

**Malt induced premature
yeast flocculation: its origins,
detection and impacts upon
fermentation**

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Abstract

Premature yeast flocculation (PYF) is a sporadic problem encountered during industrial brewing fermentations. Current hypothesis states that factors, thought to arise from fungal infection of the barley in the field and/or the malt in the maltings cause yeast to flocculate prematurely and/or heavily before the depletion of the sugars in the wort. This results in poorly attenuated worts, with higher residual extract and lower ABV, flavor abnormalities (i.e. diacetyl, SO₂), lower carbonation levels, disruption of process cycle times and potential issues with the re-use of the yeast in subsequent fermentations. Consequently, PYF generates significant financial and logistical problems both to the brewer and the maltster.

In the current study a small-scale fermentation assay was developed and optimized to predict the PYF potential of malts, as well as to investigate the importance of yeast strain in the incidence and severity of the phenomenon. Furthermore, the impacts of the PYF factor(s) (i.e. arabinoxylans, antimicrobial peptides) on yeast fermentation performance and metabolite uptake were also studied, whilst the Biolog detection system was investigated as a potential rapid tool which to detect PYF.

The results obtained suggested that our in-house assay can be successfully used to predict the PYF potential of malts 69 or 40 h post-pitching depending upon the yeast strain used. Whilst ale yeasts were not found susceptible to PYF, lager yeasts exhibited different degrees of susceptibility even to the same PYF factor(s). More specifically, the more flocculent lager yeast SMA was found to be more susceptible than the medium flocculent lager yeast W34/70. However, interestingly, the fermentation performance of a PYF+ wort could be significantly improved by using a non-flocculent and relatively insensitive to PYF lager yeast. It was also shown that worts with lower amount of glucose and maltose could be responsible for poor fermentation profiles and/or heavy PYF as well as elevated residual sugars and lower fermentability. The observation that linoleic acid (6 mg.l⁻¹) exacerbated PYF ($P = 0.047$) and made its detection more rapid was found to be contrary to the “titration hypothesis” (Axcell *et al.*, 2000) which hypothesized that the addition of fatty acids might “titrate” out antimicrobial peptides so that they can no longer bind to the yeast cells. High gravity fermentations with worts inducing PYF did not have a significant effect ($P > 0.05$) on yeast physiological characteristics or fermentation performance suggesting that the PYF+ sample used in this study was inducing PYF through the ‘bridging’ polysaccharide mechanism rather than through the antimicrobial peptides. The Biolog system can be used for the metabolic characterization of different flocculence lager yeasts incubated in different fermentation media, whilst wort composition had a significant effect in redox reduction reactions.

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Speers, A.R., Baugh, C., Cook, D.J., Eck, E., Gibson, B.R., Joy, R., MacLeod, A., **Panteloglou, A.G.**, Voetz, M., Walker, S., and Powell, C.D. Miniature fermentation method. *Journal of the American Society of Brewing Chemists*, 2011, 69, **4**, 281-287.

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List of Abbreviations

%	Percentage
±	Plus or Minus
°C	Degrees Celsius
ABV	Alcohol by volume ($ABV = \left(\frac{1.05(Starting\ SG - Final\ SG)}{Final\ SG} \right) \frac{100}{0.79}$)
A ₆₀₀	Absorbance at 600 nm
ATP	Adenosine triphosphate
cm	Centimeter(s)
FAN	Free amino nitrogen
g	Gram(s)
h	Hour(s)
HMW	High-molecular weight
Kg	Kilogram(s)
l	Litre(s)
LMW	Low-molecular weight
LPD	Lipid transfer protein(s)
m	Meter(s)
M	Molar concentration (mol.l ⁻¹)
m/v	Mass concentration
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
mm	Millimetre(s)
mm ²	Square millimetre(s)
mM	Millimolar(s)
mEq	Milliequivalents
MW	Molecular weight
M	Mol
nmol	Nanomol
nm	Nanometer(s)
N	Normality (mEq.l ⁻¹ or mol.l ⁻¹)
Pa	Pascal ($1\ Pa = \frac{kg}{ms^2}$)
ppm	Parts per million
psi	Pounds per square inch ($1\ psi = 6.89 \times 10^3\ Pa$)
PYF	Premature yeast flocculation
rpm	Revolutions per minute
s	Second
SD	Standard deviation ($\sigma = \sqrt{\frac{1}{N-1} \sum_{i=1}^N (x_i - \bar{x})^2}$)
SG	Specific gravity ($SG = \frac{\text{density of a substance}}{\text{density of a reference substance}}$)
UV	Ultra violet (wavelength in the range from 10 to 400 nm)
v/v	Volume concentration
YPD	Yeast extract, peptone and glucose medium
µg	Microgram
µl	Microlitre(s)
µm	Micrometer(s)
w/v	Mass concentration

Chapter 1

Introduction

1.1 The Brewing Process (Overview)

The brewing process consists of three phases: wort manufacture, fermentation and post-fermentation processing (Figure 1.1). However, the precise details of each step depends on the nature and characteristics of the final product (beer) being made as well as the plant used (Boulton & Quain, 2003).

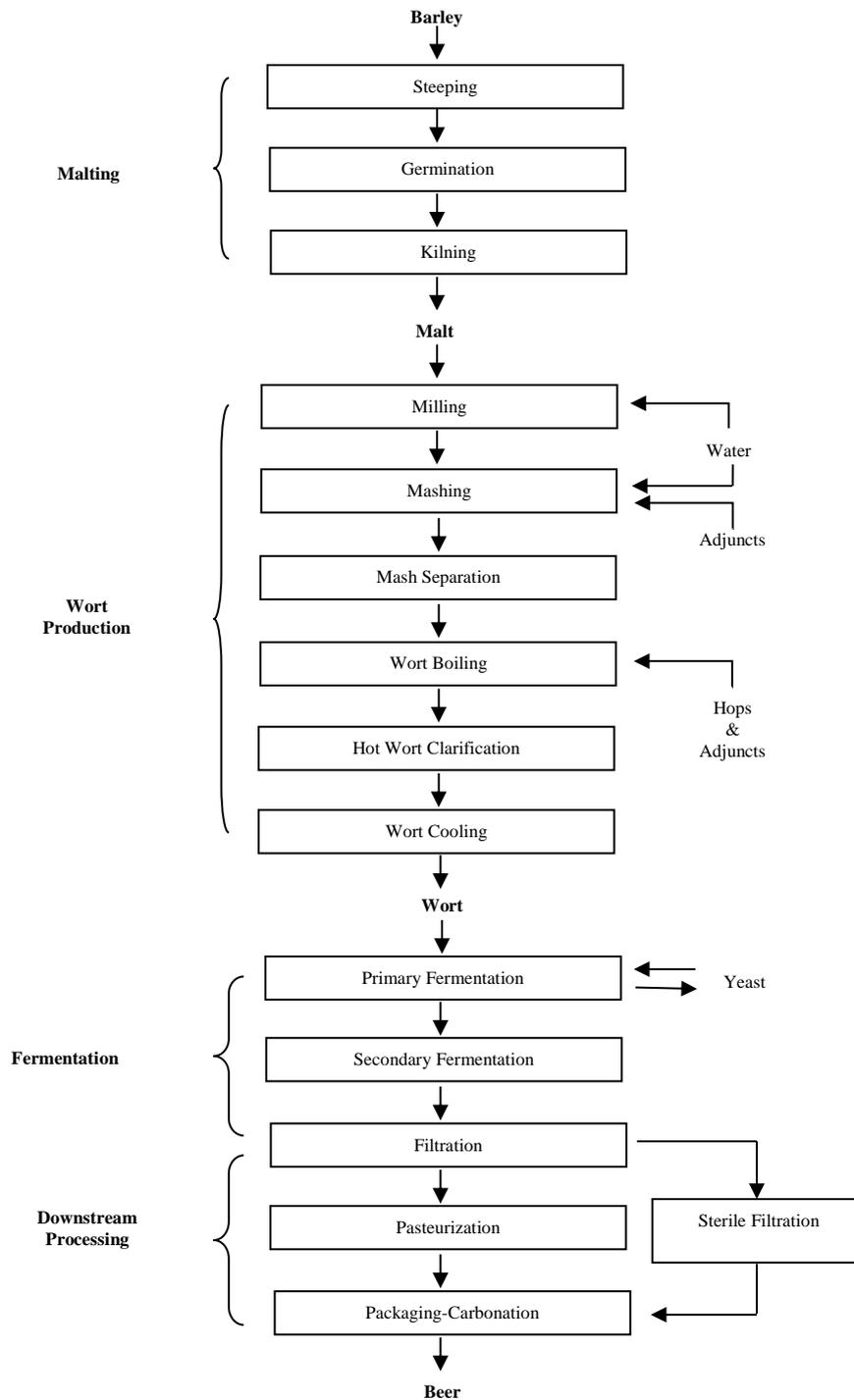


Figure 1.1: Schematic diagram of the malting and brewing processes.

1.1.1 Key Ingredients in Beer Production

The key ingredients utilised for the production of most beers are barley, water, hops and yeast. However, alternative sources of extract (adjuncts) may also be used (e.g. rice starches, hydrolysed corn syrup or sucrose) either to introduce necessary characteristics to the final product or to reduce the cost (Hornsey, 1999; Boulton & Quain, 2003; Briggs *et al.*, 2004). Adjuncts may replace a proportion of the fermentable carbohydrates provided that they do not negatively affect product quality and in particular flavour (Bamforth, 2003).

1.1.1.1 Water

Water is the main component of beer, comprising 90 – 94% (Hornsey, 1999), and so breweries often stress the purity and originality of their brewing liquor (Preedy, 2009). Brewing water has to be potable, pure, and free of pathogens or hazardous components (Bamforth, 2003; Boulton & Quain, 2003; Preedy, 2009). Besides that, it needs to have the correct balance of ions (Preedy, 2009). The principal ions in most brewing liquors are bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), chloride (Cl^-), sulphate (SO_4^{2-}), potassium (K^+), sodium (Na^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}). HCO_3^- , CO_3^{2-} , Ca^{2+} and Mg^{2+} are of major importance based on their ability to influence the pH during mashing and wort boiling, whilst chloride, sulphate and particularly the balance between them is regarded as important with respect to the final flavour of the beer (Hornsey, 1999). More specifically, Ca^{2+} reacts with malt phosphate (PO_4^{3-}), producing insoluble calcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$), and protons to reduce the pH of the mash, and thus to optimise the actions of significant mash enzymes such as α -amylase and proteases. Besides that, Ca^{2+} precipitates oxalic acid ($\text{C}_2\text{H}_2\text{O}_4$), a malt component responsible for the blocking of

dispense pipes (“beer stone”), inhibits colour formation during wort boiling, facilitates protein coagulation and favourably affects yeast flocculation and beer clarification (Fix, 1999; Hornsey, 1999; Bamforth, 2003). On the other hand, the formation of bicarbonates from carbonates, under acidic conditions during mashing, removes protons (H^+) and increases the pH of the wort reducing extract formation at concentrations > 100 ppm (Hornsey, 1999; Boulton & Quain, 2003). Too low a mash pH causes low amylase activity and problems with run-off, whilst increased pH-values causes extraction of phenolic substances giving rise to a final product with a harsh (astringent) character and haze problems (Hornsey, 1999). Desirable mash pH is generally regarded as in the range pH 5.2 – 5.4, although higher values are encountered. Mg^{2+} ions also act to reduce wort pH, by interactions with malt phosphate similar to Ca^{2+} ions (although they are not as effective in this regard due to the relatively more soluble nature of magnesium sulphate). They are also important co-factors of the enzymes catalysing the dissimilation of pyruvate ($C_3H_4O_3$) during fermentation, as well as being an essential component of many other enzymes (e.g. ATP; Boulton and Quain, 2003).

1.1.1.2 Barley

Barley (*Hordeum vulgare* or *Hordeum distichon*) is the cereal grain most often malted (Briggs *et al.*, 2004). It belongs to the grass family, the *Gramineae*, and is grown in more extremes of climate than any other cereal (Bamforth, 2003). Barley intended for use in brewing should have the ability to undergo even germination within a given period of time, have good disease resistance and have plump and consistently sized grains containing an appropriate balance of starch and nitrogen, (the content of the latter being preferably low; Boulton and

Quain, 2003). Two types of barley are used for malting and brewing; two- and six-row barley. In two-row barley, two rows of kernels develop, one on either side of the ear, whilst six-row barley has three rows on either side of the ear. Six-row barleys may have a higher proportion of cell-wall material in their endosperms that must be efficiently dealt with if problems are to be avoided in the brewery, and they are generally capable of producing higher levels of enzymes (Bamforth, 2003). Barley varieties also differ in their suitabilities for malting with some planted in autumn and some others in spring. The barley grain has a complex structure (Figure 1.2), and is a single-seeded fruit (Briggs *et al.*, 2004).

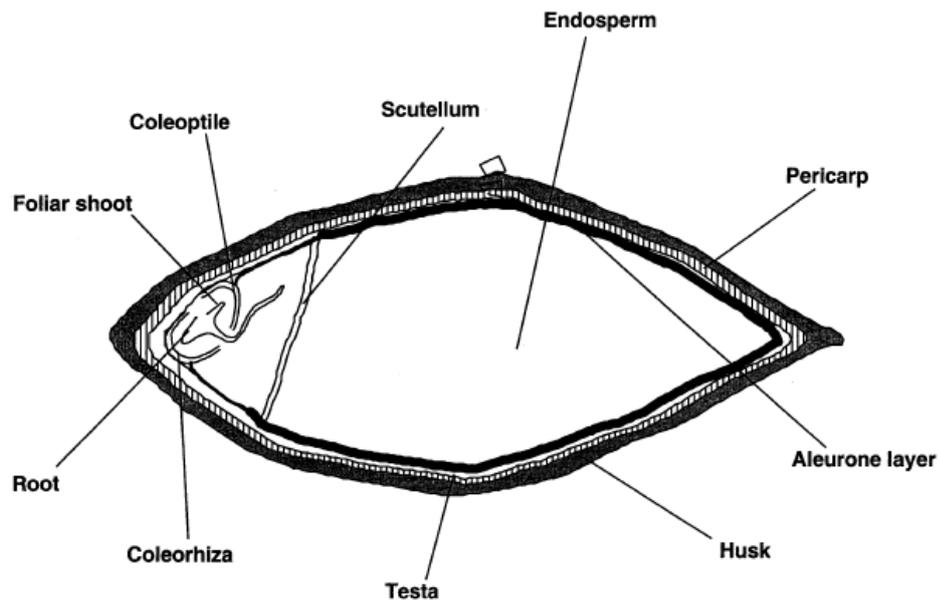


Figure 1.2: Diagrammatic representation of a cross-section through a barley grain (Adapted from Boulton and Quain, 2003).

Its dimensions vary, usually within the following ranges: length: 6 – 12 mm, width: 2.7 – 5.0 mm and thickness 1.8 – 4.5 mm (Briggs *et al.*, 2004). Barley endosperm consists of a protein mesh in which starch grains, both large and small, are embedded. Starch accounts for 55 – 65% of the total grain weight, with 75 – 80% of the starch being in the form of branched polymer,

amylopectin (D-glucose, α -(1→4) and α -(1→6) linkages), and 20 – 25% as amylose (D-glucose, with predominantly α -(1→4) linkages). The protein components of the barley grain can be categorised according to their solubility, as per Osborne's classification, into globulins, albumins, hordeins and glutelins. Globulins and albumins are relatively water soluble and include enzyme proteins, whilst the hordein and glutelin fractions are predominantly structural (and correspondingly insoluble in water) and are partially degraded during malting. The utilisation of the starch and nitrogenous components of the endosperm is facilitated by amylases and proteases, which are secreted from the aleurone layer of the grain. Barley grains contain other significant components which contribute to wort functionality (e.g. sucrose, vitamins, minerals, polyphenols, nucleotides and lipids; Boulton and Quain, 2003).

1.1.1.1 Hops

The hop plant (*Humulus lupulus L.*) is a member of the family *Cannabinaceae* that grows in temperate regions of the world (Hornsey, 1999; Boulton & Quain, 2003). Hops give beer its typical bitterness and in many cases also impart hop aroma, and have preserving (anti-microbial) effects (Bamforth, 2003; Lodolo *et al.*, 2008; Preedy, 2009). Hops contain a range of chemical components (water, resins, essential oils, cellulose and lignin as structural products, proteins and amino acids, lipids, waxes, and tannins; Hornsey, 1999). However, the flavour-active components of hops are resins and essential oils (Boulton & Quain, 2003). Resins constitute about 10 – 20% of the hop dry weight and this fraction incorporates the bittering substances. The bitter character imparted by hops is due to chemicals known as α -acids, of which humulone, cohumulone and adhumulone are the most prevalent, accounting for

2 – 15% of the hop cone weight. During wort boiling the α -acids are isomerised to cis and trans forms to impart bitterness to beer (Boulton & Quain, 2003). On the other hand, the essential oils, which account for 0.05 – 2% of the cone weight and comprise a complex mixture of more than 250 components, are added towards the end of the boiling stage or even post fermentation to give a range of spicy, citrus, as well as estery aromas and tastes (Boulton & Quain, 2003).

1.1.1.2 Yeast

Yeast is a single-celled eukaryotic organism, about 5 – 10 μm in diameter and roughly spherical in shape or oval, which reproduces by cell division (i.e. the daughter cell grows from the mother cell as a bud before separating as a distinct cell leaving a “bud scar” behind the mother cell; Fix, 1999; Bamforth, 2003). Brewing yeast strains are heterotrophic, facultative anaerobes - requiring oxygen only during the initial growth phase, and are divided into two species: *Saccharomyces cerevisiae* and *Saccharomyces uvarum* (formerly termed *Saccharomyces carlsbergensis*; Hornsey, 1999; Briggs *et al.*, 2004). The name *Saccharomyces cerevisiae* has been reserved for yeasts that make ales at temperatures in the range 18 – 22°C rising at the surface of the fermenting vessel. On the other hand, *Saccharomyces uvarum* yeasts ferment the wort at temperatures typically 6 – 15°C, flocculate to the bottom of the fermentation vessel and have been traditionally used in the production of lager-style beers (Bamforth, 2003).

1.1.2 The Malting Process (An Overview)

Malting is the “limited germination of cereal grains or, occasionally, other seeds or pulses (peas and beans), under controlled conditions” (Briggs *et al.*, 2004). During malting the barley grain undergoes controlled germination. This is initiated by wetting the grains (steeping). The increased moisture content activates enzyme synthesis as grains begin to mobilize their starch reserves to provide carbon and energy for the development of the embryo (germination). At an appropriate point the germination is arrested by the application of heat (kilning), which stabilises the grain such that in malt the relevant enzymes and reserve materials are available for subsequent extraction and further degradation to release fermentable sugars during wort production (Boulton & Quain, 2003). A major requirement during malting is the comprehensive hydrolysis of the endosperm cell walls, which leads to the softening of the grain and facilitates subsequent milling and extraction. Besides that, there needs to be a substantial breakdown of protein, to eliminate potential haze-forming material and to release the foaming polypeptides, but mainly to produce amino-acids, which the yeast will require as building blocks to make its own proteins. However, what the brewer does not want is significant degradation of the starch, for it is this that he wants to break down in the brewery to yield fermentable sugars (Bamforth, 2003).

1.1.2.1 Steeping

Malting is initiated by steeping. During this stage barley grains are soaked in water with periods of exposure to air (Boulton & Quain, 2003). During steeping, water, which might contain a biocide to minimise surface microbial growth (Boulton & Quain, 2003), enters the grain through the micropyle, the

small opening at the embryo end of the grain, and distributes through the starchy endosperm (Bamforth, 2003). The purpose of this stage is to increase the moisture content of the grain from 11 – 12% to 43 – 46% within a period of two days, as kernels would not germinate if the moisture content is below 32% (Bamforth, 2003). The homogeneous distribution of water across the entire bed of grain initiates germination (as evidenced by chitting - the emergence of the coleorhiza from the proximal end of grains; Bamforth, 2003).

1.1.2.2 Germination

The primary aim of germination is to develop the enzyme activities which can hydrolyse the cell walls, proteins, and the starch of the grain and to ensure that these act to soften the endosperm by removing the cell walls and about half of the protein, whilst leaving the bulk of the starch behind (Bamforth, 2003). Germination begins with the exposure of the embryo to moisture. This triggers the synthesis of plant hormones (gibberellins) which migrate into the aleurone layer and initiate the synthesis of endosperm-degrading enzymes (i.e. endoglucanases, pentosanases, amylases and proteases). These enzymes diffuse into the endosperm and hydrolyse the starch to glucose ($C_6H_{12}O_6$), and the reserve proteins to amino acids. In the scutellum the nutrients are transformed into transportable form and transported to the embryo so as to be utilised in the growth of the new plant (Briggs *et al.*, 2004). During germination the temperature is kept between 13 – 16°C and the humidity is maintained at high levels to avoid undue drying out of the grain (Bamforth, 2003; Boulton & Quain, 2003). At an appropriate point germination is arrested by the application of heat (kilning; Hornsey, 1999).

1.1.2.3 Kilning

Kilning comprises the drying of the “green malt” to such a low level of moisture that it is stabilized, whilst germination is arrested and enzymatic digestion halted (Bamforth, 2003). The enzymes of the malt, however, must not be destroyed as they are required to generate fermentable sugars during mashing. Bamforth (2003) indicated that often it is important that cell wall and protein degrading enzymes survive too because they may not have completed their job in the maltings (particularly in undermodified malts where a shorter germination process is employed). Subsequently, they may also be needed to deal with proteins and polysaccharides present in unmalted adjuncts that the brewer may use in mashing. For that reason drying is performed gently. During kilning, temperatures are slowly increased from 25 – 30°C to 60 – 70°C (for lager malts and up to 105°C in the case of some ale malts). More specifically, air is blown through the malt bed to facilitate the removal of moisture and the water content of the malt is gradually reduced to approximately 4%. Kilning serves several functions. The most important, though, is that it renders the malt into a stable form in which it might be stored for long periods, whilst also reducing the surface microbial load. In addition, flavour and colour reactions take place which impact on beer quality. These reactions occur mainly during the final high temperature, low moisture phase of kilning known as curing (Boulton & Quain, 2003). Roasted green malt products (e.g. cara pils and crystal malt) can be used to introduce relatively sweet, toffee-like characters. Alternatively, intense heating of pale kilned malt generates products such as black malt which can deliver potent burnt and smoky notes (Bamforth, 2003). These speciality products are manufactured in roasting drums and finished at

higher temperatures than those experienced in conventional kilning (e.g. 135 – 220°C).

1.1.3 Wort Manufacture

1.1.3.1 Milling

The aim of milling is to produce a particle size distribution that is best suited to the particular brewhouse and for the type of malt used (Bamforth, 2003). Milling reduces the size of the grist particles, and, hence, exposes the malt endosperm to enzymes during wort production (mashing) so that the greatest conversion of starch to fermentable sugar is achieved within the shortest possible period of time (Briggs *et al.*, 2004). If the particle size after milling is too large then the enzymatic degradation is inefficient, whilst with too small a particle size wort separation is impeded. Well-modified malts can be milled more coarsely to permit faster separation without sacrificing extract performance. Consequently, a relatively well-modified malt will need less intense milling than a relatively undermodified malt to generate the same particle size distribution (Bamforth, 2003). Milling may be wet or dry depending on the composition of the grist and the preference of the particular brewery (Boulton & Quain, 2003).

1.1.3.2 Wort Production

The purpose of mashing is economically to prepare wort of the correct composition, flavour and colour in the highest practical yield, and within the shortest period of time (Briggs *et al.*, 2004). During mashing, which is essentially the enzymatic stage of brewhouse operation (Bamforth, 2003), the milled malt or a mixture of malts and other prepared grist materials (e.g.

adjuncts, salts and, where allowed, supplementary enzymes) are mixed intimately with brewing water (liquor) to enable the action of enzymes (Bamforth, 2003; Briggs *et al.*, 2004). It is essential that the particles be efficiently hydrated and that careful control is exerted over process times and temperatures (Bamforth, 2003). Mashing involves a ramped temperature profile in which first a low-temperature stand (where utilised) is provided for maximum activity of the more heat-sensitive enzymes (i.e. proteases and β -glucanases). This is followed by a second higher-temperature stand for starch gelatinisation and amylolysis, whilst a final even higher-temperature short stand may be incorporated to denature the enzymes which cause problems further downstream. Low-temperature rests (45 – 50°C) are used with undermodified malts (i.e. when the breakdown of proteins and β -glucans is to be encouraged), whilst mashing temperatures between 64 – 68°C are used to maximize rapid starch conversion and production of fermentable sugars (Boulton & Quain, 2003; Briggs *et al.*, 2004). The elevated temperature during mashing gelatinises starch granules, in other words disrupts their crystalline structure, rendering them susceptible to attack by amylase enzymes. Increasing the mash temperature increases the rate of chemical and enzyme catalysed reactions and accelerates the rates of denaturation and precipitation of proteins (including the inactivation of enzymes). In addition, the increased temperature accelerates dissolution and diffusion processes, accelerates mixing, and at least above a certain temperature causes the gelatinisation of starches and disrupts the cellular structure of unmodified cereal endosperm tissues (Briggs *et al.*, 2004). Both α - and β -amylases from malt are active during mashing. However, the latter is more heat labile and its activity does not persist for a long period of

time in high temperature mashes. Similarly, limit dextrinase is moderately heat labile and denatures at higher temperatures during mashing (Boulton & Quain, 2003). Malt α -amylase is a mixture of different molecules (isoenzymes), with slightly differing properties, is produced during malting and requires Ca^{2+} ions for activity. On the other hand, β -amylase is predominantly present in bound forms in barley, is released during malting and is of particular importance when raw barley is used as a mash tun adjunct (Boulton & Quain, 2003; Briggs *et al.*, 2004). The concerted action of amylases, which have optimum activity at approximately pH 5.3, produces predominantly maltose, together with glucose, maltotriose and significant amounts of higher dextrans (Boulton & Quain, 2003). During mashing Ca^{2+} ions may be added in order to lower the pH of the mash. Ideally a mash should be at pH 5.2 – 5.6 for the appropriate balance to be struck between the various reactions that are occurring. Acids may occasionally be used directly or introduced indirectly (e.g. through the use of lactic acid bacteria during malting) as an alternative strategy to lowering mash pH using Ca^{2+} addition (Bamforth, 2003). It is essential to maintain a low pH, especially during mashing and to a lesser extent during the copper boil (Section 1.1.3.4), for efficient starch breakdown and proteolysis (Boulton & Quain, 2003).

1.1.3.3 Wort Separation

The separation of the resultant wort from the residual, “spent”, grain is performed in modern breweries either in a vessel called a lauter tun, or using mash filters. Using mash filters, wort separation can be completed in a shorter period of time than when using lauter tun filtration; which can take up to two h (Bamforth, 2003). In order to facilitate the recovery of as much fermentable

sugars as possible the mash bed is washed (sparged) with hot water (63 – 68°C; Bamforth, 2003; Briggs *et al.*, 2004). Too much water will excessively dilute the wort, whilst temperatures higher than 70°C will extract substances (e.g. β -glucans) which may cause problems further downstream (Bamforth, 2003).

1.1.3.4 Wort Boiling

Following wort separation, the sweet wort is boiled with hops in a copper (kettle, hop-boiler) for a period of 1.5 – 2 h or sometimes even longer (Briggs *et al.*, 2004). Wort boiling is a very energy intensive stage of the brewing process, and, hence, the brewer makes every effort to conserve energy input and loss (Bamforth, 2003). The consequence of this stage is to remove the water, and thus to concentrate the wort to the desired degree for yeast fermentation (Briggs *et al.*, 2004). Most brewers tend to evaporate between 4 and 10% of the wort per h (Bamforth, 2003; Briggs *et al.*, 2004). Wort boiling removes the unwanted volatile substances, originating from malts and hops (Bamforth, 2003), and sterilizes the wort, or at least destroys the ‘vegetative’ forms of microbes probably within the first 10 – 15 min (Briggs *et al.*, 2004). Despite the fact that spores may survive, after boiling the wort is handled under aseptic conditions (Briggs *et al.*, 2004). The intense heat during wort boiling inactivates any of the more robust enzymes that may have survived mashing and wort separation. In addition, it coagulates proteins (by cross linking with tannins (polyphenols) from malts and hops – producing the “hot break”), isomerizes the bitter α -acids from hops into bittering compounds and increases the wort colour through Maillard reactions (i.e. reactions between reducing sugars - sugars with an aldehyde (R-CHO) group or capable of

forming one in solution through isomerism - and proteins, peptides, amino acids or amines; Belitz *et al.*, 2004, Bamforth, 2003). Following boiling the wort is separated from the trub and other residual solids using a “hop back” (in the minority of modern production which uses whole hop cones), a vessel analogous to a lauter tun, a centrifuge or a “whirlpool” (a cylindroconical tank where the wort is set into a rotational flux forcing the trub into a conical pile at the centre of the vessel; Bamforth, 2003).

1.1.4 Fermentation

The common denominator in the production of all alcoholic beverages is fermentation (Bamforth, 2003). Fermentation is “the cumulative effect of yeast growth on wort, ultimately resulting in the spent growth medium, beer” (Lodolo *et al.*, 2008). During fermentation cooled and aerated hopped wort that has been run into fermentation vessel is pitched with yeast as soon as possible (Hornsey, 1999). Significant underpitching leads to slow initial fermentations, whilst overpitching (e.g. twice the normal rate which accounts between $15 - 20 \times 10^6$ live cells per ml wort) results in excessive nutrient competition. This, results in poor yeast growth and increased levels of certain esters (i.e. ethyl acetate; Hornsey, 1999), a broad-spectrum of off-flavours, increased risk of autolysis, problems during clarification, and losses of hop flavour and aroma. Besides that, unsaturated fatty acids that carry over to the finished beer will promote beer staling (Fix, 1999). Wort temperature during pitching is also important. Thus, if the temperature of the wort is more than 5°C cooler than the temperature at which the yeast has been held, then assumedly ‘cold shock’ will take place resulting in an extended lag phase (Hornsey, 1999). Following pitching, the wort is fermented by yeast to produce immature or green beer; a

process known as primary fermentation. Primary fermentation is followed by a much slower secondary fermentation where far less yeast remains in suspension. Secondary fermentation completes flavour development and product maturation (Hornsey, 1999; Lodolo *et al.*, 2008). During fermentation, yeast consumes the nutrients of the wort and produces alcohol, carbon dioxide (CO₂), and a range of flavour active compounds (e.g. esters, higher alcohols and acids that contribute to flavour), thus decreasing wort sugar levels and the pH of the fermenting wort (Bamforth, 2003; Boulton & Quain, 2003; Briggs *et al.*, 2004). The pH drop of the fermenting wort (from 5 to as low as 3.8) is associated with the secretion of organic acids (e.g. succinate, lactate, and acetate) by the yeast. Yeast can also produce medium-chain-length fatty acids, such as octanoic and decanoic acids, which can impart flavours to beer described as “goaty” and “wet dog” (Bamforth, 2003).

1.1.4.1 Primary Fermentation

Primary fermentation can be further divided into two stages: the initial period (‘lag phase’), lasting only a few h after pitching, and the Embden-Meyerhof-Parnes (EMP) or glycolysis stage (Fix, 1999). During the first few h after pitching nothing visibly happens in the fermentation vessel. This is the lag phase of growth, which can last anywhere from 6 to 15 h, and is an integral part of the growth cycle of the yeast inoculated into wort (Hornsey, 1999). During the lag phase of growth, although there are no outward manifestations of metabolic activity, several important physiological and biochemical events occur. The yeast is adjusting itself to the wort environment, in particular the high osmotic pressure of the dissolved sugars, and activates certain enzyme systems (e.g. inducing synthesis of carriers that will permit maltose and

maltotriose to enter the cell; Hornsey, 1999). It is also engaged in cell-wall preparation as well as oxygen, nitrogen, and sugar uptake (Fix, 1999). Sufficient dissolved oxygen in the wort permits the synthesis of membrane sterols and fatty acids leading to rapid cell growth (Hornsey, 1999; Bamforth, 2003). On the other hand, more than sufficient oxygen will allow yeast to undergo aerobic respiration via the oxidation and decarboxylation of pyruvate ($C_3H_4O_3$) and ultimately Krebs cycle producing carbon dioxide, water and energy (Equation 1.1; Bamforth, 2003), but not ethanol (C_2H_5OH).

Equation 1.1: Glucose oxidation during aerobic respiration.



The initial or lag phase of fermentation is also marked by yeast growth, resulting from cell division via budding, a build-up of energy reserves, and acidification – activities important for an orderly fermentation (Hornsey, 1999). Once the cell membranes are prepared, yeast cells start taking in amino acids, peptides, and sugars in a definite order governed by the size of the molecule, the concentration of the sugar, and the availability of enzyme systems required for metabolism (Hornsey, 1999).

Of particular importance, however, are two inhibitory effects associated with wort composition and fermentation conditions; maltose inhibition and “shock excretion”. More specifically, worts with large non-grain components (i.e. significant amounts of glucose or fructose) can create a number of problems with the yeast cell’s activities – the most important being the inhibition of the yeast’s ability to transport maltose through the cell wall. This problem can lead to a long and disordered fermentation. On the other hand, “shock excretion”

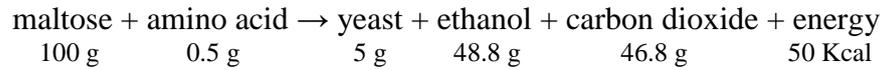
refers to the situation where adverse fermentation conditions, most notably high starting gravities and/or high fermentation temperatures, create osmotic-pressure effects on the cell wall. This process can cause yeasts to actually reject essential nutrients, mainly wort nitrogen, inhibiting yeast growth and resulting, again, in lengthy and disordered fermentations (Hornsey, 1999).

Once anaerobic conditions are established in the fermenter, true fermentation begins. That is, yeast converts the fermentable sugars, ultimately glucose ($C_6H_{12}O_6$), to ethanol (C_2H_5OH), carbon dioxide (CO_2) and energy through the EMP pathway (Equation 1.2; Bamforth, 2003).

Equation 1.2: Main products during brewing fermentative process.



The main interest during brewing fermentations, as opposed to fermentations where biomass is the main objective (e.g. pharmaceutical fermentations), is the production of ethanol and carbon dioxide. Thus, during brewing fermentations sugar concentrations are high and oxygen levels are low, but controlled (see Chapter 3 for details on yeast oxygen requirements). Under these conditions minimal excess yeast biomass is produced, because the more sugars end up in the new yeast cells, the less will be converted into alcohol (Equation 1.3; Bamforth, 2003). Nevertheless, high gravity fermentations (i.e. > 15 to 20°P) increase yeast stress due to increased osmotic pressure caused by higher levels of alcohol and carbon dioxide (Van Nierop, 2005; Gibson, 2011). On the other hand, high-gravity brewing presents tremendous opportunities for enhancing brewery capacity and maximizing the amount of beer produced per unit of expenditure on items such as energy (Bamforth, 2003).

Equation 1.3: Example of yield during brewing fermentative process.

The fermentation process initiates with the utilization of glucose in the glycolytic pathway to pyruvate ($\text{C}_3\text{H}_4\text{O}_3$), the major branch point between the fermentation process and the citric acid cycle (Krebs cycle). During fermentation, a net of two ATP (adenosine triphosphate) molecules are formed as pyruvate is converted via acetaldehyde ($\text{C}_2\text{H}_4\text{O}$) to ethanol and carbon dioxide (Hornsey, 1999; Lodolo *et al.*, 2008). This process takes one mole of glucose, or fructose ($\text{C}_6\text{H}_{12}\text{O}_6$), and yields two moles each of ethanol and carbon dioxide (Fix, 1999). Carbon dioxide affects yeast fermentation performance, flocculation kinetics as well as the carbonation levels of the final product (Lodolo *et al.*, 2008; Gibson, 2011). High glucose concentrations (i.e. > 0.4%) in the presence of oxygen would also allow yeast metabolism to be fermentative rather than oxidative; a phenomenon known as the “Crabtree Effect”. This will accelerate yeast growth and decrease sugar uptake, resulting in the formation of ethanol and carbon dioxide even under aerobic conditions. On the other hand, if the sugar content is lower than 0.4% and oxygen still available then yeast will revert fermentation to respiration (Pasteur effect; i.e. the phenomenon whereby fermentation is inhibited by respiration or glycolytic rates decrease under aerobic conditions; Briggs *et al.*, 2004) releasing carbon dioxide without alcohol production (Equation 1.1; Hornsey, 1999).

The lag phase of fermentation is followed by a short phase of accelerating growth which leads to a phase of exponential or logarithmic growth. During this period, yeast density increases by four- to six-fold. Therefore, it is at this

stage that cell growth is at its highest level, with cells multiplying by budding and rapidly producing ethanol and carbon dioxide (Hornsey, 1999). Logarithmic growth normally persists for 48 – 60 h, after which a phase of decelerating growth (retardation phase) is entered before cells reach the stationary phase. The latter signifies the end of the primary fermentation. During the stationary phase of growth only a small number of new cells are produced, counteracted by the number becoming moribund. However, Hornsey (1999) indicated that yeast required for subsequent fermentations (repitching) should be cropped at the end of the exponential phase, as cells recovered later on during fermentation will be less viable and are more likely to contain contaminating microorganisms. Once the fermentable sugars have been utilised the yeast will separate from the fermenting wort (green beer) by a natural process termed flocculation (see Section 1.2 for details), and in some cases (e.g. in the incidence of stuck or sluggish fermentations) by centrifugation.

1.1.4.2 Secondary Fermentation

When primary fermentation is complete the beer must be rendered into a form suitable for consumption (Bamforth, 2003). Most beers are subjected to post-fermentation processing, termed secondary fermentation, so as to produce a stable final product (Boulton & Quain, 2003). This is achieved through a number of downstream processes which include ‘conditioning’; also known as ‘maturation’ or ‘ageing’ which involves two different temperature stands (“warm” and “cold”), filtration and pasteurisation/sterile filtration (Bamforth, 2003; Boulton & Quain, 2003). “Warm conditioning” involves the removal of some of the undesirable by-products of primary fermentation (e.g. sulphur compounds (H_2S), acetaldehyde ($\text{C}_2\text{H}_4\text{O}$) and diacetyl ($\text{C}_4\text{H}_6\text{O}$)) by yeast. This

is generally performed at higher temperatures, relative to the fermentation temperatures (Lodolo *et al.*, 2008), takes place slowly, and requires cells to be in a relatively good metabolic condition (Hornsey, 1999). “Warm conditioning” allows the decarboxylation of alpha-acetolactate to diacetyl and the reduction of diacetyl to less-flavour active products (i.e. acetoin and butanediol; Lodolo *et al.*, 2008). Subsequently, the beer is chilled to between 0 – 1°C to ensure appropriate colloidal stability of beer; a process termed “cold conditioning”. This is often carried out in conjunction with process aids which selectively precipitate haze-forming proteins and/or polyphenols (Bamforth, 2003). Following a period of minimum three days in “cold conditioning” the beer is filtered, to remove any residual suspended particles, and consequently pasteurized/filter sterilized. Following pasteurization/filter sterilization the gases in beer (i.e. O₂, CO₂) are adjusted. More specifically, O₂ may be removed by purging an inert gas (e.g. N₂) to the beer vessel, whilst CO₂ may be introduced by injection. Following that, the beer is packaged and stored until distribution (Bamforth, 2003).

1.2 Yeast Flocculation

Yeast flocculation is a reversible, asexual and calcium dependent process in which cells adhere to one another to form flocs (Stratford, 1989; Stratford & Brundish, 1990; Bony *et al.*, 1998; Govender *et al.*, 2008). Flocculation is distinct from aggregates, which arise via budding and non-separation of daughter cells (Briggs *et al.*, 2004). Lager yeasts (*Saccharomyces uvarum*), which account for the majority of modern beer production, separate from the fermenting medium by sedimentation, a process encouraged by chilling the “green beer” (Briggs *et al.*, 2004), whilst ale yeasts (*Saccharomyces*

cerevisiae) rise to the surface of open or dish bottom fermentation vessels by coalescing around gas bubbles (Stratford, 1989, 1992a; Verstrepen *et al.*, 2003; Damas-Buenrostro *et al.*, 2008). Following that, the resultant yeast head can be removed by skimming or suction (Briggs *et al.*, 2004). Flocculation is of considerable importance to the brewer as it provides an effective, environmentally friendly, simple and cost free way to separate yeast cells from green beer at the end of fermentation (Soares & Vroman, 2003; Verstrepen *et al.*, 2003). Brewing yeast disperses, replicates, ferments as single cells and then flocculates rapidly following the depletion of nutrients, and in particular sugars, in the wort (Stratford & Carter, 1993). Early or premature flocculation leaves unattenuated sweet beer, whilst late or poor flocculation requires yeast cells to be removed by fining, filtration or centrifugation (Stratford, 1992a; Stratford & Carter, 1993; Damas-Buenrostro *et al.*, 2008), which are time-consuming and expensive procedures (Govender *et al.*, 2008). Besides that, inadequate flocculation results in poor cropping, such that there may be insufficient yeast for re-pitching, and “green beer” with unacceptably high residual yeast counts (Briggs *et al.*, 2004). Consequently, the timing of flocculation is an important factor influencing the quality of the final product (Axcell, 2003).

The flocculation characteristics of yeast strains are of major significance in brewing (Verstrepen *et al.*, 2003; Damas-Buenrostro *et al.*, 2008) as the number of suspended yeast cells in wort during both primary and secondary fermentation affects the speed of fermentation, flavour formation, maturation and filtration (Jibiki *et al.*, 2001; Jin *et al.*, 2001). A fit for purpose yeast for the modern brewing industry should therefore exhibit strong flocculation

characteristics towards the end of the primary fermentation (Verstrepen *et al.*, 2003). The efficiency of flocculation is determined by the timing of flocculation onset as well as by the rate of flocculation in conjunction with the ratio of flocculent to non-flocculent cells (Stratford & Keenan, 1987, 1988). Flocculation, usually a property of the late exponential or stationary phase (Mill, 1964), is under genetic control (Johnston & Reader, 1983 ; Stratford & Keenan, 1987; Sampermans *et al.*, 2005). Although desirable, flocculation is therefore a complex process strongly influenced by the expression of specific genes, including *FLO* genes, cell wall protein genes (*CWP*, *TIR* and *DAN* genes), and mitochondrial genes (Stratford, 1992a, b; Verstrepen *et al.*, 2003).

The *FLO* family includes 12 genes, 5 of which have been recognized as dominant zymolectin-encoding (structural) genes (*FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11*; Damas-Buenrostro *et al.*, 2008). *FLO1* is a dominant gene situated at the right arm of chromosome 1 (Verstrepen *et al.*, 2003), whilst *FLO5* and *FLO9* are highly homologous to *FLO1* (Russell *et al.*, 1980; Sieiro *et al.*, 1997). *FLO8*, originally reported as a structural gene, is currently identified as a transcriptional activator of *FLO1* and *FLO11* (Teunissen & Steensma, 1995; Lo & Dranginis, 1996; Verstrepen *et al.*, 2003), whilst *FLO2* and *FLO4* are allelic (copies) to *FLO1*, *FLO3* is semi-dominant, and *FLO6* and *FLO7* are respectively recessive to *FLO1* (Teunissen & Steensma, 1995).

1.2.1 Yeast Flocculation Mechanism

Numerous hypotheses have been proposed to explain the mechanism of flocculation in *Saccharomyces cerevisiae* (Soares & Vroman, 2003). These include the early colloidal theory (Kryut, 1952), the calcium-bridging theory

(Mill, 1964), and the lectin-like theory (Miki *et al.*, 1982a). The early colloidal theory was based on the assumption that in aqueous solution cells behave as negatively charged colloids (Kryut, 1952). The observation that inorganic salts promoted yeast flocculation was explained as surface-charge neutralization leading to aggregation and sedimentation of the cells. However, the specific requirement by most yeast strains for calcium in floc formation discredited the colloidal theory and led to the bridging hypothesis. According to this theory, calcium ions (Ca^{2+}) linked adjacent yeast cells by coupling to carboxyl groups (Mill, 1964). As the inhibition of flocculation by specific wort sugars (i.e. mannose; $\text{C}_6\text{H}_{12}\text{O}_6$) could not be explained by this theory, Miki *et al.* (1982a) proposed the lectin-like theory of flocculation. According to the lectin-like theory (Miki *et al.*, 1982a) yeast flocculation occurs when the α -mannan residues (polysaccharides of D-mannose; Kaur *et al.*, 2009) of mannoproteins interact with lectin-like proteins of adjacent cells forming large aggregates or flocs. More specifically, the N-terminal part of the lectin-like proteins bind the mannose chains (receptors) that are present in the cell walls of flocculent and non-flocculent neighbouring cells (Taylor & Orton, 1975; Stratford, 1992b; Straver *et al.*, 1993; Straver *et al.*, 1994; Teunissen & Steensma, 1995; Bony *et al.*, 1998; Kobayashi *et al.*, 1998; Soares & Vroman, 2003; Verstrepen *et al.*, 2003; Sampermans *et al.*, 2005; Van Mulders *et al.*, 2010). In this adhesion process, calcium ions are thought to ensure the correct conformation of these lectins (Taylor & Orton, 1975; Miki *et al.*, 1982a; Stratford, 1992b; Verstrepen *et al.*, 2003), whilst a recent crystallization and structural study of flocculins showed that Ca^{2+} is directly involved in carbohydrate binding (Veelders *et al.*, 2010). The lectin-like proteins (zymolectins), which specifically bind sugars

and are present only in flocculent cells (Soares & Vroman, 2003), are synthesized by yeast in preparation for flocculation and are located on the external surface of the yeast cell wall. Conversely, the mannan residues are always present on the yeast cell wall (Martinez *et al.*, 1993). Since the mannose residues are always present in the cell wall of both flocculent and non-flocculent cells (Stratford & Carter, 1993; Bony *et al.*, 1998), a critical flocculation determining factor is clearly the presence or absence of flocculins (Verstrepen *et al.*, 2003). Despite the fact that the lectin type cell-cell interaction (Miki *et al.*, 1982a) has been proposed to explain brewing yeast flocculation, cell surface hydrophobicity has been identified as the second major factor responsible for flocculation onset (Straver *et al.*, 1993; Vidgren & Londesborough, 2011). This observation was recently supported by Strauss *et al.* (2006) who reported the accumulation of hydrophobic carboxylic acids (i.e. 3-hydroxy (OH) oxylipins) on the cell surfaces of *Saccharomyces cerevisiae* during flocculation onset.

Stratford (1992a) proposed that flocculation takes place when the *FLO* genes become active and the flocculins are formed. The possession of genes producing different lectin-like proteins presumably underpins the *NewFlo* and *Flo1* phenotypes (see Section 1.2.2 for details). Strains that do not possess any of these genes are not flocculent under any circumstances. Thus, there is evidence that a gene termed *FLO1* encodes for a cell surface protein, which has been implicated in flocculation (Briggs *et al.*, 2004). Transfer of this gene from a flocculent yeast strain to a non-flocculent type is accompanied by the acquisition of a flocculent phenotype (Teunissen & Steensma, 1995). Stratford (1992c) suggested that after growth limitation, yeast cells become fimbriated

which corresponds with a sharp increase in cell surface hydrophobicity. The increase in cell's surface hydrophobicity results in the release of agglutinin. This gives rise to fimbriae-associated glutin ligands, and finally in the formation of flocs. If agitation is applied, removal and redistribution of the fimbriae may lead to more compact flocs (Figure 1.3).

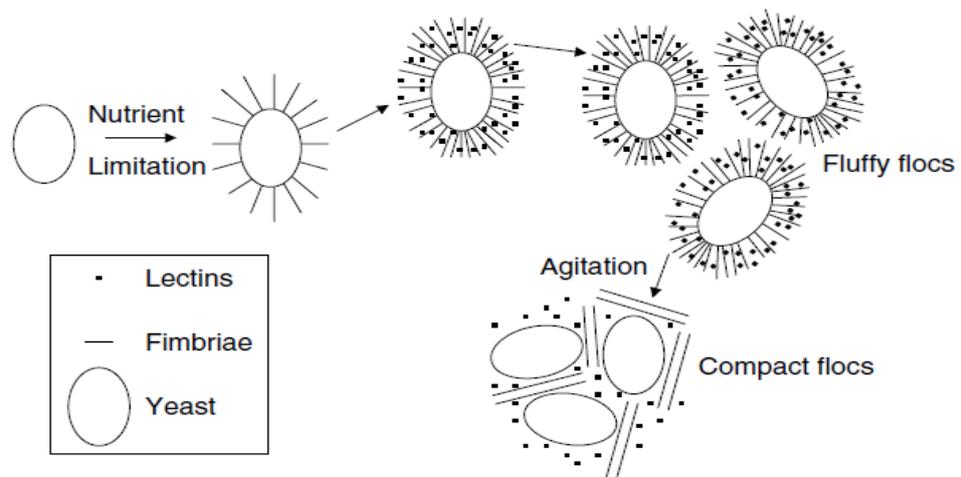


Figure 1.3: Model for flocculation of brewing yeast cells during fermentation (Adapted from Straver, 1993).

1.2.2 The Onset of Flocculation

Flocculation in brewer's yeast is stimulated by nutrient starvation and/or stress conditions (Stratford, 1992c; Straver *et al.*, 1993; Smart *et al.*, 1995). Yeast flocculation occurs when the sugars in the wort have been exhausted (Smit *et al.*, 1992), probably because prior to that, sugars (e.g. mannose) occupy the flocculin binding sites so that they can no longer bind to the mannose residues of other cells (Verstrepen *et al.*, 2003; Briggs *et al.*, 2004). Stratford (1992c) and Verstrepen *et al.* (2003) indicated that the presence of mannose and derivatives in wort inhibits flocculation, particularly with regard to the *Flo1* phenotype (which accounts for the majority of lab strains and includes strains

containing *FLO1*, *FLO4*, *FLO5*, *FLO8* and *TUP1* genes) due to its ability to block the flocculin binding sites of the cells (Lo & Dranginis, 1996).

