RHEOLOGICAL AND COLLOIDAL PROPERTIES OF COMMERCIAL BREWING YEAST SUSPENSIONS

by

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ABSTRACT

A three part study was carried out to examine rheological, colloidal and floc microstructural aspects of industrial brewing yeast strains. Following a review of the literature, the rheological properties of four yeast strains (two flocculent ale and lager types and their non-flocculent variants) were examined. In related colloidal studies, orthokinetic flocculation rates of these strains as well as their cell surface charge were determined. Floc microstructure was characterized using both light and scanning electron microscopy. In a summary chapter, the cell floc model (a modification of Hunter's elastic floc model) was used to the explain the rheological and colloidal behaviour of brewing yeast suspensions.

Flow behaviour studies of the commercial yeast suspensions suspended in a calcium-containing sodium acetate buffer revealed that yeast flocculent characteristics had an important influence on their suspension flow behaviour. As cell concentrations increased, suspension flow properties become increasingly non-Newtonian and could be described by the Casson model at low rates of shear and the Bingham model at shear rates above 100 s^{-1}. The cell floc model was proposed to explain the Bingham flow behaviour of these suspensions. The Bingham yield stress in these suspensions was believed to be a function of the orthokinetic capture coefficient, cell volume and the energy to break up doublet cells. Increasing temperature tended to lower the Bingham yield stress in lager strains and increase the yield stress in ale strains. A semi-empirical explanation for the viscosity increase of deflocculated cell suspensions and the estimation of pseudo-capture coefficients was presented.

Furthermore, studies of the flow behaviour of yeast strains suspended in decarbonated ale and lager beer revealed that: 1) suspensions of flocculent strains show

higher yield stress values than their non-flocculent variants, 2) ale strain suspensions tended to have higher yield values than the lager strains and 3) yeast dispersed in beer had higher yield stress values than when suspended in buffered calcium suspensions. This last observation was believed to reflect the influence of ethanol on the cell binding process which has important implications for future measurements of yeast flocculation.

Colloidal studies revealed for the first time, that the orthokinetic rate of flocculation of brewing yeast cells could be modelled by a first order equation, as predicted by fundamental colloid theory. While subject to considerable variation, measured rate constants led to the calculation of orthokinetic capture coefficients. Yeast cell zeta potential values generally agreed with literature data but could not be employed in the DLVO model of colloid flocculation to explain measured orthokinetic capture coefficient values. Examination of the cell zeta potential data indicated that the data had non-normal distributions.

SEM examination of the four industrial yeast strains suggested that a number of distinct structures mediated cell-to-cell interaction and that intra-strain differences occurred. These findings, along with the observation of non-normal surface charge distributions, indicated that these industrially pure strains had undergone substantial variation. Treatment of the flocculent cells with pronase tended to reduce cell-to-cell contacts.

In the summary chapter the cell floc model was employed to describe the rheological behaviour of the yeast suspensions. Estimation of the force needed to separate doublet yeast cells were made using critical shear rate data (i.e., the point at which Bingham flow begins). This estimate was similar to that reported for single antibody bonds and may be due to the presence of lectin-like structures on the yeast cell wall.

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CHAPTER I

A REVIEW OF THE MICROBIOLOGICAL AND COLLOIDAL ASPECTS OF YEAST FLOCCULATION

A. Introduction

Yeast flocculation has been defined by Stewart and Russell (1981) as, "...the phenomenon wherein yeast cells adhere in clumps and either sediment rapidly from the medium in which they are suspended or rise to the medium's surface". The process is reversible and should not be confused with chain formation that occurs in some yeasts. The flocculation characteristics of brewing yeasts are of prime concern when brewers select strains for beer production. Strongly flocculating yeasts can prematurely sediment out of beer fermentations, thereby causing flavour and microbiological problems. Conversely, weakly flocculating yeast strains are not suitable in conventional beer production as they remain in the beer during aging and also cause flavour and filtration difficulties.

Understanding and measuring yeast flocculation has been a goal of brewing scientists since the last century. A substantial research effort in the last 20 years has resulted in publication of well over 100 articles on the flocculation of brewing yeast. Yet in spite of this effort the literature contains apparent contradictions due in part to strain variability and the various empirical procedures employed in the measurement of flocculation (Calleja, 1984). There is an unfortunate tendency for each researcher to devise his or her own empirical assay, and no quantitative method has been recommended

by either the American Society of Brewing Chemists (ASBC, 1986) or the Institute of Brewing (IOB, 1986). In discussing this subject, Stewart and Russell (1981) noted the urgent need for standardization of these flocculation tests.

In spite of these difficulties, substantial gains have been made in understanding the biochemical and genetic basis of yeast flocculation. Aside from progress by brewing scientists, there have also been advances in colloid theory which can be usefully related to the study of this complex process. In this chapter our present knowledge of brewing yeast flocculation, as well as pertinent areas of colloid science, will be reviewed.

B. Brewing Yeast Flocculation

During fermentation, wort components are metabolized by brewing yeasts to produce beer that is normally referred to as either ale or lager. Ale is normally made with *Saccharomyces cerevisiae* that rises to the top of the fermenter at the cessation of fermentation while lager is made with *S. uvarium (carlsbergensis)* which settles to the bottom of the tank towards the end of the fermentation. Barnett, Payne and Yarrow (1983) stated that both types of yeast should be characterized as variants of *S. cerevisiae*. However, the strains differ in their DNA profiles, ability to ferment melibiose, (ale strains lack melibiase activity) and their maximum growth temperature (lager strains do not grow above 34 ^{o}C [Webb, 1977]) and for these reasons, Stewart (1990) has argued that the two types of yeast should be classified as separate species. Due to this on-going debate, the terms "ale" and "lager" will be employed in this thesis where possible in order to clearly distinguish these two types of brewing yeasts.

At the start of a fermentation, yeast is added or "pitched" into the wort at a cell concentration of about 10^7 cells per mL and the suspended cell concentration increases

about three to four-fold during the fermentation. At the end of this process the cells form flocs and separate from the medium. It is presumed that ale flocs rise to the top of the fermenter (rather than settle out of the beer) due to their affinity for bubbles of CO_2 which rise to the surface after being secreted by the cell during fermentation. Since the rate of yeast separation from a beer is proportional to the square of the floc's effective diameter, the time of onset and degree of flocculation is an important characteristic in commercial strains.

1. Mechanism of flocculation

In view of the extensive research effort to elucidate the mechanism of yeast flocculation it is not surprising that there have been numerous reviews of the subject (Burns, 1937; Bunker, 1961; Rainbow, 1966; Windisch, 1968; Geilenkotten and Nyns, 1971; Stewart, 1975; Stewart and Russell, 1981; Kirsop, 1982; Calleja, 1984; Rose, 1984; Hunt, 1985; Esser, Hinrichs and Kues, 1987; and finally, Stewart and Russell, 1987). The purpose of the following discussion on the mechanism of flocculation, like that of the review by Stewart and Russell (1981), is not to exhaustively cite all of the literature but to provide a contemporary view of our understanding of the process. Particular emphasis will be paid to the literature of the last 10 years.

It is worth noting that our present understanding of flocculation is far from complete and is obscured by the genetic complexity of the yeasts involved. Research has been complicated by the polyploid or aneuploid nature of most strains and the vast number of strains in use. For example, over 1000 ale strains are known (Stewart, Russell and Garrison, 1975a). It is also generally agreed that there are at least 3 dominant flocculation genes present in brewing yeasts (Stewart and Russell, 1987).

In early research, Helm, Nohr and Thorne (1953) demonstrated that flocculation developed towards the end of the primary fermentation, as the yeast cells reached the late stationary phase of growth. Just recently, it has been reported that new daughter cells have a lower degree of flocculence than their mother cell and that they slowly regained their flocculent character as they passed through subsequent cell divisions (Bielecki and Brzeski, 1989). This increase in flocculence towards the end of a fermentation cycle therefore, may be due in part to an increase in the ratio of mother to daughter cells.

In 1963, Masschelein et al. reported that this increase in flocculence was associated with a reduction in the mannan / protein ratio of the cell wall while Patel and Ingledew (1975b) positively correlated this increase with increased levels of intracellular, acid-soluble glycogen. The expression of flocculation was likewise related to the types and amount of carbohydrates present in the fermentation media (Amri et al., 1979a). Nishihara, Toraya and Fukui (1976a, b) noted that magnesium, energy generation and cytoplasmic protein synthesis were all necessary to induce floc formation. Finally, while examining the growth response of yeast to various fatty acids, Lands and Graff (1981) reported an inverse relationship of flocculation to unsaturated fatty acid concentration.

One should note that while the preceding observations were related to the nutritional status of the yeast, it was also observed that sugars, particularly mannose, caused a rapid dispersion of cells (Eddy, 1955). This deflocculation was presumed to be due to the direct action of the sugar on the cell wall.

Certain wort peptides have also been implicated in the flocculation of brewing yeasts. Kijima (1954) noted that proteins added to lager yeasts in the stationary phase enhanced flocculation. Stewart and co-workers in the early and mid 70's (Stewart and Garrison, 1972; Stewart et al. 1973; 1975a) first reported the process of co-flocculation wherein two ale strains, non-flocculent when separate, flocculated when mixed together.

This mechanism was found to require the presence of an acidic inducer peptide which had a molecular weight of less than 10 kDa. Apparently, this wort peptide was not metabolized by the cell but was bound directly to the cell wall (Stewart et al., 1973).

In a related finding, an "acid polysaccharide containing protein" has been shown by Fujino and Yoshida (1976) to induce an early or premature flocculation of brewing yeast. They also demonstrated that the addition of α-methyl-d-mannoside prevented this premature flocculation. These researchers hypothesized that the premature inducer caused a lectin-like coagulation. Recently, Herrera and Axcell (1989) reported on the isolation of a malt-husk factor possessing lectin-like specificities which was capable of surviving the malting and brewing process. This lectin-like factor was shown to be responsible for premature lager yeast flocculation and was associated with malts that caused this defect during beer fermentation. Apparently these components also attached themselves in some manner to the cell wall, rather than being metabolized by the cell.

Protein synthesis has also been implicated in the development of yeast flocculation characteristics, as cyclohexamide was shown to inhibit flocculation (Baker and Kirsop, 1972; Lands and Graff, 1981). According to the former researchers, the affected proteins were involved in mannan formation, although cyclohexamide may also have acted in a more direct fashion by preventing the formation of cell wall proteins directly involved in flocculation.

Comparisons of cell wall components from flocculent and non-flocculent cells have shown the existence of a protein unique to flocculating cells (Stewart et al., 1976; Holmberg, 1978). The former research group identified a protein of 37 kDa while the latter researcher isolated a protein of 13 kDa. In a recent study of flocculent and non-flocculent cells of *Kluyveromyces marxiamus,* Teixeria et al. (1989) reported on a 37 kDa protein that was only associated with flocculent cell walls.

As differences in the flocculation character of brewing yeasts almost certainly arise from differences in the cell walls of yeasts, various workers have investigated cell wall composition. These studies have permitted only a limited understanding of the conditions necessary for flocculation.

According to Rose (1984), 80% of the cell wall (dry weight basis) consisted of equal amounts of β−glucan and phosphomannan which formed an inner and outer layer, respectively. The balance was made up of protein, chitin, in the outer layer and possibly, lipid in the inner layer. Reports of differences in cell wall composition between flocculent and non-flocculent strains have been contradictory, although it is now generally recognized that flocculent cell walls possess a higher level of mannan than non-flocculent walls (Stewart and Russell, 1981; and Amri et al., 1982). Higher levels of phosphorus in flocculent strains have also been reported by various researchers (Mill, 1966; Lyons and Hough, 1970; 1971). More recently, Amory, Rouxhet and Dufour (1988) employed a sensitive x-ray photoelectron spectroscopy (XPS) technique to analyse the yeast cell wall and found that surface phosphorus concentration declined when flocculation properties increased. Other workers, using different methods, reported no difference in the phosphorus content of flocculent and non-flocculent cells (Griffin and MacWilliam, 1969; Jayatissa and Rose, 1976; Stewart and Russell, 1981).

Surprisingly, the electron microscope has been used by only a limited number of investigators to examine the cell wall's surface. In a study of isolated cell walls using a scanning electron microscope (SEM), Lyons and Hough (1970; 1971) reported no differences in the structure of flocculent and non-flocculent cells. This finding was confirmed by use of transmission electron microscopy (Miki et al., 1982a). In a recent SEM study (Simpson and Hammond, 1989), no differences were found between flocculent and non-flocculent yeasts either before or after acid washing. Johnson et al. (1989) also

failed to distinguish between an isogenic strain S646-1BFLOl (genetically flocculent) and S646-8Dflol (non-flocculent) using SEM. In contrast to these results, Day, Poon and Stewart, (1975) found striking differences between flocculent and non-flocculent strains using a shadow casting technique. They reported that flocculent cells were covered with an extensive layer of hairlike protuberances they termed fimbriae. These fimbriae could be sheared off by a laboratory blender whereupon the cells lost their ability to flocculate. The fimbriae were best observed after an ether wash, which may explain why these structures have not been observed by other researchers.

It has been repeatedly reported that extracellular calcium ions (Ca^{2+}) are essential for flocculation (Mill, 1964a, b, 1966; Taylor and Orton, 1973, 1975; Stewart and Russell, 1981; Calleja, 1984; Rose, 1984) and that flocculation can be prevented or reduced by the addition of chelating agents such as ethylene-diamine-tetra-acetic acid (EDTA) or cations of varying charge (K^{+}, Na^{+}, Ba^{2+}, Sr^{2+}, etc.). Kihn et al. (1988b) reported that the inhibition of calcium mediated flocculation by other cations was dependent on their ionic radius and charge. The optimal radius for flocculation was shown to be close to that of calcium (115 pm). It appears that micro-amounts of Ca^{2+} ranging from 10^{-8} to 7.5 x 10^{-6} M (Taylor and Orton, 1973; Williams and Wiseman, 1973, respectively) are sufficient to cause flocculation in susceptible strains. It is also noteworthy that flocculent cell walls have been shown to bind calcium more strongly than non-flocculent cell walls (Stewart et al., 1975a).

Contradictory findings that cations such as magnesium or manganese (Stewart and Goring, 1976) or magnesium, barium or strontium (Kirsop, 1982) can cause flocculation have been suggested to be due either to glassware contamination (Taylor and Orton, 1973) or intracellular leakage of Ca^{2+} to the cell wall (Stratford 1989a). In this later study, Stratford observed that Rb^{+}, Cs^{+}, Mn^{+2}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Al^{3+},

Cr^{3+} and Fe^{3+} cations at a level of 5.0mM at a pH near 7.0 caused flocculation in susceptible strains. It was also observed that these same ions caused a rapid efflux of Ca^{2+} from the cell (Stratford, 1989a). Thus, the positive effect of other cations was hypothesized to be due to their indirect action on calcium levels rather than to any direct effect.

A nonspecific type of flocculation caused by heavy metal ions has also been reported. Flocculation of both flocculent and non-flocculent strains of *S. cerevisiae* by tin was demonstrated by Nishihara et al. (1982) while nickel has been employed to flocculate strains at pH values above 7.0 (Weeks, Munro and Spedding, 1983).

Based on the importance of calcium in the flocculation process, Mill (1964a, b; 1966) first suggested that calcium-bridges linked flocculent cells, presumably by joining carboxyl or phosphate groups at the surface of two cells. These bridges were believed to be then stabilized by hydrogen bonds. This mechanism has been endorsed by many researchers (Lyons and Hough, 1970; 1971; Stewart et al., 1975b; Jayatissa and Rose, 1976; Beavan et al., 1979) but it fails to explain the specific inhibition of flocculation by mannose and its derivatives.

The overall charge of the yeast cell wall has also been examined in order to understand the mechanism of yeast flocculence. Yeast cell walls are known to possess a net negative charge which is affected by the pH and ionic strength of the surrounding medium. The isoelectric point of yeast cell walls is normally below pH 3.5 (Beavan et al., 1979) while the pH of maximum flocculation is in the range of 3.5-4.5 (Helm et al., 1953). However, during fermentation a decline in electrophoretic mobility has been related to an increase in flocculence (Eddy and Rudin, 1958b; and Beavan et al., 1979). Lawrence et al. (1989) confirmed this decline in both ale and lager strains by measurement of the zeta potential exhibited by yeast cells.

The source of the negative surface charge involved in the flocculation process is controversial. Based on measurements of the cell's mobility (Beavan et al., 1979) and zeta potential (Bowen and Cooke, 1989), it was concluded that phosphodiester groups account for the majority of the surface charge while carboxyl groups account for the remaining balance. However, carboxyl groups have been correlated with the acquisition of flocculating ability (Beavan et al., 1979). As well, excision of phosphodiester groups with hydrofluoric acid was shown to increase flocculation while esterification of carboxyl groups has been shown to prevent the process (Jayatissa and Rose, 1976). Finally, researchers have shown that uptake of the dye alcian blue (which binds to phosphodiester linkages) was not correlated with flocculation ability (Stewart et al., 1976).

In considering the evidence, Stewart and Russell (1981) and Rose (1984) concluded that carboxyl groups were probably active participants in brewing yeast flocculation. Recently, however, other researchers have argued that the carboxyl contribution to surface charge is minimal at brewing pH values (Amory and Rouxhet, 1988; Bowen and Cooke, 1989; Mozes et al., 1989; Mestdagh, Rouxhet and Dufour, 1990).

Regardless of the preceding carboxyl / phosphate debate, the hydrogen bond has been implicated in floc stability (Rose, 1984). A report by Mill (1964a) that deflocculation could be caused by urea, high temperature, or high dielectric constants all indicate the participation of hydrogen bonds. Mill's research has since been confirmed by; (1) Taylor and Orton (1973, 1975, 1978) who disassociated flocs with high temperatures, (2) Nishihara et al. (1977, 1982) and Kamada and Murata (1984) who reduced flocculation with the application of urea and (3) by Amory and Rouxhet (1988) who found enhanced flocculation by the addition of ethanol which reduced the medium's dielectric constant.

Recently, hydrophobic interactions have also been reported to be involved in stabilization of yeast flocs (Kamada and Murata, 1984; Hinchcliff et al., 1985; Amory et

al., 1988; Mozes et al., 1989; and Mestdagh et al., 1990). The last four reports confirmed that ale strains possess a more hydrophobic surface than lager cells and positively correlated hydrophobicity with cell surface protein. The latter three reports, from the University of Louvain, noted that wall hydrophobicity was related to the degree of flocculence.

The apparent contradiction in the literature, where Mill (1964a) and Taylor and Orton (1973, 1975, 1978) noted deflocculation with increasing temperature (evidence for hydrogen bonding) while Kamada and Murata (1984) noted an increase in flocculation with increasing temperature (attributed to hydrophobic effects but also indicative of a lack of H-bonding) underscores the need for caution in interpreting the findings reported in the literature. At the present time, no definite statements can be made regarding the relative importance of hydrogen bonding or hydrophobic interactions in flocculation.

Aside from studies involving the measurement of anionic groups on the cell surface, chemical modifications of cell wall structures have been shown to reduce flocculation ability of yeast cells. Proteinases have repeatedly and irreversibly reduced or prevented flocculation (Eddy and Rudin, 1958a; Lyons and Hough, 1971; Russell, Garrison and Stewart, 1973; Nishihara et al., 1977; Miki et al., 1982a). The reason for this loss of flocculence has been attributed to protein hydrolysis by Nishihara et al. (1977) but it has been demonstrated that proteinases can also remove the outer mannan-phosphate-protein layer from the cell wall (Russell et al., 1973). In an extensive study, Nishihara et al. (1977) reported that reagents known to act on disulphide bonds, carboxyl and phosphate groups, phenolic hydroxyl groups of tyrosine, amino groups and imadazole groups of histidine all prevented floc forming ability. The importance of disulphide bonds in flocculation was later confirmed by Miki et al. (1982a). Since some of these groups were modified in the presence of 8 M urea, it was suggested that they are important in the

maintenance of a higher order structure of proteins involved in flocculation (Nishihara et al., 1977). Alternately, these results may be due to an alteration in the overall surface charge of the yeast cell surface that may occur as a result of these rather harsh chemical treatments.

In an attempt to devise a flocculation model which accounts for the involvement of calcium, protein structures and hydrogen bonding, as well as the inhibitory effect of mannose, Taylor and Orton (1973, 1978) first suggested that adjacent cell binding mechanisms may involve a substance similar to that of concanavalin-A. This protein is known to bind to cell wall mannan after being activated by ionic calcium which changes the conformation of the molecule to its active configuration.

Elucidation of this lectin-like adhesion (LLA) model was presented by Miki et al. (1981, 1982a, b) who proposed that lectin-like proteins bind to α–mannan in bilateral or unilateral manner as shown in Figure I.1. These researchers presented convincing evidence for this model; however, they did not isolate the lectin nor determine its location.

Further support for LLA model was provided by Kihn et al. (1988b) and Hussain et al. (1986) who demonstrated that flocculation could be prevented or reduced by the addition of various mannose derivatives. The former researchers also presented evidence that the receptor has a hydrophobic region near its binding site. Additional confirmation of the LLA model came from Nishihara and Toraya (1987) who noted that flocculation can occur between a flocculent ale strain and several non-flocculent ale and lager strains as well as *Candida tropicalis* (as outlined in Fig. I.1). These researchers implicated mannans and proteins in this process which can be explained by the LLA model.

