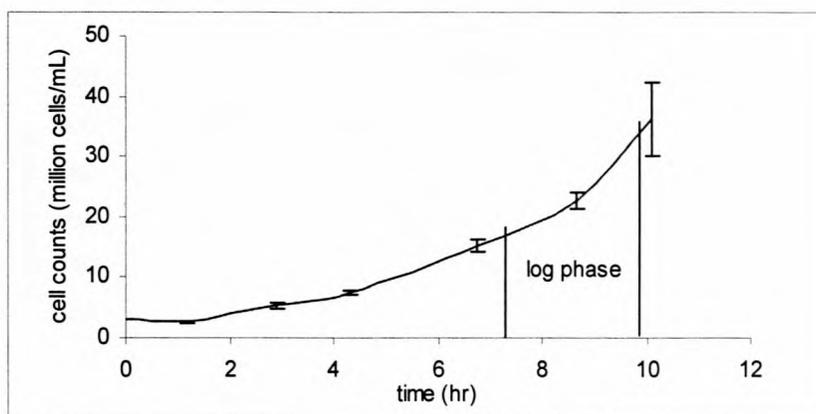
Gramicidin S does not dissolve easily in water and to aid dissolution 25% acetonitrile was used as solvent. This was adopted as the dilution solution for the assay to ensure the concentration of acetonitrile was the same in all the wells. The impact of the acetonitrile on yeast growth in the assay was determined to establish the relative inhibitory effect of the solvent and the sample being tested, in this case gramicidin S. Assuming growth in water was 100%, growth at 25% acetonitrile was 88% on average (Fig. 3). Although acetonitrile at 25% contributes to the inhibitory activity it was considered to be acceptable for the assay.



*Figure 3* Impact of increasing levels of acetonitrile on yeast growth in the antiyeast assay. Error bars represent SEM of duplicate analyses of triplicate experiments (n = 16).

### Propagation of log phase yeast

Yeast was propagated in MYGP media and the propagation was monitored by cell counts to ensure cells were in log phase and to check the variation between batches of cryogenically stored yeast. Previous growth curves over 20 hours indicated log phase occurred between 6 and 8 hours and stationary phase was reached after 12 hours (data not shown). Using the same growth conditions, growth curves were repeated between 0 and 10 hours with three different batches of cryogenically stored yeast. No significant differences were observed between these (Fig. 4) and the previous data. Log phase yeast was used after 7.5 hours, but before 10 hours based on this data.

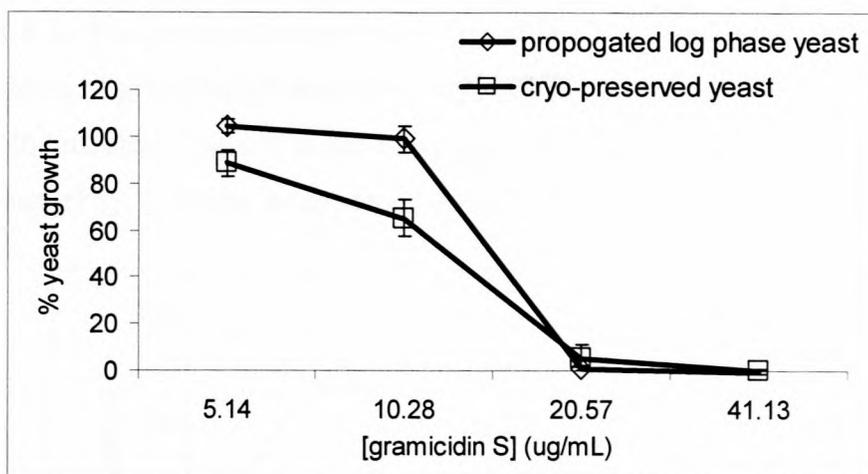


**Figure 4** Comparison of growth curves of four different batches of cryogenically stored *S. cerevisiae* (SAB Ltd. lager brewing yeast). Storage times varied from two weeks to six months. Analysis was done up to 12 months apart. Error bars represent the SEM (standard error of the mean) ( $n = 4$ ). The selected time range for the propagation of log phase yeast is indicated ( $>7.5$  hours  $<10$  hours).

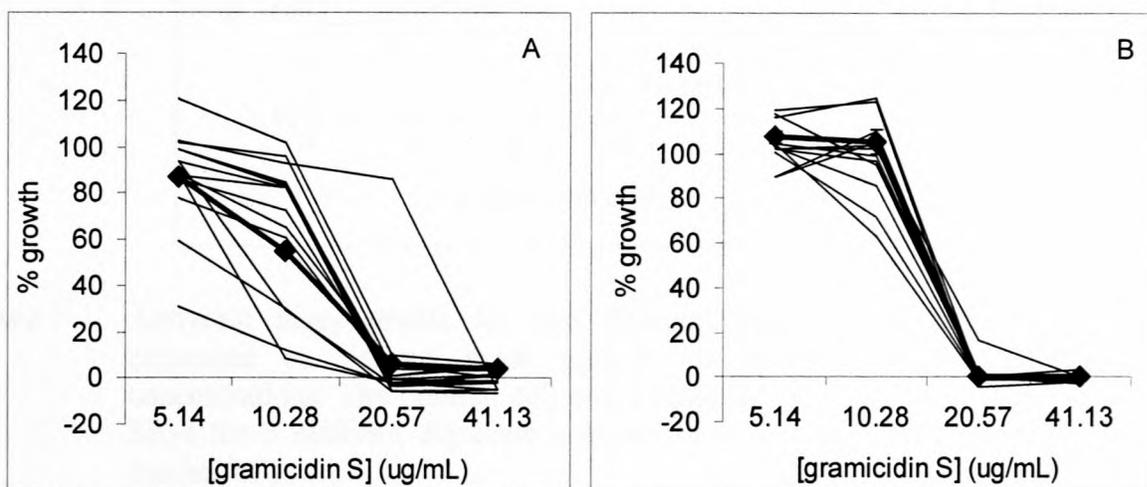
### Laboratory propagated log phase *versus* cryogenically stored yeast

Microorganisms in log phase are considered to be more sensitive to damage because they are growing rapidly and cells are delicate and small (Boeira *et al.*, 1999 and Thevissen *et al.*, 1999). Yeast directly out of cryo-preservation and propagated log phase yeast were compared to see if there actually was a difference in sensitivity. The advantage of using yeast directly from cryo-preservation is that no propagation is required, making the assay time shorter. Yeast from the same batch of cryogenically stored yeast was used directly in the assay and to propagate yeast for the assay. Viability of yeast directly out of cryo-preservation was only about  $26 \pm 5\%$  compared to propagated log phase yeast at  $76 \pm 5\%$  as determined by growth on MYGP culture plates. Counts were adjusted to compensate for this difference ensuring results were due to the viability of the yeast and not the initial cell count.

Fifteen sets of data were compared each using a different batch of yeast but all with gramicidin S as the positive control. According to the results yeast used directly from cryo-preservation without propagation was significantly more sensitive to the inhibitory effects of gramicidin S than the propagated log phase yeast (Fig. 5). However, results from cryo-preserved yeast (Fig. 6A) were less consistent than those of propagated log phase yeast (Fig. 6B) as can be seen by the spread of results. Therefore, propagated log phase yeast was selected as the yeast of choice.



**Figure 5** Antiyeast assay results for gramicidin S expressed as percent growth (% growth) relative to the negative control comparing propagated log phase yeast and yeast directly from cry-preservation, using the same ratio of viable cells ( $0.086 \times 10^6$  cells/mL). The error bars represent SEM from 12 different experiments ( $n = 12$ ).

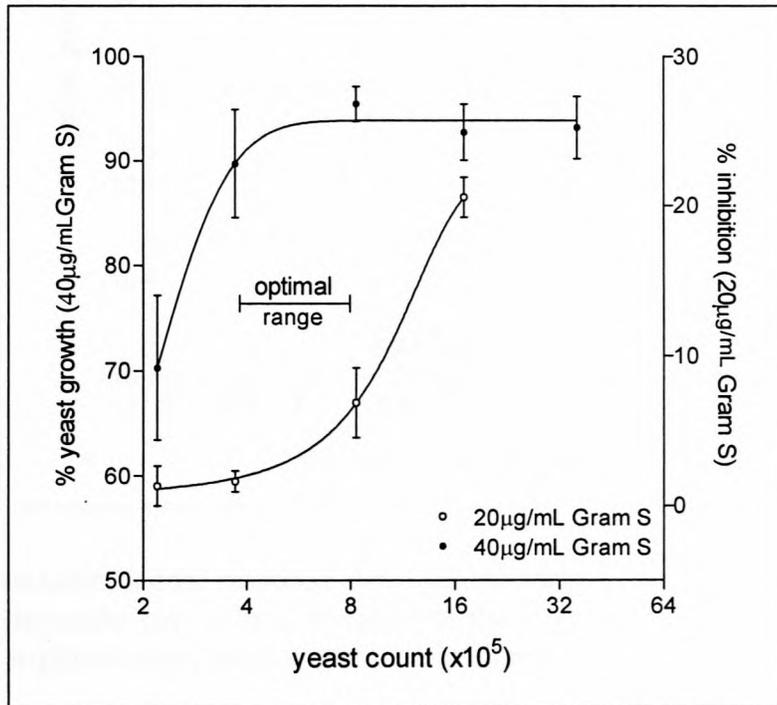


**Figure 6** Antiyeast assay results for gramicidin S expressed as percent growth (% growth) relative to the negative control of **A**: yeast direct from cryo-preservation and **B**: propagated log phase yeast. Individual curves and average (-♦-) curve are shown to indicate the spread of results.

### Cell counts

In the literature antimicrobial assays with yeast used log phase yeast with cell concentrations ranging from 1 to  $1.8 \times 10^6$  cells/mL (Boeira *et al.*, 1999 and Thevissen *et al.*, 1999). Cell counts ranging from 0.22 to  $3.6 \times 10^6$  cells/mL (log phase yeast) were used to compare two different concentrations of gramicidin S (Fig. 7). At the low and high cell counts, the difference in percentage growth between the yeast subjected to the two gramicidin S concentrations was small compared to the differences observed at cell counts ranging from  $3.6$  to  $8.3 \times 10^5$  cells/mL. The assay was therefore most sensitive to concentration differences

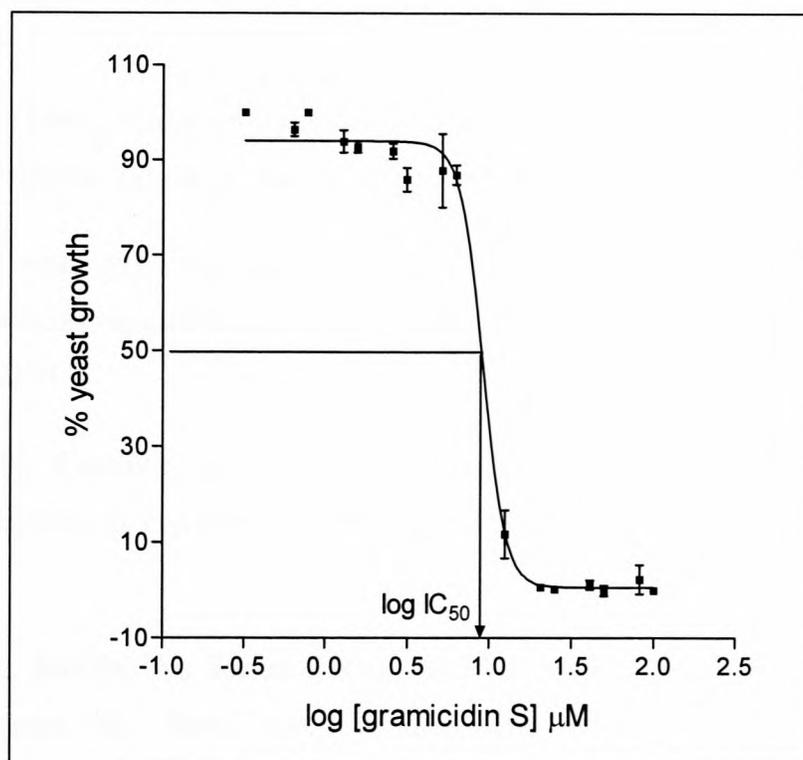
of gramicidin S in this latter cell count range. In addition the change in percent growth for each concentration of gramicidin S was relatively low across this cell count range contributing robustness to the method. The cell count range  $3.6$  to  $8.3 \times 10^5$  cell/mL was hence selected as the optimal range (Fig. 7) for the assay, based on its sensitivity and robustness.



*Figure 7* Antiyeast assay results for two different concentrations of gramicidin S expressed as percent yeast growth (% growth) versus different cell concentrations. The optimal cell count range is indicated. Error bars represent SEM for 3 different duplicate analyses ( $n = 6$ ) each analysed with different batches of yeast.

### Determining dose-response of yeast towards gramicidin S

Data from the antiyeast assay expressed as percent growth was used to generate a dose response curve by fitting the data to a sigmoidal curve with variable slope. From this the concentration required to inhibit 50% of the yeast growth ( $IC_{50}$ ) can be determined (Fig. 8) which in this case was  $12.3 \pm 1.1 \mu\text{g/mL}$  gramicidin S. Gramicidin S  $IC_{50}$  values varied  $<10\%$  and was used as the positive control for the antiyeast assay to validate subsequent assays (Chapters 7-9).



*Figure 8* Gramicidin S dose response curve generated from the antiyeast assay data fitted to sigmoidal curves used to calculate the  $\text{IC}_{50}$  value. Error bars represent SEM for triplicate analyses of triplicate experiments ( $n = 9$ ).

## Conclusions

A microtitre plate antiyeast assay was optimised, using gramicidin S as positive control, for the use of brewing yeast (SAB Ltd. lager yeast strain (*Saccharomyces cerevisiae*)) as the test organism. The set up of the microtitre plates in which the assay was performed was optimised to ensure consistent growth in all the wells. Log phase laboratory propagated yeast was shown to be most suitable for the assay compared to yeast directly out of cryo-preservation, although less sensitive, the log phase yeast gave better repeatability. Cell counts impacted on both robustness and sensitivity of the assay and the cell count range of  $3.6$  to  $8.3 \times 10^5$  cells/mL was selected as optimal in both regards. Using dose response data from the antiyeast assay it was possible to calculate  $\text{IC}_{50}$  value of gramicidin S with high repeatability ( $12.3 \pm 1.1 \mu\text{g/mL}$ ). The antiyeast assay was optimised to provide a tool for the investigation into the presence and possible impact of antimicrobial factors in barley or malt on the brewing process and specifically on yeast performance in fermentation. The application of this method is reported in Chapters 7 to 9.

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# Chapter 7

## *Screening of barley, malt and wort extracts for antiyeast activity*

### **Abstract**

The previously described microtitre plate antiyeast assay (Chapter 6) was used for the detection of antimicrobial factors in barley, malt and wort extracts. The preparation of extracts for the assay is described and the cell count parameter of the antiyeast assay was verified. Antiyeast activity was detected in both barley and malt extracts and comparative results indicated that activity probably increased during the malting process. All the malt extracts screened, except those from one of the malts, had antiyeast activity. Levels of antiyeast activity could be differentiated. For certain malts, which cause premature flocculation of yeast leading to atypical fermentations, the presence of antiyeast factors appeared to be related to yeast sugar uptake inhibition. However, malts that were not associated with premature yeast flocculation, possessing strong antiyeast activity or not, fermented well and sugar uptake was normal. Neither antiyeast nor antibacterial activity was detected in wort extracts. However, this lack of antiyeast activity appeared to be associated with the physical removal of the active factors during wort preparation and/or subsequent extraction under laboratory conditions. The presence of trub, the precipitate formed during boiling and cooling of wort, led to growth inhibition of Gram-negative and Gram-positive bacteria in laboratory prepared wort. However, yeast (laboratory propagated or brewery collected) growth inhibition was seemingly masked by the additional yeast nutrients in trub (metal ions and lipids). Initial exploration revealed yeast (brewery collected) growth inhibition in brewery collected wort compared to filtered brewery wort. We observed that addition of malt derived antiyeast factors to wort impacted negatively on yeast sugar uptake during laboratory 100 mL fermentation.

### **Introduction**

Poor malt quality has been positively identified as the source of a number of brewing related issues such as poor filtration in brewhouse (Prentice and Sloey, 1960), atypical fermentations

(Haikara, 1983, Kruger *et al.*, 1982 and Van Nierop *et al.*, 2004), beer flavour instability (Etchevers *et al.*, 1977) and poor beer foam (Gardner, 1973). Microbial contamination of the grain has been associated with poor malt quality and several brewing related issues (Gjertsen *et al.*, 1965 and Schwarz *et al.*, 2001) such as atypical fermentations due to the premature flocculation of yeast resulting in incomplete fermentations (Axcell *et al.*, 1986 and Stratford, 1992). The type and extent of infestation varied according to growing region, susceptibility of the barley variety and climate (Etchevers *et al.*, 1977). Apart from growth of microorganisms on the grain in the field and on the dry stored barley pre-malting, growth occurring during malting and malt storage may also contribute to the malt quality (Andersen *et al.*, 1967 and Prentice and Sloey, 1960). The conditions during malting were well suited for further microbial growth on barley with regard to temperatures, moisture and airflow. Some microorganisms increased immensely during steeping and germination (Kotheimer and Christensen, 1961), although many of these were killed off during the kilning process when the malt was dried. However, the total microbial load on malt was comparable to that in barley even if the composition was not (Petters *et al.*, 1988).

Plants produce antimicrobial factors as part of their innate immune/defence system (Broekaert *et al.*, 1997). Most of these factors were proteins or peptides and their presence in barley seeds has been well established (Broekaert *et al.*, 1995, Heinemann *et al.*, 1996, Mundy and Rodgers, 1989 and Ponz *et al.*, 1983 and 1986). These antimicrobial factors were either constitutive or induced to cope with sufficiently pathogenic microbial infestation (Carr and Klessig, 1989) or other forms of stress such as drought, cold or chemical exposure (Kader, 1997 and Torres-Schumann *et al.*, 1992). The constitutive type defence responses were in place before the plant tissue was challenged, while the induced plant defence responses were activated when plant tissue was challenged by pathogens (Carr and Klessig, 1989). The mode of action of plant antimicrobial factors produced as part of the plant defence system was mostly by permeabilising the microbial plasma membranes causing leakage of ions and even proteins, damaging the transport systems embedded in the membranes (Guihard *et al.*, 1993) or membrane lysis. Early work demonstrated that brewing yeast was inhibited by a barley factor (9.8 kDa protein) originating from the grain endosperm, causing sugar uptake inhibition at low concentrations and cell death at higher concentrations (Okada and Yoshizumi, 1973).

In addition to plant antimicrobial factors, microorganisms occurring on plants also produced antimicrobial factors. Aside from proteins and peptides these included toxins, by-products of the primary metabolic pathways (organic acids, ammonia, hydrogen peroxide), secondary

metabolites or idiolytes (Sahl and Bierbaum, 1998). Competition between microorganisms for the same source of nutrients stimulated the production of these antimicrobial factors. Such microorganisms and the antimicrobial factors they produced have been shown to occur naturally on barley and malt (Vaughan *et al.*, 2001). Gram-positive bacteria produced by far the widest range of microbial antimicrobial factors. Gram-negative bacteria, filamentous fungi and yeast also produced antimicrobial factors although less prevalent and usually active on a much narrower range of organisms (Jack *et al.*, 1995). Like many of the protein plant factors, these factors were mostly membrane active (Jack *et al.*, 1995 and Sahl and Bierbaum, 1998). The occurrence of microbial contamination of the grain and presence of antimicrobial factors may impact on malt quality, directly or indirectly. The direct impact would be the inhibition of the yeast during fermentation by the antimicrobial factors on the malt. The indirect impact may be a lot more complex, whereby the occurrence of microbial contamination and the stress response to that is indicative of a range of other malt quality aspects that may not necessarily affect yeast directly.

The previously optimised antiyeast assay was applied to investigate antiyeast activity in barley, malt and wort. Barley and malt extract preparation was optimised and the assay conditions for this application were verified. The presence of antiyeast factors in wort was further investigated.

## **Materials and Methods**

### **Malt extract preparation**

Malt samples were milled to a flour consistency in a 3100 Perten hammer mill with a 0.2 mm sieve (Huddinge, Sweden). Duplicate 5 g aliquots were weighed out in disposable 50 mL centrifuge tubes (Sterilin, Staffs, U.K.). 30 mL 0.05 M sulphuric acid (BDH, Poole, England) was added to each tube and incubated on ice for three hours with vigorous shaking every 15 minutes to re-suspend all the malt. The tubes were centrifuged at 4000 g for 15 min. The supernatant was dialysed against distilled water using 1 kDa cut-off dialysis tubing (Spectro/Por®, Rancho, Dominguez, California, U.S.A) pre-blocked by soaking tubing in 2 % casamino acid (Sigma, St. Louis, USA) for three hours at room temperature. Post-dialysis the content of the dialysis tubing was made up to 45 mL with distilled water and centrifuged at 6500 g for ten minutes. The supernatant was filtered through a 0.45 µm acetate syringe filter (Osmonics, Warren, Indiana, U.S.A.) and 4.5 mL aliquots were dispensed into ten mL vacutainer tubes (Preanalytical Solutions, Plymouth, U.S.A.) before freeze drying (Labconco,

Kansa City, Montana, U.S.A). The dried aliquots were centrifuged at 3000 g for three minutes to collect the dry material at the bottom of the tube for easier re-suspension. Dried samples were stored at -20°C until use and then re-suspended in 1000 µL 25% acetonitrile (HiPerSolv™, BDH, Poole, England). Finally, the samples were centrifuged at 6500 g for 15 minutes before using the supernatant in the assay. Each tube or aliquot contains extract from 0.5 g malt. The same extraction procedure was applied to 0.05 M sulphuric acid without the malt as a negative reagent control.