In contrast, efficient flocculation of yeast strains exhibiting the *NewFlo* phenotype, often associated with brewer's yeast (Soares & Vroman, 2003; Briggs *et al.*, 2004), requires the absence of mannose as well as glucose, sucrose and maltose (Stratford, 1992c; Verstrepen *et al.*, 2003). *Flo1* phenotype strains are constitutively flocculent, producing a flocculin protein (i.e. lectin) that appears to be associated with fimbriae-like structures but is not an integral part of them (Axcell, 2003), whilst brewing yeasts belonging to the *NewFlo* phenotype exhibit a cyclic behaviour and flocculate only in the stationary phase (Stratford & Assinder, 1991; Stratford & Carter, 1993; Soares & Mota, 1996; Patelakis *et al.*, 1998; Soares & Vroman, 2003). *Flo1* and *NewFlo* yeasts use interactions between lectin-like proteins and cell surface mannans. Despite the fact that the groups differ in the nature of lectins, both phenotypes use common carbohydrate receptors (i.e. the side chains of the outer mannose chain of cell wall mannoproteins), and have an obligate requirement for Ca^{2+} ions for flocculation to occur (Ca^{2+} ensures that the lectin-like proteins (zymolectins) are in the correct configuration for binding to mannose receptors; Briggs *et al.*, 2004).

MI (mannose insensitive flocculation) yeast strains, the third category of flocculent yeast cells are insensitive to mannose, sucrose (Stratford & Assinder, 1991; Masy *et al.*, 1992) or other sugars (Vidgren & Londesborough, 2011). The MI phenotype is characterised by an apparent lack of binding specificity for mannose, preventing flocculation inhibition on mannose (Masy

et al., 1992; Bossier *et al.*, 1997; Nishihara *et al.*, 2002), and is not dependent on calcium for floc formation (Vidgren & Londesborough, 2011). Onset of flocculation in the MI strains has been suggested to be controlled by both a change in cell surface hydrophobicity and an increase in ethanol concentration (Dengis *et al.*, 1995). In these cells, flocculation occurs via direct (non-lectin like) protein – protein interaction. MI strains are top-fermenters and have a highly hydrophobic cell envelope, which possibly promotes both the formation of flocs and encourages formation of a yeast head (Briggs *et al.*, 2004). Vidgren and Londesborough (2011) indicated that although the MI phenotype is much less common than the *Flo1* and *NewFlo* phenotypes, both ale and lager strains of MI phenotype have been described (Table 1.1).

Table 1.1: Current view of flocculation phenotypes (Compiled from Briggs *et al.*, 2004 and Vidgren & Londesborough, 2011).

Genes	Character	Inhibitors	Comments
<i>FLO1</i> , <i>FLO5</i> , <i>FLO9</i> , <i>FLO10</i>	Strong <i>Flo1</i> phenotype	Only mannose	Heavily flocculent throughout fermentation
<i>Lg-</i> <i>FLO1</i>	<i>NewFlo</i> phenotype	Mannose, glucose, sucrose, maltose and maltotriose (not galactose)	Flocculation at end of primary fermentation
<i>FLONL</i> , <i>FLONS</i>	Like <i>NewFlo</i> phenotype	Mannose, glucose, sucrose, maltose, maltotriose and galactose	Flocculation at end of primary fermentation
Not known	Mannose- insensitive (MI) flocculation (Ca- independent)	Not inhibited by sugars	Cells require presence of ethanol for flocculation to occur

Recent publications (Bayly *et al.*, 2005; Govender *et al.*, 2010) have reported the characteristics of *Flo11* dependent flocculation in wine strains of *Saccharomyces cerevisiae*. The role of *Flo11* in the flocculation of lager brewing strains remains to be elucidated. Jin and Speers (1998) indicated that sugars like galactose and fructose do not inhibit flocculation, whilst Straver (1993) and Straver *et al.* (1994) suggested that there are cases where flocculation is not solely dependent on the presence of flocculins. Miki *et al.* (1982b) also reported that concanavalin A, treatment with proteinase K, and reduction of disulphide bonds by mercaptoethanol were found to inhibit flocculation.

1.2.3 Factors Influencing the Flocculation of Commercial Yeast Strains

During a particular industrial fermentation process, flocculation can be affected by multiple parameters. For a given strain, flocculation depends on a combination of four main factors: i) genotype (presence of flocculation [*FLO*] genes and their regulatory elements) ii) wort nutritional status (in particular the content and profiles of sugars, free amino nitrogen (FAN) and divalent cations), iii) environmental conditions (temperature, presence of alcohol, pH, dissolved oxygen, osmotic pressure and shearing forces) and iv) physiological state of cells (cell surface hydrophobicity, vitality, membrane integrity, starvation, generation number, etc.; Soares & Vroman, 2003; Verstrepen *et al.*, 2003; Damas-Buenrostro *et al.*, 2008; Vidgren & Londesborough, 2011). A number of cellular and extra-cellular conditions have been shown to affect flocculation capacity including culture temperature, ethanol, specific nutrient limitation, wort composition and petite formation (Lawrence, 2006).

1.3 Premature Yeast Flocculation

Premature yeast flocculation (PYF) is a sporadic, but potentially serious problem in the brewing and malting industries (Jibiki *et al.*, 2006; Lake & Speers, 2008; Kaur *et al.*, 2009; Panteloglou *et al.*, 2010; Panteloglou *et al.*, 2011). PYF has been defined as the phenomenon where flocculent yeast (i.e. yeast with lectin-like proteins (zymolectins) on the cell surface) settle out of the fermentation medium abnormally early and/or heavily during primary fermentation leaving a residual extract (Koizumi *et al.*, 2009) and low end-of-ferment cell counts (Van Nierop *et al.*, 2004). The early or premature flocculation of the yeast cells hampers complete fermentation (Ishimaru *et al.*, 1967; Stratford, 1992c), and results in a poorly attenuated wort (Axcell *et al.*, 2000) and a final product with undesirable flavour characteristics (Stratford, 1992c; Koizumi & Ogawa, 2005; Koizumi *et al.*, 2008; Lake & Speers, 2008; Koizumi *et al.*, 2009). The total diacetyl content of the beer will increase, resulting in a final product with a detectable diacetyl flavour (Inagaki *et al.*, 1994). In many modern brewing processes, detectable diacetyl is regarded as a quality defect and commercial practice frequently involves a ‘diacetyl stand’ as a part of the fermentation/maturation process, whereby diacetyl produced in primary fermentation is taken up and metabolised by yeast cells in suspension. PYF slows this process due to the lower suspended cell counts (Van Nierop *et al.*, 2004; Panteloglou *et al.*, 2012). PYF has been also reported to increase susceptibility to microbial infections (Jin *et al.*, 2001; Nakamura, 2008), and gives rise to lower carbon dioxide evolution rates during fermentation, and a final product with lower alcohol content and increased sulphur dioxide (Lake & Speers, 2008). Consequently, PYF results in financial losses to brewers

(Axcell *et al.*, 2000), as the beer requires additional blending or processing and, in severe cases, disposal (Lake & Speers, 2008). Axcell (2003) suggested that in the incidence of PYF brand identity may be compromised, potentially resulting in a negative consumer reaction.

The onset of PYF may occur at the same time as normal flocculation or slightly earlier. However, the rate and extent of premature flocculation is more dramatic leading to a marked reduction in the number of yeast cells in suspension at the end of the fermentation process (i.e. after around 8 days). This, ranges from 2 to $> 20 \times 10^6$ cells.ml⁻¹ as opposed to 20×10^7 cells.ml⁻¹ of fermenting wort in normal fermentations (Figure 1.4). The premature removal of yeast may be a purely physical event associated with a factor or factors that aggregate the cells. Alternatively, the yeast could perceive starvation as a result of a factor that interacts with the yeast membrane and inhibits sugar uptake, thus triggering the flocculation mechanism prematurely (Van Nierop *et al.*, 2004). Besides that, PYF is considered by some to be an extreme example of a condition that is present to some degree in all worts (Herrera & Axcell, 1991b). Kaur *et al.* (2009) indicated that there are two different definitions related to PYF. One group defines acute or primary PYF whereby the early flocculation of yeast cells during primary fermentation results in a final product with unacceptably high levels of residual fermentable sugars (Figure 1.4A), whilst the second school of thought recognises a more subtle, chronic PYF, termed secondary PYF, where the cell count in suspension during maturation-secondary fermentation is at a sub-optimal level so that the removal of undesirable flavour components such as diacetyl (butterscotch flavour; Bamforth, 2003) is incomplete (Figure 1.4).

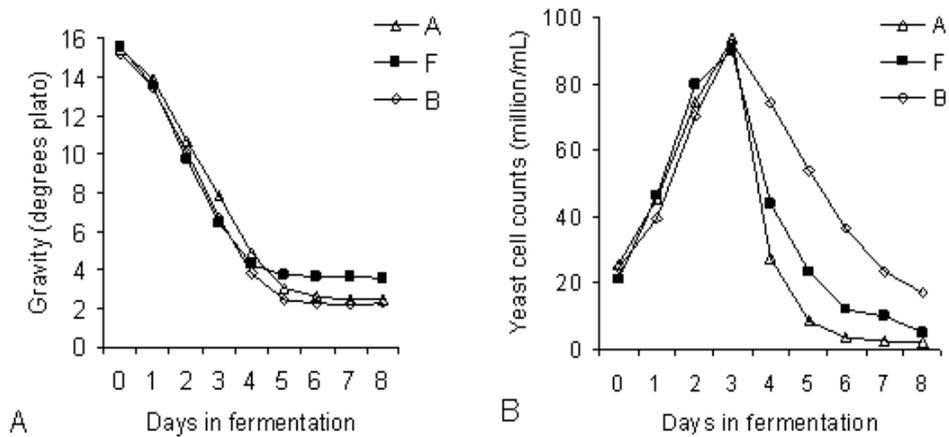


Figure 1.4: 2L EBC fermentation results of wort prepared from malts A, F and B where A and F are PYF+ malts and B is a PYF- malt (Adapted from Van Nierop, 2005). Fig. A monitors gravity through fermentation and Fig. B monitors cell counts. Each value was the average of duplicate fermentations, variations of cell count method was < 15% and std. dev. for the gravity readings was 0.04.

1.3.1 Causes of PYF

1.3.1.1 Wort Deficiency

Axcell (2003) proposed that the majority of brewers, at the onset of abnormal yeast growth or flocculation patterns, react by assuming that there is a deficiency in the wort caused by changes in the malt during the malting process. Axcell (2003) and Axcell *et al.* (1986) indicated that zinc (Zn^{2+}) as well as the combination of zinc and manganese (Mn^{2+}) are essential for efficient yeast fermentations. Biotin ($C_{10}H_{16}N_2O_3S$) is also an essential cofactor for brewer's yeast and biotin-deficient worts have resulted in spectacular failure of yeast growth. However, a biotin deficiency is more likely to be due to inappropriate wort preparation rather than an intrinsic defect in malt. Besides that, oxygen deficiencies and low vitality of yeast can also give rise to slow and incomplete fermentations. However, rather than a deficiency, several compounds present in wort have also been shown to produce tailing fermentations or impact on yeast flocculation patterns. These compounds, as

well as the processes responsible for early flocculation or incomplete fermentations are discussed, in chronological order, in Section 1.3.1.2.

1.3.1.2 Attempts to Purify and Characterise PYF+ Factors

In the late 1900s Jago reported that certain strains of brewing yeast used in the dough making process showed a poor carbon dioxide production (Okada *et al.*, 1970). Lecourt (1928) demonstrated that this phenomenon was induced by a toxic substance located in the protein fraction of wheat and barley which affected only the bottom-fermenting yeasts. In the late 1950s, researchers at Kirin in Japan started to report on the impact of several substances that caused premature flocculation of their yeast (Kudo, 1958, 1959; Kudo & Kijima, 1960). More specifically, Kudo (1958) and Kudo and Kijima (1960) after acid hydrolysis of the spent grain reported a substance, which they termed “Barmigen”, as responsible for PYF. “Barmigen”, an acid hydrolysate (0.5% HCl) of spent grain under pressure (3 kg.cm^{-2}), was identified as a reddish-brown, relatively HMW humic acid-like substance. In a concentration of $1 \mu\text{g.ml}^{-1}$ of this substance, bottom-fermenting yeast suspended in a buffer solution of pH 4.4 flocculated and settled to give a clear supernatant liquid within a period of five min. Kudo (1959) isolated a substance from six-row Japanese barley malt with a similar effect on the yeast cells during fermentation as that caused by “Barmigen”. The substance, which he named “Treberin” and was found in higher quantities in six-row barley compared with two-row barley varieties, was a water-soluble gum compound which on acid hydrolysis yielded glucose, xylose ($\text{C}_5\text{H}_{10}\text{O}_5$) and arabinose ($\text{C}_5\text{H}_{10}\text{O}_5$) indicating a gum like polysaccharide.

Okada *et al.* (1970), working in the Central Research Institute of Osaka in Japan, extracted, using dilute (0.05 N) sulphuric acid, a substance from the endosperm of wheat and barley which was toxic to brewing yeast. The substance inhibited yeast growth at lower concentrations (i.e. 0.6 – 1 u) and caused the death of the cells at toxicity level above 1 u (one unit of toxicity was defined as the lowest amount of the extract which could inhibit the yeast growth in 10 ml wort medium). However, the toxic effect of the substance was not observed in the presence of divalent metal ions such as Ca^{2+} , Zn^{2+} and Fe^{2+} at a concentration of 5×10^{-3} M or above. In a subsequent study the same year, Okada and Yoshizumi (1970) reported that the toxic substance identified by Okada *et al.* (1970) was a basic protein with an isoelectric point higher than pH 10 and a molecular weight of the order of 9.8 kDa (estimated by the Archibald method). It was also suggested that the protein inhibited yeast growth by combining with the acidic groups (i.e. carboxyl and phosphoric acid; H_3PO_4) located on the cells surface. Okada and Yoshizumi (1970) proposed that the inhibition of the toxicity after neutralization with Ca^{2+} , Zn^{2+} and Fe^{2+} was due to the competitive binding of the acidic groups on the cells surface. Okada and Yoshizumi (1973) suggested that the toxin of Okada and Yoshizumi (1970), able to absorb both onto the cell wall and the cell membrane, inhibited yeast respiration and fermentation and caused the death of the cells within a short period of time (6 min) at a concentration of 4 mg.l^{-1} . This was seen as the ability of the toxin to bind to the cell membrane, causing changes in the permeability of the membrane and resulting in the death of the cells. Nevertheless, at lower concentrations (i.e. 0.4 mg.l^{-1}), the protein-toxin

inhibited only the sugar uptake and had no lethal effect on the yeast cells (Okada & Yoshizumi, 1973).

Morimoto *et al.* (1975) isolated a PYF-inducing factor from wort and malt. The factor, isolated using ethanol precipitation, was called EP and was thought to be a mixture of arabinoxylan (i.e. a chain of D-xylopyranose units where the OH groups in the 2- and 3-position are glycosidically linked to L-arabinofuranose; Belitz *et al.*, 2004), α -glucan (a polysaccharide of D-glucose monomers linked by glycosidic bonds), and a glycoprotein (a protein that contains oligosaccharide chains (glycans) covalently attached to polypeptide side-chains) consisting of two polysaccharides. A factor isolated from malt (G-50) was primarily made up of the carbohydrates arabinose ($C_5H_{10}O_5$), xylose ($C_5H_{10}O_5$) with some glucose ($C_6H_{12}O_6$), and an unidentified component. The glucan components had little PYF-inducing activity, but the arabinoxylan moiety with the protein was closely associated with PYF induction. The same authors (Morimoto *et al.*, 1975) showed that when a solution of EP was mixed with dilute sodium hydroxide (NaOH) at room temperature for a period of 3 h, the PYF activity of EP was abolished. It was also reported that ferulic acid ($C_{10}H_{10}O_4$) was liberated in this digest, and it was speculated that this component of EP might be directly related to PYF interactions. Nevertheless, no MW data for either fraction were provided.

Fujii and Horie (1975) isolated a factor from wort which caused the early flocculation of yeast during primary fermentation. The factor was a HMW glycoprotein with a negative charge called EFS1. The carbohydrate portion contained, in decreasing concentrations, galactose ($C_6H_{12}O_6$), arabinose,

glucose, xylose, and mannose ($C_6H_{12}O_6$), whilst the contents of polyphenol (i.e. large multiples of phenol structural units; C_6H_5OH) and inorganic and organic phosphorus (P) were 0.1%, 1.5% and 0.8% respectively. Approximately 0.7 g of the EFS1 factor was present per l of 10.8°P wort, but only 10 mg.l⁻¹ were required to induce PYF, whilst 20 mg.l⁻¹ or more of the EFS1 factor induced a distinct PYF pattern. EFS1 appeared to induce the early flocculation phenomenon at a lower concentration than the substances reported by previous researchers (Kudo, 1958, 1959; Kudo & Kijima, 1960; Morimoto *et al.*, 1975). Besides that, the EFS1 preparation was found to be highly homogeneous. Fujii and Horie (1975) observed that a HMW fraction prepared from three times more normal wort than the amount of early flocculent wort used to prepare EFS1, induced also early flocculation in the presence of LMW fraction of either normal or early flocculent wort. This suggested that the difference between normal and early flocculent wort was due to a difference in their contents of an “early flocculation-inducing substance”. It was also found that a mixture of 9 parts of normal malt and 1 part of early flocculent malt could induce the early flocculation phenomenon, suggesting that the level of early flocculent activity in PYF+ wort was very high, as well as that the phenomenon could not be prevented simply by using a mixture of a small amount of early flocculent malt and a much larger amount of normal malt. However, more recent studies (e.g. Nakamura *et al.*, 1997; Jibiki *et al.*, 2006) have shown blending away to be effective. Besides that, the success of blending away may lie in other factors such as the severity of PYF and whether or not surface washing is employed (see Section 1.3.3 for details). The fact that treatment with pronase, a nonspecific protease that breaks down most proteins

into individual amino acids, caused loss of activity suggested for the first time that the protein component of the EFS1 was directly related to PYF. Interestingly, the addition of trypsin or pepsin did not reduce the PYF activity.

In 1976 a small peptide (< 10 kDa), which was high in glutamic ($C_5H_9NO_4$) and aspartic ($C_4H_7NO_4$) acids, was shown to be associated with hung fermentations involving certain ale yeast strains (Stewart *et al.*, 1976). However, the fact that the peptide was insensitive to heat suggested that the hung fermentations were not due to true PYF, but rather due to another component in the fermenting medium. Fujino and Yoshida (1976), using concanavalin A-Sepharose affinity chromatography, reported that the substance responsible for the tailing fermentations was extracted from an acid polysaccharide and proposed that PYF was a kind of lectin-like coagulation. The lectin-like coagulation put forward from the former researchers (Yoshida *et al.*, 1979) involved the binding between a lectin-like protein, located on the surface of the yeast cells, and the substance from the wort inducing PYF. The same authors (Fujino & Yoshida, 1976) described also two additional PYF factors. The first factor, termed FA, when hydrolysed with 1 N hydrochloric acid (HCl) and chromatographed on paper chromatography, was found to contain glucuronic acid ($C_6H_{10}O_7$), glucose, galactose, mannose, xylose and arabinose. On the other hand, the second factor called FB, following the same treatment as FA, was found to contain glucose, galactose, mannose and arabinose. Both fractions (FA and FB) contained also an identified compound, and the same amino acid compositions; except that only FA contained cysteine ($C_3H_7NO_2S$). Following elemental analysis, it was also concluded that FA and FB fractions contained nitrogen, (3% and 4% respectively), suggesting that

they were glycoproteins. Treatment of the FB hydrolysates with pronase did not reduce its PYF activity, indicating that either the protein moiety was not directly associated with PYF activity or that the protein moiety was resistant to pronase degradation. Since FA was not the prominent PYF inducing factor, it was proposed that arabinoxylan was not directly responsible for the PYF activity. It was also demonstrated that premature flocculation caused by a polysaccharide-containing protein was delayed by the addition of the nonfermentable sugar α -methylmannoside ($C_7H_{14}O_6$) or various other fermentable sugars.

Yoshida *et al.* (1979) were the first to associate undermodified malts with PYF. They proposed that the application of pressure in the grain during steeping (2 kg of barely were suspended in 10 l of H_2O in a 12 l stainless-steel pressure vessel and were subjected three times to a pressure of $1.5 \text{ kg}\cdot\text{cm}^{-2}$ for a period of 10 s) was responsible for both the abnormal germination and premature flocculation. More specifically, it was suggested that the high pressure during steeping restricted respiration, possibly because parts of the embryonic organs were destroyed when the water was forced into the embryo. The impacts of high pressure during steeping were influenced by the degree and duration of pressure, the phase of steeping at which the grain experienced high pressures, and the barley variety used.

Axcell *et al.* (1986), studying the cause of poor fermentability ratings of certain malts, associated the occurrence of heavy, and sometimes PYF, hypothesized a factor originating from the malt husk and produced during steeping. More specifically, Axcell *et al.* (1986) proposed that at a certain stage

during steeping the turgor pressure within the embryo cells may be such that the fluid “leaks” out of the cells and subsequently becomes associated with the husk. Thus, during mashing the factor in this exudate is extracted from the husk and subsequently affects yeast flocculation. The same authors also suggested that with lower steep-out moistures this factor was not released to the same extent and subsequently was either removed with the rootlets during malt polishing or it remained in the kernel where it was further metabolized during germination. Besides that, Axcell *et al.* (1986) also showed that when husk extract from malt inducing PYF was added to wort made from “normal” malt, premature or heavy flocculation of the yeast occurred. On the other hand, when husk extract of the “normal” wort was added to a fermentation carried out with the non- PYF-inducing wort, it did not alter the flocculation profile, suggesting that “normal” malt husk extract lacks factor(s) causing PYF. However, further experiments showed that addition of higher concentrations of “normal” malt husk extract resulted in premature flocculation.

Herrera and Axcell (1989) investigated the effect of barley lectins (i.e. sugar-binding proteins or glycoproteins of non-immune origin that agglutinate cells or precipitate glycoconjugates) on yeast flocculation. This was initiated by previous results which suggested that the isolated husk factor(s), putatively responsible for PYF, bound to yeast cell walls (Fujino & Yoshida, 1976). On the basis that barley contains lectins, which by definition bind specific sugars, the authors proposed that lectins, if present in adequate concentrations, would produce premature flocculation by binding to yeast cell walls. The first part of their research concluded that a barley lectin, isolated by affinity chromatography and shown to survive both the malting and brewing processes,

was not implicated in premature flocculation. In the second part of their study, Herrera and Axcell (1989) used yeast that had undergone premature flocculation as a form of affinity chromatography column to isolate the factor(s) in malt husk responsible for premature flocculation. Glucose, mannose, α -methylmannoside, and N-acetylglucosamine ($C_8H_{15}NO_6$) were able to release the factor(s) from prematurely flocculated yeast. These factors caused the early flocculation of a lager yeast strain (*Saccharomyces uvarum* 2036) when added back to normal fermentations. Furthermore, PYF could be prevented by treating (i.e. malt extract was incubated with 200 mM solutions of monosaccharides for 1 h at room temperature before pitching), the flocculation factors from malt husk with the above sugars. Lactose ($C_{12}H_{22}O_{11}$), however, was unable to release any flocculation factor(s) from the yeast and was also unable to prevent early flocculation when incubated with the malt husk factor, suggesting that the factor had lectin-like sugar specificities. Thus, whilst Fujino and Yoshida (1976) proposed that the substance producing PYF, an acid polysaccharide-protein, was binding to the lectin-like proteins located on the cell wall, Herrera and Axcell (1989) suggested the opposite. That is, that the substance causing PYF is likely to be a type of lectin that binds to sugars on the yeast cell wall. Herrera and Axcell (1991a) reported that a malt husk extract termed CMHE, which was easily obtained after a mild aqueous extraction procedure, was associated with PYF activity during lager yeast (*Saccharomyces uvarum* 2036) fermentations. CMHE contained a HMW polysaccharide (PAS I) and four protein bands of varying molecular size. PAS I had a MW > 100 kDa and following paper chromatography analysis, and GC-MS quantification, was found to contain arabinose (27%), xylose (17%),

mannose (17%), galactose (16%), rhamnose ($C_6H_{12}O_5$; 14%) and glucose (12%) together with an acidic sugar component. SDS-PAGE electrophoresis, followed by staining with Coomassie Brilliant Blue of the protein migration patterns, indicated MW of approximately 42.6 kDa for the higher protein band and approximately 13.1, 15.1 and 17.5 kDa for the three bands of lower MW. The proteins present in CMHE husk extracts were also found to be components of normal worts (i.e. worts not inducing PYF). PAS I and the different MW proteins were separated using gel filtration chromatography and the PYF activity of the individual components were tested using the same lager yeast strain (*S. uvarum* 2036). Whilst addition of the different protein components of CHME to fermentations in control wort did not affect the flocculation pattern of *S. uvarum* 2036, supplementation of the control wort with 30 mg of PAS I induced heavy flocculation. Interestingly, PYF was heavier after the supplementation of the PAS I to control worts, than was the case with fermentation profiles obtained after the supplementation of CHME malt extract (containing both PAS I and the various MW proteins). The properties (i.e. solubility and ease of extraction using H_2O) and sugar composition of the malt husk extract (PAS I) led Herrera and Axcell (1991a) to propose that PAS I was a gum type polysaccharide, rather than a hemicellulose as the latter does not dissolve in H_2O . Besides that, the presence of multiple sugars in significant amounts suggested that PAS I consisted of more than one polysaccharide. Herrera and Axcell (1991b) used immunogold electron microscopy to demonstrate that their isolated HMW polysaccharide (PAS I; Herrera and Axcell, 1991a) bound significantly to the surface of flocculent yeast cells grown in a PYF+ wort. On the other hand, cells incubated with anti-42.5 kDa

protein and anti-LMW protein antibodies showed weak binding to the surface of the same flocculent yeast. The same authors, using enzyme-linked immunosorbent (ELISA) assays showed that the protein components of the PYF factor(s), either high or LMW weight in size, were present in similar concentrations in premature flocculent and normal wort. On the other hand, the ELISA mean absorbance values for the CMHE PAS I component were approximately 65% higher than those for regular wort (AMHE). The results obtained confirmed previous results of the same authors (Herrera & Axcell, 1991a) suggesting that PAS I was the premature flocculation factor, as well as that PAS I was binding to the yeast cell in a lectin-like type of interaction. However, later studies of Axcell *et al.* (2000) identified a protein fraction which by binding to the yeast cells was causing their premature flocculation. The protein was found in the outer tissues of barley malt and could be obtained through simple water-washing of the whole grain. The fact that the protein could not be found on the surface of the barley, but was produced by the grain during the steeping process, led Axcell and co-workers to suggest that PYF might have its origins in microbial contamination of the grain. The molecular weight and basic nature of the protein was reminiscent of barley lipid transfer protein – produced by the plant in response to a particular stress either in the field or in the malting plant. Through this observation the authors proposed the “antimicrobial peptide theory” of PYF (Section 1.3.2.2). Van Nierop *et al.* (2004) also concluded that the breakdown of malt husk arabinoxylans by fungal enzymes, thought to be produced by the fungi in order to generate assimilable nutrients, resulted in the formation of the PYF factors. More specifically, it was shown that PYF⁺ compounds could be enzymatically

generated through the addition of glucanases and xylanases to malt husks prior to mashing. However, interestingly, it was found that the PYF activity was lost in the event of excessive arabinoxylan degradation. Van Nierop *et al.* (2004) further suggested that the arabinoxylan components must be of a particular size in order to induce PYF. The same authors also showed that the removal of the husk from previously PYF+ malts resulted not only in the removal of PYF, but also in delayed flocculation, thus supporting the notion that a certain component of the husk is required for normal flocculation performance; Herrera and Axcell (1991b), while sugar uptake remained comparable to the PYF+ control malt. Van Nierop *et al.* (2004) also showed that malt husk factors pre-treated with extracellular fungal extracts (i.e. *Aspergillus niger*) displayed PYF+ activity. Besides that, in contrast with the results obtained by Yoshida *et al.* (1979), the addition to wort of arabinoxylans from wheat endosperm (which have a lower glucuronic acid content than barley arabinoxylans) induced PYF. Following on from the “antimicrobial peptide hypothesis” (Axcell *et al.*, 2000; Van Nierop *et al.*, 2004), Van Nierop *et al.* (2008) proposed that antimicrobial compounds, and in particular antiyeast compounds (e.g. α -thionin, LTP-1a and other ns-LTP’s) extracted from malt using 0.05 M sulphuric acid (H_2SO_4), were responsible for PYF fermentations and/or gushing, a quality defect of finished beer long associated with infection by *Fusarium* spp. on barley and the presence of hydrophobic peptides (see Chapter 3 for further details).

Koizumi *et al.* (2008) purified a PYF factor by using yeast as an affinity column with which to concentrate the factor and then fractionating the eluted extract using anion-exchange chromatography. The purified factor was

composed mainly of arabinose and xylose, with some galactose, glucose, rhamnose and galacturonic acid ($C_6H_{10}O_7$) and was described as ‘pectin-like’ material (Koizumi *et al.*, 2008). The MW of the active polysaccharide was estimated to be < 40 kDa and, interestingly, it was shown that when the factor was digested, using Sanzyme 1000 (containing various carbohydrate hydrolases including β -xylosidase, β -galactosidase, β -glucosidase, cellulose, (1 \rightarrow 4)- β -xylanase and (1 \rightarrow 3)- β -glucanase), the PYF activity was retained even in fractions with MW < 5 kDa. Concanavalin A affinity chromatography was used to identify the minimum digested unit that possessed PYF activity, and it was found that as little as 0.3 ppm of this fraction could induce significant PYF. This result was in disagreement with Van Nierop *et al.* (2004) who proposed that the PYF activity could be lost upon subjecting the barley husk to excessive enzymatic digestion (e.g. by using endo-xylanase M3 from *Trichoderma longibrachiatum*). Nevertheless, Koizumi *et al.* (2008) proposed that relatively small fragments of PYF factors may bridge cells together through Ca^{2+} ion bridges, as seen with pectin (a chain-like polymer of α -D-galacturonic structural units joined by 1 \rightarrow 4 linkages; Belitz *et al.*, 2004). The fact that the MW of the PYF factor(s) was estimated to be < 40 kDa, as opposed to previous studies of Herrera and Axcell (1991a) who estimated the MW of the PYF factor(s) to be > 100 kDa, was presumed to be due to the differences in the purity of the PYF factor(s). One possible reason for the discrepancy in reports of the active MW range of the PYF bridging polysaccharide might be that the activity is dependent on the charge of the fragments as well as molecular size – in which case the uronic and glucuronic acid contents of the factors might be a significant variable.

Most recently, Koizumi *et al.* (2009), using linkage analysis, suggested that the PYF factor was a complex polysaccharide mainly composed of a highly substituted glucuroarabinoxylan-associated arabinogalactan protein with rhamnogalacturonan I, as is seen in maize and rice seed. They also postulated, that in such a complicated substance both the PYF-active and inactive polysaccharides, separated by concanavalin A affinity chromatography, were present suggesting that the PYF activity may result from minor structural changes. The same authors, upon enzymatic digestion and separation of the PYF factor(s), using anion exchange and concanavalin A affinity chromatography, suggested that severe PYF was inducible at a PYF-active polysaccharide concentration as low as 30 ppb. Besides that, it was also proposed that there were no differences between the PYF factor prepared from a North American cultivar and that from a Japanese cultivar, suggesting that the structural feature of the PYF factor might be conserved across samples. In addition, Koizumi *et al.* (2009) proposed that there are two possibilities for the production of PYF-active polysaccharides. One is that PYF-active polysaccharides are synthesized from a PYF-inactive polysaccharide during ripening or malting by enzymes such as glycosyltransferases which might be produced endogenously or secreted by fungi on the surface of the grain. Alternatively, PYF-active polysaccharides may pre-exist in barley husk. In this case, formation of normal malt or PYF depends on whether the polysaccharide is extracted into the wort. It is also possible that an endogenous barley enzyme could work in concert with secreted fungal enzymes.

A summary of historical developments in the attempt to identify PYF factors is included in Table 1.2.

Table 1.2: History of factors indicated or associated with premature yeast flocculation since 1960 (Partially reproduced from Lake and Speers, 2008).

Factor Description	Size ^a	Effect	Year
Barnigen: humic acid-like substance containing ash (11%), carbon (47.56%), hydrogen (4.92%), nitrogen (3.14%)	HMW	Caused flocculation in buffered solution	1958 & 1960
Treberin: gum based polysaccharide containing glucose, xylose, and arabinose	Not given	Associated with PYF	1959
EPS1: glycoprotein with a negative charge; sugar components in decreasing order: galactose>arabinose>glucose>xylose>mannose	HMW	Associated with PYF	1975
EP: a mixture of arabinoxylan, α -glucan, and glycoprotein consisting of two polysaccharides	HMW	Associated with PYF	1975
G-50: gum based polysaccharide containing arabinose (44%), xylose (34%), glucose (15%), and an unidentified component (7%)	HMW	Associated with PYF	1975
Peptide high in glutamic and aspartic acids	< 10 kDa	Associated with hung fermentation; not specifically PYF	1976
FB: glycoprotein composed mainly of glucose, galactose, and mannose with traces of xylose and arabinose; minor amount of nitrogen constituents also detected	Not given	Associated with PYF	1976
FA: glycoprotein composed mainly of mannose, xylose, arabinose with traces of galactose, and glucose; nitrogen with uronic and ferulic acid also detected	Not given	Associated with slight PYF	1976
Barley lectin	MW = 20.7 kDa	Not associated with PYF	1989
PAS I: gum based polysaccharide composed of arabinose (27%), xylose (17%), mannose (17%), galactose (16%), rhamnose (14%), and glucose (12%), with an acidic sugar component	> 100 kDa	Associated with PYF	1991
Lipid transfer protein	MW \approx 10 kDa	Associated with PYF	2000
Arabinoxylan products of husk degradation by endo-xylanase and <i>Aspergillus niger</i>	HMW	Associated with PYF	2004
Complex polysaccharides containing arabinose (31%), xylose (21%), galactose (12%), rhamnose (9%), and mannose (3%)	\approx 40 kDa	Associated with PYF	2004
Malt extracts tentatively identified as antimicrobial peptides (i.e. α -thionin, LTP1a and possibly other ns-LTPS)	Not given	Associated with PYF,	2008
Pectin-like polysaccharides: composed mainly of arabinose, xylose, and galactose, with rhamnose and galacturonic acid	< 40 kDa	Severe PYF	2008
Complex polysaccharides: composed of a highly substituted glucuronoarabinoxylan-associated arabinogalactan protein with rhamnogalacturonan I	< 40 kDa	Severe PYF	2009

^a MW = molecular weight; HMW = high molecular weight

1.3.2 Theories Associated with the Occurrence of PYF

The periodic occurrence of PYF has been associated with certain harvests, years and regions of barley production (Armstrong & Bendiak, 2007). PYF arises during brewery fermentations; however the causative factor(s) have been shown to originate from the malted barley (Herrera & Axcell, 1991a). The link between the incidence of PYF and particular harvest conditions suggested the likely involvement of barley and malt microbes in PYF (Axcell *et al.*, 2000), and since it has been shown that surface washing of PYF+ malt can diminish the severity of PYF (Van Nierop *et al.*, 2004; Jibiki *et al.*, 2006), the action of microbes on the barley husk has been a key focus of research (Van Nierop *et al.*, 2004; Van Nierop *et al.*, 2006; Van Nierop *et al.*, 2008). In addition, PYF activity could be induced by treating the barley husk with fungal enzyme extracts (Van Nierop *et al.*, 2004). Two main theories have been proposed to account for this phenomenon. These have largely been based upon the characterisation of purified extracts from PYF+ malts (as reviewed in Section 1.3.1.2), which retain PYF activity, coupled with process knowledge and theories as to how the isolated factors might arise. Here we shall refer to these theories as “The Bridging Polysaccharide Mechanism” and “The Antimicrobial Peptide Hypothesis”.

1.3.2.1 The ‘Bridging’ Polysaccharide Mechanism

Van Nierop *et al.* (2006) proposed that ‘wet’ conditions on the grain’s surface either in the field, due to higher rainfall, or during steeping will dramatically increase the grain’s microbial load (Figure 1.5A). Although barley’s microflora (bacteria, wild yeast and filamentous fungi originating from the air and the soil; Van Nierop *et al.*, 2006) varies from region to region, microbes, and in

particular fungi, will secrete enzymes (e.g. proteinases, endo-xylanases, β -glucanases) in an attempt to generate nutrients for assimilation and this in turn facilitates the breakdown of the grain's outer layers. Cellulose is relatively resistant to enzymatic degradation and as a consequence does not appear to be associated with PYF (Morimoto *et al.*, 1975). Therefore, the degradation of the barley husk, predominantly comprising arabinoxylans and cellulose (Van Nierop *et al.*, 2004), will produce predominantly a wide range of acidic HMW arabinoxylans (Figure 1.5B and 1.5C).

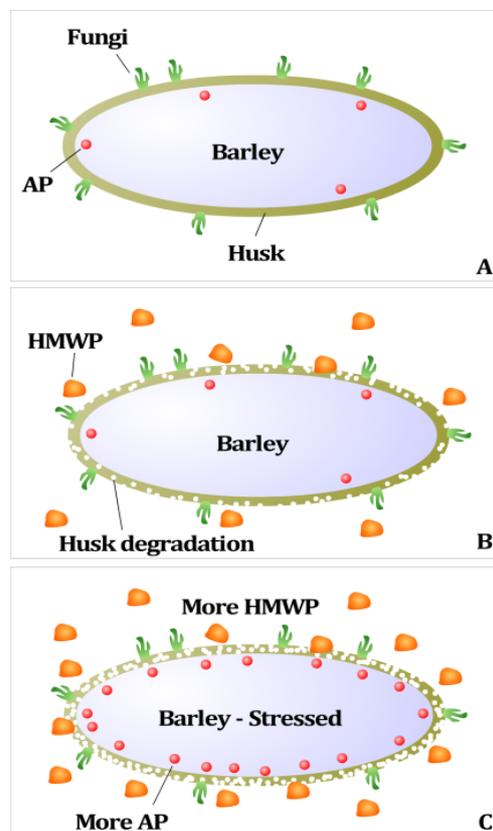


Figure 1.5: Proposed mechanism of premature yeast flocculation factor(s) generation from barley husk by fungi (Adapted from Van Nierop *et al.*, 2004). Initial infestation by fungi (A), fungal enzymatic degradation of the husk (B) and production of more antimicrobial peptides (AP) by barley (C). HMWP = high-molecular-weight-polysaccharides.

The HMW polysaccharides are hypothesized to bind to the yeast cells via zymolectins, adhering glycoproteins which act as cell-surface receptors and are activated upon wort sugar's depletion; Kaur *et al.* (2009), leading to cross-bridging between adjacent yeast cells (Figure 1.6). The cross-bridging is suggested to lead to the formation of flocs that accelerate sedimentation and the effective removal of the yeast from the fermenting wort. Koizumi and Ogawa (2005) proposed that PYF is caused by the formation of larger-than-normal cell clumps mediated by the PYF factor, which results in faster-than-normal sedimentation.

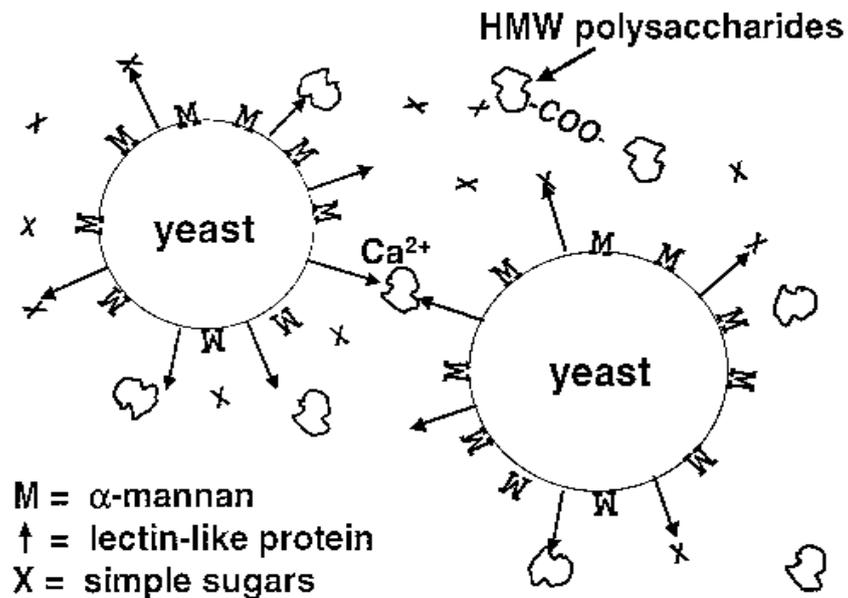


Figure 1.6: Proposed mechanism of premature yeast flocculation by high-molecular weight polysaccharides (Adapted from Van Nierop *et al.*, 2004).

1.3.2.2 The ‘Antimicrobial Peptide’ Hypothesis

Although commonly referred to as the ‘antimicrobial peptide’ hypothesis (Axcell *et al.*, 2000; Van Nierop *et al.*, 2004), the origin of such peptides implicated in PYF has never been categorically proven (Lake & Speers, 2008; Porter *et al.*, 2010). Barley in the field and/or in the maltings responds to

microbial attack by producing basic peptides (e.g. thionins, defensins and non-specific lipid transfer proteins) with antimicrobial properties. Thionins, defensins and ns-LTPs are cationic antimicrobials which are relatively small (5 – 10 kDa), stable due to multiple disulphide bridges and capable of persisting through both the malting and brewing processes. Van Nierop *et al.* (2004) proposed that the antimicrobial peptides are not only active against the barley microflora, but may also have anti-yeast activity. Amphipathic polypeptides are able to disrupt membrane integrity and function and may impair sugar uptake by yeast during industrial fermentations, thus contributing to abnormally high residual extract and problems with poor attenuation. It has been suggested that their action on membranes leads to cell lysis (Van Nierop *et al.*, 2006) and that they may disrupt yeast membrane integrity, leading to impairment of sugar uptake and resulting in leakage of cell constituents. Impairment of sugar uptake may result in an induction of the starvation response in yeast which has been linked to the regulation of the onset of flocculation (Axcell, 2003). Axcell (2003) proposed that the antimicrobial peptides can cause poor attenuation, but they do not necessarily give rise to premature flocculation. This is facilitated by the action of the HMW polysaccharides (i.e. natural materials associated with the husk or result from the degradation by bacteria or fungi of the external tissues of barley husk). More specifically, the acidic residues (e.g. glucuronic acid) of these HMW carbohydrates might bind to the cationic antimicrobial peptides and act as ‘pseudo fimbriae’ which then cross-link with other yeast cells, generating flocs, and giving rise to PYF (Figure 1.7).

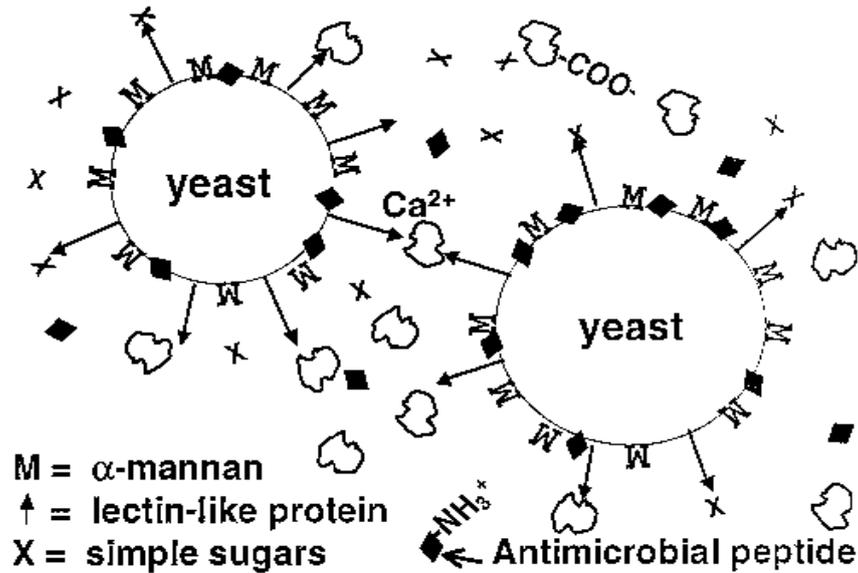


Figure 1.7: Proposed mechanism of premature yeast flocculation by HMW polysaccharides in association with antimicrobial peptides (Adapted from Van Nierop *et al.*, 2004).

This may explain the phenomenon in high-glucose worts, reported by Axcell *et al.* (2000), where substantial quantities of maltose and maltotriose remained in the fermented wort. Under such conditions, PYF is not normally observed, but worts of low fermentability are produced. In this case perhaps the residual sugars are blocking the lectins and preventing cell-to-cell aggregation. However, as these fermentations do not attenuate properly, antimicrobial peptides may still bind to yeast cells interfering with sugar uptake (Axcell, 2003). Besides a possible direct effect, Van Nierop *et al.* (2008) indicated that the antimicrobial peptides can also have an indirect impact on the final product due to microbial infection (e.g. mycotoxin contamination, introduction of off-odours, inconsistency in brewhouse performance leading to slower processing and flavour instability as well as haze in beer).

Despite the current theories regarding the occurrence of PYF further work is required to verify them and to ascertain whether PYF arises from one or a

combination of factor(s) present in PYF+ worts. However, both of the foregoing hypotheses explain most of the observations reported previously in the literature and go some way towards explaining why both the malting and the brewing process may either minimise or accentuate these problems. For example, one malting plant may provide more anaerobic conditions than another, and this may lead to the rapid growth of certain microorganisms and generate a response by the germinating barley. In another example, wort produced in one brewery may contain more lipid material than one from another location and this lipid may then be able to 'titrate' out the antimicrobial peptides so that they cannot subsequently bind to the yeast (see Chapter 4 for further consideration of this suggestion).

1.3.2.3 The Effect of the Malting Process on the Incidence of PYF

Despite the fact that the sporadic occurrence of PYF has been associated with the presence of fungi on the surface of grains, the effect of the steeping process on the barley grains has also been implicated in the occurrence of "tailing" fermentations (Yoshida *et al.*, 1979; Axcell *et al.*, 1986). More specifically, Yoshida *et al.* (1979) demonstrated that when barley was subjected to higher pressure during steeping, respiration was restricted leading to under modified malts and PYF worts during fermentation. The Japanese malt varieties (Betzes and Fuji Nijo) tested by Yoshida *et al.* (1979) responded differently to the applied pressure. More specifically, Betzes was more susceptible when PYF was initiated under one application of pressure at 1 kg.cm^{-2} for 10 s, whereas three applications of pressure at $1.5 \text{ kg.cm}^{-2} 10 \text{ s}^{-1}$ for three subsequent times only induced slight PYF in Fuji Nijo malts. In addition, a slight increase in water absorbed by the kernel was also noted with the application of pressure.

Higher steeping pressure increased the moisture content of the endosperm and the embryo. Thus, Yoshida *et al.* (1979) suggested that the steeping pressure may significantly affect the quality and quantity of the polysaccharide fractions responsible for PYF. However, Lake and Speers (2008) indicated that it is difficult to determine whether the increased pressure produced PYF factor(s) or simply caused poor quality malt that displayed less than optimal fermentation. Axcell *et al.* (1986) also associated the poor fermentation profiles with the uptake of water during the steeping process. More specifically, they suggested that the PYF factor may be released during malting by forcing water into the grain at a late stage of steeping (see Section 1.3.1.2 for further details).

1.3.3 Strategies for the Alleviation or Prevention of PYF

Several studies have concluded that the PYF factor(s) are water extractable and consequently may be easily removed from the surface of the grain by simple washing (Axcell *et al.*, 1986; Axcell *et al.*, 2000; Jibiki *et al.*, 2006). Jibiki *et al.* (2006) reported that surface washing and drying of malts led to a substantial improvement in the suspended yeast cell counts of PYF+ fermentations, although these were still only around 50% of the cell counts for the PYF negative control. In agreement with this observation, Axcell *et al.* (2000) proposed that wet milling of malt before mashing as well as the discarding of steep water may alleviate the problem. Where available, the use of a washing screw or washing drum in the maltings prior to steeping can clean the surface of the grain and reduce the microbial loading entering the malting process (Panteloglou *et al.*, 2012).

In addition to issues surrounding barley quality and surface washing of the grain, the PYF status of malts has been reported to be sensitive to process conditions in the maltings (Axcell *et al.*, 1986). Irrespective of the origins of PYF this should not come as a surprise since the operational conditions employed in a maltings (e.g. process temperatures, airflows, hydrostatic pressures) have a strong influence on both microbial growth and the stress experienced by malting barley and its consequential stress response in the form of anti-microbial peptides. Axcell *et al.* (1986) investigated a situation where the incidence of PYF was specific to the maltings at which a South African barley (variety *Clipper*) was malted. By transferring samples between two maltings at various steps of the process it was ascertained that in this specific instance the problem originated in the steeping process at Caledon maltings. It was then hypothesized that high pump pressures during steep-out might trigger the leakage of a factor which might otherwise have remained in the kernel and been metabolised during germination. Walker *et al.* (2008) commented on the significance of maintaining aerobic conditions during malting, and in particular suggested the adequate carbon dioxide extraction during air-rests and through maintaining fresh, as opposed re-circulated, air during germination. Based upon the observation that turbid worts (i.e. those with higher lipid content) offered some protection against PYF relative to the use of very bright worts, Axcell *et al.* (2000) proposed that wort fatty acids might bind to the amphipathic antimicrobial peptides and effectively ‘titrate’ them out (see Chapter 4 for further consideration).

Other practical strategies available to the brewer faced with a consignment of PYF+ malt include the option to blend. Results presented by Jibiki *et al.*

(2006), as opposed to the results of Fujii and Horie (1975); Section 1.3.1.2) indicated that the blending of PYF+ wort with PYF- wort alleviated the severity of PYF in some instances and at low ratios of PYF+ malt (10 or 25%). It was an interesting feature of their results that ability to blend away the issue satisfactorily was highly dependent upon the specific PYF+ sample utilised. Nakamura *et al.* (1997) commented that where the practical blend ratio of PYF+ malt had been limited to 5%, the ‘dead-stocks’ of PYF+ malts at his brewery had swollen. As a practical measure to brew acceptable quality beer with higher blend ratios two steps were recommended. Firstly a protocol labelled ‘green transfer’ wherein brews based on > 50% PYF fermentation were mixed after 7 days of fermentation in a ratio of 3:1 with PYF- fermentation 3 days post pitching. This protocol increased suspended yeast cell counts during maturation and eased problems with vicinal diketone (VDK) maturation. Secondly, an increase in fermentation temperature (from 10 to 12.5°C) was reported to improve assimilation of VDK and hence offer another potential practical strategy for brewing with higher proportions (40%) of PYF+ malt.