Finally, the discovery of galactose-specific lectins in *S. cerevisiae* (Basu et al., 1986; Kundu, Basu and Chakrabarti, 1988) and *Kluyveromyces bulgaricus* (Al-Mahmood et

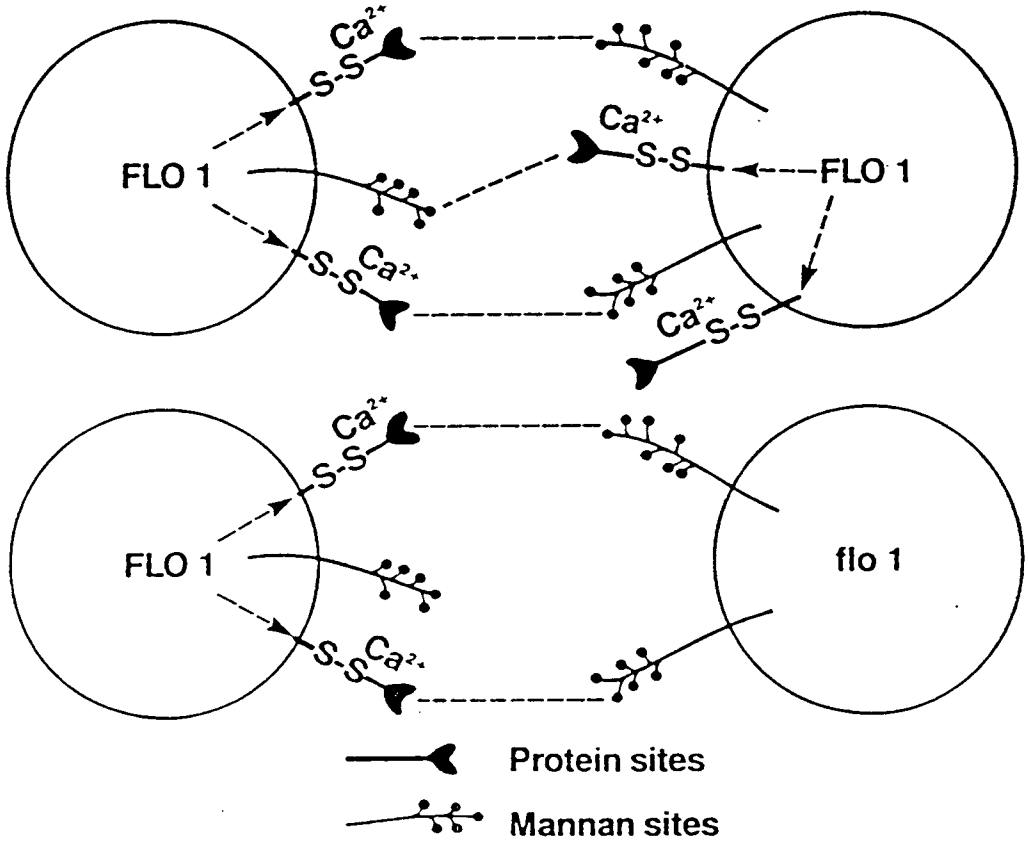


Figure I.1. A model for *Saccharomyces cerevisiae* flocculation (adapted from Miki et al.,1982a by Stewart, 1989). Uppercase letters denote wild genes.

al., 1988) provides support, albeit mild, for the presence of mannose-specific lectins in flocculent cells.

Since microbial lectins have often been associated with various surface appendages (Sharon and Lis 1989) it is tempting to suggest that the flocculation promoting fimbriae reported by Day et al. (1975) contain the mannose-specific lectin postulated by Miki and others. However, the definitive isolation of flocculation promoting lectins, as well as determination of their location remains to be discovered. In summary, three mechanisms have been invoked to explain the flocculation of susceptible strains of brewing yeast. These are; the LLA model, the cross bridging of anionic groups on adjacent cells (calcium salt-bridging) and surface charge neutralization. The last process however, (described by classic colloid theory) does not account for the specific requirement for calcium in flocculation. The calcium-bridge model, while accounting for this need, fails to account for the inhibitory effect of mannose and its derivatives. Thus, at present, most researchers believe the LLA model best explains brewing yeast flocculation. Future research is required to explain the role of electrostatic and hydrophobic interactions, London dispersion forces and hydrogen bonding in this type of protein-sugar interaction during yeast cell flocculation.

It may well be that more than one of these models is operative at once, in a specific strain. In fact recently, Kihn, Masy and Mestdagh, (1988a) have argued that competition between electrostatic repulsion and LLA governs the extent of cell flocculation.

2. Measurement of flocculation

The assay of brewing yeast flocculation has been, almost without exception, studied by either a direct or indirect measurement of yeast floc settling rates. Since these rates are dependent on factors such as the floc volume concentration and the floc density,

almost all published methods are empirical. As mentioned previously, each paper which examines the phenomenon of yeast flocculation almost always revises an old method or devises a new one. To quote Calleja (1984), "many a worker makes matters worse by adding to the clutter his own version of one of the many methods already available". In this review, flocculation assays were somewhat arbitrarily divided into two sections; those methods published prior to 1981 and techniques reported in the last 10 years.

i. Methods employed prior to 1981 - While some research in the area was carried out before 1937, in that year Burns published what is often regarded as the first modern comprehensive study and review of yeast flocculation. He proposed a sedimentation test where the amount of settling of 5 g of pressed yeast suspended in 100 mL of acetate buffer was measured over 10 min. Helm et al. (1953) refined Burns' method and devised a procedure in which the yeast was washed twice in distilled water containing 0.510 g / L of $CaSO_4$. One gram of this centrifuged wet yeast was suspended in 10 mL of pH 4.5 acetate buffer containing 6.80 g / L Na-acetate, 4.05 g / L acetic acid and 150 mg / L calcium as $CaSO_4$. The suspension was then held at 20 ^{o}C and the level of sediment recorded after 10 min. Acetate buffer was apparently selected for this test as it gave a similar pH optimum to acidified water. Kato and Nishikawa (1957) further modified the above procedure of Helm et al. (1983) by diluting their acetate buffer (hereafter referred to as Helm's acetate buffer) and determining the density of the sediment by spectrophotometric means. Specifically, 9 mL of a 2.5% pressed yeast suspension was added to 1 mL of Helm's acetate buffer, then mixed and allowed to stand at 20^{o}C for a given time. The turbidance of the bottom 2 mL of sediment was determined at 800 nm, after suspension in 18 mL of 0.0025N HCl. This procedure was then repeated with a water blank (in place of the Helm's acetate buffer). A "sedimentation percent" (SP) was

then calculated as follows:

$$SP=[\ 100\ (b - a)\ /\ [4\ a\ t] \qquad (I.1)$$

where a is the reading of the blank, b is the turbidance of the suspended sediments and t is the elapsed time. This procedure was used by a Japanese research group in a series of studies while recording the turbidance at 420 nm rather than 800 nm (Nishihara, et al., 1976a, b, 1977, 1982).

In 1957, Hough advocated yet a different series of tests for classifying yeasts based on their flocculating ability. He recommended using a visual examination of yeast suspended in either a pH 3.5 or 5.0 buffer containing 0.05% calcium from calcium chloride. Yeasts which failed to flocculate in the pH 3.5 buffer were tested further by suspension in a pH 3.5 buffer containing 2% ethanol.

Mill (1964a, b) measured flocculence by suspending washed cells (4 mg dry wt/mL) in a 0.05 M Na-acetate buffer (pH 4.6), agitating the suspension vigorously and measuring the turbidance in a colorimeter. The sedimentation rate (SR) was determined by measurement of the maximum rate of turbidance decline. Mill recommended using the logarithm of SR as a measure of flocculence.

In studies of strongly flocculent yeasts used in continuous tower fermenters, Greenshields et al. (1972) reported a modification of Helm's method where the amount of sediment over 15 min was measured at 1.0 min intervals, and the rate of increase of sedimentation was used to determine what they termed a modified Burns number:

$$M_B = [R_{0\text{-}1} + R_{1\text{-}5} + R_{5\text{-}10} + R_{10\text{-}15}]\ /\ 10 \qquad (I.2)$$

where M_B is the modified Burn's number and $R_{0\text{-}1}$, 1-5, 5-10, 10-15 are the sedimentation rates calculated over times of 0 to 1, 1 to 5, 5 to 10 and 10 to 15 min. This test value was found to discriminate between highly flocculent yeasts better than the procedure of Helm et al. (1953). In the same paper they reported on a spectrophotometric method in which 0.1 g of centrifuged yeast was added to 4 mL of Helm's acetate buffer in a standard 1-cm cuvette, mixed and the turbidance at 670 mm recorded. This method was reported to give similar results to their modified (M_B) values. Further research on this second spectrophotometric method (Greenshields, et al., 1974) demonstrated that the decline in turbidance could be characterized by a (pseudo) second order relationship:

$$a_0 * k_{sed} = (a_0 - a_e) / (t_0 - t_e) \qquad (I.3)$$

where a_0 and a_e are the initial and equilibrium turbidance at 670 nm, k_{sed} is a constant and t_0 and t_e are the initial and equilibrium measurement times.

Patel and Ingledew (1975a) compared the methods of Kato and Nishikawa (1957) and Greenshields et al, (1972) to a modified Helm's method and found their assay superior for use with Canadian lager strains. In their method, yeast harvested after a 72 h fermentation was washed twice in a 0.05 M phosphate buffer and then suspended in pH 4.8 Helm's acetate buffer at a concentration of 1.15 g dry wt / mL. The suspension was incubated at 20°C in a 15 mL graduated centrifuge tube and the volume above the cleared boundary recorded for intervals up to 3 h.

In a unique procedure Taylor and Orton (1973, 1975, 1978) measured what they termed a flocculation intensity (F) and a floc dissociation temperature (T_F). These parameters were measured in a cylindrical stirred chamber placed in a spectrophotometer and the transmitted light intensity measured as the temperature was raised from 25 to 60 °C. A Tris buffer of pH 7.6 containing various levels of calcium and other ions was

used to suspend the yeast. Resulting plots of the effect of temperature on transmittance at 550 nm showed a rapid increase in flocculation (i.e., a decrease in transmittance) until a plateau was reached in the region of 50 to 60 ^{o}C. The effect was reversible and the data could be used to obtain a T_F value at the onset of the plateau region. The flocculation intensity or F value was calculated using the ratio of the intensity at 28 ^{o}C (I_{28}) to equilibrium intensity (I_e):

$$F = (I_{28} / I_e) - 1 \qquad (I.4)$$

Measurement of yeast flocculation in buffers was criticized by Gilliland (1951) as artificial, and he examined ale yeast after fermentation in wort for three days at 77 ^{o}F (25 ^{o}C). The yeast was resuspended and allowed to settle for 1 min and the remaining yeast in suspension (YIS) examined with a colorimeter. Chester (1963) and Porter and Macaulay (1965) employed a variation of this method where the suspended yeast was allowed to settle in an E.E.L. colorimeter equipped with a "Chance OB - 4" filter (wavelength not stated) and the time measured for the reading to drop from 30 to 20 units.

Yoshida (1962) also modified Gilliland's method by adapting it for estimation of the flocculence of bottom yeasts. In his procedure, lager yeast was fermented at 11 ^{o}C and the YIS was plotted against the percentage attenuation at daily intervals. The resulting curves characterized a yeasts' flocculating power.

Two variants of Gilliland's method have been routinely used by a major Canadian brewery. In the first method 15 mL of wort was fermented at 25 ^{o}C for three days, then resuspended and the sediment examined visually (Stewart, Russell and Goring, 1975b). In the second test, the YIS and percent attenuation during a fermentation was measured daily and the data graphically analysed according to Yoshida (1962). This method has the

advantage of being an "*in vivo*" style test that can be employed with commercial scale fermentations (Stewart et al., 1975b).

Ironically, Amri et al. (1979a, b) modified Gilliland's method for use with defined media. Using this procedure, Amri and co-workers examined the effects of various precursors on yeast flocculation.

Woof (1962) also criticized the use of buffers for the measurement of yeast and flocculation. He made use of a haemocytometer to measure the YIS during fermentation in a brewery wort. A plot of YIS against time resulted in a nearly vertical line followed by an almost horizontal line which were connected by a short elbow. Woof suggested that 2 parameters a "Terminal Time" and a "Terminal Count" could be obtained by extrapolation of the vertical and horizontal lines, respectively. Ginterova and Janotkova (1965) published a method that was almost identical in which the YIS was determined by use of a colorimeter. Holmberg and Kielland-Brandt (1978) used a method similar to that of Woof (1962) to study flocculence in temperature-sensitive mutants of *S. cerevisiae*.

ii. Recent methods - It is readily apparent that each of the researchers cited above had their own preferred assay and thus, comparison between reports is difficult. While researchers since 1981 have also employed their individual methods, some researchers active in the area have recognized some of the basic principles of colloid science and developed assays of a more fundamental nature. The previous lack of use of colloidal techniques is understandable since in the past, brewing scientists were concerned with the biological rather than the physical aspects of yeast flocculation.

During the examination of lectin-mediated flocculation, Miki, Poon and Seligy (1982b) developed a technique similar to Woof (1962). These authors measured a critical cell density (cCD) which they defined as the number of cells remaining in suspension after three to five min of settling. Miki et al. (1982b) stated that this cCD value was a property

of each strain which was independent of the initial cell concentration and appeared "to reflect the intensity and stability of flocculation interactions".

Kihn et al. (1988b) argued that the cCD values observed by Miki et al. (1982a) were dependent on the degree of agitation of the yeast cell suspension and suggested that at equilibrium the following relationship held:

$$\text{[free (suspended) cells]} \underset{\mathbf{b}}{\overset{\mathbf{a}}{\Leftrightarrow}} \text{[flocculated (settled) cells]} \qquad \text{(I.5)}$$

where rate constant **a** in I.5 is the flocculation process constant and **b** in I.5 the deflocculation constant due to shear. Both processes are dependent on the rate of shear or agitation. An increase in the rate of agitation will thus shift the equilibrium to the left.

Hunt (1985) and Davis and Hunt (1986) reported a novel sedimentation / light extinction technique in which they related the extinction coefficient at 632 nm to the inverse of the cube root of the aggregation number (# of cells per floc) by means of fractal theory. By measurement of the turbidance of a thin beam focused at a known depth below a column of sedimenting flocs, a population distribution could be obtained. Application of this technique in future studies might prove useful since floc size distributions could be obtained with only a minor modification of most existing methods.

In his text entitled, "Microbial Aggregation", Calleja (1984,) reviewed a number of flocculation assays reported prior to 1981 and introduced a method of measuring the aggregation number by a haemocytometric method. This technique, while tedious, does give a floc size distribution which can be used for calculation of fundamental flocculation constants. Calleja (1984) also outlined basic colloid theory and noted the importance of shear on the rate of flocculation, a factor often ignored or poorly controlled by past researchers.

This lack of control of the shear history of flocculating yeast supensions, while apparent to colloid scientists, has just been appreciated by researchers in the brewing field. In the last four years, researchers have noted the influence of agitation on the rate of flocculation (Stratford and Keenan, 1987, 1988; Stratford, Coleman and Keenan, 1988; Kihn et al., 1988b and Stratford, 1989a, b, c). Both Stratford's and Kihn's groups have detailed procedures involving either stirring, vortexing or orbital shaking of flocculating yeast suspensions followed by spectrophotometric examination of the supernatant. While these researchers controlled the length and degree of agitation, it should be noted that both methods involve mixing of the suspensions in a *turbulent* flow field rather than a *laminar* flow regime normally employed by colloid scientists. The significance of this point will be expanded upon in a later section of this chapter, but the use of turbulent flow in flocculation studies results in empirical rather than fundamental data being obtained from these types of experiments.

3. Genetic factors

The genetic nature of yeast flocculence has been examined repeatedly since the 1950's and has recently been reviewed (Johnston and Reader, 1983; Stewart and Russell, 1987; and Esser et al., 1987). As previously mentioned, the genetic control of brewing yeast flocculence is complex. Presently, 11 genes which influence flocculence have been tentatively identified as shown in Table I.1. The comment of Rose in 1984, that much confusion exists with regard to the genetics of flocculence still holds true today. Along with uncertainty as to the number of genes that control the process, little is known about the gene products and their mode of action.

Research by Hodgson, Berry and Johnston (1985) illustrated that the gene products of FLO1 and FLO5 have different chemical susceptibilities to degradation. They

Table I.1. Genes of *Saccharomyces cerevisiae* involved in flocculation (adapted from Esser et al.,1987).

Gene*	Degree of Flocculence**	Remarks	Reference
FLO1 FLO2 FLO4	XX	- allelic, lectin specificity to mannose	Russell et al.,1980
flox	XX	-possibly allelic to FLO1	Esser et al.,1987
flo6	XX		
flo7	XX	- linked to FLO1	Johnston & Reader, 1983
FLO5	XXX		Johnston & Reader,1983
flo3	X		
FLO8	XXX?		Yamashita & Fukui,1983
NewFLO	?	-broad lectin specificity	Stratford,1989c
TUP1	?		Holmberg & Kielland-Brandt,1978
fsul I & 2	?	-suppressor of FLO1	Lipke & Hull-Pillsbury, 1984

* Uppercase letters denote wild genes. ** Graded on a three point scale.

demonstrated that the FLO1 product (or products) are stable after incubation at 70 ^{o}C but that they can be degraded by chymotrypsin, whereas FLO5 product(s) are heat labile but stable to the action of chymotrypsin.

Mitochondrial DNA (mtDNA) may also be involved in the mediation of flocculence since respiratory mutants show altered flocculation ability (Johnston and Reader, 1983). Hinrichs, Stahl and Esser (1988) have argued that mtDNA influences either the modulation of nuclear genes or the alteration of cell wall components. It is noteworthy that workers have succeeded in transferring the FLO1 gene to non-flocculent strains (Barney, Jansen and Helbert, 1980; Watari et al., 1987, 1989).

Aside from the complexity and degree of flocculence, the stability of brewing yeast strains is also of paramount importance to the brewer. Strains are known to easily mutate over successive generations and become less and less flocculent (Chester, 1963). In a study of a production lager strain, it was demonstrated that highly flocculent yeast strains can be selected from poorly flocculent yeast crops by repeatedly collecting cells that have settled out early in the fermentation (Norstedt and Bergtsson, 1969). Mutations of this type are strain dependent and have been reported to occur in production strains at a rate of 2×10^{-3} per cell division (Chester, 1963; Thompson,1970). Diploid FLO1 strains have been reported to mutate at a rate of 10^{-3} per cell division while haploid strain mutations of 10^{-2} per cell division have been observed (Johnston and Reader, 1983). The rate of non-flocculent to flocculent mutations was reported to occur at much lower rates of less than 8×10^{-5} per cell division (Lewis, Johnston and Martin, 1976).

Apparently, much remains to be discovered regarding the genes that govern flocculence. The existence of multiple genes as well as the possibility of more than one flocculation mechanism underscores the difficulty in studying the genetics of brewing yeast flocculation.

C. Colloidal Flocculation

Apart from the volume of literature in the brewing sciences concerned with yeast flocculation, examination of the phenomenon in light of current colloidal theory can help clarify our understanding of how yeast cells associate. While largely ignored in the past, two central tenets of colloid science, "DLVO" (after Derjaguin, Landau, Verwey and Overbeek [Shaw, 1980]) and kinetic flocculation theories can be used to: (1) predict the energy of interaction as two yeast cells approach one another and (2) predict the rate at which cells flocculate, respectively. A third area of colloid science, concerned with the flow behaviour or rheological properties of particulate suspensions, can also help provide an insight into the flocculation state of brewing yeast cells. Comprehensive explanations of these and other colloid theories are available in several texts (Hiemenz, 1977; Dickinson and Stainsby, 1982; Hunter, 1987).

Prior to preceding to a discussion of these various colloid theories, their applicability to the study of yeast cells (5-10 μm in diameter), should be demonstrated. Colloid particles are normally defined as having diameters ranging from 1 nm to 1 μm, however the upper limit of 1 μm is not sharply defined (Shaw, 1980). Also, numerous research teams have employed both DLVO and kinetic theories to explain the behaviour of cells of 5-10 μm in diameter (Wilkins, Ottewill and Bangham, 1962; Brooks et al., 1967; Curtis, 1970; Curtis, 1973; Hornby, 1973; Hahn and Eppler, 1976; van de Ven and Mason, 1977; Goldsmith et al., 1982 and Duszyk, Kawalec and Doroszewski, 1986).

1. DLVO theory

In order to examine the energies involved when two charged cells approach each other, one must first understand the concept of the double layer. In an ionic medium,

negatively charged particles will attract cations to their surfaces. The concentration of these ions will decline with distance from the particle surface and this will result in an electrostatic potential occurring as shown in Figure I.2. While variations of this diagram exist, the one portrayed below based on the Stern model, has been used by many researchers (Dickinson and Stainsby, 1982). In this model, counter-ions are assumed to be adsorbed on the surface of the particle forming a Stern layer about the radius of the adsorbed ion. Also indicated is the shear boundary (the shear plane during flow) and the zeta potential (ζ), the potential between this shear plane and the particle surface.

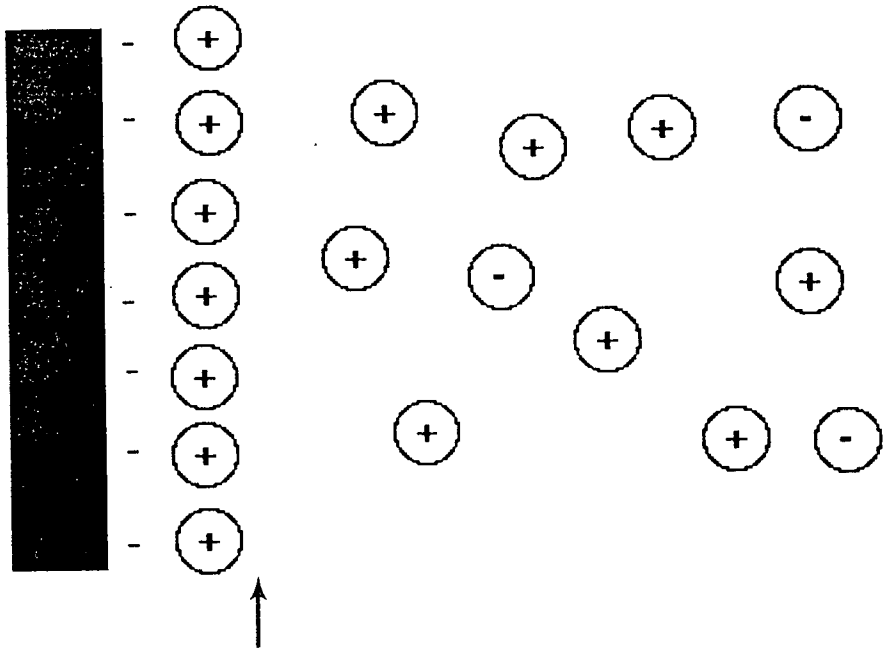
Building on the double layer concept, different equations for estimating the energies of attraction and repulsion have been developed (see, for example: Brooks et al., 1967; Rutter and Vincent, 1984; Ho, 1986; Krekeler, Zieher and Klein, 1989). The specific equations cited below apply to large spheres with small double layers at 'long range' distances of greater than 2 nm (Rutter and Vincent, 1980). The contribution of electrostatic repulsion (V_R) to the interaction energy is then:

$$V_R = 2\pi \varepsilon \varepsilon_o r \psi^2 \ln (1 + e^{-\kappa h}) \qquad (I.6)$$

where ε_o is the permittivity of free space, ε is the dielectric constant of the medium, r is the particle radius, ψ is the surface potential (often taken to be ζ), κ is the reciprocal of the double layer length and h is the distance between the two particles. The attractive potential energy, V_A, due to van der Waals interactions can also be estimated by:

$$V_A = Af (h, T) r / 12h \qquad (I.7)$$

where Af(h,T) is the Hamaker function (dependent on distance and temperature, but normally taken as a constant).