### **Barley extract preparation**

Barley (80 g) was milled as per the malt extraction method and placed into the 500 mL beakers for the R. Chaix M.E.C.A. mash bath (Nancy, France) with overhead paddle stirrers at a temperature of at 55°C. 200 mL Mash water (deionised water with 1.2 mM CaCl<sub>2</sub>.2H<sub>2</sub>O (Sigma, St. Louis, USA) and with pH adjusted to 3.0-3.1, using a dilute solution of lactic acid, (Sigma, St. Louis, USA)) was added to the milled barley as well as 200 µL Bioferm L (Kerry Bio-Science, Co. Cork, Ireland) containing α-amylase and 65 µL Bioglucanase 500 (Kerry Bio-Science, Co. Cork, Ireland) containing β-glucanase. The mixture was stirred at 100 rpm for one hour and the beakers were removed from the mash bath. Concentrated sulphuric acid was added to the mixture to give a final concentration of 0.05 M and extracted as per the malt extraction method described above, commencing with the three hour incubation on ice with vigorous shaking every 15 minute to re-suspend all the barley. In addition barley was extracted in the same way without enzyme and malt was likewise extracted at 55°C with and without enzyme. As control, milled malt was also re-suspended in 200 mL mash water, left to stand at 4°C for one hour and extracted as described earlier commencing with the addition of concentrated sulphuric acid.

### **Mash extraction**

The mashing process whereby malt is extracted prior to boiling and wort preparation was performed according to Van Nierop *et al.*, (2004) (Chapter 4 and 5). The milled malt was mixed with mash water (described earlier) and taken through the mashing temperature profile; 60 minutes at 63±1°C, 72±1°C for 20 minutes and finally five minutes at 76±1°C (temperatures were increased at a rate of 1°C/min). Samples were taken five minutes after mashing in, at the end of the 63°C stand, end of the 72°C stand and end of the 76°C stand. The solids were removed by filtering through cheesecloth and the filtrate was extracted by adding concentrated sulphuric acid to a final concentration of 0.05 M. The acidified mixture was

shaken for three hours at 4°C and further extracted as per the malt extraction method described above.

### **Wort extraction**

All malt wort (16 degrees plato (°P) where 1°P is defined as 1 g solids per 100 g solution) was prepared according to Van Nierop *et al.*, (2004) (Chapter 4 and 5). Concentrated sulphuric acid was added to the wort to a final concentration of 0.05 M. The mixture was shaken for three hours at 4°C and further extracted as per the malt extraction method described above. To test the effectiveness of the extraction, known amount of malt extract (with antimicrobial activity) was added to 30 mL wort and extracted alongside wort without malt extract added. In addition, the same malt extract was added to 0.05 M sulphuric acid in water and extracted.

### **Assay Method**

The antiyeast assay was performed as previously described (Chapter 6). The concentration of the barley, malt and wort extracts used in the assay was related back to the amount of grain it was extracted from. For example, each aliquot or tube of extract contains extract from 0.5 g barley or malt. This was re-suspended in 1000 µL of 25% acetonitrile, hence the concentration was 0.5 g barley or malt extracted per mL. Only 20 µL was used per well in the assay (undiluted or in doubling dilutions) and the total volume per well was 100 µL. The concentration in the well was therefore 0.01 g barley or malt extracted per 100 µL or 0.1 g/mL. Any deviation from these concentrations was noted where applicable.

### **100 mL fermentations**

All-malt wort (13°P) preparation was adapted from Van Nierop *et al.*, (2004) (Chapter 4 and 5). Malt extract (as prepared for the antiyeast assay) from 200 g malt was added pre-boil to wort prepared from 100 g malt. Carbohydrate profiles and wort free amino nitrogen were determined according to EBC methods 8.7 and 8.10 (European Brewing Convention, 1998). Brewery yeast was collected as described previously (Van Nierop *et al.*, 2004) (Chapter 4 and 5) and added to wort at  $20 \times 10^6$  yeast cells/mL determined by counting cells in a Hawksley–Cristallite Haemocytometer with improved Neubauer ruling (Boeco, Hamberg, Germany). Duplicate fermentations were performed in 100 mL sterile glass cylinders with foil caps and incubated at 13°C for 8 days. A 1 mL sample was removed daily from the cylinders at a standard depth and the relative amount of yeast was determined by comparing the light dispersion (optical density, OD) by the yeast cells in suspension at 600 nm using a dual beam

UV/visible spectrophotometer (Shimatzu, UV-1600, Tokyo, Japan). The gravity (g of solids per 100 mL solution) in degrees Plato (°P) was determined using an Anton Paar Beer Analyser (Anton Paar, Graz, Austria) at the beginning and end of the fermentation.

## **2L EBC tall tube fermentations**

Brewery yeast was collected aseptically from the yeast storage vessel within six hours of cropping (harvesting yeast from the bottom of the fermentation vessel after it has precipitated/flocculated) and stored at 5°C for no longer than 12 hours.

Wort with a gravity of 16°P was prepared with 40% dextrose syrup/60% malt, using 12 x 2 L bucket mash bath with overhead paddle stirrers as described by Kruger *et al.* (1982). The wort (2 L aliquots) was steamed for 30 minutes in 5 L round flasks with cotton wool plugs covered in aluminium foil and stored at 5°C for no longer than two weeks.

EBC 2L fermentations were done using a modification of Kruger *et al.* (1982) and Phaweni *et al.* (1992). The stand time after pitching and first wort aeration was changed from 2 to 4 hours. Fermentations were also monitored daily for yeast counts and gravity. Yeast cell counts were determined by counted cells in a Haemocytometer as described above.

## **Laboratory filtered and unfiltered wort as growth medium**

Wort was prepared according to the method described in Chapter 4, which was adapted from the method of Kruger *et al.* (1982) to give an all-malt wort with a gravity of 13°P. At the end of the boil, wort was filtered through folded filter paper (Schleicher & Schuell, Dassel, Germany) to attain “filtered” wort while omission of this filtration step was used to attain “unfiltered” wort. Wort (50 mL) was dispensed into 250 mL Erlenmeyer flasks which was stoppered with cotton wool and covered with foil and steamed to sterilise as described previously. Cryogenically preserved SAB Ltd. lager brewing yeast (*Saccharomyces cerevisiae*) was grown up in MYGP broth (3 g each of malt and yeast extract, 5 g peptone and 10 g glucose (Biolab Diagnostics, Midrand, South Africa) dissolved in 1 L water and autoclaved with pressure to attain 121°C for 15 minutes) as per the antiyeast assay method. Brewery isolated bacteria of the genus *Acetobacter* and pure culture *Micrococcus luteus* from plate colonies were grown up in nutrient broth (Biolab Diagnostics, Midrand, South Africa, made up as per manufacturers instructions) overnight at 30°C, shaking at 150 rpm. All three cultures were diluted in their growth media to an optical density of 0.3 at 600nm. In addition brewery collected yeast (as described for 2L EBC tube fermentations) was also diluted to the

same specifications using MYGP broth. A 0.2 mL aliquot of each of the diluted cultures were inoculated separately into filtered and unfiltered wort in the 250 mL flasks. The flasks with yeast were incubated at 25°C and those with bacterial cultures at 30°C, while being shaken at 150 rpm. Samples (1 mL) were removed from all the flasks at time intervals and the optical density at 600nm determined. The values were used to produce growth curves for each of the organisms in both filtered and unfiltered wort.

### **Brewery filtered and unfiltered wort as growth medium**

Brewery wort with a gravity of 16°P (40% dextrose syrup/60% malt) was collected from a brewery ex-paraflow (cooling) en route to the fermentation vessel (i.e. wort as used in fermentation). The wort was used as is (unfiltered), filtered through Schleicher & Schuell (Dassel, Germany) filter paper or through a 0.45 µm acetate syringe filter. Each wort (15 mL) was dispensed into 100 mL Erlenmeyer flasks which was stoppered with cotton wool and covered with foil and steamed to sterilise as described previously. Brewery collected yeast, as described for 2 L EBC fermentations, was inoculated into each wort at a cell count of  $20 \times 10^6$  cells/mL. The flasks were incubated at 25°C while being shaken at 150 rpm. Samples (1 mL) were removed from all the flasks after 12 and 24 hours and the optical density at 600nm determined.

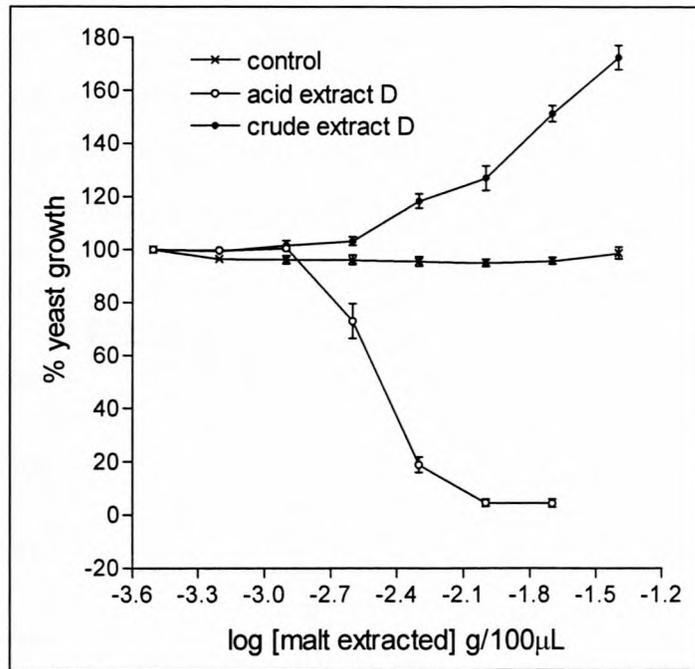
## **Results and Discussion**

### **Malt extract preparation for the antiyeast assay**

Crude cold-water extracts of milled malts were tested for antiyeast activity using the antiyeast assay, but little or no inhibition was detected. On the contrary growth was enhanced relative to the control believed to be due to the additional nutrients in the crude extract, overshadowing any antimicrobial factors that may have been present (Fig.1).

Several extraction procedures were tested and an adaptation of the original method for barley by Okada and Yoshizumi (1970) was found to be most suitable for malt. They used an acid extraction of milled barley followed by a pH adjustment and ethanol precipitation. The method was adapted as follows: milled malt was used, acid extraction was done at a lower temperature, samples were dialysed (1 kDa cut-off) to remove the acid thus neutralising the pH, samples were concentrated by freeze drying and filtration was used to further clarify and sterilise the samples. The extract prior to the ethanol extraction was found to be the most active and sufficiently “purified” to eliminate growth enhancement in the assay as seen

previously with crude malt extracts (Fig.1). While the acid step removed the contaminating proteins (Roberts and Selitrennikoff, 1986), the dialysis not only removed the acid, which was antimicrobial in itself, but presumably also most of the sugars and other potential nutrients.



*Figure 1* Comparison of antiyeast assay results, expressed as percent growth of yeast relative to the negative control, of different malt extracts: acid extract adapted from Okada and Yoshozumi (1970) and crude cold water extract of malt D. Error bars represent standard error of the mean (SEM) for three different duplicate analyses (n = 6) each analysed with different batches of yeast.

### Acceptable number of extracts and antiyeast analyses

Initially all malts were extracted in triplicate and each extract was analysed by three different batches of yeast (n = 9). Antiyeast assay results from 10 different malts was used to determine, based on goodness of fit ( $R^2$ ) and  $IC_{50}$  values (concentration that causes 50% inhibition of growth), that doing only duplicate extractions and using two different batches of yeast (n = 4) had minimal impact on the values.  $R^2$  was reduced on average by 1.4% and  $IC_{50}$  values varied <10%. This format of two extractions and two batches of yeast was more practical and adopted as standard practice for the method.

### Cell counts

Cell counts for the antiyeast assay was previously optimised (Chapter 6) using a pure, known antimicrobial peptide (gramicidin S). It was shown that the cell count had a major impact on the antiyeast assay and hence the optimisation was repeated using malt extracts, because the



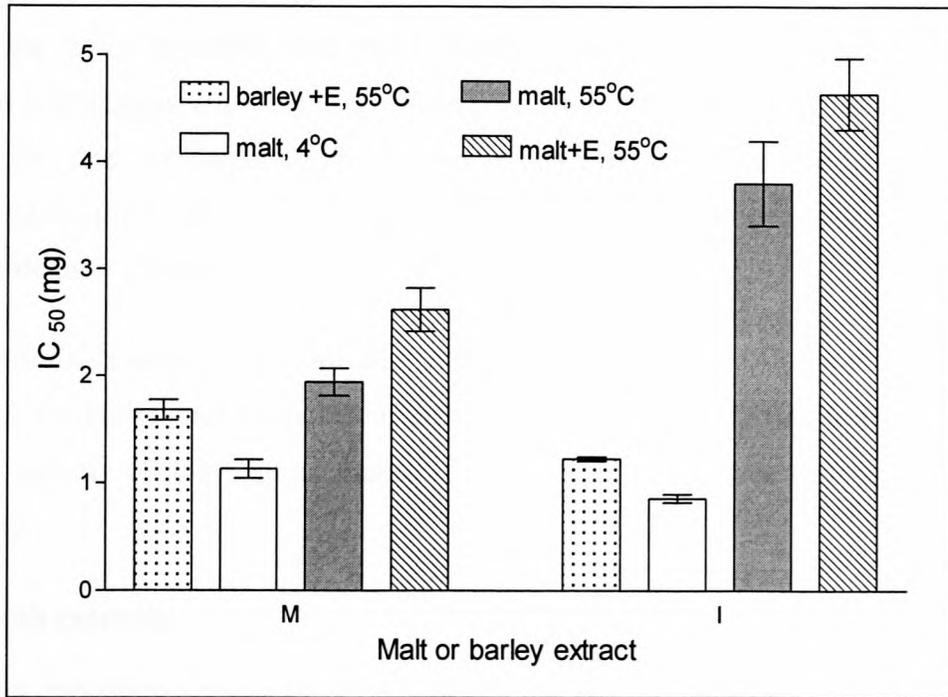
procedure. No antiyeast activity was found in the barley extracts, suggesting there was no activity present, activity was not extracted or the assay was not sensitive enough to detect activity. Okada and Yoshizumi (1973), however, were able to show that barley extracts inhibited the growth of *S. cerevisiae* in wort. Antimicrobial activity could be retrieved when active malt extract was added to the barley extraction mixture, indicating the added factors were not bound to the barley material (results not shown).

In order to improve the extraction process of antimicrobial factors from barley, commercially available enzymes used in barley brewing were employed to breakdown the starch granules (a process which normally occurs during malting). With the aid of these enzymes ( $\alpha$ -amylase and  $\beta$ -glucanase) antimicrobial activity was extracted from the two barley samples. Comparison of the  $IC_{50}$  values for the barley enzyme aided extract (at 55°C) and malt extract (at 4°C<sup>1</sup>) of the two corresponding barley and malt samples<sup>2</sup> showed an increase in antiyeast activity across the malting process (Fig. 3, first two bars only for M and I). However, this increase was only statistically significant ( $P < 0.05$ ) for the less active barley-malt pair (M in Fig. 3). The barley I may already have been primed in the field by pathogens to have a higher basal level of antimicrobial factors (Carr and Klessig, 1989), while growth of pathogens during the germination stage (Carr and Klessig, 1989) of the malting process may have caused the increase of antimicrobial activity from barley to malt in the case of the barley-malt pair M. However, it should be noted that the small increase in antiyeast activity from barley to malt, could also be attributed to the barley extraction process.

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<sup>1</sup> The activity of malts I and M in this experiment at 4°C differ to those reported in Fig. 5 due to the differences in extraction procedures (see Materials and methods)

<sup>2</sup> Barley and malt samples M and I are very different w.r.t. variety, where they were grown and where they were malted.



**Figure 3** Comparison of antiyeast activity, expressed as IC<sub>50</sub> values (in mg of malt or barley extracted), of different barley and malt extracts prepared at 4 and 55 °C with and without starch degrading enzymes (E). Error bars represent SEM of duplicate analyses of duplicate experiments (n = 4).

The optimal temperature of the enzymes, used to release the antiyeast factors from the barley matrix, was 55°C (Wainwright, 1997) and therefore the extraction mixtures were incubated at this temperature. In addition, calcium was added (as calcium chloride) to help stabilise  $\alpha$ -amylase (Briggs *et al.*, 1981) and the pH was adjusted to 5.2±0.5 for optimal enzyme activity. The impact of temperature and enzyme in the presence of calcium and pH adjustment was also determined for the malt extraction as controls. Significantly less antiyeast activity was extracted from malt in the presence of raised temperature (Fig. 3). This loss is probably the consequence of enzymatic degradation of the active factors (known to be heat resistant and to contain a protein component (Chapter 9) by malt enzymes. Malt contains higher levels of proteases and starch degrading enzymes than barley (Briggs 1981), which may explain the impact of malt exposure to 55°C at which temperature these enzymes would be active (Wainwright, 1997).

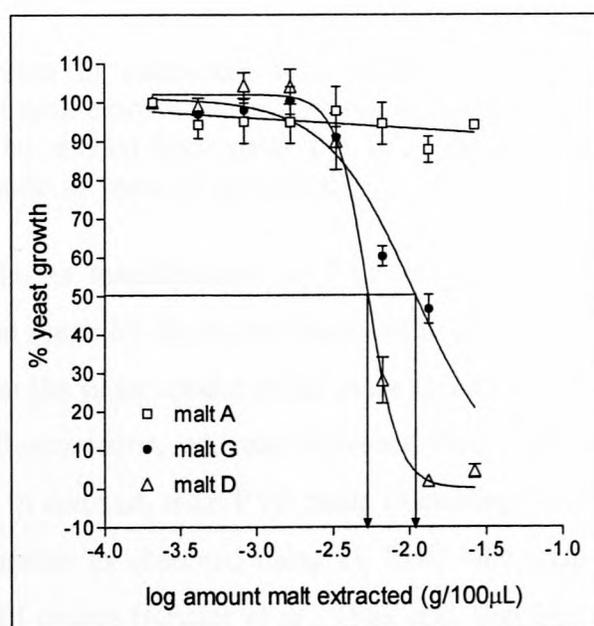
An interesting result is the further significant decrease ( $P < 0.01$  for M,  $P < 0.05$  for I) in antiyeast activity of malts at 55°C when  $\alpha$ -amylase and  $\beta$ -glucanase were added (Fig. 3). The enzymes themselves did not impact on the antiyeast assay (results not shown). This could either indicate that carbohydrates are associated with the antiyeast activity/factors or that they

aid in the protection of antimicrobial factors from proteases. The latter may explain why barley, containing fewer proteases, was not affected to such a great extent as malt by the addition of starch degrading enzymes (Fig. 3). In particular, the retention of barley antiyeast activity may be due to larger carbohydrate polymers associated with protein-like antimicrobial factors, protecting them from protease action, but requiring the starch degrading enzymes to enable their release.

Although interesting results came from this experiment, the small difference in antiyeast activity between the barley and its malt, the difficulty in extracting activity from barley and the increase of activity during malting makes it more feasible to screen different malts for antiyeast activity.

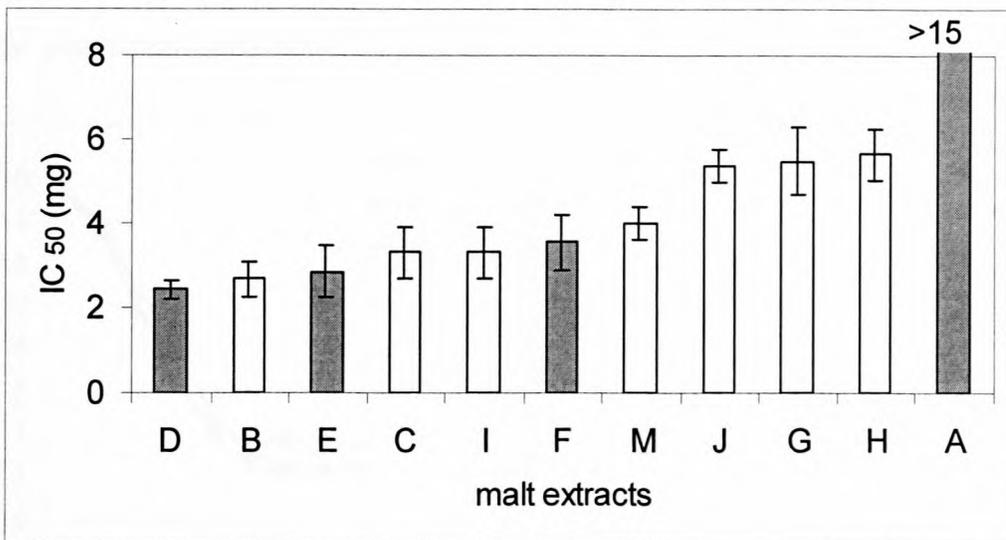
### Screening of malt extracts

A range of malts, including some associated with atypical fermentations and others known to ferment well, were selected, extracted and analysed using the optimised antiyeast assay.  $IC_{50}$  values calculated from the sigmoidal curves fitted to the dose-response data (Fig. 4) indicate differences in antimicrobial activity or growth inhibition between malts (Fig. 5). All the malt extracts, except one (A), had antiyeast activity with  $IC_{50}$  between 2.4 and 5.7 (in mg malt extracted per 100  $\mu$ L assay volume).



*Figure 4* Dose-response of *S. cerevisiae* towards three different malt extracts tested in the antiyeast assay. The arrows indicate log  $IC_{50}$  values for the calculation of  $IC_{50}$  values reported below. Error bars represent SEM of triplicate experiments analysed in duplicate ( $n = 6$ ). The goodness of fit ( $R^2$ ) for the two active extracts (G and D) was  $>0.99$ .

Several of these malts cause premature yeast flocculation (PYF) during fermentation (Fig. 5, shaded bars), which has been positively linked to microbial infection of the grain (Axcell *et al.*, 1986, Herrera and Axcell, 1991 and Stratford, 1992). We previously speculated a connection between the presence of PYF factors and malt antimicrobial peptides, both produced by barley in response to microbial infestation (Axcell *et al.*, 2000 and Van Nierop *et al.*, 2004). These results suggest there to be a possible link, since malts D, E and F (with high levels of antiyeast activity) all cause PYF (Fig. 5). However, an exception to this was the PYF-positive malt A extract, which yielded little or no antiyeast activity.