According to the ‘bridging polysaccharide’ hypothesis (Fujino & Yoshida, 1976) the induction of PYF is associated with interactions between lectin-like proteins located on the yeast cell surface and part of the polysaccharide inducing PYF. Thus, Axcell *et al.* (2000) proposed that the rousing of yeast cells and/or the increase of pitching rate might leave sufficient normal yeast cells to complete the fermentation. In this context it is interesting that Armstrong and Bendiak (2007) noted in their practical experiences of brewing with PYF+ malts in New Zealand, that the same malt which presented PYF in

industrial-scale batch fermentations could perform normally in another brewery which operated a stirred continuous fermentation (Coutts') process. In the same paper it is stated that rousing of yeast after the incidence of PYF achieved nothing – the yeast appearing 'turned off' and no longer interested in the remaining fermentables! Whether this statement applies to all instances of PYF is not clear and may well depend upon the type of PYF encountered.

Sugihara *et al.* (2008) reported the use of tannic acid to alleviate PYF issues in brewery fermentations. The mode of action was not related to wort clarity, but appeared to be linked to the ability of tannic acid to bind to the yeast cell surface during fermentation and thus disrupt flocculation. Addition rates of 25 – 100 mg.L⁻¹ were effective in increasing suspended yeast cell counts and lowering residual extract in fermentations using two PYF+ malts, each blended at 30% of grist. In addition, Axcell *et al.* (1986) suggested that due to the risk involved, the purchase of malt from a supplier whose malt repeatedly gives poor ratings should be avoided wherever possible.

Chapter 2

Materials and Methods

2.1 Yeast Strains

Three lager (W34/70, SMA and Industrial) and three ale (NCYC 1332, NCYC 2359 and M2) brewing yeast strains were used in this study. The ale yeast strains and W34/70 (ex Weihenstephen) were obtained from the National Collection of Yeast Cultures (NCYC), the SMA from the VLB Research Institute (Berlin, Germany) and the 'Industrial' yeast strain was provided by a large multinational brewing company. The ale yeast strains were selected to exhibit varying degrees of flocculence. W34/70 is a medium flocculent yeast strain, whilst SMA is a highly flocculent strain. The Industrial lager yeast strain was of interest because it was thought to be relatively insensitive to PYPF.

2.2 Growth and Storage

2.2.1 YPD (Yeast Extract-Peptone-Glucose)

Yeast strains were maintained and grown on YPD (1% [w/v] yeast extract, 2% [w/v] neutralised bacteriological peptone, 2% [w/v] glucose) media. All media components were supplied by Fisher Scientific (Fisher Scientific UK Ltd, Loughborough, UK). Media were prepared using RO water and were steam sterilised immediately following preparation by autoclaving at 121.1°C and 29.8 Psi for 15 min in an Astell autoclave (Astell Scientific, Kent, UK).

2.2.2 Slope and Plate Storage of Yeast Strains

Yeast strains were grown on YPD slopes and YPD plates at 25°C, for later storage at 4°C on YPD slopes and YPD plates prior to use. Slopes were prepared by making 2% [w/v] YPD agar and aliquoting 10 ml volumes into 25 ml sterile glass universal bottles. The bottles were rested at an angle to set as a

slope. The YPD plates were also made using 2% [w/v] YPD agar which was poured into sterile petri dishes and allowed to set.

2.2.3 Cryogenic Storage of Yeast Strains

Stock cultures of each strain were cryogenically maintained in cryovials (Fisher Scientific UK Ltd, Loughborough, UK). Yeast cells were grown aerobically on YPD and were re-suspended in YPD containing 25% [v/v] glycerol as a cryoprotectant to maintain cell viability. Following that, the tubes were stored in a freezer at -80°C.

2.2.4 Yeast Propagation

Cell suspensions were achieved by selecting representative colonies from YPD slopes and inoculating into cooled (25°C) autoclaved YPD media in two stages. For the first stage of propagation, a loop of yeast cells was aseptically transferred into 10 ml YPD in 25 ml sterile universal bottles. Cultures were aerobically propagated at 25°C for 24 h in a Certorat BS-1 shaken incubator (Sartorius UK Ltd, Surrey, UK) at 120 rpm. The transfer of the yeast cells in the second stage of propagation took place whilst cells were in the log phase (after 24 h of propagation). Cells at the log phase (10 ml) were transferred aseptically into sterile YPD (100 ml) in 250 ml pre-sterilised conical flasks fitted with non-absorbent cotton wool plugs covered in aluminium foil. Following that, the culture (110 ml) was aerobically propagated for a further 72 h at 25°C with continuous shaking at 120 rpm.

2.3 Cell Density and Viability Determination of Yeast Populations

Cell counts were performed using methylene blue stain. Methylene blue (10 mg; Hopkin & Williams Ltd, London, UK) and sodium citrate (2 g; Fisher

Scientific UK Ltd, Loughborough, UK) were diluted in sterile RO water to a final volume of 100 ml). Cells were added to methylene blue stain at a ratio 1:6, and following a static incubation of 5 min at room temperature cell counting was performed in a Neubauer counter chamber (haemocytometer) with improved ruling (Weber Scientific International Ltd, Hamilton, USA) at a $\times 40$ magnification according to the method of the Society (ASBC, 2004). Viable cells remained unstained, whilst non-viable cells were stained blue. At least 300 cells were counted in order to calculate cell density. The total number of yeast cells per ml of yeast culture or fermentation broth was calculated using Equation 2.1.

Equation 2.1: Formula for the calculation of cell density of yeast cultures and fermentation broths.

$$\text{Number of cells/ml} = \frac{a + b}{2} \times 5 \times 10^4 \times \text{dilution factor}$$

Where: a = number of yeast cells in the upper area of the haemocytometer

b = number of yeast cells in the lower area of the haemocytometer

The number of viable cells was expressed as a percentage of the total population (Equation 2.2).

Equation 2.2: Calculation of the percentage viability of yeast cell populations.

$$\text{Viability (\%)} = \frac{\text{total cells} - \text{dead cells}}{\text{total cells}} \times 100$$

To eliminate the possibility of counting some yeast cells twice, the counting technique was standardized. Cells touching or resting on the top and right

boundary lines of the haemocytometer were not counted, whilst cells touching or resting on the bottom or left boundary lines were counted. Yeast cells that were budded (daughter cells) were counted as one cell if the bud was less than one-half the size of the mother cell and as two cells when the bud was equal or greater than one-half the size of the mother cell (Section 2.6.3). To obtain an accurate yeast cell count, no fewer than 75 cells on the entire (1 mm²) ruled area and no more than about 48 cells in one of the 25 squares were counted. Counts from both sides of the slide agreed to within 10%.

2.4 Samples

2.4.1 Barley and Malt Samples

Malt samples (Table 2.1) used in this study were sourced from various malting and brewing companies around the world and were either brewery PYF+ or samples chosen to be their controls.

Table 2.1: Barley and malt samples used in this study.

Barley Variety	Harvest Year	Region of Production	PYF Abbreviation
Scarlett	2007	France	PYF1+
Prudentia	2007	Spain	PYF1-
Scarlett	2007	France	PYF2+
Scarlett	2007	France	PYF2-
Quench	2009	U.K	PYF3+
Prestige	2009	Europe	PYF3-
Nectararia	2009	Hungary	PYF4+

2.5 Wort Preparation

2.5.1 Mash Bath Calibration

Prior to full operation, the Brewing Research Foundation mash bath (Brewing Research Foundation, Surrey, UK) was calibrated so as to achieve the required time-temperature profiles using RO water.

2.5.2 Mill Calibration

The Bühler Miag disk mill DLFU (Bühler AG, Uzwil, Switzerland) was calibrated and adjusted for coarse grinding (1 mm) according to the Analytica-European Brewery Convention (EBC) method (EBC, 2006).

2.5.3 Mashing, Filtration and Wort Stabilization

Mashing was performed in a Brewing Research Foundation mash bath (Brewing Research Foundation, Surrey, UK) to give an all-malt wort with a gravity of 11°P unless otherwise stated. Barley malt (75 ± 0.1 g) was milled to a flour consistency in a Bühler Miag disk mill DLFU (Bühler AG, Uzwil, Switzerland) with 1 mm gap between the discs. Milled malt (70.0 ± 0.5 g) was placed in a 500 ml stainless steel beaker containing 360 ml brewing liquor (1.2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; Sigma-Aldrich Co., Dorset, UK) in RO water adjusted to pH 2.9 – 3.1 with 10% w/w lactic acid (Fisher Scientific UK Ltd, Loughborough, UK) pre-heated at 63°C. After a period of 60 min at 63°C, the mash was raised to 72°C at a rate of 1°C per min, and was maintained at that temperature for 25 min. Following that, the temperature was raised to 76°C (1°C per min) where it was maintained for 5 min. Consequently, the mash was cooled to room temperature (25°C) and was filtered through 320 mm grade 1 Whatman folded filter papers (Whatman Plc, Kent, UK). Without disturbing the cake, the first

100 ml of the filtrate were returned into the funnel (Fisher Scientific UK Ltd, Loughborough, UK) and when the cake appeared to be dry, after approximately 2 h, a further 100 ml of sparging water (prepared as mash water) at 68°C were added to the funnel. After filtration, the wort was gently boiled for 1 h and re-filtered to separate the “hot trub” (denatured proteins which have precipitated from the boiled wort together with polyphenols and other relatively hydrophobic insoluble matter). The specific gravity of the wort was determined using a DMA 5000 M model Anton Paar density-alcolyzer meter (Anton Paar GmbH, Graz, Austria) at $20 \pm 0.1^\circ\text{C}$ and the gravity was adjusted to the required °P with RO water using Equation 2.3. The wort was then stabilized by autoclaving in an Astell autoclave (Astell Scientific, Kent, UK) at 121.1°C and 29.8 Psi for 15 min. The sterilized wort was stored at 4°C for no longer than two weeks or in a freezer at -20°C until further use.

Equation 2.3: Formula used to calculate the dilution water required to standardize wort gravity.

$$\text{Volume of RO water (ml)} = \frac{V_w - G_w}{G_f}$$

Where: V_w = the volume (ml) of the wort after 1 h of boiling

G_w = the gravity (°P) of the wort after 1 h of boiling

G_f = the required gravity (°P) of the wort for fermentation

2.5.4 Analyses conducted on Wort Samples

The carbohydrate and free amino nitrogen (FAN) compositions of worts prior to fermentation (Sections 2.6.3 and 2.6.4 respectively) were determined to ensure that any subsequent differences observed in the flocculation and/or

fermentation profiles were most likely not caused by major nutritional differences between wort samples.

2.6 Laboratory Scale Fermentations

2.6.1 Premature Yeast Flocculation (PYF) Assay

The in-house PYF assay involved running small-scale fermentations and was based on similar existing methods (Fujino & Yoshida, 1976; Van Nierop *et al.*, 2004; Van Nierop, 2005; Jibiki *et al.*, 2006). Yeast cells in the stationary phase (after 4 days of propagation) were aseptically transferred into 250 ml centrifuge tubes and were centrifuged at 5,000 rpm for 5 min at 15°C. Following centrifugation, the supernatant was discarded and the cells were re-suspended in an equal amount of sterile RO water to obtain a 50% [w/v] yeast slurry. Viable cell counts were performed using methylene blue stain as described in Section 2.3.1. The appropriate amount of 50% [w/v] yeast slurry (viability > 98%) to achieve a pitching rate of 20×10^6 live cells.ml⁻¹ wort (Equation 2.4) was added to 200 ml of autoclaved sterile wort in 500 ml Schott bottles (Fisher Scientific UK Ltd, Loughborough, UK). The mixture (wort and approximately 2 ml yeast slurry) was shaken 35 times (clockwise and anticlockwise) to oxygenate the wort (this will give approximate 8 ppm concentration of dissolved oxygen; Fisher, 2009) as described by Phaweni *et al.* (1992). Following shaking, the wort was allowed to stand for 5 min to allow the foam formed during shaking to drain. The mixture was swirled, to ensure suspension, and then transferred into 250 ml pre-sterilised dropping funnels (Fisher Scientific UK Ltd, Loughborough, UK; Figure 2.1). The funnels were plugged with non-absorbent cotton wool plugs and placed in a SANYO MIR-253 static incubator (SANYO GmbH, München, Germany) at

15°C for a total period of 96 h. A summary of the stages, from wort production to fermentation, of our in-house fermentation assay used to predict the PYF potential of malts is illustrated in Figure 2.2.

Equation 2.4: Calculation of the volume of yeast slurry needed to be added to 200 ml of wort to achieve the desired pitching rate (i.e. 15 or 20 × 10⁶ live cells.ml⁻¹).

$$\frac{\text{Pitching rate}}{\text{Viable cells per ml yeast slurry}} = \text{ml of yeast slurry per 200 ml wort}$$



Figure 2.1: Photograph of small-scale fermentations used in the PYF assay (Panteloglou *et al.*, 2011).



Figure 2.2: Experimental overview of the in-house small-scale fermentation assay used to predict the PYF potential of malt samples (Panteloglou *et al.*, 2011).

2.6.2 Premature Yeast Flocculation Assay Sampling

2.6.2.1 Determination of Cell Concentration in Suspension

Yeast cell density analysis was assessed at specific time intervals between 0 - 92 h post pitching by removing a 2 ml aliquot from the fermentation broth at a standard depth (4 cm) below the surface of the fermentations. Following dilution (0.5 ml fermenting wort: 2 ml sterile RO water) the cell density was assayed by measuring absorbance at 600 nm (A_{600}) using a UV/visible Cecil CE 2021 spectrophotometer (Cecil Instruments Ltd, Cambridge, UK).

2.6.2.2 Microscopic Yeast Cell Counting

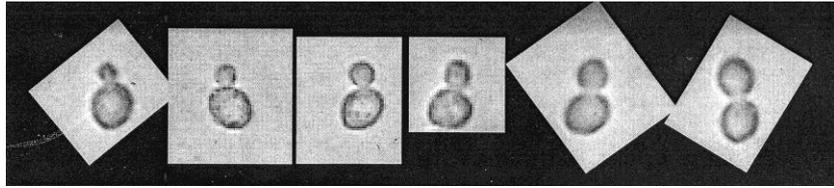
Yeast cell density was also on occasions assessed microscopically by removing a 2 ml aliquot from the fermentation broth at a standard depth (4 cm) below the surface of the fermentation and counting cells according to the method described in Section 2.2.4.1.

2.6.2.3 Budding Index

The percentage of the cells exhibiting a bud, termed budding index, was calculated using the Equation 2.5. The calculation of the budding index in the fermentation broths through fermentation progression is illustrated in Figure 2.3.

Equation 2.5: Calculation to determine the budding index of cells populations.

$$\text{Budding index} = \frac{\text{cells exhibiting a bud}}{\text{total number of cells}}$$



Budding Index	1	1	1	1	1	1
Cell Count	1	1	1	1	2	2

Figure 2.3: Example illustrating the calculation of the budding index in the fermentation broths obtained during fermentation progression (Image courtesy of Dr Stephen Lawrence, The University of Nottingham, UK).

2.6.3 Mini Fermentations

Fermentations were performed in glass hypovials (Figure 2.4) according to the method of Quain *et al.* (1985). 120 ml hypovials (International Bottle Company Ltd, Hertford, UK) containing a magnetic flea were autoclaved in an Astell autoclave (Astell Scientific, Kent, UK) at 121.1°C and 29.8 Psi for 15 min prior to use. 100 ± 1 ml of sterile 15°P, diluted with RO water from an initial 18°P all-malt wort, PYF+ and PYF- wort were aseptically transferred into pre-sterilised mini-fermenters.

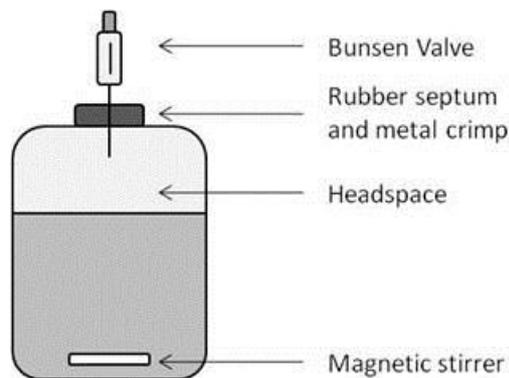


Figure 2.4: Miniature fermentation vessel schematic (Quain *et al.*, 1985).

Following wort addition, the hypovials were plugged with pre-sterilised non-absorbent cotton wool plugs and were saturated with air at 15°C in a SANYO

MIR 253 static incubator (SANYO GmbH, München, Germany) for a total period of 24 h. Yeast cells in the stationary phase, obtained from a 50% [w/v] yeast slurry with a viability > 98%, were added to 100 ± 1 ml wort (approximately 1 ml of 50% [w/v] yeast slurry) to achieve a pitching rate of 20×10^6 live cells.ml⁻¹ (20×10^9 live cells were added per 100 ml wort). Following pitching, the hypovials were sealed with suba seals and metal crimp seals using a hand-held crimper. Pre-sterilised needles were placed on the top of the fermenters so as to allow the building up of the pressure as well as the partial removal of the CO₂ during fermentation progression. Fermentations were conducted in a Sanyo MIR 253 static incubator (SANYO GmbH, München, Germany) at 15°C for a total period of 162 h with continuous stirring (180 rpm), unless otherwise stated, using a flatbed 15-place immiscible magnetic stirrer (Sigma-Aldrich Co., Dorset, UK). Fermentation progression was monitored by measuring weight loss, pH, gravity, ethanol yield, FAN and fermentable sugars (sucrose, fructose, glucose, maltose and maltotriose) over time.

2.6.3.1 Sampling from the mini fermentation vessels

At pre-determined time points (0, 3, 8, 18, 24, 40, 48, 68, 92, 124 and 162 h post-pitching) the fermentation vessels were opened and following mixing, unless otherwise stated, 1 ml aliquots were transferred into two separate 5 ml bijoux bottles. The bijoux bottles were kept on ice (4°C) for determination of the total and viable cells (Section 2.3.1) as well as for the calculation of budding index (Section 2.6.2.3). Following sampling, the remaining contents of the fermentation vessels were transferred into two 50 ml centrifuge tubes and centrifuged at 5,000 rpm for 5 min at 4°C to remove the yeast cells. After

centrifugation, the fermentation broths were decanted into two centrifuge tubes and following pH determination (Section 2.6.2) were frozen at -20°C until required for analysis (Section 2.7).

2.7 Mini Fermentations Analysis

2.7.1 Weight Loss

Weight loss during the mini-fermentations was assessed by weighing the mini-fermenters using a Sartorius M-power AZ3102 analytical balance (Sartorius UK Ltd, Surrey, UK) at $15 \pm 0.1^{\circ}\text{C}$. Weight loss measurements were performed in triplicate at least every 2 h daily but not overnight until constant weight through fermentation progression.

2.7.2 pH Determination

The pH of the fermentation broth samples were measured at $15 \pm 0.1^{\circ}\text{C}$ using an FEP20 Mettler-Toledo pH meter (Mettler-Toledo International Inc., Greifensee, Switzerland) previously calibrated with standard solutions of known pH (4.0 and 7.0 at $15 \pm 0.1^{\circ}\text{C}$).

2.7.3 Free Amino Nitrogen Analysis

The free amino nitrogen (FAN) content of wort samples was determined using the American Society of Brewing Chemists (ASBC) ninhydrin method of analysis (ASBC, 1992), which measures amino acids, ammonia, as well as some end-group α -amino nitrogen in peptides and proteins. Wort samples were diluted 1:100 in RO water and aliquots of 2 ml diluted wort were transferred to test tubes in triplicate. A 1 ml volume of ninhydrin colour reagent (10 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$; 6 g KH_2PO_4 ; 0.5 g ninhydrin; 0.3 g fructose in 100 ml RO water; stored in an amber bottle at 4°C for a maximum of two weeks; Sigma-

Aldrich Co., Dorset, UK) was added to each test tube and was heated for 16 min in boiling water. Following 20 min of cooling at 20°C in a water bath, 5 ml of the dilution solution (2 g KIO₃ in 1 l of 96% [v/v] ethanol; stored at 4°C; Sigma-Aldrich Co., Dorset, UK) were added to each tube. The contents of each tube were thoroughly mixed and the absorbance was read at 570 nm against RO water within 30 min of addition of the dilution solution. Glycine solution (2 mg.ml⁻¹; Sigma-Aldrich Co., Dorset, UK) was used as a standard, and RO water was used as a sample in the preparation of the reagent blank. Average absorbance readings of the glycine standards and triplicate experimental samples were used in the calculations. The FAN contents of samples (worts, fermentation broths) were calculated using the formula obtained from the glycine calibration curve (Figure 2.5) and using Equation 2.6.

Equation 2.6: The calculation of the samples free amino nitrogen (FAN).

$$\text{Free amino nitrogen (mg/l)} = \frac{y - 0.047}{0.2325}$$

Where: y = the mean absorbance of three replicate measurements

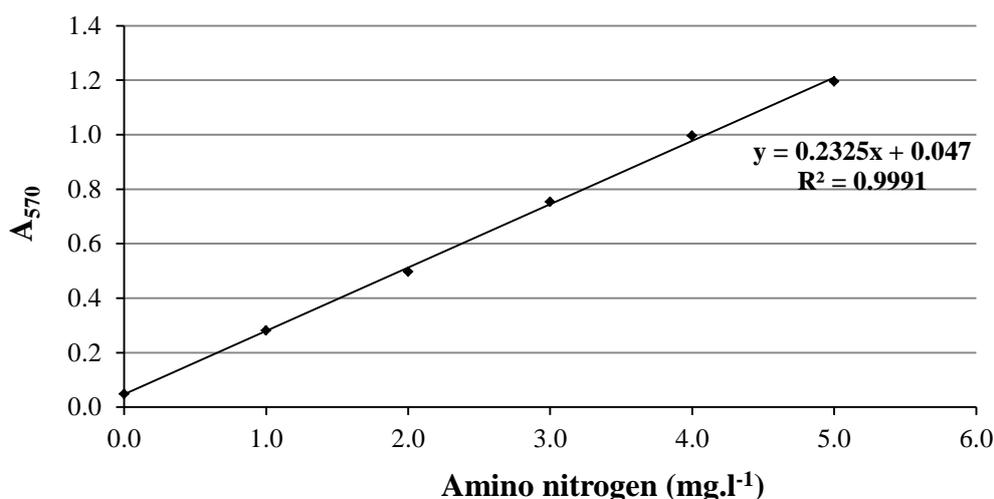


Figure 2.5: Glycine standard curve for the calculation of the free amino nitrogen in worts and samples (fermentation broths) obtained during fermentation progression.

2.7.4 Fermentable Sugars Analysis

Wort fermentable sugars were analysed using high pressure liquid chromatography (HPLC). Wort samples (1 ml) were passed through a C18 solid phase extraction cartridge (Strata-X 33 μm Polymeric Reversed Phase 30 $\text{mg}\cdot\text{m}^{-1}$ cartridge Phenomenex, Utrecht, Netherlands) previously conditioned with 1 ml methanol and equilibrated with 1 ml dH_2O . The first half of the sample that passed through the cartridge was discarded, and the second half of the sample was collected into glass vials containing 100 μl of melizitose, which acted as internal standard; 100 $\text{mg}\cdot\text{ml}^{-1}$, in preparation for analysis. Samples were arranged in a random running order before being placed in the automatic sampler. A random running order was used to ensure that any systematic variation in the instrument response over time was not biased towards particular samples. A 20 μl aliquot of the sample was injected onto an amino HPLC column (250 mm \times 4.6 mm i.d., 5 μm particle size, Spherisorb NH₂; Phenomenex, Utrecht, Netherlands) and the sugars were eluted using acetonitrile: water (80:20, [v/v]) at a flow rate of 5 $\text{ml}\cdot\text{min}^{-1}$ into an Optilab 903 Refractive Index Detector (Wyatt Technology Corporation, Santa Barbara, USA). Samples were analysed in triplicate and peak areas were recorded for each compound. The retention order of the compounds is given in Table 2.2. Fermentable sugars concentrations were determined by reference to standards of known concentration (Table 2.2). The ratio of the area of the fermentable sugar to the internal standard (melizitose) was used to normalise individual samples. The concentration of the fermentable sugars in the wort samples or fermentation broths was calculated using equations obtained from the calibration curves for standard series (Table 2.3 and 2.4 respectively).

Table 2.2: Retention time of fermentable sugars in the amino column used for their separation during the HPLC analysis.

Fermentable sugar	Retention time (min)
Fructose	2.35
Glucose	2.83
Sucrose	4.03
Maltose	4.73
Melzitose	7.07
Maltotriose	8.57

Table 2.3: Example of the equations used to determine the fermentable sugars of interest using HPLC analysis.

Fermentable sugar	Equation	Transformed equation
Fructose	$y = 0.11x - 0.03$	$x = (y + 0.03) / 0.11$
Glucose	$y = 0.09x - 0.02$	$x = (y + 0.02) / 0.09$
Sucrose	$y = 0.11x - 0.06$	$x = (y + 0.06) / 0.11$
Maltose	$y = 0.09x - 0.12$	$x = (y + 0.12) / 0.09$
Maltotriose	$y = 0.11x - 0.18$	$x = (y + 0.18) / 0.11$

Table 2.4: The composition of the standard stock solutions during the quantification of the fermentable sugars of interest in worts and fermentation samples using HPLC analysis.

Fermentable sugar	Std stock solution (mg/ml)	Dilution factor	Amount in 10 ml working solution	Std 1	Std 2	Std 3
				(mg/ml)	(mg/ml)	(mg/ml)
Fructose	60	×6	1	6.00	3.00	1.50
Glucose	60	×6	1	6.00	3.00	1.50
Sucrose	120	×12	1	12.00	6.00	3.00
Maltose	180	×24	1.33	24.00	12.00	6.00
Maltotriose	150	×30	2	30.00	15.00	7.50

2.7.5 Amino Acids Analysis

Amino acids were isolated from samples and derivatized (chemically modified) using the EZ:faast™ amino acid kit (Phenomenex, Utrecht, Netherlands). Using the amino acids concentrates supplied with the EZ:faast™ analysis kit, standard solutions of the targeted amino acids were made (i.e. 50, 100 and 200 nmol.ml⁻¹). Wort (25 µl) was combined with 100 µl of 20 nmol norvaline (Phenomenex, Utrecht, Netherlands) which acted as an internal standard. This solution was mixed and passed through the EZ:faast™ solid phase extraction absorbent (contained within a pipette tip) which was subsequently washed with 200 µl propanol (Phenomenex, Utrecht, Netherlands). A solution of propanol and sodium hydroxide (200 µl; Phenomenex, Utrecht, Netherlands) was then used to remove the absorbent, and the amino acids retained on it, from the pipette tip. 50 µl chloroform (Phenomenex, Utrecht, Netherlands) and 100 µl iso-octane (Phenomenex, Utrecht, Netherlands) were then sequentially added to the solution to derivatize the amino acids. This was required so as to produce compounds more suitable for the GC-MS (Gas Chromatography-Mass Spectrometry) analysis than the amino acids themselves. Using a Pasteur pipette the amino acids were recovered in the upper organic layer, dried under a stream of nitrogen gas and re-dissolved in 100 µl iso-octane:chloroform (80:20 [v/v]; Phenomenex, Utrecht, Netherlands). Subsequently, the samples were transferred into a GC vial insert, which was placed inside a vial, and capped. Where necessary, samples were stored at -20°C for a maximum of 24 h, whilst prior to analysis were assigned a random running order. This was used to ensure that any

systematic variation in the instrument response over time was not biased towards particular samples.

For GC-MS, 1 μl of the sample was injected in splitless mode (split closed for 10 s) using an AS3000 auto-sampler (Fisher Scientific UK Ltd, Loughborough, UK). The injector of the trace GC ultra gas chromatograph (Fisher Scientific UK Ltd, Loughborough, UK) was maintained at 250°C, with an initial oven temperature of 90°C which was increased to 320°C at a rate of 20°C.min⁻¹ (transfer line from the oven to mass spectrometer was held at a constant temperature of 300°C). Helium (8 psi) was used as the carrier gas to elute the amino acids from the ZB-AAA column (10 m \times 0.25 mm internal diameter, 0.1 μm film thickness; Phenomenex, Utrecht, Netherlands). The DSQ II mass spectrometer (Fisher Scientific UK Ltd, Loughborough, UK) was operated in selected ion mode recording ions 101, 114, 116, 130, 144, 146, 155, 156, 158, 172, 180, 184, 243 and 244 with a dwell time of 0.03 s (Table 2.5), whilst preliminary (dummy) runs were performed in full ion mode so as to allow the selection of the appropriate ions and windows of detection. After that, the DSQ II mass spectrometer was operated in selected ion mode (Table 2.5) and the wort samples were analysed in triplicate. The ratio of the amino acid to the internal standard (norvaline) was used to normalise individual samples. The concentration of the amino acids in the wort samples was calculated using equations obtained from the calibration curves for standard series (Table 2.6).

Table 2.5: Retention times, ions for quantification and windows of detection used in the GC-MS amino acid analysis.

Amino acid	Retention time (min)	Ion for quantification	Ion detected in selective ion mode	Window of detection
Alanine	2.15	130	101, 114, 130, 144, 158	0.0 – 2.42
Glycine	2.35	144	101, 114, 130, 144, 158	0.0 – 2.42
Alpha aminobutyric acid	5.80	184	84, 101, 114, 156, 184, 244	5.51 – 7.15
Valine	2.70	158	158, 172	2.42 – 3.00
Beta aminoisobutyric acid	2.81	116	116, 130, 144, 158, 172	2.42 – 3.00
Leucine	3.06	172	116, 130, 156, 172	3.00 – 3.35
Isoleucine	3.15	172	116, 130, 156, 172	3.00 – 3.35
Threonine	3.49	101	101, 144, 146, 156, 180, 243	3.35 – 3.74
Serine	3.55	146	101, 144, 146, 156, 180, 243	3.35 – 3.74
Proline	3.66	156	101, 144, 146, 156, 180, 243	3.35 – 3.74
Aspartic acid	4.74	130	101, 116, 130, 146, 172, 244	4.23 – 5.51
Methionine	4.78	101	101, 116, 130, 146, 172, 244	4.23 – 5.51
Glutamic acid	5.32	172	101, 116, 130, 146, 172, 244	4.23 – 5.51
Phenylalanine	5.34	146	101, 116, 130, 146, 172, 244	4.23 – 5.51
Lysine	7.40	116	116, 155, 170, 172, 180	7.15 – 7.93
Histidine	7.69	180	116, 155, 170, 172, 180	7.15 – 7.93
Tyrosine	8.14	116	107, 130, 206, 244	7.93 – 9.00
Tryptophan	8.58	130	107, 130, 206, 244	7.93 – 9.00

Table 2.6: Example of the equations used to determine the amino acids of interest using GC-MS analysis.

Amino acid	Equation	Transformed equation
Alanine	$y = 0.0294x + 0.0101$	$x = (y - 0.0101) / 0.0294$
Glycine	$y = 0.002x - 0.0009$	$x = (y + 0.0009) / 0.002$
Alpha aminobutyric acid	$y = 0.0404x + 0.007$	$x = (y - 0.007) / 0.0404$
Valine	$y = 0.0267x + 0.0148$	$x = (y - 0.0148) / 0.0267$
Beta aminoisobutyric acid	$y = 0.0059x + 0.0014$	$x = (y - 0.0014) / 0.0059$
Leucine	$y = 0.0797x - 0.0648$	$x = (y + 0.0648) / 0.0797$
Isoleucine	$y = 0.034x + 0.0146$	$x = (y - 0.0146) / 0.034$
Threonine	$y = 0.0067x + 0.0018$	$x = (y - 0.0018) / 0.0067$
Serine	$y = 0.0094x - 0.0027$	$x = (y + 0.0027) / 0.0094$
Proline	$y = 0.0393x + 0.0278$	$x = (y - 0.0278) / 0.0393$
Aspartic acid	$y = 0.0035x - 0.0002$	$x = (y + 0.0002) / 0.0035$
Methionine	$y = 0.004x - 0.0002$	$x = (y + 0.0002) / 0.004$
Glutamic acid	$y = 0.003x - 0.0022$	$x = (y + 0.0022) / 0.003$
Phenylalanine	$y = 0.0026x - 0.0007$	$x = (y + 0.0007) / 0.0026$
Lysine	$y = 0.0014x - 0.0021$	$x = (y + 0.0021) / 0.0014$
Histidine	$y = 0.011x - 0.0204$	$x = (y + 0.0204) / 0.011$
Tyrosine	$y = 0.003x - 0.0023$	$x = (y + 0.0023) / 0.003$
Tryptophan	$y = 0.0705x - 0.0701$	$x = (y + 0.0701) / 0.0705$

2.7.6 Alcohol and Gravity Determination

Wort samples collected during laboratory scale fermentations were filtered and centrifuged at 5,000 rpm for 5 min at 20°C. Following centrifugation, 10 ml of the supernatant were removed and used for alcohol determination following filtration through sterile 0.45 µm filters (Sartorius UK Ltd, Surrey, UK) to degas the wort and remove particulate matter. Wort specific gravity (SG) and ethanol content (% [v/v]) were determined using a DMA 5000 M model Anton Paar density-alcolyzer meter (Anton Paar GmbH, Graz, Austria). The density meter was rinsed prior to sampling using ethanol and between samples using

dH₂O. Gravity values were converted into °P using Equation 2.7.

Equation 2.7: Calculation to convert wort specific gravity into °Plato.

$$^{\circ}\text{Plato} = \frac{(\text{specific gravity} \times 1000) - 1000}{4.07}$$

Where: °Plato = % w/w sucrose in solution

$$\text{Specific gravity (SG)} = \frac{\text{gravity of sample}}{\text{gravity of water}}$$

2.7.7 Fermentability

The fermentability of worts (the proportion of the wort dissolved solids (extract) which can be fermented) was calculated as a percentage according to Equation 2.8.

Equation 2.8: Calculation of the fermentability of wort samples.

$$\text{Fermentability (\%)} = \frac{\text{original gravity} - \text{final gravity}}{\text{original gravity}} \times 100$$

Where: Original gravity = the gravity of the wort before pitching

Final gravity = the gravity of the wort when it is fully attenuated

The original gravity, normally expressed in °P, measures the concentration in weight/weight terms as g of solids per 100 g of wort. Final gravity is the gravity of the wort when it is fully fermented such that adding more yeast or leaving it longer will lead to no further fall in gravity. This lowest gravity is often called the attenuation limit gravity and when it is reached the beer is said to be fully attenuated (Briggs *et al.*, 2004). However, the alcohol formed in the fermentation has a lower density than water and so it decreases the final

gravity. Therefore, the final gravity does not show the amount of extract left in the fermented wort. The attenuation limit gravity referred to above is therefore called the apparent attenuation limit and what is calculated by equation 2.9 is the apparent attenuation of the wort. To measure the real attenuation the alcohol must be removed, e.g. by distillation before determining the gravity. The real attenuation is approximately 80% of the apparent attenuation. The true factor published by Balling in 1880 was 0.81. In modern practice the real attenuation can be obtained from the apparent attenuation by the use of tables (Briggs *et al.*, 2004).

2.8 Biolog Phenotype Microarrays

Phenotype microarrays were conducted using the Omnilog system (Biolog Inc., Hayward, USA). The Omnilog system (Figure 2.6) is a methodology for the metabolic characterization of micro-organisms for various research purposes (DeNittis *et al.*, 2010b). Phenotype MicroArrays (PMs) are a new and high-throughput technology which allows the simultaneous testing of a large number of cellular phenotypes, the observable characteristics or traits of an organism. PMs can directly assess the effects of genetic changes on cells and particularly gene knock-outs (Bochner *et al.*, 2001).

The method utilises the 96 well plate format (Figure 2.16) in which each well tests a different cellular phenotype, whilst an automated instrument continuously monitors and records the response of the cells in all the wells of the array. Cells incubated at a specific temperature grow, respire and upon respiration they reduce a dye resulting in the formation of a purple colour, usually tetrazolium violet (Figure 2.7; DeNittis *et al.*, 2010b). Respiration

constitutes an accurate reflection of the physiological state of the cell even though it does not necessarily indicate growth (i.e. cell division; Outeiro & Giorgini, 2006). Under physiological conditions the reduction of the dye is irreversible and thus the accumulation in the well over a period of time amplifies the signal and integrates to give a signal proportionate to the amount of respiration over time. On the other hand, partial or total loss of a function will result in partial or no growth and therefore in reduced or very little purple colour formation. By measuring cell respiration, PM technology offers the possibility to study directly the impact of oxidants, metals or even different nutrient sources, which influence the physiological state of the cell and their respiration(Outeiro & Giorgini, 2006).

The evolution of these changes, expressed by the index *average well colour development* (AWCD) can be plotted as a curve (AWCD curve, similar to a growth curve) that represents the temporal evolution of the metabolic activity of the population under study (DeNittis *et al.*, 2010b). The instrument cycles microplates in front of a colour CCD camera to read and provides quantitative and kinetic information about the response of the cells in the PMs.



Figure 2.6: The OmniLog instrument.

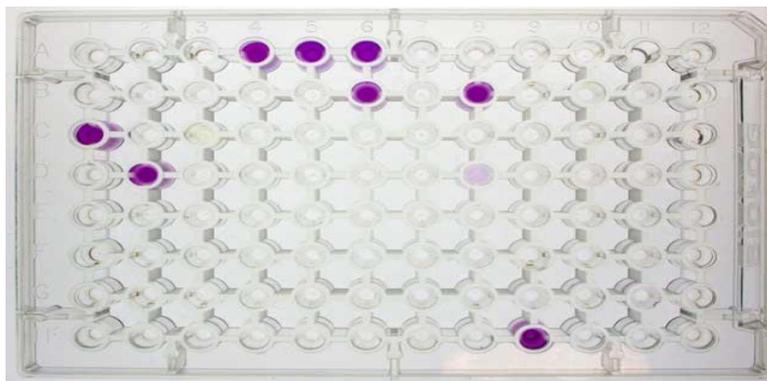


Figure 2.7: Plate used for the incubation of the yeast strains in the Omnilog instrument.

2.8.1 Incubation of Yeast Cells

Yeast cells were recovered from cryostorage and maintained on YPD agar slopes at 4°C. Cell suspensions were achieved by selecting representative colonies from the YPD agar slopes and aseptically inoculating into cooled (25°C) autoclaved YPD agar plates at 25°C for 96 h.

2.8.2 Preparation of Cell Suspensions

The suspended cell count of each individual yeast strain was adjusted to 62% transmittance using a 3587 portable Biolog turbidimeter (Biolog Inc., Hayward, USA). For that reason, selective representative colonies from the YPD agar plates were added, using dry sterile cotton swabs, to 13 ml sterile RO water in a 25 ml pre-sterilised glass tube to a final turbidity corresponding to 62% transmittance in the Biolog portable turbidimeter. The swab, containing the yeast cells obtained from the YPD agar plates, was rubbed against the dry inner wall of the glass tube above the meniscus so as to avoid the formation of the clumps. Following that, the glass tubes were covered with aluminium foil and were set aside (15°C) for no more than 15 min.

2.8.3 Wort Dilutions, Yeast Incubation and Absorbance Readings

18°P all-malt wort, previously kept at -20°C, was thawed and diluted with sterile RO water to 15 and 11°P final concentration respectively. 5 ml from each dilution was aseptically added to 25 ml pre-sterilised Universal bottles containing 160 to 640 µl of dye-D (Biolog Inc., Hayward, USA) and 0 to 40% IFY (i.e. 0 – 3.2 ml of dye-D when 11°P wort required; Inoculating Fluid for Yeast - a proprietary Biolog buffer used to stabilize the signal; based on % final volume; Biolog Inc., Hayward, USA). Following homogenization, 95 µl of each mixture were added to each of three replicate wells, of approximately 200 µl volume, containing 30 µl 62% transmittance yeast suspensions. The plates were then incubated in the dark in the Omnilog instrument (Biolog Inc., Hayward, USA) at 25°C for a maximum period of 70 h and periodically, every 5 min, submitted to absorbance readings of the colour in the wells with the Biolog E-MAX Reader (Biolog Inc., Hayward, USA).

Chapter 3

Development of a small-scale Assay to Predict the Premature Yeast Flocculation Potential of Malts

Data from this Chapter have been presented in a paper entitled *Malt induced premature yeast flocculation: current perspectives*, which was been published in the Journal of Industrial Microbiology and Biotechnology (2012, 39, 6, 813 – 822).

3.1 Introduction

Premature yeast flocculation (PYF) is a recurring problem in the brewing and malting industries associated with certain harvests, conditions and regions of barley production (Panteloglou *et al.*, 2010). Despite several decades of research into the phenomenon its precise nature and mechanisms have not been fully understood and elucidated. In part, this is because PYF is a ‘catch-all’ syndrome which can have different origins. Furthermore, there are complex interactions in the malting and brewing processes which together mean that the PYF status of a malt sample is hard to predict at a generic level. Whether or not PYF is observed depends not only on the barley quality, but on process factors in the maltings (e.g. process temperatures, airflows, hydrostatic pressures) and to a substantial extent on the brewing yeast strains concerned (Panteloglou *et al.*, 2012). Lake *et al.* (2008) ascribed part of this confusion as being a consequence of brewer’s poor ability to differentiate between PYF malt and poorly fermenting malts which are of low quality for other raw material or processing reasons. These are normally the result of a lack of fermentable sugars caused either by not generating the enzymes necessary to liberate these nutrients or by over modifying and thus diminishing the carbohydrate profile (Axcell *et al.*, 1986).

Despite these issues, the detection of malts responsible for PYF during fermentation is of major importance both to the maltster and the brewer (Panteloglou *et al.*, 2010). Inagaki *et al.* (1994) indicated that one important characteristic of the malt which cannot be evaluated by chemical or physical analysis is the prediction of its PYF-inducing ability. Standard malt analysis cannot predict all aspects of the performance of a malt in the brewery and in particular cannot predict the ‘hung’ or ‘stuck’ fermentations synonymous with PYF (Kruger *et al.*, 1982; Axcell *et al.*, 1984; Sampermans *et al.*, 2005). The majority of malt analysis evaluates, directly or indirectly, the modification of the grain during the malting process (i.e. the extent of protein and starch breakdown as well as the accompanying enzyme activities developed). The former factors contribute to the amount of extract (sugars) that can be recovered from the grain and indicate the value of the malt rather than predicting PYF (Van Nierop, 2005).

Various methods for the prediction of the PYF potential of malts have been reported (Ishimaru *et al.*, 1967; Baker & Kirsop, 1972; Fujino & Yoshida, 1976; Inagaki *et al.*, 1994; Mochaba *et al.*, 2001; Koizumi & Ogawa, 2005). Overall there appear to be three different types of PYF assays. The first type of PYF assays, and most widely used (Fujino & Yoshida, 1976; Kruger *et al.*, 1982; Herrera & Axcell, 1989, 1991a; Inagaki *et al.*, 1994; Nakamura *et al.*, 1997; Van Nierop *et al.*, 2004; Fisher, 2009), is based on small-scale fermentation tests (e.g. the ‘Kirin’ test; ‘Asahi’ test), the second type uses malt extracts rather than fermentations (Mochaba *et al.*, 2001; Koizumi & Ogawa, 2005), whilst the third category of the PYF assays employs sensitive

microbiological assays to predict the PYF potential of barley or malt samples (Van Nierop *et al.*, 2008; Kaur *et al.*, 2009).

Predicting the PYF potential of malts using the first type of PYF assays (small-scale fermentation tests, also known as ‘fermentability tests’ – Axcell, 2003), involve the fermentation of a boiled extract of raw materials, as used in the brewing process, in small fermentation vessels with similar aspect ratios and geometry to the brewery fermentation tanks (e.g. 100 ml volume and 25 cm high glass cyllindroconical ‘dropping funnels’ – Van Nierop *et al.*, 2004) at a constant temperature for a specific period of time. The use of glass cylinders is not arbitrary (Lake & Speers, 2008). Ishimaru *et al.* (1967) demonstrated that among numerous shapes and sizes tested, glass cylinders mimicked industrial fermentations the best. Fermentation assays rely on suspended yeast cell counts and residual extract and, depending on the precise experimental conditions (i.e. yeast strain, pitching rate and fermentation temperature) take several days to be completed (Panteloglou *et al.*, 2010) and more than two weeks if malting is required. In this type of PYF assays a set of duplicate fermentations run in parallel including positive and negative PYF control malts (i.e. malts exhibiting severe PYF and malts having normal fermentability and flocculation properties respectively; Van Nierop *et al.*, 2004). In general, the use of fermentation assays for routine monitoring of PYF status is expensive and inconsistent (Kaur *et al.*, 2009). Although they can distinguish between malts inducing PYF and malts exhibiting normal fermentation profiles, the PYF fermentation assays are time consuming (e.g. the ‘Improved Kirin test’ takes up to eight days to be completed – Inagaki *et al.*, 1994) and in some cases they do not predict the real performance of the malts in the brewery. “A

positive result with the fermentability test does not necessarily translate into a problem in the brewery” (Axccl *et al.*, 1986; Axccl *et al.*, 2000). Besides that, fermentation tests cannot determine the compounds causing PYF (e.g. arabinoxylans, antimicrobial peptides), and only indicate fermentation performance, which may be influenced by factors other than PYF (e.g. wort composition – sugars, amino acids, vitamins, inorganic ions, lipids, yeast strain, oxygen levels; Lake & Speers, 2008). However, the results obtained from the PYF fermentation tests can give useful information about potentially problematic malts (Axccl, 2003). Van Nierop *et al.* (2004) conducting parallel fermentations in 2 l EBC tall tubes and 100 ml cylindroconical vessels found that the small-scale fermentation tests have a comparable ability to detect PYF. The same authors (Van Nierop *et al.*, 2004) suggested that 100 ml fermentation assays provided adequate wort for any remaining analyses (e.g. residual gravity). Kirin brewery has used small-scale fermentation assays (e.g. the ‘Kirin’ test) since 1974 in order to establish a malt evaluation system to test malts before brewing as well as to evaluate the various maltsters. The introduction of this malt evaluation system made possible the purchase of high quality malt necessary for the production of high quality beer (Inagaki *et al.*, 1994). Besides Kirin, SABMiller uses fermentations assays as part of their routine methods of analysis (Kruger *et al.*, 1982; Van Nierop *et al.*, 2004). Kruger *et al.* (1982) suggested that perfect-grade malt could be used according to an internally developed fermentation test. Fermentations carried out using the same yeast and the same wort in the brewery and in 2 l fermentation tubes showed that the fermentation patterns obtained in the laboratory were very similar to those obtained in the brewery (including 330 hl and 2640 hl

cylindroconical vessels, and 1000 hl horizontal fermenters). Although tall-tube fermentations are reliable tools for the detection of PYF, they are time-consuming, labour intensive and require greater amounts of raw materials (Van Nierop *et al.*, 2004; Jibiki *et al.*, 2006; Lake & Speers, 2008). Thus, in the last decade several reports of downscaling or speeding-up fermentation tests for the detection of PYF have been developed (Lake & Speers, 2008).

The ‘Asahi test’ (Jibiki *et al.*, 2006) is such a PYF fermentation laboratory test which is widely used in the industry. Using a 50 ml fermentation conducted in a graduated cylinder at 21°C, PYF+ malts can be distinguished from negative controls on the basis of suspended yeast cell counts and apparent extract after two days (40 and 48 h post-pitching respectively). Following up on this report Lake *et al.* (2008) investigated the occurrence of PYF in fermentations conducted in tall tubes, test tubes and cuvettes to determine whether the assay size could be reduced further. They concluded that a 15 ml test tube fermentation assay supplemented with 4% [w/v] glucose and conducted at 21°C mimicked tall-tube fermenters the best. The fermentation assay predicted the PYF status of the malts within a period of 48 h similarly to the Asahi test. Besides measuring yeast cells in suspension (A_{600}) and gravity drop through fermentation progression, which lasted less than 72 h, Lake *et al.* (2008) also determined the minimum shear rate (i.e. between 4 and 7.5 s⁻¹) required to keep yeast in suspension when downscaling the PYF fermentation assay from 200 to 15 ml volume. Nakamura *et al.* (1997) presented a method to detect PYF through the use of a novel mashing technique that uses enzymes coupled with a 48 h fermentation. In this method the process time is reduced, as barley can be used directly, thus avoiding time dedicated to malting. Although the

method correlated well with traditional techniques to detect PYF, there is always the risk that using enzymatic methods may accentuate or provide false indications of PYF+ barley, especially if the PYF factors are generated enzymatically by fungal infection. Besides that, if the PYF factors are generated or enhanced during mashing, then an analysis of unmalted barley may not be representative of the final malt to be used in the brewery (Lake *et al.*, 2008).

Despite the different PYF fermentation assays, a major current drawback is that there is no standard method for a laboratory fermentation assay (mashing regimes, control malts, yeast strain), which makes results from different research groups harder to compare (Van Nierop, 2005; Lake *et al.*, 2008; Panteloglou *et al.*, 2012). Selection of yeast strain is just one significant aspect which should be standardised (Panteloglou *et al.*, 2010; Panteloglou *et al.*, 2012). The lager yeast SMA is one strain which has been proposed for widespread adoption, based upon its susceptibility to PYF (Panteloglou *et al.*, 2010; Porter *et al.*, 2010; Panteloglou *et al.*, 2011; Speers *et al.*, 2011; Panteloglou *et al.*, 2012). Van Nierop (2005) observed that not all the yeast strains are sensitive to flocculation changes, whilst Jibiki *et al.* (2006) concluded that lager strains are more susceptible to PYF than ale yeasts. The same authors (Jibiki *et al.*, 2006) also examined the difference in sensitivity of yeast crops originating from the same lager strain but different breweries to the same PYF+ and PYF- worts produced at the same time. The results obtained suggested that the same yeast strain may behave differently when fermented with the same PYF+ malt in different breweries. Besides that, it was also concluded that the sensitivity of yeast crops to PYF+ malts varied even within

the same brewery, indicating that the differences in crops were more influential than the differences in the breweries. Variability in the performance of malt samples in PYF tests can also arise because of the lack of homogeneity in the samples submitted. Samples containing a high proportion of fines and husk material, through breakage, give more PYF+ test results than samples which have been aspirated to remove such material (Voetz & Woest, 2011).

In the second type of PYF assays, those that do not require fermentation, the PYF factor(s) are extracted either from the barley or the malt and the PYF potential is predicted by measuring the rate of yeast sedimentation/flocculation after a specific period of time. Koizumi and Ogawa (2005) reported a rapid (3 h) assay which involved the extraction of barley or malt samples with water, precipitation of HMW material with ethanol and then re-suspension of these materials in water. The PYF activity of such extracts was assayed using a suspension of late-logarithmically growing yeast cells in a cuvette, with the ratio $A_{600 \text{ sample}}/A_{600 \text{ water}}$ 3 min after re-suspension of yeast being used as an index of PYF status. Results were correlated against a laboratory scale fermentation test ($R^2 = 0.85$).