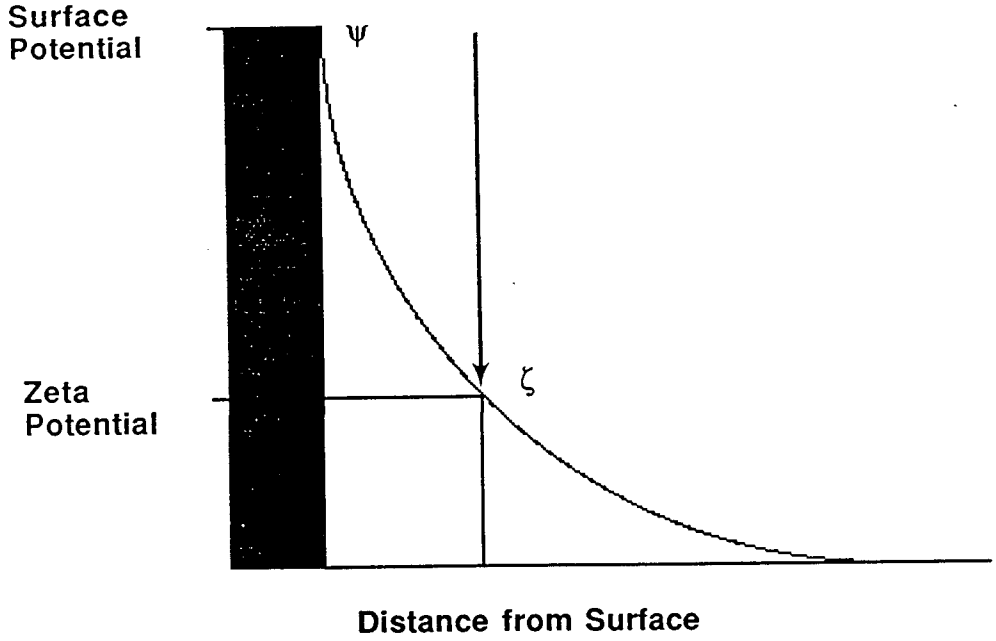
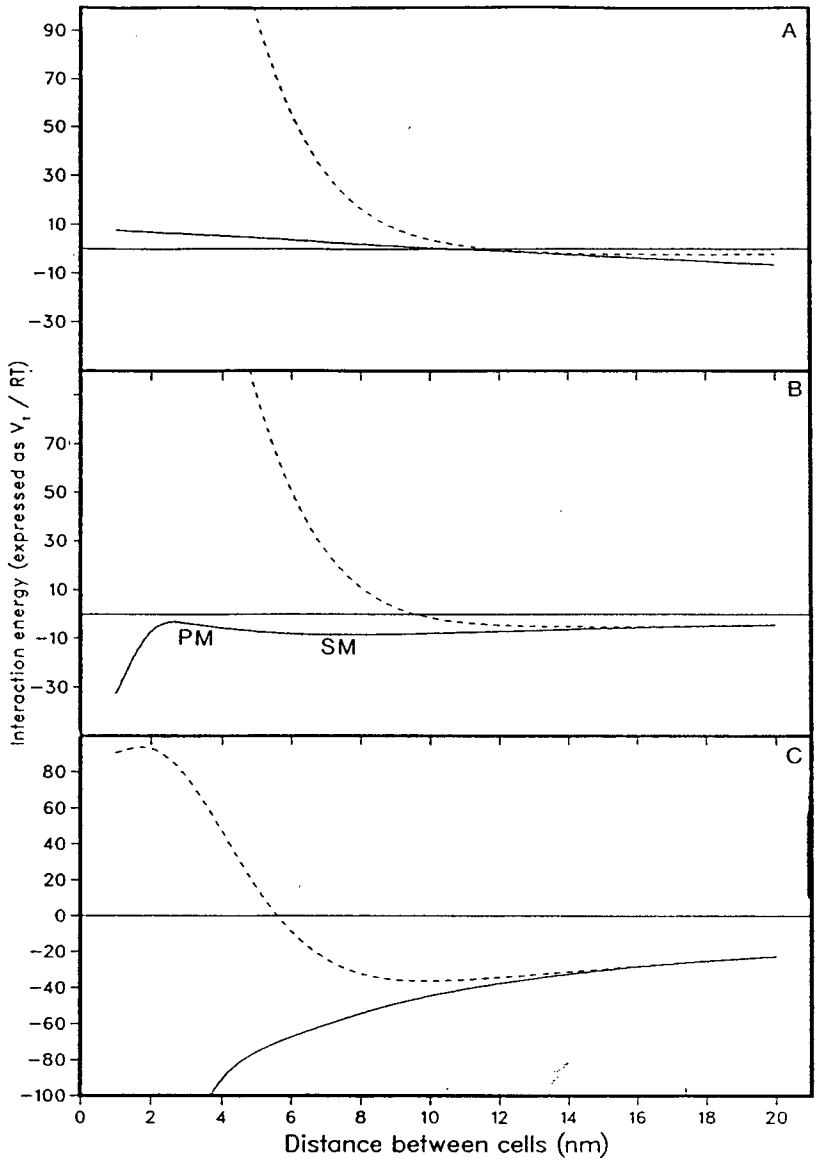


Figure I.2. The Stern model of decay of the electrostatic potential across an electrical double layer.

Summation of the V_R and V_A terms leads to the construction of so called DLVO curves (Figs. I.3a-3c), which relate the change in the potential energy of interaction with distance. Calculation of the potential energies of particles interacting under various conditions can give one a useful insight into the flocculation process. DLVO curves often have a primary minimum close to the particle, followed by a primary maximum where positive repulsion forces predominant and then a secondary minimum at a greater separation where a relatively small negative energy of attraction occurs. Some colloid scientists reserve the term flocculation to apply to the reversible approach of particle to the secondary minimum and the term aggregation for particles tightly bound in the primary minimum. However, these terms will be taken to be equivalent in this discussion.

DLVO theory has a number of limitations which, if not noted could lead to erroneous conclusions. First, the use of various expressions for the estimation of V_R and V_A terms are subject to debate regarding the assumption of constant charge or constant potential during particle interactions (Sculley, 1986). Secondly, the theory only applies at distances greater than 2 nm (Rutter and Vincent, 1984). Due to retardation effects, the theory also begins to overestimate the attractive energy above 10 nm (Hunter, 1987). As well, the values used for the Hamaker function, surface potential and dielectric constant are estimates and these values dramatically affect the total interaction energy, V_T. Finally, the application of the theory assumes that the particles are smooth spheres. Since yeast cells are rough prolate spheroids, application of the model may result in violation of some of its assumptions. For example, any fimbriae present can affect the values used for the dielectric constant, Hamaker function and the zeta potential.

Aside from this (large scale) type of electrostatic repulsion and van der Waals attraction there also exist other types of interactions that can influence the flocculation of yeast cells. The first of these interactions is due to the presence of polymers on the cell



Figures I.3a-3c. Potential energy curve of typical brewing yeast cells for Hamaker values of (a) 5 X 10^{-22}, (b) 10^{-21} and (c) 5 x 10^{-21} J (@25 ^{o}C, Ionic strength=0.02 M and cell radius=4.5 µm, solid line indicates a zeta potential of -5 mV, dotted line indicates a zeta potential of -15 mV). Note different scales on ordinate axis & example of primary minimum (PM) & secondary maximum (SM) in Fig. 3b.

wall. When these polymers prevent the approach of adjacent cells (steric repulsion) flocculation may be prevented. Alternately, when the polymer chains are more attracted to themselves than the suspending solvent, flocculation may occur by interpenetration of adjacent polymer chains. Bridging of cells by extracellular polymers such as fimbriae may also occur. In flocculent ale or lager cells this may be due to either calcium salt bridging or lectin-like adhesion. In such cases, the adhesion structures may bridge cells at distances where DLVO repulsion would otherwise prevent approach.

While it is worthwhile recognizing the preceding concerns, it is still useful to estimate DLVO interaction energies. Figures I.3a-3c represent such calculations using Equations I.6 and I.7. The calculation assumed the cells were suspended in a 'typical' flocculation assay buffer (Speers, Durance and Tung, 1989), using various estimates for the Hamaker function (Lips and Jessup, 1979) and zeta potentials reported for brewing yeasts (letting $\zeta=\psi$ as suggested by Lawrence et al., 1989). These curves indicate that changes of the possible Hamaker and surface charge values, can dramatically affect the attractive energies between the cells. According to these estimates, the yeast cells would only approach to the limit of the secondary minimum (> 6 nm).

Despite the aforementioned limitations, DLVO theory is still useful for determining the qualitative importance of the van der Waals and electrostatic repulsion forces in yeast cell flocculation. In such flocculation studies, the effect of the suspending medium on yeast flocculation rates and the corresponding DLVO interaction energies could be compared. Correlation of these factors would indicate the importance of electrostatic repulsion in flocculation.

2. Kinetic theory

The second branch of colloid science that can aid in clarifying our understanding of brewing yeast flocculation is that concerned with the rate at which particles collide and combine. There are essentially three mechanisms by which particles can associate: (1) by perikinetic aggregation due to Brownian motion, (2) by orthokinetic aggregation due to fluid flow and (3) so-called ballistic aggregation due to the settling of cells or flocs. Due to the relatively large size of yeast cells, perikinetic aggregation does not normally occur in brewing. One would expect that orthokinetic, and possibly ballistic, aggregation are responsible for the majority of brewing yeast cell flocculation.

At present, only the rate equation for aggregation in laminar shear flow has been determined. The rate at which particles combine in shear was first examined by von Smoluchowski (1917). His method was later corrected by van de Ven and Mason (1977), who considered the effect of hydrodynamic resistance, van der Waals attraction and electrostatic repulsion on doublet formation. These researchers found the following expression applied:

$$(N_t / N_o) = e^{-(4\alpha_o \dot{\gamma} \varphi_o / \pi)\, t} \qquad \text{(I.8)}$$

where N_t is the number concentration of particles at time t, N_o is the initial number concentration of particles, α_o is the orthokinetic capture coefficient, $\dot{\gamma}$ is the shear rate and φ_o is the initial volume fraction of particles. The expression has been said to hold for up to an 80% reduction of particle numbers (Gregory, 1982). The orthokinetic capture coefficient was shown to be dependent on van der Waals attractive forces, electrostatic repulsive forces and highly dependent on the rate of shear (van de Ven and Mason, 1977). In the absence of repulsive forces, the capture coefficient rapidly declined with increasing

shear. If the values for the zeta potential, Hamaker function, particle size, volume fraction, medium viscosity and shear rate are known, then an estimate of the capture coefficient can be calculated (van de Ven and Mason, 1977).

A different model for cell aggregation has been proposed by Bell (1981) who considered only the effect of antibody (or lectin) mediated bonding on the capture coefficient. In his model, the capture coefficient was shown to be dependent upon the number of cell wall receptors, their mobility and rate of bond formation. By using typical cell antibody parameters, Bell demonstrated that his capture coefficient could be calculated from theory. His results showed that the capture coefficient declined rapidly with increasing shear rates.

The value of the capture coefficient is thus the key parameter in orthokinetic flocculation. Examining the dependence of this value on the rate of shear should allow one to determine whether brewing yeast flocculation is governed by DLVO interactions or lectin-mediated aggregation.

As indicated previously, brewing researchers have used only empirical means to determine the rate of flocculation. While difficult, measurement of the decline of the total number of yeast flocs with time (in a defined shear field) could provide estimates of the capture coefficient values. Knowledge of these fundamental values would allow one to determine the applicability of either van de Ven and Mason's DLVO model or Bell's antibody / lectin model. Studies such as this would help resolve the current controversy over the mechanism of brewing yeast flocculation.

3. Suspension rheology

Knowledge of the principles of rheology, which is the study of deformation and flow of materials, can be useful in the examination of flocculent brewing yeast suspensions.

The rheological properties of such suspensions are very sensitive to floc structures which in turn are highly dependent on the attractive forces within the floc. Floc structure is determined by a number of physico-chemical factors and, in the case of a cellular suspension, by a number of microbiological concerns. Figure I.4 depicts the complexity of the relationships between these factors and the suspension viscosity. Of all the factors in Figure I.4, the shear rate has the greatest effect on suspension viscosity.

At present, no theory has been devised that is able to predict the many-particle interactions during shear that, in turn, determine the resistance to flow of these suspensions (Russell, 1980; Barnes, Hutton and Walters, 1989). However, a group of researchers at the University of Sydney led by Hunter has developed an "elastic floc model" which explains the non-Newtonian behaviour of colloidal suspensions semi-empirically (Hunter, 1982, 1984). The model assumed that the energy being consumed during (laminar) flow of suspensions was primarily due to the deformation of particles or flocs in the shear field. At shear rates above $\dot{\gamma}_c$, a critical shear rate, all flocs were considered to be broken down into single units where Bingham flow occurs. In Bingham flow, the apparent viscosity rapidly declined with increasing rates of shear due to a linear relationship of the shear rate with the shear stress:

$$\sigma = \sigma_y + \eta_\infty \dot{\gamma}, \ (\dot{\gamma} > \dot{\gamma}_c) \qquad \text{(I.9)}$$

where σ is the shear stress, σ_y is the yield stress and η_∞ is the plastic or infinite shear viscosity. In their examination of the magnitude of energies involved during flow, van de Ven and Hunter (1977) demonstrated that E_d, the energy required to deform the flocs was the dominant term and that the Bingham yield stress was the product of the number of collisions occurring between flocs and E_d:

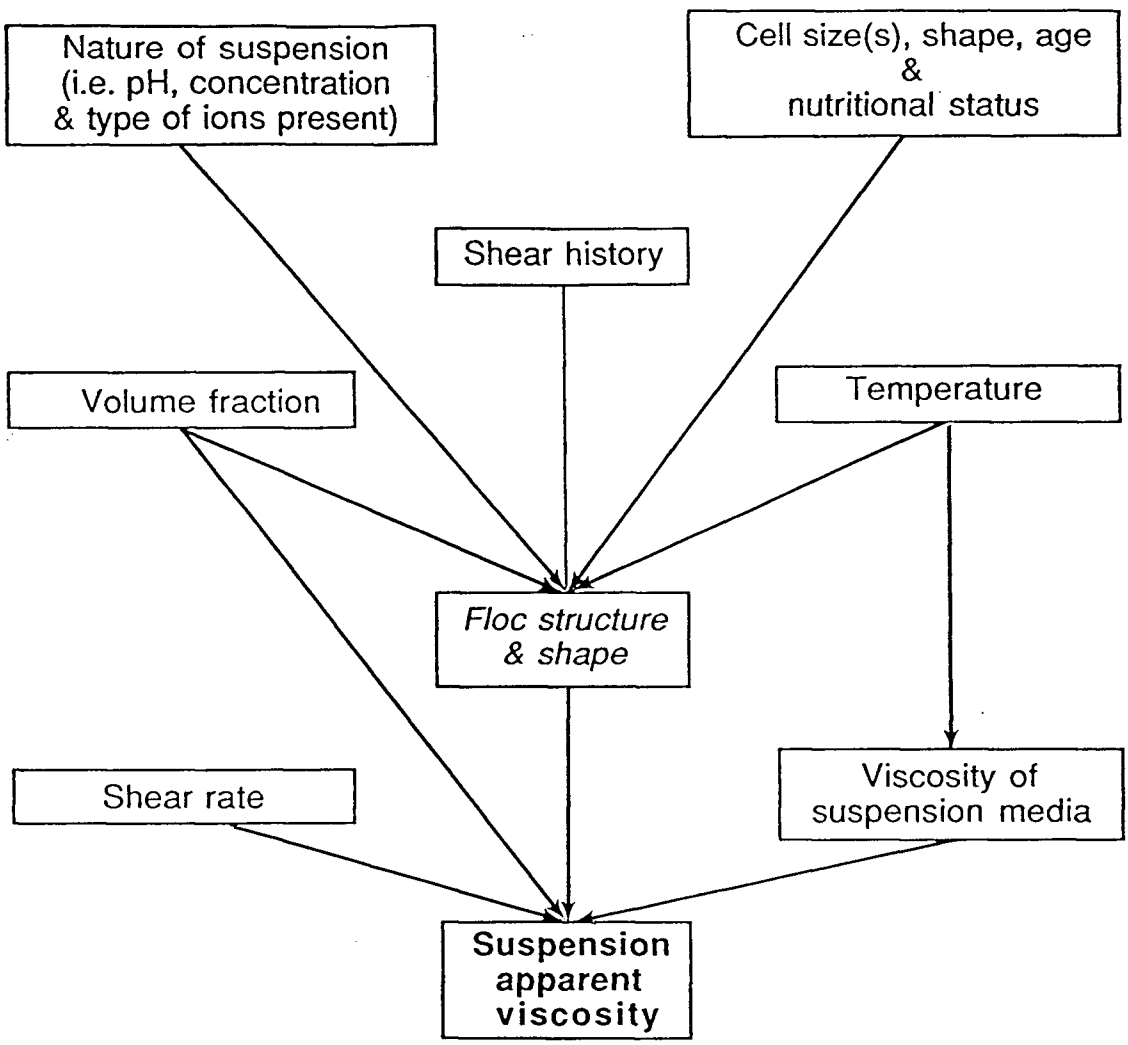


Figure I.4. Schematic representation of various factors affecting the apparent viscosity of yeast suspensions.

$$\sigma_y = 3\,\alpha_o\,\varphi_f^2\,(E_d)\,/\,\pi^2\,r^3 \quad (\dot{\gamma} > \dot{\gamma}_c) \tag{I.10}$$

where φ_f is the floc volume. The elastic floc model successfully accounted for the effect of interparticle DLVO energies, volume fraction and shear rate on the suspension viscosity. Additionally, measurement of the onset of the critical shear rate provided a value of the force required to "rupture" doublets (Hunter, 1984). The elastic floc model has been criticized (Russell, 1980) but it does account for a sizeable body of data.

While only limited reports of the rheological properties of yeast suspensions exist, it is reasonable to assume that the viscometric properties of these flocculated suspensions should reflect the flocculation state of the brewing yeast cells. Like other areas of colloid science, rheology has been under utilized by researchers studying brewing yeast flocculation. Rheological measurement of the energies and forces involved in flow of these suspensions, along with consideration of the forces and energies associated with various theories of brewing yeast flocculation, may provide additional information on this phenomenon.

i. Effect of volume fraction and temperature - Various expressions have been devised to predict the change in viscosity with increasing volume concentration. Many researchers have attempted to extend Einstein's treatment, normally applied to dilute dispersions, to higher volume concentrations (Russell, 1980):

$$\eta_r = 1 + k_1\phi + k_2\phi^2 + \ldots \tag{I.11}$$

where η_r is the relative viscosity (the ratio of suspension to continuous phase viscosities), k_1 is a constant equal to 2.5, ϕ is the volume fraction, k_2 is a constant equal to 6.2 at low shear and 5.2 at high shear rates. The literature contains numerous examples of the application of variations of Einstein's equation to specific systems.

Unfortunately, most models have not been shown to have a general application to colloidal systems. One model, albeit semi-empirical, has been generally accepted and employed by colloid rheologists. It was developed by Krieger and co-workers (for a review see Krieger, 1983) and is based on doublet flow between non-interacting spheres. The model has been successful in predicting the effect of shear stress and volume concentration on suspension viscosity. This "master equation" predicts a constant viscosity at low or "zero" rates of shear followed by an asymptotic decline in viscosity to a infinite shear viscosity (Woods and Krieger, 1970; Papir and Krieger, 1970):

$$\eta_r = \eta_{r\infty} + (\eta_{ro} + \eta_{r\infty}) / (1 + 0.431\sigma / \sigma_c) \qquad (I.12)$$

where σ_c is a critical shear stress, while η_{ro} and $\eta_{r\infty}$ are the relative limiting viscosities at zero and infinite shear rates. These limiting relative viscosities could in turn, be accurately predicted by the Krieger-Dougherty equation:

$$\eta_{ro \text{ or } r\infty} = 1 / (1 + \phi / p)^{-[\eta]p} \qquad (I.13)$$

where $[\eta]$ is the intrinsic viscosity or the relative viscosity extrapolated to zero concentration and p is the maximum packing fraction. The packing fractions and intrinsic viscosities were found to be 0.68 and 2.67 $m^3\ kg^{-1}$ at zero shear and 0.57 and 1.67 $m^3\ kg^{-1}$ in the high shear region, respectively. It should be noted that Krieger's theory assumes that the suspended colloids exhibit minimal interparticle interaction, a condition one might not expect to occur in brewing yeast suspensions.

The effect of temperature on the viscosity of most fluids is often modelled with an expression first developed by Arrhenius:

$$\eta = A\, e^{(\Delta E / RT)} \qquad (I.14)$$

where, A is the frequency factor, ΔE is the activation energy, R is the universal gas constant and T is the absolute temperature. This relationship has been successfully applied to a wide variety of both simple fluids and complex dispersions over various temperature ranges. Water viscosity is strongly dependent on temperature with a increase of one C^o resulting in a 2.5% reduction of viscosity at 20 ^oC (Bourne, 1982). Since water is a major constituent of many fluid foods, it is common to observe a decline in apparent viscosity with increasing temperature in aqueous suspensions and solutions. However, the opposite trend has been observed in some guar (Prentice, 1984) and xanthan gum (Speers and Tung, 1985) solutions. Measurement and examination of the activation energy can be a useful tool to help understand the extent of molecular associations within a fluid (Tung, 1989).

ii. Time dependence - When reviewing the effect of time on the rheological properties of colloidal suspensions, it is useful to distinguish between different types of time dependent behaviour and clarify a number of terms employed to describe these properties.

The term thixotropy is applied to systems which exhibit a reversible decrease in shear stress or apparent viscosity at a constant temperature and shear rate. This decline in viscosity is believed to be due to a temporary breakdown in the structure of the fluid. It is commonly observed in many fluid food systems when they are subjected to shear after a period at rest. The phenomenon can also be observed when the shear rate of a system is suddenly increased to a higher rate. In both instances the decline in the measured viscosity is due to a corresponding loss of structure of the system. An important feature of

thixotropic systems is that they will completely recover their initial structure given a sufficient resting time.

Rheopectic fluids or "anti-thixotropic fluids" are systems that exhibit an increase in their apparent viscosity or shear stress during shearing at a constant rate. This type of behaviour can be envisaged in a system when prolonged shearing results in greater interactions among components in the fluid which in turn cause increased resistance to flow.

Systems are termed rheodestructive when they experience an irreversible loss of structure during shearing at a constant shear rate. This behaviour is observed in systems which do not have the ability to reform their structure after shear. Some fluids such as liquid egg albumen may exhibit only partial recovery of time dependent loss of structure even after an extended period of rest (Tung, Watson and Richards, 1971).

When a time dependent but non-rheodestructive fluid is sheared at increasing shear to a maximum value and then back to zero shear, hysteresis can often be observed when their shear stress and shear rate values are plotted. Hysteresis in rheograms of this type are evidence that the apparent viscosities being measured are not equilibrium values. If this type of experiment is carried out over sufficiently long ramp times, then one can expect the "up-curve" and "down-curve" portions of the plot to coincide, indicating that equilibrium values are being measured.