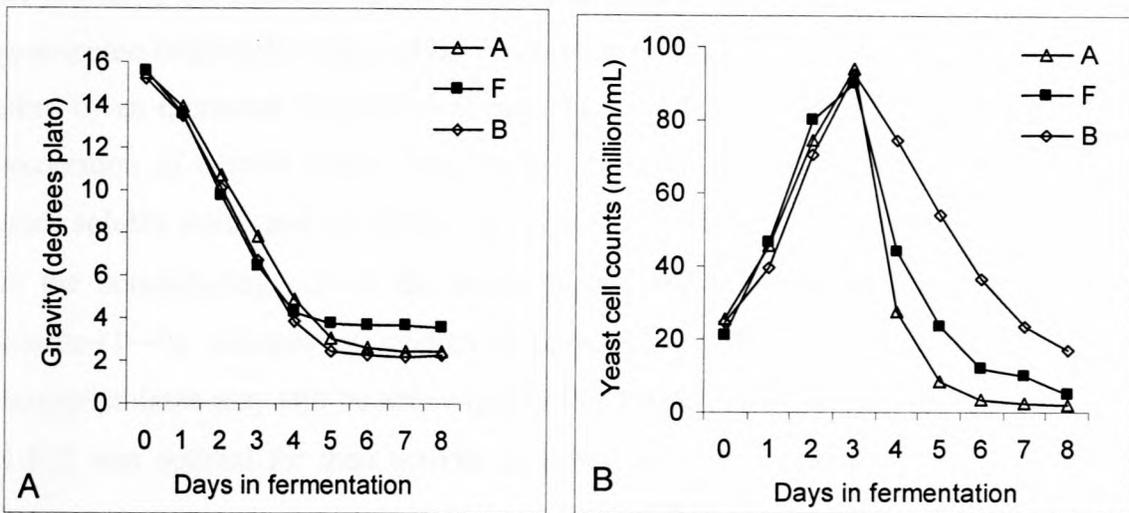


**Figure 5** Comparison of calculated IC<sub>50</sub> values (in mg of malt extracted) between different malt extracts; error bars represent SEM of six different experiments (n = 6). The shaded bars show the IC<sub>50</sub> values of malts that cause premature flocculation of yeast in fermentation.

Malt A is atypical in its manifestation of PYF in that it caused the physical effect of premature flocculation (seen by the earlier decrease in cells counts in suspension (Fig. 6A)), but does not impact on the sugar uptake of the yeast (seen by normal gravity or residual sugar values at the end of fermentation, whereas increased values are associated with sugar uptake inhibition (Fig. 6B)). In contrast, most PYF malts (including D, E and F tested here) do cause a decrease in sugar uptake as observed using 2L EBC tube fermentations with daily gravity measurements and cell counts (Kruger *et al.*, 1982 and Van Nierop *et al.*, 2004) (results for malt F are shown in Fig.6). When a specific malt exhibits PYF it seems that the presence of antimicrobial factors results in sugar uptake inhibition. Yeast sugar uptake inhibition during assay conditions was observed by Okada *et al.* (1970) using barley antimicrobial extracts. This has also been observed intermittently during atypical (non-PYF) fermentations in the

breweries. However, it was found that the other PYF-negative malts, be they strongly antimicrobial or not, fermented well and sugar uptake was normal (Malt B, Fig.6). The differences in cells counts (Fig. 6A, between A and F) and gravities (Fig. 6B, between A and F) reported here are considered to be sufficiently different to impact on product quality (personal communication Dr. Cameron – Clarke).

The possible association of PYF during fermentation with malt antimicrobial activity as proposed by Axcell *et al.* (2000) and Van Nierop *et al.* (2004) was supported by the results presented here. The association between barley antiyeast activity and sugar uptake inhibition reported by Okada *et al.*, (1970) was also supported by the data on the PYF positive malts and suggests the presence of such factors during the fermentation and hence in wort.



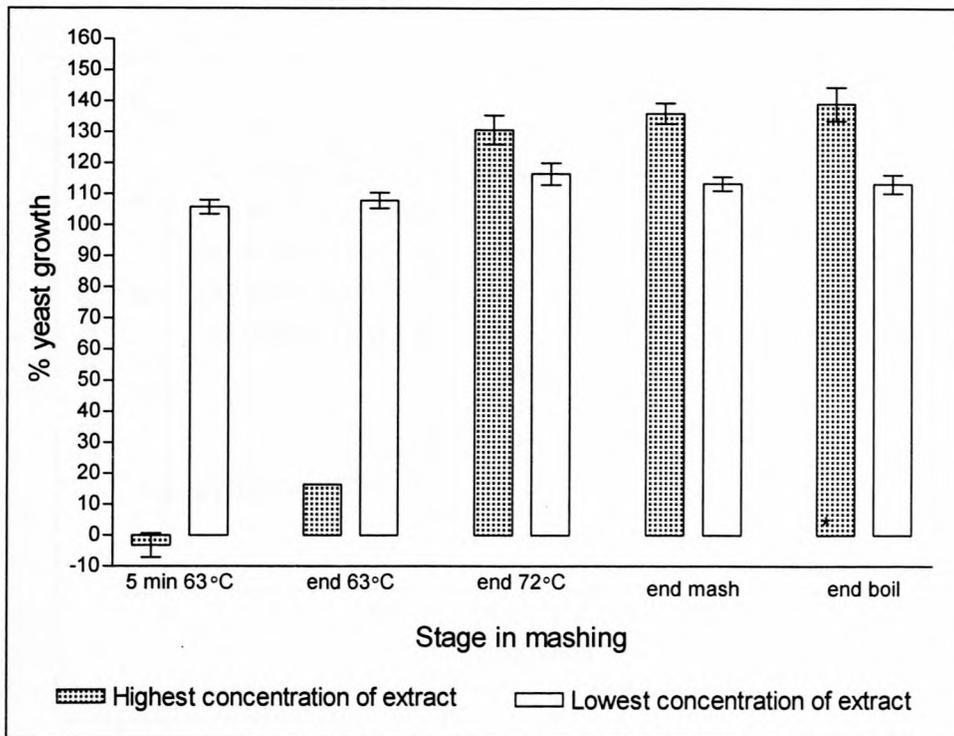
**Figure 6** 2L EBC fermentation results of wort prepared from malts A, F and B where A and F are PYF malts and B is not. Fig. A monitors gravity through fermentation and Fig. B monitors cell counts. Each value was the average of duplicate fermentations, variations for cell count method was <15% and standard deviation for the gravity readings was 0.04.

**Wort extract preparation for the antiyeast assay**

An attempt to extract the antiyeast factors from laboratory prepared wort (boiled) using an adaptation of the malt extraction method was unsuccessful as no antiyeast activity or antibacterial activity (*M. luteus* as target organism) was observed in these extracts (results not shown). Malt extracts with antiyeast activity have been shown to be antibacterial and antifungal as well (Chapter 9). To test the wort extraction method, active malt extract was added to wort. The antiyeast activity was preserved and could be recovered from the wort, demonstrating that the extraction process was applicable. These results may indicate that a.)

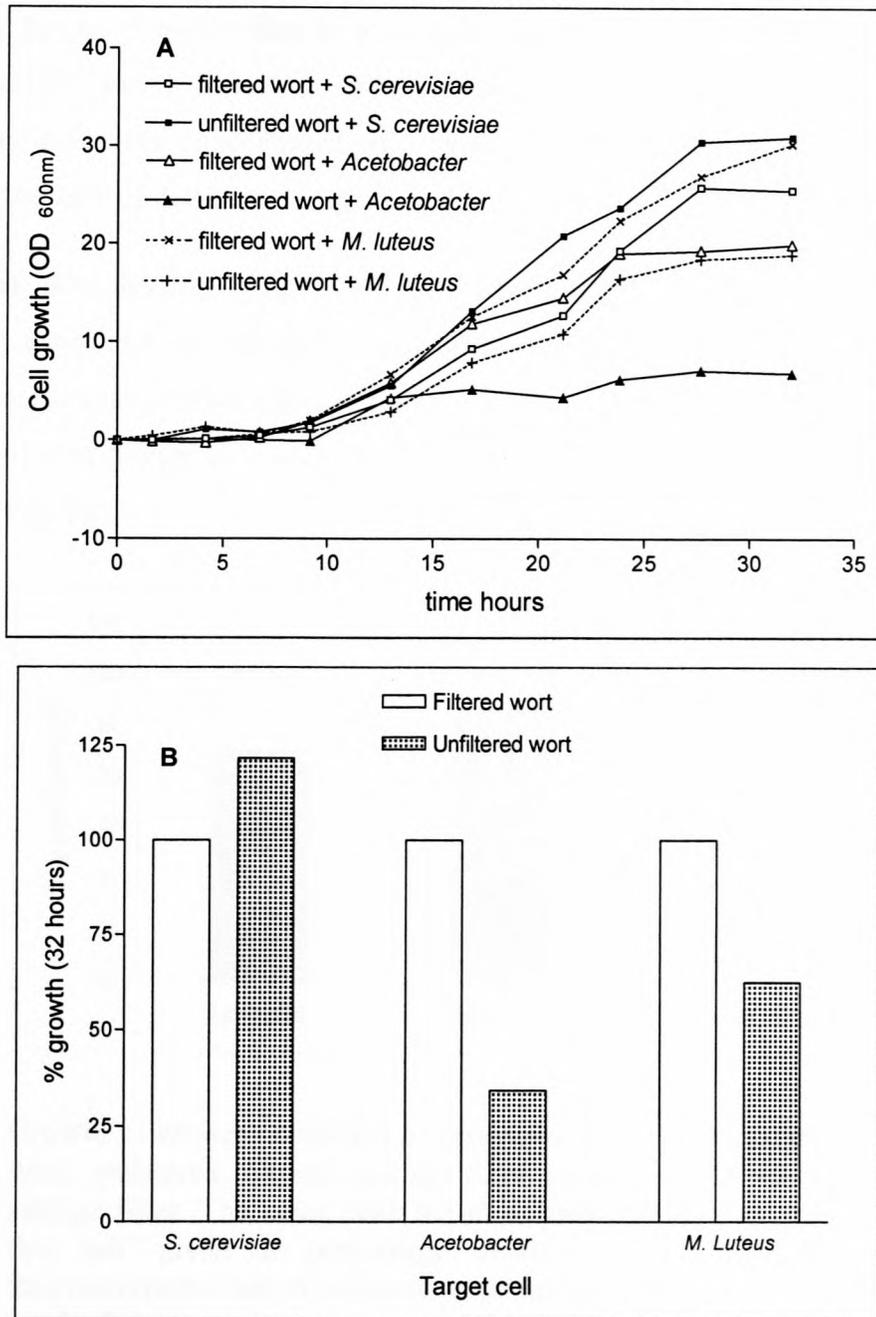
the antiyeast factors did not survive the wort preparation process, b.) the activity was retained, but was physically separated from the wort due to the laboratory wort preparation or the subsequent extraction procedure or c.) low levels of activity survived, but could not be detected by our assay. Options a, b and c were examined in more detail.

To trace the possible loss of antiyeast activity prior to the wort boil of malt B, samples were taken during wort preparation or mashing. During mashing, milled malt in mash water (see Materials and Methods) was exposed to various temperature stands to allow for the enzymatic degradation and extraction of predominantly the fermentable sugars from the malt. Antiyeast activity was lost between the end of the first temperature stand at 63°C and the end of the second temperature stand at 72°C as seen by the decreased difference between the percent yeast growth in the presence of the highest and lowest concentration of extract based on the dose response (sigmoidal) curve (Fig. 7). Any remaining antiyeast activity thereafter may be masked by an increased availability of nutrients seen by the increased growth at the highest concentration of extract added (Fig. 7). The function of these temperature stands was to degrade soluble starch and dextrans by the action of malt  $\beta$ -amylase (63°C), releasing maltose from the non-reducing end of the starch chains, and  $\alpha$ -amylase (72°C), attacking the  $\alpha$ -glucosido-(1→4) linkages at random (Briggs *et al.*, 1981). In addition, proteases and carboxypeptidases may still be active during this temperature stand since the pH of the mash (pH 5.2) was optimal for their activity and they are only inactivated at 70°C even if their optimum temperatures were much lower (45-50°C) (Wainwright, 1997). Although the loss of antiyeast activity may have been partly due to the raised temperature, it is likely that enzymatic degradation of the antimicrobial factors contributed to the loss as was discussed for barley extraction earlier.



*Figure 7* Survival of antiyeast activity during the mashing of malt B shown by comparing yeast growth in the presence of the highest and lowest concentration of mash extract, derived from the sigmoidal dose response growth curve. Error bars represent SEM of duplicate extracts analysed with duplicate batches of yeasts ( $n = 4$ ). The experiment was repeated with similar results for malt F.

To determine if antiyeast factors are physically separated from the wort, due to the laboratory wort preparation or the subsequent extraction procedures, filtered and unfiltered wort prepared from malt B was compared as growth media for laboratory propagated yeast, brewery collected yeast, brewery isolated bacteria belonging to the genus *Acetobacter* (known beer spoilage Gram-negative bacterium) and pure culture of *M. luteus* (Gram-positive bacterium, previously used by Du Toit and Rautenbach (2000) as antimicrobial test organism). Growth in the presence (unfiltered) or absence (filtered) of trub, the precipitate formed during boiling and cooling of wort (hot and cold break), was monitored over 32 hours and plotted as growth curves in Fig. 8A. Interestingly, growth of both bacteria was inhibited in the unfiltered wort compared to the filtered wort, but this effect was not observed for yeast (Fig. 8B). On the contrary, growth was enhanced for yeast in unfiltered wort (Fig. 8B), which was most likely due to the added metal ions and lipids found in the trub which are known to enhance yeast growth (Meireles de Sousa, 1989, Kreder, 1999 and Rees and Stewart, 1997).

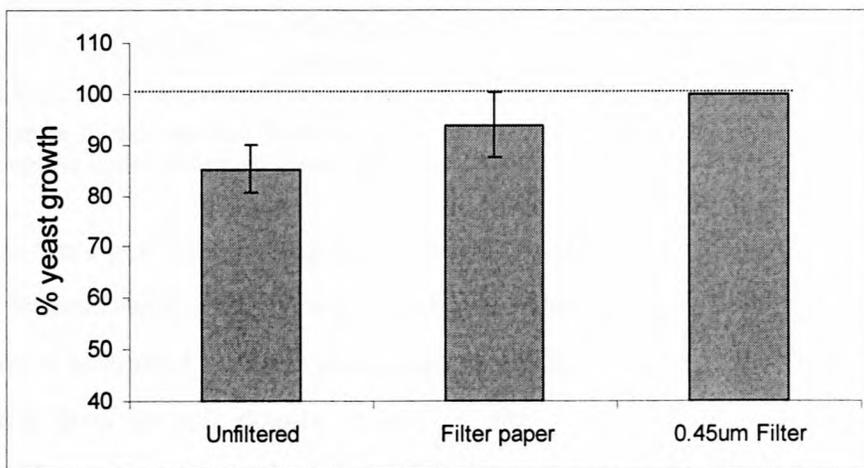


**Figure 8** A. Growth curves of laboratory propagated *S. cerevisiae*, brewery isolated *Acetobacter* species and pure culture *M. luteus* in filtered and unfiltered all malt wort. Cell growth is related to optical density at 600 nm (OD<sub>600nm</sub>). B. Comparison of the 32 hour growth of the three target cells in filtered and unfiltered wort. Values represent duplicate experiments, no error bars are shown but values varied <10% in all cases.

Brewery collected yeast was also tested because it was found to be far more sensitive to the inhibitory effect of malt antimicrobial factors than the laboratory propagated yeast (Chapter 8), but again growth was enhanced by the unfiltered wort (results not shown). The malt antimicrobial factors have been shown to have a broad range of activity, inhibiting barley

derived bacteria and fungi, as well as yeast (Chapter 9). It is therefore possible that the yeast inhibition by factors in wort B may be overshadowed by the enhanced growth (Figs. 1 and 7). Enhanced growth, due to added nutrients, also overshadowed any inhibition that may have been present when more concentrated wort extracts were analysed by the antiyeast assay to determine if the lack of detection in wort was due to very low levels of activity.

Brewery wort (40% dextrose syrup/60% malt), which differs significantly to laboratory wort (100% malt) especially the filtration procedures during preparation, was also tested with brewery collected yeast. In this case preliminary investigations revealed that yeast growth was inhibited relative to filtered wort (Fig. 9) although differences were not as great as those seen for bacteria (Fig. 8).



*Figure 9* Growth of brewery collected *S. cerevisiae* in the presence of brewery collected wort; unfiltered, filtered through filter paper and filtered through a 0.45  $\mu\text{m}$  syringe filter. The latter, with the most growth, was used as 100% growth and the rest given a percentage relative to that. Error bars represent minimum/maximum of duplicate experiments with two batches of yeast and one batch of wort.

Findings have indicated that antimicrobial activity is present in brewery wort which may therefore impact on yeast fermentation. Conditions are quite different in a brewery fermentation to those of the laboratory assays used. However, monitoring and comparing small scale fermentations with differing levels of antimicrobial activity may provide an indication as to the impact on yeast during fermentation despite the inherent limitations of the method. Fermentations (100 mL) were performed using existing analyses to determine if any differences could be detected. Wort made from relatively low antiyeast activity malt (G) with/without malt extracts (A, G, and B) added during wort preparation prior to the boil (Table 1) were compared. Due to the added extract, the free amino nitrogen (FAN) and

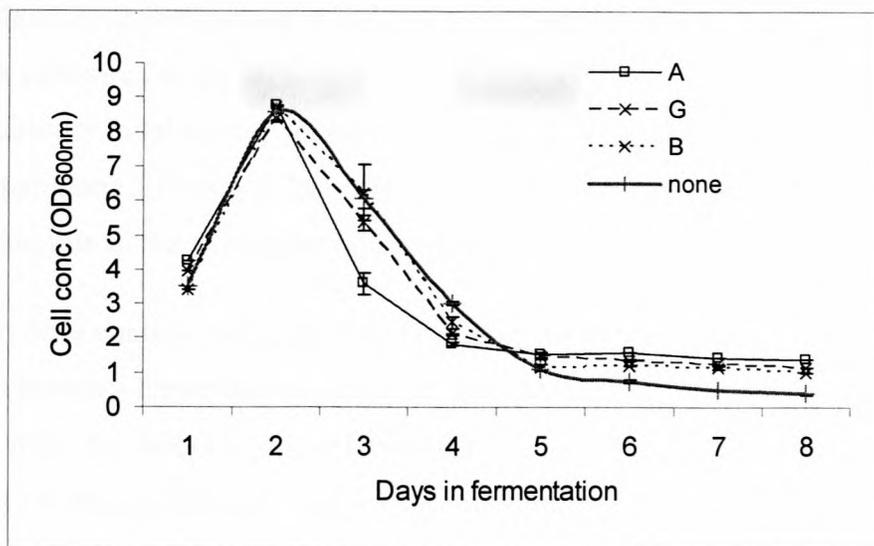
fermentable carbohydrate profiles were compared for all the worts to ensure any fermentation differences were not due to nutrient availability; the sugars were comparable and the FANs were not limiting (results not shown).

Table 1 Antimicrobial activity and description of malts used in 100 mL fermentations. SEM of at least 22 analytical values are reported.

Malt	IC <sub>50</sub> ±SEM (mg)	Description
A	Not active	Least antiyeast malt tested so far, known to cause premature flocculation of yeast in fermentation.
G	5.5 ± 0.79	Malt that ferments well*, relatively low antiyeast activity. This malt was used to prepare the wort.
B	2.7 ± 0.40	Malt that ferments well*, consistently high antiyeast activity.

\* Based on absence of any obvious fermentation abnormalities such as premature flocculation of yeast and inhibited sugar uptake under laboratory fermentation conditions.

Fermentations were performed using brewery yeast, cell growth was monitored daily (Fig. 10) and end of ferment wort gravity was measured. Malt A with least antimicrobial activity detected so far was known to cause premature yeast flocculation, seen here by the earlier and more dramatic drop in cell counts on day 3, the other fermentations did not flocculate prematurely. The worts with malt extract added differed to the control with no addition. The end of ferment cell counts correlated (linear correlation  $R^2 = 0.94$ ) to the remaining sugar concentration (gravity). The malt extracts appear to have introduced or increased the concentration of a factor (or factors) that led to incomplete fermentations, leaving high number of cells in suspension and high concentrations of sugars. All the malt extracts tested had a negative impact on fermentation relative to the control (differences are sufficient to have an impact as discussed for Fig. 6). The degree of impact could not be differentiated between the malt extracts which may be explained by the large amount of extract added (extract from 2 g of malt per 1 g of malt used to make the wort). It is also possible that all malts may have some degree of antimicrobial activity since in this case even malt A was observed to have an effect. The impact was also shown to be greater when double the amount of extract was added (results not shown). It is important to recall that the impact cannot be due to small components such as metal ions since the extract goes through a 1 kDa dialysis stage to remove such components. Clearly should any antimicrobial activity survive the brewing process there may well be an impact on fermentation.



*Figure 10* Yeast cell concentrations throughout the fermentation of 100 mL wort prepared from malt G with and without malt extracts (A, G and B) added. Cell counts were determined by measuring the optical density at 600nm (OD<sub>600nm</sub>). Curves represent average values for duplicate fermentations with the error bars showing the minimum and maximum values. Brewery yeast was used for the fermentations.

## Conclusions

All the research to date was based on antimicrobial factors extracted from barley not malt or wort. Extraction of malt was easily done using acid precipitation at 4°C, but it was necessary to aid the extraction of antiyeast factors from barley with enzymes to digest the starch matrix. Extracts of all the malts, except one, and both barley samples had antiyeast activity. From these results it was concluded that screening of malts for antiyeast activity as a measure of malt quality has potential application in the brewery industry. Not only do malts differ in their antiyeast activity, but there was also evidence that the presence of antimicrobial factors in malt can, in certain conditions, impact on yeast sugar uptake during fermentation. Furthermore the brewing process uses malted barley and not raw barley, therefore it is of greater interest to examine malt quality and malt associated antiyeast factors.