Examples of the third, and most sensitive type of the PYF assays are the methods of Van Nierop *et al.* (2008) and Kaur *et al.* (2009). Van Nierop *et al.* (2008) used an antimicrobial assay to determine the antiyeast activity of the antimicrobial peptides that are present in the barley/malt and adversely impact brewing fermentation. Using malt extracts, from a series of commercial lager-type two row barley, Van Nierop *et al.* (2008) monitored the growth (A_{600}) of a lager brewing yeast strain of *Saccharomyces cerevisiae* (*S. pastorianus*) in a

96-well plate over a 24 h incubation using a microtitre plate reader at $23 \pm 1^\circ\text{C}$. The IC_{50} values (i.e. the concentration of extracted malt that causes 50% inhibition of yeast growth), calculated from the sigmoidal curves fitted to the dose response, indicated differences in antimicrobial activity or growth inhibition. As IC_{50} values lower than four ($\text{IC}_{50} < 4$) indicated the problematic malts that were used in the study, it was suggested that this value should be used as a preliminary threshold to be refined by a larger study. The assay differentiated malt samples according to their anti-yeast activity and malts which were associated with PYF fermentations and/or gushing, a quality defect of finished beer long associated with poor microbial quality of barley (Panteloglou *et al.*, 2012), showed the highest anti-yeast activities. The extracts used in this study were shown to contain peptides, tentatively identified as α -thionin, lipid transfer protein 1 (LTP-1a) and other non-specific lipid transfer proteins (ns-LTPs).

The implication of barley and malt microbes in PYF led Kaur *et al.* (2009) to propose an assay based upon terminal restriction fragment length polymorphism (T-RFLP) screening of microbial populations. A test set of 32 malt samples (including 18 PYF+ malts) were included in the study and microbial community fingerprint patterns were generated by T-RFLP analysis (based on 16S rRNA and 26/28S rRNA genes for bacterial and fungal communities respectively). The resultant data were analysed using multivariate statistical techniques and correlations sought between microbial strains and PYF status. Some fungal taxa were reported to be strongly associated with PYF+ assignments made using conventional fermentation tests.

The development, and validation, of a small-scale PYF fermentation assay for the prediction of the PYF potential of the malts is reported in this Chapter. Besides that, the importance of PYF to the performance of subsequent fermentations as well as the significance of wort composition on the PYF phenomenon is also discussed.

3.2 Experimental

3.2.1 Malts

3.2.1.1 Control Malts

Two control barley malts from a similar region (France) and crop year (2007) were used throughout these experiments. The malts were however prepared from different barley varieties (Scarlett and Prudentia for the PYF1+ and PYF1- malts respectively). The Scarlett malt sample was known to have caused PYF in brewery fermentations, whilst the Prudentia sample was a control malt giving rise to normal fermentations profiles.

3.2.1.2 Ring-Trial Malts

Two trial malts were used to validate the in-house small-scale fermentation assay. The malts ('Alpha' and 'Beta') were sourced as part of a ring-trial and were provided from the Institute of Brewing and Distilling (IBD) in a collaborative study between various research labs worldwide as a part of the PYF Network Scheme convened by Campden-BRi. The study was a 'blind' trial and no sample details, besides the PYF status of the samples, were provided by the IBD to collaborating laboratories until after conclusion of the study.

3.2.1.3 Unknown PYF Status Malts

The PYF status of three malts was predicted relative to our PYF+ and PYF- control malts using the in-house PYF assay. The malts were malted from the barley varieties Gairdner, Harrington and Jinyang barley variety and were provided from an international Korean brewery. Jinyang malt was sourced in 2009 from South Korea, whilst Gairdner and Harrington in 2008 from Australia and Canada respectively. Jinyang malt exhibited normal fermentation profiles in industrial scale fermentations when a medium flocculent lager yeast strain was used and only 30% of this malt was employed in the production of the wort. However, the same malt (Jinyang) exhibited strong PYF profiles, with elevated residual sugars and diacetyl levels, when a more flocculent lager yeast strain was used. On the other hand, Gairdner and Harrington exhibited normal fermentation profiles in industrial scale fermentations with Gairdner worts giving rise to a slightly higher residual gravity when comparing to Harrington worts. A summary of the malt samples used in this study is shown in Table 3.1 below.

Table 3.1: Barley variety, harvest year and region of production for the malts used in this study.

Barley Variety	Harvest Year	Region of Production	PYF Code
Scarlett	2007	France	PYF1+
Prudentia	2007	France	PYF1-
‘Alpha’	Unknown	Unknown	Unknown
‘Beta’	Unknown	Unknown	Unknown
Gairdner	2008	Australia	PYF-
Harrington	2008	Canada	PYF-
Jinyang	2009	South Korea	PYF+

3.2.2 Wort Preparation

Worts were prepared from control (Section 3.2.1.1), trial (Section 3.2.1.2) and unknown PYF status (Section 3.2.1.3) malts using a standardized laboratory mashing procedure as described in Section 2.5.

3.2.3 Wort Composition Analyses

FAN and fermentable sugar spectrum analyses were performed as described in Sections 2.7.3 and 2.7.4 respectively.

3.2.4 Yeast Strains and Propagation Conditions

Two lager brewing yeast strains were used in this study (W34/70 and SMA). W34/70 (ex Weihenstephan) and SMA were obtained from the National Collection of Yeast Cultures (NCYC) and from the VLB Research Institute (Berlin, Germany) respectively. W34/70 is a medium flocculent yeast strain, whilst SMA a highly flocculent yeast strain (Section 2.1). Yeast propagation was performed in an orbital shaken incubator at 120 rpm for 4 days at 15°C as described in Section 2.2.4.

3.2.5 Premature Yeast Flocculation (PYF) Assay

Full details of the PYF assay procedures may be referenced on Section 2.5.1. In brief, the procedure involved conducting small-scale (200 ml) fermentations in 250 ml 'dropping funnels' within a temperature controlled incubator (15°C) and using worts prepared from control (Section 3.2.1.1), trial (3.2.1.2) and unknown PYF status (Section 3.2.1.3) malts using a standardized laboratory mashing procedure (see Section 2.5 for details). The fermentations were carried out at a pitching rate of 20×10^6 live cells.ml⁻¹ after the

supplementation of the 11°P all-malt worts with 4% [w/v] glucose for a maximum period of 92 h.

3.2.6 The Importance of PYF to the Performance of Subsequent Fermentations using re-pitched yeast

Yeast cells were harvested from the bottom of a PYF+ fermentation previously performed in a 250 ml 'dropping funnel' as described in Section 3.2.5. This was achieved by discarding the fermentation broth and washing the precipitated yeast cells by adding 10 ml of sterile RO water in duplicate. Following washing, the culture (precipitated cells and 20 ml sterile RO water) was used for the preparation of a 50% [w/v] yeast slurry. The resultant yeast slurry was used for the pitching and fermentation of 11°P PYF- worts as described in Section 3.2.5.

3.2.7 Premature Yeast Flocculation Assay Measurements

3.2.7.1 Monitoring Suspended Yeast Cells Counts

Cell concentration in suspension was assessed at specific time intervals between 0 – 92 h post-pitching by measuring absorbance at 600 nm (A_{600} ; Section 2.6.2.1) and on occasions microscopically (Section 2.6.2.2).

3.2.7.2 Gravity Drop and Residual Gravity

The gravity drop, during fermentation progression, and the residual gravity of the fermenting broths were determined at 15°C using a DMA 5000 M model Anton Paar density-alcolyzer meter as described in Section 2.7.6.

3.2.7.3 Fermentability

The fermentability of the worts (the proportion of the wort dissolved solids which can be fermented) was calculated as a percentage according to Equation 2.8; Section 2.7.7.

3.2.8 Statistical Analysis

The statistical significance of the different malt types and yeast strains on yeast flocculation, residual gravity and ethanol yield was assessed using ANOVA and the statistical program Minitab (version 15, Minitab Inc., State College, USA). In each instance the null hypothesis (H_0) was that no significant difference existed between data sets. If the P value generated by the test was less than 0.05 then the null hypothesis of no significant difference was rejected and the alternative hypothesis (H_a) of significance was adopted. Whilst ANOVA can indicate that an overall significant difference exists between data sets, *post-hoc* analysis is required to assess which sample means differed statistically from one another. Pair-wise comparison of means was completed using the Tukey test at the significance level $P < 0.05$.

3.3 Results and Discussion

3.3.1 Wort Fermentable Sugars and FAN Compositions

Worts prepared from the PYF1+ and PYF1- malt samples used throughout these experiments were of similar composition with respect to fermentable sugar spectrum and free amino nitrogen (FAN; Figures 3.1 and 3.2 respectively).

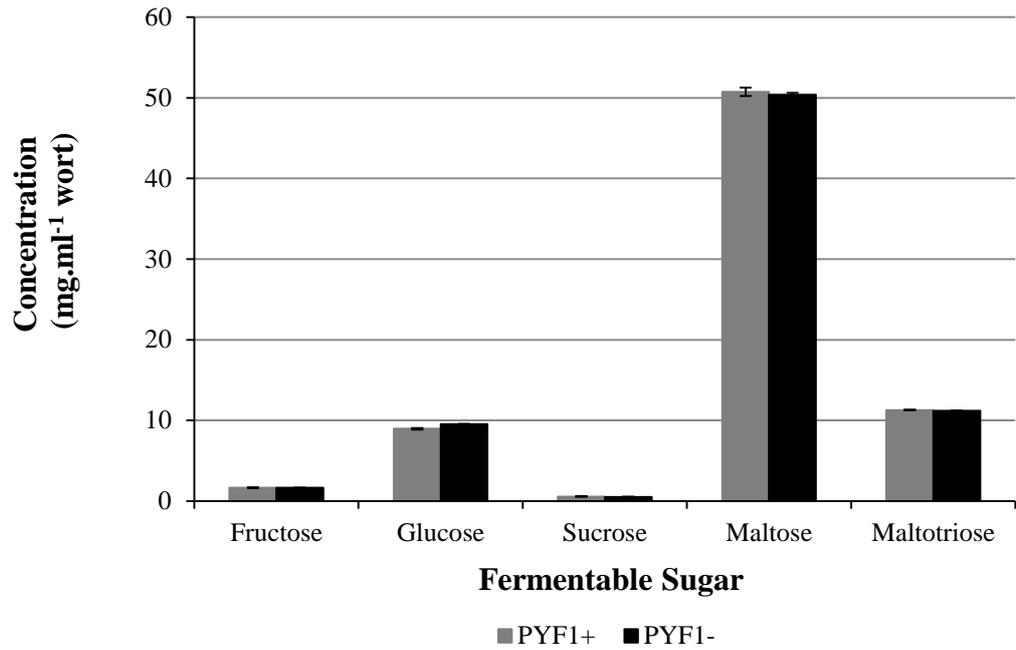


Figure 3.1: Fermentable sugar composition for all-malt worts (11°P) prepared from PYF1+ (Scarlett) and PYF1- (Prudentia) control malts. Data are the mean of three replicates \pm SD.

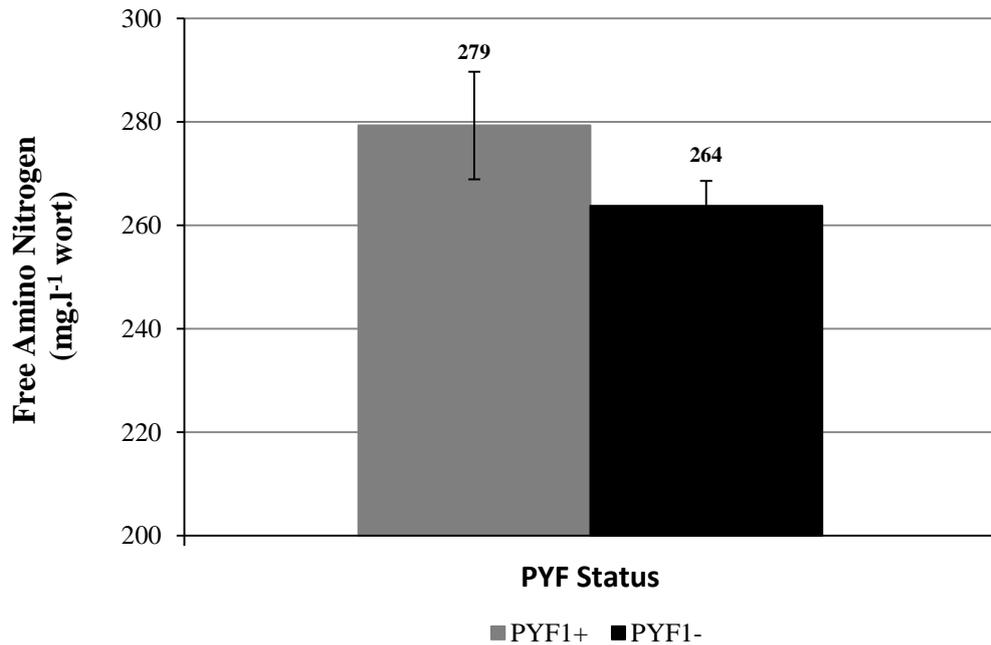


Figure 3.2: Free amino nitrogen (FAN) composition for all-malt worts (11°P) prepared from PYF1+ (Scarlett) and PYF1- (Prudentia) control malts. Data are the mean of three replicates \pm SD.

Van Nierop *et al.* (2004), conducting 21 EBC fermentations with 13°P PYF+ and PYF- control worts at the pitching rate of 20×10^6 live cells.ml⁻¹, proposed

that the amounts of individual wort fermentable sugars (i.e. sucrose, fructose, glucose, maltose and maltotriose) must not vary from one another by more than 15%, whilst their FAN concentrations should be higher than 200 mg.l^{-1} so as to be used in the same PYF assay. On that basis, it could be assumed that they would ferment similarly other than for differences caused by PYF.

Nevertheless, Axcell (2003) suggested that deficiency in other nutritional aspects of the worts (e.g. zinc, biotin as well as the combination of zinc and manganese) can also affect yeast growth and flocculation performance, whilst Bamforth (2003) indicated that besides zinc (needed for the reduction of pyruvaldehyde to ethanol during primary fermentation) most brewers will also specify and quantify the oxygen dosed in before fermentation. Boulton and Quain (2003) indicated that failure to provide oxygen at the start of fermentation results in slow fermentation rate, incomplete attenuation and poor yeast growth. According to the same authors (Boulton & Quain, 2003) oxygen is required in brewery fermentations so as to allow yeast to synthesize sterols and unsaturated fatty acids, which are essential components of membranes. However, the quantity of oxygen required for fermentation is yeast-dependent. “Some yeast strains are satisfied when the brewer air-saturates the wort – bubbles air into the wort after cooling, which introduces approximately 8 ppm wort. Some strains are happy with half that level, others demand oxygen saturation (16 ppm), and some require even higher amounts of oxygen” (Bamforth, 2003).

3.3.2 Predicting the PYF Potential of Malts using a Medium Flocculent Brewing Lager Yeast Strain

Using the small-scale fermentation test (Section 2.7.1) with W34/70 yeast, a medium flocculent brewing lager yeast strain, PYF1+ worts could be differentiated from PYF1- worts on the basis of suspended yeast cell counts (Figures 3.3 and 3.4) and residual gravity (Figure 3.5). Monitoring suspended yeast cells, using either the absorbance at 600 nm (Figure 3.3) or microscopic cell counting (Figure 3.4) after a number of serial dilutions, the PYF potential of the malts could be predicted within a period of 64 or 69 h through fermentation progression respectively. At that period of time, the number of suspended yeast cells in the PYF1+ fermentations was significantly lower ($P < 0.0001$) than the number of suspended yeast cells in the PYF1- fermentations (Figures 3.3 and 3.4).

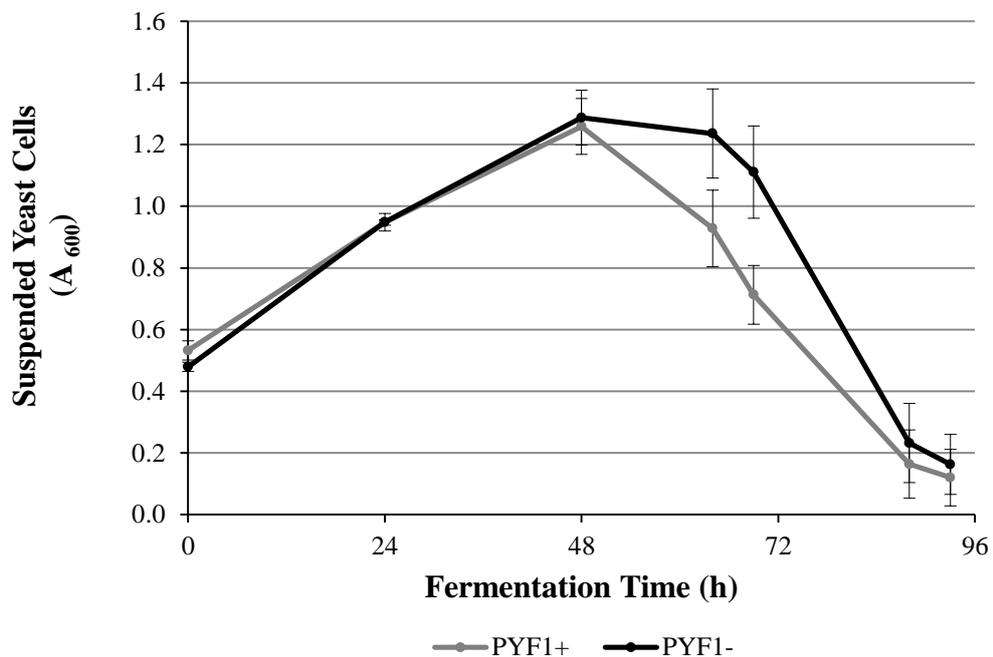


Figure 3.3: Fermentation profiles for PYF1+ (Scarlett) and PYF1- (Prudentia) control worts fermented at 15°C using W34/70 yeast at a pitching rate of 20×10^6 live cells.ml⁻¹. Data are the mean of four replicate fermentations \pm SD. The number of suspended yeast cells approximately 4 cm below the fermenting broths was determined using the A₆₀₀.

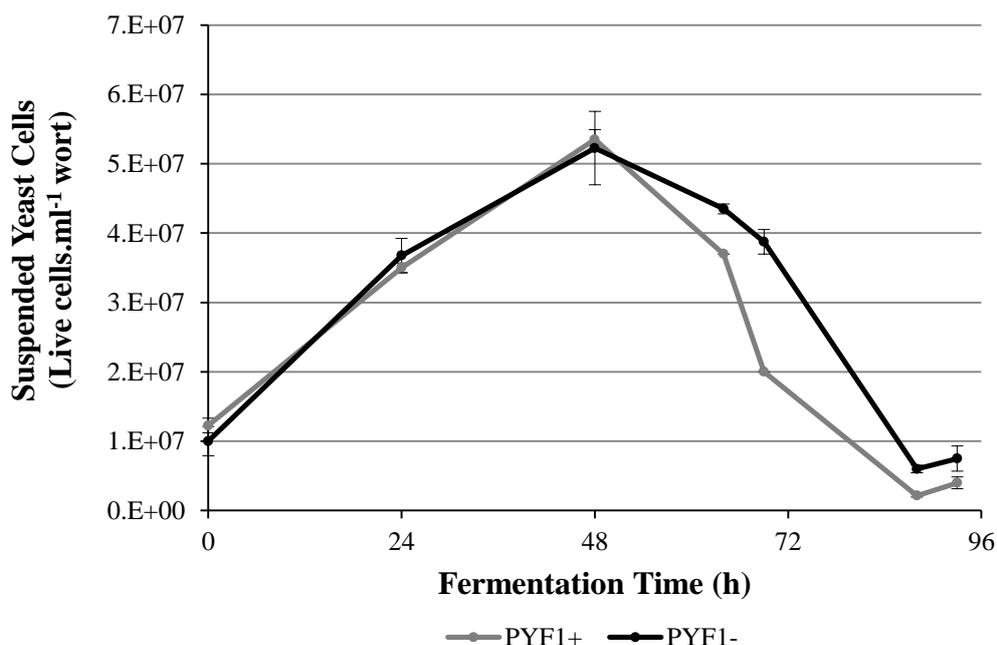


Figure 3.4: Fermentation profiles for PYF1+ (Scarlett) and PYF1- (Prudentia) control worts fermented at 15°C using W34/70 yeast at a pitching rate of 20×10^6 live cells.ml⁻¹. Data are the mean of two replicate fermentations \pm SD. The number of suspended yeast cells approximately 4 cm below the fermenting broth was determined using cell counting (microscopically).

In addition, PYF1+ fermentations had a significantly higher residual gravity ($P < 0.05$), than was the case for the PYF1- fermentations 96 h post-pitching. At that time, there was on average a 0.5°P elevation in the residual gravity when comparing the PYF1+ with the PYF1- fermentations (Figure 3.5). Consequently, PYF1+ fermentations had a lower apparent fermentability (86.8%), than was the case for the PYF1- fermentations (90.1%). Therefore, fermentations with worts inducing PYF besides having a statistically significant lower suspended yeast cells count after 64 or 69 h of fermentation (depending with the method used for the determination of the suspended yeast cells) also resulted in elevated residual gravity and lower apparent fermentability 96 h post-pitching.

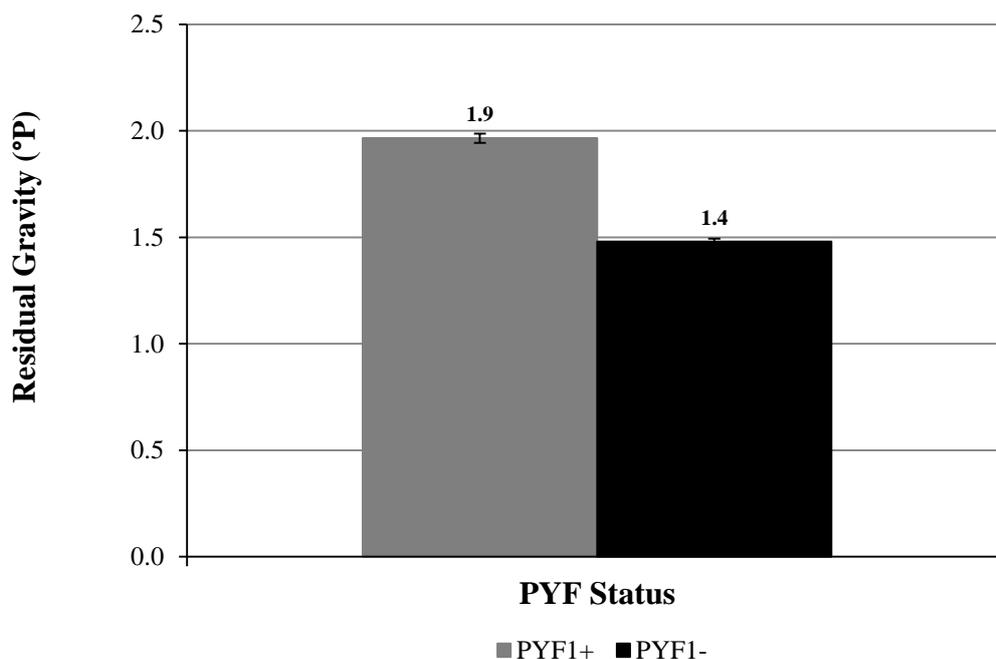


Figure 3.5: Residual gravity 96 h post-pitching for PYF1+ (Scarlett) and PYF1- (Prudentia) control worts fermented at 15°C using W34/70 yeast at a pitching rate of 20×10^6 live cells.ml⁻¹. Data are the mean of two replicate fermentations \pm SD.

3.3.3 Predicting the PYF Potential of Malts using a Highly Flocculent Brewing Lager Yeast Strain

Using the small-scale fermentation tests (Section 2.6.1) with the highly flocculent but PYF sensitive brewing lager yeast strain SMA in fermentations conducted under the same experimental conditions (pitching rate of 20×10^6 live cells.ml⁻¹ and using 11°P all-malt worts supplemented with 4% [w/v] glucose) the PYF potential of the same PYF1+ and PYF1- malts (produced from Scarlett and Prudentia barleys respectively) could be predicted 40 h post-pitching. At that time, the number of suspended yeast cells, as indicated by A_{600} readings, in the PYF1+ fermentations was significantly lower ($P < 0.0001$) than the number of suspended yeast cells in the PYF1- fermentations (Figure 3.6).

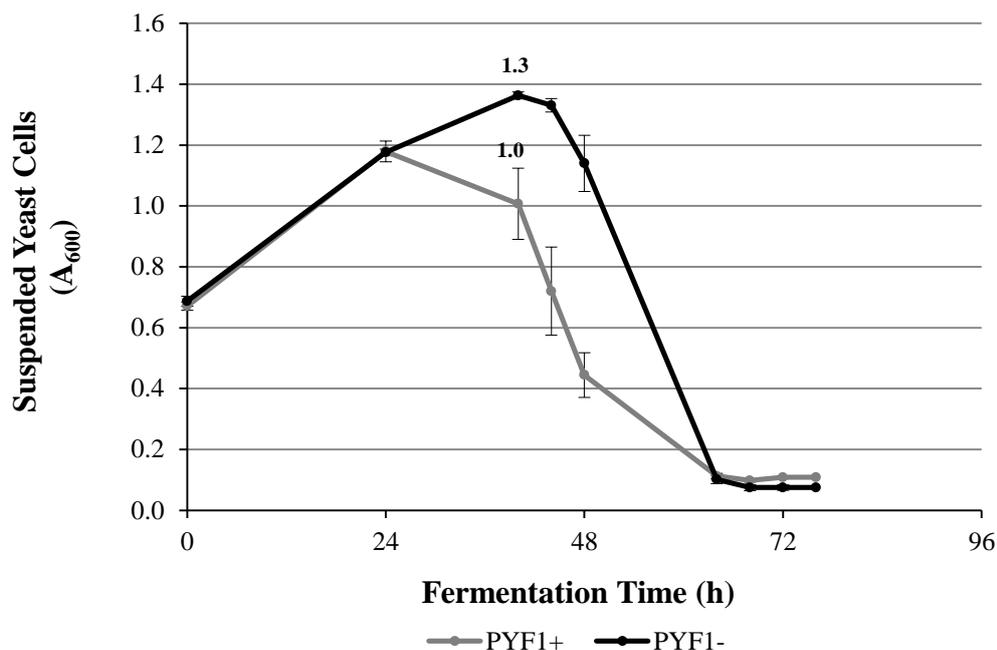


Figure 3.6: Fermentation profiles for PYF1+ (Scarlett) and PYF1- (Prudentia) control worts fermented at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹. Data are the mean of five replicate fermentations \pm SD.

Thus, the use of a highly flocculent but PYF sensitive yeast strain resulted in more rapid discrimination between PYF1+ and PYF1- malts, reducing the time of analysis by 24 h (i.e. from 64 to 40 h; Figures 3.3 and 3.6 respectively). The results obtained suggested that the highly flocculent yeast strain SMA was more susceptible to PYF factor(s) than the medium flocculent yeast strain W34/70. Besides that, PYF1+ fermentations also resulted in a significantly higher residual gravity ($P < 0.005$) and consequently a lower apparent fermentability (1.8°P and 87.4% respectively) than was the case with the PYF1- fermentations (1.2°P and 91.4% respectively) (Figure 3.7).

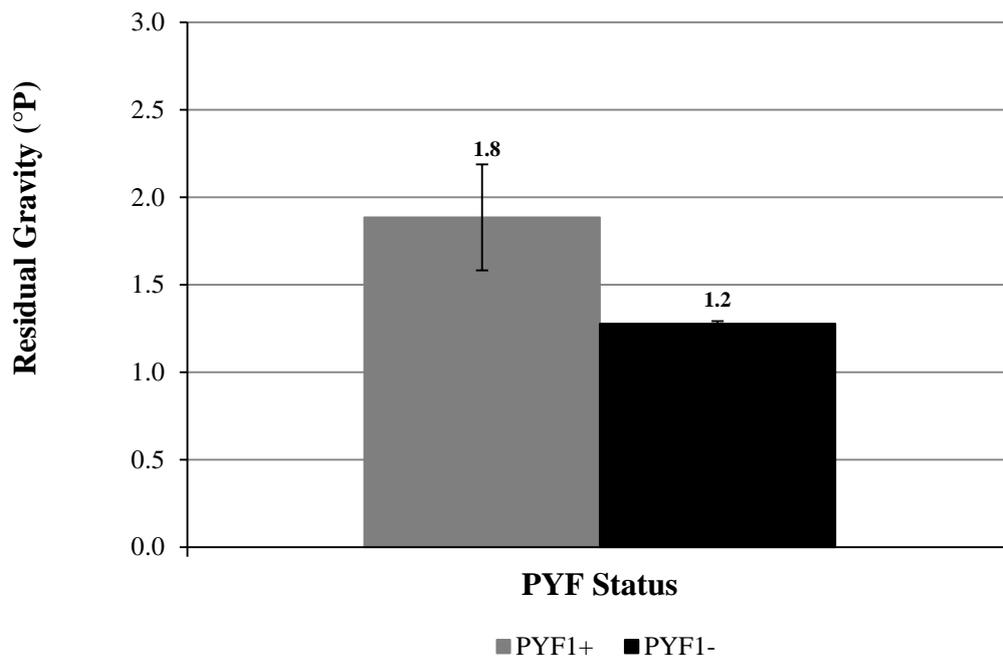


Figure 3.7: Residual gravity 72 h post-pitching for PYF1+ (Scarlett) and PYF1- (Prudentia) control worts fermented at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹. Data are the mean of five replicate fermentations \pm SD.

PYF1+ and PYF1- control worts fermented with the highly flocculent but PYF sensitive yeast strain SMA resulted in better attenuation when comparing with the medium flocculent yeast strain W34/70. In addition, the impact on residual gravity of PYF1+ relative to PYF1- worts was on average slightly higher in the SMA fermentations when comparing with the W34/70 fermentations (0.61 and 0.49°P respectively; Figures 3.7 and 3.5 respectively). These data are in agreement with the results obtained from Armstrong and Bendiak (2007) who, taking into consideration real extracts of bright beer, proposed that the more flocculent yeast strains are definitely more susceptible to PYF than the less-flocculent or non-flocculent strains.

Monitoring gravity drop (Figure 3.8) through the four days of the small-scale fermentation test, using the same PYF1+ and PYF1- control worts as well as the same fermentation conditions (pitching rate of 20×10^6 live cells.ml⁻¹ and

using 11°P all-malt worts supplemented with 4% [w/v] glucose), provided a fermentation profile. In this instance there was a significant difference ($P < 0.001$) in the gravity 72 and 96 h post-pitching. At these periods of time, PYF1+ fermentations had a higher gravity, equal with 0.9 and 0.7°P respectively, than the PYF1- fermentations. However, the initial gravity drop to 24 h post-pitching was quicker in the PYF1+ fermentations (Figure 3.8). This suggested that the cause of PYF in this particular sample did not influence the onset of fermentation and that for other variable reasons, due to the provenance of the samples, it actually fermented slightly quicker. This result also suggested that the general nutritional status of the PYF1+ worts was good and that problems arose only towards the end of the fermentation.

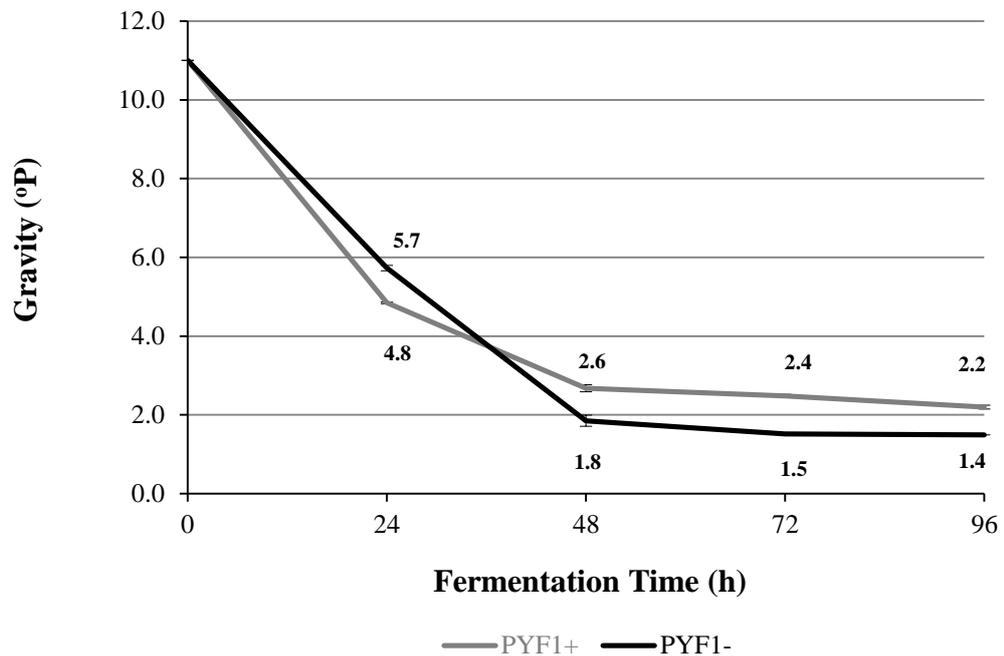


Figure 3.8: Gravity drop observed during PYF1+ (Scarlett) and PYF1- (Prudentia) fermentations conducted at 15°C using the highly flocculent PYF sensitive yeast SMA at a pitching rate of 20×10^6 live cells.ml⁻¹. Data are the mean of two replicate fermentations \pm SD.

However, Van Nierop *et al.* (2004), Panteloglou *et al.* (2010) and Eck *et al.* (2011) indicated that although high residual sugars at the end of the primary

fermentation have been frequently associated with PYF on an industrial scale, this is not always observed in the small-scale PYF fermentation assays and consequently this parameter cannot be used as a definitive indicator of PYF. This is thought to be due to the different types of PYF which have been observed/ postulated. According to Kaur *et al.* (2009), one group defines acute (primary) PYF whereby the early flocculation of yeast cells during primary fermentation results in a final product with unacceptably high levels of residual fermentable sugars (residual gravity). According to the same authors (Kaur *et al.*, 2009), the second school of thought recognises a more subtle, chronic PYF, termed secondary PYF, where the cell count in suspension during maturation/secondary fermentation is at a sub-optimal level so that the removal of undesirable flavour components such as diacetyl (butterscotch flavour) is incomplete.

Results presented here suggest that measuring both the number of suspended yeast cells during primary fermentation, using either the absorbance at 600 nm (A_{600}) or microscopic cell counting after a number of serial dilutions, as well as the residual gravity at the end of the primary fermentation offers a better understanding on which to base predictions of the malts true PYF potential in the brewery (Panteloglou *et al.*, 2010; Eck *et al.*, 2011). However, it should be borne in mind that differences in brewery practise and in particular the specific yeast strain utilised also play a significant role in determining whether or not the PYF potential is actually realised and exhibited in individual breweries.

3.3.4 Predicting the PYF Potential of Ring-Trial Malts using the in-house PYF Assay

Worts obtained from ring-trial malt samples ('Alpha' and 'Beta'; Section 3.2.1.2) had similar fructose, maltose and maltotriose compositions to those noted previously for the in-house PYF1+ and PYF1- control worts (Figure 3.9). Beta worts were slightly deficient in glucose and sucrose (Figure 3.9) and had also a significantly (based on standard deviations) lower amount of FAN than was the case with the other three malts used in this study (Figure 3.10).

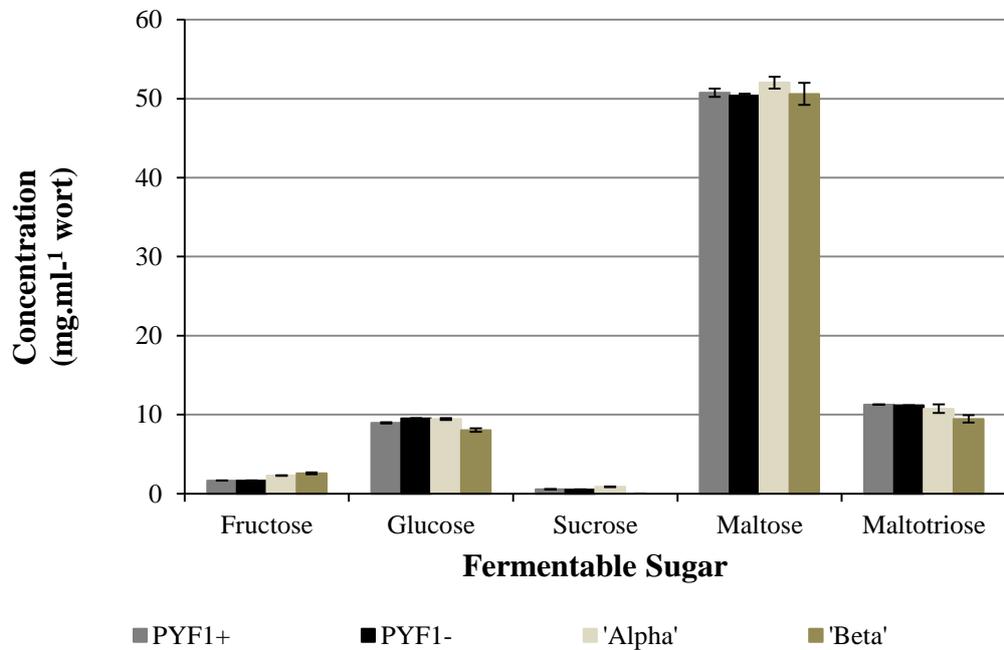


Figure 3.9: Fermentable sugar composition for all-malt worts (11°P) prepared from PYF1+ (Scarlett) and PYF1- (Prudentia) control malts and the IBD ring-trial malt samples 'Alpha' and 'Beta'. Data are the mean of three replicates \pm SD.

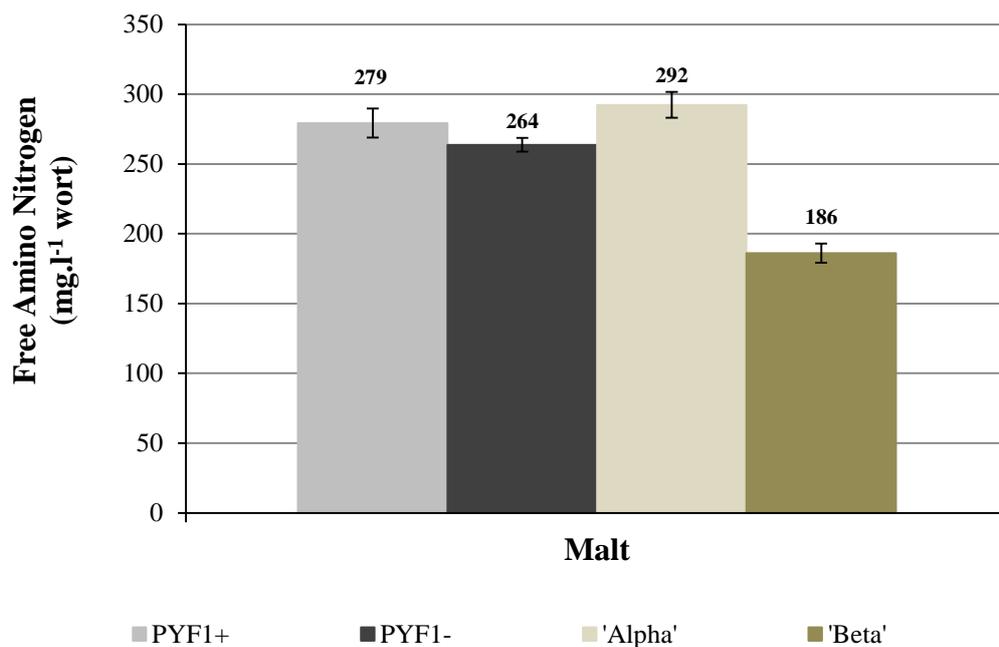


Figure 3.10: Free amino nitrogen content for all-malt worts (11°P) prepared from PYF1+ (Scarlett) and PYF1- (Prudentia) control malts and the IBD ring-trial malt samples 'Alpha' and 'Beta'. Data are the mean of three replicates \pm SD.

However, as stated earlier (Van Nierop *et al.*, 2004), differences in the worts fermentable compositions lower than 15% would be unlikely to affect significantly the fermentation progression. In addition, Hornsey (1999) and Boulton and Quain (2003) proposed that in order to achieve a good and rapid fermentation the FAN content of the wort should not be less than 100 mg.l⁻¹ (preferably between 150 – 200 mg.l⁻¹). The same authors (Boulton & Quain, 2003) also indicated that a half to one third of FAN in wort arise from the action of proteases (mainly carboxypeptidases) during mashing, whilst the remainder being derived directly from the malt and is formed during malting. Malt carboxypeptidases have maximal activity at temperatures between 40 and 60°C and are inactivated at 70°C.

Using the highly flocculent but PYF sensitive lager yeast strain SMA the PYF potential of the malts could be differentiated 40 h through fermentation

progression (Figure 3.11). At that time the number of suspended yeast cells, as indicated by A_{600} readings, in fermentations conducted using the ‘Alpha’ and ‘Beta’ worts was significantly lower ($P < 0.0001$) than the number of suspended yeast cells in the fermentations conducted using our control PYF1- sample.

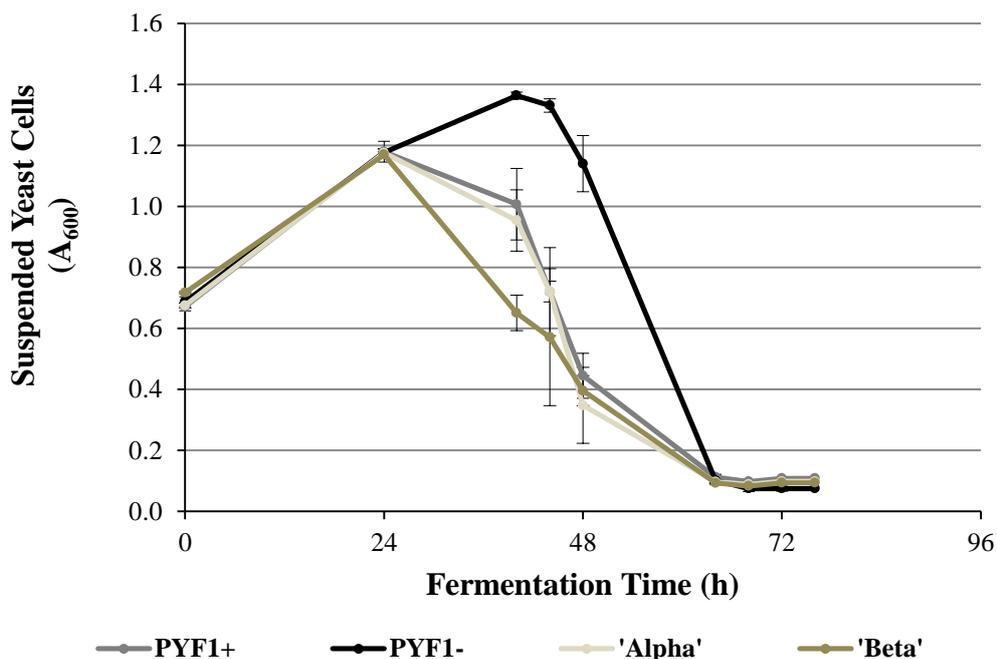


Figure 3.11: Suspended yeast cell profiles of PYF test fermentations conducted using the IBD ring-trial samples ‘Alpha’ and ‘Beta’ in addition to in-house PYF1+ (Scarlett) and PYF1- (Prudentia) control malts.

Fermentations were conducted at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹. Data are the mean of five replicate fermentations \pm SD.

‘Alpha’ and ‘Beta’ worts had a higher and significant higher ($P < 0.005$) residual gravity respectively than our PYF1- control fermentations. ‘Beta’ worts exhibited dramatically more severe PYF profile than our PYF1+ control sample (Figure 3.11) - finishing the fermentation with 1.0°P higher residual sugars than our PYF- control wort (Figure 3.12). This result confirms that PYF+ malts can exhibit different degrees of severity in respect to PYF when fermented with the same brewing yeast strain.

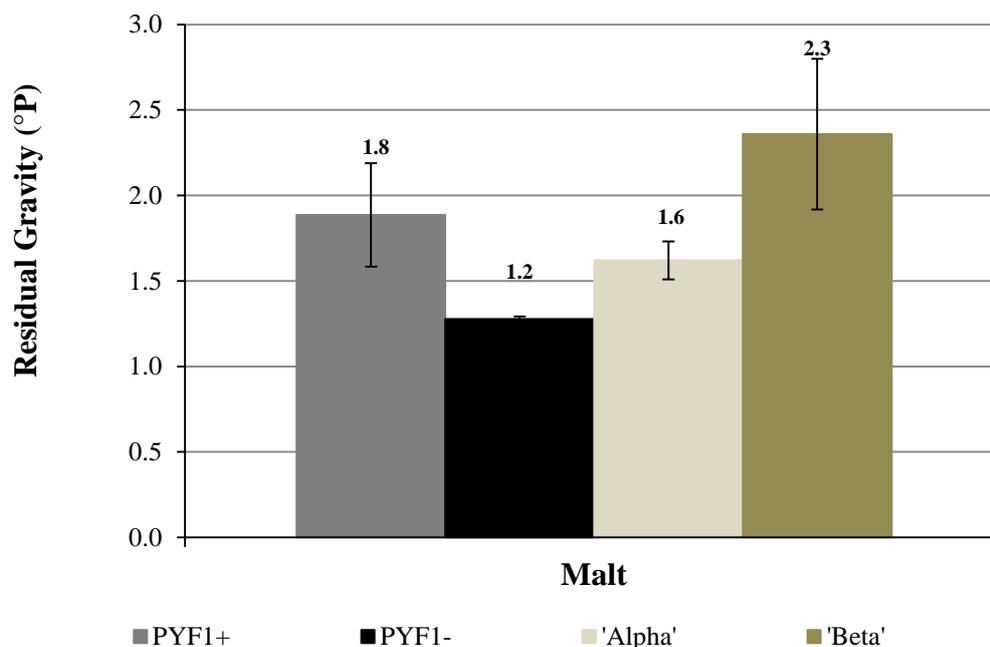


Figure 3.12: Residual gravity 96 h post-pitching for the IBD ring-trial samples 'Alpha' and 'Beta' in addition to in-house PYF1+ (Scarlett) and PYF1- (Prudentia) control worts.

Fermentations were conducted at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹. Data are the mean of five replicate fermentations \pm SD.

Consequently, 'Alpha' and 'Beta' worts had a lower fermentability than the PYF1- control worts (Table 3.1). Accordingly, both of the IBD malts circulated in the ring-trial were found by the in-house small-scale fermentation test to be PYF1+. Their PYF potential though was different ('Beta' worts exhibited a stronger PYF potential than 'Alpha' worts; Figure 3.11).

Table 3.2: Residual gravity and apparent fermentability for control and ring-trial worts.

Wort	Residual Gravity (°P)	Apparent Fermentability (%)
'Alpha'	1.6	89.2
'Beta'	2.3	84.2
PYF1+	1.8	87.4
PYF1-	1.2	91.4

The results obtained were in agreement with the results from the majority of the research labs (80%) which participated in the trial, which in turn were consistent with the PYF problems that had been presented by the malts when brewed on an industrial scale. Thus, it was concluded that the in-house small-scale fermentation assay (Panteloglou *et al.*, 2010) could be successfully used for the prediction of the PYF potential of different malt samples.

3.3.5 The Importance of PYF to the performance of Subsequent Fermentations using re-pitched yeast.

Figure 3.13 shows the fermentation profiles of PYF1+ and PYF1- worts in fermentations conducted at 15°C at the pitching rate of 20×10^6 live cells.ml⁻¹ after the supplementation of the 11°P all-malt worts with 4% [w/v] glucose. Using the in-house small-scale PYF assay (Section 2.7.1) and the medium flocculent brewing lager yeast strain W34/70 the PYF potential of our control PYF1+ and PYF1- malts could be differentiated within a period of 64 h (Figures 3.3 and 3.4). At that period of time the number of suspended yeast cells, as measured by A_{600} readings, was significantly lower ($P < 0.0001$) than was the number of suspended yeast cells in the PYF- fermentations (Figure 3.13). However, when the PYF1- worts were pitched with yeast cells cropped from a previous PYF1+ fermentation (see Section 3.2.6 for further experimental details), the incidence of PYF was heavier than in the original PYF1+ fermentations (Figure 3.13).

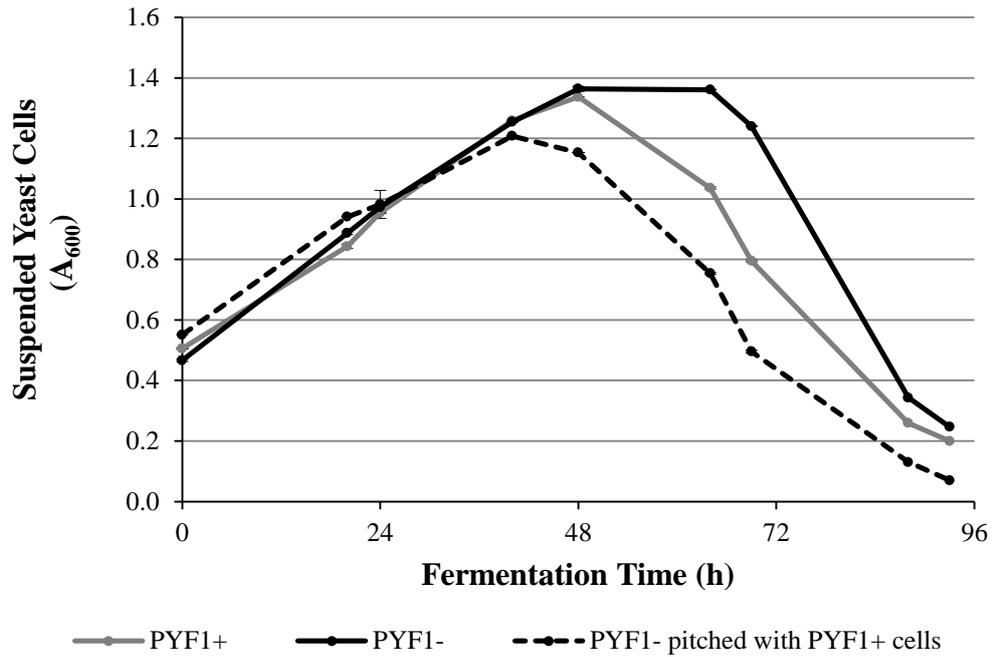


Figure 3.13: Fermentation profiles for PYF1+ (Scarlett), PYF1- (Prudentia) and PYF1- (Prudentia) worts pitched with PYF+ cells.