Modelling the change of viscosity of food materials with time has been carried out by a number of authors. For example, Tung et al. (1971) examined the time dependence of egg albumen using a power-law type function:

$$\log (\eta - \eta_e) = a - b \log(t) \qquad (I.15)$$

where η_e is the equilibrium apparent viscosity at a fixed rate of shear, a and b are constants and t is time. A wide variety of food materials was also examined by DeKee and co-workers, (DeKee and Turcotte, 1980; DeKee, Turcotte and Code 1980; DeKee, Code and Turcotte, 1983). They used a similar model:

$$\log (\eta) = a - \dot{\gamma} t \qquad (I.16)$$

to examine the time dependent flow behaviour of banana puree, blood, honey, corn oil, yoghurt and mayonnaise. Various other relations have been presented to model the time dependence of foodstuffs although the majority of these expressions have only an empirical basis.

In the case of flocculent cell suspensions it may be possible to explain their change in viscosity with shearing time on a semi-theoretical basis using orthokinetic aggregation theory previously described. When dispersed cell suspensions are sheared at low rates of shear, one would expect singlet cells to aggregate according to Equation I.8. As these single cells form doublets, the energy required for flow increases, which is reflected in a higher apparent viscosity. If this energy requirement is primarily due to increases in floc volume or doublet separation energy, it is possible to derive an expression relating the increase in viscosity to the capture coefficient, shear rate and volume fraction:

$$\eta_t = (\eta_o - \eta_e)\, e^{(4\, \alpha_o\, \dot{\gamma}\, \phi_o / \pi)\, t} + \eta_e \qquad (I.17)$$

This semi-empirical derivation is detailed in Appendix I and assumes: (1) that the viscosity increase can be attributed to single cells or flocs forming doublets, (2) that (as assumed by Hunter [1982]) the high particle concentration does not seriously affect the

rate law, (3) that as the floc volume increases, shear forces cause floc breakup until an equilibrium is reached, and finally, (4) that the rate for constant doublet formation is much larger than the rate constant for doublet breakup.

D. Summary

In the first portion of this review, reports on brewing yeast flocculation were detailed and existing adhesion models discussed. Also, a brief summary of our understanding of the genetic factors involved in brewing yeast flocculation has been given. Examination of the methods used for measuring yeast flocculation revealed their empirical nature. The limitations of the methods employed by brewing researchers to study the process have contributed to the current controversy in the literature. Present areas of colloidal theory which might be useful to those assaying brewing yeast flocculation were also reviewed. Specifically, (1) the energies of interaction between colloid particles (yeast cells), (2) the significance of orthokinetic flocculation rates and (3) the rheological properties of flocculent suspensions appear to hold the greatest promise for a more clear understanding of yeast flocculation in fermentation media.

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CHAPTER II

RHEOLOGICAL STUDIES

A. Introduction

The overall objective of this research was to determine if rheological and colloidal theory were adequate to describe the flocculent behaviour of commercial brewing yeast strains. Four specific techniques; steady shear rheometry, zeta potential measurement, scanning electron and light microscopy were employed to to examine the adequacy of these theories to describe commercial brewing yeast flocculation.

The purpose of this chapter is to present findings on the effect of temperature, cell concentration, shearing time, suspension media and yeast strain on the viscometric behaviour of industrial brewing yeast suspensions. This will be done following a review of previous research in the field. It is also particularly important to be cognisant of the potential errors that may occur during rheological measurement and analysis. As will become evident in the following discussion, without the proper consideration and correction of these sources of error in viscometric studies, one can easily draw erroneous conclusions from the data.

Considering the extensive interest that has been shown in the flocculation behaviour of brewing yeast, it is surprising that few investigators have tried to relate flocculation properties to the viscosity of cell suspensions. In the limited research that has been undertaken in this area, most workers have not considered the effect of cell flocculence on suspension viscosity. The lack of coordinated research on this topic is probably a

reflection of the specialized training and background of brewing microbiologists which seldom includes the subject of rheology, rather than an indication of the topic's importance.

1. Flow behaviour of yeast suspensions

One of the first accounts regarding the viscosity of yeast suspensions was made by Luers and Heuss in 1921. They reported (in German) that the relative viscosity and flocculence of brewing yeast suspensions were affected by yeast strain, pH and the ionic composition of the suspending medium. As the volume concentration of the suspensions increased, they noted large increases in relative viscosity (as measured by capillary viscometry). Later in 1936, Eirich, Bunzl and Margaretha also examined the effect of volume concentration on the apparent viscosity of "killed hardened cells" (presumably *Saccharomyces cerevisiae*) also using a capillary viscometer. Both the studies of Eirich and co-workers and Luers and Heuss should be viewed with caution since the suspensions may have been shear rate dependent; however, this was not considered by these early researchers.

The next reports on yeast suspension rheology were made by two Japanese groups; (Koga, Kuribayashi and Nunonura, 1959; Aiba, Kitai and Ishida, 1962) who studied the rheological properties of *S. cerevisiae*. Both groups noted that the increase in relative viscosity (η_r) due to an increase in a suspension's (cell) volume concentration (ϕ) could be modelled by an extended Einstein relation (Russell, 1980):

$$\eta_r = 1 + 2.5\phi + k_2\phi^2 \qquad \text{(II.1)}$$

where η_r is the relative viscosity, ϕ is the volume fraction and k_2 is a constant. Both Koga et al. (1959) and Aiba et al. (1962) observed Newtonian flow behaviour at cell concentrations of 4.5×10^9 cells / mL and volume fractions of 0.21, respectively.

These (presumably non-flocculent) cell suspensions were found by Aiba et al. (1962) to exhibit a more gradual increase in relative viscosity than that predicted by Einstein's equation (Eq. II.1). This difference was speculated to be due to either inaccuracies in their measurement of yeast cell volume concentration or the deformability of the yeast cell walls.

In 1976, Shimmons, Svrcek and Zajic examined the effect of cell concentration on the apparent viscosity (@ 2200 s^{-1}) of bakers yeast and described the changes using the extended Brinkman equation:

$$\eta_r = [2.02 / (1-\phi)] + (1.36 / \eta_S)\,\phi \qquad \text{(II.2)}$$

where η_S is the suspension viscosity. This model was criticized by Reuss et al. (1979) who, in extensive rheological studies of *S. cerevisiae* and *Candida utilis* found the increase in viscosity with increases of cell volume concentration could be modelled with the following expression:

$$\eta_r = 1 / [1-(\phi / p)^a] \qquad \text{(II.3)}$$

where p is the maximum packing fraction (taken to be 0.63) and a is a constant equal to 0.5 (Reuss et al., 1979; Reuss, Debus and Zoll, 1982). They found this expression to be applicable over volume fractions of up to 0.60, where Newtonian behaviour was observed. The similarity between the Krieger-Dougherty equation (Krieger, 1983) and the above expression II.3 is noteworthy. One should recall that Krieger's theory assumes the suspended colloids exhibit minimal interparticle interaction, a condition one might not expect to occur in brewing yeast suspensions.

Aside from the reports concerning the concentration dependence of yeast suspension viscosity, within the last 20 years several researchers have noted that these suspensions

often possess a shear rate dependency. Labuza et al. (1970) measured the flow properties of bakers yeast (*S. cerevisiae*) and noted pseudoplastic shear thinning behaviour in the range of 1 to 100 s^{-1}, at yeast concentrations above 10.5% (w/w) at 25 ^{o}C. The power-law model was found to apply to these suspensions:

$$\eta = m \dot{\gamma}^{(n-1)} \qquad \text{(II.4)}$$

where m is the consistency coefficient, $\dot{\gamma}$ is the shear rate and n is the flow behaviour index. Similarly, Rao and Hang (1975) reported that *Candida utilis* suspensions exhibited Newtonian flow at low concentrations but could be modelled according to the Casson model at solids concentrations of 29.2% at 25.8 ^{o}C in the shear rate range of 5 to 50 s^{-1}. Like the Bingham and power-law models, the Casson model predicts a rapid decline in viscosity with increasing shear rates:

$$\eta^{0.5} = (\sigma_y / \dot{\gamma})^{0.5} + \eta_\infty^{0.5} \qquad \text{(II.5)}$$

Miyasaka, Rha and Sinskey (1980) also employed the Casson model to describe the flow behaviour of suspensions of temperature sensitive mutants of *S. cerevisiae*.

More recently, Lenoel et al. (1987) reported that suspensions of brewing yeast (strain not specified) displayed Newtonian behaviour below a concentration of 40% (w [pressed yeast]/v) and Bingham behaviour above those concentrations. The range of shear rates employed in this research was not clearly specified but the viscometric measurements were carried out at 0 ^{o}C.

It is difficult if not impossible to quantitatively compare the reported viscosities in the above studies. Factors that affect yeast suspension viscosity, as discussed in Chapter I (see Fig. I.4), varied in each of the previous studies. However, it is apparent that at higher volume concentrations the yeast suspensions exhibited pseudoplastic

behaviour. Since the fit of different flow models (i.e., Bingham, Casson and power-law) to the viscometric data of these suspensions was justified empirically rather than theoretically, caution should be used in choosing the "best" model to predict the change in viscosity with shear rate. Only the Bingham model (Eq. I.9) can be applied to these suspensions with some theoretical justification within the framework of Hunter's "elastic floc model" previously discussed.

2. Rheometry

While measurement of the viscosity of Newtonian fluids is considered routine by many researchers, measurement of the rheological properties of non-Newtonian fluids is not as straightforward. When these fluids are measured in coaxial cylinder viscometers, one must consider several corrections to the initial "raw" or Newtonian shear rate data.

When materials possessing a yield stress (such as Bingham or Casson fluids) are sheared at low speeds within coaxial cylinders, a phenomenon called "plug flow" can occur. Below a critical shear rate ($\dot{\gamma}_{c,pg}$) a portion or "plug" of the material is incompletely sheared between the cylinders, resulting in inaccurate shear rate determinations. While corrections can be made to these low shear stress data (Nguyen, 1983), normally this flow deviation is accounted for by calculating the critical shear rate and discarding information below this value (Speers, 1984). Also, when non-Newtonian fluids are measured with a coaxial cylinder viscometer, shear rates must be corrected for the velocity profile deviation from that of Newtonian flow. These two types of corrections have been reported in many articles and differ according to which model one fits to the experimental data. These procedures present a challenging problem because in principle, it is necessary to know the proper flow model in order to determine the true velocity

profile and hence the appropriate correction to the Newtonian shear rate. A full discussion and derivation of these two types of corrections are presented in Appendix II.

One further correction which one should recognize is that due to slip at the fluid-fixture interface. When one suspects slip has occurred, the data can be corrected by using the methods of either Mooney (1931) or Yoshimura and Prud'homme (1988). These techniques require measurement of the fluid in either two or three coaxial cylinder combinations of different bob and cup radius ratios (Yoshimura and Prud'homme, 1988; Mooney, 1931; respectively). A convenient test for the presence of wall slip is to measure the flow behaviour of the material of interest in two different bob and cup cylindrical fixtures and examine the linearized flow curves by an analysis of covariance procedure. If no significant differences are found between the two flow curves, one can conclude that slip at the fluid-fixture boundary has not occurred.

B. Materials and Methods

In examining the relationship between the flocculation properties of brewing yeast and the viscosity of their suspensions, commercial samples of brewing yeast were examined in a series of four different trials. Due to the limited viability of the yeast samples, experiments designed to compliment the rheological investigations were undertaken in conjunction with the trials. The complete experimental plan is portrayed in Figure II.1. Commercial yeast strains as opposed to "pure" strains were used due to the high cell concentrations and large suspension volumes required for these rheological examinations.

At each phase of the research, commercial samples of brewing yeast were obtained from 3 different plants of the Labatt Brewing Company (London, ON). The yeast strains

Phase I

- Examine the effect of shearing time, temperature, concentration and cell strain on the flow behaviour of industrial brewing strains by coaxial cylinder viscometry,
- Measure the settling rates of different yeast strains at varying cell concentrations.

⇓

Phase II

- Study effects of varying pH, suspension media and shear rates on cell suspension viscosity with a cone and plate viscometer,
-Assess the effect of the above variables on the orthokinetic flocculation rate at 200 s^{-1},
-View the brewing strains using the Scanning Electron Microscope (SEM).

⇓

Phase III

- Test for the presence of slip using coaxial cylinder fixtures of different radius ratios,
- Examine the effect of ethanol on the yeast cell surface (SEM).

⇓

Phase IV

- Measure the zeta potential of the brewing strains in beer and various pH and ionic conditions,
- Determine orthokinetic flocculation rates of differing strains, shear rates and cell concentrations in various media,
- View the effect of pronase on the cell surface using SEM,
- Examine the effect of high shear on the flocculation behaviour of flocculent brewing strains.

Figure II.1. Research plan for rheological and related studies on brewing yeast samples from four separate trials.

were classified as: a non-flocculent lager (NFL), a flocculent lager (FL), a non-flocculent ale (NFA) and a flocculent ale strain (FA). The non-flocculent strains were reported by the brewery to be variants of the flocculent strains. All samples were received within 2 days of collection from the fermenter and kept between 0 and 4 ^{o}C during shipment and storage. The yeast slurries were shipped in 11 L stainless steel containers encased in a styrofoam^{TM} insulated container similar to that described by Casey et al. (1983). The samples were analysed within three weeks of delivery at which point, minimal changes in viability (as measured by a methylene blue method, American Society of Brewing Chemists [ASBC, 1976]) were evident. Also, minimal budding was observed in the yeast samples.

Prior to analysis, the cells were repeatedly washed with distilled-deionized water, ethylene-diamine-tetra-acetic acid (EDTA), distilled-deionized water, and finally, a pH 4.00 sodium acetate buffer containing 1.0 mM calcium as described in Figure II.2. The buffer was made by adjusting 0.1 M acetic acid with 0.1 M sodium acetate (Na-acetate) to a pH of 4.00. The final ionic strength of the buffer was 0.02 M. Acetate buffers of pH 3.5, 5.0, 5.5 were of varying ionic strength. This elaborate washing procedure was employed to eliminate any wort substances that might affect the flocculation process. Calcium was added due to the requirement for this cation during flocculation.

1. Coaxial cylinder viscometer study

In the first portion of this research the yeast cells were suspended in a 0.1 M sodium acetate buffer containing 0.1 mM calcium and the viscosity of the resulting suspension was measured as a function of shear rate, temperature, concentration and shearing time.

Initially, three of the four yeast strains were studied; NFL, FL and FA. With the first strain examined, NFL, the following cell concentrations were tested; 0.1, 0.25, 0.5,

Store yeast slurry @ 4 °C

⇓

Suspend in distilled-deionized water /
centrifuge (3000 xg) for 15 min (**x2**)

⇓

wash / centrifuge in 10.0 mM EDTA (**x2**)

⇓

wash / centrifuge in distilled-deionized water (**x2**)

⇓

wash / centrifuge in pH 4.00 Na-acetate buffer
(with 1.0 mM Ca as $CaCl_2$)

Figure II.2. Yeast washing procedure (modified from Stewart, Russell and Garrison, 1975 and ASBC, 1986).

1.0, 2.5 and 4.25 x 10^9 cells / mL. This range was used in order to determine the approximate cell concentrations which would permit measurement across wide shear rate (0.66 - 1005 s^{-1}) and shear stress (0.1 - 20 Pa) ranges within the temperatures selected for the study (5, 15, 25 and 35 °C). The two other lager and ale strains were then examined at concentrations of 0.75, 1.0, 1.75 and 2.5 x 10^9 cells / mL at all four of the above temperatures.

The flow behaviour of each of the cell suspensions was measured using a coaxial cylinder viscometer (Brabender Rheotron, C. W. Brabender Instruments Inc., South Hackensack, NJ). The viscometer was interfaced with an Apple II microcomputer (Apple Computer Inc., Cupertino, Ca) which contained a AI13 analog to digital board (Interactive

Structures Inc., Bala Cynwyd, PA) which was programmed to record the average of three shear stress signals (measured within 0.003 s) every three s. The collected data were saved to floppy disk and later transferred to the university Amdahl computer for further analysis.

Yeast suspensions were made up to the desired concentration by suspending a pellet of the washed yeast in the pH 4.00 buffer and measuring the cell concentration by haemocytometer according to ASBC standard methods (1976). The suspensions were then loaded into the "A1" coaxial cylinder fixture (1.0 mm gap size, r_b/r_c = 0.964) and allowed to equilibrate to the desired temperature. The viscometer cup was then removed from the tempering jacket assembly and the sample gently stirred to ensure homogeneity. The suspension was then reloaded into the viscometer and sheared at a Newtonian shear rate equivalent of 1005 s^{-1} for 2.0 min in order to deflocculate the sample. This shear rate value was somewhat arbitrarily selected while recognizing that high shear rates may damage cell wall structures(s). This method of cell deflocculation was apparently successful as the shear stress signal reached equilibrium during this time. Following this period of high shear the viscometer drive unit was suddenly stopped and the shear stress signal allowed to stabilize for a period of 2 to 3 s. The sample was then suddenly sheared at the lowest measurable Newtonian shear rate equivalent (in the range of 0.66 to 24.2 s^{-1}) and the shear stress signal recorded until an equilibrium was reached. The suspended cells were then sheared at a high shear rate (≈1005 s^{-1}) for 2.0 min, allowed to rest for 2 to 3 s and sheared at the next highest rate of shear, again measuring the shear stress signal until an equilibrium was obtained. This cycle of high shear, followed by the measurement of the shear stress response was repeated until all of the measurable Newtonian shear rate equivalents (0.66, 1.30, 2.53, 4.99, 10.0, 24.2, 75.8, 147, 284, 508, 1005 s^{-1}) had been evaluated. At this point the cycle was reversed and the

shear stress responses were measured at lowering shear rates (i.e., ≈508, 284 s^{-1} etc.) until the lowest measurable shear rate was tested. No evidence of cell settling was evident in any of these experiments.

Each shear stress - shear rate data set was examined for changes of shear stress values with time. In cases where such changes were observed, Equation I.17 was fitted to the data in order to relate the change in apparent viscosity to time, (see Chapter I & Appendix I). Equilibrium values were also fitted by the Newtonian, Bingham and Casson models and the flow parameters corrected for plug and non-Newtonian flow within the gap according to the methods outlined in Appendix II.

2. Settling behaviour

In conjunction with this first rheological study the settling behaviour of the suspensions was also examined. Specifically, samples of all three strains at concentrations of 1.0, 2.5, 5.0 and 10 x 10^8 cells / mL were suspended in the pH 4.00 Na-acetate buffer, stirred for 30 min in a 500 mL beaker, and then allowed to settle in a 100 mL graduated cylinder at room temperature. The level of interface subsidence was measured at 10, 20, 30, 45, 60, 120, 180 and 240 min, and the final settled volume was measured at 48 h. The rate of of settling was then determined using a "mean-zero" linear regression technique (Fox and Guire, 1976). In this procedure the intercept (i.e., the initial interface level) was forced through zero.

3. Cone and plate rheometer examinations

In Phases II and IV of this research, four yeast strains (NFL, FL, NFA and FA) were obtained, washed and suspended at a concentration of 2.5 x 10^9 cells / mL as detailed previously. In the last portion of this research (Exps. 17-20, Table II.1), a more

Table II.1. Media used during cone and plate rheometry.

Treatment		Calcium level (mM)	Yeast strain*	pH
Number	Medium			
1	buffer	1.0	NFL	4.00
2	buffer	1.0	FL	4.00
3	buffer	1.0	NFA	4.00
4	buffer	1.0	FA	4.00
5	buffer	1.0	FL	3.50
6	buffer	1.0	FA	3.50
7	buffer	1.0	FL	5.00
8	buffer	1.0	FA	5.00
9	buffer	1.0	FL	5.50
10	buffer	1.0	FA	5.50
11	buffer + 0.5 M KCl	1.0	FL	4.00
12	buffer + 0.5 M KCl	1.0	FA	4.00
13	buffer + 0.01 M EDTA	0.0	FL	4.00
14	buffer + 0.01 M EDTA	0.0	FA	4.00
15	buffer + 5% EtOH**	1.0	FL	4.00
16	buffer + 5% EtOH	1.0	FA	4.00
17	Lager***	7.25×10^{-4}	NFL	3.95
18	Lager	7.25×10^{-4}	FL	3.95
19	Ale***	8.25×10^{-4}	NFA	4.00
20	Ale	8.25×10^{-4}	FA	4.00

*Yeast codes refer to non-flocculent lager (NFL), flocculent lager (FL) and non-flocculent ale (NFA) yeast strains. ** Concentration = 5% (w/w) of ethanol. *** For composition of the beers, see Table II.2.

accurate cell count was undertaken by counting a larger suspension volume, 0.16 mm^3, rather than 0.02 mm^3 as recommended by the ASBC (1976). The flow behaviour of the four strains were examined in the pH 4.00 calcium containing Na-acetate buffer and in commercial samples of decarbonated lager and ale. As well, the steady shear viscometric properties of the flocculent ale and lager strains were measured in the calcium containing Na-acetate buffer system at pH 3.50, 5.00 and 5.50. Additionally, these two flocculent strains were suspended in the pH 4.00 Na-acetate / Ca buffer system containing either 0.5M KCl or 5% (w/w) ethanol. Finally, the two strains were also suspended in the pH 4.00 Na-acetate buffer system (without Ca) containing 0.01 M EDTA. Details of these experiments are set forth in Table II.1.

The ale and lager beers were obtained from the Creston and New Westminster, BC plants of the Labatt Brewing Company (London, ON) within one week of bottling. The samples were held in darkness at 4 ^oC before analysis. In order to decarbonate the samples they were filtered twice through Whatman 2V filter paper (Fisher Scientific Inc., Dartmouth, NS). The properties of the ale and lager samples as supplied by the brewery are presented in Table II.2. The elements were analysed by atomic absorption spectrophotometry, the ions by ion specific electrode, ethanol by gas chromatography, apparent and real extract (for a detailed explanation of these terms see ASBC, 1976) by densitometer.

Viscometric evaluations were undertaken using a Carri-Med CS500 rheometer (Carri-Med Ltd., Dorking, Surrey, GBR) equipped with a 2^o, 6 cm diameter cone and plate fixture. The rheometer was interfaced to an IBM-compatible microcomputer for control, data acquisition and analysis. After repeated shearing in a 5 mL syringe (i.e., until deflocculation was observed microscopically), the yeast suspensions were loaded on to the rheometer platen and allowed to equilibrate at 15 ^oC. The instrument was operated

Table II.2. Composition of beers used in Phase IV of this study.