None of the laboratory prepared worts that were extracted retained any antiyeast activity. Although antiyeast activity was seemingly lost during wort preparation (mashing), antibacterial activity (against Gram-negative and Gram-positive bacteria) but not antiyeast activity was retained in the wort trub (precipitate). On the contrary the presence of trub seemed to enhance yeast growth presumably due to the additional yeast nutrients in the trub (metal ions and lipids) possibly overshadowing any antiyeast activity that may have been

present. Preliminary investigations did reveal growth inhibition of brewery collected yeast by brewery wort compared to the same wort with its trub removed. These results suggest the lack of antiyeast activity in laboratory prepared wort and wort extracts may be the consequence of the wort preparation, differing to brewery wort preparation, and the removal of precipitates (including trub) due to the wort extraction procedure.

Brewery wort does contain more trub than laboratory wort and malt antimicrobial factors may play a role in brewery fermentations based on these results. In addition, collection of brewery yeast slurry from the bottom of the fermentation vessel for re-pitching may result in the accumulation of trub components and a concentration of antimicrobial factors impacting on brewery fermentations and explaining the increased sensitivity of brewery collected yeast compared to laboratory propagated yeast (Chapter 8).

It was shown that should antiyeast and antibacterial factors be present in wort and brewery yeast slurry it would impact on yeast performance, sugar utilisation and thus the brewing process, specifically the fermentation. Based on the results and likelihood that malt derived antimicrobial factors play a role in yeast fermentation performance, further investigation of the antimicrobial factors was warranted. The limited information available on antiyeast factors in malt convinced us to characterise and attempt to identify the antiyeast factors in malt (Chapter 9).

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## Chapter 8

### *Screening of brewery yeast and brewing yeast strains against malt extracts to assess yeast quality*

#### **Abstract**

Yeast quality, as measured by sensitivity of brewery yeast and different lager brewing yeast strains towards known antimicrobial barley malt extracts was assessed by an adaptation of the microtitre plate antiyeast assay (Chapter 6). It was found that yeast obtained directly from a brewery was much more sensitive towards the malt extracts than the same yeast strain propagated in the laboratory. Sensitivity was also seen to change during the course of a laboratory scale fermentation inoculated with brewery yeast. Different brewing yeast strains of *Saccharomyces cerevisiae* were propagated in the laboratory and challenged with antimicrobial extracts from a number of different malts. The susceptibility of these different brewing yeast strains, when challenged with malt extracts varying in antimicrobial activity, were compared and differences were found between the yeast strains in terms of their sensitivity. Results indicate that this application of the antiyeast assay has potential as a yeast assessment method, possibly as a measure of membrane integrity, since antimicrobial activity is membrane associated and membrane integrity is critical for yeast vitality.

#### **Introduction**

Plants contain antimicrobial factors as part of their innate immune/defence system (Broekaert *et al.*, 1997), which is either constitutive or induced to cope with sufficiently pathogenic microbial infestation (Carr and Klessig, 1989) or other forms of stress such as drought, cold or chemical exposure (Torres-Schumann *et al.*, 1992). The most common factors associated with plant defence were proteins (Batalia *et al.*, 1996, Boller, 1993 and Van Loon and Van Strien, 1999) and peptides (Broekaert *et al.*, 1997 and Florack and Stiekema, 1994). The factors extracted from barley malt (or malt) were identified to be 5-14 kDa peptides (Chapter 9).

Since plant antimicrobial factors usually act on the microbial plasma membrane, using the antimicrobial assay to expose yeast to malt antimicrobial extracts would assess yeast

susceptibility to these factors, presumably as a function of membrane integrity that is critical for yeast functioning. Yeast is subject to ageing and stress factors (Heggart *et al.*, 1999) such as osmotic pressure, high ethanol and carbon dioxide concentrations (Hammond, 1999 and White *et al.*, 2003) and stress due to yeast handling in the plant that include mechanical stress (Stafford, 2003), high physical pressure and oxidative stress (Martin *et al.*, 2003). These factors impact on the physiological status of the yeast, decreasing viability (dead and alive) and vitality (health) resulting in growth inhibition, decreased genetic stability and of particular interest in this case; changing cell membrane stabilisation and functioning (Casey and Ingledew, 1983 and Stewart *et al.*, 1999).

When antimicrobial factors act on the microbial plasma membrane, a number of different effects can occur including leakage of ions and in some cases even proteins by membrane permeabilisation, damage to the transport systems embedded in the membranes (Guihard *et al.*, 1993) or total membrane disruption resulting in cell lysis. The latter was observed when yeast was exposed to the antimicrobial malt extracts as determined by monitoring the presence of an intercellular yeast enzyme extracellularly (Chapter 9). Several existing quality measurements were based on membrane integrity and functioning such as capacitance where an applied radio-frequency permits the build up of charge due to the dielectric nature of the cell's (intact) membrane and vital stains that were actively taken up by functioning membranes only (Heggart *et al.*, 1999).

In order to study malt antimicrobial factors, an antiyeast assay, using laboratory propagated log phase brewing yeast as test organism for malt extracts, was optimised for execution in a microtitre plate (Chapter 6). The assay was used, not only to screen malts for antiyeast activity, but also to investigate the origin and relevance of these factors to the beer brewing process and malt quality (Chapters 7 and 9). The assessment of yeast quality and prediction of fermentation performance is notoriously difficult as variations in yeast vitality are very subtle and there is a continued need to develop more accurate means of measuring yeast quality (Heggart *et al.*, 1999). The utilisation of the optimised microtitre plate antiyeast assay as a yeast quality assay to compare the susceptibility of different brewery yeast and brewing yeast strains to malt antimicrobial factors is reported here.

# Experimental

## Antiyeast analysis

Malt extract preparation and the antiyeast assay were performed as previously described (Chapter 6). Cryogenically stored *Saccharomyces cerevisiae* SAB Ltd. lager brewing yeast strain (S) and other SABMiller lager strains (T, P and M) were propagated as described previously (Chapter 6). All yeast propagation was performed in MYGP broth; 3g each of malt and yeast extract, 5 g peptone and 10 g glucose dissolved in 1L water, autoclaved 15 minutes, pressurised to attain 121°C. All yeast cell counts were performed in a Hawksley–Cristallite Haemocytometer with improved Neubauer ruling (Boeco, Hamberg, Germany).

## EBC 2L fermentations

Fermentations were performed as adapted by Van Nierop *et al.* (2004) from Kruger *et al.* (1982) and Phaweni *et al.* (1992) using brewery yeast and wort aseptically collected from breweries C and D. The two different yeasts were fermented in the same wort ex-brewery D. The brewery wort used was 40% adjunct wort, where 60% of the fermentable sugars come from malt and the rest from maize derived maltose syrup.

## Results and Discussion

The antiyeast assay format as used to screen malts (Chapter 6 and 7), was applied to different yeasts used at the same yeast counts ( $3.6 \times 10^5$  cell/mL) as prescribed for the antiyeast assay. Yeast viability (percent alive) was not taken into account, as these were not expected to vary much in freshly propagated yeast.

When comparing brewery yeast to laboratory propagated log phase yeast (all strain S), brewery yeast was found to be much more sensitive to the inhibitory impact of malt extracts. This trend was observed for all six samples of brewery yeast tested on three different malt extracts (A, C and D), a selection of which is shown in Fig. 1. According to the  $IC_{50}$  values (for all six brewery yeast tested) for malt extract D, between 3 and 7 fold less malt extract was required to inhibit 50% of the yeast growth compared to laboratory propagated log phase yeast.

Although brewery yeast was more sensitive, laboratory propagated log phase yeast was found to be more discriminating when comparing sensitivity to extracts from three different malts (Fig. 2), these findings support the use of propagated yeast for the assay. The inhibitory

potential of the known antimicrobial peptide, gramicidin S, used as a positive control for the antiyeast assay, was not influenced by the different yeasts, be it laboratory propagated or brewery yeast, hence no results were included for gramicidin S.

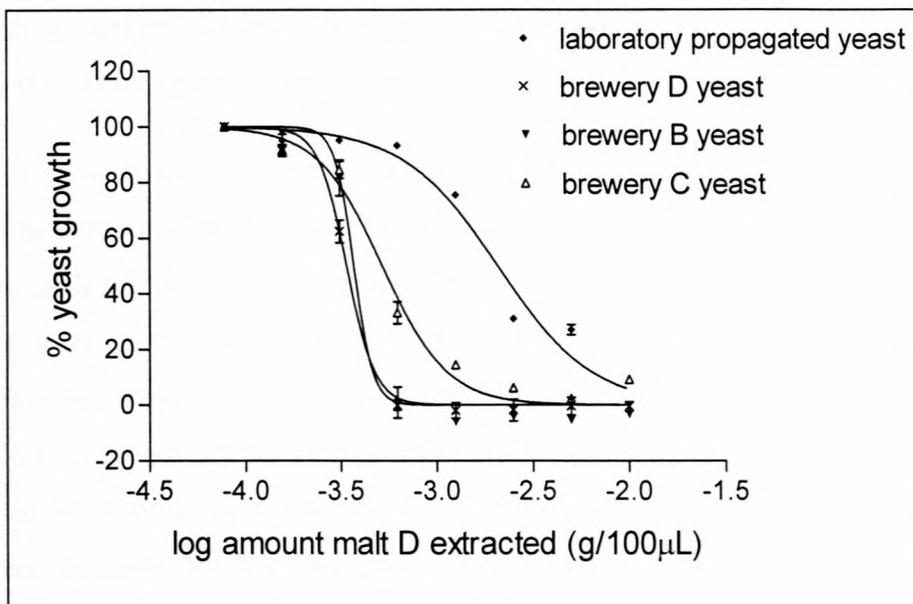


Figure 1 Antiyeast assay results expressed as dose response curves comparing laboratory propagated and brewery yeast samples (from breweries C, D and B). All data for yeast strain S. The error bars represent SEM for duplicate analyses of duplicate experiments (n = 4).

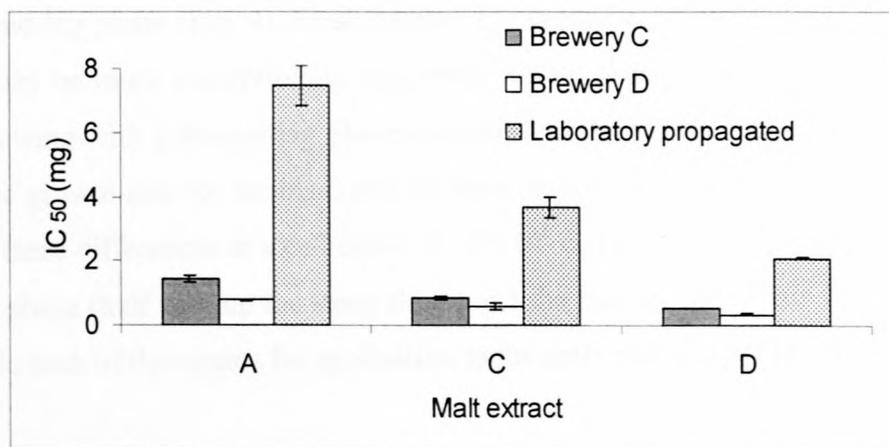
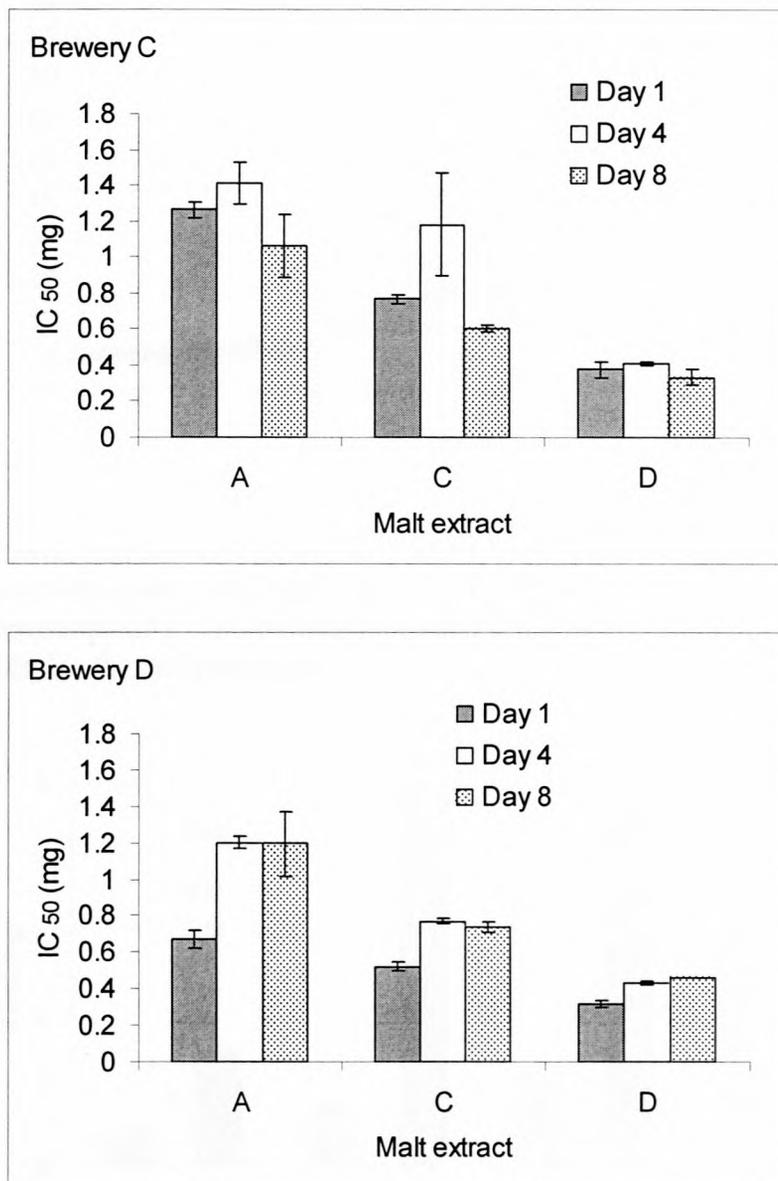


Figure 2 Antiyeast assay results showing relative susceptibilities of yeast from brewery C and D and laboratory propagated yeast to three different malt extracts (A, C and D). All data for yeast strain S. IC<sub>50</sub> expressed in mg malt extracted. Error bars represent minimum and maximum of duplicate analyses (n = 2).

The brewery yeast samples tested were in stationary phase, having been collected from the bottom of fermentation vessels at the end of fermentation after they had flocculated. To determine what impact growth phase had on the susceptibility of yeast to malt extracts, brewery yeast samples were re-propagated in MYGP medium as per the method use to propagate yeast for the antiyeast assay. In all cases the susceptibility decreased upon propagation in ideal conditions with no fermentation related stress, but the sensitivity was still far greater than assay yeast (results not shown).

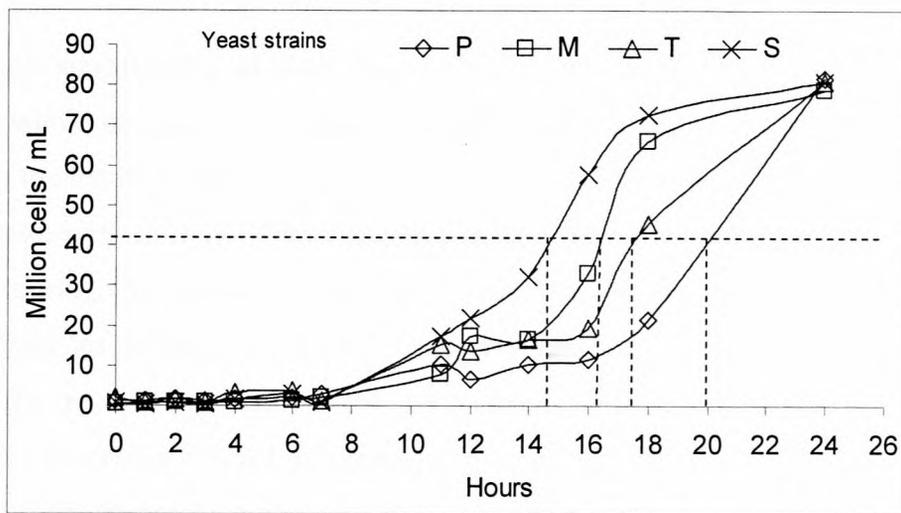
Changes in yeast susceptibility were examined further by comparing yeast from different stages of the fermentation process. Yeast was sampled on days 1, 4 and 8 from 2L EBC (laboratory scale) fermentations inoculated with brewery yeast (Fig. 3). All the yeasts at all the stages were more susceptible to inhibition by all three malt extracts than the log propagated yeast (results not shown) supporting the finding that brewery yeast was more susceptible than laboratory propagated yeast (refer to Figs. 1 and 2). Susceptibility of these yeasts to all the antimicrobial malt extracts changed during the fermentations. However, the trends were different for the two yeasts as brewery C yeast appeared to become more susceptible by the end of fermentation, while brewery D yeast improved (Fig. 3).

In order to test the influence of strain variability, four of the major strains of *Saccharomyces cerevisiae* used within SABMiller, including strain S, were compared for malt antimicrobial susceptibility. For a true comparison and reproducibility, each strain was propagated as described for the antiyeast assay and growth curves were used to establish when each strain was in mid-log phase (Fig. 4). Fresh batches of cryogenically stored yeast were used as some strains may be more susceptible to long term storage than others. Strain S, used so far, was rapid growing with a shorter lag phase compared to the slower growing strain P. During log phase the growth rate for strains S and M were most rapid as seen by the slope of the curve. Despite these differences at a cell count of  $40 \times 10^6$  cells/mL each strain was at approximately mid-log phase (half way up the steep slope) and the time at which this was reached was used to sample each of the strains for application to the antiyeast assay (Fig. 4).

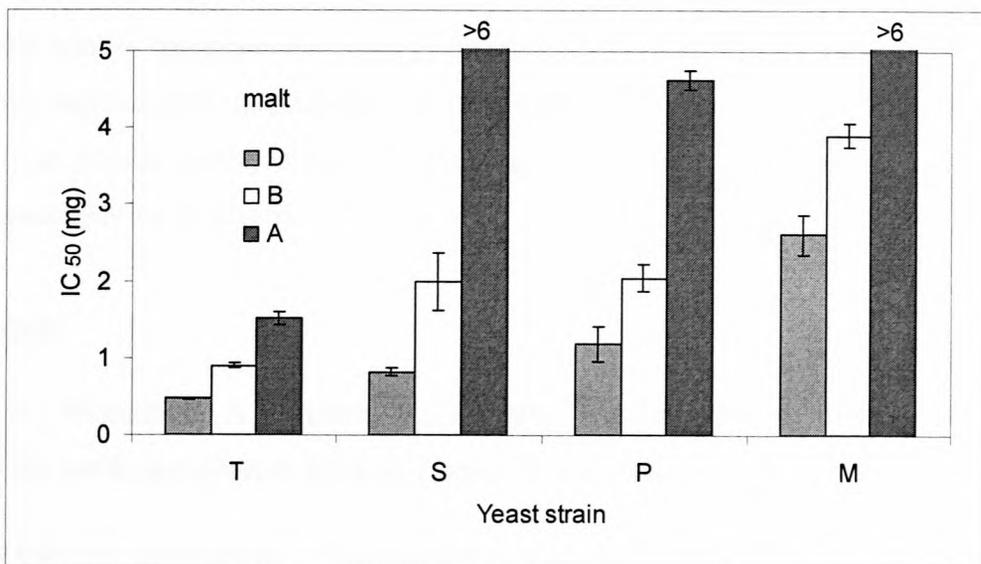


*Figure 3* Antiyeast assay results comparing yeast taken at different times from 2L EBC fermentations of brewery wort inoculated with brewery yeast (ex-brewery C and D, strain S yeast). Yeast was assayed against three different malt extracts (A, C and D). Error bars represent minimum and maximum of duplicate analyses from duplicate fermentations ( $n = 2$ ).

Three different malt extracts were used to challenge each yeast strain in the antiyeast assay. Differences between yeast strains were detected; yeast strain T for instance was found to be far more susceptible to the malt antimicrobial factors than yeast strain M (Fig. 5). Yeast strains S and P display similar sensitivity to malt extracts although their growth curves were most different. Malt extract A as observed previously (Chapter 7), was the least antimicrobial malt and results vary as the data fit to the sigmoidal curve used to get an  $IC_{50}$  value was very poor, in spite of this strain T was sensitive to this extract and clear inhibition of yeast growth was noted.



*Figure 4* Growth curves of different SABMiller yeast strains from cryogenically preserved yeast, propagated in MYGP medium. Cell counts were determined microscopically. The dotted lines represents the times at which cells would be taken for the antiyeast assay.



*Figure 5* Antiyeast assay results comparing different yeast strains and the impact of different malt extracts. Error bars reflect SEM of duplicate analyses of duplicate experiments (n = 4). A repeat experiment with malt D gave similar results.

## Conclusions

The antiyeast assay previously used to screen malts for antimicrobial activity (Chapter 7) was successfully used to differentiate yeasts using known antimicrobial malt extracts. Brewery yeast was found to be more sensitive to the inhibitory impact of malt derived antimicrobial factors than laboratory propagated log phase yeast as standardised for the antiyeast assay. The assay yeast, however, was more discriminating between malt extracts, supporting its selection for the antiyeast assay. Different brewery yeast samples (same strain) directly from the

brewery varied significantly in their susceptibility to malt antimicrobial factors. Further investigation would be required to determine if these differences reflect yeast history in terms of how it was treated in the plant, how many fermentations it has been through and the malt it may have been exposed to via wort. Susceptibility of yeast to antimicrobial factors changed considerably during the course of laboratory scale fermentations inoculated with brewery yeast. These changes differed with different brewery yeasts and it may be possible to use this to examine the process as well as the yeast itself. Different brewing yeast strains of *Saccharomyces cerevisiae* reacted differently to malt antimicrobial extracts, clearly indicating that one strain can be much more susceptible to antimicrobial peptide induced membrane damage which may be useful in strain selection for different brewing purposes.

The significance of these results in terms of yeast performance and relevance as a measure of yeast quality has yet to be determined. Wider range yeast screening and fermentation monitoring would be required as well as anchoring this measurement to some established existing yeast quality measurements as a point of reference. The advantage of this type of method above sophisticated methods such as flow-cytometry is the cost and simplicity and the advantage over simple methods such as staining is that little experience is required and improved quantification is gained.