Fermentations were conducted at 15°C using W34/70 yeast at a pitching rate of 20×10^6 live cells. ml^{-1} . Data are the mean of two replicate fermentations \pm SD.

Besides the heavier flocculation, fermentations conducted with our standard PYF1- worts and yeast cells cropped from PYF1+ fermentations had also higher residual gravity (Figure 3.14) and consequently lower apparent fermentability even when compared with our PYF+ control worts (84.8% and 89% respectively).

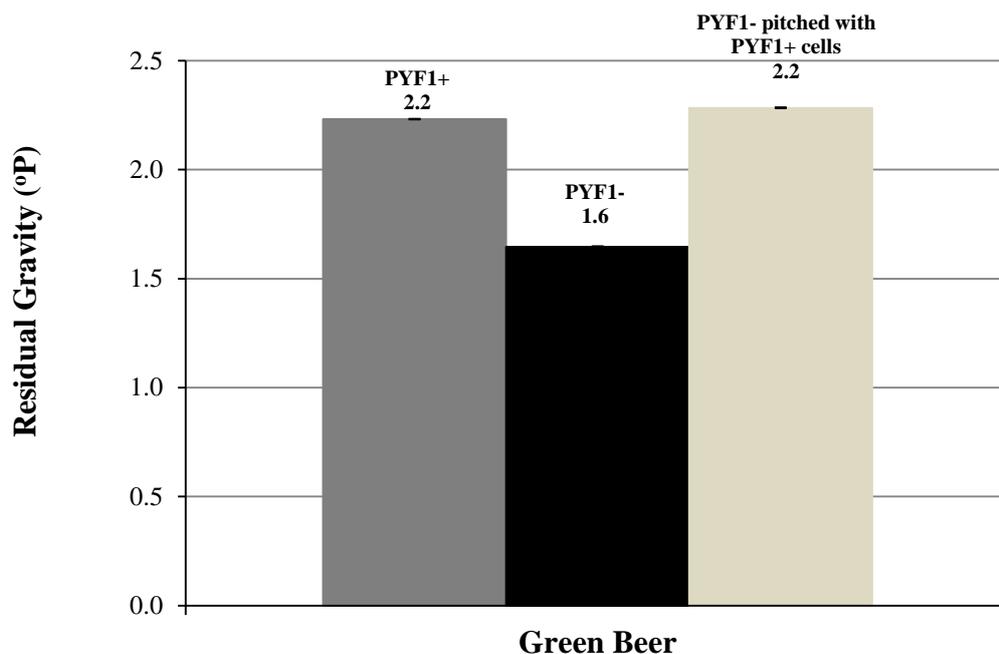


Figure 3.14: Residual gravities 96 h post-pitching of laboratory scale (200 ml) PYF-test fermentations utilising PYF1+ (Prudentia) and PYF1- (Scarlett) control worts.

Fermentations were conducted at 15°C using W34/70 yeast at a pitching rate of 20×10^6 live cells.ml⁻¹. PYF+ and PYF- data are the mean of two replicate fermentations \pm SD.

These data suggested that for this particular PYF+ sample the PYF factor(s) had caused a longer term effect on the yeast cells such that the incidence of PYF was more marked in subsequent fermentations. Besides that, the total diacetyl (2,3-butanedione) content of the beer fermented with the PYF- worts and yeast cells cropped from a previous PYF+ fermentation will also increase, resulting in a final product (beer) with detectable diacetyl flavour (Panteloglou *et al.*, 2012). Diacetyl, which has a distinct butterscotch character and is produced as a result of yeast metabolism during fermentation, derives from pyruvate via the intermediary of α -acetolactate (a precursor of valine biosynthesis during fermentation; Hornsey, 1999; Bamforth, 2003; Boulton & Box, 2003; Briggs *et al.*, 2004). Alpha-acetolactate is excreted into wort where it spontaneously oxidatively decarboxylates to form diacetyl. During the

warm phase of conditioning diacetyl is assimilated by yeast and reduced to less flavour-active metabolites acetoin and 2,3-butanediol (Boulton & Box, 2003). In many modern brewing processes, detectable diacetyl is regarded as a quality defect and commercial practice frequently involves a ‘diacetyl stand’ as a part of the fermentation/maturation process, whereby the diacetyl produced in primary fermentation is taken up and metabolised by yeast cells in suspension (Panteloglou *et al.*, 2012). Boulton and Box (2003), studying the formation and disappearance of diacetyl during lager fermentations, suggested that diacetyl has a flavour threshold of approximately 0.05 ppm above which considered undesirable. Bamforth (2003) indicated that diacetyl removal, by adding a charge of freshly vigorous yeast – known as “krausening” or by rising the temperature in the end of the primary fermentation, continues until the reduction of diacetyl below 0.01 – 0.1 ppm. Thus, the occurrence of PYF slows diacetyl removal owing to the lower suspended cell counts (Panteloglou *et al.*, 2012).

3.3.6 The Importance of Wort Composition on PYF Phenomenon

Figure 3.15 shows the fermentation profiles of three worts of unknown PYF status (Section 3.2.1.3) alongside our control PYF1+ and PYF1- worts. Using the small-scale fermentation tests (Section 2.6.1) and the highly flocculent but PYF sensitive lager yeast strain SMA Gairdner and Harrington worts exhibited normal fermentation profiles (similar to our standard PYF- worts). On the other hand, Jinyang worts, fermented under the same experimental conditions (pitching rate of 20×10^6 live cells.ml⁻¹ of wort after the supplementation of 11°P all-malt worts with 4% [w/v] glucose), exhibited PYF profiles (Figure 3.15). In particular, there was a significant difference ($P < 0.001$) in the

number of suspended yeast cells between Harrington and Jinyang worts 44 h post-pitching. At that period of time the number of suspended yeast cell counts in Jinyang fermentations was statistically lower than the number of suspended yeast cells in the Harrington fermentations. In addition, Jinyang wort fermentations also resulted in higher residual gravity and consequently lower apparent fermentability 72 h post-pitching (Table 3.2).

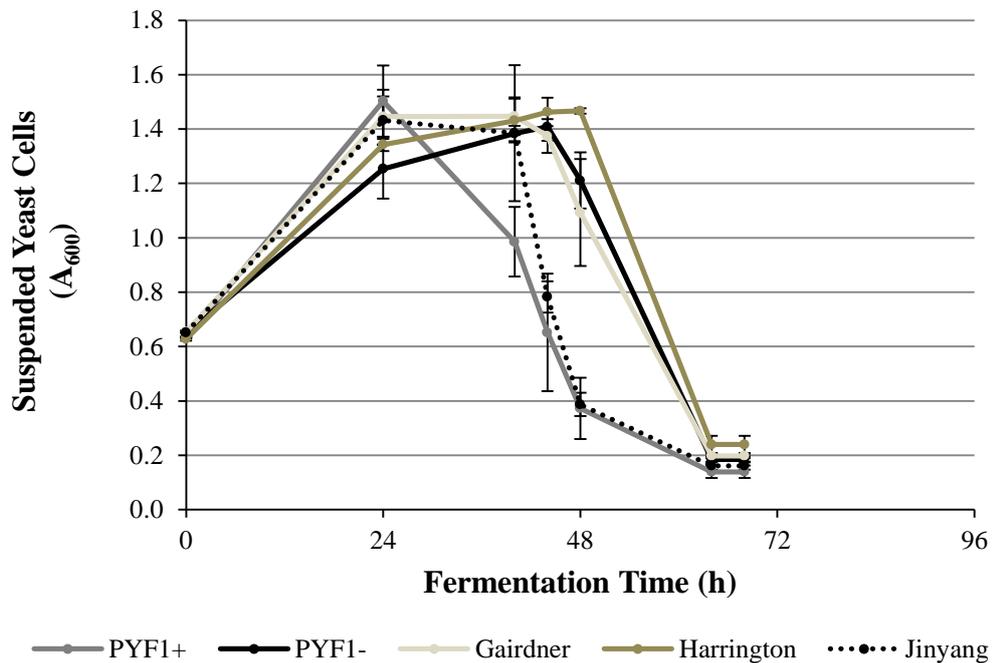


Figure 3.15: Suspended yeast cell profiles for 3 malts of unknown PYF status run alongside the PYF1+ and PYF1- control malts.

Fermentations were conducted at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells. ml^{-1} . Data are the mean of two replicate fermentations \pm SD.

Table 3.3: Residual gravity and apparent fermentability for unknown PYF status malts.

Wort	Residual Gravity (°P)	Apparent Fermentability (%)
Jinyang	1.6	89.0
Gairdner	1.2	91.9
Harrington	1.5	89.8

Besides the differences that were observed in the fermentation profiles between the different worts (Figure 3.15), only the Harrington wort, found to be PYF- according to the in-house PYF assay (Figure 3.15), had a FAN composition lower than 200 mg.l^{-1} . Jinyang and Gairdner worts, predicted as PYF+ and PYF- respectively during the PYF fermentation assay, had FAN compositions higher than 200 mg.l^{-1} of wort (Figure 3.16). On the other hand, worts prepared from the Jinyang malt (found to be PYF+ using the in-house assay) contained a lower amount of maltose (Figure 3.16). This suggested that the poor fermentation profiles, with respect to suspended yeast cell counts of the Jinyang worts during the in-house small-scale fermentation assay (Figure 3.14), might not be due to the presence of the PYF factor(s) but rather due to the lower concentration of glucose and maltose (Figure 3.16). Such an interpretation would be consistent with the views put forward by Lake and Speers (2008) who considered that part of the confusion surrounding PYF is caused by the brewers poor ability to differentiate between PYF malt and poorly (e.g. undermodified) fermenting malts. However, whilst poorly modified malt could lead to poor fermentation performance, a poorly optimised mashing schedule for example could also lead to the same effect. Thus, a positive result in the laboratory test may not necessarily translate/lead to a PYF+ malt.

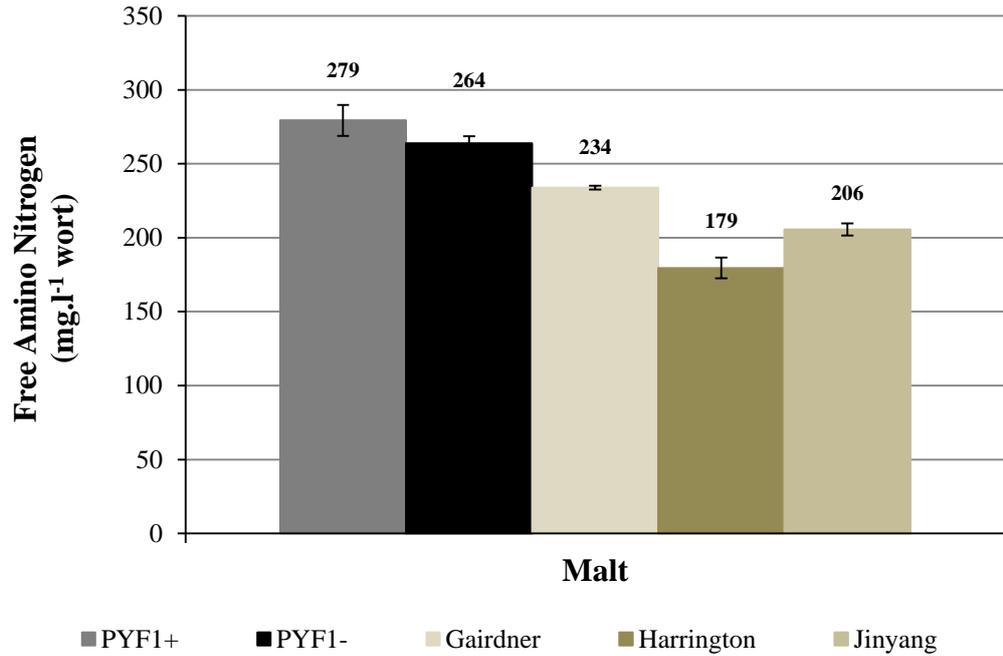


Figure 3.16: Free amino nitrogen content for all-malt worts (11°P) prepared from PYF1+ (Scarlett) and PYF1- (Prudentia) control malts and 3 malts of unknown PYF status. Data are the mean of three replicates ± SD.

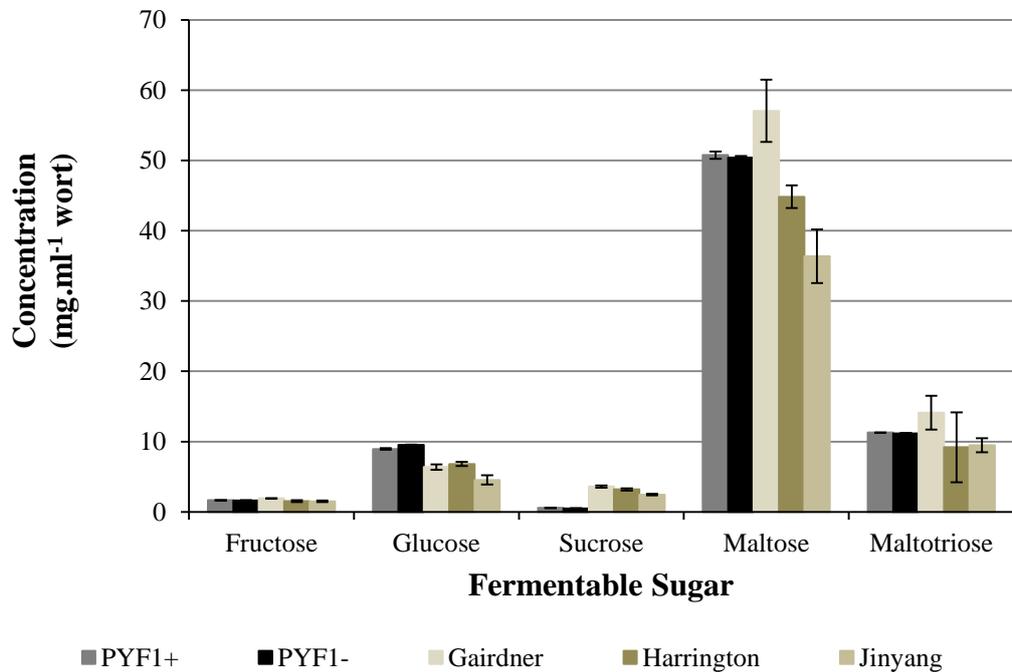


Figure 3.17: Fermentable sugar composition for all-malt worts (11°P) prepared from PYF1+ (Scarlett) and PYF1- (Prudentia) control malts and 3 malts of unknown PYF status. Data are the mean of three replicates ± SD.

3.4 Conclusion

The PYF potential of malt samples was successfully differentiated using an in-house small-scale fermentation assay 69 or 40 h post-pitching depending upon the yeast strain used. The highly flocculent yeast strain SMA was found more susceptible to PYF factor(s) than the less flocculent yeast strain W34/70, whilst a range of PYF+ malts sourced from the industry exhibited different degrees of PYF severity when fermented with the same brewing lager yeast strain. Moreover, worts with lower amount of glucose and maltose could be responsible for poor fermentation profiles, heavy PYF as well as elevated residual sugars and lower fermentability at the end of the primary fermentation.

Chapter 4

Optimization of a Small-scale

Fermentation Test to Predict the

Premature Yeast Flocculation Potential of

Barley Malts

Data from this Chapter formed the basis of a paper entitled *Optimization of a Small-scale Fermentation Test to Predict the Premature Yeast Flocculation Potential of Malts*, which has been published in the Journal of Institute of Brewing and Distilling (2010, 116, 4, 413 – 420).

4.1 Introduction

The development of a rapid fermentation assay that permits the detection of PYF and/or the potential of malts to cause PYF fermentations is essential to allow remedial strategies in the brewery to be actioned (Kruger *et al.*, 1982). The objective of this work was to identify appropriate conditions for the small-scale fermentation assay (Section 2.7.1) that would enable a more rapid differentiation between PYF+ and PYF- malts. The aim was both to achieve a reduction in the time required for detection as well as to enhance the current knowledge of the mechanisms involved in the PYF process. To achieve this, several variables for the fermentation test were assessed which were hypothesized to have an impact on the flocculation process.

The variables investigated included the addition of divalent metal cations. Calcium (Ca^{2+}) was selected due to its key involvement in the mechanism of yeast flocculation (calcium ensures that zymolectins are in the correct configuration for binding to the mannose receptors of adjacent yeast cells; Briggs *et al.*, 2004), and likewise supplementation of wort with differing concentrations of Zn^{2+} was investigated, since it was previously shown that Ca^{2+} , Zn^{2+} and Fe^{2+} inhibited the antiyeast activity of Okada's PYF toxin at an amount of substance equal with 5×10^{-3} M or more (Okada *et al.*, 1970). Okada's toxin, previously isolated from the endosperm of wheat and barley

with a dilute (0.05 N) sulphuric acid solution was thought to react with the functional site(s) (i.e. carboxyl and phosphoric acidic groups) of the cell wall and cell membrane causing changes in the permeability of the membrane. As a result, the toxin inhibited the uptake of glucose when present at 0.4 mg.l^{-1} and caused the death of the cells within a short period of time (6 min) when present at 10 mg.l^{-1} (Okada & Yoshizumi, 1973). The toxin was also found to be active against a variety of flocculent brewing yeast strains, whilst non-flocculent yeast strains appeared to be less sensitive. Okada's toxin was later identified as a protein with a MW of 9.8 kDa, with an isoelectric point higher than pH 10, and which was resistant to proteolysis and heat. Okada and Yoshizumi (1970) suggested that neutralization of the toxicity by the divalent ions (i.e. Ca^{2+} , Zn^{2+} and Fe^{2+}) might be due to the competitive binding with the acidic groups on the cell surface. Three years later, in 1973, Okada and Yoshizumi (1973) proposed that Ca^{2+} ions protected the yeast cell from toxicity rather than through chemical binding with the toxin. The toxic effect was also inactivated by trypsin, but not by chymotrypsin and carboxypeptidase (Okada & Yoshizumi, 1970). Axcell *et al.* (2000) indicated that the molecular weight and the basic nature of the protein was reminiscent of some antimicrobial peptide groups such as the thionins and the non-specific lipid transfer proteins (ns-LTP's).

The production of 'turbid' worts (i.e. worts containing higher amount of lipids or fatty acids) by varying the laboratory mash filtration protocol or by adding pure linoleic acid ($\text{C}_{18}\text{H}_{32}\text{O}_2$; 18:2) prior to pitching were also investigated as factors that might play a role in the action of PYF. Axcell *et al.* (2000) reported that breweries with brighter wort production (i.e. those with a lower

lipid content), typically experienced more fermentation problems, with respect to PYF, and that these problems were not alleviated by the addition of protein-based yeast foods (previously reported able to act as nucleation sites (Axcell *et al.*, 1988). Axcell *et al.* (2000) hypothesized that the strong cationic and amphiphathic character of the antimicrobial peptides would allow them to bind to lipids or fatty acids of the worts leading to a “titration effect” in which the peptides were no longer available to bind to yeast cells. Non-specific lipid transfer proteins (ns-LTP’s) and thionins are strongly cationic with an amphipathic character (see Section 1.3.2.2 for details). This helps them to interact with membranes, in that this can take place at the interface between hydrophobic aliphatic acyl chains and the polar head groups in contact with the aqueous environment. The interaction of antimicrobial peptides with lipid membranes depends on their hydrophobic – hydrophilic balance (their ability to evoke ion-channel activity depends on their secondary structures and self-assembly), whilst the antimicrobial activity, insertion and disruption function must follow from membrane interaction (Axcell *et al.*, 2000).

Finally, the worts were supplemented with different levels of added glucose in order to achieve worts of different gravities. This had also been proposed as a mechanism of enhancing fermentation vigour, and maintaining yeast cells in suspension (Jibiki *et al.*, 2006; Lake & Speers, 2008; Speers *et al.*, 2011) which can be an issue with the small-scale laboratory fermentation tests due to reduced CO₂ and hence, the reduced shear rates typically encountered (Boswell *et al.*, 2002; Lake & Speers, 2008).

4.2 Experimental

4.2.1 Malts

Two control barley malts from a similar region (France) and crop year (2007) were used throughout these experiments. The malts were however prepared from different barley varieties (Scarlett and Prudentia for the PYF1+ and PYF1- malts respectively). The Scarlett malt sample was known to have caused PYF in brewery fermentations, whilst the Prudentia sample was a control malt giving rise to normal fermentations profiles.

4.2.2 Wort Preparation

Worts were prepared from control (Section 4.2.1) malts to give an all-malt wort with a gravity of 11°P using a standardized laboratory mashing procedure as described in Section 2.5.

4.2.3 Wort Composition Analyses

FAN and fermentable sugar spectrum analyses were performed as described in Sections 2.7.3 and 2.7.4 respectively.

4.2.4 Yeast Strains and Propagation Conditions

Two lager brewing yeast strains were used in this study (W34/70 and SMA). W34/70 is a moderately flocculent yeast strain, whilst SMA a more flocculent yeast strain (Section 2.1). W34/70 was obtained from the National Collection of Yeast Cultures (NCYC), whilst SMA was sourced from the VLB Research Institute (Berlin, Germany). Yeast propagation was performed in an orbital shaken incubator at 120 rpm for 4 days at 15°C as described in Section 2.2.4.

4.2.5 Premature Yeast Flocculation (PYF) Assay

Full details of the PYF assay procedures may be referenced in Section 2.6.1. In brief, the procedure involved conducting small-scale (200 ml) fermentations in 250 ml ‘dropping funnels’ within a temperature controlled incubator (15°C) and using worts prepared from control PYF+ and PYF- malts (Section 4.2.1) using a standardized laboratory mashing procedure (see Section 2.5 for details). The fermentations were conducted at a pitching rate of 20×10^6 live cells.ml⁻¹ after the supplementation of the 11°P all-malt wort with 4% [w/v] glucose.

4.2.6 Premature Yeast Flocculation Assay Measurements

4.2.6.1 Monitoring Suspended Yeast Cells in Suspension

The determination of the cell concentration in suspension was assessed at specific time intervals between 0 – 92 h post-pitching by measuring absorbance at 600 nm (A_{600} ; Section 2.6.2.1).

4.2.6.2 Residual Gravity

The residual gravities of the fermenting broths were determined at 15°C using a DMA 5000 M model Anton Paar density-alcolyzer meter meter as described in Section 2.7.6.

4.2.6.3 Fermentability

The fermentability of the worts (that is the proportion of the wort dissolved solids (extract) which can be fermented) was calculated as a percentage according to Equation 2.8; Section 2.7.7.

4.2.7 Statistical Analysis

The statistical significance of the impacts of different malt samples and yeast strains on yeast flocculation and residual gravity were calculated using ANOVA and the statistical program Minitab (version 15, Minitab Inc., State College, USA). In each instance the null hypothesis (H_0) was that no significant difference existed between data sets. If the P value generated by the test was less than 0.05 then the null hypothesis of no significant difference was rejected and the alternative hypothesis (H_a) of significance was adopted. Whilst ANOVA can indicate that an overall significant difference exists between data sets, *post-hoc* analysis is required to assess which sample means differed statistically from one another. Pair-wise comparison of means was completed using the Tukey test at the significance level $P < 0.05$.

4.2.8 Optimization of the Small-scale PYF Fermentation Assay

Experimental design software (Design Expert version 8.01, Statease, Minneapolis, USA) was used to devise a robust experiment with which to investigate the impacts of five factors on the ability of the fermentation test to distinguish PYF+ from PYF- malts at various time points post-pitching. A D-optimal design was selected which required thirty seven fermentation tests (nineteen PYF+ and eighteen PYF-worts) performed over two weeks of trials (Blocks 1 and 2). The investigated factors and ranges are shown in Table 4.1.

Table 4.1: D-optimal experimental design used for the optimization of the in-house PYF fermentation assay.

Run	Block	Calcium Chloride (g.l ⁻¹)	Zinc (mg.l ⁻¹)	Linoleic Acid (mg.l ⁻¹)	Glucose (% w/v)	Malt Type	Filtration
1	Week 1	0.182	0.2	6.0	4.0	PYF+	Recycling
2	Week 1	0.182	0.2	6.0	4.0	PYF+	Recycling
3	Week 1	0.000	0.2	0.0	0.0	PYF+	Recycling
4	Week 1	0.000	0.2	0.0	4.0	PYF-	Recycling
5	Week 1	0.091	0	6.0	4.0	PYF+	Not Recycling
6	Week 1	0.182	0.1	6.0	0.0	PYF-	Recycling
7	Week 1	0.091	0.0	6.0	4.0	PYF+	Not Recycling
8	Week 1	0.046	0.1	3.0	2.0	PYF-	Recycling
9	Week 1	0.000	0.2	0.0	0.0	PYF+	Recycling
10	Week 1	0.182	0.0	0.0	4.0	PYF-	Not Recycling
11	Week 1	0.000	0.1	6.0	0.0	PYF+	Not Recycling
12	Week 1	0.000	0.0	6.0	0.0	PYF-	Not Recycling
13	Week 1	0.182	0.2	0.0	0.0	PYF-	Recycling
14	Week 1	0.182	0.2	0.0	2.0	PYF+	Not Recycling
15	Week 1	0.182	0.2	6.0	4.0	PYF-	Not Recycling
16	Week 1	0.000	0.0	3.0	4.0	PYF+	Recycling
17	Week 1	0.091	0.0	0.0	0.0	PYF+	Not Recycling
18	Week 1	0.000	0.0	0.0	4.0	PYF-	Not Recycling
19	Week 1	0.000	0.2	6.0	2.0	PYF-	Not Recycling
20	Week 2	0.182	0.1	0.0	4.0	PYF+	Not Recycling
21	Week 2	0.182	0.0	0.0	0.0	PYF-	Not Recycling
22	Week 2	0.091	0.0	0.0	4.0	PYF+	Recycling
23	Week 2	0.000	0.0	6.0	0.0	PYF+	Recycling
24	Week 2	0.000	0.2	6.0	0.0	PYF-	Recycling
25	Week 2	0.000	0.1	6.0	4.0	PYF-	Recycling
26	Week 2	0.000	0.2	3.0	4.0	PYF+	Not Recycling
27	Week 2	0.091	0.2	3.0	2.0	PYF+	Recycling
28	Week 2	0.000	0.0	0.0	2.0	PYF+	Not Recycling
29	Week 2	0.000	0.2	0.0	0.0	PYF-	Not Recycling
30	Week 2	0.182	0.2	6.0	0.0	PYF+	Not Recycling
31	Week 2	0.137	0.05	4.5	2.0	PYF-	Not Recycling
32	Week 2	0.000	0.2	0.0	0.0	PYF-	Not Recycling
33	Week 2	0.182	0.0	6.0	4.0	PYF-	Recycling
34	Week 2	0.000	0.0	0.0	0.0	PYF-	Recycling
35	Week 2	0.000	0.0	6.0	0.0	PYF+	Recycling
36	Week 2	0.182	0.0	3.0	0.0	PYF+	Recycling
37	Week 2	0.182	0.2	0.0	4.0	PYF-	Recycling

Calcium chloride (CaCl₂·2H₂O) was added to the mashing liquor at 0 – 0.182 g.l⁻¹; zinc (0 – 0.2 mg.l⁻¹) was added as zinc sulphate (ZnSO₄·12H₂O), glucose (0 – 4% w/v) and linoleic acid (0 – 6 mg.l⁻¹) were added to the wort immediately prior to fermentation. Satisfactory dispersion of linoleic acid was

achieved by first dispersing in methanol (3 ml), and then into wort (25 ml) of the appropriate PYF status. This was then diluted further into experimental worts to yield the desired range of final concentrations. To investigate the impact of wort clarity (turbidity) the first 100 ml of the filtrate were either recycled through the mash bed during laboratory wort filtration or were not, the latter procedure thereby creating more turbid worts. During each fermentation suspended yeast cell counts were monitored at time intervals of 0, 24, 40, 44, 48, 64, 68 and 72 h post pitching using the procedures described in Section 4.2.6.1.

4.3 Results and Discussion

4.3.1 Wort Fermentable Sugars Composition and FAN Content

The importance of wort composition on yeast growth and fermentation performance has been discussed in Chapter 3; Section 3.1. Worts prepared from the PYF1+ and PYF1- malt samples used throughout these experiments were of similar composition with respect to fermentable sugar spectrum (sucrose, fructose, glucose, maltose and maltotriose) and FAN (Figures 3.1 and 3.2 respectively). On that basis it could be assumed that they would ferment similarly, other than for differences caused by PYF (Van Nierop *et al.*, 2004).

4.3.2 Predicting the PYF Potential of Malts using the PYF Assay

The development of a small-scale fermentation test to predict the PYF potential of malts was described in Chapter 3. The results obtained were found to be dependent upon the yeast strain used, and in particular its flocculation characteristics. Thus, using the medium flocculent brewing lager yeast strain W34/70 it was not possible to distinguish PYF1+ from PYF1- fermentations

until 64 or 69 h post-pitching (depending upon the method used for the determination of the suspended yeast cells; Figures 3.3 and 3.4 respectively). After these time periods, the number of suspended yeast cells in the PYF1+ fermentations was found to be significantly lower ($P < 0.0001$) than the number of suspended yeast cells in the PYF1- fermentations. Besides that, PYF1+ fermentations had a statistically significantly higher residual gravity, and, hence, a lower fermentability than when compared with the PYF1- fermentations 96 h post-pitching (Figure 3.5).

On the other hand, when the highly flocculent but PYF sensitive yeast SMA was used under the same fermentation conditions (pitching rate of 20×10^6 live cells.ml⁻¹ and using 11°P all-malt worts supplemented with 4% [w/v] glucose) significant differences ($P < 0.0001$) in A_{600} were obtained just 40 h post-pitching (Figure 3.6). Thus, the use of a more flocculent yeast strain (SMA) resulted in more rapid discrimination between PYF1+ and PYF1- malts, reducing the time of analysis by 24 h.

4.3.3 Optimization of the PYF Fermentation Assay

The five experimental factors (Table 4.2) varied across a D-optimal design space, which was specifically developed for SMA, and were used to model A_{600} data at each time point (i.e. 0, 24, 40, 44, 48, 64 and 68 h post-pitching). The derived model for suspended yeast cell count 40 h post-pitching indicated that the addition of linoleic acid ($0 - 6$ mg.l⁻¹) to wort had a significant effect ($P = 0.047$) on the model, whereas the addition of zinc and glucose before pitching, the different filtration processes after mashing as well as varying calcium levels in the brewing liquor did not have a significant effect

($P < 0.05$) on the ability of the test to distinguish PYF1+ from PYF1- fermentations 40 h post-pitching (Table 4.2).

Table 4.2: Impact of the factors investigated on the ability of the fermentation assay to distinguish between PYF+ and PYF- malts 40 h post-pitching.

Factor	Investigated levels	<i>P</i> -value
Zn ²⁺ (added as Zinc sulphate)	0 – 0.2 (mg.l ⁻¹)	0.196
Linoleic acid	0 – 6 (mg.l⁻¹)	0.047
CaCl ₂ ·2H ₂ O (in the Brewing liquor)	0 – 0.182 (g.l ⁻¹)	0.939
Glucose	0 – 4 % (w/v)	0.281
Turbidity	Recycling vs. Non-Recycling (of the first 100 ml wort during mash filtration)	0.731

The impact of linoleic acid supplementation on PYF1+ fermentations was to reduce the concentration of yeast cells in suspension at 40 h post-pitching, and this reduction was found to be significant ($P < 0.05$). Since this effect was not observed in the PYF1- fermentations, differentiation of PYF status was enhanced (Figure 4.1).

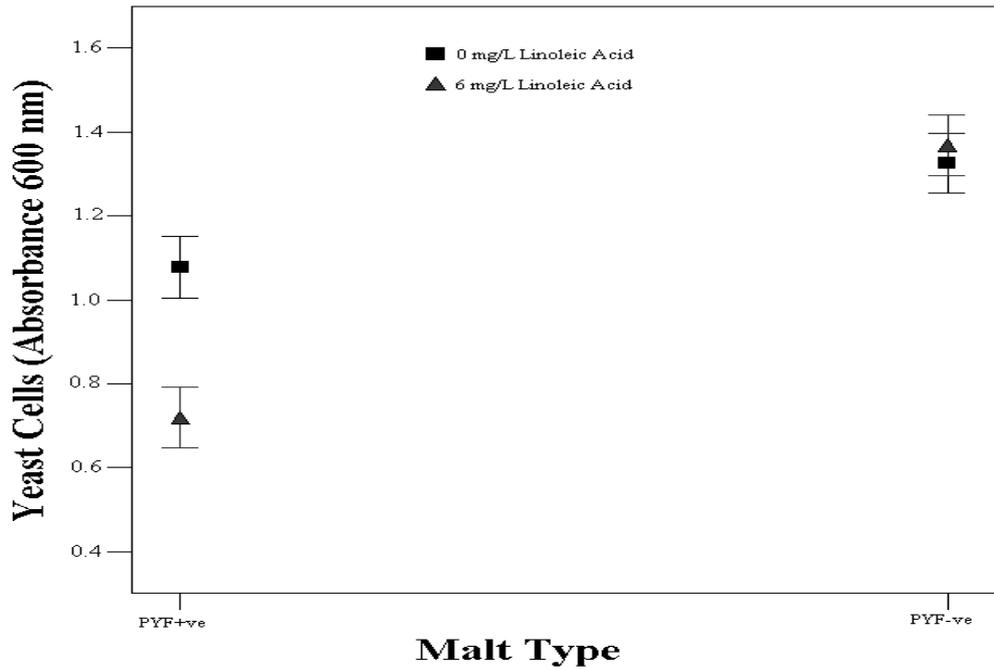


Figure 4.1: A Design Expert interaction plot showing the effect of 0 and 6 mg.l⁻¹ linoleic acid on suspended yeast cell counts (as indexed by A₆₀₀) in PYF1+ (Scarlett) and PYF1- (Prudentia) fermentations 40 h post-pitching.

These data suggested that the addition of 6 mg.l⁻¹ linoleic acid to wort prior to pitching, and the use of the highly flocculent but PYF sensitive yeast strain SMA, in the small-scale fermentation test analysis permitted the PYF status of malts to be determined after just 40 h of fermentation. This method resulted in a substantial reduction in the fermentation assay time from 64 h (Figure 3.3) to 40 h (Figure 4.2).

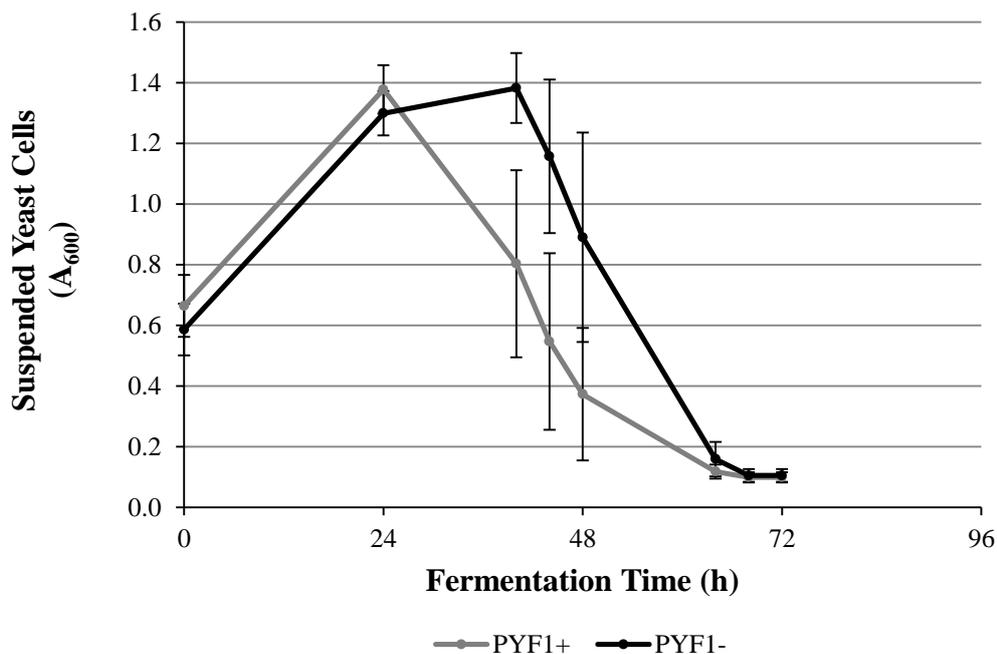


Figure 4.2: Fermentation profiles showing the effect of adding 6 mg.l⁻¹ linoleic acid to PYF1+ (Scarlett) and PYF1- (Prudentia) worts fermented at 15°C using SMA yeast at the pitching rate of 20 × 10⁶ live cells.ml⁻¹.

PYF1+ and PYF1- data are the mean ± SD of seven and nine fermentations respectively.

Despite the more rapid discrimination that was reproducibly achieved in this experiment the mode of action of linoleic acid is not yet understood and requires further elucidation. Boulton and Quain (2003) indicated that fermentations with trub-rich worts (i.e. lipid-rich worts) were associated with faster rates and increased yeast growth compared to bright worts, whilst Gibson (2011) reported that linoleic acid has a significant effect on fermentation performance and beer quality (i.e. faster fermentation rates, improved yeast growth and viability and increased levels of ethanol, but not higher alcohols). Hornsey (1999) proposed that cloudy worts, containing anywhere from 5 to 40 times the unsaturated fatty acid content of clear worts, contributed positively to yeast viability and inhibited the formation of some less pleasant acetate esters during fermentation. Bamforth (2003) proposed that lipids, that are present in high amounts in the so-called cold break produced in

the brewhouse, promote a vigorous fermentation. This is because of the ability of the lipids, and some other wort solids (e.g. trace metals), to form nucleation sites (i.e. sites for the creation of bubbles) for gas release which keeps yeast in suspension and therefore in contact with wort for fermentation. The nucleation sites prevent the accumulation of carbon dioxide (CO₂) that tends to inhibit yeast metabolism. More recently, Gibson (2011), based on numerous published data, suggested also that the use of cloudy worts improves fermentation performance, not through any nutritional effect of the lipids but, rather, through solid particles acting as nucleation sites for CO₂ bubble formation. As a consequence, suspended cell increase, and the inhibitory effect of dissolved CO₂ is reduced. Due to higher suspended cell counts the contact of yeast cells to the medium is also intensified and therefore, metabolic rate rises. Stewart and Martin (2004) found that the use of turbid worts improved fermentation performance over and above that seen in clear wort containing diatomaceous earth (kieselguhr) as a CO₂ nucleation factor, despite the reduction in dissolved CO₂ being identical in both cases. The results of Stewart and Martin (2004) were later confirmed by Kuhbech *et al.* (2007) who noted an increase in fermentation performance in the presence of trub which could not be matched by the addition of other particles without nutritive effect such as PVPP, kieselguhr and activated carbon. However, the increase in fermentation performance did not occur as clearly for all yeast vitalities and was not as great as that of hot trub. Gibson (2011) proposed that improved fermentation performance in trub-rich wort may also be influenced by the presence of bound ionic metals such as copper (Cu²⁺) or zinc (Zn²⁺), which otherwise may be lost through clarification.

On the other hand the suspended particles in the wort, which can consist of as much as 50% lipids, can have a negative effect on foam stability, and more specifically in beer staling (Hornsey, 1999). Gibson (2011) indicated that the principal objection of the use of turbid worts in brewing fermentations relates to potential reduction on ester synthesis, which impart a fruity or floral aroma to beer, by the yeast cells due to repression of the ATF genes which encode for alcohol acetyltransferases.

Therefore, in our experiments, it is possible that either the fermentation cycle was shifted forward by the addition of linoleic acid and/or that 18:2 promoted a more vigorous fermentation enabling the earlier and clearer differentiation of a malt sample's PYF status. Kock *et al.* (2000) observed the accumulation of hydrophobic carboxylic acids (i.e. 3-hydroxy 8:0 and 3-hydroxy 10:0 oxylipins) on the cell surface of *Saccharomyces cerevisiae* ATCC 26602, a known flocculent strain, during the initiation of flocculation. One year later, in 2004, Strauss *et al.* (2004) showed that the addition of linoleic acid during fermentation with *Saccharomyces cerevisiae* UOFS Y-2330, led to yeast uptake of the fatty acid with peak cellular accumulation occurring during the first 8 h of flocculation. *Saccharomyces cerevisiae* UOFS Y-2330 yeast strain exhibited both *Flo1* and *NewFlo* behaviour probably due to a switch in sensitivity of the yeast to flocculate in the presence of glucose as well as pH which may in turn influence the availability of calcium ions (Strauss *et al.*, 2003). During the first 8 h of flocculation 16:1 (palmitoleic acid) was probably converted to 18:1 (oleic acid) via an elongase enzyme, which was then further desaturated to 18:2 via a Δ desaturase enzyme. Strauss *et al.* (2004) suggested that this was a response limited to their strain of interest (*Saccharomyces*

cerevisiae UOFS Y-2330), and although they did not propose a link between fatty acid uptake and flocculation *per se*, in light of the current data it is tempting to speculate that 18:2 addition might accelerate flocculation onset under certain conditions.

The absence of an impact of wort turbidity as well as the observation that linoleic acid exacerbated PYF and made its detection more rapid is also contrary to the “titration hypothesis” (Axcell *et al.*, 2000) which hypothesized that the addition of fatty acids might “titrate” out antimicrobial peptides so that they can no longer bind to the yeast cells. In the current study linoleic acid appeared to exacerbate the impact of PYF, which perhaps indicates that the sample used was inducing PYF via the HMW arabinoxylan bridging mechanism, as opposed to disruption of yeast cell membrane function as is thought to occur with the antimicrobial peptides.

The effect of linoleic acid on yeast flocculation was confirmed in subsequent fermentations conducted under the same experimental conditions using the same PYF1+ and PYF1- worts (Figure 4.3). More specifically, fermentations conducted with the PYF1+ worts after the supplementation of 6 mg.l⁻¹ linoleic acid, prior to pitching, had a significantly lower number ($P < 0.0001$) of suspended yeast cell counts 44 h post-pitching than was the case with the PYF1- worts supplemented with the same amount (6 mg.l⁻¹) of the nutrient (18:2).

Despite the fact that the differences between the PYF1+ and PYF1- fermentations, with respect to the number of suspended yeast cells, were found to be statistically significant 4 h later (i.e. 44 h post-pitching), when compared

with our previous experiments, (i.e. significant differences 40 h post-pitching) it was confirmed that the addition of 18:2 prior to pitching exacerbated the flocculation of the yeast cells in the PYF1+ fermentations. Consequently, the results obtained suggested, once again, that in the current malt sample (PYF1+) PYF was induced through the HMW polysaccharides (i.e. barley/malt degradation products) rather than the antimicrobial peptides (i.e. ns-LTPs, thionins, defensins) which have been hypothesized by Van Nierop *et al.* (2004) as the second type of PYF factor(s).

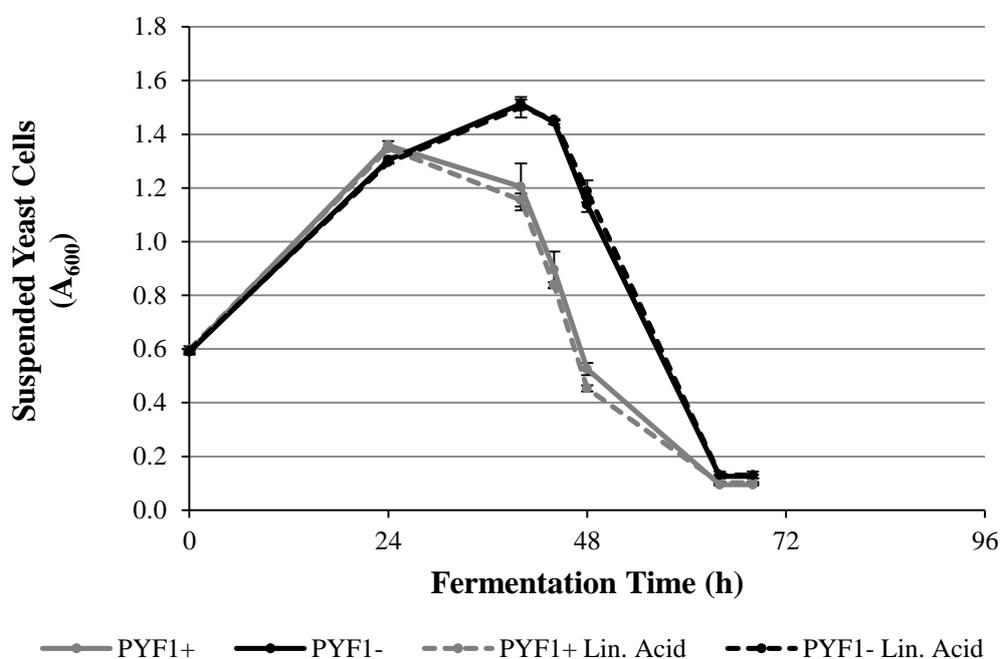


Figure 4.3: Fermentation profiles showing the effect of adding 6 mg.l⁻¹ linoleic acid to PYF1+ (Scarlett) and PYF1- (Prudentia) worts fermented at 15°C using SMA yeast at the pitching rate of 20 × 10⁶ live cells.ml⁻¹. Data are the mean of two replicate fermentations ± SD.

The complexity of the PYF phenomenon was reflected by the fact that factors such as varying calcium or zinc salt addition to the mashing liquor or wort had relatively minor impacts upon the differentiation of PYF+ from PYF- malts and that these effects were not statistically significant across the design space as a whole. This suggested either that there are complex interactions between

the factors which could not be adequately modelled, or simply that the effect of linoleic acid addition was substantially greater than any other effects occurring over the design space used.

4.4 Conclusions

By supplementing the worts with 6 mg.l⁻¹ linoleic acid and using a highly flocculent PYF sensitive yeast strain during the small-scale fermentation tests, the PYF potential of malts could be predicted 40 h post-pitching. These adaptations reduced the required time of analysis by 24 h. Besides shortening the required time of analysis, the consideration of the mechanism by which addition of linoleic acid enhanced the early distinction between PYF+ and PYF- malts may prove useful in further elucidating the underlying causal factors of this complex phenomenon.

Chapter 5

The Importance of Yeast Strain in the Incidence of Premature Yeast Flocculation Phenomenon

Data from this Chapter were presented at the 74th American Society of Brewing Chemists Meeting, which took place in Sanibel Island in Florida 11th - 15th June 2011. The paper has been included in the Proceedings of the 74th American Society of Brewing Chemists Annual Meeting (Oral Presentation 17).

5.1 Introduction

One factor which has made the facts around PYF hard to establish over the years is the variable impact of PYF on different yeast strains (Axcell, 2003; Van Nierop *et al.*, 2004; Panteloglou *et al.*, 2010; Panteloglou *et al.*, 2012). Thus, a PYF+ malt can be dispatched in apparently identical condition to two different breweries – one of which will experience severe PYF, whilst the other may observe no negative impacts whatsoever.

Jibiki *et al.* (2006) using a 50 ml laboratory fermentation test and EBC Congress wort fermented at 21°C concluded that ale yeasts (i.e. NCYC 1681, NCYC 1026, NCYC 1078, NCYC 1301, Weihenstephan 184) of varying degrees of flocculence were not sensitive to PYF+ malts. On the other hand, all of the lager yeasts examined (i.e. Asahi, Weihenstephan 34/70, Weihenstephan 195, Weihenstephan 71, SMA and NCYC 1324) as well as the non-flocculent mutations of Asahi (i.e. Lager1-Mutant) and Weihenstephan (i.e. W34/70-Mutant) were found sensitive to the same PYF+ malts. Armstrong and Bendiak (2007) commented on the apparent impact of yeast strain and concluded from retrospective analysis of brewery trend data, mainly with regards to real extract of bright beer, that the more flocculent yeast strains involved definitely showed more susceptibility to PYF than the less-flocculent or non-flocculent strains.

Evans and Kaur (2009) observed that “it is the associations between the preferred yeast strains of the major brewing companies and PYF susceptibility which have resulted in problems for brewers such as Kirin, Asahi, SABMiller and Anheuser Busch (now AB InBev)”.

Thus, whilst previous studies (Jibiki *et al.*, 2006) investigated the sensitivity of ale and lager yeast strains on the same PYF factor(s) (i.e. using the same PYF+ worts), this study aims to develop understanding of how different yeasts respond to the presence of different PYF factor(s). For that reason trial fermentations were conducted using our in-house PYF assay (Section 2.6.1) with industrial provided PYF+ and PYF- worts and using lager and ale yeast strains of varying degrees of flocculence. The differences in yeast response to fermentation conditions were evaluated by monitoring suspended yeast cell counts at different time points during fermentation and by determining residual gravity and alcohol yield.

5.2 Experimental

5.2.1 Malts

Two PYF+ and two PYF- malts were used in this study. The first pair of PYF+ and PYF- malts were produced from the same barley variety (Scarlett), region (France) and crop year (2007), whilst the second pair of malt samples were manufactured from different barley varieties (Quench and Prestige) and were sourced from different regions (UK and Europe; Table 5.1). The third PYF+ malt (PYF4+) was a mixture of different barley varieties produced in central Europe (Table 5.2). In each case PYF+ malts were known to have caused PYF, whilst PYF- samples exhibited normal fermentation profiles in industrial scale

fermentations.

Table 5.1: Barley variety, harvest year and region of production for PYF+ and PYF- malts used in this study.

Barley Variety	Harvest Year	Region of Production	PYF Status
Scarlett	2007	France	PYF2+
Scarlett	2007	France	PYF2-
Quench	2009	U.K	PYF3+
Prestige	2009	Europe	PYF3-
Nectaria	2009	Hungary	PYF4+

Table 5.2: Barley varieties comprising PYF4+ malt.

Barley Variety	% Percentage
Scarlett	13
Cristalia	17
Cellar	20
Nectaria	50

5.2.2 Wort Preparation

Worts were prepared from control (Section 5.2.1) malts to give an all-malt wort with a gravity of 11°P using a standardized laboratory mashing procedure as described in Section 2.5.

5.2.3 Wort Composition Analyses

FAN and fermentable sugar spectrum analyses were performed as described in Sections 2.7.3 and 2.7.4 respectively.