Analysis*	Lager	Ale
Na	20.	21.
K	314.	276.
Mg	69.	59.
Ca	29.	33.
SO_4^{-2}	87.	114.
PO_4^{-3}	319.	165.
Cl^{-1}	248.	251.
Cu	28.	61.
Fe	61.	38.
pH	3.95	4.00
Ethanol	4.70	4.88
Apparent extract	1.802	1.531
Real extract	3.50	3.29

* Elements and ion levels in mg / L. All values averaged from duplicate measurements.

in a programmed shear rate mode to linearly ramp the shear rate from 0 to 1000 s^{-1} and back to 0 s^{-1} over a period of 20 min while recording the shear stresses imposed. All measurements were undertaken in duplicate, and stored on floppy disk for analysis. These data (100 data points/ramp) were transferred to the university Amdahl computer and the linear portion of the flow curves fitted by the Bingham model. In cases where covariance analysis indicated that the intercept of this model (the Bingham yield stress) was not significantly different from zero ($p>0.05$), Newtonian flow was assumed and the viscosity calculated.

4. Examination for viscometric slip

In Phase III of this research the possibility of slip during viscometric evaluation was considered. Specifically, the flow properties of the flocculent ale strain (FA) were measured using the Brabender Rheotron linked to a microcomputer as previously described.

After washing according to the procedure outlined in Figure II.2, the cells were suspended in the pH 4.00 buffer at a concentration of 2.5×10^9 cells / mL. Viscometric evaluations were undertaken at 15 ^oC using the "A1" and "A2" Rheotron fixtures, using the same technique as previously described. The bobs of A1 and A2 fixtures had diameters of 5.4 and 5.0 cm, respectively. Both fixtures used an identical cup with a diameter of 5.6 cm. The resulting equilibrium flow data were subjected to an analysis of covariance procedure (Fox and Guire, 1976) to test for possible differences between the two (Bingham) flow curves.

5. High shear effects

In the last experimental portion of this thesis, the effect of high shear on the flocculation behaviour of the flocculent brewing strains was examined. Cell suspensions of the four brewing strains were prepared as previously described and suspended in the calcium-containing Na-acetate buffer (pH 4.00) at a concentration of 2.5 x 10^9 cells / mL. Their flow behaviour was measured both before and after blending at 15 ^oC in a Waring Blendor (at maximum speed for 60 s) using the cone and plate rheometer procedure detailed earlier. All measurements were undertaken at 15 ^oC.

C. Results and Discussion

1. Examination for viscometric slip

The viscometric testing of a flocculent ale suspension (@15 ^oC and 2.5 x 10^9 cells/mL) using both the A1 and A2 Rheotron fixtures showed no evidence of slip. Visual examination of plots of shear stress against shear rate as well as an analysis of covariance test of the linear portion of the flow curves failed to distinguish any differences ($p>0.05$). As this suspension (@15 ^oC and 2.5 x 10^9 cells/mL) exhibited one of the largest Bingham yield stress values determined, one can conclude that slip between the suspensions and the different fixtures employed was not a concern in this study.

2. High shear effects

The purpose of this study was to determine if the loss of cell surface fimbriae reported by Day, Poon and Stewart, (1975) would affect cell suspension rheology. Stewart and Russell (1981) noted that these surface hairs could be removed from the cell wall by blending in a laboratory blender at maximum speed for approximately 30 s.

Viscometric evaluation of the four yeast strains both before and after the application of a high shear treatment failed to show any evidence of rheodestruction. Comparison of the flow curves from before and after blending, showed that in all four cases the data overlapped one another. Since it was impossible to distinguish the paired flow curves before and after high shear, it was concluded that rheodestruction did not occur. This finding suggests that no loss or destruction of cell fimbriae occurred in the flocculent strains. This may be due to the lack of cell fimbriae, or differences in shearing procedure from that of Stewart and Russell (1981). Examination of the cells by light microscopy after high shear did not show any evidence of cell lysis.

3. Coaxial cylinder flow behaviour

i. Steady shear - Examination of the data collected using the Brabender Rheotron revealed that the yeast suspensions could be described as Newtonian fluids at the lower cell concentrations tested. In the case of non-flocculent ale and flocculent lager strains, non-Newtonian behaviour was observed at high volume concentrations (2.5×10^9 cells/mL) as shown in Figure II.3. In such cases the variation in viscosity with shear rate could be modelled by the Bingham Equation (Eq. I.9) at shear rates above about 20 s^{-1}. However, a curvilinear trend in the low shear rate region was observed in some of the experiments as was also apparent in Figure II.3. At shear rates below 20 s^{-1} the Casson model (Eq. II.5) was found to fit the limited amount of data available.

As destruction of the flocculent cell fimbriae by blending was a concern, the viscometric measurements were examined for signs of rheodestruction. If such destruction had occurred, one would expect that; (1) the shear stress values measured at constant rates of shear would show a gradual decline after reaching a peak value, and (2) the flow curves as derived from the equilibrium shear stress data would exhibit

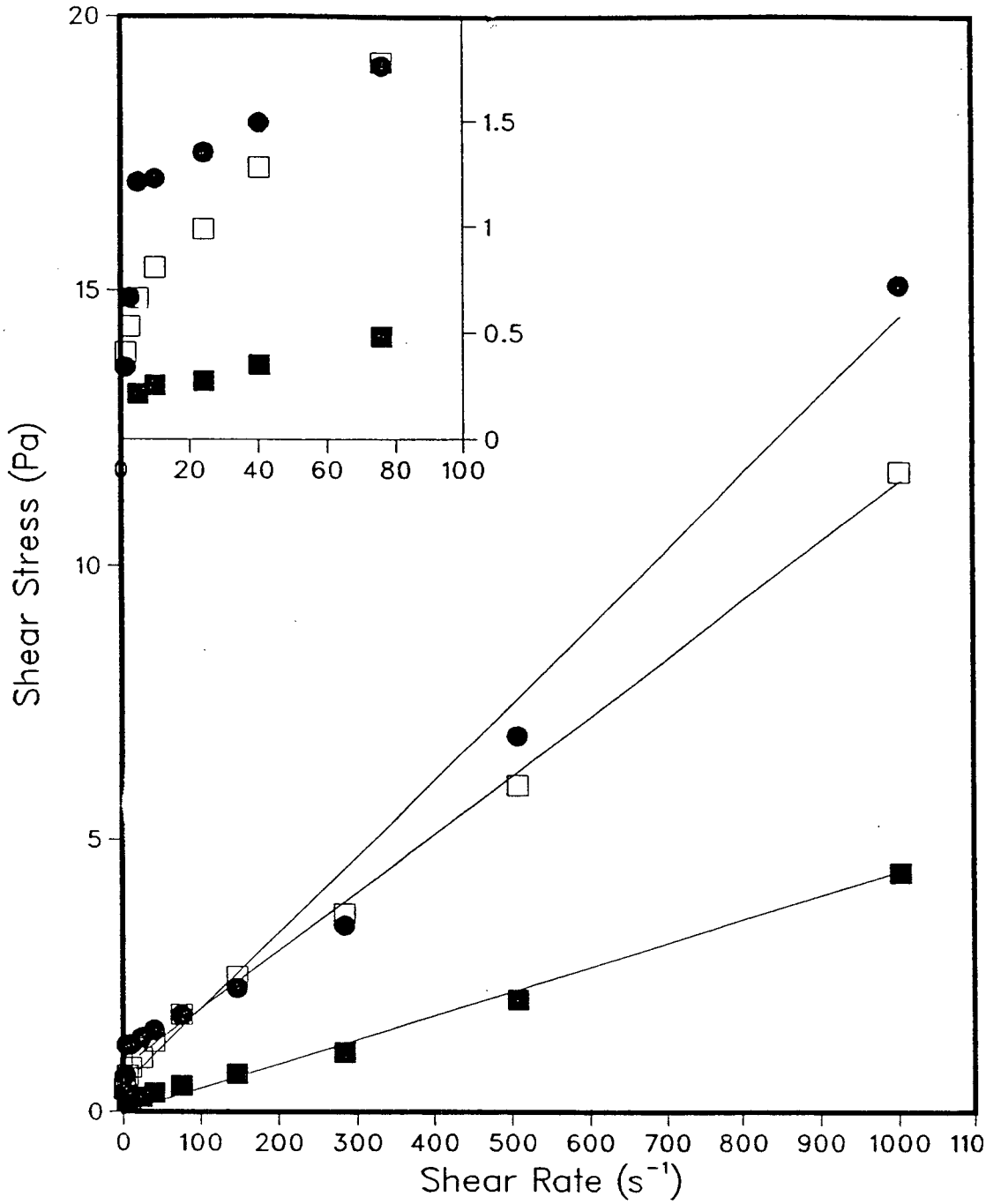

Figure II.3. Flow behaviour of a non-flocculent lager yeast (■), flocculent lager yeast (□) and a non-flocculent ale yeast (●) as measured by coaxial cylinder viscometry (@ 15 ^{o}C and 2.5 x 10^{9} cells/mL). Inset illustrates flow behaviour at low shear rates.

hysteresis. While visual examination of the data did not support this conclusion, an analysis of covariance was undertaken on the "up-curve" and "down-curve" data measured at the highest concentrations and the lowest temperature. In general, no significant difference was observed between the two curves ($p>0.05$). However, in a few cases, a significant difference was observed ($p<0.05$) between these curves. This difference was believed to be due to shear stress signal noise decreasing during the course of the experiment. In a study not reported here, an extraordinary warm up-time, up to 3 h, was required for the shear stress signal to stabilize. Nevertheless, as a precaution, all the "down-curve" data were eliminated from further consideration.

The remaining shear stress - shear rate values ($\dot{\gamma}>20\ s^{-1}$) were then fitted by the Bingham flow model. In cases where a non-significant ($p>0.05$) intercept was evident, it was concluded that Newtonian flow behaviour occurred and the data over the entire shear rate range were employed to calculate Newtonian viscosities. Where a significant intercept was present ($p<0.05$), the Bingham model was employed to best explain the variation of shear stress with shear rate. A similar procedure was used with the low shear rate data ($\dot{\gamma}<20\ s^{-1}$) to determine if the Casson model best fitted the data. Table II.3 displays the Newtonian, Bingham and Casson parameters which best fitted the up-curve data.

While the fit of the Bingham and Newtonian models to the data is in complete accord with predictions of Hunter's elastic floc model, the "Casson fit" to the yeast suspensions at the low shear rates can only be said to qualitatively agree with the model. In a few of the thicker suspensions, limiting low and high shear rate viscosities were observed. In these instances, the flow behaviour in the ranges between these shear rate plateaux did not agree with the Krieger-Dougherty model (Eq. I.13) and appeared to be best predicted by the Cross equation (1965). However, this tentative observation should be confirmed

Table II.3. Values of the Newtonian, Bingham and Casson parameters for brewing yeast suspensions as measured by coaxial cylinder viscometry.

Yeast Code*	Conc. (cells/mL) x 10^8	Temp. (°C)	Model: Newtonian η (mPa·s)	Casson σ_y (Pa)	Casson η_∞ (mPa·s)	Casson r^{2**}	Bingham σ_y (Pa)	Bingham η_∞ (mPa·s)	Bingham r^{2**}
NFL	1.0	5.0	0.85	-	-	-	-	-	-
		35.0	0.68	-	-	-	-	-	-
	2.5	5.0	1.12	-	-	-	-	-	-
	5.0	5.0	1.79	-	-	-	-	-	-
		15.0	0.823	-	-	-	-	-	-
		25.0	0.675	-	-	-	-	-	-
		35.0	0.475	-	-	-	-	-	-
	10.0	5.0	2.06	-	-	-	-	-	-
		15.0	1.27	-	-	-	-	-	-
		25.0	1.01	-	-	-	-	-	-
	25.0	5.0	4.03	-	-	-	-	-	-
		15.0	4.41	-	-	-	-	-	-
		25.0	2.41	-	-	-	-	-	-
		35.0	1.17	-	-	-	-	-	-
	42.5	5.0	-	-	-	-	1.17	58.2	0.985
		15.0	18.4	-	-	-	-	-	-
		25.0	11.8	-	-	-	-	-	-
		35.0	10.3	-	-	-	-	-	-

Table II.3. (Continued) Values of the Newtonian, Bingham and Casson parameters of brewing yeast suspensions as measured by coaxial cylinder viscometry.

Yeast Code*	Conc. (cells/mL) x 10^8	Temp. (°C)	Model: Newtonian	Casson			Bingham		
			η (mPa·s)	σ_y (Pa)	η_∞ (mPa·s)	$r2^{**}$	σ_y (Pa)	η_∞ (mPa·s)	$r2^{**}$
FL	7.5	5.0	1.82	-	-	-	-	-	-
		15.0	1.49	-	-	-	-	-	-
		25.0	0.884	-	-	-	-	-	-
		35.0	0.691	-	-	-	-	-	-
	10.0	5.0	2.67	-	-	-	-	-	-
		15.0	1.58	-	-	-	-	-	-
		25.0	1.39	-	-	-	-	-	-
		35.0	1.02	-	-	-	-	-	-
	17.5	5.0	-	-	-	-	0.123	4.62	0.998
		15.0	-	-	-	-	0.162	3.94	0.999
		25.0	-	-	-	-	0.064	3.51	0.999
		35.0	3.17	-	-	-	-	-	-
	25.0	5.0	-	0.274	15.6	0.970	0.816	10.7	0.998
		15.0	-	-	-	-	0.739	9.13	0.999
		25.0	-	-	-	-	0.464	7.39	0.998
		35.0	-	-	-	-	0.283	6.27	0.994

Table II.3. (Continued) Values of the Newtonian, Bingham and Casson parameters of brewing yeast suspensions as measured by coaxial cylinder viscometry.

Yeast Code*	Conc. (cells/mL) x 10^8	Temp. (°C)	Model: Newtonian η (mPa·s)	Casson σ_y (Pa)	Casson η_∞ (mPa·s)	Casson r^2	Bingham σ_y (Pa)	Bingham η_∞ (mPa·s)	Bingham r^{2**}
NFA	7.5	5.0	1.50	-	-	-	-	-	-
		15.0	1.14	-	-	-	-	-	-
		25.0	0.896	-	-	-	-	-	-
		35.0	0.969	-	-	-	-	-	-
	10.0	5.0	4.06	-	-	-	-	-	-
		15.0	1.24	-	-	-	-	-	-
		25.0	1.34	-	-	-	-	-	-
		35.0	1.03	-	-	-	-	-	-
	17.5	5.0	4.28	-	-	-	-	-	-
		15.0	4.38	-	-	-	-	-	-
		25.0	16.8	-	-	-	-	-	-
		35.0	-	-	-	-	0.782	2.75	0.985
	25.0	5.0	-	0.204	53.8	0.751	0.470	14.0	0.983
		15.0	-	0.030	116.	0.908	0.992	8.39	0.985
		25.0	-	0.036	168.	0.934	2.17	9.35	0.927
		35.0	-	-	-	-	1.80	5.00	0.997

*Yeast codes refer to non-flocculent lager (NFL), flocculent lager (FL) and non-flocculent ale (NFA) yeast strains. **All regressions were significant ($p<0.05$, $n\geq4$ for Casson and $n=7$ for Bingham models, each data point was an average of ≥ 50 measurements).

with studies over a much wider shear rate range. Further theoretical and experimental research is needed before one can claim any fundamental basis for the application of either the Casson or Cross models.

In reviewing the data and model parameters it was apparent that the non-Newtonian character of the yeast suspensions is most pronounced in the flocculent lager, less so in the non-flocculent ale and least evident in the non-flocculent lager strain. Also, the trend towards non-Newtonian behaviour follows the increase in suspended cell concentration. It is noteworthy that the concentration and temperature at which the onset of non- Newtonian behaviour was detected was limited by viscometer sensitivity.

Temperature affected the flow behaviour of the lager and ale suspensions differently. In the case of lager yeasts, increasing temperatures resulted in an decrease in suspension consistency as evidenced by the Newtonian viscosity or the Bingham yield stress values. This trend is commonly observed in colloid suspensions. In this case it is believed to be due to a decrease in cell-cell attractions at higher temperatures. In contrast, the ale suspensions exhibited yield stress values which increased with increasing temperature. This increase in the case of the ale strain was both unexpected and notable. Since yield stress values are indicative of increased cell-cell interactions, this trend may be due to increased hydrophobic interactions (as the strength of these interactions increase in the temperature range examined). This explanation concurs with the general belief that the hydrophobic nature of ale yeast cell walls are responsible for the "top fermenting" characteristics of these cells. As well, hydrophobic interactions have recently been reported in ale cells by numerous authors (Hinchcliff et al., 1985; Amory, Rouxhet and Dufour, 1988; Mozes et al., 1989; Mestdagh, Rouxhet and Dufour, 1990).

ii. Time dependence - Aside from the examination of the steady shear behaviour, the rate of change in viscosity with time during shearing at a constant rate was also

examined. Figure II.4 shows typical "start up" curves for the flocculent lager yeast. A relatively large amount of variation was present in some of the data sets, especially at the low concentrations and shear rates.

If the time dependence of the shear stress or viscosity can be explained by the formation of doublets as discussed earlier, then appropriate transforms of the viscosity and time data (i.e., ln $[\eta_t-\eta_e]$ vs. t) should result in linear behaviour. Statistical examination of the transformed data at concentrations of 2.5 x 10^9 cells / mL and shear rate ranges from 4.98 to 150 s^{-1} confirmed this linear behaviour ($p<0.05$, $n\approx30$, $r^2\geq0.87$). No systematic variation of the residuals was observed with time. However, the range of shear rates that could be evaluated by Equation I.17 were restricted by two considerations. First, at high shear rates only limited data were collected due to the rapid rate of viscosity increase. Second, at low shear rates, low signal to noise ratios were encountered (especially in the case of the low viscosity, non-flocculent lager yeast). Where these two factors were not of concern, first estimates of the orthokinetic capture coefficient were calculated. In these calculations, volume fraction values were estimated from the settling experiment by extrapolating the final settled volume to 2.5 x 10^9 cells / mL and by dividing by 0.7, an approximate value for closest packing.

The derived capture coefficients should be considered pseudo-capture coefficients in view of the numerous assumptions made in their calculation. The value of the coefficients as shown in Figure II.5 are subject to uncertainty but the trend to an equilibrium value at the higher shear rates agrees with the findings of Duszyk, Kawalec and Doroszewski (1986). In their study, an electronic particle counter technique was used to examine lectin-bound thymus cells and they reported a constant value of 0.0007 for the capture coefficient over shear rates of 400 to 2400 s^{-1}. This value is of the same magnitude as

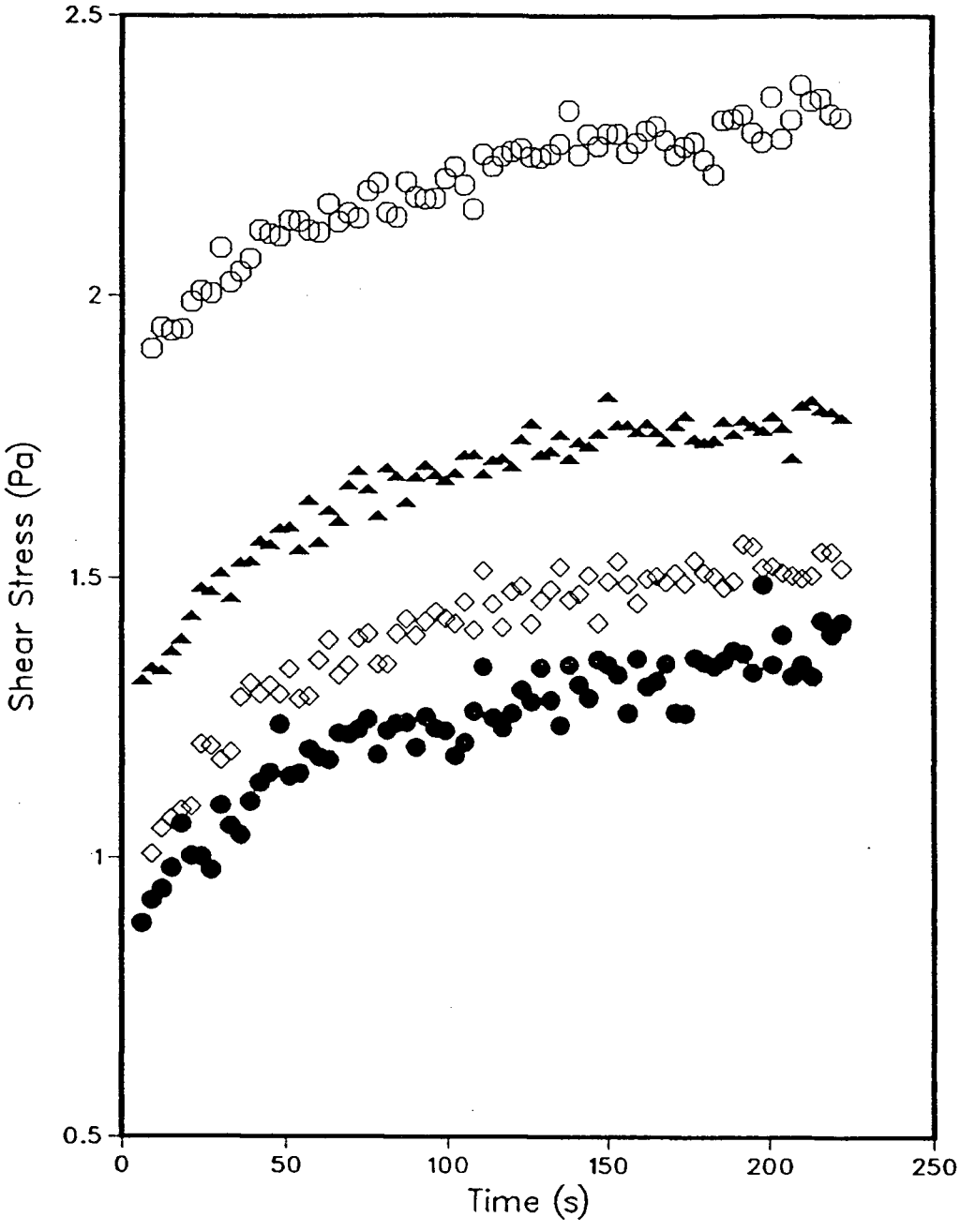

Figure II.4. Effect of a sudden application of shear on the shear stress of a shear-deflocculated, flocculent lager yeast suspension (o=146, ▲=75.8, ◊=40.3 and ●=24.2 s^{-1}, @5 ^{o}C and 2.5 x 10^{9} cells/mL).