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## Chapter 9

### *Partial characterisation of antimicrobial factors in barley malt*

#### **Abstract**

The antiyeast factors in barley malt (Chapter 7) were partially characterised and evaluated. The antimicrobial activity was found to originate from the plant tissue and displayed a broad range of activity although the activity was most potent against yeast for the malt extracts tested. Antimicrobial activity was associated with the presence of a 5-14 kDa fraction containing a cationic heat resistant peptide(s) that causes cell lysis and has a pH stability optimum of  $\pm 5$ . Liquid chromatography-electrospray mass spectrometry (LC-ESMS) revealed that the cationic fraction of one malt extract with antimicrobial activity contained LTP1 (equivalent to LTP1a reported in Chapter 3), some proteins with a similar molecular weight to LTP1 (9-10 kDa) and other peptides (and other compounds) with molecular weights ranging from 0.4-4.5 kDa. Exposure to extreme pHs and certain reagents caused irreversible damage and this had to be taken into consideration for the selected purification procedures. The heat stability of malt extracts varied. Those with the strongest antimicrobial activity were the most stable, but stability was also inversely related to amount of LTP1 in extract. The active fraction was more heat stable in wort than in water, probably due to protection by association to residual carbohydrates.

#### **Introduction**

Poor malt quality associated with microbial contamination of barley/malt has been related to process and beer quality issues such as inconsistent brewhouse (wort preparation) performance (Stars *et al.*, 1993), atypical fermentations including premature yeast flocculation (Fujii and Horie, 1975, Morimoto *et al.*, 1975, Herrera and Axcell, 1991), beer flavour instability, presence of off flavours and haze in beer (Etchevers *et al.*, 1977), decreased beer foam quality (Evans *et al.*, 1999) and gushing; the uncontrolled release of carbon dioxide from beer when it is opened (Casey, 1996 and Gardner, 1973). Although coupled to atypical microflora or comparatively high microbial load on the grain, most of these quality issues

were not predicted by the conventional barley/malt analyses (European Brewing Convention. *Analytica* 1998). Microbial contamination of plant tissue could elicit plant defence responses resulting in the generation of antimicrobial factors. In addition, the microflora itself could generate antimicrobial factors. Standard malt evaluation does not assess the presence of barley or malt antimicrobial factors, which may or may not play a role in terms malt quality, be it process or aspects of the final beer product.

Most if not all bacteria are able to produce a heterogeneous array of inhibitory molecules including toxins, bacteriolytic enzymes, by-products of the primary metabolic pathways (organic acids, ammonia, hydrogen peroxide), secondary metabolites or idiolytes, antibiotics, peptides or polypeptides (Sahl and Bierbaum, 1998). Although these compounds were mostly active against other related bacteria, some were active against filamentous fungi and a few against yeast including *Saccharomyces cerevisiae* (Atanassova *et al.*, 2003, Besson *et al.*, 1984, Latoud *et al.*, 1987 and Magnusson *et al.*, 2003). Singh *et al.* (1971) reported the incidence of antibacterial compounds in fungi such as *Candida albicans*, *Rhizopus nigricans*, *Aspergillus leukensis*, *Penicillium funiculosum* and *Trichoderma viride*, most of these compounds were found to be sterols, phenolic compounds and tannins. Several *Aspergillus* species produce antifungal peptides although these have not been shown to be active against yeast (Ng, 2004). Microorganisms found naturally on barley and malt (Vaughan *et al.*, 2001) such as *Lactobaccillus lactis*, *Enterococcus* (Hartnett *et al.*, 2002) and *Aspergillus* (Singh *et al.*, 1971) are known to produce antimicrobial compounds. Many bacterial antimicrobial peptides were membrane active causing cell lysis (Sahl and Bierbaum, 1998 and Shai, 1995) or interfered with cell wall synthesis (Debono and Gordee, 1994 and Hsu *et al.*, 2003). Unlike plant antimicrobial factors, which were generally active against a broad range of organisms, antimicrobial factors of microbial origin mostly acted on a relatively narrow range of related organisms (Sahl and Bierbaum, 1998).

Plants contain antimicrobial factors as part of their defence system (Broekaert *et al.*, 1997), which was either constitutive or induced to cope with sufficiently pathogenic microbial infestation (Carr and Klessig, 1989) or other forms of stress such as drought, cold or chemical exposure (Torres-Schumann *et al.*, 1992). The constitutive type defence responses were in place before the plant tissue was challenged, while the induced plant defence responses were activated when plant tissue was challenged by pathogens (Carr and Klessig, 1989).

The most common factors associated with plant defence were proteins (Batalia *et al.*, 1996, Boller, 1993 and Van Loon and Van Strien, 1999) and peptides (Broekaert *et al.*, 1997 and Florack and Stiekema, 1994). Other antimicrobial factors included chemicals such as alkaloids, unsaturated lactones, sulphur compounds, saponins, phenolic compounds (Carr and Klessig, 1989), active oxygen and nitrogen species such as hydrogen peroxide and peroxy nitrite also occurred (Broekaert *et al.*, 1997 and García-Olmedo *et al.*, 2001). Typical antimicrobial peptides such as thionins, defensins and nonspecific lipid transfer proteins (ns-LTPs) have been studied in barley seeds. These peptides were generally between 5 and 10 kDa, consisted of 45 to 95 amino acid residues, contained 3 or 4 disulphide bonds, they were generally basic and amphipathic (Ponz *et al.*, 1983 and 1986, Molina *et al.*, 1993a and b, Broekaert *et al.*, 1995 and Heinemann *et al.*, 1996). The wide range of antimicrobial factors that occurred in the various plants inhibited the growth of bacteria (gram positive and/or gram negative) and/or fungi. Some were more potent than others and some inhibited a broader range of organisms than others (Cammue *et al.*, 1995, Osborn *et al.*, 1995 and Terras *et al.*, 1992).

The mode of action of these antimicrobial factors was mostly by permeabilising the microbial cell membranes causing leakage of ions, in some cases even proteins and damaging the transport systems embedded in the membranes (Guihard *et al.*, 1993). Antimicrobial factors could be microstatic, inhibiting growth and/or microcidal, killing microorganisms. Some of the antimicrobial factors may not act individually, but synergistically with other factors. A factor's antimicrobial activity may otherwise be increased by the synergistic effect with other factors (Blocket *et al.*, 1993, Dubreil *et al.*, 1998, Molina *et al.*, 1993b and Terras *et al.*, 1993). Examples of synergism between two components include iturin A (antibiotic and antifungal) with surfactin (strong surfactant), both lipopeptides extracted from *Bacillus subtilis* (Maget-Dana *et al.*, 1992), as well as magainin 2 with PGLa, both antimicrobial peptides isolated from *Xenopus laevis* (Matsuzaki *et al.*, 1998).

Characterising the antimicrobial factors from malt, be they of microbial or plant origin, was warranted in view of evidence that such factors may be present and play a role in yeast fermentation in the brewing process (Chapter 7). It was established in Chapter 7 that malts differed in the level of antiyeast activity. Okada and Yoshizumi (1970) previously identified a 9.8 kDa barley endosperm peptide (with similarities to non-specific lipid transfer protein or ns-LTP) that caused sugar uptake inhibition at low concentrations and cell death at higher concentrations. Apart from these facts, limited information was available on antiyeast factors

in malt and brewing. The aim of this paper was to partially characterise the antimicrobial factors extracted from malt, examining their origin, specificity, nature and stability.

## **Materials and methods**

### ***Samples for antiyeast analysis***

#### **Whole malt extraction**

Unmilled malt was extracted as per an adaptation of the malt extract method (Chapter 7), 10 g whole malt was used instead of 5 g milled malt.

#### **Washed malt extraction**

Whole malt was washed and dried as described by Van Nierop *et al.* (2004a) (also see Chapter 4), and then extracted as per malt extraction method (Chapter 7). The wash was retained and extracted as per wort extraction method (Chapter 7).

#### **Biomass extraction**

Microflora from barley was propagated as for the antimicrobial assay described below and grown for five days before removing the biomass by centrifugation (10000 g, 15 minutes) and extracting the supernatant as per wort extraction method (Chapter 7).

#### **Boiled malt extracts**

Aliquots of various malt extracts in deionised water were boiling suspended in a beaker of water for up to 60 minutes. Acetonitrile (HiPerSolv™, BDH, Poole, England) was added post-boil to obtain 25% as used in the antiyeast assay for antiyeast analysis. Alternatively the malt extracts were added to wort that was boiled as before and then extracted as per wort extraction method (Chapter 7).

### ***Characterisation***

#### **Protein degradation**

Various malt extracts were treated with Pronase (Roche Diagnostics GmbH, Mannheim, Germany) protease cocktail, 0.014 g Pronase was added per malt extract aliquot re-suspended in 750 µL deionised water where one aliquot represented extract from 0.5 g malt. After

incubation at 40°C for one and a half hours, 250 µL acetonitrile was added to attain the 25% used in the antiyeast assay. Pronase in deionised water was treated the same way and tested on the assay as a control.

### **Arabinoxylan degradation**

Xylanase M3 from *Trichoderma longibrachiatum* (Megazyme, Bray, Ireland), at 10 U per aliquot dissolved in 750 µL distilled water, was used to treat malt extracts at 50°C for one hour. Enzyme activity was destroyed by boiling reaction mixture for 10 minutes. Acetonitrile (250 µL) was added after cooling the aliquot to attain the 25% for the antiyeast assay. Prior to analysis the aliquot was centrifuged at 6500 g for 15 minutes to remove any precipitate formed. Xylanase without malt extract was treated the same way as a control.

### **pH stability**

Malt extracts were dissolved in buffers ranging in pH from 4 to 9 (prepared by mixing 1% acetic acid with 1% ammonia (Sigma, St.Louis, USA) in varying ratios). After one hour the samples were frozen and repeatedly freeze dried (Labconco, Kansa City, Montana, U.S.A) to remove all traces of the buffer components prior to antiyeast analysis. Buffers without malt extracts were freeze dried and tested as controls.

### **Reagent sensitivity**

Any volatile reagents used for the purification procedures (see later) were tested by dissolving malt extracts in the reagent being tested and after one hour the extract was frozen and repeatedly freeze dried to remove all volatile components of the reagent prior to antiyeast analysis. Reagents without malt extracts were freeze dried and tested as controls.

### **Size partitioning**

Malt extracts, in deionised water, were dialysed in different cut-off dialysis tubing (Spectro/Por® tubing (Rancho, Dominguez, California, U.S.A)); 5 and 12-14 kDa. The dialysates were then freeze dried and analysed for antiyeast activity. Otherwise, malt extracts in deionised water, were filtered through ultrafiltration membranes (Amicon Inc., Beverly, MA, U.S.A.) with 3 and 10 kDa cut-off limits and fractions above and below the membranes were freeze dried and used for antiyeast analysis.

## **Lipid transfer protein 1 analysis**

LTP1 concentration was determined in malt extracts by quantitative double sandwich ELISA using polyclonal LTP1 antibodies and purified barley LTP1 as described previously (Evans and Hejgaard, 1999 and Van Nierop *et al.*, 2004b (Chapter 3)) Antibodies and LTP1 was donated by Dr. E. Evans (University of Tasmania, Sandy Bay, Australia).

## ***Antimicrobial analysis***

### **Microflora from barley**

Microflora from barley was propagated by adding 10 barley grains to 200 mL MRS broth, plate count broth and potato dextrose broth prepared as per product instructions (Biolab Diagnostics (PYT) Ltd., Halfwayhouse, South Africa) in 1 L Erlenmeyer flasks, incubated for 48 hours at 37°C and shaking at 150 rpm. The various broths with propagated microbes were diluted with the same broth to achieve an optical density (OD) of 0.3 at 600 nm (measured with a dual beam UV/visible spectrophotometer, Shimadzu, UV-1600, Tokyo, Japan). The diluted solutions (100 µL) were then added to 30 mL of the same broth and used in the antiyeast assay format with various malt extracts.

### **Antibacterial assay**

*Micrococcus luteus* was propagated in Luria broth (Sigma, St.Louis, USA, made up as per manufacturers instructions) as described by Du Toit and Rautenbach (2000), cells were diluted in potato dextrose broth (Biolab Diagnostics, Midrand, South Africa, made up as per manufactures instructions) until the optical density was between 0.2 and 0.3 at 600nm. The cells were used in the antiyeast assay format instead of yeast cells in MYGP. Everything else in the assay was the same as for the antiyeast assay.

### **Antiyeast assay**

Malt extract preparation and the antiyeast assay were performed as previously described (Chapter 6 and 7). Yeast was propagated in MYGP broth; 3 g each of malt and yeast extract, 5 g peptone and 10 g glucose (Biolab Diagnostics, Midrand, South Africa) dissolved in 1 L water and autoclaved 15 minutes pressurised to attain 121°C.

## **Yeast lysis**

Yeast cells in MYGP (6 mL), as used for the antiyeast assay, were added to an aliquot of malt extract. This was transferred to a 25 mL Erlenmeyer flask, shaken at 25°C for two hours and filtered through 0.45 µm acetate syringe filters (Osmonics, Warren, Indiana, U.S.A.). The adenylate kinase leakage from yeast cells was measured as described by Cameron–Clarke *et al.* (2003).

## ***Purification procedures***

### **Gel filtration**

Sephadex G10 (Pharmacia LKB Biotechnology, Uppsala, Sweden) was swollen in deionised water and packed into a glass Pasteur pipette. Malt extract (1 aliquot), dissolved in 200 µL deionised water, was loaded onto the column and eluted with deionised water. The first 550 µL was discarded. The next 500 µL was collected and analysed for antiyeast activity.

### **Thin layer chromatography**

Thin layer chromatography (TLC) was performed on malt extracts according to Atherton *et al.* (1989) using Kieselguhr 60-F254 TLC plates (Merck, Darmstadt, Germany) developed in butan-1-ol: pyridine: acetic acid: water (90:80:60:72). The impact of the reagent on the antimicrobial activity was tested by adding the developing solution directly to the malt extract. The solution was removed by repeated freeze drying after which the extract was re-analysed for antiyeast activity.

### **Cation exchange**

CM52 pre-swollen carboxymethyl cellulose (Whatman International, Ltd., Maidstone, England) was equilibrated in deionised water or buffers ranging in pH from 4 to 9 (prepared by mixing 1% acetic acid with 1% ammonia in varying ratios). Malt extract dissolved in corresponding buffer was added to pre-equilibrated resin in test tubes and gently mixed. Centrifugation (five minutes, 3000 g) was used to separate the resin from the liquid containing the extract components not bound to the resin. The bound components were eluted with 1.0 M ammonium acetate pH 6.0 (Sigma, St.Louis, USA) and removed by centrifugation. The unbound and bound fractions were freeze dried repeatedly to remove all traces of reagents used. The dried fractions were dissolved in 25% acetonitrile and analysed by the antiyeast assay. Malt extracts, treated as above but without the resin, were used as control. The pH of

buffers and malt extracts in buffer was measure using a pH meter (744 pH meter, Metrohm, Herisau, Switzerland) and confirmed with pH indicator paper (Macherey-Nagel, Düren, Germany).

### **Liquid chromatography electrospray mass spectrometry**

*Liquid chromatography-* The cationic fraction of malt D (3 mg) was dissolved in 2 mL 50% acetonitrile containing 0.1% formic acid (Sigma, St.Louis, USA). The sample was filtered through a 0.22  $\mu\text{m}$  nylon syringe filter (Osmonics, Warren, Indiana, U.S.A.). To achieve separation 1  $\mu\text{L}$  was injected on to a 150  $\mu\text{m}$  x 150 mm Waters Atlantis dC18 capillary column (Waters Corporation, Milford, MA, USA), packed with 3  $\mu\text{m}$  particles, using a 2% acetonitrile solution containing 0.1% formic acid. The column was connected to a Waters CapLC XE capillary liquid chromatography system. Gradient elution was employed at a ca. 300 nL/min flow rate. Mobile phase A contained 2% acetonitrile and 0.1% formic acid. Mobile phase B contained 98% acetonitrile and 0.1% formic acid. The gradient was run over 40 minutes from 20% B to 90% B.

*Mass spectrometry-* A Waters QTOF API Ultima Quadropole-Time-Of-Flight mass spectrometer with nano-electrospray ionization in the positive mode was used. A capillary voltage of 3.5 kV and a cone voltage of 35V were applied. The source temperature was set to 80 °C. The TOF analyzer was used for MS analysis. Calibration of the TOF analyzer was performed using the fragment ions of glucorino-fubrino peptide B (Human) for the 333 to 1285  $m/z$  mass range. The pusher frequency of the TOF analyzer was set for the 100 to 2000  $m/z$  mass range.

## **Results and Discussion**

It was established that the antimicrobial factors, active against brewing yeast, in the malt extracts were already present on barley (Chapter 7). The malting process appeared to contribute to the antimicrobial activity, either *via* the expression of antimicrobial plant peptides during the germination process and/or *via* microbial factors introduced by growing microflora.

## ***Origin of antimicrobial activity***

To establish if these antiyeast factors were produced by the plant tissue in response to microbial contamination and/or by the biomass on the grain itself, a number of different experiments were performed.

Whole malt (unmilled) was extracted to establish if the antiyeast factors were on the outside of the grain, but no antiyeast activity was observed (Table 1). Washed grain was dried and extracted, but no significant decrease in antiyeast activity was observed (Table 1). The retained wash was also extracted and it tested negative for antiyeast activity (Table 1).

Biomass residing within the husk layers of the grain, however, could still contribute to the antiyeast activity. Barley was used to inoculate different growth media that encourages the growth of a wide range of fungi and bacteria. The biomass was removed and the media were extracted. No detectable antiyeast activity was secreted into the media by the biomass (Table 1). These findings confirmed that the antiyeast activity found in barley and malt was of plant origin.

*Table 1* Antiyeast activity of malt F extracted in a number of different ways. Where applicable the standard error of the mean (SEM) was quoted for duplicate analyse of duplicate extracts (n = 4)

<b>Material extracted</b>	<b>Antiyeast activity</b>
Milled malt	4.03 ±0.29
Whole (un-milled) malt	No activity
Washed malt (milled)*	4.31±0.55
Retained malt wash*	No activity
Growth media (biomass ex-barley removed)	No activity

\*The water used to wash malt was retained and extracted

## Characterisation of activity

### Specificity

Plant derived antimicrobial factors have a broader range of antimicrobial activity compared to antimicrobial factors of microbial origin (Rao, 1995). Microflora from barley M and I was grown up in three different growth media and maximum growth inhibition of the microflora by malt extract D was recorded (Table 2). It was found that the antiyeast malt extracts (and gramicidin S) inhibited the growth of mixed cultures of bacteria and fungi extracted from barley. Similar activity was observed for malt extracts D and F (results not shown). Although the barley microflora was tested in the antiyeast assay format, the assay was not optimised for these different organisms, hence the results are not quantitative but they do indicate there was a degree of inhibition of both bacteria and fungi (Table 2).

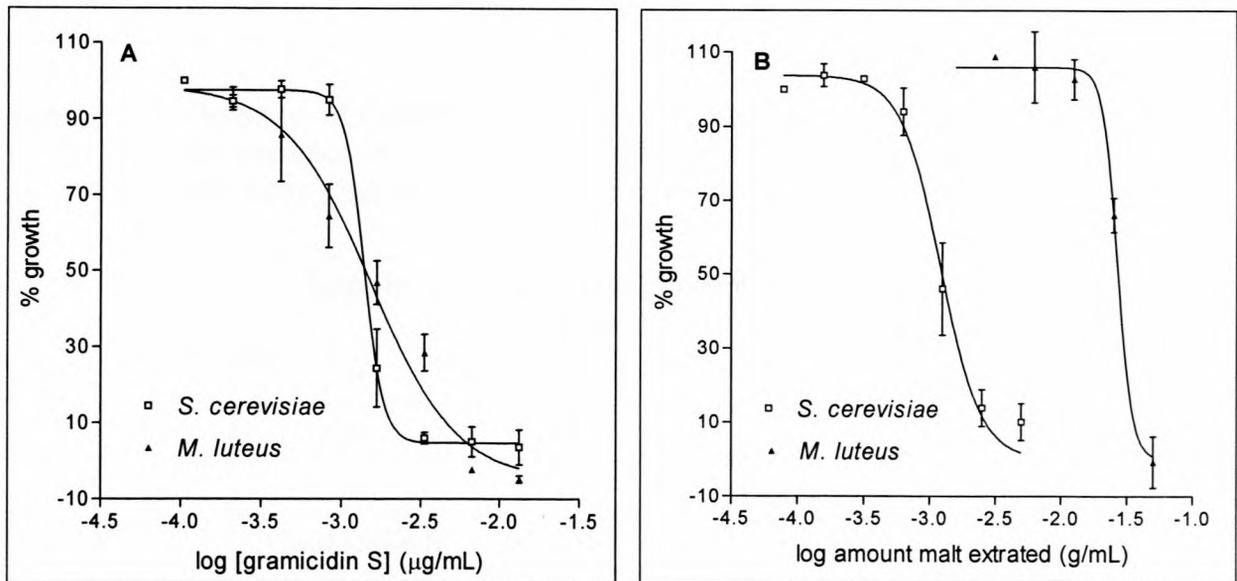
*Table 2* Relative inhibition of barley microflora grown up in different media was reported for malt extract D. All the different microflora tested, were inhibited up to 100% by gramicidin S, used as control for the assay.

<b>Growth media for barley microflora</b>	<b>Microflora from barley M (Detected inhibition)</b>	<b>Microflora from barley I (Detected inhibition)</b>
MRS broth (bacteria)	-	+/-
PCB <sup>1</sup> (bacteria)	+	++
PDB <sup>2</sup> (fungi)	+	+

<sup>1</sup>PCB- plate count broth

<sup>2</sup>PDB- potato dextrose broth

Application of *M. luteus* as test organism in the antiyeast assay format was quantitative and up to 100% inhibition was observed, as for yeast. Relative to yeast, *M. luteus* was much less sensitive to the inhibitory effect of malt antimicrobial factors, whereas gramicidin S had similar IC<sub>50</sub> (concentration required to inhibit microbial growth by 50%) values towards both organisms under these assay conditions (Fig.1). Results are shown for malt extract D in Figure 1, but malt extracts B and F were also tested yielding similar results.