5.2.4 Yeast Strains and Propagation Conditions

Three lager (W34/70, SMA and ‘Industrial’) and two ale (NCYC 1332 and M2) brewing yeast strains were used in this study. The ale yeast strains and

W34/70 (ex Weihenstephen) were obtained from the National Collection of Yeast Cultures (NCYC), the SMA from the VLB Research Institute (Berlin, Germany) and the ‘Industrial’ yeast strain was provided by a large multinational brewing company. Ale and lager yeast strains were selected to exhibit varying degrees of flocculence (Table 5.3). The ‘Industrial’ lager yeast strain was of interest because it was thought to be relatively insensitive to PYF and is not identified for reasons of commercial sensitivity. Yeast propagation was performed in an orbital shaken incubator at 120 rpm for 4 days at 15°C as described in Section 2.2.4.

Table 5.3: Source and relevant flocculence of yeast strains used in this study.

Yeast Strain	Source	Yeast Type	Relevant Flocculence
W34/70	NCYC	Lager	Medium
SMA	VLB Research Institute	Lager	High
‘Industrial’	Industry	Lager	Non-flocculent
NCYC 1332	NCYC	Ale	Non-flocculent
M2	NCYC	Ale	Flocculent

5.2.5 Premature Yeast Flocculation (PYF) Assay

PYF assays were conducted as described in Section 2.6.1. In brief, the procedure involved conducting small-scale (200 ml) fermentations in 250 ml ‘dropping funnels’ within a temperature controlled incubator (15°C) and using worts prepared from PYF+ and PYF- malts (Section 5.2.1) using a standardized laboratory mashing procedure (see Section 2.5 for details). The fermentations were conducted at a pitching rate of 20×10^6 live cells.ml⁻¹ after the supplementation of 11°P all-malt worts with 4% [w/v] glucose for a maximum period of 92 h.

5.2.6 Premature Yeast Flocculation Assay Measurements

5.2.6.1 Monitoring Suspended Yeast Cell Counts

The determination of the cell concentration in suspension was assessed at specific time intervals between 0 – 92 h post-pitching (i.e. 0, 24, 40, 44, 48, 52, 64, 68 and 92) by measuring absorbance at 600 nm (A_{600} ; Section 2.6.2.1).

5.2.6.2 Residual Gravity and Alcohol Yield

The residual gravity and alcohol yield of the fermenting broths were determined at 15°C using a DMA 5000 M model Anton Paar density-alcolyzer meter as described in Section 2.7.6.

5.2.6.3 Fermentability

The fermentability of the worts (the proportion of the wort dissolved solids (extract) which could be fermented) was calculated as a percentage according to Equation 2.8; Section 2.7.7.

5.2.7 Replicates of Malts and Yeast Strains used in this study.

The number of replicates for each combination of malts and yeast strains used in this study is shown in Table 5.4.

Table 5.4: Replicates for each combination of malts and yeast strains used in this study.

Figure	Samples	Yeast Strain	Replicates
5.3 – 5.5	PYF2+, PYF2-	W34/70, SMA	3
5.6 – 5.9	PYF2+, PYF2-	SMA, 'Industrial'	3
5.10	PYF2+, PYF2-	NCYC 1332	3
5.11	PYF2+, PYF2-	M2	3
5.12 – 5.13	PYF2+, PYF2-	NCYC 1332, M2	3

5.2.8 Statistical Analysis

The statistical significance of the different malt types and yeast strains on yeast flocculation, residual gravity and ethanol yield was performed using ANOVA and the statistical program Minitab (version 15, Minitab Inc., State College, USA). In each instance the null hypothesis (H_0) was that no significant differences existed between data sets. If the P value generated by the test was less than 0.05 then the null hypothesis of no significant difference was rejected and the alternative hypothesis (H_a) of significance was adopted. Whilst ANOVA can indicate that an overall significant difference exists between data sets, *post-hoc* analysis is required to assess which sample means differed statistically from one another. Pair-wise comparison of means was completed using the Tukey test at the significance level $P < 0.05$.

5.3 Results and Discussion

5.3.1 Wort Fermentable Sugars and FAN Composition

The importance of wort fermentable sugars and FAN on yeast growth and fermentation performance was discussed in Chapter 3 (Section 3.3.1). Worts prepared from the Scarlett (PYF2+, PYF2-) and Quench malts (PYF3+) were of similar composition with respect to fermentable sugars (fructose, glucose, sucrose, maltose and maltotriose) and FAN (Figures 5.1 and 5.2 respectively). On the other hand, worts prepared from the Prestige malt (PYF3- control malt) had lower amount of maltose, whilst worts prepared from the PYF4+ malts had a lower amount of maltotriose and FAN compared to those previously noted from the Scarlett and Quench worts. However, as the differences in maltose concentrations between the worts used in this study were less than 15% and their FAN contents were all greater than 200 mg.l⁻¹ wort, it could be assumed

that they would ferment similarly other than for differences caused by PYF (Van Nierop *et al.*, 2004).

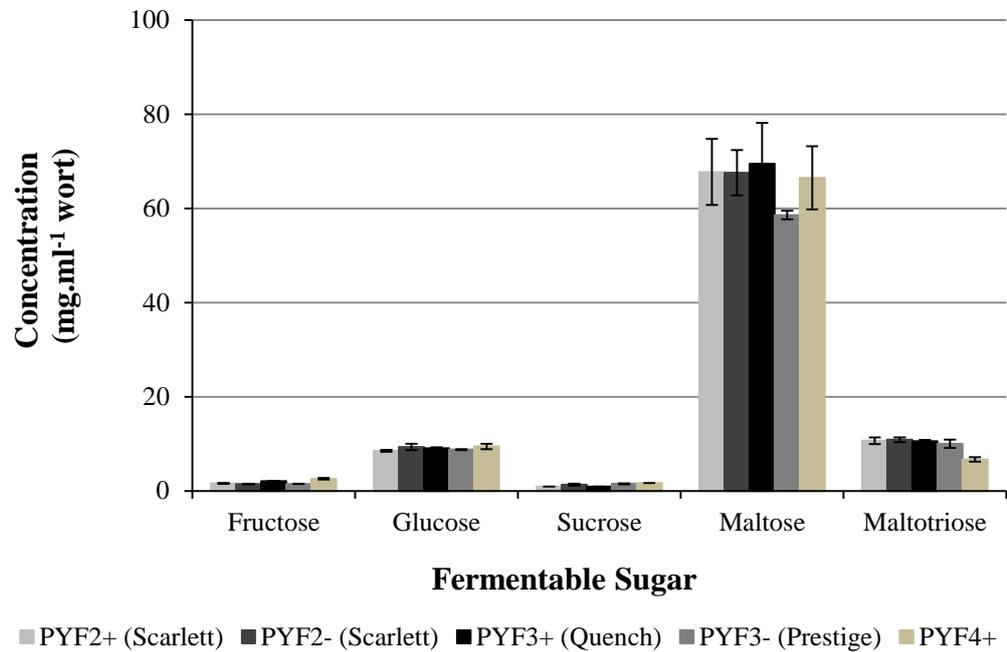


Figure 5.1: Fermentable sugar composition for all-malt worts (11°P) prepared from PYF+ and PYF- control malts. Data are the mean of three replicates ± SD.

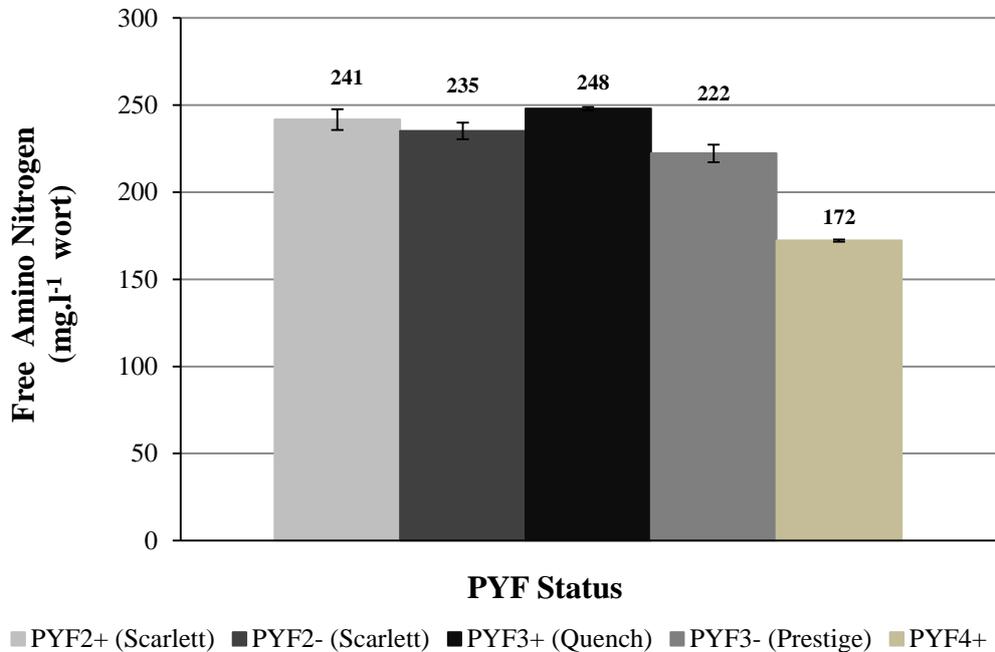


Figure 5.2: Free amino nitrogen (FAN) composition for all-malt worts (11°P) prepared from PYF+ and PYF- malts. Data are the mean of three replicates ± SD.

5.3.2 Predicting the PYF Potential of Malts using the in-house PYF Fermentation Assay

Figure 5.3 shows the time course of suspended yeast cells counts in PYF2+ and PYF2- worts produced from malts of the barley variety Scarlett. Using the small-scale fermentation tests (Section 2.6.1) with the medium flocculent lager yeast strain W34/70, PYF2+ worts could be differentiated from PYF2- worts 69 h post-pitching. At that period of time the number of suspended yeast cells in the PYF2+ fermentations was significantly lower ($P < 0.0001$) than the number of suspended yeast cells in the PYF2- fermentations. However, when the more flocculent lager yeast strain SMA was used to predict the PYF potential of the same PYF2+ and PYF2- worts in fermentations conducted under the same experimental conditions, the PYF potential of the malts was predicted 29 h earlier (40 h post-pitching). At that period of time the number of suspended yeast cells in the PYF2+ fermentations, as indicated by A_{600} readings, was significantly lower ($P < 0.0001$) than the number of suspended yeast cells in the PYF2- fermentations (Figure 5.3). Therefore, using the medium (W34/70) and the highly flocculent (SMA) lager yeast strains the PYF potential of a second pair of industrial provided PYF+ and PYF- worts, besides the PYF1+ and PYF1- control malts used in Chapters 3 and 4 belonging to Scarlett and Prudentia barley varieties respectively, could be successfully predicted using our in-house PYF assay and A_{600} readings 69 and 40 h post-pitching respectively (Figure 5.3).

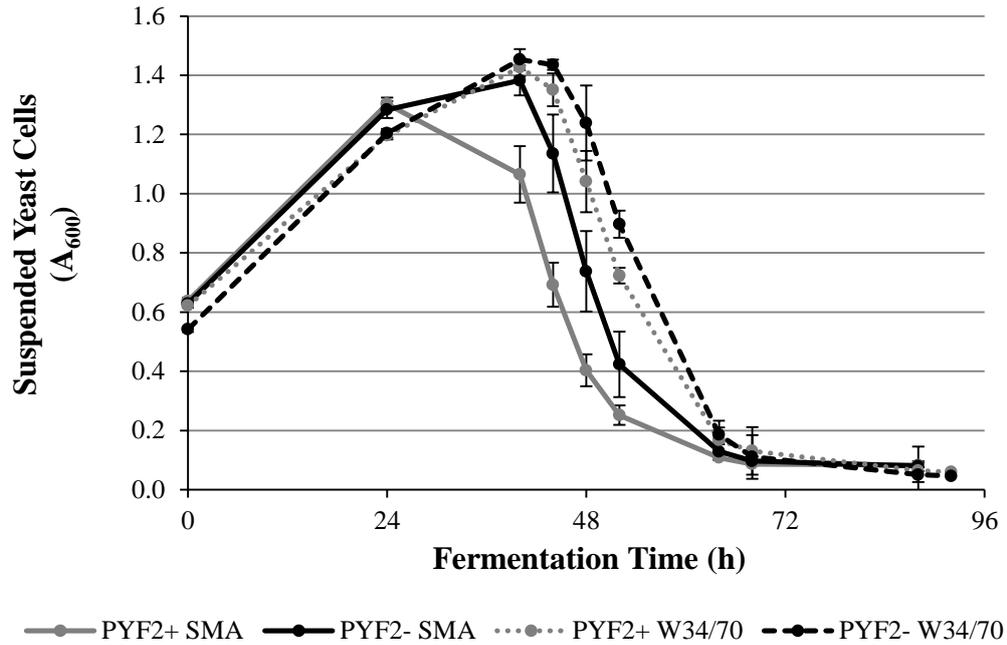


Figure 5.3: Fermentation profiles for PYF2+ (Scarlett) and PYF2- (Scarlett) worts fermented at 15°C using W34/70 and SMA yeast strain at a pitching rate of 20×10^6 live cells.ml⁻¹. Data are the mean of three replicate fermentations \pm SD.

Besides the differences that were observed between the PYF2+ and PYF2- fermentations, with respect to suspended yeast cell counts, when the medium (W34/70) and highly flocculent (SMA) lager yeast strains were used (Figure 5.3), there was also little effect of wort PYF status on residual gravity (Table 5.5). In this instance the mean residual gravity in the PYF2+ fermentations showed a small increase, whilst the mean alcohol yield showed a small decrease when compared with the PYF2- fermentations. Consequently, PYF2+ worts had lower fermentability values than the PYF2- worts (Table 5.5). However, the effect of PYF status on residual gravity was statistically significant only when the PYF2+ worts were fermented with W34/70 yeast. In addition, PYF2+ worts fermented with W34/70 yeast had a significant higher ($P > 0.05$) alcohol yield 92 h post-pitching than the PYF2+ worts fermented with SMA yeast (Table 5.5).

Table 5.5: Residual gravity, apparent fermentability and ethanol yield for PYF2+ and PYF2- worts fermented with W34/70 and SMA yeast.

Worts & Yeasts Utilised	Residual Gravity (°P)	Apparent Fermentability (%)	Alcohol Yield (% v/v)
PYF2+ W34/70	1.5	89.9	4.5
PYF2- W34/70	1.3	91.3	4.7
PYF2+ W34/70	1.3	90.9	4.8
PYF2- W34/70	1.2	91.7	4.8

5.3.3 Sensitivity of Lager Brewing Yeast Strains to PYF Factor(s)

The brewing lager yeast strains (W34/70, SMA and ‘Industrial’) used in these experiments had different degrees of susceptibility to the same PYF factor(s) (Figures 5.3 and 5.4). Fermentations conducted at 15°C using the in-house PYF fermentation assay at the pitching rate of 20×10^6 live cells.ml⁻¹ after the supplementation of the 11°P all-malt worts with 4% [w/v] glucose showed that the flocculent and highly-flocculent brewing lager yeast strains (W34/70 and SMA respectively) were both susceptible to the PYF factor(s) (Figure 5.3). However, their degree of susceptibility to these PYF factor(s) was different. Fermentations carried out with the PYF2+ worts and the W34/70 yeast strain resulted in less severe PYF profiles than was the case with the SMA yeast strain. This result suggested that the more flocculent yeast strain SMA was more susceptible to PYF than the less flocculent yeast strain W34/70. The results obtained are in agreement with Armstrong and Bendiak (2007) who concluded that the more flocculent strains were more sensitive to PYF than the less-flocculent or non-flocculent lager yeast strains. On the other hand, the use of the ‘Industrial’, non-flocculent, lager yeast strain in fermentations conducted using the same PYF2+ and PYF2- worts (produced from malts belong to

Scarlett barley variety) under the same experimental conditions showed little sensitivity to the same PYF factor(s) (Figure 5.4). In this instance the ‘Industrial’ yeast strain gave similar suspended cell count profiles when fermented with the PYF2+ and PYF2- worts.

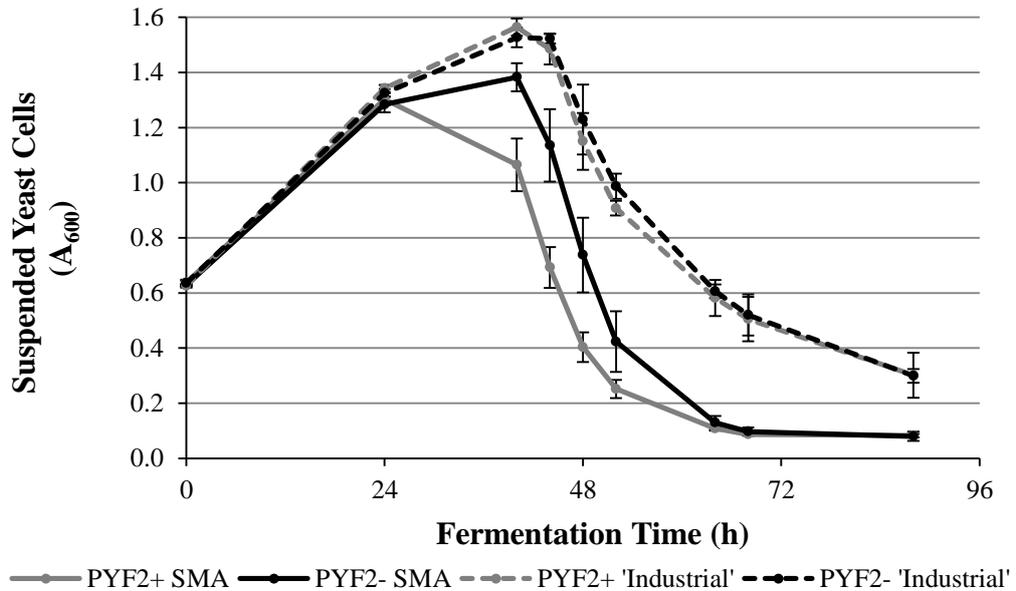


Figure 5.4: Fermentation profiles for PYF2+ (Scarlett) and PYF2- (Scarlett) worts fermented at 15°C using SMA and the Industrial yeast strain at a pitching rate of 20×10^6 live cells.ml⁻¹. Data are the mean of three replicate fermentations \pm SD.

Besides the similarities that were observed between the PYF2+ and PYF2- fermentations, with respect to suspended cell counts, when the ‘Industrial’ yeast strain was used (Figure 5.4), there was also a minimal effect of the PYF status on residual gravity and alcohol yield of the PYF2+ worts 89 h post-pitching (Table 5.7). However, this effect was not found to be significant ($P > 0.05$).

Table 5.6: Residual gravity and ethanol yield for PYF2+ and PYF2- worts 92 h-post pitching fermented with SMA and the ‘Industrial’ yeast.

Wort	Barley Variety	Yeast Strain	Residual Gravity (°P)	Alcohol Yield (% v/v)
PYF2+	Scarlett	SMA	1.5	4.5
PYF2-	Scarlett	SMA	1.3	4.7
PYF2+	Scarlett	‘Industrial’	1.2	4.6
PYF2-	Scarlett	‘Industrial’	1.1	4.6

Data are the mean of three replicate fermentations.

Thus, whilst Jibiki et al. (2006) showed that the non-flocculent lager yeast strains Weihenstephan 71 and NCYC 1324 were susceptible to the PYF factor(s), the results from this study suggested that the non-flocculent lager yeast strain ‘Industrial’ was insensitive to a particular PYF+ malt (PYF2+). These results support the prior practice-based observations that a PYF+ malt and wort would only give rise to latent PYF in a brewery operating with a yeast strain sensitive to the factor. Nevertheless, brewers would not swap yeast strains simply to achieve the desired attenuation, because of the key links between yeast strain and brand quality characteristics.

However, for large scale operations brewing different beer qualities across multiple sites, knowledge of the relative susceptibilities of each yeast strain might assist in the logistics of how best to utilise stocks of PYF+ malts in problem harvest years. These results, besides highlighting the complexity of the phenomenon and the importance of the yeast strain on the severity of PYF during brewing fermentations, could be used to explain why malt from the same barley variety, harvest year and region of production which has been also malted under the same conditions (i.e. process temperatures, airflows) would

behave differently when brewed in different breweries. On the other hand, when the ‘Industrial’ lager yeast strain was fermented under the same experimental conditions (pitching rate of 20×10^6 live cells.ml⁻¹ and using 11°P all-malt worts supplemented with 4% [w/v] glucose) with a second pair of PYF+ and PYF- worts (PYF3+ and PYF3- respectively), produced from malts belong to Quench and Prestige barley varieties respectively; Table 5.1, significant differences in the fermentation profiles were observed 48 h post-pitching (Figure 5.5). That is, the number of suspended yeast cells in the PYF3+ fermentations was significantly lower ($P < 0.0001$) than the number of suspended yeast cells in the PYF3- fermentations. Despite the differences that were observed in the suspended cell counts between the PYF3+ and PYF3- fermentations, the fermentation performance of PYF3+ wort was significantly improved when the ‘Industrial’, rather than the SMA, yeast strain was used.

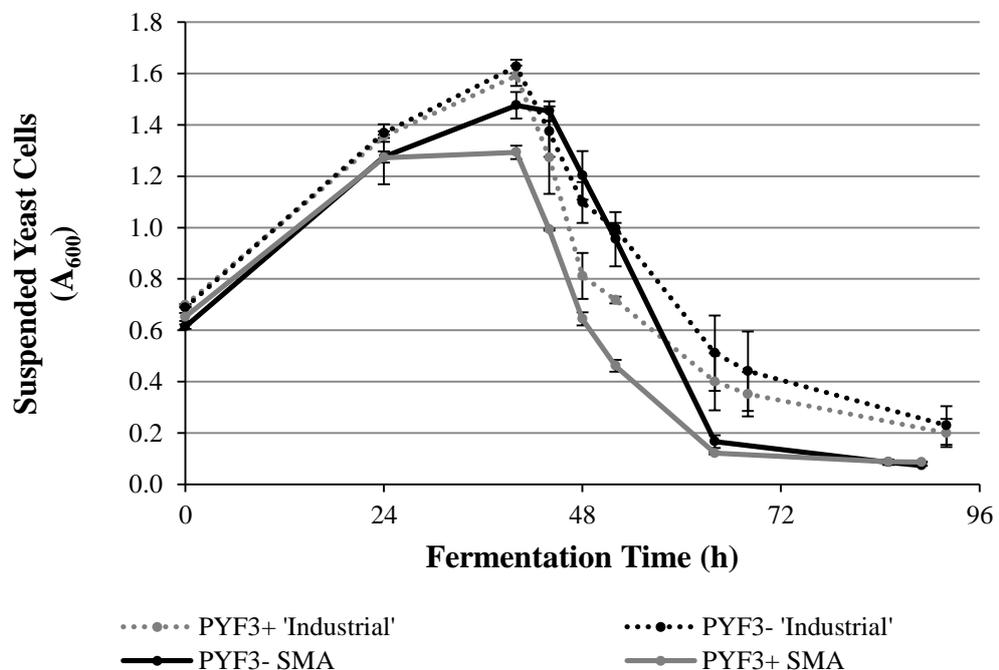


Figure 5.5: Fermentation profiles for PYF3+ (Quench) and PYF3- (Prestige) worts fermented at 15°C using SMA and the ‘Industrial’ yeast at a pitching rate of 20×10^6 live cells.ml⁻¹. Data are the mean of three replicate fermentations \pm SD.

These results suggested that barley variety, harvest year and region of production have a significant effect on the severity of PYF. In particular, it was shown that worts which are not directly matched (i.e. produced from malts belong to different barley varieties and regions of production) would ferment differently irrespective of their PYF status. It was also shown that the fermentation of wort derived from a second PYF+ malt, exhibiting PYF both in industrial and small-scale (200 ml) fermentations, was significantly improved when using a non-flocculent lager yeast strain which appeared to be relatively insensitive to PYF. However, whereas the 'Industrial' yeast strain had indistinguishable fermentation profiles in PYF2+ and PYF2- worts in the first experiment, in this case there was a significant impact of PYF status on the suspended yeast cell count profile. This suggests that the sensitivity of the yeast is linked to the nature of the specific factor(s) present in each individual PYF sample (e.g. the size and charge of 'bridging' polysaccharides or the presence of antimicrobial peptides) as well as to the identity of the strain itself. Besides improving the fermentation performance, with respect to the suspended yeast cell counts of the PYF2+ and PYF3+ worts (Figures 5.4 and 5.5 respectively), the 'Industrial' yeast improved the fermentation performance of a third PYF+ wort (PYF4+; Figure 5.6). In this instance the 'Industrial' yeast fermented with the PYF4+ worts gave more similar suspended cell count profiles to the PYF3- worts, previously found PYF- during our in-house PYF fermentation assay (Figure 5.4) than was the case with the SMA yeast. However, this improvement took place only 40 – 48 h post-pitching, delaying the onset of PYF in the PYF4+ worts by around 8 h.

Thus, whilst fermentation with the ‘Industrial’ yeast strain significantly improved the fermentation profiles of PYF2+ and PYF3+ worts (Figures 5.4 and 5.5 respectively) throughout the fermentation progression (i.e. 0 – 92 h post-pitching), it did not have the same effect in the PYF4+ worts (Figure 5.6). The results obtained support the view that there is not a single PYF factor but rather a variety of factors able to induce different degrees of PYF (Van Nierop *et al.*, 2004; Koizumi *et al.*, 2009). Van Nierop *et al.* (2004) claimed that there is no single PYF factor but, rather, a range of arabinoxylan fragments with varying molecular weights of similar but not identical compositions. Koizumi *et al.* (2009), based on sugar composition analysis, indicated that the structural features of a PYF factor (derived from North American and Japanese cultivar after enzymatic digestion and separation with concanavalin A) is common in barley. The same authors (Koizumi *et al.*, 2009) suggested the possibility that the PYF factors are a group of polysaccharides with unique structure recognized by a lectin-like protein on the yeast cell surface (see Section 1.3.1.2 for details).

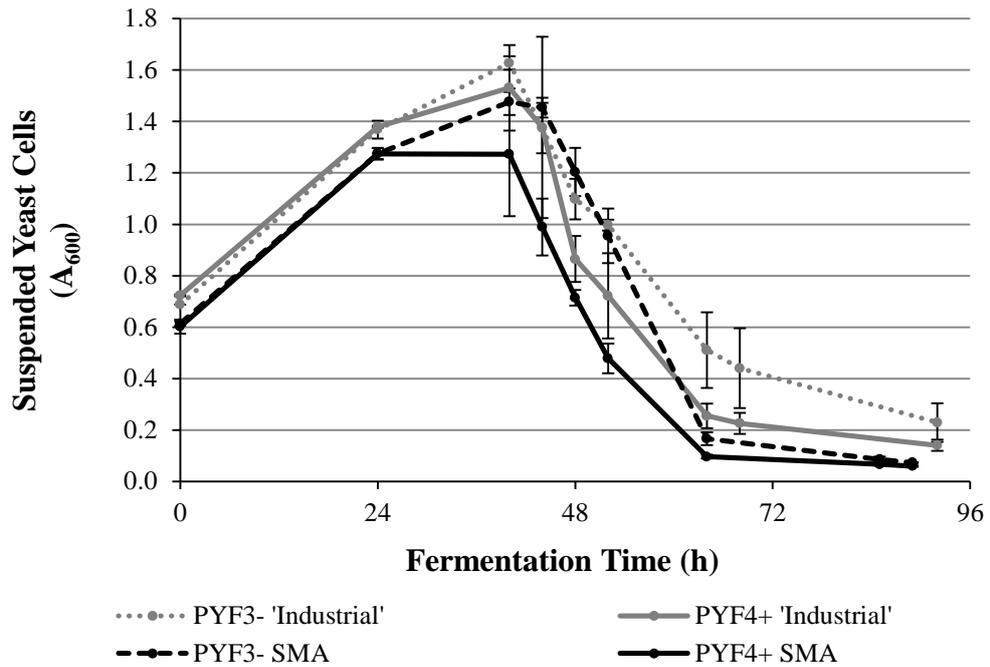


Figure 5.6: Fermentation profiles for PYF3± and PYF4± worts fermented at 15°C using SMA and the 'Industrial' yeast at a pitching rate of 20×10^6 live cells.ml⁻¹. Data are the mean of three and two replicate fermentations ± SD respectively.

5.3.4 Sensitivity of Ale Brewing Yeast Strains to PYF Factor(s)

Figures 5.7 and 5.8 show the suspended yeast cell profiles of PYF2+ and PYF2- worts (produced from malts of variety Scarlett) fermented with the ale brewing yeast strains NCYC 1332 and M2 in our in-house PYF assay. For each strain the suspended cell profiles showed minimal differences when comparing fermentations of PYF2+ with PYF2- worts. That is, the number of suspended yeast cells in the PYF2+ fermentations, as indicated by A_{600} readings at any given time point, was not found to be significantly different ($P > 0.05$) from the number of suspended yeast cells in the PYF2- fermentations.

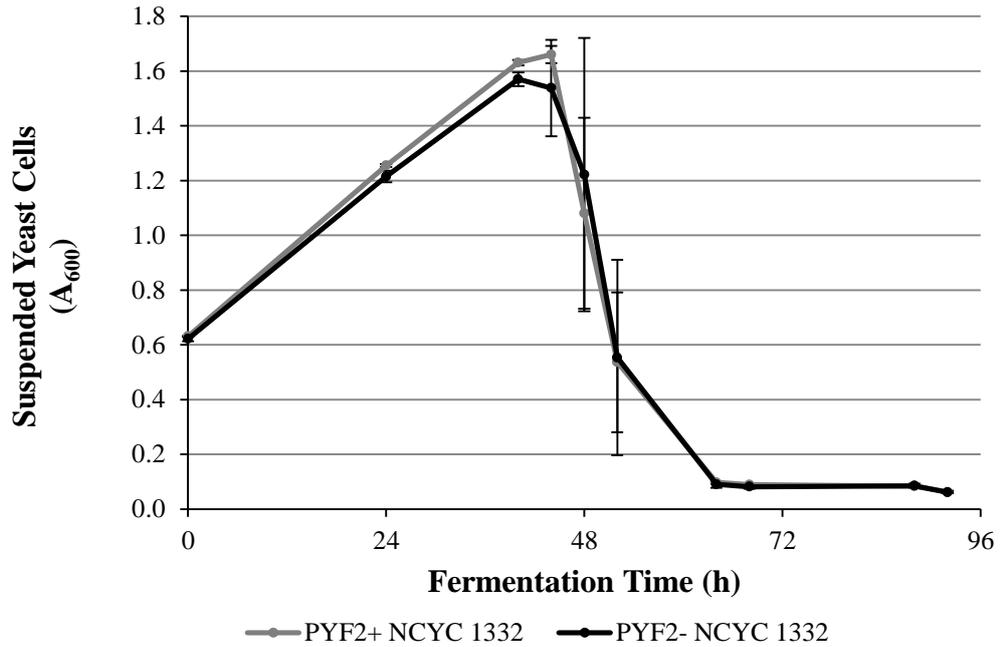


Figure 5.7: Fermentation profiles for PYF2+ (Scarlett) and PYF2- (Scarlett) worts fermented at 15°C using NCYC 1332 yeast at a pitching rate of 20×10^6 live cells.ml⁻¹. Data are the mean of three replicate fermentations \pm SD.

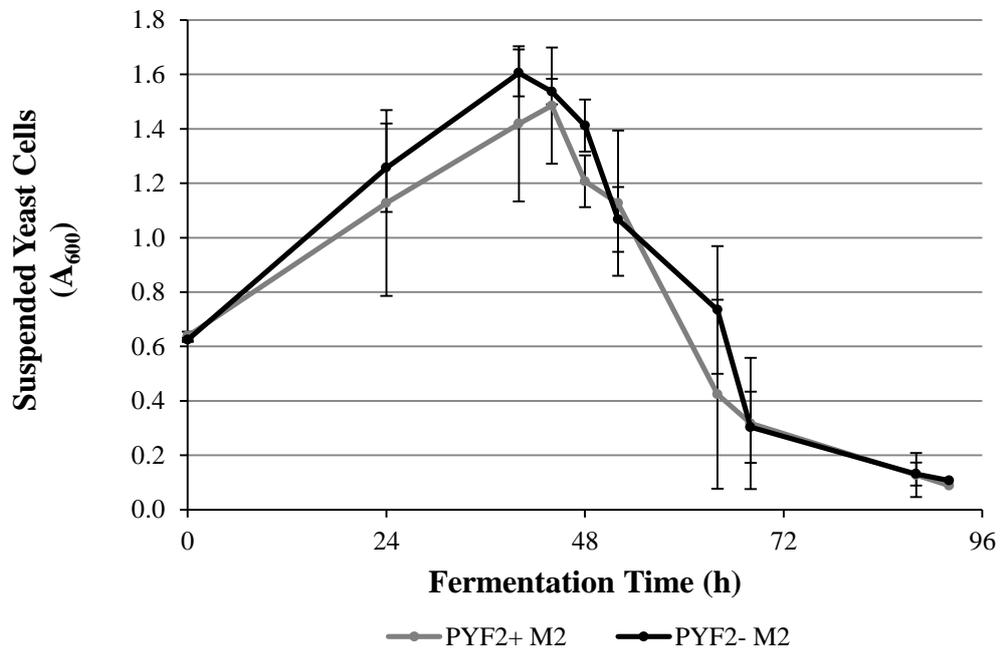


Figure 5.8: Fermentation profiles for PYF2+ (Scarlett) and PYF2- (Scarlett) worts fermented at 15°C using M2 yeast at a pitching rate of 20×10^6 live cells.ml⁻¹. Data are the mean of three replicate fermentations \pm SD.

Although the mean residual gravity and ethanol yield showed small differences between the PYF2+ and PYF2- fermentations 89 h post-pitching when the NCYC 1332 and M2 strains were used, these differences were not significant ($P > 0.05$; Table 5.7).

Table 5.7: Residual gravity and ethanol yield for PYF2+ and PYF2- worts 92 h post-pitching fermented with NCYC1332 and M2 yeast.

Wort	Yeast Strain	Residual Gravity (°P)	Alcohol Yield (% v/v)
PYF2+	NCYC 1332	1.1	4.6
PYF2-	NCYC1332	1.0	4.4
PYF2+	M2	1.1	4.6
PYF2-	M2	1.0	4.7

Data are the mean of three replicate fermentations.

The results obtained suggested that none of the ale yeast strains used in this study, either flocculent (M2) or non-flocculent (NCYC1332), were found susceptible to the same PYF factor(s) (i.e. PYF2+ worts). These results are in agreement with the results of Jibiki *et al.* (2006) who concluded that the ale yeasts (i.e. NCYC 1681, NCYC 1026, NCYC 1078, NCYC 1301, Weihenstephan 184), irrespective of their degree of flocculence, were not susceptible to PYF. However, why ale yeasts are not susceptible to PYF requires further elucidation. In view of the fact that Herrera and Axcell (1991b) and Koizumi *et al.* (2008) showed that the PYF factor(s) contain pectin-like material, it could be hypothesized that PYF involves lectin-like interactions between yeast cells and PYF factor(s). Thus, the fact that some of the top fermented ale yeasts express the MI type of flocculation (Vidgren & Londesborough, 2001; see Section 1.2.2 for details) it could be proposed as the

most possible reason of ale yeasts being insensitive to PYF. In addition, taking into consideration that the different yeast strains contain different combinations of *FLO* genes (Van Mulders *et al.*, 2010), resulting in different flocculation characteristics; Stratford and Assinder (1991); Sieiro *et al.* (1995), it could be also hypothesized that differences in *FLO* gene characteristics between lager and ale yeasts as well as the fact that ale yeasts are more hydrophobic than lager strains (Amory & Rouxhet, 1988), and consequently rise easily to the surface of the fermentation vessels by adhering to the gas bubbles, as also possible reasons why ale yeasts are not susceptible to PYF factor(s).

5.4 Conclusions

The results obtained suggested that the specific yeast strain utilised has an important role in the PYF phenomenon. Whilst none of the ale yeast strains used in this study were found to be susceptible to the PYF factor(s), the lager yeast strains exhibited different degrees of susceptibility even to the same PYF factor(s). In particular, the more flocculent yeast strain (i.e. SMA) exhibited a higher degree of susceptibility than the less-flocculent yeast strain (i.e. W34/70). Besides that, it was shown that the fermentation performance of a PYF+ wort could be significantly improved by using a non-flocculent brewing lager yeast strain which is relatively insensitive to PYF. However, the improvement in the fermentation profiles varied among the different PYF+ samples. The former results could be used to explain why malt supplied from the same producer (i.e. barley from the same variety, harvest year and region of production) and malted under the same conditions can give rise either to 'normal' or PYF worts. Thus, besides the PYF potential of the barley/malt

samples, the yeast strain has also an important role on the incidence and severity of the phenomenon.

Chapter 6

Impacts of Premature Yeast Flocculation

Factor(s) on Fermentation and Metabolite

Profiles

Data from this Chapter were presented at the 74th American Society of Brewing Chemists Meeting, which took place in Sanibel Island in Florida 11th - 15th June 2011. The paper has been included in the Proceedings of the 74th American Society of Brewing Chemists Annual Meeting (Oral Presentation 17).

6.1 Introduction

Two main theories have been used to explain the occurrence of PYF in the brewing industry; the “bridging polysaccharide theory” (Section 1.3.2.1), and the “antimicrobial peptide hypothesis” (Section 1.3.2.2). According to the second theory of PYF (“antimicrobial peptide hypothesis”; Axcell *et al.*, 2000) the grain responds to microbial attack, or to other related stress, by producing anti-microbial peptides (AP). Defensins, thionins and non-specific lipid transfer proteins (ns-LTP) are groups of antimicrobial peptides which have been proposed as possible candidates for the PYF factor. These cationic antimicrobial peptides are relatively small (5 – 10 kDa), and can survive both the malting and brewing processes. Their structures are usually stabilised by the presence of multiple disulphide bridges. Van Nierop *et al.* (2008) suggested that a direct impact of antimicrobial peptides would be associated with the inhibition of yeast metabolism during fermentations, whilst there were also indirect impacts on barley/malt quality aspects (mycotoxin contamination, off-odours and inconsistent brewhouse performance). With respect to inhibition of yeast metabolism, Van Nierop *et al.* (2004) showed that fermentations with PYF+ worts resulted in a slower uptake of maltose and maltotriose by yeast. On the other hand, Lake and Speers (2008) observed that it was not clear if the reduction in maltose and maltotriose uptake reported by Van Nierop *et al.*

(2004) was due to insufficient yeast cells in suspension, caused by PYF, or due to a direct effect of the antimicrobial peptides themselves. More recently, Porter *et al.* (2010), searching for differences in wort peptide profiles in three different worts using fast protein liquid chromatography (FPLC), did not manage to find any antimicrobial peptides or even simple peptide differences between control and PYF+ malt samples.

One objective of the work reported in this Chapter was to further characterise the impacts of PYF factor(s) on yeast fermentation performance and metabolite profiles, in order to see if any effects consistent with the antimicrobial peptide hypothesis could be found. To achieve this, 33 PYF+ and 33 PYF- high gravity (15°P) mini-fermentations (100 ml) were conducted within a period of seven days using the highly flocculent but PYF sensitive yeast strain SMA under stirred and unstirred conditions. Mechanical agitation (180 rpm) could be employed in these experiments so as to keep yeast cells in suspension and maintain homogeneity (which can be a problem in small-scale fermentations due to reduced carbon dioxide evolution; Boswell *et al.*, 2002; Lake *et al.*, 2008). Besides conducting stirred and unstirred mini-fermentations, the standard PYF assay (Section 2.6.1) was also used as a control test to confirm the PYF status of the malts.

6.2 Experimental

6.2.1 Malts

Two industrial malts from the same barley variety (Scarlett), region (France) and crop year (2007) were used in this study. One was known to have caused PYF, whilst the other malt exhibiting normal fermentation profiles in brewery

fermentations.

6.2.2 Wort Preparation

PYF⁺ and PYF⁻ worts were prepared from control malts (Section 6.2.1) using a standardized laboratory mashing procedure as described in Section 2.5. However, in order to obtain the PYF factor(s) in higher concentrations a “thicker” mash was employed (120 ± 0.5 g of milled malt was added to 360 ml brewing liquor), resulting in approximately 18°P gravity. This was subsequently standardized using RO water to 15°P as per the detail given in Section 6.2.6.

6.2.3 Wort Composition Analyses

FAN and fermentable sugar spectrum analyses in 11°P PYF²⁺ and PYF²⁻ worts were performed as described in Sections 2.7.3 and 2.7.4 respectively.

6.2.4 Yeast Strain and Propagation Conditions

One lager yeast strain was used in this study (SMA). For the first stage of propagation, a loop of yeast cells was aseptically transferred into 30 ml YPD in 250 ml sterile Universal bottles and the cultures were aerobically propagated in a Certorat BS-1 shaken incubator at 25°C for 24 h at 120 rpm. The transfer of the yeast cells in the second stage took place while cells were in the log phase. Cells at the log phase (30 ml) were transferred aseptically to 500 ml of sterile YPD into 1 l pre-sterilised conical flask with non-absorbent cotton wool plugs covered in aluminium foil. The culture (approximately 530 ml) was aerobically propagated in a Certorat BS-1 shaken incubator for further 72 h at 25°C under continuous shaking at 120 rpm.

6.2.5 Premature Yeast Flocculation Assay

PYF assays were conducted as described in Section 2.6.1. In brief, the procedure involved conducting small-scale (200 ml) fermentations in 250 ml ‘dropping funnels’ within a temperature controlled incubator (15°C) and using worts prepared from PYF2+ and PYF2- malts (Section 6.2.1) using a standardized laboratory mashing procedure (see Section 2.5 for details). The small-scale fermentation tests were conducted at a pitching rate of 20×10^6 live cells.ml⁻¹ after the supplementation of 11°P all-malt worts with 4% [w/v] glucose for a maximum period of 92 h.

6.2.5.1 Monitoring Suspended Yeast Cell Counts

Cell concentration in suspension was assessed at specific time intervals between 0 – 92 h post-pitching (i.e. 0, 24, 40, 44, 48, 52, 64, 68 and 92) by measuring absorbance at 600 nm (A_{600} ; Section 2.6.2.1).

6.2.5.2 Residual Gravity and Alcohol Yield

The residual gravity and alcohol yield of the fermenting broths were determined at 15°C using a DMA 5000 M model Anton Paar density-alcolyzer meter as described in Section 2.7.6.

6.2.5.2.1 Fermentability

The fermentability of the worts (the proportion of the wort dissolved solids which can be fermented) was calculated as a percentage according to Equation 2.8; Section 2.7.7.

6.2.6 Stirred Laboratory Fermentations (Mini Fermentations)

Fermentations were performed in glass hypovials according to the method of Quain *et al.* (1985) as described in Section 2.6.3. In brief, 100 ± 1 ml of sterile

15°P wort, diluted with RO water from initial 18°P all-malt wort, was transferred aseptically into each of 33 pre-sterilised mini-fermenters (120 ml hypovials) containing a magnetic flea. Following wort addition, the hypovials were plugged with pre-sterilised non-absorbent cotton wool plugs and were saturated with air at 15°C in a Sanyo static incubator for a total period of 24 h. Yeast cells in the stationary phase, obtained from a 50% [w/v] slurry with a viability > 98%, were added to 100 ± 1 ml wort (approximately 1 ml 50% [w/v] yeast slurry per 100 ml wort) to achieve a pitching rate of 20 × 10⁶ live cells per ml. Following pitching, the hypovials were sealed with suba seals and metal crimp seals using a hand-held crimper. Pre-sterilised needles were placed on the top of the fermenters so as to allow the build up of the pressure as well as the partial removal of the CO₂ during the fermentation. After that, the mini fermenters were transferred into a 15°C Sanyo static incubator for a total period of 162 h. Fermentations were conducted at 15°C both under stirred and unstirred conditions. In the mechanically agitated fermentations homogeneity was achieved by gentle agitation (180 rpm) using a flat bed 15-place magnetic stirrer. Samples were taken at 0, 3, 8, 18, 24, 40, 48, 68, 92, 124 and 162 h post-pitching and fermentation progression was monitored by measuring weight loss (CO₂ evolution), pH, gravity content, ethanol yield, FAN and fermentable sugars (sucrose, fructose, glucose, maltose and maltotriose) over time using destructive time point sampling (3 reps per time point).

6.2.6.1 Sampling from the mini fermentation vessels

At pre-determined time points (0, 3, 8, 18, 24, 40, 48, 68, 92, 124 and 162 h post-pitching) the fermentation vessels were opened and following mixing 1 ml aliquots were transferred into two separate 5 ml bijou bottles. The bijou

bottles were kept on ice (4°C) for determination of the total and viable cells (Section 2.6.2.2) as well as for the calculation of budding index (Section 2.6.2.3). Following sampling, the remaining contents of the fermentation vessels were transferred into two 50 ml centrifuge tubes and centrifuged at 5,000 rpm for 5 min at 4°C to remove the yeast cells. Following centrifugation, the fermentation broths were decanted into two centrifuge tubes and following pH determination (Section 2.7.2) were frozen at -20°C until required for analysis.

6.2.7 Mini Fermentation Analysis

6.2.7.1 Weight Loss

Weight loss during the fermentations was determined by weighing the PYF2+ and the PYF2- mini-fermenters in a Sartorius balance (Sartorius UK Ltd, Surrey, UK) at $20 \pm 0.1^\circ\text{C}$. Weight measurements were taken every 4 h during the first two days of fermentation (but not overnight) and at pre-determined time points beyond 48 h post-pitching.

6.2.7.2 Cell Density and Budding Index

Cell suspensions were diluted to an appropriate volume (100 μl aliquot diluted $10 \times$ with methylene blue) and density was measured using a counting chamber and standard light microscope at $\times 40$ magnification. To determine the budding index, a minimum of 500 cells were scored microscopically, and the number of budded cells was calculated as a percentage of the total using Equation 2.5; Section 2.6.2.3.

6.2.7.3 pH Determination

The pH of the fermenting wort was measured using a Mettler Toledo pH meter

(at $20 \pm 0.1^\circ\text{C}$) which had previously been calibrated with standard solutions of known pH (4.0 and 7.0 at $20 \pm 0.1^\circ\text{C}$).

6.2.7.4 Specific Gravity and Ethanol Determination

Fermenting wort (40 ml) was transferred to 50 ml falcon tubes and centrifuged at 3,500 rpm for 10 min at 4°C . Following centrifugation, the supernatant was transferred into 50 ml falcon tubes and the specific gravity and ethanol content of the fermenting broth were measured using a DMA 5000 M model Anton Paar density-alcolyzer meter. Specific gravity measurements were converted to $^\circ\text{P}$ according to Equation 2.7; Section 2.7.6.

6.2.7.5 Wort Amino Acid Analysis

The amino acid profiles of the PYF2+ and PYF2- worts were analysed using the EZ:faastTM amino acid kit (Phenomenex, Macclesfield UK) as described in Section 2.7.5.

6.2.8 Statistical Analysis

The statistical significance of the different malt types and fermentation conditions (i.e. stirred vs. unstirred) on yeast flocculation, fermentation performance and metabolite uptake was assessed using ANOVA and the statistical program Minitab (version 15, Minitab Inc., State College, USA). In each instance the null hypothesis (H_0) was that no significant differences existed between data sets. If the P value generated by the test was less than 0.05 then the null hypothesis of no significant difference was rejected and the alternative hypothesis (H_a) of significance was adopted. Whilst ANOVA can indicate that an overall significant difference exists between data sets, *post-hoc* analysis is required to assess which sample means differed statistically from

one another. Pair-wise comparison of means was completed using the Tukey test at the significance level $P < 0.05$.

6.3 Results and Discussion

6.3.1 Wort Composition: Fermentable Sugars, FAN and Amino Acids

The PYF2+ and PYF2- worts used throughout these experiments were matched in terms of barley variety (Scarlett), harvest year (2007) and region of production (France). Besides that, as mentioned in Chapter 5 (Section 5.3.1), they had similar composition with respect to fermentable sugars (fructose, glucose, sucrose, maltose and maltotriose) and FAN (Figures 5.1 and 5.2 respectively). Thus, it could be assumed that any differences in their fermentation performance were not due to differences in bulk nutrients but rather due to the presence of the PYF factor(s) (Van Nierop *et al.*, 2004).

In spite of the noted similarities in terms of fermentable sugars and FAN content, the 11°P all-malt PYF2+ and PYF2- worts had different profiles of amino acids before pitching (Figure 6.1). More specifically, PYF2+ worts contained significantly lower ($P < 0.05$) amounts of asparagine, proline and valine when compared with the PYF2- worts (Figure 6.1). However, both worts contained the amino acids proline and alanine in the highest concentrations, whilst alpha and beta aminobutyric acid were the amino acids that were present at relatively low concentrations. The results obtained are in disagreement with Gibson *et al.* (2009) who reported that asparagine and proline were the amino acids present in the highest concentrations, whilst threonine, serine, glutamate, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, aminobutyric acid, lysine, histidine and

arginine are commonly present in lower concentrations in brewery worts. The differences observed in the relative amino acid concentrations could arise from a number of sources, in particular the barley variety and crop year, malting process conditions and the different mash protocols that are used during wort production. Boulton and Quain (2003) indicated that all the free amino acids that are present in the wort can be assimilated by yeast during fermentation, other than proline which requires oxygen, and as a consequence its assimilation is usually limited or absent during fermentation. However, this was recently challenged by Gibson *et al.* (2009) who showed a significant reduction in proline during a fourth-generation 3,375 hl industrial-scale wort fermentation with the lager yeast strain CB11, whilst Wang and Brandriss (1987) indicated that if no other amino acid is present, then proline utilization may be supported under anaerobic conditions. Nevertheless, the brewer would not specify the individual amino acid content of the wort before fermentation, but rather would be interested in the FAN content which was reasonably well matched between the two malts (Hornsey, 1999).