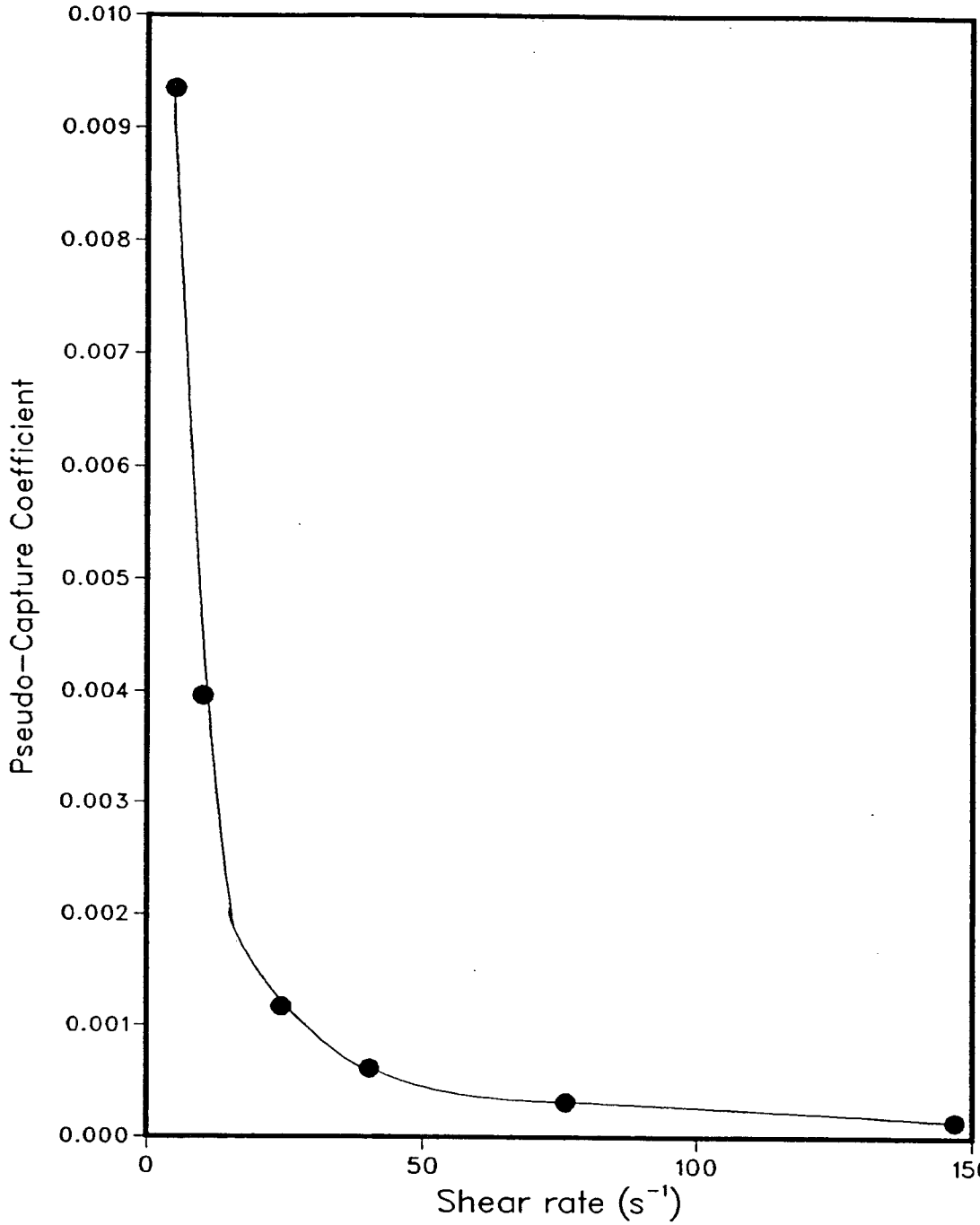

Figure II.5. Dependence of the pseudo-capture coefficient of a flocculent lager yeast on the rate of shear (@ 5 ^{o}C and 2.5 x 10^{9} cells/mL).

the values determined at the higher shear rates of this experiment. No other estimates of yeast orthokinetic capture coefficients have been reported to date.

4. Settling behaviour

Table II.4 displays the settling velocity of the three strains at each of the four concentrations examined as well as the final settled volume. Examination of the settling velocity of the yeast cell suspensions revealed that there were no substantial differences between the settling rates of the three strains although the settling velocities did decrease with increasing cell concentration. This finding agrees with collaborative studies of the ASBC (1978, 1986). They thus recomended visual examination techniques rather than settling type procedures for the determination of yeast flocculation. Additionally, relationships between interface settling velocity and particle concentration predicted by Ekdawi and Hunter (1985) were found to be not applicable.

5. Cone and plate rheometer examinations

During the second and final Phases of this research, a cone and plate viscometer was used to investigate the effect of different suspension media on four yeast strains (see Fig. II.1 & II.2). In general, most of the yeast suspensions exhibited Bingham flow behaviour in the medium and upper shear rate range examined. As no substantial differences were observed between duplicate runs or between up and down ramps, the data for each yeast strain and suspension medium were pooled prior to analysis. One suspension exhibited Newtonian flow behaviour while a few others had a paste-like consistency and corresponding high yield stresses. In these latter cases during the initial application of "controlled" shear rates, erratic rates of shear were observed. This behaviour may have been due to; 1) the rheometer being unable to control the amount of

Table II.4. Settling rates of brewing yeast cell suspensions at room temperature.

Yeast code*	Concentration (cells/mL) x 10^8	Settling rate (mm/min)	Coefficient of determination**	Final settled volume (%)
NFL	1.0	0.099	0.997	2.0
	2.5	0.101	0.997	4.5
	5.0	0.099	0.971	9.5
	10.0	0.075	0.998	21.0
FL	1.0	0.102	0.990	2.0
	2.5	0.092	0.986	6.0
	5.0	0.089	0.987	12.5
	10.0	0.081	0.983	23.0
NFA	1.0	0.122	0.996	3.0
	2.5	0.098	0.966	7.0
	5.0	0.084	0.974	12.5
	10.0	0.082	0.961	24.0

*Yeast codes refer to non-flocculent lager (NFL), flocculent lager (FL) and non-flocculent ale (NFA) yeast strains. **All regressions were significant ($p<0.05$, $n=8$).

torque directed to the sample or, 2) the yeast suspension first sticking and then slipping within the cone and plate fixture, or a combination of these two factors. When this occurred, the data were discarded and the term "too viscous to measure" (with this rheometric technique, i.e., TVTM) was used to describe the flow behaviour of these suspensions. Also, the onset of the Bingham flow behaviour or critical shear rate differed depending on the yeast strains and suspension media employed. Table II.5 displays the results of these cone and plate experiments.

It was apparent that the non-flocculent strains had lower yield stress values than the flocculent strains as observed in the earlier coaxial cylinder flow study. This observation was especially evident in Figure II.6 where the difference in flow behaviour between the flocculent and non-flocculent ale strains suspended in ale is evident. As well, these results indicated that the nature of the suspension medium can have a dramatic effect on the flow behaviour of these yeast suspensions. In examining these data one should be aware that the first 16 and the last four experiments were carried out with yeast collected at different times. Additionally, in the latter set of experiments, the accuracy of the suspended cell concentration measurement was improved. Due to these considerations only comparisons within experiments 1-16 or 17-20 should be made.

It can be stated that ethanol and KCl solutions both drastically increased the Bingham yield stress of flocculent ale and lager suspensions respectively. This observed increase was presumably due to either an increase in the binding strength of the lectin-like bonding or the DLVO-type interaction energies. The EDTA buffer also caused a reduction of observed Bingham yield stress values. This may be due to the chelation of the available calcium which would in turn, cause a reduction of lectin binding energies.

While the pH of the buffer had a substantial effect on the viscometric behaviour of the suspensions it was confounded by variations in ionic strength. Thus, few conclusions

Table II.5. Results of cone and plate viscometry.

Treatment*			Yeast strain**	Bingham Model		
No.	pH	Medium	Other		Yield stress (Pa)	Plastic viscosity (mPa·s)	r^{2***}
1	4.00	Buffer		NFL	0.1	6.0	0.999
2	4.00	Buffer		FL	2.4	22.5	0.998
3	4.00	Buffer		NFA	0.7	26.9	0.999
4	4.00	Buffer		FA	8.7	7.8	0.996
5	3.50	Buffer		FL	TVTM****		
6	3.50	Buffer		FA	51.0	43.0	0.998
7	5.00	Buffer		FL	1.7	11.4	0.994
8	5.00	Buffer		FA	35.7	38.5	0.873
9	5.50	Buffer		FL	7.7	5.9	0.994
10	5.50	Buffer		FA	56.5	135.	0.872
11	4.00	Buffer	0.5M KCl	FL	3.1	44.3	0.972
12	4.00	Buffer	0.5M KCl	FA	TVTM		
13	4.00	Buffer	0.01 M EDTA	FL	Newtonian Viscosity 2.8 (mPa·s)		
14	4.00	Buffer	0.01 M EDTA	FA	2.80	6.9	0.994
15	4.00	Buffer	5% EtOH	FL	TVTM		
16	4.00	Buffer	5% EtOH	FA	14.6	9.3	0.972
17	3.95	Lager		NFL	0.9	4.6	0.999
18	3.95	Lager		FL	1.7	11.5	0.996
19	4.00	Ale		NFA	1.2	5.9	0.997
20	4.00	Ale		FA	3.8	8.0	0.992

* The first 16 experiments were carried out with yeast samples gathered in Phase II of this research while the last four experiments were undertaken in Phase IV. (Thus different yeast samples were used in experiments 17-20). **Yeast codes refer to non-flocculent lager (NFL), flocculent lager (FL) and non-flocculent ale (NFA) yeast strains. ***All regressions were significant ($p<0.05$, $n\approx360$). ****TVTM - Too viscous to measure with this rheometric method.

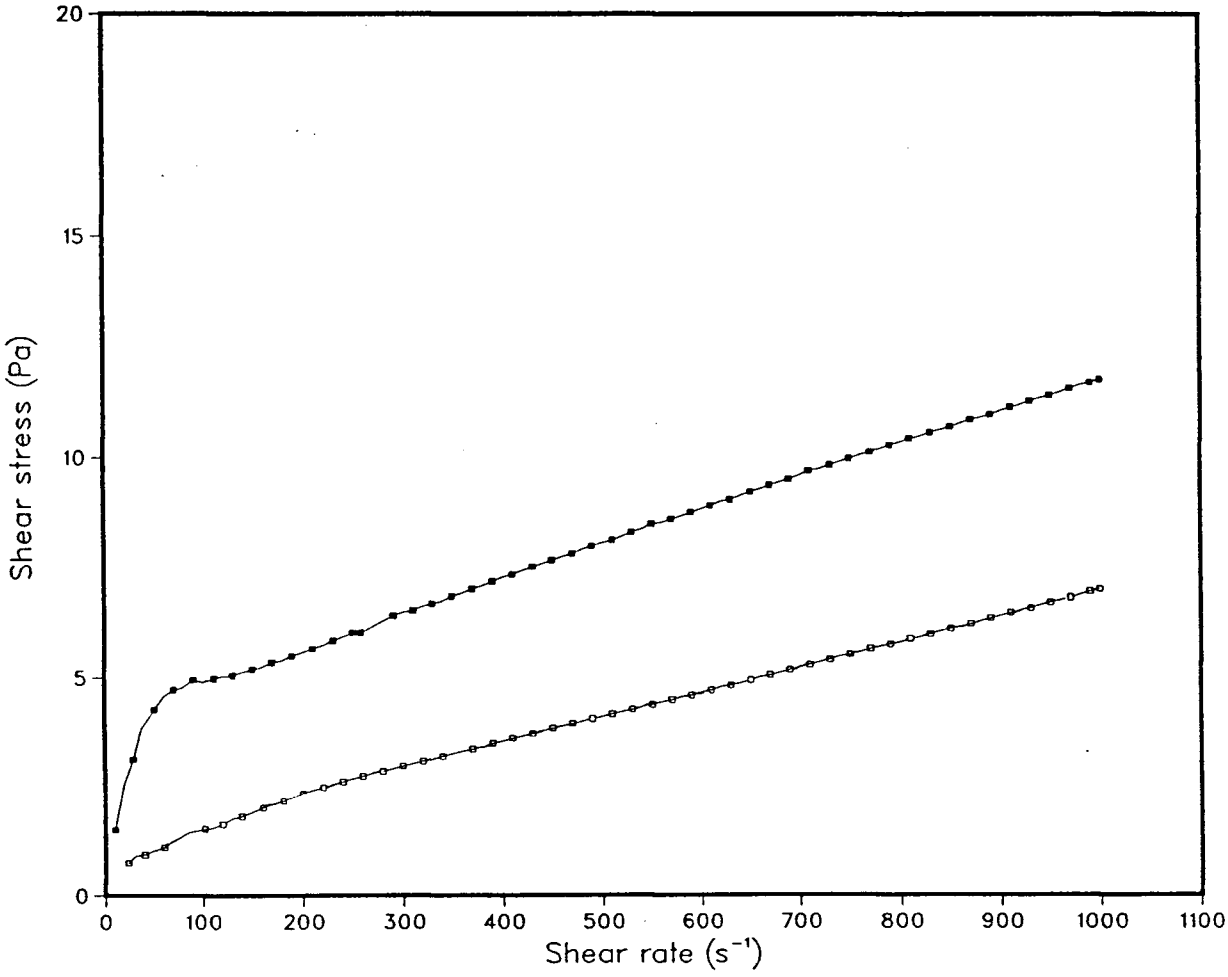

Figure II.6. Flow behaviour of flocculent (■) and non-flocculent (□) ale yeast suspended in ale and measured by cone and plate viscometry (@15 ^{o}C and 2.5 x 10^{9} cells/mL).

can be drawn from these data. The intent of shifting the pH and ionic strength of the buffer solutions was to alter the zeta potential of the cell wall, to measure this change in zeta potential and to relate it to the flow properties of the yeast suspensions. Unfortunately, equipment breakdowns prevented measurement of the cell zeta potential using the yeast strains sampled during this phase of the experiment.

The large change in the Bingham plastic viscosity observed in experiments 1-16 was not expected. Since these values were, according to Hunter, dependent on floc volume concentration, these changes may be due to floc size (and volume) changes or error inherent in the ASBC method of measuring cell concentration.

Inspection of the flow behaviour of the four yeast strains suspended in beer (Exp. 17-20 in Table II.5) lead to a number of observations. First, as previously observed, the flocculent strains showed higher yield stress values than their non-flocculent variants. The ale strains also tended to have higher yield stresses than the lager strains. These increased values were believed to be due, in part, to the presence of 4.70-4.88 % (v/v) ethanol.

Ethanol seemed to have a substantial effect on yeast flocculation in these experiments and this finding agrees with that of Amory et al. (1988). This increase may have been due to changes in the conformation of the lectin (i.e., protein) at the active site allowing for stronger binding. Alternately, the reduction of the dielectric constant may have reduced the double layer repulsion allowing for increased "DLVO" interactions. The importance of ethanol in flocculation suggests that the addition of 4.00-5.00% ethanol in future "standard" flocculation buffers would provide results that would more closely parallel the actual brewing yeast flocculation process.

6. Cell floc model

The flow behaviour of each yeast suspension examined could be described by either the Bingham or Newtonian flow model at shear rates above 100 s^{-1}. This observation, as well as the presence of curvilinear flow behaviour below a critical shear rate is explained by Hunter's "elastic floc model". If this model (Eq. I.10) is valid, it is interesting to consider which of the energy terms contribute most to the Bingham yield stress.

Hunter postulated that the individual flow units, or flocs, were present as either singlets or doublets within the Bingham flow regime. In sheared yeast suspensions one would expect these flocs to be individual yeast cells. It should also be recalled that the flocculent and non-flocculent cell variants are believed to differ only in their flocculation character and thus, could be expected to have similar cell wall structures. Therefore, the substantially different yield stresses between comparable[1] yeast suspensions were most likely due to variation in separation energy rather than differences in the deformation energy of the cells. While it is possible that the deformation energies contributed to yield stress values, differences in the energy required to separate cell doublets and the orthokinetic capture coefficient may account for the inter-strain differences observed in the Bingham yield stress values of the suspensions.

Close examination of the Bingham flow curves (Fig. II.3) shows a slight curvilinear deviation from the model. This trend may be due to either small increases in the deformation energies as the cells collide at higher velocities, or to variation in the orthokinetic capture coefficient. The constant value of the capture coefficient in the shear rate region of 100-1000 s^{-1} shown in Figure II.5 suggests that the deformation energy may be the cause of this small curvilinear behaviour.

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[1] E.g., compare values of flocculent and non-flocculent strains of ale and lager yeasts @ 15 ^{o}C and 2.5 x 10^{9} cells / mL, (Tables II.3 & II.5).

It would appear that Hunter's elastic floc model fitted the flow behaviour of these yeast suspensions. However, it appears that the energy needed to separate doublet cells, rather than the floc deformation energy was the dominant contributor to the Bingham yield stress. In recognition of this energy contribution, the phrase, "cell floc model" has been proposed to describe the flow of this type of cellular suspension (Speers, Durance and Tung, 1989). However, without further supporting data, this model should be employed with caution.

Examination of the microstructural nature of the yeast flocs while in shearing flow as well as data on the rate of orthokinetic flocculation would further elucidate this proposed cell floc model. This information gathered in Phases II-IV of this research, will be considered in the last chapter of this thesis.

D. Conclusions

Research findings presented in this chapter demonstrated that the flocculent characteristics of brewing yeast strains had an important influence on their suspension flow behaviour. As cell concentrations increased, suspension flow properties became increasingly non-Newtonian. No evidence of slip was detected in these initial coaxial cylinder viscometer studies.

A modification of the elastic floc model, termed the cell floc model, has been proposed to explain the Bingham flow behaviour of these suspensions. The presence of a Bingham yield stress in these suspensions was believed to be a function of the orthokinetic capture coefficient, cell volume and the energy to break up doublet cells. Increasing temperature tended to increase the Bingham yield stress in ale strains (possibly due to hydrophobic interactions) and to reduce yield stress in lager strains. A

semi-empirical explanation for the viscosity increase of deflocculated cell suspensions has also been presented, allowing for the estimation of pseudo-capture coefficients.

High shear blending of the strains followed by viscometric evaluation failed to show any signs of rheodestruction. This finding indicates that; 1) no fimbriae were present in the strains tested or 2) at the shearing rate used, no flocculation-promoting fimbriae were destroyed.

Finally, examination of the flow behaviour of yeast strains suspended in beer revealed that suspensions of flocculent strains showed higher yield stress values than their non-flocculent variants and ale strain suspensions tended to have higher yield values than the lager strains. As well, the addition of ethanol to the pH 4.00 buffer resulted in a large increase in yield stress of the flocculent lager strain. This last observation was believed to reflect the influence of ethanol on the cell binding process and has important implications for future measurements of yeast flocculation.

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CHAPTER III

COLLOIDAL STUDIES

A. Introduction

As mentioned previously, very little research has been undertaken on the fundamental properties of flocculating yeast suspensions. Carefully controlled studies to gather information on the rates of flocculation of yeast cells in defined shear fields are needed in order to gain a clear understanding of this process. Fundamental investigations such as these, coupled with information about cell surface charges might aid in resolving some of the controversies discussed in Chapter I.

The purpose of this chapter is to present and discuss findings regarding; 1) the effects of cell strain and shear rate on the rate of orthokinetic flocculation and 2) the effect of cell strain and suspension media on yeast cell surface charges.

1. Orthokinetic flocculation

Brewing researchers have recently noted the effect of *turbulent flow* conditions on yeast flocculation (Stratford and Keenan, 1987, 1988; Stratford, Coleman and Keenan, 1988; Stratford and Wilson, 1990). However, the author is not aware of any research reporting flocculation rates of brewing yeasts subjected to *steady* shear in laminar flow fields. Other cells such as sheep leucocytes (Wilkins, Ottewill and Bangham; 1962), embryonic chicken cells (Curtis, 1969, 1970; Hornby, 1973a, b), algae and bacteria (Hahn and Eppler, 1976), rat thymocytes (Duszyk and Doroszewski, 1986; Duszyk, Kawalec

and Doroszewski, 1986) and hybridoma cells (Aunins and Wang, 1990) have been subjected to steady shear and their flocculation rates measured.

The expression describing rate of flocculation in a defined steady shear field was first described by von Smoluchowski (1917) and modified by van de Ven and Mason (1977) as discussed in Chapter I:

$$(N_t / N_o) = e^{-(4\alpha_o \dot{\gamma} \varphi_o / \pi) t} \qquad (I.8)$$

where N_t is the number concentration of particles at time t, N_o is the initial number concentration of particles, α_o is the orthokinetic capture coefficient, $\dot{\gamma}$ is the shear rate and φ_o is the initial volume fraction of particles.

The orthokinetic capture coefficient is the key variable which determines the flocculation ability of a yeast strain in a given environment. The value of α_o is determined by the forces acting on the cells as they approach one another in shearing flow. Essentially, two theories have been developed which estimate the values of the capture coefficient. The first theory considers the DLVO forces acting on the cells as they approach each another (van de Ven and Mason, 1977) while the second considers the effect of lectin-like binding structures present on the cell wall (Bell, 1981).

In van de Ven and Mason's 1977 treatment, two dimensionless numbers, C_a and C_r, (the attractive forces and repulsive forces, respectively) were presented:

$$C_a = A / 36 \pi \eta \dot{\gamma} r^3 \qquad (III.1)$$

$$C_r = 2 \varepsilon \varepsilon_o \psi^2 / 3 \eta \dot{\gamma} r^2 \qquad (III.2)$$

where A is the Hamaker function, η is the viscosity, r is the cell radius, ε_0 is the permittivity of free space and ε is the dielectric constant of the medium, ψ is the surface potential (assumed to equal the zeta potential). Van de Ven and Mason (1977) calculated and graphically presented the dependence of the capture coefficient on the logarithm of C_a at various ratios of C_r to C_a, different double layer thickness as described in Chapter I and the London wavelength (the wavelength of interaction, see Hunter, [1987] for a detailed explanation). Using this method they showed that at high ratios of C_r / C_a, the orthokinetic capture coefficient could decline to zero and then increase from this minimum as the shear rate was further increased. Duszyk and Doroszewski (1986) confirmed van de Ven and Mason's 1977 findings and recalculated capture coefficients at conditions encountered in the shearing flow of cellular suspensions.

While there are a number of uncertainties in the values used in the preceding DLVO calculation of the capture coefficient, perhaps the greatest uncertainty lies in assigning a value to the Hamaker function. As noted in Chapter I, the function is taken to be a constant but values varying from 10^{-19} to almost 10^{-24} J have been used. A summary of Hamaker values used in, or calculated from, cell flocculation studies is provided in Table III.1.

In a theoretical treatment of lectin-mediated adhesion, Bell (1981) argued that the the orthokinetic capture coefficient was dependent upon and proportional to $\dot{\gamma}$ at low shear rates and $\dot{\gamma}^2$ at high rates of shear. In contrast to Bell's theory, Duszyk, Kawalec and Doroszewski, (1986) measured the lectin-mediated aggregation of thymus cells, and found that the orthokinetic capture coefficient was independent of the rate of shear at high lectin concentrations. At low lectin concentrations, however, the orthokinetic capture coefficient was shown to be dependent on the shear rate. In their study, the cell

Table III.1. Values used for the Hamaker function in studies from the literature*.