**Figure 1** Comparison of *M. luteus* and *S. cerevisiae* as test organisms in the antimicrobial assay method described for yeast (Chapter 6). The inhibitory effect of (A) the antimicrobial peptide, gramicidin S, and (B) an extract from malt D was tested on both organisms. Error bars represent SEM of triplicate analysis from one assay using one batch of bacteria/yeast ( $n = 3$ ).

### Type of activity

Antimicrobial peptides usually act on the cell membrane causing inhibition of growth by damaging the membrane leading to cell death by lysis (Shai, 1999 and Epanand and Vogel, 1999). To test the impact of the malt extract on yeast membranes, yeast was exposed to the extract under the same conditions as used for the antiyeast assay. After removing the yeast, the activity of adenylate kinase, an intra-cellular yeast enzyme, was measured as an indicator of cell lysis (Cameron-Clarke *et al.*, 2003). The antimicrobial extracts were all added at the same concentration based on 50% growth inhibition of the most potent extract in the antiyeast assay. Exposure to malt extracts caused a significant increase (2 to 4 times) in adenylate kinase activity relative to the negative control, indicating lysis (Table 3). Gramicidin S, a highly lytic peptide (Staudegger *et al.*, 2000 and Prenner *et al.*, 1999), caused a 13-fold increase in adenylate kinase activity when added at a 50% inhibitory concentration. Other malt extracts were also tested and gave similar results, even the extract from malt A, known to have the lowest level of antiyeast activity of all the malts screened to date (Chapter 7).

**Table 3** Detection of extracellular adenylate kinase activity, indicative of yeast lysis, in the presence of various malt extracts, positive control for lysis (gramicidin S) and assay reagents or negative controls (deionised water and 25% acetonitrile).

<b>Sample</b>	<b>Adenylate kinase activity</b>	<b>Comment</b>
B	+	Lytic
D	+	Lytic
F	+	Lytic
Gramicidin S	+++	Highly lytic
Water	–	No activity
25% acetonitrile	–	No activity

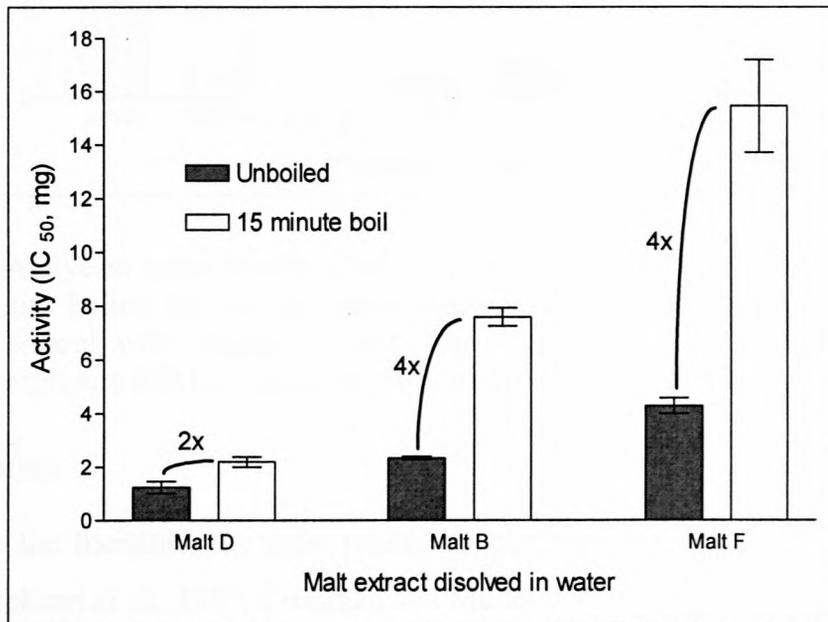
In the antiyeast assay, optical density was measured after approximately 24 hours incubation, re-reading the plates at 48 hours revealed there was still no growth at high extract concentrations (0.1 g malt extracted/mL) of the potent malt extracts B and F (results not shown). This result was indicative of a cytotoxic action by the active factor(s), which would result in total cell death.

### ***Nature of antimicrobial factor(s)***

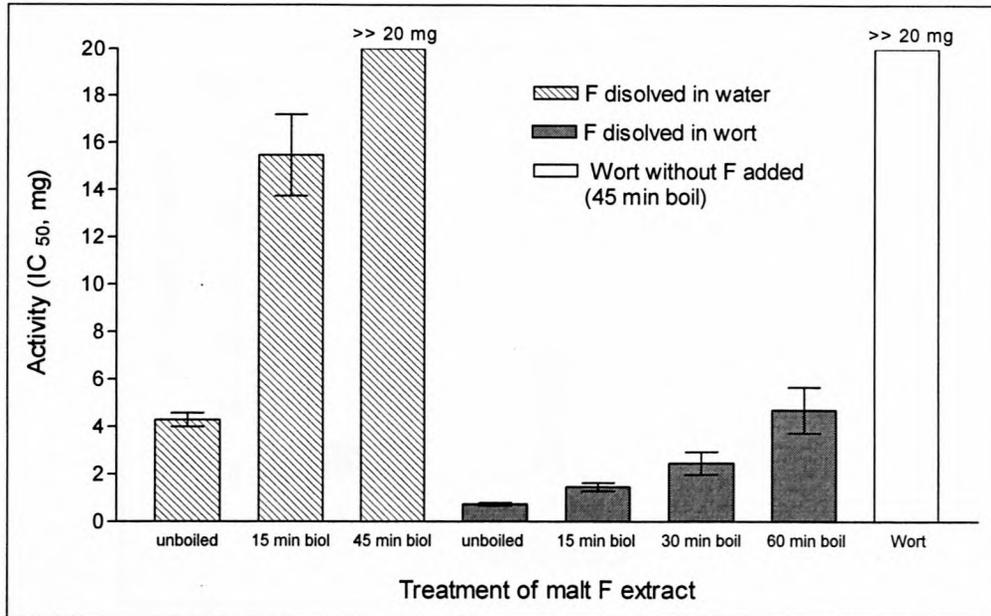
#### **Temperature stability**

Work by Okada and Yoshizumi (1970) on the antiyeast activity of barley extracts did not include any findings on the temperature stability of these factors. As they proposed that these factors impacted on yeast during fermentation, the temperature stability of the factors would be of importance since the wort preparation involves a number of temperature stands during mashing (63-76°C) and a 60 minute boil. In Chapter 7 it was shown that temperatures as low as 55°C during malt extraction negatively impacted on the antiyeast activity. It was proposed that this was due to enzymatic degradation rather than temperature. It was also shown that antimicrobial activity could be carried through to fermentation *via* the wort trub or precipitate suggesting some degree of temperature stability. To determine the sensitivity of malt extracts with antiyeast activity to temperature, extracts were dissolved in water or wort and boiled for varying period of time (Figs. 2 and 3).

Initial investigation revealed that temperature stability varies between malt extracts (Fig. 2) and the  $IC_{50}$  values increase to varying degrees on boiling. Through further examination into malt extract F, it was determined that antiyeast activity survived better in wort than in water (Fig. 3), the wort environment possibly being more conducive to stabilising the factors. Overall malt antimicrobial factors were found to be temperature resistant, but not heat stable. Therefore, if antimicrobial activity was present prior to the boil potentially some antimicrobial activity may be carried through to the wort.



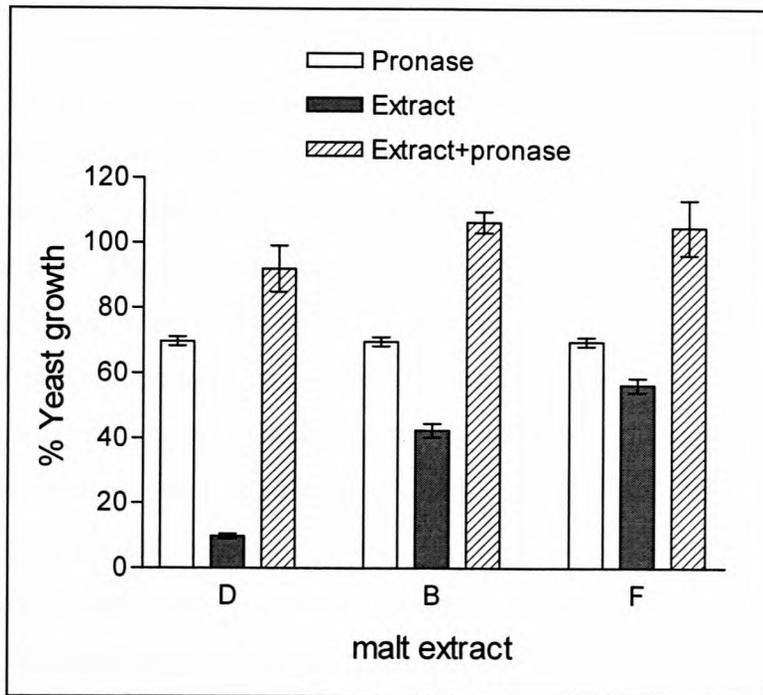
*Figure 2* Antiyeast assay results of malt extracts D, B and F dissolved in deionised water and either not boiled (unboiled) or boiled for 15 minutes. The numbers (2x, 4x) indicate the activity lost on boiling. Error bars represent SEM for duplicate analyses of duplicate experiments (n = 4).



**Figure 3** Antiyeast assay results of malt extract F dissolved in deionised water or wort and boiled for various times ranging from zero to 60 minutes. As negative control wort without extract added was boiled for 45 minutes. Error bars represent SEM of duplicate analyses for duplicate experiments (n = 4).

### Enzyme stability

According to the literature, the most prominent plant antimicrobial factors are proteins or peptides (Broekaert *et al.*, 1997, Creelman and Mullet, 1997, Lamb and Dixon, 1997 and Van Loon and Van Strien, 1999). To determine if antimicrobial activity from malt was due to protein material, a cocktail of proteases (Pronase) was used to treat a number of different malt extracts. In each case the antimicrobial activity was significantly reduced or totally lost, therefore the antiyeast activity was most probably due to peptide and/or protein factor(s) (Fig. 4). The Pronase on its own, used as a control for the assay, was also mildly antimicrobial, but that activity was no longer detected when in the presence of the malt extract. This discrepancy may be due to the inhibition of proteases by peptides, as observed for LTP1 and malt endoproteinases (Jones, 1997).



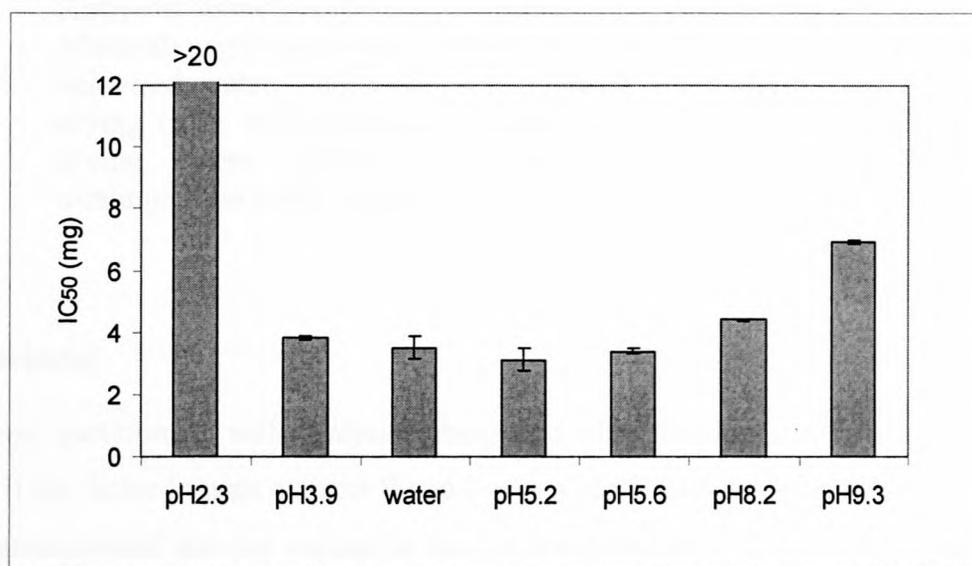
*Figure 4* Antiyeast assay results expressed as percent yeast growth in the presence of the same concentration of malt extracts treated/not treated (at 40°C) with Pronase protease cocktail and Pronase on its own. One set of extracts of each of the malts was analysed in duplicate or triplicate and SEM for each average is shown.

The malt extract F (malt F causes premature yeast flocculation or PYF) was also treated with xylanase, but this did not influence the antiyeast activity of the extract. Xylanase was selected because the phenomenon of malt associated PYF has been positively linked to arabinoxylan and was destroyed by xylanase digestion (Van Nierop *et al.*, 2004a; Chapter 5). In addition it was previously postulated that the PYF factor may be associated with an antimicrobial peptide (Axcell *et al.*, 2000 and Van Nierop *et al.*, 2004a, Chapter 5). These results may not support this postulation, but screening of PYF malts for antiyeast activity indicated a possible connection between the presence of antimicrobial factors, PYF and observed sugar uptake inhibition (Chapter 7).

In Chapter 7 it was reported that the addition of a cocktail of  $\beta$ -glucanase and  $\alpha$ -amylase during malt I and M extraction at 55°C (optimum temperature for enzyme activity) also caused reduction of antiyeast activity. This loss was attributed to the breakdown or loss of protective factors making the antimicrobial factor more temperature sensitive or susceptible to proteolysis by enzymes present in malt.

## pH stability

The pH stability of antimicrobial factors in malt extracts B and F was determined by exposing the extracts to different pH solutions. The volatile buffers were used and removed by freeze drying, thereby avoiding false antimicrobial activity in the antiyeast assay due to exposure of yeast to different pHs. The active malt extracts B and F were tested and found to be stable over the range pH 4-8, with a pH stability optimum of  $\pm 5$  (Fig. 5, results for F shown). Removing the buffer components and re-suspending in deionised water did not re-establish antimicrobial activity, suggesting the damage/denaturation was irreversible. It should be noted that acid extraction was used during malt extract preparation for the antiyeast assay, temporarily reducing the pH to below 2. However, the malt extraction was performed on ice, whilst the pH treatments were performed at room temperature, probably increasing the damaging effect. Without any extreme pH treatment it is likely that in the brewing process the antiyeast activity is in fact greater.

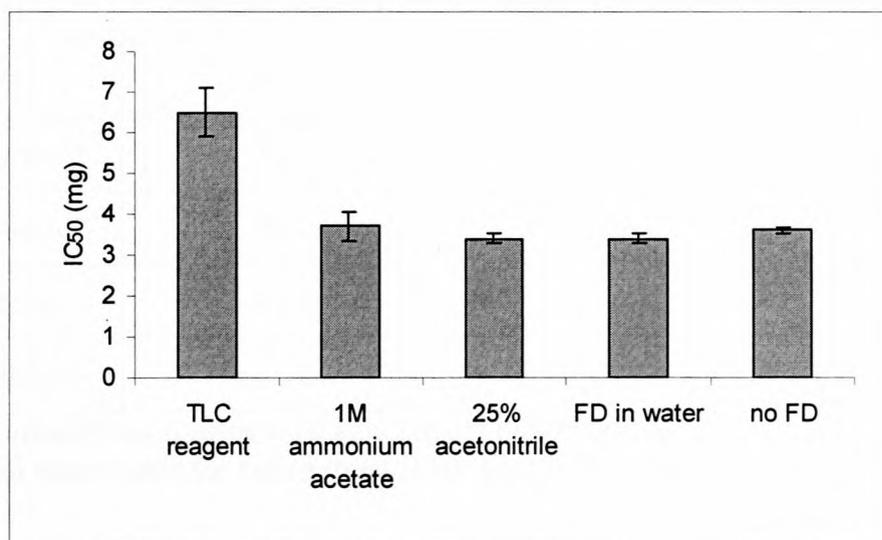


*Figure 5* Antiyeast assay results of malt extract F exposed in different pHs. The volatile components were removed by freeze drying prior to antiyeast analysis. The extract, exposed to deionised water, was used as control. Error bars represent minimum/maximum values for duplicate analyses.

## Reagent sensitivity

Since a number of different reagents are used for various purification methods the stability of antimicrobial activity in malt extracts to these volatile reagents was determined (Fig. 6). The TLC reagent was found to irreversibly damage antiyeast activity explaining the lack of success of the method (see later). As expected, 25% acetonitrile had no impact as previously

determined (Chapter 6). Ammonium acetate also had no significant impact on antiyeast activity and was a suitable reagent to use in purification procedures (see later).



*Figure 6* Antiyeast assay results of malt extract F re-suspended in TLC reagent (see Materials and methods), 1.0 M ammonium acetate, 25% acetonitrile or deionised water. The reagents and water were removed by repeated freeze drying (FD). Malt extract F, re-suspended in water with and without freeze drying, were assayed as positive controls. Error bars represent minimum/maximum of duplicate analyses.

### **Size of factor(s)**

Simple size partitioning with dialysis tubing and ultrafiltration membranes was used to establish if the factor in malt extracts B and F was a protein or a small protein/peptide (Table 4). The antimicrobial activity resided in the fraction between 5 kDa and 14 kDa due to the dialysis results but the ultrafiltration results were inconsistent, the activity being retained or filtered through the membrane. Both dialysis and ultrafiltration are not exact methods and the lack of repeatability using the 10 kDa cut-off ultrafiltration membranes may be due to the proximity of the size of the factors and the cut-off. The latter was supported by the detection of LTP 1 (9-10 kDa) in a cationic fraction of one malt extract with antimicrobial activity (see later) and the size of the barley antiyeast factor (9.8 kDa) reported by Okada and Yoshizumi (1970).

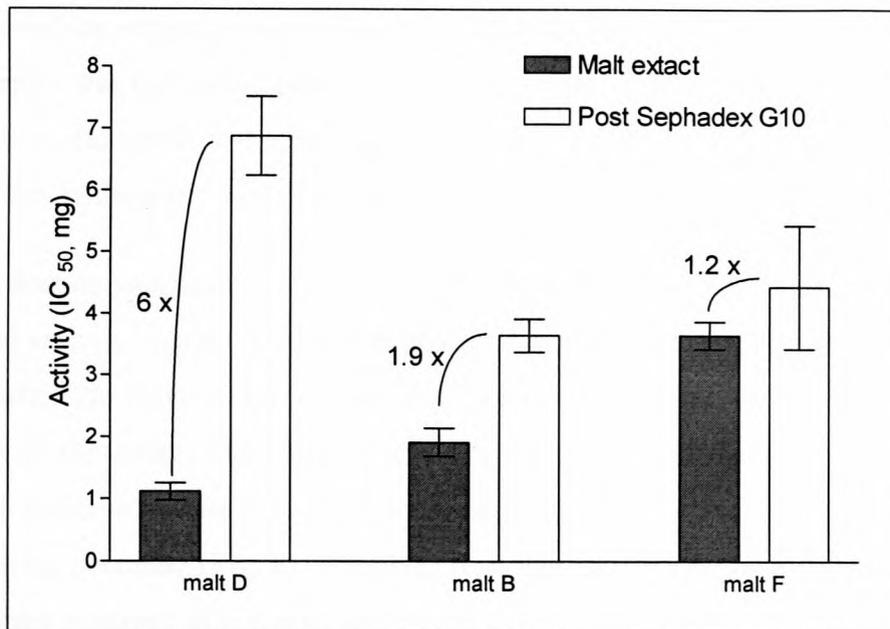
**Table 4** Antiyeast activity after malt extracts D, B and F re-suspended in deionised water were dialysed or filtered using ultrafiltration. Results indicate duplicate experiments. Antiyeast activity defined as IC<sub>50</sub> (in mg malt extracted) <4.3.

Size (kDa)	Malt extract D	Malt extract B	Malt extract F
>5 (dialysis)	+ ; +	++ ; ++	+ ; +
<10 (ultrafiltration)*	NA	+ ; -	+ ; -
>10 (ultrafiltration)*	NA	- ; +	- ; -
>12-14 (dialysis)	± ; ±	- ; -	- ; -
Pre filtration/dialysis	++ ; ++	+++ ; +++	++ ; ++

\*Results with ultrafiltration using a 10 kDa cut-off membrane gave very mixed results, it was repeated several more times for malt extract B but again results were inconsistent.

### ***Partial purification***

Gel filtration of the antimicrobial extracts in deionised water revealed differential loss of antiyeast activity (Fig. 7), the extent of which was inversely related to the activity loaded onto the gel filtration column and the LTP1 present (discussed later (Fig. 9)). Hence, malt extract D with high antimicrobial activity and low LTP1 concentration, lost the most activity on the resin. This may be due to differential binding of the antimicrobial factor to the dextran-type resin (Sephadex G10), and to the LTP1 present. It was found that cationic antimicrobial peptides, such as magainin 2 tended to be totally retained on Sephadex G10, when water was used as an eluant (personal communication, Dr. M. Rautenbach).



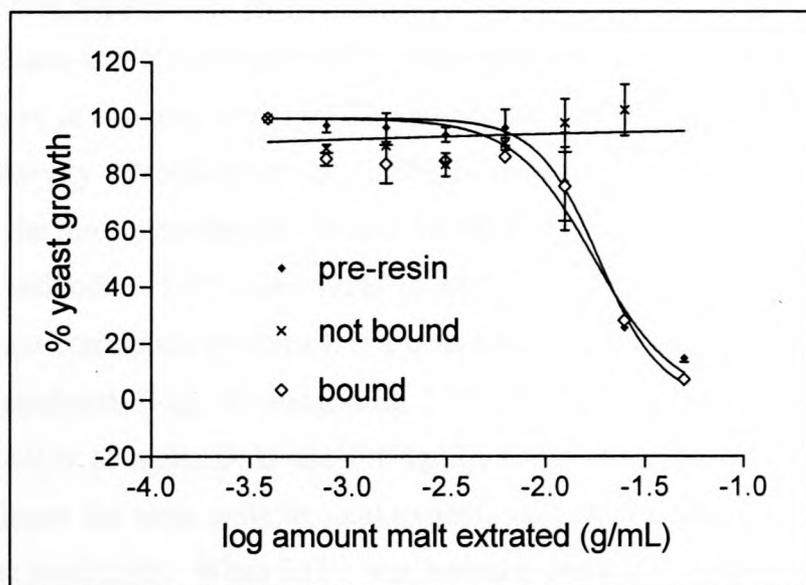
*Figure 7* Antiyeast assay results of different malt extracts before and after gel filtration (Sephadex G10) in deionised water. The numbers (6x, 1.9x, 1.2x) indicate the antiyeast activity lost on the column. Error bars represent SEM for duplicate experiments analysed in duplicate (n = 4).