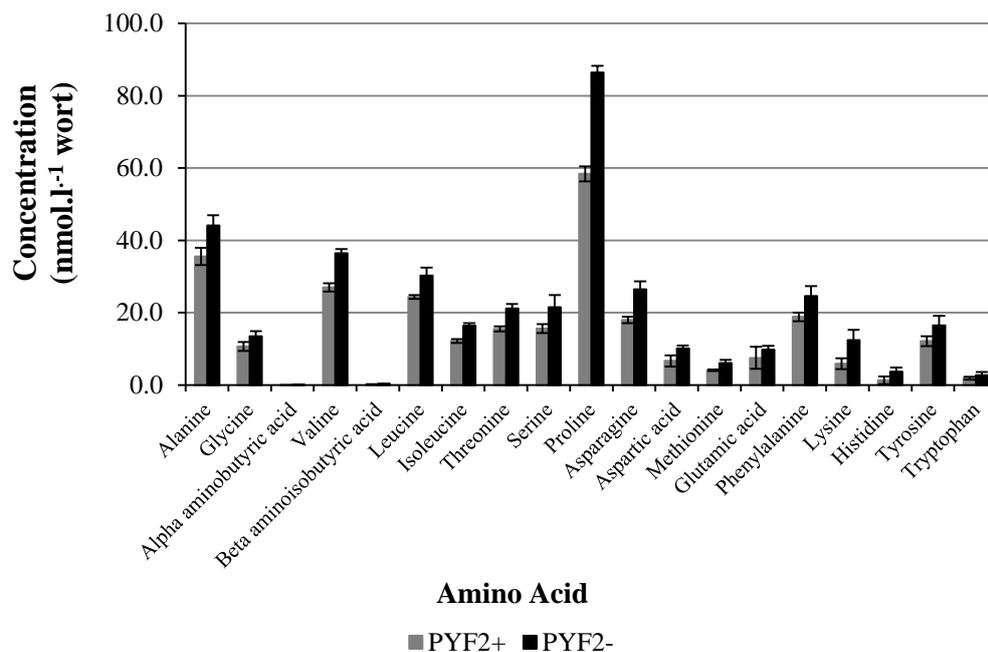


Figure 6.1: Amino acid composition for all-malt worts (11°P) prepared from PYF2+ and PYF2- control malts. Data are the mean of three replicates \pm SD.

6.3.2 Predicting the PYF Potential of Malts using the in-house PYF Fermentation Assay

Figure 6.2 shows the fermentation profiles of PYF2+ and PYF2- worts in fermentations conducted at 15°C at the pitching rate of 20×10^6 live cells.ml⁻¹ after the supplementation of the 11°P all-malt worts with 4% [w/v] glucose. Using the in-house small-scale PYF assay (Section 2.6.1) and the highly flocculent but PYF sensitive lager yeast strain SMA the PYF potential of the malts could be differentiated 44 h post-pitching. At that period of time the number of suspended yeast cells, as indicated by A_{600} readings, in the PYF2+ fermentations was significantly lower ($P < 0.0001$) than the number of suspended yeast cells in the PYF2- fermentations.

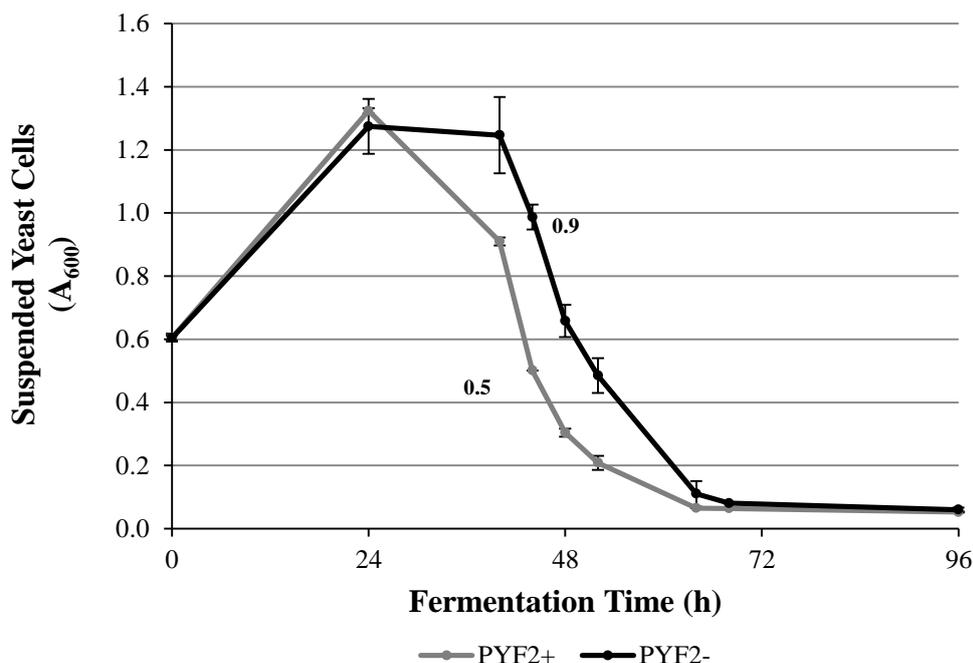


Figure 6.2: Fermentation profiles for 11°P PYF2+ and PYF2- worts fermented in 250 ml ‘dropping funnels’ at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹. Data are the mean of three replicate fermentations \pm SD.

Besides the significant differences that were observed in the suspended yeast cell counts between the PYF2+ and PYF2- fermentations 44 h post-pitching (Figure 6.2), there was also a minor effect of the PYF status on the residual gravity and alcohol yield of the worts 92 h post-pitching (Table 6.1). However, this effect was not significant ($P > 0.05$). Thus, whilst in Chapter 5 small-scale fermentations conducted with the SMA yeast strain and the PYF2+ and PYF2- worts indicated significant differences in the residual gravity and alcohol yield towards the end of the fermentation (i.e. 92 h post-pitching), in this study these differences were not found to be statistically significant. These results, besides highlighting the inconsistency of brewing yeast fermentations, also suggest that the determination of the residual gravity and/or alcohol yield during the PYF fermentation assays is not in itself sufficient to predict the PYF potential of malts (Panteloglou *et al.*, 2010). Thus, as already mentioned in Chapter 3,

measuring both the number of suspended yeast cells during primary fermentation (by using either the absorbance at 600 nm (A_{600}) or microscopic cell counting after a number of serial dilutions) as well as the residual gravity and ethanol yield at the end of the fermentation offers a better understanding on which to base predictions of the malt's true PYF potential in the brewery.

Table 6.1: Residual gravity, fermentability and ethanol yield for PYF2+ and PYF2- worts 96 h post-pitching fermented with SMA yeast.

Wort	Residual Gravity (°P)	Fermentability (%)	Alcohol Yield (% v/v)
PYF2+	1.2	92.0	4.7
PYF2-	0.9	93.7	4.9

Data are the mean of three replicate fermentations.

6.3.3 Impact of PYF Factor(s) on Yeast's Physiological Characteristics

6.3.3.1 Impact of PYF Factor(s) on Cell Cycle Progression

Laboratory brewing strains of *Saccharomyces cerevisiae* undergo asexual reproduction via an asymmetric form of cell division called 'budding'. During 'budding' one cell gives rise to one 'daughter' cell that is genetically identical to the original 'mother' cell. Cell division is an on-going process in that the progeny of cell division – the 'virgin' daughter cells – themselves divide becoming mother cells and so on. However, cell division is not a linear, never-ending process, but rather a process which slows or stops ('arrests') when growth nutrients become limiting (i.e. during 'stationary phase' – G0), when cells age and become senescent or when cells die (Boulton & Quain, 2003).

Cells sampled from the 15°P PYF2+ and PYF2- fermentations immediately after pitching had a budding index of 15 and 24% in the stirred and unstirred fermentations respectively (Figure 6.3). These values increased to a maximum of 52 and 56% in the PYF2+ and PYF2- stirred fermentations and to 66 and 62% respectively in the unstirred fermentations after 18 h of exposure to fresh oxygenated wort. Following 40 h of yeast addition to worts, the budding index was reduced to 20 and 18% in the PYF2+ and PYF2- stirred fermentations and to 29 and 23% in the samples obtained from the unstirred fermentations. The budding index reached a minimum in the stirred and unstirred fermentations towards the end of the sampling period (i.e. 162 h post-pitching). The results obtained suggested that the concentration (15°P wort) of the PYF factor(s) used in these experiments did not have any obvious impact on the cell cycle progression of the stirred and unstirred PYF2+ fermentations. However, interestingly, the budding index was on average higher in the unstirred fermentations than was the case with the stirred fermentations where the overall yeast growth was higher (Figure 6.4). This result suggested that the yeast cells in the stirred fermentations were replicating quicker, but at a lower budding index, than the yeast cells in the unstirred fermentations.

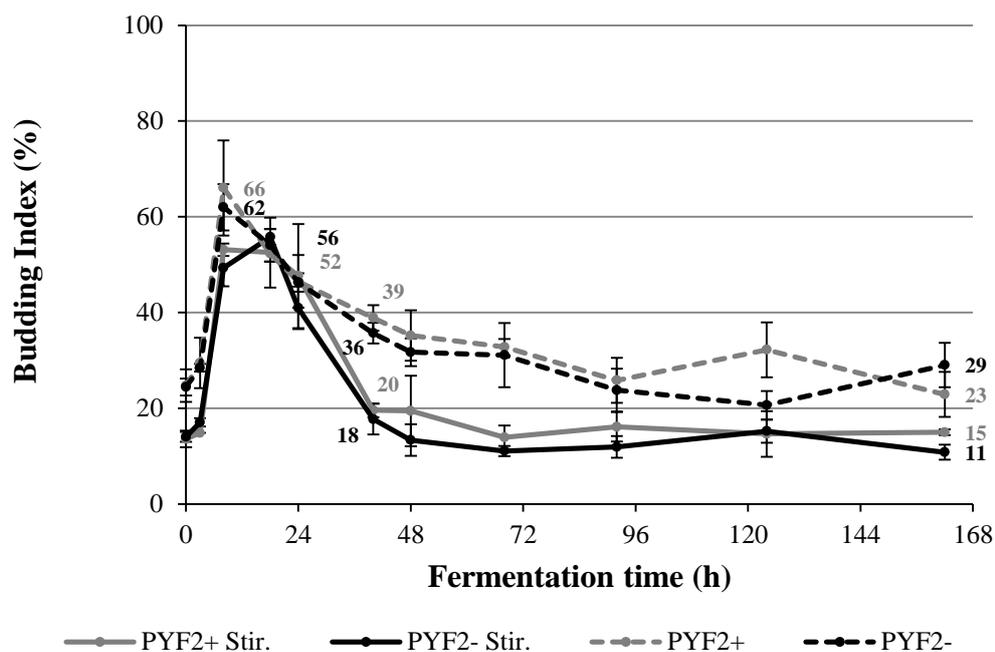


Figure 6.3: Changes in budding index for 15°P PYF2+ and PYF2- worts fermented in 120 ml mini-fermenters at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹ under both stirred and unstirred conditions. Data are the mean of three replicate fermentations \pm SD.

6.3.3.2 Impact of PYF Factor(s) on Cell Density

The increase in the budding index within the first 18 h of fermentation progression was followed by an increase in the cell density as a result of cell division (Figure 6.4). Cell density was increased in PYF2+ and PYF2- stirred fermentations from 20 to 120×10^6 cells.ml⁻¹ of wort, with the exponential growth occurring between 3 and 48 h post-pitching. On the other hand, cell density increased from 20 to 80×10^6 cells.ml⁻¹ in the unstirred fermentations during the first 48 h of fermentation. The results obtained indicated that there were not significant differences ($P > 0.05$) in the cell density between the PYF2+ and PYF2- fermentations throughout the sampling period (i.e. 0 – 168 h post-pitching). Thus, it was suggested that whilst the PYF factor(s) did not affect the yeast growth, fermentation progression was quicker in the stirred fermenters when compared with the unstirred fermentations. Boswell *et al.*

(2002) indicated that mechanical agitation could lead to a more rapid process with concomitant savings in fermentation time and cost as well as increasing the reproducibility of products between batches. Stratford and Keenan (1987), on the premise that brewery fermentations are indirectly 'mixed' through the upward motion of CO₂ bubbles generated in the lower region of the vessel (Boswell *et al.*, 2002), showed that relatively gentle mixing (70 – 120 rpm) triggered flocculation. The same authors (Stratford & Keenan, 1987) demonstrated that the more vigorously flocculent yeast strains were shaken the better they flocculated. Indeed, without agitation, a flocculating culture was unable to flocculate, whilst the rate of flocculation increased in parallel with increasing mechanical agitation. Mixing may also alter the yeast cell surface leading to changes in flocculation kinetics or colloidal stability, and since it depends on the rate of CO₂ evolution, the liquid depth, vessel size, and aspect ratio, mixing may also strongly influence the flavour characteristics of the beer. Besides that, where metabolic activity is lower, at the initial and later stages of the fermentation, flow and mixing (resulting from the CO₂ evolution) are reduced resulting in increasing heterogeneity (Boswell *et al.*, 2002).

The fact that PYF could not be detected in the unstirred mini fermentation experiments could be attributed to the gentle mixing that was employed before sampling (see Section 6.2.6.1 for details) in order to enable the accurate determination of the yeast cells in the PYF+ and PYF- fermenting broths (especially from the point that SMA yeast exhibited strong flocculation characteristics in the mini fermenters). Besides that, key aspects able to affect the PYF detection are the addition of glucose, which as already mentioned increases fermentation vigour; Jibiki *et al.*, 2006, Lake *et al.*, 2008, as well as

the aspect ratio of the vessel. More specifically, Lake *et al.* (2008) showed that when downscaling a fermentation PYF assay by reducing the fermenter height, the rate of fermentation must be increased (e.g. by increasing the fermentation temperature by 9°C) so as to maintain adequate shear rates.

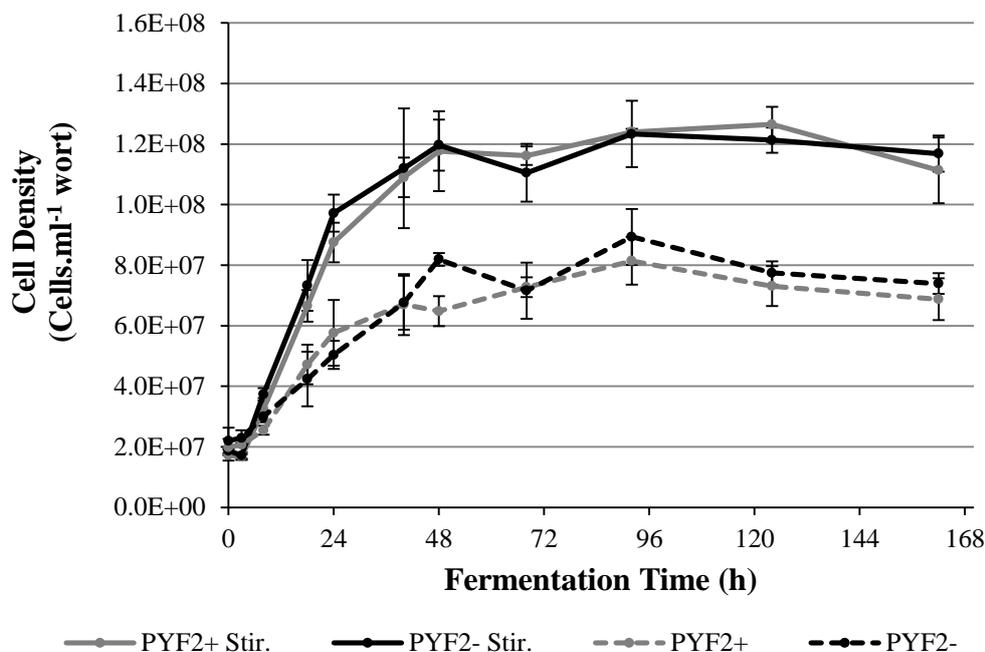


Figure 6.4: Changes in cell density for 15°P PYF2+ and PYF2- worts fermented in 120 ml mini-fermenters at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹ under both stirred and unstirred conditions. Data are the mean of three replicate fermentations \pm SD.

6.3.3.3 Impact of PYF Factor(s) on Yeast Viability

In addition to the similarities that were observed with regard to cell density and budding index (Figures 6.3 and 6.4 respectively), the number of viable cells in the PYF2+ and PYF2- stirred and unstirred fermentations were not statistically different from one another ($P > 0.05$) at every measurement time-point during fermentation progression (Figure 6.5). These results showed that the concentration and nature of PYF factor(s) used in these experiments did not have a detrimental effect on yeast viability. Hence, the PYF sample used in these experiments most likely presented the ‘bridging’ polysaccharide type of

PYF. Thus, in addition to the PYF1+ control wort used in Chapter 4 (produced from Scarlett barley), a second PYF+ sample (PYF2+) appeared to present the ‘bridging’ polysaccharide type of PYF (since the presence of antimicrobial peptides would be anticipated to have impacted on yeast viability; Van Nierop *et al.*, 2004). In addition, it was also apparent that stirring increased the viable cell counts in both PYF2+ and PYF2- fermentations, relative to the unstirred fermentations. However, a decline in cell viability was observed in both fermentation systems (stirred and unstirred) onwards of 96 h post-pitching. This decline, which was more obvious in the non-stirred fermentations, was enhanced by a combination of exposure to inhibitory concentrations of ethanol and exhaustion of substrates during this period as opposed to the effect of mechanical damage due to agitation alone (Boswell *et al.*, 2002).

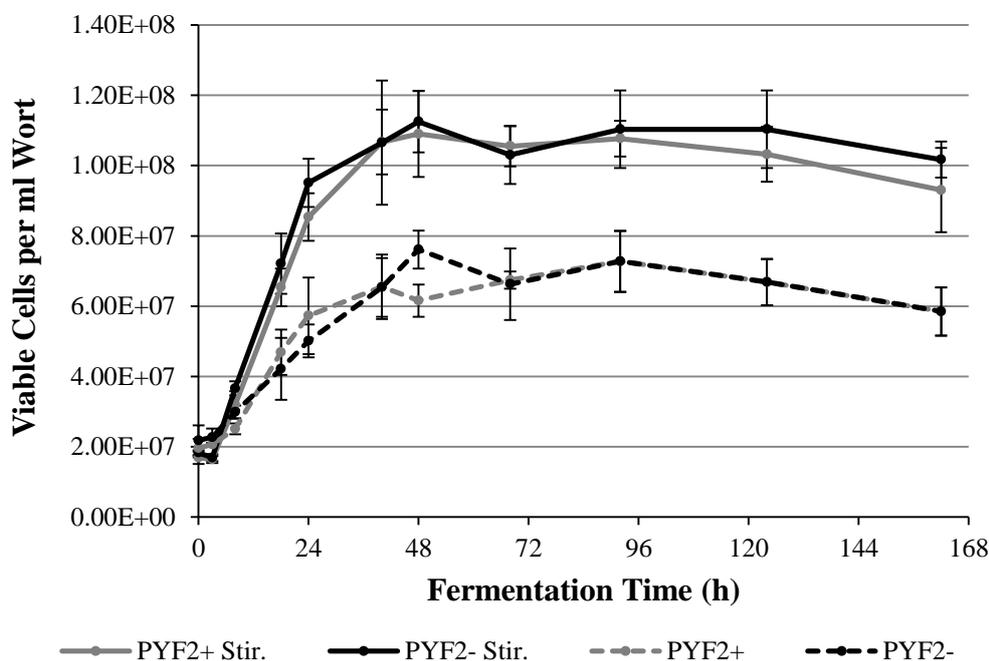


Figure 6.5: Changes in viable cells for 15°P PYF2+ and PYF2- worts fermented in 120 ml mini-fermenters at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹ under both stirred and unstirred conditions. Data are the mean of three replicate fermentations \pm SD.

6.3.4 Impact of PYF Factor(s) on Fermentation Progression

6.3.4.1 Impact of PYF Factor(s) on Gravity Drop

Measurements of the reduction in wort specific gravity, or a derived unit, as sugar is utilised by yeast is the most commonly applied method of gauging fermentation progress (Boulton & Quain, 2003). Yeast cells after four days of propagation at 25°C were added into sterile 15°P PYF2+ and PYF2- all-malt worts with the maximum sugar utilization taking place within the first two days of fermentation (i.e. up to 48 h post-pitching; Figure 6.6).

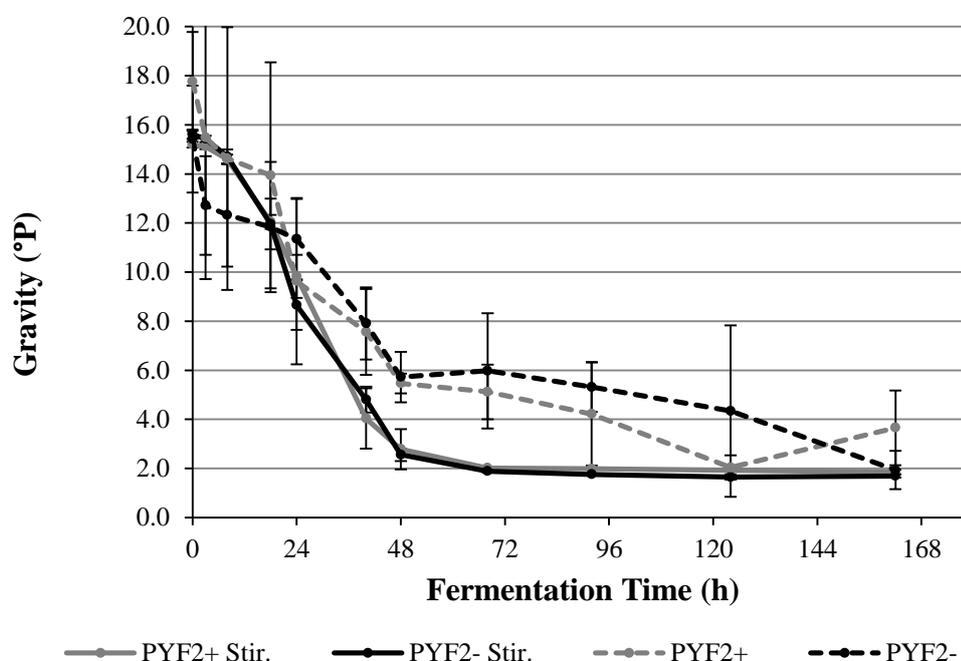


Figure 6.6: Changes in gravity for 15°P PYF2+ and PYF2- worts fermented in 120 ml mini-fermenters at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹ under both stirred and unstirred conditions. Data are the mean of three replicate fermentations \pm SD.

During that period of time, the SMA yeast cells were exhausting the wort's fermentable sugar in the stirred fermentations at a similar rate. The decline in gravity in the PYF2+ and PYF2- stirred fermentations reached a plateau at the same time during fermentation progression (i.e. approximately 72 h post-pitching). On the other hand, the utilization of sugars was more rapid in the

PYF2- unstirred fermentations when compared with the PYF2+ unstirred fermentations. However, there were no significant effects of PYF status in either the stirred or unstirred fermentations (i.e. the differences in sugar utilization between the PYF2+ and PYF2- stirred and unstirred fermentations were not significant ($P < 0.05$) at any time through fermentation progression). The results obtained are in agreement with Porter *et al.* (2010) who monitored the decline in apparent extract in three different PYF+ and PYF- worts during small-scale fermentations (3.5 ml cuvette size fermenters). These fermentations though, albeit conducted with the SMA yeast strain were performed at 21°C at the pitching rate of 15×10^6 live cells.ml⁻¹. Thus Porter *et al.* (2010) suggested that PYF was not caused by the impairment of sugar uptake, resulting from the action of antimicrobial peptides on cells membrane as stated in the “antimicrobial peptide hypothesis” by Van Nierop *et al.* (2004), but rather was likely caused by the presence of the ‘bridging’ polysaccharides present in their PYF+ worts.

Nevertheless, the results obtained from the present study suggested that fermentation progression was quicker in the stirred fermentations than was the case in the unstirred fermentations. This is in agreement with Boswell *et al.* (2002) who studied the effect of agitation intensity during small-scale (500 ml) fermentations, conducted with the lager yeast strain NCYC1324. The authors showed that fermentations carried out above 0.03 kW.m⁻³ specific power input (a value normally found in 400 m³ cylindroconical vessels at the maximum CO₂ evolution rate) increased fermentation rate and decreased attenuation time from 168 to 100 h. The same authors (Boswell *et al.*, 2002) associated the increased fermentation rate and the reduced fermentation time with the

enhanced turbulence at the scale of cells, leading to higher mass transfer rates coupled to metabolic processes. The higher error bars (Figure 6.6) observed in the PYF2+ and PYF2- unstirred fermentations are, assumedly, due to the lack of mixing which results in a more variable fermentation progression.

6.3.4.2 Impact of PYF Factor(s) on Alcohol Production

In line with the similarities that were observed in the sugar utilization (Figure 6.6), samples taken from the PYF2+ and PYF2- stirred fermentations 0 – 48 and 92 – 162 h post-pitching had similar alcohol contents ($P < 0.05$; Figure 6.7). On the other hand, whilst there were not statistically significant differences in the alcohol content between the PYF2+ and PYF2- unstirred fermentations at every time during fermentation progression, the ascent to full attenuation in the unstirred fermentations was slower than in the stirred fermentations. The more rapid alcohol yield in the PYF2+ and PYF2- stirred fermentations, as already mentioned; Boswell *et al.* (2002), was due to mixing which by keeping yeast in suspension increased the number of yeast cells and resulted in more rapid conversion of sugars to alcohol.

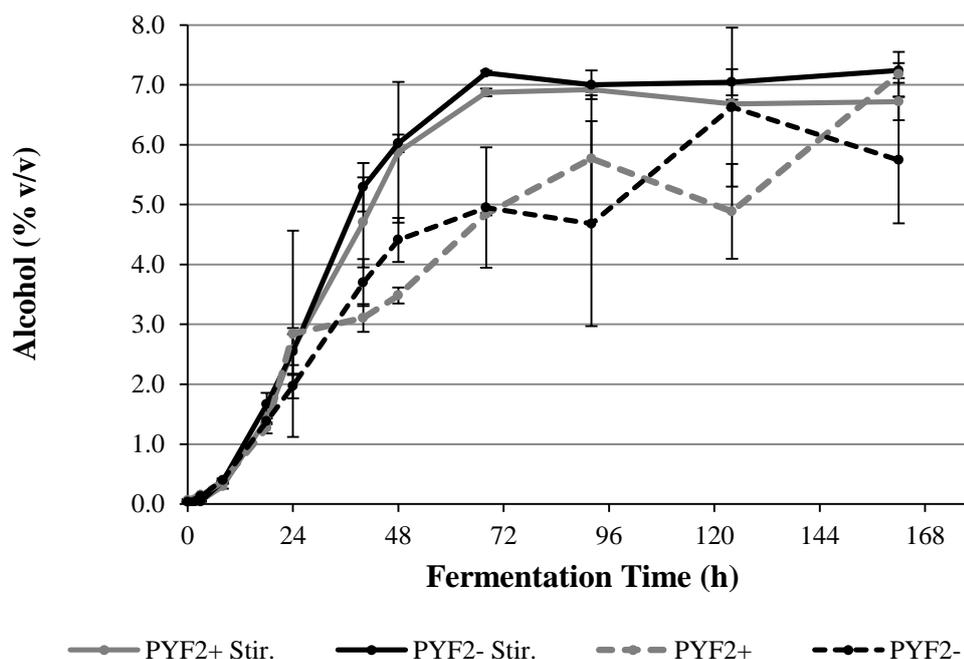


Figure 6.7: Changes in alcohol content for 15°P PYF2+ and PYF2- worts fermented in 120 ml mini-fermenters at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹ under both stirred and unstirred conditions. Data are the mean of three replicate fermentations \pm SD.

6.3.4.3 Impact of PYF Factor(s) on Carbon Dioxide Evolution

Formation of carbon dioxide (CO₂) during brewing yeast fermentations is stoichiometric (Boulton & Quain, 2003). Daoud and Searle (1990) studied the CO₂ evolution in laboratory and pilot scale (1.5 and 100 hl respectively) trial fermentations. At laboratory scale, the former authors (Daoud & Searle, 1990) demonstrated correlation coefficients of 0.9944 between CO₂ evolved and ethanol production and 0.99 between CO₂ evolved and carbohydrate utilisation. On the other hand, in the 100 hl fermentations, no gas evolution was observed until wort became saturated (i.e. after 9 – 10 h of fermentation). Following wort saturation, rates of approximately 1.0 g of CO₂ per litre per degree gravity drop were measured. Stassi *et al.* (1987) and Stassi *et al.* (1991), using thermal mass flow meters to measure CO₂ evolution rates both at laboratory and production scale brewing fermentations, noted also a correlation

between CO₂ formation and decline in gravity. CO₂ evolution was also correlated with the formation of ethanol, the extent of yeast growth, the decline in wort pH and the concentration of dissolved sulphur dioxide (SO₂). Therefore, the profile of CO₂ evolution can be used to monitor fermentation progress (Boulton & Quain, 2003). Nevertheless, the major potential problem is that there is little or no opportunity to gather data during early fermentation. This is because during the first few h of fermentation, the period where the critical processes of oxygen assimilation and yeast sterol synthesis take place (Hammond, 2000), little or no CO₂ formation occurs and even when gas evolution begins there is the period of inertia due to saturation of the wort (Boulton & Quain, 2003).

Figure 6.8 shows the fermentation progression of PYF2+ and PYF2- worts in stirred and unstirred fermentors monitored in terms of percentage weight loss (due to CO₂ evolution). The results obtained confirmed that fermentation progression was quicker ($P < 0.05$) in the stirred fermentations than was the case with the unstirred fermentations. Furthermore, CO₂ evolution was significantly higher in the PYF2- stirred fermentations 68 – 76 h post-pitching when compared with the PYF2+ stirred fermentations, whilst CO₂ evolution was statistically the same at every time point during the PYF2+ and PYF2- unstirred fermentations. Besides that, whilst small differences between the CO₂ mean values were found to be significant in the stirred fermentations, much bigger differences were insignificant in the unstirred fermentations. The great variability in the CO₂ evolution in the unstirred fermentations was due to the lack of mechanical agitation which, as already mentioned, results in more

consistent-reproducible fermentations in which case smaller trends can be identified.

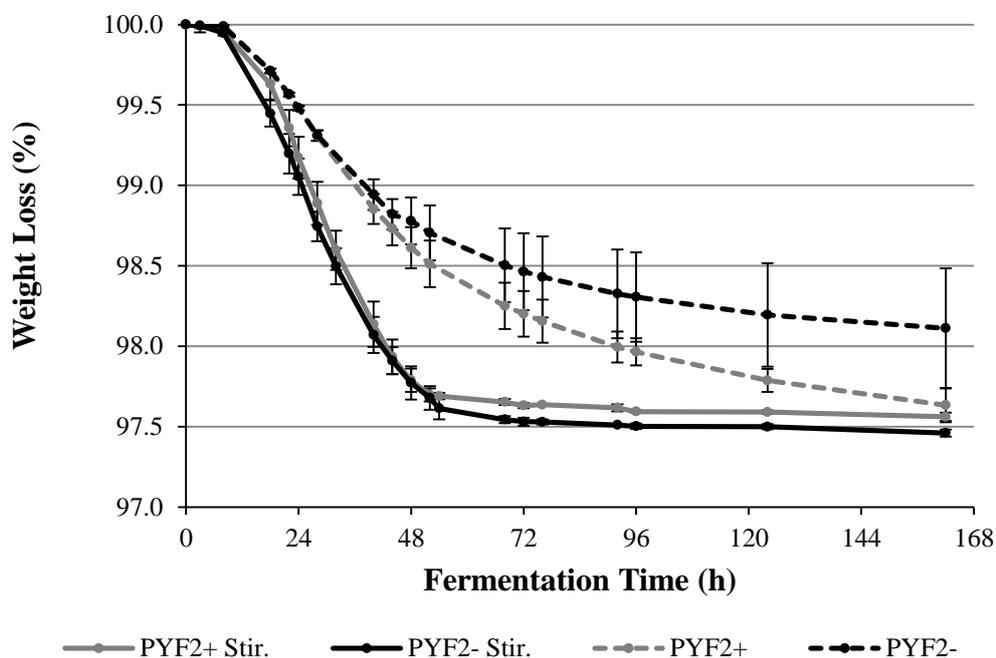


Figure 6.8: Changes in weight loss for 15°P PYF2+ and PYF2- worts fermented in 120 ml mini-fermenters at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹ under both stirred and unstirred conditions. Data are the mean of three replicate fermentations \pm SD.

6.3.4.4 Impact of PYF Factor(s) on pH

The fermentation of wort to beer is accompanied by a drop in pH, typically from just over pH 5.0 to around pH 4.0. This change is a consequence of yeast metabolism, involving excretion of several organic acids (including: pyruvate (100 – 200 ppm), citrate (100 – 150 ppm), malate (30 – 50 ppm), acetate (10 – 50 ppm), succinate (50 – 150 ppm), lactate (50 – 300 ppm) and 2-oxoglutarate (0 – 60 ppm) and proton expulsion in response to assimilation of wort sugars (Boulton & Quain, 2003). The majority of organic acids derive directly from pyruvate or from the branched tricarboxylic acid cycle which is characteristic of the repressed, anaerobic physiology of brewing yeast during fermentation

(Wales *et al.*, 1980). Excretion of organic acids into beer by yeast can be explained by the lack of any mechanism for further oxidation, the need to maintain a neutral intracellular pH and the fact that they are not required for anabolic reactions (Boulton & Quain, 2003). The most dramatic changes in pH occur during the early fermentation and the minimum value is achieved before wort attenuation is complete. Often, there is a modest increase in pH from the mid-point onwards. In this regard, therefore, pH is not a particularly useful monitor of overall fermentation progression, and certainly it is of no value in identifying the end-point. Nevertheless, the rapid decrease, which occurs in the first few h after pitching, can be monitored for the early identification of non-ideal performance (Boulton & Quain, 2003).

Yeast cells in the stationary phase, after 4 days of propagation at 25°C, were pitched into sterile 15°P all-malt PYF2+ and PYF2- worts with initial pH values of 4.9 and 4.8 respectively (Figure 6.9). The mean pH values were reduced to 3.8 and 3.1 after two days of fermentation, to final values of 4.09 and 4.0 in the PYF2+ and PYF2- stirred fermentations respectively towards the end of the sampling period (i.e. 162 h post-pitching). The results obtained indicated that there were significant differences in the pH of the two fermentations only 0 – 3 h post-pitching. During that period of time PYF2- worts had a significantly higher ($P < 0.05$) mean pH value than was the case with the PYF2+ fermentations. On the other hand, the 15°P PYF2+ and PYF2- worts used in the unstirred fermentations had initial pH values of 4.8 and 4.6 respectively. Following 48 h post-pitching the mean pH values were reduced to 4.1 and 4.1 in the PYF2+ and PYF2- fermentations reaching final mean values of 4.1 and 4.3 towards the end of the sampling period (i.e. 162 h post-

pitching). The greater pH reduction in the stirred fermentations was due to mixing which, by keeping yeast in suspension, enabled a more vigorous fermentation and resulted in higher yeast growth and therefore greater excretion of organic acids. The results obtained suggested that there were not statistically significant differences in the mean pH values between the PYF2+ and PYF2- unstirred fermentations 0 – 162 h post-pitching. Thus, it could be concluded that the PYF factor(s) used in these experiments either did not have a pronounced effect on the pH of the PYF2+ stirred and unstirred fermentations and/or that the occurrence of PYF is irrelevant to the pH of the fermenting broths.

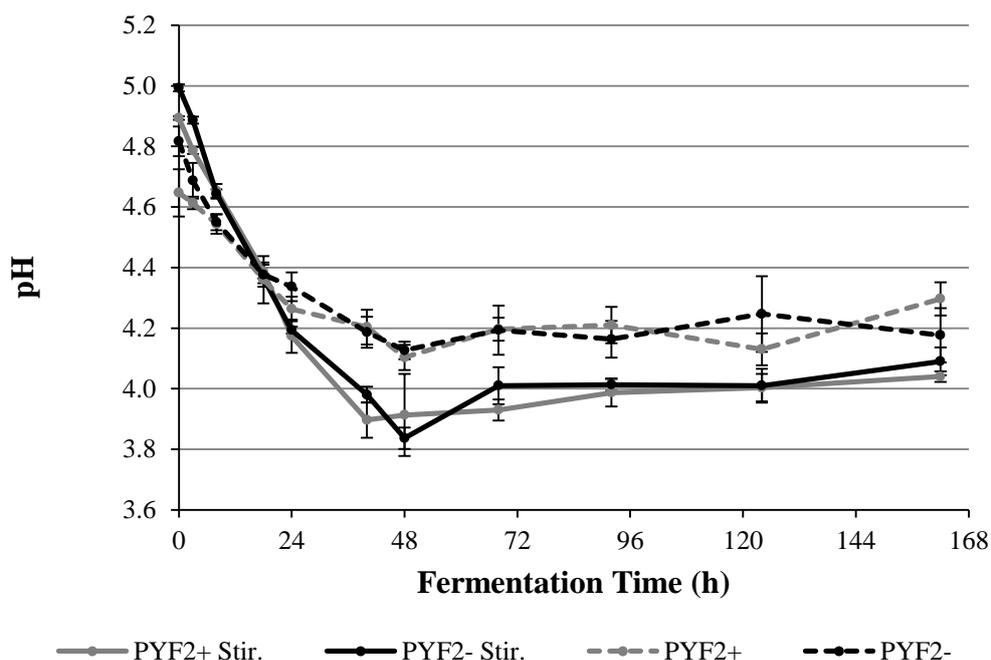


Figure 6.9: Changes in pH for 15°C PYF2+ and PYF2- worts fermented in 120 ml mini-fermenters at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹ under both stirred and unstirred conditions. Data are the mean of three replicate fermentations \pm SD.

6.3.4.5 Impact of PYF Factor(s) on the Assimilation of Individual Sugars

Standard brewery wort contains approximately 90% carbohydrates (as a percentage of wort solids). This fraction principally consists of the fermentable

sugars sucrose, glucose, fructose, maltose and maltotriose (Gibson *et al.*, 2008; Gibson *et al.*, 2010). Despite the fact that there is some variability between individual strains, brewing yeast can utilise a wide variety of carbohydrates (Hammond, 2000; Boulton & Quain, 2003). More specifically, ale strains of *Saccharomyces cerevisiae* ferment glucose, sucrose, fructose, maltose, raffinose, maltotriose and occasionally trehalose. On the other hand, lager strains of *S. cerevisiae* are able to ferment also the disaccharide melibiose, whilst *S. cerevisiae* var. *diastaticus* can utilise dextrans (i.e. oligomers of glucose; Boulton and Quain, 2003; Briggs *et al.*, 2004).

The 15°P all-malt PYF2+ and PYF2- worts used throughout these experiments had similar fermentable sugar contents before pitching (Chapter 5; Figure 5.1). Maltose and maltotriose were the most abundant fermentable sugars, whereas glucose, fructose and sucrose were present in much lower concentrations. Although sucrose was present at very low concentrations in the PYF2+ and PYF2- worts before pitching (approximately 0.5 mg.l⁻¹; Figure 5.1) it could not be detected in significant amounts during the analysis (data not shown as sucrose was hydrolysed prior to the first sampling point at 3 h). This is because sucrose, which is hydrolysed by an invertase that is secreted into the periplasm, is normally depleted within the first h of fermentation resulting in a transient increase of glucose and fructose (Hornsey, 1999; Boulton & Quain, 2003; Briggs *et al.*, 2004). The next fermentable sugar to be consumed during the fermentation was glucose. Lagunas (1993) indicated that glucose, diminishing from the wort more or less at the same time as fructose – after 24 h (Boulton & Quain, 2003), is the preferred substrate and its presence in the medium inactivates or represses carriers for the uptake of other sugars. Glucose was

present in the stirred fermentations until 24 h post-pitching, whilst in the unstirred fermentations until 40 h post-pitching (Figure 6.9). During these periods of time the highly flocculent but PYF sensitive yeast strain (SMA) used in these experiments was utilizing glucose in the PYF2+ and PYF2- stirred and unstirred fermentations at the same rate ($P > 0.05$). However, glucose utilization was quicker in the stirred fermentations when compared with the unstirred fermentations as a result of keeping the yeast cells in suspension and maintaining homogeneity.

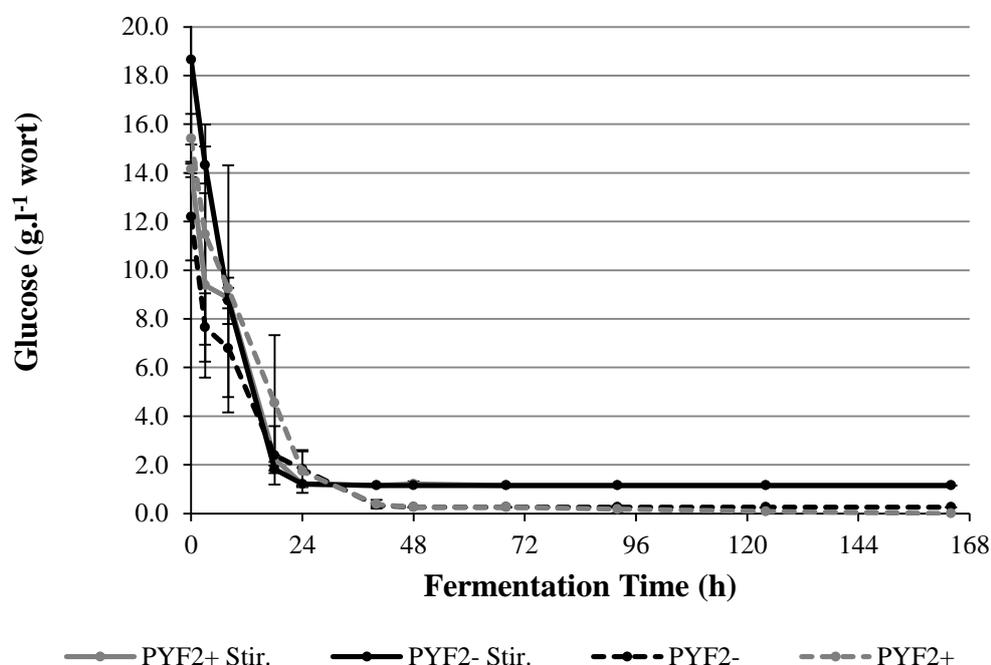


Figure 6.10: Changes in glucose assimilation for 15°P PYF2+ and PYF2- worts fermented in 120 ml mini-fermenters at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹ under both stirred and unstirred conditions. Data are the mean of three replicate fermentations \pm SD.

Fructose, the third sugar to be depleted, was present in the stirred fermentations until approximately 40 h post-pitching (Figure 6.10). Although the mean fructose concentration in the PYF2+ stirred fermentations showed a small increase 18 and 24 h post-pitching, this increase was not found to be

statistically significant. Thus, during the first 40 h of fermentation SMA cells were utilizing fructose in the PYF2+ and PYF2- stirred fermentations at the same rate. On the other hand, fructose was present in the unstirred fermentations until 120 h post-pitching (Figure 6.11).

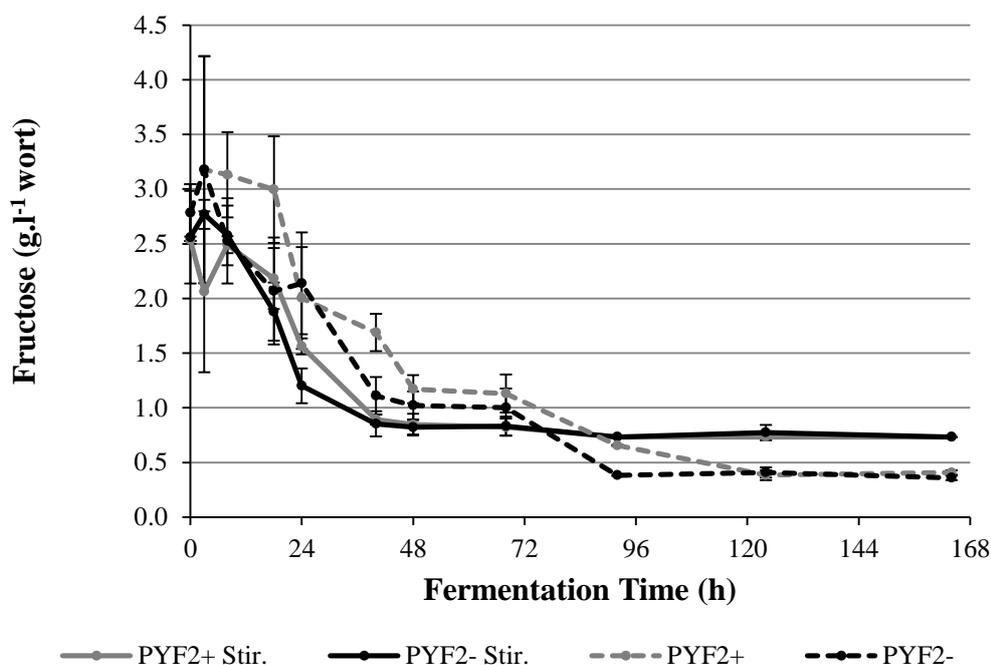


Figure 6.11: Changes in fructose assimilation for 15°P PYF2+ and PYF2- worts fermented in 120 ml mini-fermenters at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹ under both stirred and unstirred conditions. Data are the mean of three replicate fermentations \pm SD.

Despite the fact that fructose was initially utilised at a slower rate in the PYF2+ unstirred fermentations than was the case in the PYF2- unstirred fermentations, the differences observed in fructose assimilation throughout fermentation progression were not found to be significant ($P > 0.05$). The higher residual glucose and lower fructose concentrations in the unstirred fermentations (Figures 6.10 and 6.11 respectively) could be attributed to the variability of the HPLC analysis – especially since the gravity drop data showed reduction to a lower level for the stirred samples (Figure 6.6).

The completion of glucose and fructose assimilation is followed by the uptake of maltose, which is the most abundant sugar in the wort, whilst maltotriose is utilised last after assimilation of all maltose (Hornsey, 1999; Boulton & Quain, 2003; Briggs *et al.*, 2004). Maltose and maltotriose will typically only be taken up after the depletion of monosaccharides in wort due to carbon catabolite repression of metabolic pathways involved in the uptake and utilization of alternative sugars (Lagunas, 1993). In the present experiment, the highly flocculent PYF-sensitive yeast cells (SMA) depleted maltose and maltotriose in the stirred and unstirred fermentations at similar rates (Figures 6.12 & 6.13). However, whilst both sugars (maltose and maltotriose) were exhausted in the stirred fermentations 72 h post-pitching the assimilation of maltose and maltotriose in the unstirred fermentations continued until the end of the sampling period (i.e. 162 h post-pitching). Higher polysaccharides (i.e. dextrins) are not utilised by brewing yeast strains, but rather contribute to beer flavour by way of imparting fullness (Boulton & Quain, 2003; Briggs *et al.*, 2004), and for that reason were not identified in this study. However, in the early 1980s attempts were made to utilise dextrins in brewing fermentations via two different strategies. The first attempt was through the introduction of appropriate enzymes into yeast cells (genetic manipulation), whilst the second attempt by addition to wort of commercial dextrinase enzymes. In the latter case dextrins were hydrolysed to assimilable sugars (Boulton & Quain, 2003).

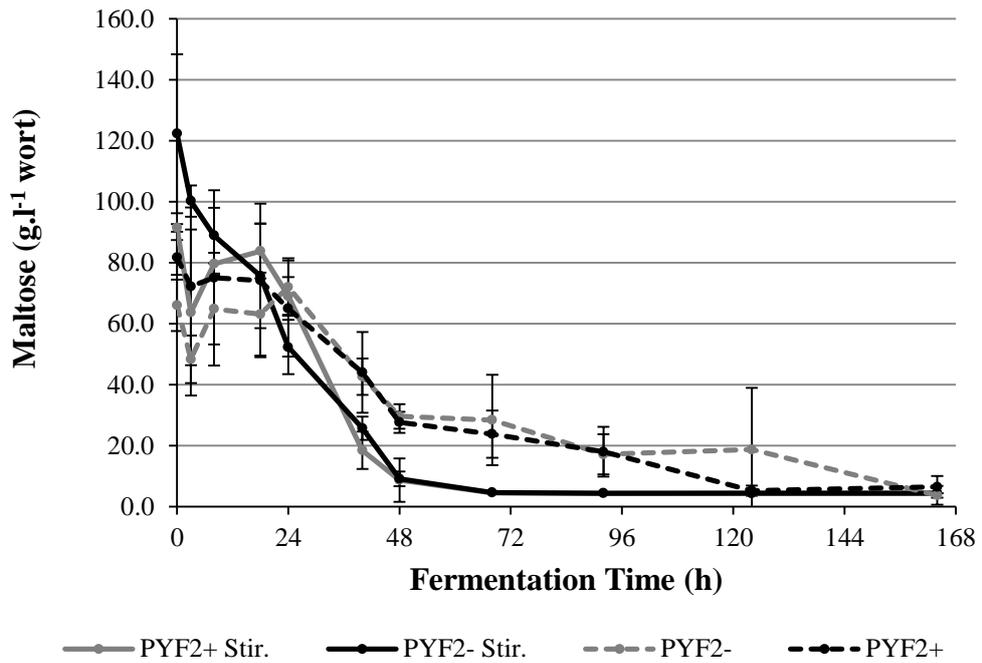


Figure 6.12: Changes in maltose assimilation for 15°P PYF2+ and PYF2- worts fermented in 120 ml mini-fermenters at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹ under both stirred and unstirred conditions. Data are the mean of three replicate fermentations \pm SD.

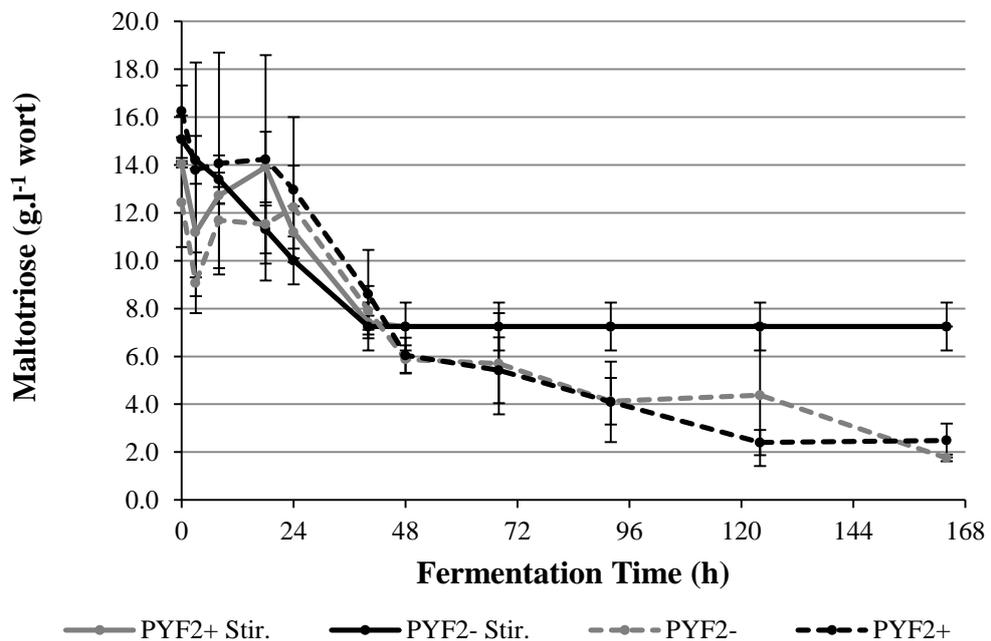


Figure 6.13: Changes in maltotriose assimilation for 15°P PYF2+ and PYF2- worts fermented in 120 ml mini-fermenters at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹ under both stirred and unstirred conditions. Data are the mean of three replicate fermentations \pm SD.