System examined	Value of the Hamaker function (J)	Reference
biological systems	$1 - 5 \times 10^{-21}$	Brooks et al., 1967
chick embryo cells/ Ca & Mg-free Hanks saline	$0.035 - 1.9 \times 10^{-23}$	Hornby, 1973a
biological cells / aqueous systems	4×10^{-21}	Visser, 1976
theoretical cell models	$1-10 \times 10^{-21}$	Nir, 1976
biological cells	$4 - 8 \times 10^{-21}$	Lips and Jessup, 1979
red blood cells / saline	$5-8 \times 10^{-21}$	Parsegian and Gingell, 1980
red blood cells / saline	7×10^{-23}	Lerche, 1983
aqueous systems	4×10^{-19}	Ho,1986
hybridoma cells	10^{-19}	Aunins and Wang, 1990

*A constant value for Hamaker function is normally employed by most researchers while recognizing this practice results in a first approximation of the Hamaker function only. (See Chapter I for a discussion of this topic).

concentration per unit volume (N_t) was determined from counts of samples collected from the outflow of a capillary tube. Bongrand et al. (1979) demonstrated that during this type of Poiseuille flow, the mean rate of shear ($\dot{\gamma}_{av}$) is dependent on the flow velocity (V) and the tube radius (r_t):

$$\dot{\gamma}_{av} = 8\ V / 3\ r_t \qquad \text{(III.3)}$$

Using the above expression as well as the data of Duszyk et al. (1986), the average rates of shear at which they carried out their experiments can be shown to be 400, 1500 and 2400 s^{-1}. The limited amount of "aggregation" data available at low lectin concentrations indicate that under their experimental conditions, orthokinetic capture coefficients actually increased with increasing mean shear rates. However, since this finding is based on measurements at only three mean shear rates, no firm conclusions can be drawn regarding the shear rate dependence of lectin-mediated cell flocculation.

2. Cell surface charge

In contrast to the paucity of information about rates of flocculation, various brewing scientists have measured electrophoretic mobilities and zeta potential of brewing yeast cells (Eddy and Rudin, 1958; Fisher, 1975; Beavan et al., 1979; Amory, Rouxhet and Dufour, 1988; Lawrence et al., 1989; Bowen and Cooke, 1989). The purpose of these studies was to understand the role of phosphate and carboxyl groups in the flocculation process. It is noteworthy that none of these researchers considered in detail the attractive forces operative between adjacent yeast cells as discussed in Chapter I.

In the first study of brewing yeast surface charge, Eddy and Rudin (1958) concluded that, "the majority of cell surface charges play no direct part in flocculation". They also

noted that the flocculation abilities of cells increased and their surface charges decreased during fermentation. In further research, Beavan et al. (1979) concluded that: 1) flocculation occurred when the mobility attributable to carboxyl groups exceeded 0.5 x 10^{-8} m^{-2} V^{-1} s^{-1} and; 2) the amount of cell surface phosphate groups declined with cell age and the level of carboxyl charged groups increased. Amory et al. (1988) found that the zeta potential at pH 4.00 could be correlated with the level of surface phosphorus. Interpretation of the data of Bowen and Cooke (1989) was complicated by substrate interference of their dipped-cell; however, further research from that laboratory (Lawrence et al., 1989) demonstrated that the zeta potential of cell surfaces declined during both ale and lager fermentations.

While prediction of DLVO forces operative between cells is complex and uncertain, estimation of these forces (as detailed in Chapter I) would help determine the relative importance of electrostatic forces in brewing yeast flocculation. In order to compare the values of the zeta potential (ζ) and electrophoretic data reported for yeast cells, one can employ the Smoluchowski form of the Henry equation. Use of this form of the Henry equation to examine electrophoretic data is valid since the ratio of the particle radius to the double layer thickness exceeds 100 (Shaw, 1980):

$$\zeta = (\mu \eta) / (\varepsilon \varepsilon_0) \qquad \text{(III.4)}$$

where μ is the electrophoretic mobility. In comparing zeta potential values of different studies, it is also important to consider the temperature at which the mobility was measured. The type of medium in which the yeast is suspended would also be expected to influence observed zeta potential of the cells.

B. Materials and Methods

Measurement of the orthokinetic rate of yeast cell flocculation and cell zeta potentials were undertaken in Phases II and IV (refer to Fig. II.1) of this research. The cells to be used were sampled and washed according to the procedure described in Chapter II (refer to Fig. II.2).

In the orthokinetic flocculation experiments, the four strains of yeast cells were examined after suspension in various media including commercial samples of decarbonated lager and ale used previously. For the zeta potential determinations, the four strains were measured while suspended in the Na-acetate / Ca buffer at pH 4.00. As well, the zeta potentials of the two flocculent strains were measured in the Na-acetate / Ca buffers at pH values of 3.50, 4.00, 5.00 and 5.50 and in the Na-acetate / Ca pH 4.00 buffer system containing either 0.5 M KCl or 5% (w/w) ethanol. These two strains were also suspended in the pH 4.00 Na-acetate buffer system (without calcium) containing 0.01 M EDTA, and their zeta potentials determined. Finally, zeta potentials of all four strains were measured after suspension in lager (NFL and FL) and ale (NFA and FA) beers (see Table II.1).

1. Orthokinetic flocculation

The rate of orthokinetic flocculation was measured by taking a cell suspension of 5 x 10^7 cells / mL (determined by a standard haemocytometric method [ASBC, 1976]) and de-flocculating the cells via repeated shearing through a 5 mL syringe equipped with a 38 mm long 21 gauge needle. Measurements of the rate of flow and syringe diameter were used to demonstrate that turbulent flow occurred within the syringe. A sample of the suspension was then loaded onto the flat platen of a Carri-Med CS 500 rheometer (Carri-

Med Ltd., Dorking, Surrey, GBR) equipped with a 4^{o}, 4.0 cm diameter cone. Use of the cone and plate fixture allowed the entire sample to be subjected to a constant shear rate. All samples were allowed to equilibrate at 15 ^{o}C for 20 s and then sheared at 200 s^{-1} for times of up to 30 min. At set periods, the cone and plate fixtures were separated and one sample carefully taken from the plate and one from the cone tip using the "bulb" end of a clean disposable Pasteur pipette. Each sample was placed on a haemocytometer slide and the cover-slip gently lowered over the suspension. Brightfield slide micrographs were then taken of the haemocytometer slides. These micrographs were then projected with either a 35 mm slide projector or a microfiche reader for measurement of N_t, the number of flocs per mL. Using these data, calculations of the flocculation rate constant ($4\alpha_o\gamma\varphi_o/\pi$) and orthokinetic rate constant (α_o) were completed according to equation I.8 discussed earlier in this chapter.

In the first set of experiments carried out in the second phase of this study, the rates of cell flocculation were measured at times of 0, 5 and 30 min. These experiments were undertaken with the four yeast strains (NFL, FL, NFA, FA) described previously, in the buffer systems detailed in Table II.1 (Treatments 1-16).

In a second set of experiments carried out in Phase II of this research, the four yeast strains were cultured on YEPD medium (20 g/L dextrose, 20 g/L peptone, 10 g/L yeast extract and 20 g/L agar [BDH Chemicals Canada, Toronto, ON] according to van Uden and Sousa, 1957) for 5 days at 30 ^{o}C. An isolated colony from each strain was then grown in 200 mL of YEPD broth at 30 ^{o}C in a 250 mL shaker flask for 7 days. The flasks were shaken at 20 revolutions per min in a shaker bath (Eberach Corporation, Ann Arbor, MI). The yeast cells were then washed according to the procedure described earlier and the rates of orthokinetic flocculation measured at times of 0, 5, 10 and 15 min and 200 s^{-1}.

Finally, in the fourth and last phase of this study, the four yeast strains were suspended in either decarbonated lager (NFL and FL) or ale (NFA and FA) as detailed in Chapter II (see Table II.1 - treatments 17-20 & Table II.2). The number concentration of flocs was then determined after shearing at 200 s^{-1} for times of 0, 5, 10 and 20 min. using the Carri-Med cone and plate rheometer. In contrast to the previous experiments, a cell concentration of 2.5 x 10^7 rather than 5 x 10^7 cells / mL was employed and the initial cell concentration was determined from suspension volumes of 0.16 mm^3 rather than 0.02 mm^3.

2. Zeta potential determination

Zeta potential determinations were carried out on the four brewing strains using a Pen Kem System 3000 Automated Electrokinetic Analyzer (Pen Kem Inc., Bedford Hills, NY). The electrophoretic velocities of particles were measured by using a He-Ne laser to project the image of the moving particles on to a rotating grating. A signal detector then sensed a fluctuation in the intensity of the light transmitted through the rotating grating. Since the frequency of these transmitted light fluctuations have been shown to be proportional to the electrophoretic mobility of the particles (Goetz, 1980), the zeta potential values (using the Smoluchowski modification of the Henry Equation [Eq. III.4]) could be calculated by means of the system software in the microcomputer interfaced with the Electrokinetic Analyzer.

The size of the tracking window (i.e., the measured volume within the electrophoretic chamber) in the Automated Electrokinetic Analyzer was 1.35 mm x 0.22 mm x 2.0 μm or 0.6 x 10^{-6} mL (Phillips, 1990). In order to measure the zeta potential of the cells, suspensions of approximately 5 x 10^6 cells / mL were pumped into the electrophoresis chamber and either eight or sixteen mobility spectra measured. The

sample in the chamber was then pumped out and a new sample measured as detailed above. This procedure was repeated three times in order to obtain a "reading". A minimum of seven such readings was taken for each combination of suspension medium and yeast tested. Using the above experimental protocol, measurement difficulties due to particle settling were minimized.

Zeta potential values were determined at 15 ^{o}C and their values adjusted to a reference temperature of 25 ^{o}C by correction of the viscosity and dielectric constant terms of Equation III.4. The following values of the dielectric constant for both aqueous and media containing ethanol (Akerlof, 1932) were used:

- 82.0, H_2O @ 5 ^{o}C,
- 78.5, H_2O @ 25 ^{o}C,
- 79.5, 5% ethanol @ 5 ^{o}C,
- 75.7, 5% ethanol @ 25 ^{o}C.

A similar viscometric correction was carried out using published values (Weast, 1984). Other substances present in the suspending media were assumed to have a minimal effect on solution viscosities and dielectric constants. Further data analyses were carried out with version 5.0 of the Systat® - Sygraph® software analysis packages (Wilkinson, 1989a, b).

C. Results and Discussion

1. Orthokinetic flocculation

Examination of the orthokinetic flocculation data collected in Phase II of this research yielded limited information as to the rate of flocculation of different yeast strains

suspended in various media. While flocculation was observed in samples sheared for 5 and 30 min, it was concluded that the majority of cell flocculation occurred early in the shearing period. As well, a high level of "scatter" was noted in the data.

In the second set of experiments the yeast strains that were isolated on YEPD agar and grown in YEPD broth were used. During the period of orthokinetic shear, a clear decline in the number of flocs was observed. When the corrected Smoluchowski equation (Eq. I.8, van de Ven and Mason, [1977]) was fitted to the orthokinetic data, it was found to explain most of the variation of floc concentration with shearing time as displayed in Table III.2. This table shows the flocculation rate constant and the coefficient of determination for the fitted functions obtained from the data of the four yeast strains cultured in the YEPD media and broth. Estimations of the orthokinetic capture coefficient in YEPD were not carried out as accurate estimates of the size of the yeast cells cultured in this media were not available.

Since the non-flocculent cells were variants of their respective flocculent strains, it was assumed they were of similar size. Based on this assumption, it is apparent from Table III.2 that the flocculent strains tended to have higher flocculation rates as one might expect. As differences in the sizes of ale and lager yeasts are known to exist, it is difficult to compare the rate constants of these strains.

In the final set of flocculation experiments carried out with the four yeast strains suspended in the ale and lager beer, a decrease in the floc concentration with shearing time was also evident. However, in three of the four strains a large amount of variation in the data was observed as evidenced by generally lower coefficient of determination values (see Table III.2). These differences were believed to be due in part to the use of lower cell concentrations (in contrast to the YEPD experiments). As well, excess sample may have been applied to the cone and plate fixture resulting in the mixing of unsheared

Table III.2. Orthokinetic flocculation rates of brewing yeast cell suspensions sheared at 200 s^{-1} and 15 °C at concentrations of 2.5 and 5 x 10^7 cells / mL.

Yeast code*	Initial cell conc -entration (cell/mL) x 10^7	Media**	Flocculation rate constant*** ($4\alpha_o \dot{\gamma} \varphi_o/\pi$, min^{-1})	Orthokinetic capture coefficient (α_o)	Coefficient of determination (r^2)****
NFL	5	YEPD	0.047	-	0.98
FL	5	YEPD	0.085	-	0.85
NFA	5	YEPD	0.037	-	0.99
FA	5	YEPD	0.100	-	0.96
NFL	2.5	Lager	0.015	0.25×10^{-3}	0.56
FL	2.5	Lager	0.043	1.0×10^{-3}	0.61
NFA	2.5	Ale	0.062	0.75×10^{-3}	0.87
FA	2.5	Ale	0.070	1.0×10^{-3}	0.98

*Yeast codes refer to non-flocculent lager (NFL), flocculent lager (FL) and non-flocculent ale (NFA) yeast strains. **YEPD (Yeast Extract, Peptone Dextrose). For Ale and Lager composition see Table II.1. ***For description of symbols refer to Equation I.8. **** All regressions were significant ($p<0.05$, $n=10$).

sample (i.e., the portion of the suspension remaining directly outside of the cone and plate) with the sheared suspension during the sampling procedure. In the case of the flocculent ale yeast (FA) a strong flocculation trend was evident as displayed in Figure III.1.

It is interesting to note that the linear trend depicted in Figure III.1 represented slightly more than a four-fold decrease in the original floc concentration. While one might expect a deviation from the orthokinetic rate law (Eq. I.8) after extended shearing, no such deviation was observed. This observation agrees with simulations undertaken by Gregory (1982).

As some limited information was available on the cell sizes of the four strains from scanning electron microscopy (SEM) observations of cells suspended in 5% (w/w) ethanol (discussed in the following Chapter), it was possible to calculate orthokinetic capture coefficients for the flocculation of the four yeast strains suspended in ale and lager beer. As relatively few cells were measured by SEM the estimates shown in Table III.2 should be viewed with caution (i.e., volume fraction, coefficient of variation ≈ 0.23, n=50). While the capture coefficients shown in Table III.2 are similar to literature findings, due to the uncertainty in the volume fraction values used in these calculations, one can only state that these observations do not disagree with existing literature findings.

While no other directly comparable orthokinetic capture coefficients are available, these results are within the same order of magnitude, (but twice as high) as the pseudo-capture coefficient value determined from stepped-shear experiments undertaken in the rheological portion of this research (see Fig. II.5). Although it is reasonable to assume that the orthokinetic values would be higher in this experiment, because of ethanol content of the yeast suspension media in the study, further comment on these differences would be speculative.

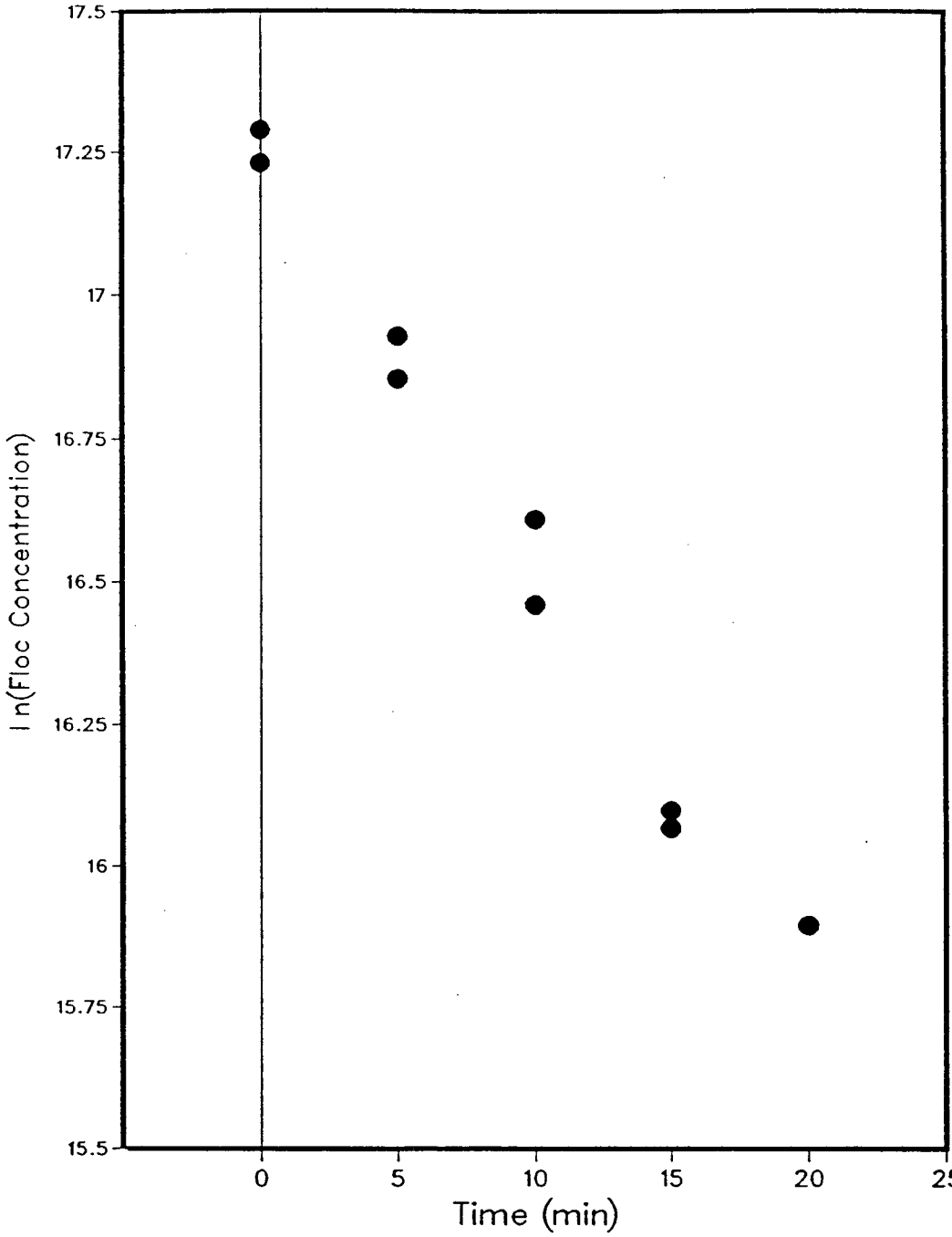

Figure III.1. Effect of shearing time on the number concentration of flocs of flocculent ale yeast in ale (@ 15 ^{o}C, 2.5 x 10^{7} cells/mL and 200 s^{-1}).

Ideally, measurement of the pseudo and true orthokinetic capture coefficients should have been carried out in concert. However, the equipment necessary to undertake such a program were located in separate laboratories, in Vancouver and Halifax. As well, limited yeast stability and finite financial resources precluded such simultaneous experimentation.

2. Zeta potential

While measuring the zeta potential values of the cells treated as described earlier, problems were encountered with the measurement of the ale and lager strains suspended in the KCl - Ca / Na acetate buffer at pH 4.00. Visual examination of these flocs within the electrophoretic chamber revealed that excessive flocculation and settling of the cells occurred and, as a result, zeta potential values for the yeast cells suspended in this KCl - buffer were not measured.

Results of the balance of the zeta potential determinations (18 in total) are displayed in Figures III.2 and III.3. It is noteworthy that each measurement by the Electrokinetic Analyzer represents a mean of the zeta potentials of a large number of cells viewed in the tracking window.

The plots depicted in Figures III.2 and III.3 are known as box plots and were invented by Tukey (Wilkinson, 1989b). The horizontal bar in these plots represent the median of the dataset, in this case zeta potential values for each suspension treatment. The upper and lower ends of the box (or hinges, UH and LH respectively) represent medians of upper and lower subgroups of the data split by the median. A high inner fence (HIF) and low inner fence (LIF) beyond which possible outliers lie are defined as:

$$HIF = UH + 1.5\ (UH - LH) \qquad \text{(III.5a)}$$

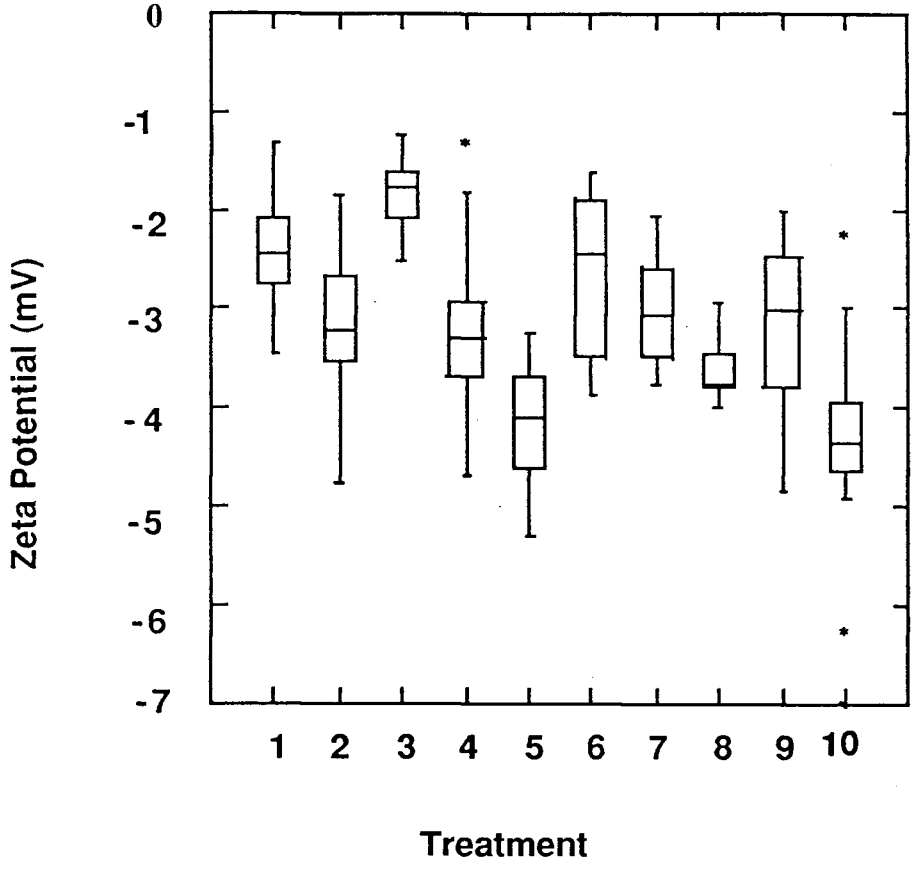

Figure III.2. Range of zeta potential values for four yeast strains in calcium-containing buffers at pH 3.5 to 5.5[1, 2]:

Treatment 1 - NFL pH 4.0,	Treatment 6 - FA pH 3.5,
Treatment 2 - FL pH 4.0,	Treatment 7 - FL pH 5.0,
Treatment 3 - NFA pH 4.0,	Treatment 8 - FA pH 5.0,
Treatment 4 - FA pH 4.0,	Treatment 9 - FL pH 5.5,
Treatment 5 - FL pH 3.5,	Treatment 10 - FA pH 5.5.