Further purification of the active factors was attempted. In an initial investigation (not reported here), a peptide with  $M_r$  4030 (as determined with ESMS) was extracted with C<sub>18</sub> resin from a crude water extract of zinc chloride stressed malt (this malt led to slow fermentations and was prepared by laboratory micro-malting in the presence of zinc). Chemical stress had been used to try and induce antimicrobial peptides since chemicals, like fungal contamination, have been associated with the activation of plant defence responses including the production of antimicrobial peptides (Carr and Klessig, 1986). However, this stress procedure was not reproducible and not enough material could be purified for antiyeast assay analysis. In addition, because the zinc chloride stress was artificial and not brewery or field related, this line of enquiry was not pursued further.

Malt extracts were separated by TLC. The dried TLC plates were overlaid with nutrient agar containing the target organism (yeast or *M. luteus*), but no clear zones with antimicrobial activity could be identified, apart from that of positive control, gramicidin S. It was subsequently found that exposure of the malt extract to the TLC solvent mixture was enough to destroy the antimicrobial activity (described earlier). This indicated either dissociation of an active complex or denaturation of the active factor or one of the active factors.

Because most of the antimicrobial peptides are cationic or positively charged, cation exchange chromatography was utilised to purify the factor(s) from malt extract D. The active antiyeast factor bound to the resin in the pH range of 4 to 7, but the most effective binding was in deionised water without pH adjustment (resulting in a pH of 4-5 according to pH paper).

Ammonium acetate was used to elute the bound fraction, since it was already established that antimicrobial activity was retained in the presence of this reagent. The antiyeast activity was determined after the reagent components were removed by freeze drying, thus not impacting on the yeast in the assay. The active fraction, be it composed of one or more components, bound to the resin indicating it to be cationic and little or no dissociation or denaturation of the active factor occurred (Fig. 8). The dose response curves and hence antiyeast activity of the malt extract exposed to reagents and freeze dried, but without the cation exchange resin step was also tested. The antiyeast activity of this treated malt D extract and the bound fraction, released from the resin were found to be similar (Fig. 8). The fraction that did not bind to the anionic resin had no antiyeast activity. The optimised cation exchange procedure was repeated on malt extracts B and F and yielded similar results.



*Figure 8* Antiyeast assay results for cation ion exchange fractions of malt extract D. Fractions represent malt extract in water not exposed to the resin (pre-resin), not bound to the resin and bound to the resin (released form the resin with ammonium acetate). Error bars represent SEM for the overall curve of duplicate analyses

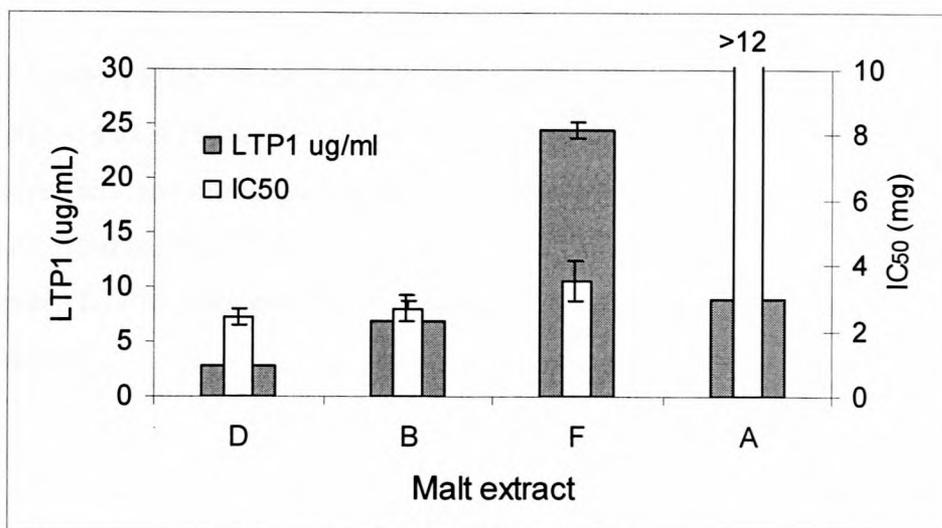
## ***Identification***

### **ELISA identification of LTP in extracts**

HPLC purification of one of the malt extracts with antiyeast activity only yielded LTP1 (equivalent to LTP1a reported in Chapter 3), as confirmed by ESMS (results not shown). The purified barley LTP1, used as a standard for the ELISA, did not have any antiyeast activity over the concentration range of 1-155 µg/mL. It may, however, work synergistically with other components as observed for wheat ns-LTP1e, which was very similar to the barley seed LTP1 and required the presence of another peptide for antimicrobial activity (Dubriel *et al.*, 1998). In addition, Lindorff-Larsen *et al.* (2001) reported that LTP1 containing a lipid-like post-translational modification (LTP1b detected and reported in Chapter 3, with a covalent linkage between Asp7 and C17 putative fatty acid) demonstrated putative antimicrobial activity.

The identification of LTP1 in HPLC fractions of malt extract and the size limit of between 5 and 14 kDa, prompted us to investigate the role and abundance of LTP1 in the malt extracts. In addition, the size suggests the factor may be the same as the 9.8 kDa barley antiyeast factor identified by Okada and Yoshizumi (1973). Although not named as a ns-LTP at the time, it had many features in common with ns-LTP and several ns-LTPs have been shown to possess antimicrobial activity (Broekaert *et al.*, 1995, Heinemann *et al.*, 1996 and Molina *et al.*, 1993b). LTP1, the most abundant of the ns-LTPs in barley seeds was measured using ELISA with available antibodies. LTP1 was found in all the extracts in varying amounts, but there was no significant correlation between LTP1 concentration and antiyeast activity based on the malt extracts analysed (Fig. 9) suggesting LTP1 is not the antimicrobial factor. The temperature stability of malts D, B and F (Fig. 2), however, seem to be inversely related to LTP1 levels. Hence the most antimicrobial extract, malt D, was the most temperature stable and contains the least LTP1. When LTP1 was boiled it denatured (Chapter 3) and if LTP was associated with the active factor(s), this may have resulted in the formation of inactive aggregates.

Ns-LTPs with no antimicrobial activity have been shown to enhance the activity of the antimicrobial peptides such as thionins (Molina *et al.*, 1993b, Gautier *et al.*, 1994 and Terras *et al.*, 1993). The antimicrobial activity in malt extracts may therefore not be due to LTP1 or ns-LTP, but enhanced by LTP1 or ns-LTP.



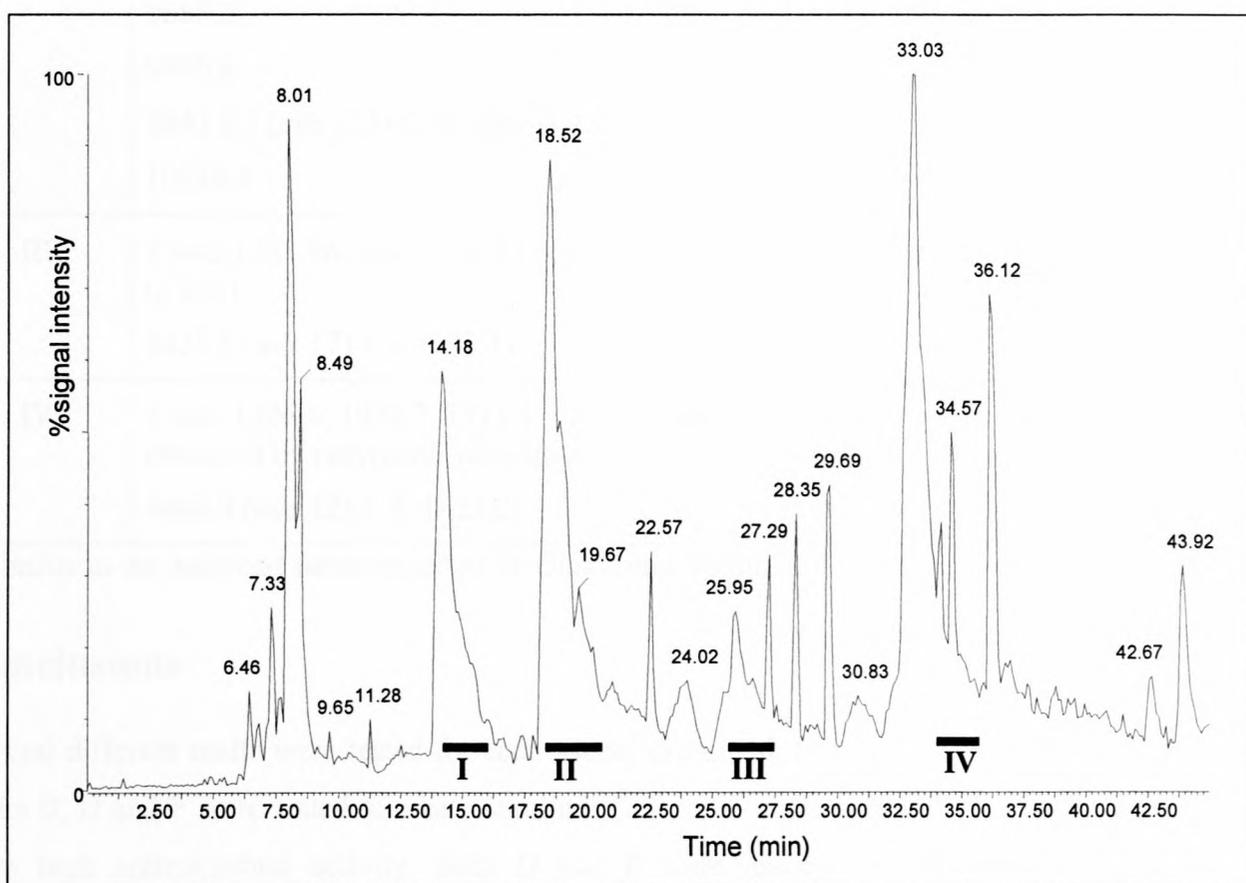
**Figure 9** LTP1 and antiyeast assay results of different malt extracts. Error bars for LTP1 values represent standard deviation (SD) of duplicate malt extracts analysed in duplicate ( $n = 4$ ) and error bars for antiyeast  $IC_{50}$  values represent SEM for triplicate malt extracts analysed in duplicate ( $n = 6$ )

### Preliminary identification of factors with LC-ESMS

The bound cation exchange fraction from malt D was subjected to LC-ESMS. The reverse phase chromatography revealed a large number of compounds in the extract (Fig. 10), some of which had typical peptide and protein mass spectra and others with spectra more indicative of polymers, possibly polysaccharides (results not shown). Preliminary mass analysis of four of the peaks (Appendix A, Figs. I-IV) showed that LTP1a (reported in Chapter 3) and/or peptides in the molecular weight range of LTP ( $M_r$  9000-10000 or 9-10 kDa) co-eluted with smaller peptides/compounds in some peak fractions (Table 5, Appendix A)

Only the peaks occurring in the retention time range of 12.5 to 37.5 minutes (corresponding to elution with 28-84% acetonitrile) on the base-peak chromatogram (Fig. 10) were examined as this was where amphipathic peptides were expected to elute (personal communication, Dr. M. Rautenbach). Under the chromatographic conditions used, LTP1a co-eluted with other smaller components in at least three of the fractions examined (Table 5, Appendix A, (I, II and III)). LTP1a co-eluted in fractions II and III with a putative peptide ( $M_r$  3426.5) suggesting that LTP1a formed different complexes which eluted at different retention times. It was therefore likely that the protein fraction of the malt antimicrobial factor was a ns-LTP, probably LTP1a, and it was strongly associated with a peptide or peptides. The association with a peptide(s) could be required for inhibitory activity as supported by the size exclusion results (Table 4), LTP1 analysis of malt extracts and antiyeast analysis of pure LTP1. In addition, a complex of LTP1a with the major peptides detected (Table 5, Appendix A (II and III)) would

give a  $M_r$  of 12400-13100, which would correspond to the predicted size of the factor, based on dialysis, of 5-14 kDa (Table 4). One of the compounds found in peak II ( $M_r$  9843, Table 5) appears to have the same molecular weight as the antiyeast factor found in barley and reported by Okada and Yoshizumi (1970). These results are preliminary, based on one run and one malt. However, LTP1a was previously detected in some of the earlier purification work (described above).



**Figure 10** LC-ESMS base-peak chromatogram of the cation exchange fraction of malt extract D. The mass spectra of the peaks designated I to IV were included in the appendix.

*Table 5* Summary of the preliminary mass analysis of some of the peptide containing fractions compounds identified using LC-ESMS.

Peak	Calculated Mr	Identification and comments
I	454.3 9684.1	Small compound? LTP1a
II	3426.5 ? ( <i>m/z</i> 1714.4, other signals too low) 2713.7 ( <i>m/z</i> 679.4; 905.6) 9685.2 9600.8 9843.5 ? ( <i>m/z</i> 1231.433 other signals too low) 10038.4	Peptide ? Peptide LTP1a LTP? LTP?*
III	? ( <i>m/z</i> 1231.96; 1372.6, 1384.6, other signals to low) 3426.5 ( <i>m/z</i> 1714.4; 1143.1)	LTP1a and others? Peptide
IV	? ( <i>m/z</i> 1026.6, 1070.7, 1711.5, other signals obscured by polymeric substances) 4866.9 ( <i>m/z</i> 1217. 8, 1623.2)	Peptides? Peptide

\* Similar to the antiyeast factor reported by Okada and Yoshizumi (1970).

## Conclusions

Several different malts were tested for each aspect examined. In most cases, where possible malts B, D and F were tested to create continuity, all three malts were selected because they have high antimicrobial activity. Both D and F were associated with premature yeast flocculation during fermentation as well as sugar uptake inhibition (Chapters 4, 5 and 7). Malt D was also associated with gushing, the uncontrolled release of carbon dioxide and foam when a bottle of beer is opened which is associated with fungal infected barley. Malt B appears to perform well under laboratory fermentation conditions (Chapter 7).

These malt derived antimicrobial factors have a broad range of inhibitory activity (also see Chapter 7) Interestingly, although *M. luteus* was as sensitive to gramicidin S (known antimicrobial peptide, positive control for assay) as *S. cerevisiae*, the latter was more sensitive

to the malt extracts. This not only makes yeast the best target organism for a sensitive antimicrobial assay, but should these factors be present during fermentation as reported in Chapter 7 they potentially pose a real threat to yeast fermentation performance.

Plant-derived antimicrobial activity is often associated with cationic peptides, which are mostly quite stable. The antimicrobial activity in malt was associated with a critical protein component as it is lost by protease exposure. The active fraction was between 5 to 14 kDa, which was in agreement with a peptide component. In addition, as first suggested in Chapter 7, a protective carbohydrate component may be present as seen by the sensitivity to starch degrading enzymes. However, the presence of more than one component may account for the temperature and solvent instability of the antimicrobial fraction.

The temperature stability of the malt extracts tested varied. The malt extracts with the strongest antimicrobial activity were also the most heat stable. Stability of malt extract F was compared in water and in wort and it was found to be far more stable in wort. The active factor may be getting a protective effect from the wort environment due to increased ionic strength or stabilising effect from other proteins or carbohydrates.

The pH stability was optimum at  $\pm 5$  and exposure to extreme pH causes irreversible loss of antimicrobial activity. The pH of the assay environment was incidentally also around 5. The irreversible loss of antimicrobial activity when exposed to TLC reagent was not due to a pH change since the pH of the reagent was  $\pm 5$ , but most likely due to the denaturing effect of butanol and pyridine.

The factor(s) was found to be cationic and most effectively bound the cation exchange resin at pH 4-5, suggesting it was most positively charged at this pH and may contain basic amino acids such as histidine with a side chain that has a pKa of 6. Preliminary results indicate that in the case of malt D the active component may be LTP1a (first reported in Chapter 3) in association with a smaller peptide(s). The association was strong enough to remain intact during chromatographic conditions.

Antimicrobial activity in malt is likely to play a significant role in yeast performance during fermentation in the brewing process (Chapter 7). Should this prove to be a potential area for improving yeast performance as well as representing a malt quality parameter then it becomes paramount to fully purify the factor(s) to further elucidate the antimicrobial activity and

develop a non-biological assay such as an ELISA for its detection. The latter would offer a more targeted and robust method.