The results obtained from this study suggested that SMA cells were utilizing the four fermentable sugars (glucose, fructose, maltose and maltotriose) in the PYF2+ and PYF2- stirred and non-stirred fermentations at broadly similar rates throughout the fermentation progression. The results with respect to maltose utilization are in disagreement with the results reported from Van Nierop *et al.* (2004). In their experiments, conducted with PYF+ and PYF- worts in 2 l EBC tall tubes fermented using a different yeast strain (SAB lager yeast strain) and without employing stirring, the uptake of maltose was delayed in the PYF+ fermentations after four days of fermentation. Besides Van Nierop *et al.* (2004), Axcell *et al.* (2000) also reported abnormal flocculation patterns in breweries using high dextrose adjuncts (40%). On analysis these worts had substantial amounts of residual maltose and maltotriose.

6.3.4.6 Impact of PYF Factor(s) on Free Amino Nitrogen Utilization

Wort nitrogen levels have a marked effect on yeast growth. Below about 100 mg.l⁻¹ yeast growth is nitrogen dependent, above this value becomes less dependent, whilst FAN levels above 220 mg.l⁻¹ have little effect (Hammond, 2000). The nitrogenous components of wort account for 4 – 5% of the total dissolved solids (Boulton & Quain, 2003) and comprise proteins, polypeptides, amino acids and nucleotides in varying amounts (Ingledew, 1975). The bulk (85 – 90%) of the total nitrogen content is in the form of amino acids, small peptides and proteins, whilst the relative proportion of each of these groups depend on the composition of the grist and the conditions of wort production (Boulton & Quain, 2003). *Saccharomyces* yeasts can utilize brewer's wort ammonium ions, amino acids, peptides, purines and pyrimidines but cannot

utilize proteins (as these yeasts do not produce extracellular proteases) nitrate, nitrite and gaseous nitrogen (Briggs *et al.*, 2004).

The PYF2⁺ and PYF2⁻ worts used in this study had similar FAN contents immediately after pitching (349.9 and 328.9 mg.l⁻¹ of wort respectively; Chapter 5 – Figure 5.2). FAN was depleted in the stirred fermentations until approximately 48 h post-pitching. During that period of time, the SMA yeast cells were utilizing the FAN content in the PYF2⁺ and PYF2⁻ worts at the same rate ($P > 0.05$). Despite the fact that there were not statistically significant differences in the FAN utilization in the unstirred fermentations, it was apparent that FAN assimilation was slower when compared with the FAN utilization in the stirred fermentations (Figure 6.14). The residual FAN levels observed in the stirred and unstirred fermentations could be possibly due to the presence of proline, which as stated, is one of the major amino acids in the wort and requires the presence of oxygen for its assimilation.

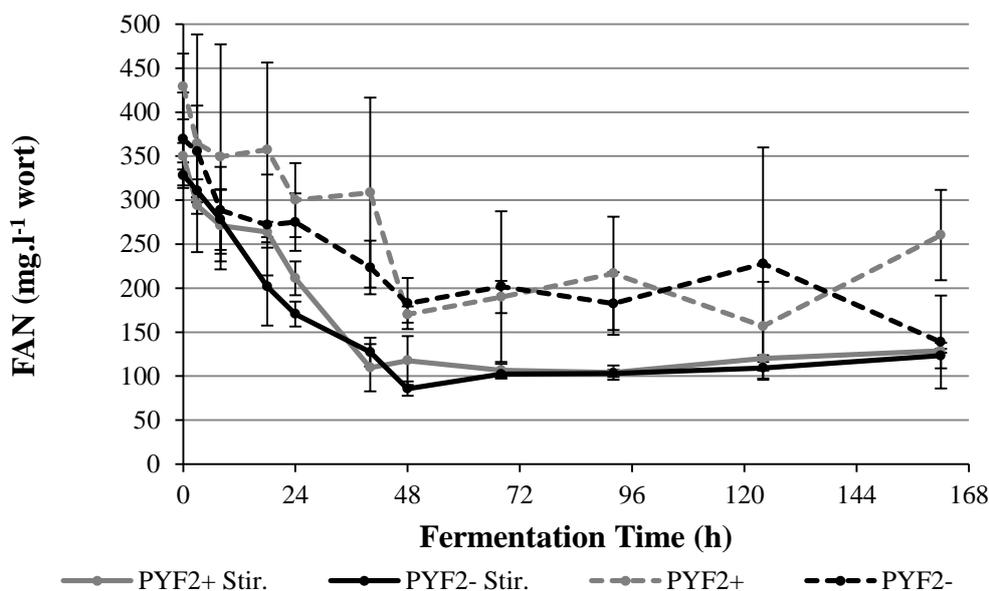


Figure 6.14: Changes in FAN assimilation for 15°P PYF2⁺ and PYF2⁻ worts fermented in 120 ml mini-fermenters at 15°C using SMA yeast at a pitching rate of 20×10^6 live cells.ml⁻¹ under both stirred and unstirred conditions. Data are the mean of three replicate fermentations \pm SD.

6.4 Conclusions

High gravity (15°P) stirred and unstirred fermentations with PYF+ and PYF- worts did not detect significant effects of the PYF factors on yeast physiological characteristics or metabolic aspects of fermentation performance. This was in spite of the fact that the PYF status of the same batches of wort was verified using our in-house PYF test. Since sugar uptake was not significantly impacted by the PYF status of the wort it is unlikely that significant amounts of antimicrobial peptides were present in this particular PYF+ sample. It could thus be suggested that the PYF+ sample used in these experiments was inducing PYF primarily through the 'bridging' polysaccharide mechanism. Interestingly, the use of the 'mini-FV' fermenters and a 15°P all malt wort meant that the incidence of PYF, even in the unstirred fermentations, was much less pronounced with regard to yeast suspended cell profiles. The same worts standardised to 11°P, supplemented with 4% glucose and fermented in the relatively tall/ thin dropping funnel used for our in-house PYF tests, clearly demonstrated PYF (in wort prepared from malt known to have caused PYF in brewery fermentations). It is possible that more significant differences in some of the parameters monitored through PYF+ and PYF- fermentations might have been observed, if the vessel design and fermentation vigour had encouraged yeast to stay in suspension better in the unstirred PYF- fermentations. The primary differences observed in these experiments were between the stirred and unstirred fermentations. Keeping yeast in suspension, by mechanical agitation, enabled a more rapid fermentation progression and cell density, viability, alcohol yield and CO₂ evolution were all significantly higher as compared with the unstirred fermentations.

Chapter 7

Assessing the sensitivities of yeast strains

to factor(s) inducing Premature Yeast

Flocculation using Phenotype

MicroArrays

7.1 Introduction

The impacts of PYF factor(s) on yeast fermentation performance and metabolite profiles were discussed in Chapter 6. The results obtained suggested that high gravity (15°P) worts inducing PYF did not have a significant effect on yeast physiological characteristics through fermentation or on fermentation performance indicators (i.e. CO₂ evolution, pH, gravity content, ethanol yield, FAN and fermentable sugars) either under stirred or unstirred conditions. In this chapter, a new approach is undertaken so as to further investigate the effects of the PYF factor(s), on yeast growth and respiration using a new tool; the OmniLog Phenotype MicroArrayTM (PM) technology.

The OmniLog Phenotype MicroArrayTM (PM) technology was created by Biolog Inc. in 2000 and since then has been used for the metabolic characterization of micro-organisms for various research purposes (DeNittis *et al.*, 2010b). Phenotype MicroArrays are a new and high-throughput technology which allows the simultaneous testing of a large number of cellular phenotypes (Bochner *et al.*, 2001), the detectable manifestations of a specific gene (Outeiro & Giorgini, 2006). They can directly assess the effects of genetic changes on cells and particularly gene knock-outs (Bochner *et al.*, 2001).

The method consists of preconfigured well arrays in which each well tests a different cellular phenotype, whilst an automated instrument continuously monitors and records the response of the cells in all the wells of the arrays. Cells incubated at a specific temperature grow, respire and upon respiration they reduce a dye (usually tetrazolium violet) resulting in the formation of a

purple colour (Bochner *et al.*, 2001; Bochner, 2003; DeNittis *et al.*, 2010a). The reduction of the dye is irreversible, and thus the accumulation in the well over a period of time amplifies the signal and integrates the amount of respiration over time. On the other hand, partial or total loss of a function will result in partial or no respiration-growth, and therefore in reduced or no purple colour formation (Bochner *et al.*, 2001; Bochner, 2003). Respiration constitutes an accurate reflection of the physiological state of the cell even though it does not necessarily indicate growth (i.e. cell division; Outeiro & Giorgini, 2006). Consequently, cell respiration can be used in some important assays that do not depend on growth (Bochner, 2003). By measuring cell respiration, PM technology offers the possibility to study directly the impact of oxidants, metals or even different nutrient sources, which influence the physiological state of the cell and their respiration. Outeiro and Giorgini (2006) used PMs to identify phenotypes-conditions able to improve or reduce the viability of yeast strains expressing human proteins involved in neurodegenerative disorders (e.g. Parkinson's disease or Huntingdon's disease). The evolution of these changes, expressed by the index *average well colour development* (AWCD) can be plotted as a curve, similar to a growth curve, that represents the temporal evolution of the metabolic activity of the population under study (DeNittis *et al.*, 2010a). The Biolog PM instrument cycles microplates in front of a colour CCD camera and provides quantitative and kinetic information about the response of the cells in the wells, whilst the data are stored directly into computer files and can be recalled and compared with other data at any time (Bochner *et al.*, 2001; Bochner, 2003).

The purpose of this study was to investigate the sensitivity of lager yeast strains, of varying degrees of flocculence, to factors inducing PYF using the Biolog system. The aim was to offer insights into the complex relationship between the various PYF factors and different yeast strains, as well as to investigate the application of a new tool which has the potential to be applied for the screening of the PYF status of malt samples without the need for fermentation tests. Besides the PMs, our in-house PYF assay (see Chapter 3 for details) was also used as a control test to confirm the PYF status of the malts used in this study.

7.2 Experimental

7.2.1 Malts

Two PYF+ and two PYF- malts were used in this study. The first pair of PYF+ and PYF- malts were produced from the same barley variety (Scarlett), region (France) and crop year (2007), whilst the second pair of malt samples were manufactured from different barley varieties (Quench and Prestige) and were sourced from different regions (UK and Europe respectively; Table 7.1).

Table 7.1: Barley variety, harvest year and region of production for PYF+ and PYF- malts used in this study.

Barley Variety	Harvest Year	Region of Production	PYF Abbreviation
Scarlett	2007	France	PYF2+
Scarlett	2007	France	PYF2-
Quench	2009	U.K	PYF3+
Prestige	2009	Europe	PYF3-

7.2.2 Wort Preparation

The PYF+ and PYF- worts were prepared from control malts (Section 7.2.1) using a standardized laboratory mashing procedure as described in Section 2.5. However, in order to obtain the PYF factor(s) in higher concentrations, comparable to wort concentrations used in Chapter 6, a “thicker” mash (120 ± 0.5 g of milled malt was added to 360 ml brewing liquor), resulting in approximately 18°P gravity, was used during wort preparation (mashing). This was subsequently standardized, using RO water, to 15 or 11°P.

7.2.3 Wort Composition Analyses

Wort FAN and fermentable sugar spectrum analyses were performed as described in Sections 2.7.3 and 2.7.4 respectively.

7.2.4 Yeast Strain and Propagation Conditions

Three lager brewing yeast strains (W34/70, SMA and ‘Industrial’) of varying degrees of flocculence were used in this study (Table 7.2). W34/70 (ex Weihenstephen) was obtained from the National Collection of Yeast Cultures (NCYC), the SMA from the VLB Research Institute (Berlin, Germany) and the ‘Industrial’ yeast strain was provided by a large multinational brewing company. As already mentioned in Chapter 5, the ‘Industrial’ yeast strain was of interest because it was thought to be relatively insensitive to PYF and is not identified for reasons of commercial sensitivity. Yeast propagation, in the cases of the PYF tests, was performed in an orbital shaken incubator at 120 rpm for 4 days at 15°C as described in Section 2.2.4.

Table 7.2: Source and relative flocculence of yeast strains used in this study.

Yeast Strain	Source	Yeast Type	Relative degree of Flocculence
W34/70	NCYC	Lager	Medium
SMA	VLB Research Institute	Lager	High
‘Industrial’	Industry	Lager	Non-flocculent

7.2.5 Premature Yeast Flocculation Assay

PYF assays were conducted as described in Section 2.6.1. In brief, the procedure involved conducting small-scale (200 ml) fermentations in 250 ml ‘dropping funnels’ within a temperature controlled incubator (15°C) and using worts prepared from PYF+ and PYF- malts (Table 7.1) using a standardized laboratory mashing procedure (see Section 2.5.3 for detail). The fermentations were conducted at a pitching rate of 20×10^6 live cells.ml⁻¹ after the supplementation of 11°P all-malt worts with 4% [w/v] glucose for a maximum period of 92 h.

7.2.5.1 Monitoring Suspended Yeast Cell Counts

Cell concentration in suspension was assessed at specific time intervals between 0 – 92 h post-pitching (i.e. 0, 24, 40, 44, 48, 52, 64, 68 and 92) by measuring absorbance at 600 nm (A_{600} ; Section 2.6.2.1).

7.2.5.2 Residual Gravity and Alcohol Yield

The residual gravity and alcohol yield of the fermenting broths were determined at 15°C using a DMA 5000 M model Anton Paar density-alcolyzer meter as described in Section 2.7.6.

7.2.6 Phenotype Microarray™ Analysis

7.2.6.1 Incubation of Culture Plates

Yeast cells were recovered from cryostorage and maintained on YPD agar slopes at 4°C. Cell suspensions were achieved by selecting representative colonies from slopes and inoculating into cooled (25°C) steam sterilized YPD media in two stages. For the first stage of propagation, a loop of yeast cells was aseptically transferred into 10 ml YPD in 25 ml sterile Universal bottles. Cultures were aerobically propagated at 25°C for 24 h in a Certorat BS-1 shaken incubator at 120 rpm. 24 h later the cells were streaked aseptically to sterile YPD agar plates and were grown aerobically for a further 72 h at 25°C in an MIR-262 Sanyo static incubator.

7.2.6.2 Preparation of Cell Suspensions

The suspended cell count of each individual yeast strain was adjusted to 62% transmittance using a 3587 portable Biolog turbidimeter as described in Section 2.8.2.

7.2.6.3 Wort Dilutions, Yeast Incubation and Absorbance Readings

18°P all-malt wort, previously kept at -20°C, was thawed and diluted with sterile RO water to 15 and 11°P final concentration respectively. 5 ml from each dilution was aseptically added to 25 ml pre-sterilised Universal bottles containing 160 to 640 µl of dye-D (Biolog Inc., Hayward, USA) and 0 to 40% inoculating fluid for yeast (i.e. 0 – 3.2 ml of dye-D when 11°P wort required). The inoculating fluid for yeast (IFY) is a proprietary Biolog buffer used to stabilize the signal. Following homogenization, 95 µl of each mixture were added to each of three replicate wells, of approximately 200 µl volume,

containing 30 μ l 62% transmittance yeast suspensions. The plates were then incubated in the dark in the Omnilog instrument (Biolog Inc., Hayward, USA) at 25°C for a maximum period of 70 h and periodically, every 5 min, submitted to absorbance readings of the colour in the wells with the Biolog E-MAX Reader (Biolog Inc., Hayward, USA).

7.3 Results and Discussion

7.3.1 Wort Fermentable Sugars Composition and FAN Content

The fermentable sugar and FAN compositions of the PYF2 and PYF3 worts were discussed in Chapter 5; Section 5.3.1. The results obtained suggested that worts prepared from the Scarlett (PYF2+, PYF2-) and Quench malts (PYF3+) were of similar composition with respect to fermentable sugars (fructose, glucose, sucrose, maltose and maltotriose) and FAN (Figures 5.1 and 5.2 respectively). On the other hand, worts prepared from the Prestige malt (PYF3) contained a lower amount of maltose. Nevertheless, as the differences in sugar concentration between the worts used in this study was less than 15%, and their FAN contents were all greater than 200 mg.l⁻¹ wort, it could be assumed that they would ferment similarly other than for differences caused by PYF (Van Nierop *et al.*, 2004). However, as already stated other aspects of nutrient deficiency (i.e. zinc, manganese, biotin) could also cause worts not to ferment properly (see Sections 1.3.1 and 3.3.1 for details).

7.3.2 Predicting the PYF Potential of Malts using the in-house PYF Fermentation Assay

The PYF potential of the PYF2 and PYF3 worts was measured in Chapter 5. More specifically, using the small-scale fermentation tests and the medium

(W34/70) and highly flocculent but PYF sensitive lager yeast strain SMA the PYF potential of the PYF2 malts could be differentiated 69 and 40 h post-pitching respectively. At these periods of time the number of suspended yeast cells in the PYF2+ fermentations, as indicated by A_{600} readings, was found to be statistically significantly lower ($P < 0.0001$) than the number of suspended yeast cells in the PYF2- fermentations. Besides that, PYF2- worts had a lower residual gravity and higher ethanol yield when compared with the PYF2+ worts (Chapter 5; Table 5.5). On the other hand, PYF2+ worts fermented with the 'Industrial' lager yeast strain, previously found insensitive to PYF in industrial-scale fermentations, under the same experimental conditions (fermentations conducted at 15°C at the pitching rate of 20×10^6 live cells.ml⁻¹ after the supplementation of 11°P all-malt worts with 4% [w/v] glucose) gave similar fermentation profiles, with respect to suspended cell counts, with the PYF2- worts. Besides that, fermentations conducted with the 'Industrial' lager yeast strain resulted also in lower elevation in residual gravity and ethanol yield between PYF2+ and PYF2- worts (Chapter 5; Figure 5.7). Small-scale fermentations conducted with the 11°P all-malt worts (after the supplementation with 4% [w/v] glucose before pitching) and the SMA yeast strain allowed also the differentiation between PYF3+ and PYF3- worts 40 h post-pitching. However, fermentations with the PYF3+ worts and the 'Industrial' lager yeast strain did not give similar fermentations profiles when compared with the fermentations conducted with the PYF3- worts (Chapter 5; Figure 5.6).

7.3.3 Phenotype Microarray™ Analysis

7.3.3.1 The Relationship between IFY concentration and Biolog Reactions

Figure 7.1 shows the average well colour development (AWCD) or average redox value, as measured in Biolog units, of the highly flocculent but PYF sensitive lager yeast strain SMA incubated in 15°P PYF2+ and PYF2- worts at 25°C after the addition of 0 and 16.66% IFY and 160 µl of dye for a total period of 70 h.

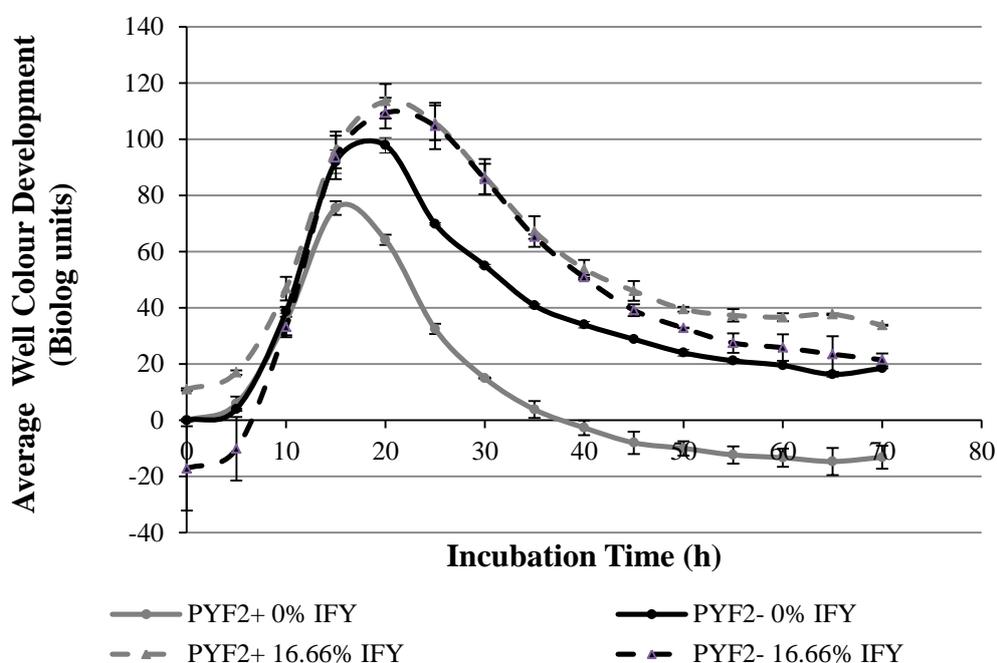


Figure 7.1: Redox potential for SMA yeast cells incubated in 15°P PYF2+ and PYF2- worts at 15°C after the addition of 0 and 16.66% IFY and 160 µl dye over a period of 70 h. The redox potential was assessed spectrophotometrically using the average colour response (Biolog units) caused by the reduction of a tetrazolium dye. Data are the mean of six replicates ± SD.

Using the Biolog detection system without addition of IFY (0%), PYF2+ worts could be differentiated from PYF2- worts on the basis of redox reduction after approximately 15 h of incubation and beyond. In this case the SMA cells in the PYF2- worts were reducing the dye at a quicker rate than was the case with the SMA cells in the PYF2+ worts. The results obtained suggested that the yeast

cells in the PYF2+ worts were in a different physiological state than the yeast cells in the PYF2- worts following 15 h of incubation and beyond. On the other hand, the incubation of the SMA yeast cells with the 15°P PYF2+ worts containing 16.66% IFY and 160 µl of dye resulted in similar degrees of redox reduction-cell respiration with the SMA yeast cells in the 15°P PYF2- worts, containing the same amount of IFY (16.66%) and dye, until approximately 40 h from the incubation onset. After more than 40 h of incubation, the SMA yeast cells in the PYF2+ worts exhibited a higher degree of redox reduction than was the case with the SMA yeast cells in the PYF2- worts. These results suggested that an attribute of the PYF2- worts, not associated with the occurrence of PYF (as these worts were previously found not to cause PYF using our in-house small-scale fermentation assay; Chapter 5; Figure 5.3), was the cause of the lower average redox values obtained 40 h post-incubation. Thus, whilst in the absence of IFY it was possible to differentiate rapidly between the 15°P PYF2+ and PYF2- worts, the signal remained more stable – especially after 20 h of incubation, when 16.66% IFY was used. Consequently, it was concluded that the amount of IFY, which as stated earlier is a buffer used to stabilize the signal, has a significant and direct effect on Biolog results.

In spite of the fact that the addition of 16.66% IFY to the 15°P worts did not allow the differentiation between PYF2+ and PYF2- worts when the highly flocculent but PYF sensitive lager yeast strain (SMA) was used (Figure 7.1), the incubation of the same gravity worts (15°P) with the medium flocculent (W34/70) and the ‘Industrial’ lager yeast strains enabled a rapid differentiation between PYF2+ and PYF2- worts (Figure 7.2).

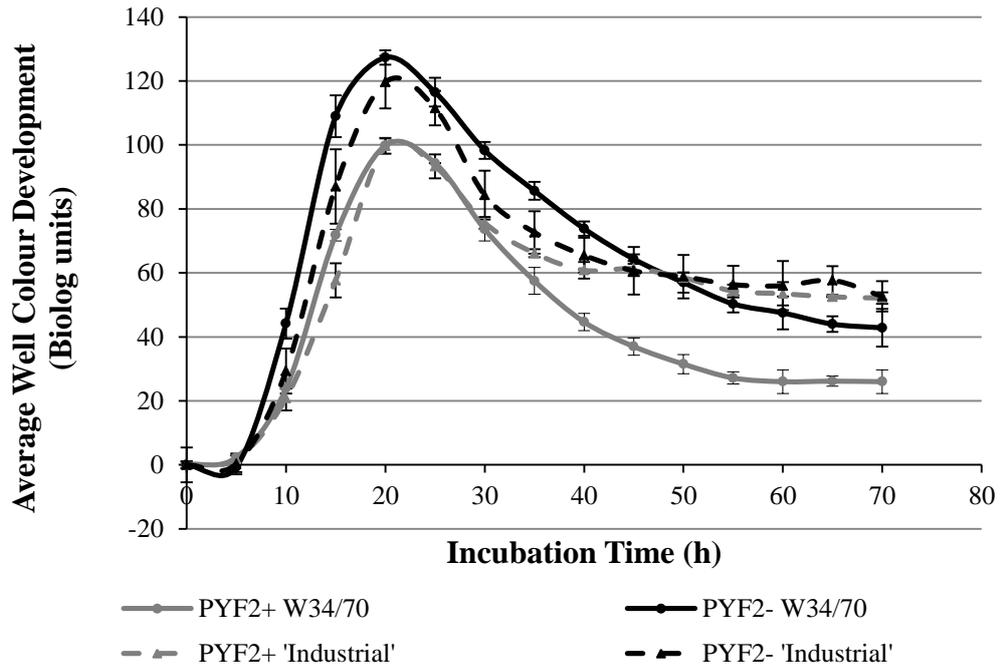


Figure 7.2: Redox reduction for W34/70 and 'Industrial' yeast cells incubated in 15°P PYF2+ and PYF2- worts at 15°C after the addition of 16.66% IFY and 160 µl dye over a period of 70 h. The redox potential was assessed spectrophotometrically using the average colour response (Biolog units) caused by the reduction of a tetrazolium dye. Data are the mean of six replicates ± SD.

More specifically, W34/70 cells incubated in the PYF2+ worts containing 16.66% IFY exhibited a lower degree of redox dye reduction, following 10 h of incubation and beyond, than was the case with the PYF2- worts incubated with the same yeast strain (W34/70) and the same amount of IFY (16.66%). Nevertheless, redox reduction reached a maximum 20 h from the onset of incubation both in the PYF2+ and PYF2- wells. At that period of time the maximum difference in redox reduction-cell respiration, expressed as average Biolog units, was observed between the PYF2+ and PYF2- worts.

Using Biolog and the 'Industrial' lager yeast strain, the 15°P PYF2+ and PYF2- worts could also be differentiated after 20 h of incubation in the 96 well plates (Figure 7.2). The average well colour development again reached a maximum after 20 h of incubation. Thus, whilst the 'Industrial' lager yeast strain was found insensitive to the PYF factor(s) in the PYF2+ worts (Chapter

5; Figure 5.4), in this study the same yeast showed sensitivity to some other attributes of the same worts. This result calls into question whether the Biolog response indicates anything of significance with respect to PYF. More specifically, taking into consideration that the 'Industrial' yeast was found insensitive to PYF, both industrially and in our in-house PYF assay, the observed differences in the rates of dye reduction would have probably been caused either by a lack of consistency in the yeast physiological state used in the assay, or, more likely, the different rates of dye reduction reflect other differences in mineral or nutrient compositions of the worts.

Having established the relationship between IFY and Biolog reactions (i.e. that IFY addition stabilizes the signal during the PM analysis; Figures 7.1 and 7.2), the sensitivity of the three lager yeast strains (SMA, W34/70, and 'Industrial') was investigated in 11°P PYF2+ and PYF2- worts containing 16.66% IFY and 160 µl dye (Figure 7.3).

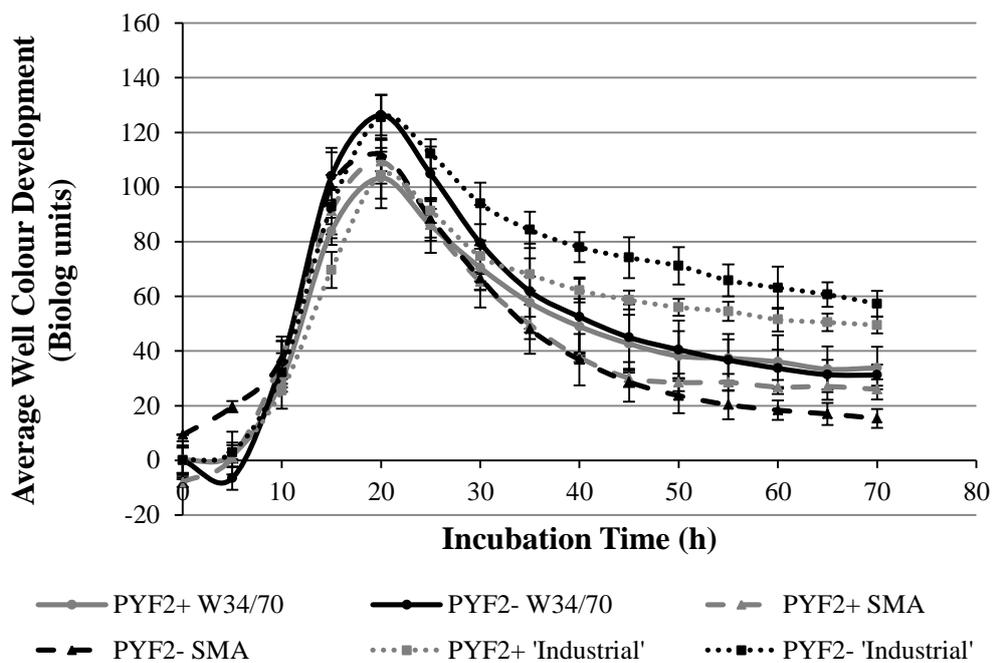


Figure 7.3: Redox reduction for SMA, W34/70 and ‘Industrial’ yeast cells incubated in 11°P PYF2+ and PYF2- worts at 15°C after the addition of 16.66% IFY and 160 µl dye over a period of 70 h. The redox potential was assessed spectrophotometrically using the average colour response (Biolog units) caused by the reduction of a tetrazolium dye. Data are the mean of six replicates ± SD.

The results obtained suggested that redox reduction reached a maximum in all the cases, irrespective of the yeast strain used, 20 h post-incubation onset. Following that period of time the redox reduction, as indicated by Biolog units, started to gradually decline, most likely due to insufficient amount of IFY needed to further stabilize the signal rather than due to the production by the yeast of various metabolites (e.g. organic acids) likely to affect the dye. Lowering pH, using concentrated HCl, to pH 2 resulted in a sequential drop in the signal. However, after a quick drop the redox response increased again and remained stable throughout the analysis. Thus, it was concluded that the bell-shaped curves are more likely to occur due to the loss of an essential component of the cell, most probably nitrogen, resulting in the lysis of the cells and consequently in the loss of the signal (Dr. Darren Greetham; *personal communication*).

SMA yeast cells incubated with the 11°P PYF2+ and PYF2- worts containing 16.66% IFY and 160 µl dye gave similar Biolog profiles 10 to 45 h from the onset of incubation (Figure 7.3). After 45 h of incubation, however, the redox reduction was lower in the PYF2- worts than was the case with the PYF2+ worts. These results were found to be in agreement with the results obtained from the 15°P experiments using the same yeast strain (SMA) and the same amount of IFY and dye (Figure 7.1). Thus, whilst the PYF2+ worts induced PYF when fermented with the SMA yeast cells, the presence of the same PYF factor(s) during the Biolog analysis did not have a significant effect on yeast respiration and growth (i.e. redox potential or average well colour development).

On the other hand, the differences in dye reduction were found to be more pronounced when the W34/70 and the 'Industrial' lager yeast strains were incubated with the 11°P PYF2+ and PYF2- worts containing 16.66% IFY and 160 µl dye (Figure 7.3). More specifically, there was a distinct difference between PYF2+ and PYF2- worts incubated with W34/70 after 20 h from the onset of incubation. After that period of time PYF2+ worts had an average lower well colour development value than the PYF2- worts, indicating a clear difference in the physiological state of the yeast cells. Nevertheless, W34/70 yeast cells in the 11°P PYF2+ and PYF2- worts were found to be in the same physiological condition, taking into consideration the average well colour development values, following 45 h of incubation and beyond.

The differences in redox reduction in the 11°P PYF2+ and PYF2- worts, containing 16.66% IFY and 160 µl of dye-D, reached also a maximum 20 h

from the onset of incubation when the 'Industrial' lager yeast strain was used (Figure 7.3). However, in this case the PYF2+ worts had a lower average well colour development when compared with the PYF2- worts 0 to 70 h during the incubation period. Thus, it could be suggested that whilst the 'Industrial' lager yeast strain was not susceptible to the 11°P PYF factor(s) in the PYF2+ worts (Chapter 5; Figure 5.4), it was found sensitive to some other attributes of the same worts (e.g. vitamins, trace elements). These attributes, therefore, will affect both yeast respiration and growth; which during the PMs are both expressed by the average well colour development (AWCD) values.

7.3.3.2 The Effects of IFY and Dye on Biolog Reactions

Figure 7.4 shows the Biolog profiles for 11°P PYF2+ and PYF2- worts incubated with the SMA lager yeast strain after the addition of 40% IFY and either 160 µl or 640 µl of the dye at 25°C over a period of 70 h. The results obtained suggested that addition of 160 or 640 µl of dye to the PYF2- worts resulted in similar Biolog profiles (i.e. average redox readings). On the other hand, the addition of 640 µl of dye to the PYF2+ worts resulted in higher degrees of average well colour development when compared with the addition of 160 µl of dye to the same worts. The continuous increase of the average well colour development readings, as opposed to the bell-shaped curves (Figures 7.1 – 7.3), observed in this case is most likely due to the higher concentrations of IFY and dye that were used in these experiments.

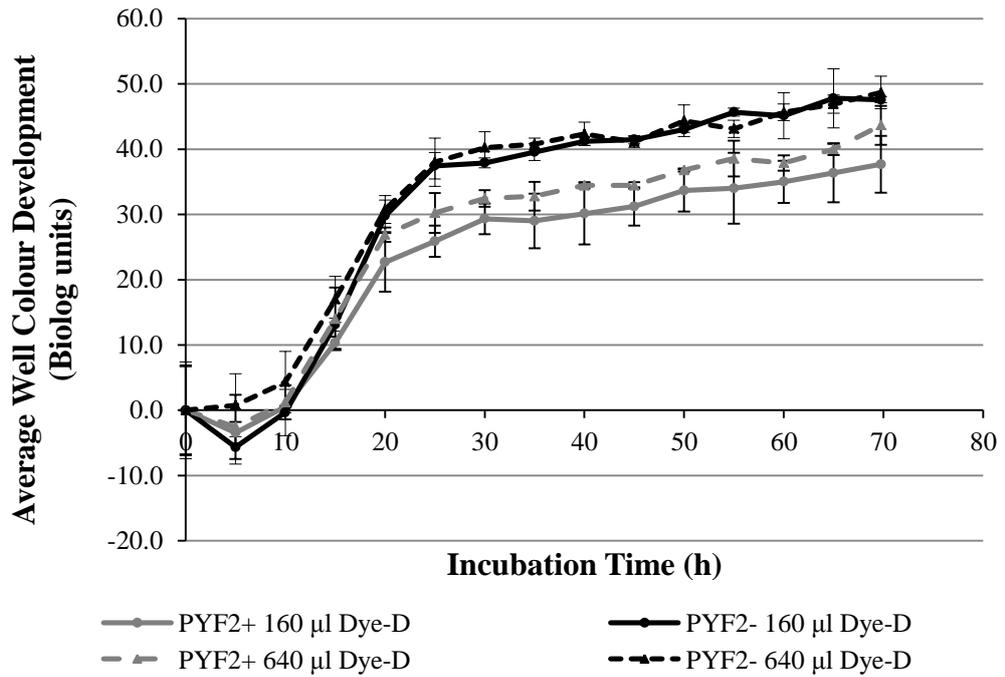


Figure 7.4: Redox reduction for SMA yeast cells incubated in 11°P PYF2+ and PYF2- worts at 15°C after the addition of 40% IFY and 160 or 640 µl Dye-D over a period of 70 h. The redox potential was assessed spectrophotometrically using the average colour response (Biolog units) caused by the reduction of a tetrazolium dye. Data are the mean of three replicates ± SD.

Whereas addition of 16.66% IFY and 160 µl of dye resulted in the maximum redox reduction after 20 h of incubation, the addition of 40% IFY and either 160 or 640 µl dye resulted in maximum recorded redox reduction values 70 h post incubation onset (and this was still increasing). However, there were significant differences in the physiological state of the yeast cells incubated with the 11°P PYF2+ and PYF2- worts, irrespective of the amount of the dye added, after 15 h of incubation. In this case, PYF2+ worts incubated either with 160 or 640 µl of the dye and 40% IFY had a lower average well colour development after 15 h of incubation and beyond than was the case with the PYF2- worts. These conditions enabled the differentiation between PYF2+ and PYF2- worts after just 20 h of incubation. However, taking into consideration that PMs might indicate differences in the physiological state of the cells rather than differences caused by the PYF factor(s), it is not possible, based on this

data, to be sure if the differences in the AWCD values between PYF2+ and PYF2- worts were due to PYF or some other attributes of the PYF2+ worts. However, the results obtained confirmed the significance of IFY in Biolog reactions, and more specifically showed that the more IFY that is used the more stable the signal. However, 40% IFY might be considered excessive, increasing the overall cost and/or resulting in wrong conclusions. For that reason the respiration and growth performance of the highly flocculent but PYF sensitive lager yeast strain (SMA) were tested using 11°P PYF+ (PYF2+ and PYF3+) and PYF- (PYF2- and PYF2+) worts supplemented with 25% IFY and 640 µl dye. However, in these experiments whilst the incubation took place at 25°C, the AWCD was monitored for a total period of 35 h in an attempt to further reduce the required time of analysis (Figure 7.5).

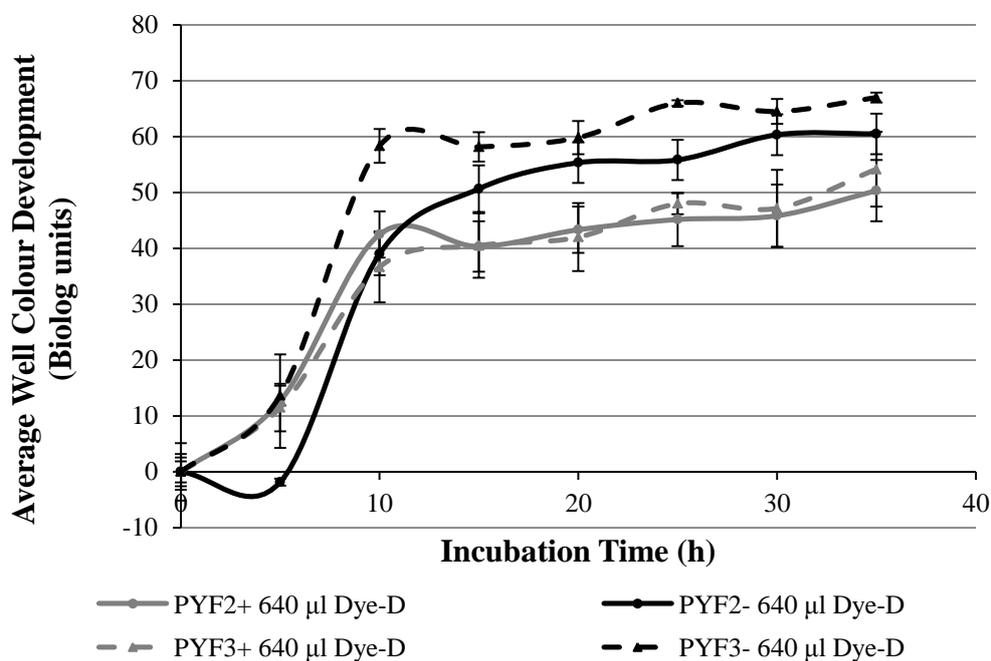


Figure 7.5: Redox reduction for SMA yeast cells incubated in 11°P PYF2 and PYF3 worts at 15°C after the addition of 25% IFY and 640 µl dye-D over a period of 35 h. The redox potential was assessed spectrophotometrically using the average colour response (Biolog units) caused by the reduction of a tetrazolium dye. Data are the mean of three replicates ± SD.

Incubation of the 11°P PYF2+ and PYF2- worts with SMA yeast strain after the addition of 25% IFY resulted in the stabilization of the signal after 20 h. Following that period of time, PYF- worts (PYF2-, PYF3-) had a higher average well colour development value than was the case with the PYF+ worts (PYF2+, PYF3+) incubated with the same yeast strain. These results suggested that SMA yeast cells were in different physiological state in the PYF+ and PYF- worts after 20 h of incubation.

7.4 Conclusions

The Biolog detection system can be used for the metabolic characterization of lager yeast strains of differing degrees of flocculence incubated in different fermentation media. The results obtained suggested that the amount of IFY has a significant and direct effect on Biolog measurements reactions. More specifically, it was shown that by increasing the amount of IFY the signal remained stable even after 20 h of incubation (i.e. the point where the maximum average redox values were observed). Besides that, it was concluded, interestingly, that whilst a lager yeast strain might be insensitive to PYF factor(s) both in small- and industrial- scale fermentations, the same yeast strain may also be sensitive to some other attributes of the same wort during the Biolog analysis. Thus, it was concluded, again, that wort composition has a significant effect not only in yeast fermentation performance but also on metabolic activity as monitored by redox dye reduction.

Chapter 8

Conclusions and Future Work

8.1 Conclusions

The objective of this thesis was the study of the PYF phenomenon in the brewing and malting industry. The aim was to investigate the origins, detection and impacts of the PYF factor(s) upon fermentation. To achieve this several steps were undertaken. These steps included the development (Chapter 3) and optimization (Chapter 4) of a small-scale fermentation assay to predict the PYF potential of malts, the study of how yeast strains of varying degrees of flocculence are impacted by PYF (Chapter 5), the investigation of the impacts of PYF factor(s) on fermentation performance and metabolite profile (Chapter 6) as well as the study of sensitivity of different yeast strains against PYF factor(s) (i.e. PYF+ worts; Chapter 7).

Using the in-house small-scale fermentation assay (Chapter 3) and the medium (W34/70) and highly flocculent (SMA) lager yeast strains the PYF potential of the malts was successfully predicted 69 and 40 h post-pitching respectively (Panteloglou *et al.*, 2010). SMA yeast was found to be more susceptible to PYF factor(s) than W34/70 yeast (Panteloglou *et al.*, 2011), supporting the previous findings of Armstrong and Bendiak (2007) who indicated that the more flocculent lager yeast strains were more susceptible to PYF, whilst a range of PYF+ malts sourced from the industry exhibited different degrees of PYF severity when fermented with the same brewing lager yeast strain. This result was found to be in agreement with earlier studies suggesting that there are varying types of PYF factor(s) and consequently different degrees of PYF (Van Nierop *et al.*, 2004). The fact that the results obtained from our in-house PYF assay were in agreement with the results obtained from the majority (80%) of the research labs who participated in a ring-trial in a collaborative

study between research labs worldwide convened by Campden-BRi indicated that our in-house small-scale fermentation assay (Panteloglou *et al.*, 2010) can be successfully used for the prediction of the PYF potential of different malt samples. The results obtained were consistent with the PYF problems that had been presented by the malts when brewed on an industrial scale. Besides that, in Chapter 3 it was also concluded that worts containing lower amount of glucose and maltose could be responsible for poor fermentation profiles, heavy and or PYF as well as elevated residual sugars and lower fermentability at the end of the primary fermentation. These findings supported the view of Axcell (2003) who highlighted the importance of wort composition both on yeast flocculation and fermentation performance.

In order to achieve a reduction in the time required for detection, as well as to enhance the current knowledge of the mechanisms involved in the PYF process, our in-house fermentation assay was optimized (Chapter 4). The results obtained suggested that supplementation of the worts with 6 mg.l^{-1} linoleic acid ($\text{C}_{18}\text{H}_{32}\text{O}_2$; 18:2) before pitching as well as the use of the highly flocculent PYF sensitive lager yeast strain SMA enabled the differentiation between PYF+ and PYF- malts just after 40 h post-pitching. This result was found to be in agreement with the findings of Jibiki *et al.* (2006) who, by using a different fermentation PYF test (i.e. 50 ml test tube), lower pitching rate (i.e. 15×10^6 live cells.ml⁻¹ instead of 20 million cells) but the same yeast strain (SMA), also showed maximum differences in the number of suspended yeast cell counts between PYF+ and PYF- fermentations at the same time point through fermentation (i.e. 40 h post-pitching). The results obtained in Chapter 4 also indicated that among the five experimental factors used to optimize the

PYF test (i.e. CaCl_2 , Zn^{2+} , 18:2, glucose and “turbid” worts), chosen on the basis that they would affect flocculation, only addition of 18:2 had a significant effect. This effect was possibly because solid particles (i.e. 18:2) act as nucleation sites for CO_2 bubble formation allowing the increase of suspended cells, due to lower CO_2 accumulation in the fermenting broth, and therefore promoting a more vigorous fermentation (Boswell *et al.*, 2002; Stewart & Martin, 2004; Kuhbech *et al.*, 2007; Gibson, 2011). However, since the production of “turbid worts” had no impact on the ability of the test to distinguish between PYF+ and PYF- worts, if the nucleation hypothesis is correct then it is something very limited to the lipid content of the nucleation sites.

Using the in-house small-scale fermentation tests the importance of varying degrees of flocculence of lager and ale yeast strains on the incidence and severity of the PYF phenomenon was also investigated (Chapter 5). The results obtained suggested that the yeast strain has an important role on the PYF phenomenon. Thus, whilst none of the ale yeasts (i.e. NCYC 1332, M2) used in this study were found to be susceptible to the different PYF factor(s), lager yeasts (i.e. W34/70, SMA and ‘Industrial’) exhibited different degrees of susceptibility even to the same PYF factor(s). More specifically, it was found that the more flocculent yeast SMA exhibited a higher degree of susceptibility than the less-flocculent yeast W34/70. This result was found to be in agreement with previous studies indicating that ale yeasts, either flocculent or non-flocculent, were not susceptible to PYF (Jibiki *et al.*, 2006). It was also shown, interestingly, that the fermentation performance of a PYF+ wort could be significantly improved, with respect to the number of suspended yeast cell

counts, residual gravity and alcohol yield, by using a non-flocculent lager yeast strain which is relatively insensitive to PYF (Panteloglou *et al.*, 2011). However, the improvement in the fermentation profiles varied amongst the different PYF+ samples. These results help to explain why malt supplied from the same producer (i.e. barley from the same variety, harvest year and region of production) and malted under the same conditions can give rise either to 'normal' or PYF worts. Thus, besides the PYF potential of the barley/malt samples, the yeast strain was found to have an important role on the incidence and severity of the PYF phenomenon.

The impacts of PYF factor(s) on yeast fermentation performance and metabolite profile were investigated using mini fermentations (120 ml) in Chapter 6. The experiments, conducted under stirred and unstirred conditions using high gravity (15°P) PYF+ and PYF- worts originating from the same barley variety, harvest year and region of production, were performed in order to see if any effects consistent with the antimicrobial peptide hypothesis (Axcell *et al.*, 2000) could be found. The results obtained suggested that 15°P fermentations with worts inducing PYF did not have a significant effect on yeast physiological characteristics (i.e. cell density, viability, budding index), metabolite uptake (i.e. sugars, FAN) or fermentation performance (i.e. CO₂, alcohol). Thus, it was suggested that the PYF+ sample used in these experiments was inducing PYF through the presence of 'bridging' polysaccharide mechanism rather than through the presence of antimicrobial peptides. Besides that, it was shown that by keeping yeast in suspension, by mechanical agitation, fermentation progression was quicker and cell density, viability, alcohol yield and CO₂ evolution were higher. Similar trends were

also observed in 500 ml brewing fermentations conducted with *Saccharomyces cerevisiae* NCYC 1324 under continuous stirring (Boswell *et al.*, 2002).

In Chapter 7 the Biolog Phenotype MicroArray system was used for the metabolic characterization of varying degrees of flocculence yeast strains incubated in different fermentation media (i.e. PYF+ and PYF- worts). The results obtained suggested that the amount of IFY, used to stabilize the signal during the analysis, has a significant as well as a direct effect on Biolog reactions. More specifically, it was shown that by increasing the amount of IFY the signal remained stable even after 20 h of incubation (i.e. the point where the maximum average redox values were observed). However, increasing the amount of IFY (i.e. > 40%) besides increasing the overall cost of the analysis resulted also in “wrong estimates”. Besides that, it was also concluded that whilst a lager yeast strain might be insensitive to PYF factor(s) both in small- and industrial- scale fermentations, the same yeast strain may also be sensitive to some other attributes of the same wort (e.g. vitamins, trace elements) during the Biolog analysis. Thus, it was concluded that wort composition has a significant effect not only on yeast flocculation and fermentation performance (Axcell, 2003) but also on the redox dye reduction used to monitor metabolic activity in the Biolog system.

8.2 Future Work

Despite systematic investigations in recent decades, progress towards the effective detection and control of PYF has been hampered by the lack of a universal diagnostic method. Thus, the establishment of a universal and reliable test, using a common lager yeast strain (e.g. SMA), and the sharing of

information and samples between industry and the various research labs are key goals in furthering our understanding of the mechanisms underlying PYF. Furthermore, developments in knowledge of the genetic and epigenetic regulation of flocculation (e.g. by using microarrays so as to detect potential differences in the expression of the *FLO* genes during PYF+ and PYF- fermentations) in commercially relevant lager brewing strains should help to explain some apparent inconsistencies observed in the incidence of this phenomenon.

In addition, the investigation of the impacts of PYF factor(s) on fermentation and metabolite profiles (see Chapter 6 for details) using the same lager yeast strain and a series of PYF+ and PYF- samples, belonging to the same barley variety, harvest year and region of production and known to have cause PYF both in industrial and small-scale fermentations, could further help towards the elucidation of the antimicrobial peptide hypothesis.

Since supplementation of 6 mg.l⁻¹ of linoleic acid and the use of the flocculent lager yeast SMA had a statistically significant impact of yeast flocculation, and therefore on the ability of our in-house PYF assay to distinguish between PYF+ and PYF- worts, the use of an unsaturated fatty acid (e.g. 18:0) would also be an interesting and promising experiment.

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