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# Appendix A

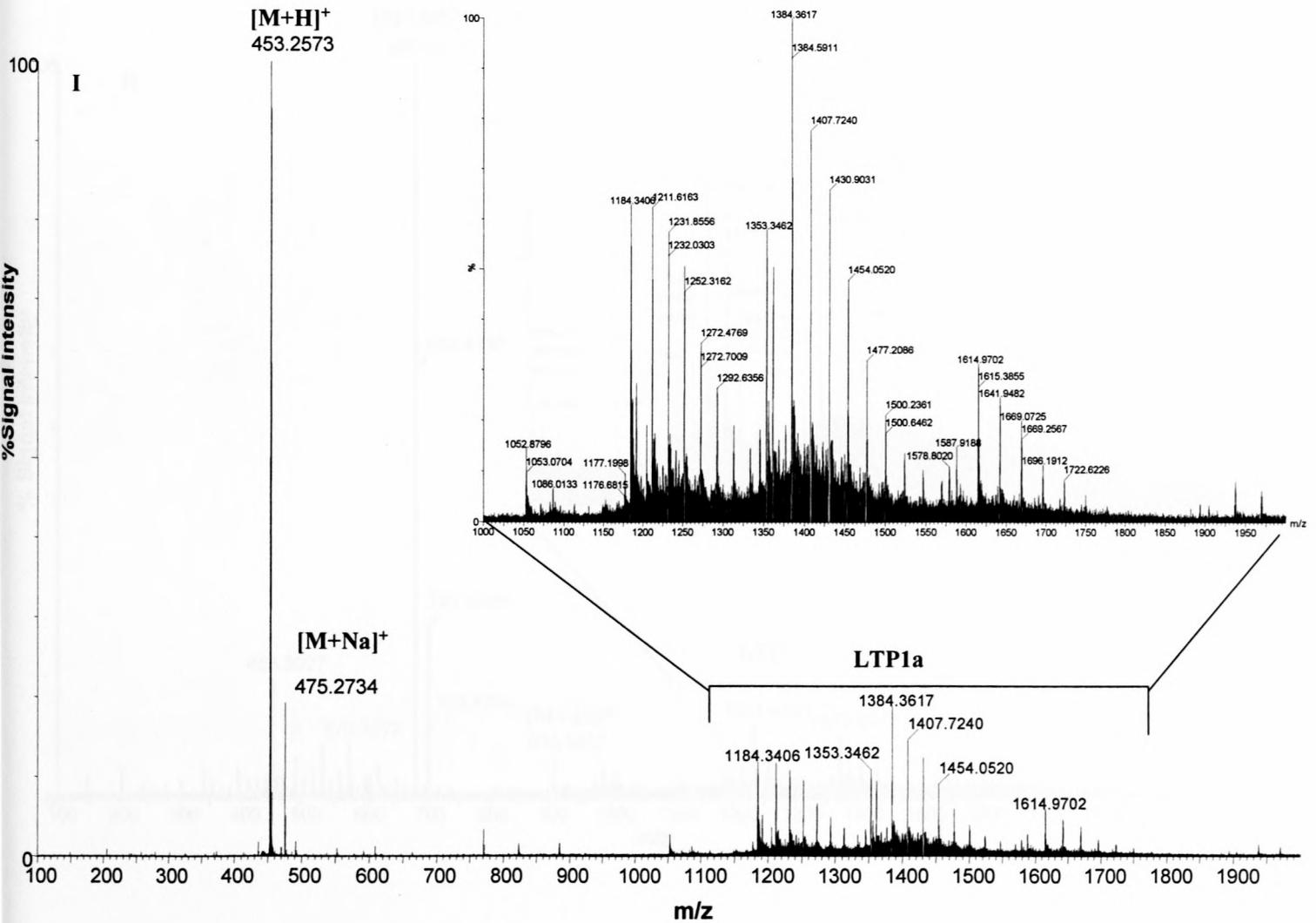
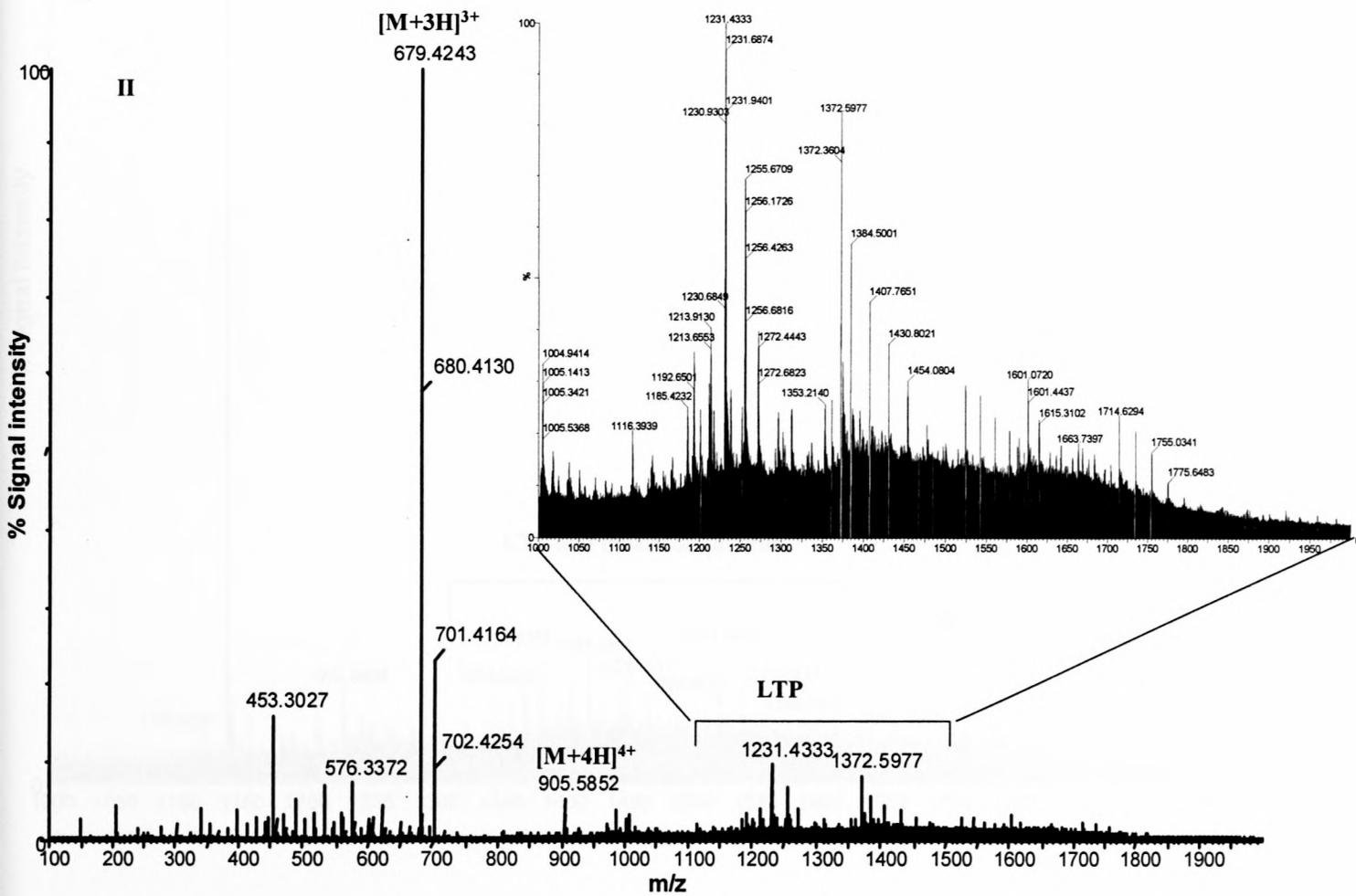
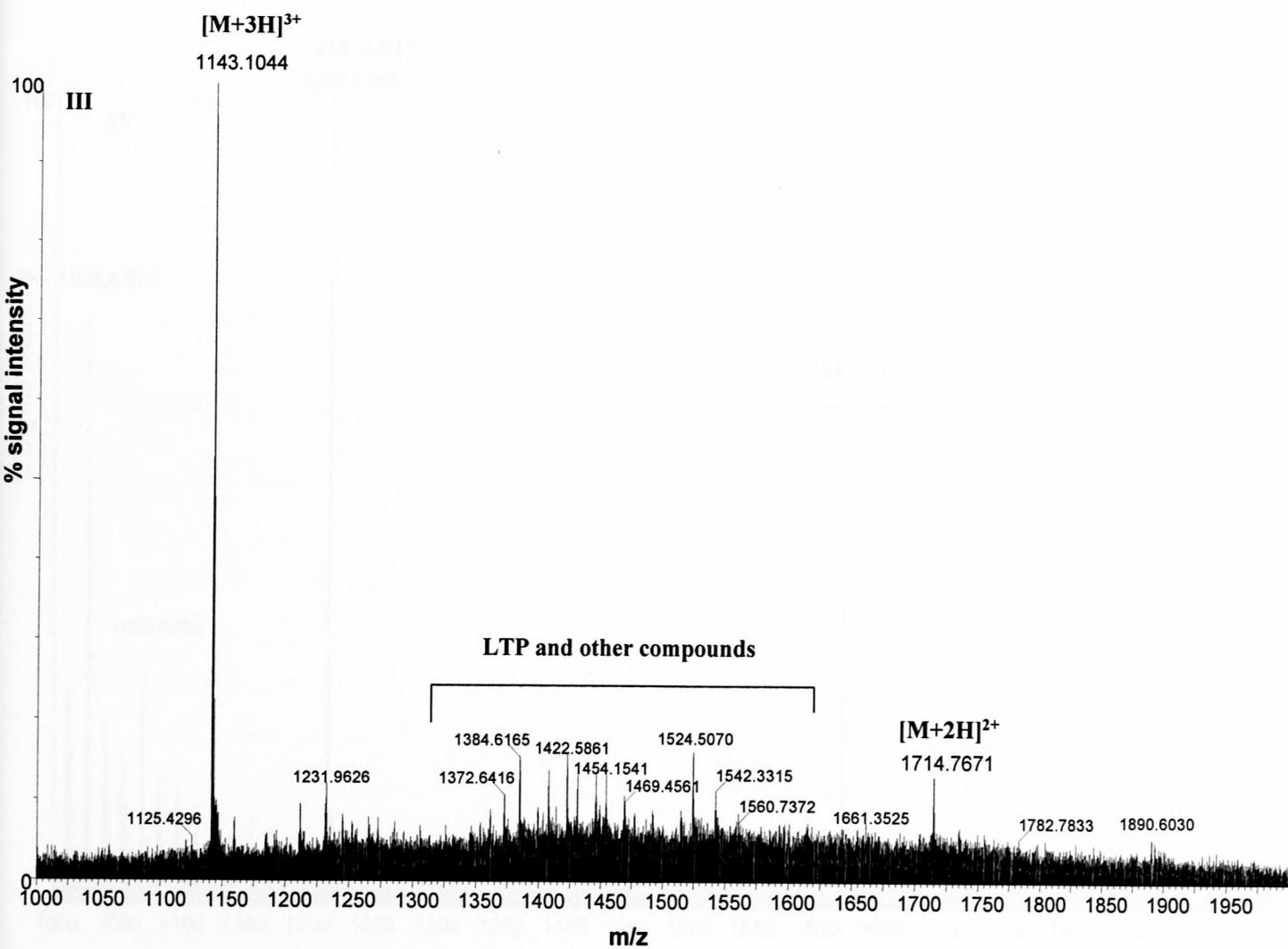


Figure I

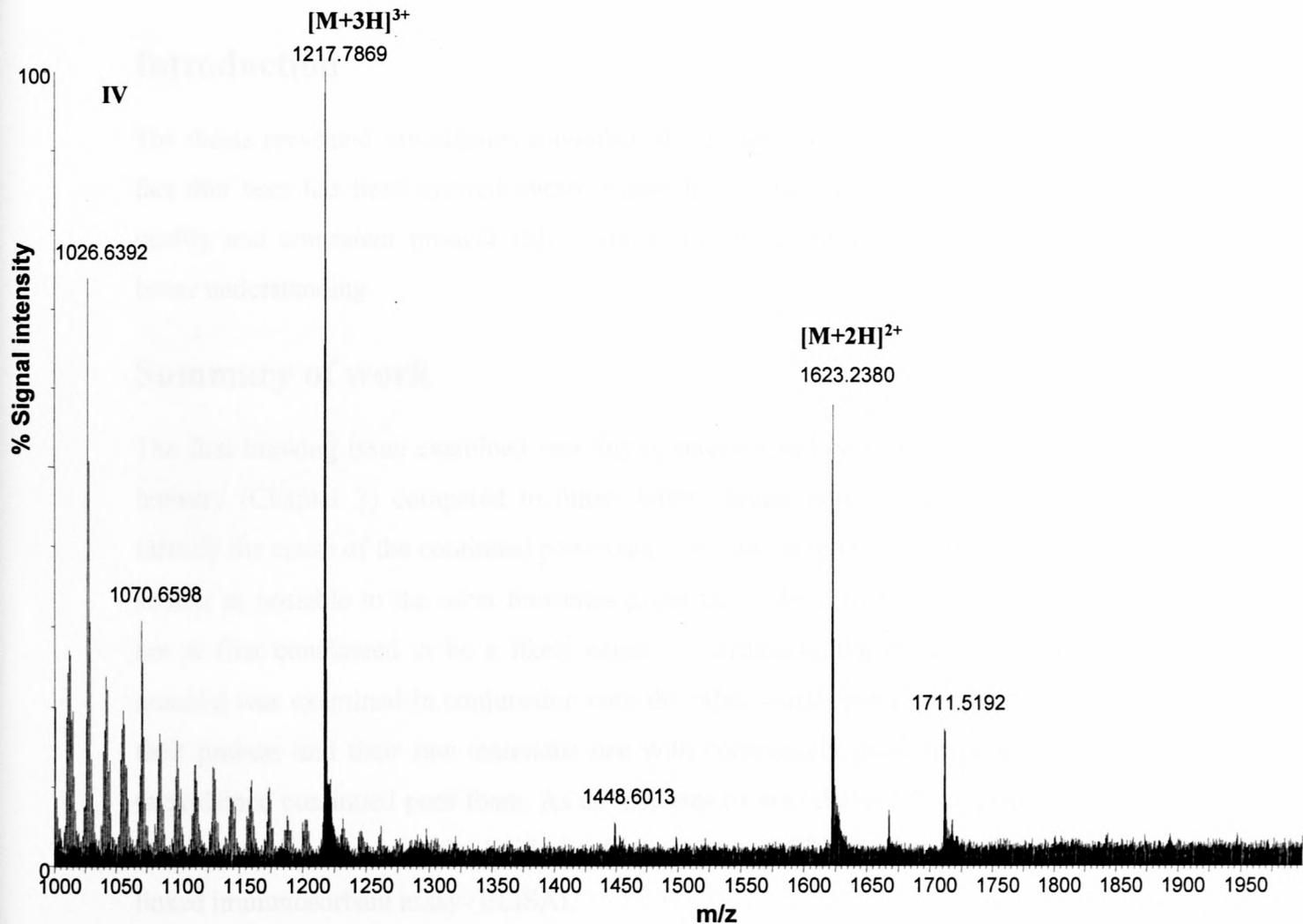
Mass spectra of the peak I (see Fig 10) from the chromatography of the cationic fraction of malt D extract.



*Figures II* Mass spectra of the peak II (see Fig 10) from the chromatography of the cationic fraction of malt D extract.



*Figures III* Mass spectra of the peak III (see Fig 10) from the chromatography of the cationic fraction of malt D extract.



**Figures IV** Mass spectra of the peak IV (see Fig 10) from the chromatography of the cationic fraction of malt D extract.

# Chapter 10

## *Summation, conclusions and future studies*

### **Introduction**

The thesis presented investigated a number of relevant brewing industry issues. Despite the fact that beer has been brewed over the ages the demand for increased volume, improved quality and consistent product still requires continued improvement and research to gain better understanding.

### **Summary of work**

The first brewing issue examined was that of unexplained poor quality foam in a particular brewery (Chapter 3) compared to others within South Africa. Initial exploration did not identify the cause of the continued poor foam. The raw materials used by the brewery were as similar as possible to the other breweries given the scale, distribution and logistics and was not at first considered to be a likely cause. Nevertheless, the malt as the source of foam material was examined in conjunction with the other variables by comparing two breweries, their process and their raw materials; one with consistently good foam and the other with unexplained continued poor foam. As an indicator of malt derived foam positive material the most abundant foam protein, lipid transfer protein 1 (LTP1) was monitored by an enzyme-linked immunosorbant assay (ELISA).

Interestingly, it was found that the foam protein levels started to diverge between the two breweries at the boiling stage of the wort preparation and a survey of a number of different breweries revealed that the boiling temperature differed due to altitude and this difference, while small, was critical not only in determining the final concentration of the foam proteins but also the conformation of the proteins. The conformation of LTP1 appeared to determine its ability to bind lipids; at lower boiling temperatures, it denatured less and bound lipids better, acting as a lipid sink and protecting the beer foam from lipid damage. Hence, the balance between lipids or free fatty acids in beer and LTP1 in the correct conformation was critical. The fatty acids are derived mainly from yeast lysis, highlighting the importance of good quality yeast and avoiding yeast stress that may result in lysis. The brewery in question

had low levels of LTP1 due to its coastal location and associated high boiling temperature. In addition the FFA levels were continually high and the LTP1 levels were insufficient to protect the foam from lipid damage.

The second brewing issue was that of malt associated premature yeast flocculation (PYF) during fermentation, which is an industry wide recurring issue (Chapters 4 and 5). The mechanism of premature flocculation has been elucidated and it is known that the fungal infection of barley plays a role. What caused a malt to become PYF positive was not understood, making prevention difficult. In addition although the factor or factors associated with PYF have been partially characterised as high molecular weight polysaccharide(s), rich in arabinose and xylose, acidic in nature and containing some nitrogenous material (Axcell *et al.*, 1986, Fujino and Yoshida, 1976 and Herrera and Axcell, 1991b)), they have not been successfully isolated. As a result a reliable and quantitative measurement method such as ELISA has not been developed and existing methods have poor reproducibility. The lack of understanding and lack of reliable assessment meant that often the first time it was discovered that a malt was PYF positive was when the yeast flocculated too early during a brewery fermentation, leaving a fermentation incomplete with high residual extract or sugars and too little alcohol.

To avoid repeating the efforts of others in trying to isolate the factor(s) and developing a reproducible and quantitative assay, a simple small scale fermentation type method was optimised as a reliable research tool to distinguish a PYF positive malt from a PYF negative malt relative to known control malts. With the assay in hand I was able confirm that PYF was associated with the malt husk and tried to induce PYF in non-PYF barley or malt. This was done successfully by fungal infection of the barley during malting or exposure of malt husk to extra-cellular fungal extracts (containing a range of polysaccharide hydrolysing enzymes including xylanases and glucanases). In addition, exposure of malt husk to commercial fungal xylanase and the addition of arabinoxylan (the polysaccharide product of xylanase action on husk) to the mash both impacted on PYF. The results indicated that the presence of fungal infection on the grain caused the enzymatic breakdown of the predominant husk component arabinoxylan, into smaller arabinoxylans of differing molecular weights. The presence of glucuronic acid residues in husk arabinoxylan would account for the acidic nature of the PYF factor previously reported (Fujino and Yoshida, 1976). These factors associate with yeast and result in the aggregation of yeast cells resulting in their flocculation before then end of fermentation when sugars are still present in high concentrations.

The source of the nitrogenous material believed to be part of the PYF factor (Herrera and Axcell, 1986) was proposed to be antimicrobial peptides (Axcell *et al.*, 2000) produced by the plant in response to microbial infection. The peptide may not be essential for PYF, but occurs simply as a result of the same trigger – microbial infestation of the grain. This area of plant defence and antimicrobial factors in barley and malt quality had not been previously investigated in the brewing industry and was undertaken as the final area of research.

To establish the importance of malt derived antimicrobial factors required the optimisation of an antimicrobial assay based on the methods already available. Brewing yeast was selected as target organism to assess any direct impact of antimicrobial factors in malt on yeast performance (fermentation). However, an indirect impact of antimicrobial factors present in malt, as a result of microbial induced stress, may reflect other aspects of malt quality. The assay was applied to measuring antimicrobial activity in barley and malt. As expected, malting appears to induce more antimicrobial activity due to the germination process and microbial growth. The antimicrobial factors were likely to be carried through to wort and fermentation impacting on yeast sugar uptake, although the levels were low and appeared to be associated with the trub (precipitate) that was found in wort and was partially transferred to the fermentation vessel.

Having established the likelihood of a direct impact on yeast in fermentation, the factor or factors associated with the inhibition of yeast were partially characterised. The antimicrobial factor was of barley and not microbial origin, residing in the grain endosperm and demonstrated a wide range of activity inhibiting not only yeast but also fungi and bacteria (gram-positive and gram-negative). The factor contained a cationic peptide fraction with sizes ranging between 5 and 14 kDa, putatively identified as LTP1 (approximately 9-10 kDa) associated with smaller 3-4 kDa peptide(s). The pH stability optimum for antiyeast activity was found to be 5.2 and exposure to extreme pHs caused irreversible damage. Starch degrading enzymes reduced the activity suggesting there may be a carbohydrate component essential for activity or protection of the protein component from heat and proteases.

## **Trends**

The theme of peptides, antimicrobial or not, was common to all the brewing issues examined. LTP1 is a peptide that is of great importance to foam. The group of peptides LTP1 belongs to, non-specific lipid transfer proteins (ns-LTPs), includes antimicrobial peptides. The antimicrobial activity of LTP1 has not been examined although our initial analyses revealed it was not antimicrobial in the purified form and there was no correlation between malt LTP1

concentration and malt antimicrobial activity. Activity may, however, be lost during purification or other components may have been required in conjunction to LTP1 for activity. Both possibilities were supported by findings in the research presented. Furthermore, there was evidence of LTP1 association with another peptide in an antimicrobial fraction. This kind of synergism between peptides was also reported in wheat where ns-LTP1e (very similar to barley LTP1) required the presence of another peptide for activity (Dubreil *et al.*, 1998). The antiyeast activity from barley observed by Okada and Yoshizumi (1970) was also associated with a peptide that had many features in common with ns-LTP.

Screening of malts for antiyeast activity revealed that all malts had some degree of activity and although we could differentiate between malts there was no obvious relationship between the level of antimicrobial activity and fermentation problems. Likewise for LTP1, all malts contain LTP1 and the levels do differ, but again with no obvious link to beer foam levels. The lack of correlation between LTP1 and antimicrobial activity may be due to the proposed role of an associated peptide required for activity. Measuring the presence of this peptide or LTP1 associated with this peptide may result in correlation with antimicrobial activity. In addition the assay measured LTP1 specifically, therefore other ns-LTPs would not be detected. Although there is evidence that LTP1 specifically is antimicrobial, this may not be the case and the other ns-LTPs present in lower concentrations may be more important for the antimicrobial activity. As in the case for LTP1 and foam, the underlying brewing process also contributed to the lack of a direct relationship between ns-LTP levels and antimicrobial activity.

PYF has been associated not only with the aggregation and flocculation of yeast but also with the inhibition of sugar uptake. Malts that resulted in both PYF and yeast sugar uptake inhibition had high levels of antimicrobial activity. The antimicrobial factors appeared to be involved in the sugar uptake inhibition. However, the presence of high levels of antimicrobial activity in non-PYF malts did not necessarily result in sugar uptake inhibition. Again there appeared to be no obvious link between the presence of antimicrobial factors and the occurrence of yeast sugar uptake inhibition, although there was evidence to support the association of antimicrobial factors with sugar uptake inhibition under certain conditions.

The major variables in the production of beer are the raw materials, yeast and process. The process impact seemed to play an underlying role in several of the issues investigated. The survival and conformation of the foam protein LTP1 was dependent on the boiling temperature, given the right process, besides having more foam material present the foam was protected against lipid damage. PYF could be induced during micro-malting, hence malting

may well play a role in the occurrence of PYF under certain conditions (e.g. the presence of the right microbial population). Antimicrobial factors were carried through the brewing process in the trub, the amount of which was process related. The contribution of more than one factor made all of these issues more complex. Although the understanding of all the brewing issues tackled in this thesis has moved forward there is a broad scope for future work.

## **Future work**

Given the evidence that there was a link between 1.) LTP1 and antimicrobial activity and 2.) PYF and antimicrobial activity, work to elucidating this relationship may further our understanding of all three areas of interest.

The association of LTP1 with antimicrobial activity and the impact of boiling temperature on its activity needs to be established to ensure the drive to minimise/maximise LTP1 considers not only foam but also yeast inhibition.

Now that it is apparent that the antimicrobial factors bind to the solids precipitated in the wort and may be carried through the process, extraction of the activity from the trub (precipitate) may be possible and would enable quantification of the antimicrobial activity at the point of impact. This would overcome any process contribution and may highlight real differences that do correlate directly to yeast performance.

In the brewing industry there has been an ongoing debate about “cloudy” and “clear” wort and the positive/negative impact on yeast performance (O’Connor-Cox et al., 1996). Investigating antimicrobial factors associated with unfiltered or cloudy worts may shed more light on this debate.

The trub was noted to be antibacterial as well and it may be possible to exploit this elsewhere in the brewing process. Should it prove that there is no significant impact of the malt antimicrobial activity on yeast in fermentation, it may be possible to utilise the antibacterial activity of trub in wort and during fermentation or during yeast handling.

The presence of antimicrobial factors in the malt grain as a result of microbial contamination and stress may be indicative of other aspects of malt quality besides a direct inhibitory effect on yeast in fermentation. Investigation of this would determine if measuring the malt antimicrobial activity is worthwhile as an assessment of malt quality.

The possible increase in antimicrobial activity during malting and the previously reported induction of PYF in malting (Axcell *et al.*, 1986) highlights the importance of the malting process. Elucidating the role or contribution of the malting process to antimicrobial activity/PYF may enable implementation of procedures to prevent or minimise their occurrence.

## **Closing remarks**

From a brewing industry point of view, several advances have been made in the areas examined. The findings concerning foam and the impact of boiling temperature on LTP1 has highlighted the need not only to try and maximise the foam positive factors such as foam proteins in the right conformation but also to balance that by reducing the amount of foam negatives. The key finding was the importance of balance and in this case location or rather reviewing the kettle design. Certainly the purchase of a kettle at one of the breweries was influenced by these findings.

The advances in the PYF research as a result of using a novel approach of avoiding the seemingly troublesome purification of the PYF factor, yet still gaining understanding of the events leading to PYF in malt, are already being applied. The assay is in use routinely to avoid purchasing PYF malts as the first line of defence and work is continuing to improve the assay or develop an alternative more robust assay for industry application. In addition, any malts that are found to cause PYF are examined further to determine at what stage the malt became PYF, be it in the field or in the malting process and how to minimise the impact of the malt in the brewing process.

The malt antimicrobial research has reached a stage where there are potential industry applications revealing several areas of interest. The importance of LTP1 in foam and the apparent association with yeast inhibition and malt antimicrobial factors could influence the management of LTP1 or foam by considering the impact on yeast. The finding that the factor may reside in the trub is of most consequence. This may be applied to compare different breweries with varying brewing equipment, evaluation of equipment design and the impact on wort production and fermentation. The application of the antimicrobial assay as a malt and yeast quality tool also has real potential for the brewing industry.

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