[1] The horizontal bars in these plots represent the median of zeta potential values while the upper and lower ends of the box (or hinges) represent medians of upper and lower subgroups of the data as split by the median. The ends of the vertical lines extending from the box represent the range of data points that are not defined as "outliers". Refer to Wilkinson (1989b) for a further explanation.

[2] For details of the yeast and suspending media used see Tables II.1 and II.2.

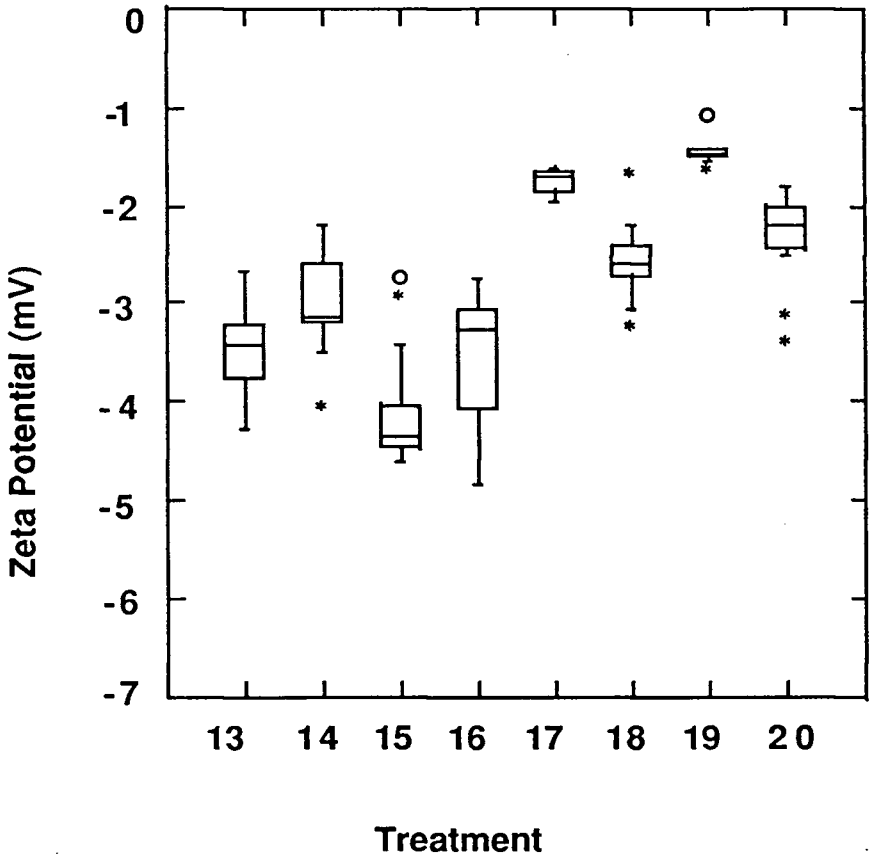

Figure III.3. Range of zeta potential values for four yeast strains in various media[1, 2]:

Treatment 13 - FL EDTA	Treatment 17 - NFL Lager,
Treatment 14 - FA EDTA,	Treatment 18 - FL Lager,
Treatment 15 - FL EtOH,	Treatment 19 - NFA Ale,
Treatment 16 - FA EtOH,	Treatment 20 - FA Ale.

[1] The horizontal bars in these plots represent the median of zeta potential values while the upper and lower ends of the box (or hinges) represent medians of upper and lower subgroups of the data as split by the median. The ends of the vertical lines extending from the box represent the range of data points that are not defined as "outliers". Refer to Wilkinson (1989b) for a further explanation.

[2] For details of the yeast and suspending media used see Tables II.1 and II.2.

$$LIF = LH - 1.5(UH- LH) \quad \text{(III.5b)}$$

High and low outer fences (HOF and LOF, respectively) are defined as:

$$HOF = UH + (UH- LH) \quad \text{(III.6a)}$$

$$LOF = LH - (UH- LH) \quad \text{(III.6b)}$$

Data points that lie between the inner and outer fences are denoted by an asterisk while data points that lie outside the outer fences are represented by circles. The vertical line in the box represents the range of data points which lie within the inner fence.

While these box plots are a new and perhaps an initially confusing way of presenting the data, they are useful to portray information on non-normal distributions. A statistical test for non-normality of the data - the Kolmgorov-Smirnov test - was carried out on each dataset and the logarithmic transforms of these datasets. In each case, the test indicated that the data were non-normally distributed ($p<0.5$). While based on limited sample numbers (from 7 - 30, mode=20), one would expect the distribution of these means[3] to approximate the normal distribution according to the central limit theorem (Zar, 1974).

The finding of non-normally distributed zeta potentials is not unreasonable since these yeast samples had gone though 50-100 cell divisions (after isolation) and might be expected to have exhibited substantial variation. While each of the yeast types used in this study was technically a *pure strain* (i.e., arising from one isolated colony), this term maybe somewhat of a misnomer in the case of industrial scale fermentations.

Examination of the median zeta potential values presented in Figures III.2 and III.3 demonstrated that yeast strain and suspension media exerted only a small effect on the

[3] Each zeta potential value is a mean of three readings which, in turn, consist of 8-16 measurements of a "swarm" of cells in the tracking window.

value of yeast cell zeta potential. These data indicate that in fact, the cells of the non-flocculent strains tended to have fewer negative surface charges, than their flocculent variants as tested by the Mann-Whitney test (treatments 17-20, $p<0.001$, Wilkinson, 1989a). This finding is contrary to the DLVO explanation of colloid flocculation. The ethanol treatment also tended to increase the surface charge on the flocculent lager yeast strain ($p<0.05$).

The excessive flocculation of cells in the KCl treatment of the flocculent variants of ale and lager strains may have been due to the high ionic strength (0.5 M KCl) resulting in the compression of the ionic double layer surrounding the cell. This would then permit the "classic DLVO" flocculation of the yeast cells as observed in the electrophoretic chamber.

In order to compare the zeta potential values reported here to literature reports of brewing yeast electrophoretic mobility one must first convert previous findings to zeta potential values. Using the Henry equation (Eq. III.4) and assuming that the viscosity and dielectric constants were equal to those of water, zeta potential values calculated from the data of Eddy and Rudin (1958), Fisher (1975) and Beavan et al. (1979) are equal to -1.3 to -15.4, -3.2 and -2.3 to -8.6 mV, respectively (at a pH 4.00 and ionic strength of 0.05 M). Lawrence et al. (1989) reported cell zeta potentials of -8.0 mV for an ale yeast fermented 70 h and -3.5 mV for a lager yeast after 100 h of fermentation. Thus, the zeta potentials determined in this study are of the same order of magnitude as the literature values.

Using this cell surface charge information, it was possible to estimate theoretical values of the DLVO capture coefficient (Duszyk and Doroszewski, 1986). Assuming "typical" parameters ($A=2.5 \times 10^{-21}$ J, $\eta=1.0$ mPa·s, $\psi=\zeta=4$ mV, $\dot{\gamma}=200$ s^{-1}, $r=3.5$ μm, $\varepsilon=81$ and $\varepsilon_o=8.85 \times 10^{-12}$ J^{-1} C^2 m^{-1}) repulsive forces would dominate during the

approach of cells in shearing flow and the orthokinetic capture coefficient would have a value of near zero ($\alpha_o << 0.0001$, Fig. 3, Duszyk and Doroszewski, 1986). With measured experimental values of capture coefficients equal to or greater than 0.0002, one can conclude that binding mechanisms other than that described by the DLVO theory are important in brewing yeast flocculation.

The obvious alternate cell binding mechanism is of course, the lectin-like aggregation (LLA) model of Miki et al. (1982) described in Chapter I. In order to compare the measured orthokinetic capture coefficients measured here with Bell's (1981) LLA method of calculating lectin mediated orthokinetic capture coefficients, information on receptor (i.e., lectin) density, mobility and bond formation rates are needed. Unfortunately, this information is not available for brewing yeast cells. Without this information, one can only state that Bell's method of calculating LLA-type capture coefficients may agree with the observed data.

E. Conclusions

Studies of the colloidal nature of brewing yeast flocculation described in this chapter yielded a number of interesting observations. For the first time, the orthokinetic rate of flocculation of brewing yeast cells has been modelled by a first order equation, as predicted by fundamental colloid theory. While subject to considerable variation, measurement of the rate constants lead to the calculation of orthokinetic capture coefficients. Measured cell zeta potential values generally agreed with literature findings but could not be employed in the DLVO model of colloid flocculation to explain measured orthokinetic capture coefficient values.

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CHAPTER IV

FLOC MICROSTRUCTURE

A. Introduction

The purpose of this chapter is to report the results of investigations on the microstructure of brewing yeast cell flocs. These flocs were examined using both brightfield light microscopy and scanning electron microscopy (SEM) in Phases II, III and IV of this study as detailed in Figure II.1. While details of our understanding of the "fine" (i.e., electron microscopic) structure of brewing yeast cell walls were commented on in Chapter I, it is worth reviewing available information on the gross microstructure of brewing yeast cell flocs.

When examining the gross morphology of brewing yeast flocs, researchers have been concerned with the packing density or number of cells within the floc. Brohan and McLoughlin (1984) examined the structure of brewing yeast flocs while in (presumably) turbulent flow and reported the following relationship:

$$\rho_{fl} - \rho_m = a / (D / 1.0)^b \quad (\text{or} = aD^{-b}) \qquad \text{(IV.1)}$$

where ρ_{fl} and ρ_m are the density of the floc and the suspending medium, a and b are parameters and D is the floc diameter. Recently, the structure of colloidal aggregates have been modelled as fractals and have been shown to possess the property of scale invariance (Sander, 1986). That is, they have a similar structure when viewed at different

size scales. This theory can be employed to relate the number of cells in a floc (N) to the floc diameter (D):

$$N = \alpha D^{\beta} \qquad \text{(IV.2)}$$

where α is the fractal constant and β is the fractal dimension. Hunt (1985) applied fractal theory to the structure of settling yeast flocs and reported the relationship:

$$(\rho_{fl} - \rho_m) = 6 \alpha V_c (\rho_c - \rho_m) / \pi D^{3-\beta} \qquad \text{(IV.3)}$$

where V_c is the settling velocity of the floc and ρ_c is the density of the cell. Letting the term $(6 \alpha V_c(\rho_c - \rho_m)/\pi)$ be constant, then the fractal dimension (β) can be equated to the value 3 - b (from Eq. IV.1). Values of β calculated from the data reported by Brohan and McLoughlin (1984) range from 0.29 to 1.84 depending on the flocculent nature of the yeast type. Data from Hunt (1985) reported by Davis and Hunt (1986) indicated that the fractal dimension β ranged from 1.75 to 2.25. As one might expect sheared flocs to be less tightly packed, it is understandable that Davis and Hunt (1986) found higher fractal dimensions for their slowly settling flocs.

Other than these studies on the fractal dimension of yeast flocs, little is known about size and structure of yeast flocs when subjected to shear. Hunter's elastic floc model (van de Ven and Hunter, 1977) predicts only the presence of singlets and doublets at medium and high volume fractions and shear rates. At shear rates below that of the Bingham flow regime one might expect the size of the yeast flocs to be a function of the rate of shear, chemical composition of the surrounding fluid and the flocculation tendencies of the yeast strain.

B. Materials and Methods

1. Scanning electron microscopy studies

Four commercial yeast strains, non-flocculent and flocculent lager and non-flocculent and flocculent ale strains (NFL, FL, NFA and FA, respectively) were collected and washed as described in Chapter II. Samples were examined during Phases II, III and IV of this study. In each sampling the yeasts were subjected to a different treatment prior to examination by SEM (model JXA-35, JOEL Ltd., Tokyo, JPN).

Cell samples collected during Phase II of this study were suspended in distilled water at a concentration of 10^6 cells / mL. Prior to viewing, the non-flocculent and flocculent yeast samples had been collected from the fermenter and stored at 4 ^{o}C for 21 and 17 days, respectively. Ten mL of these suspensions were then separately filtered on to a 1.0 μm polycarbonate filter (Nuclepore Corp. Ltd., Pleasantown, CA) and washed repeatedly with distilled water. The samples were plunge-frozen in Freon 22 that was cooled with liquid nitrogen, then stored in liquid nitrogen and freeze-dried. Freeze-drying was carried out using a Virtis model 10-010 automatic freeze drier (Virtis Company, Gardner, NY) for 24 h at -60 ^{o}C and 13.3 Pa. Following mounting on specimen holders, the samples were gold coated with an Edwards model 306A gold-coater (Edwards High Vacuum Limited, London, GBR) to a thickness of about 30 nm. The specimens were examined with the SEM and photo-micrographs taken.

The second set of samples (obtained during Phase III of this research) were examined after storage for 14 days at 4 ^{o}C following collection from the fermenter. The preparation technique described above was used and differed only in the composition of the final yeast suspension and washing solution. In this study the samples were

suspended in 5% (w/w) ethanol prior to filtration and washed with this solution rather than distilled water.

In the third and final SEM examination, the yeast samples were received from the brewery, stored for 14 days at 4 ^{o}C and washed as described in Chapter II. The four strains were then treated with 200 µg / mL of pronase (proteinase type XIV, from *Streptomyces griseus*, Sigma Chemical Co., St. Louis, MO) dissolved in 50 mM sodium phosphate, 50 mM EDTA at pH 7.50 as described by Hodgson, Berry and Johnston (1985). The suspension was incubated at 30 ^{o}C for one h prior to SEM preparation. Hodgson et al. (1985) noted a decline in flocculence after flocculent yeast cells had been subjected to this pronase treatment. After deposition on the polycarbonate filters, the samples were repeatedly washed with distilled water.

2. Light microscopy studies

The aggregation number of sheared cell flocs was examined by loading de-flocculated cell suspensions onto the flat platen of a Carri-Med CS 500 rheometer (Carri-Med Ltd., Dorking, Surrey, GBR) equipped with a 4^{o}, 4.0 cm diameter cone. All samples were allowed to equilibrate at 15 ^{o}C for 20 s and then sheared at 200 s^{-1} for 20 min. The bottom fixture (i.e., platen) was then lowered and one sample carefully taken from the plate and one from the cone tip using the "bulb" end of a clean disposable Pasteur pipette. Each sample was placed on a haemocytometer slide and the cover-slip gently lowered over the suspension. Brightfield slide micrographs were then taken of both fields of the haemocytometer slide at 100 times magnification. These micrographs were then projected with either a 35 mm slide projector or a microfiche reader for measurement of the size and aggregation number (N, the number of cells per floc) of the four yeast strains. Flocs consisting of more than 10 cells were not evaluated.

C. Results and Discussion

1. Scanning electron microscopy studies

Examination of the electron micrographs from the first experiment suggested a number of interesting but tentative conclusions. Micrographs of each of the four lager and ale strains are shown in Figures IV.1-2. and IV.3-4., respectively.

In general, the non-flocculent lager cells were distributed as distinct cells rather than flocs. In cases where the cells appeared to be adjacent to one another, little evidence of attachment was visible (Fig. IV.1). In contrast, the flocculent lager yeast cells, shown in Figure IV.2, tended to be grouped in flocs of 4 to 5 cells and appeared to be joined by long fibre-like structures. Also visible in the lower right of Figure IV.2 is an apparently dead cell indicating that dead cells may still participate in the flocculation process.

Examination of the non-flocculent and flocculent ale strains, shown in Figures IV.3 & IV.4 show a loose fibre-like attachment in the case of the non-flocculent strain and a mucus-like attachment between large regions of the flocculent ale cells. This appearance was similar but not as extensive as junctions between ale cells examined after acid washing (Simpson and Hammond, 1989).

The structures present in Figures IV.2 to IV.4 were interesting, but caution must be used in drawing conclusions from these micrographs. First, some or all of these structures may be artifacts of the preparation method and second, by its nature, this technique viewed only a small portion of the cell population. As these yeast populations had been through a minimum of 50 generations after isolation, it was not unreasonable to expect some cell-to-cell variation. In fact, intra-strain differences, in the appearance of cell attachments were noted. In one case, the rough strand-like appearance typical of the

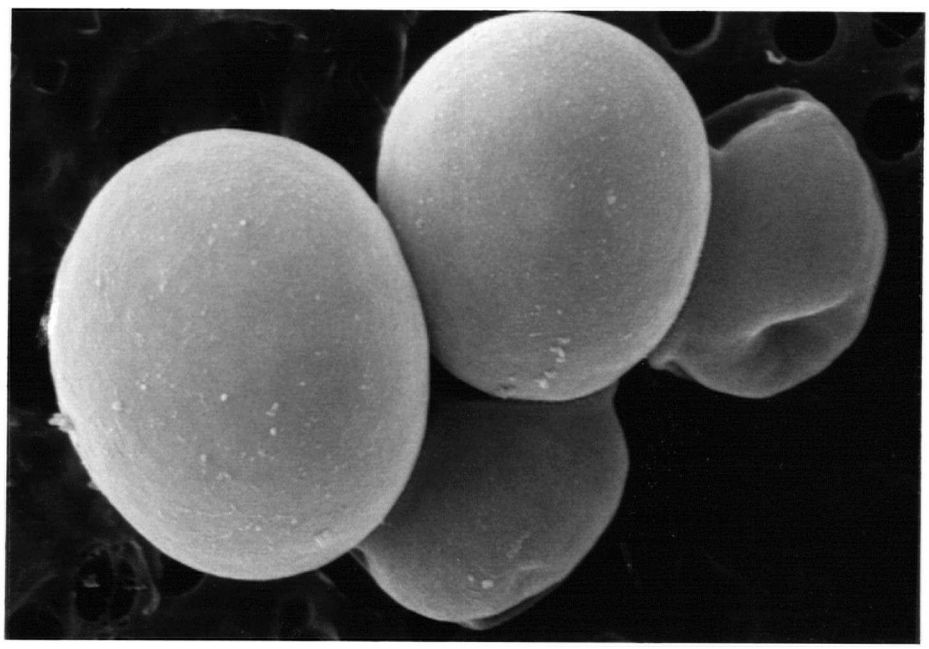

Figure IV.1. SEM micrograph of water washed non-flocculent lager yeast cells (bar=1μm).

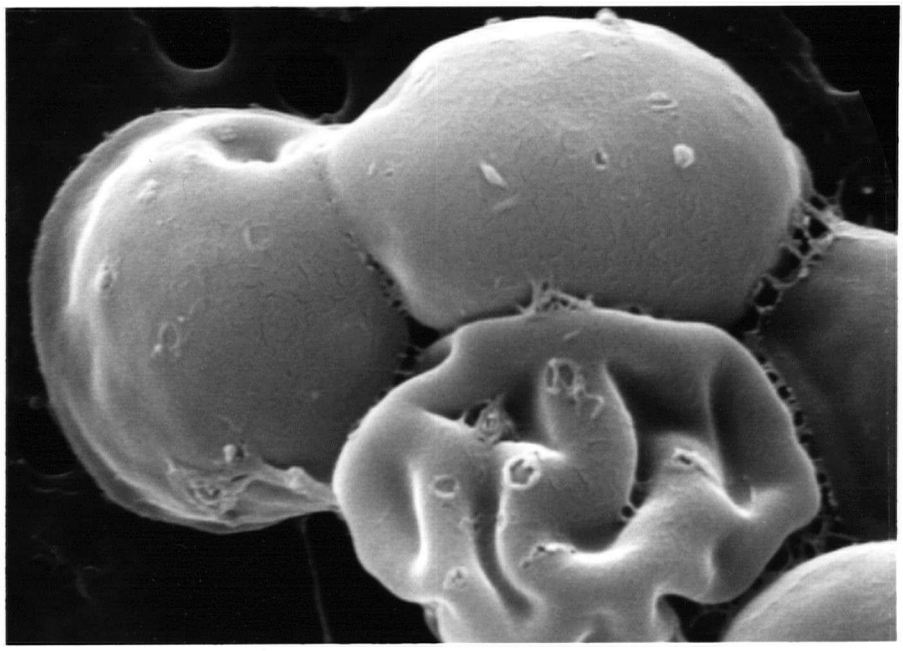

Figure IV.2. SEM micrograph of water washed flocculent lager yeast cells (bar=1μm).

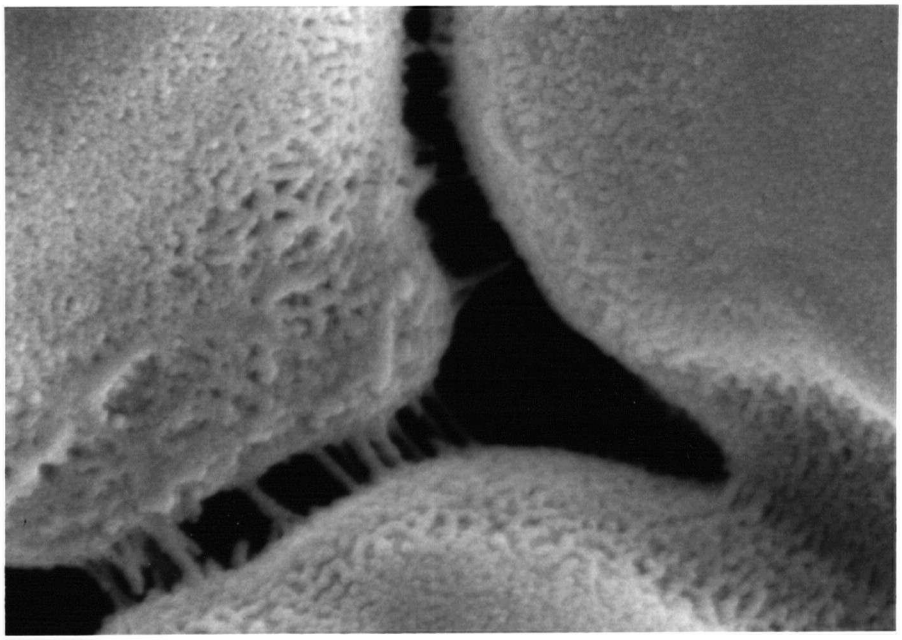

Figure IV.3. SEM micrograph of water washed non-flocculent ale yeast cells (bar=1μm).

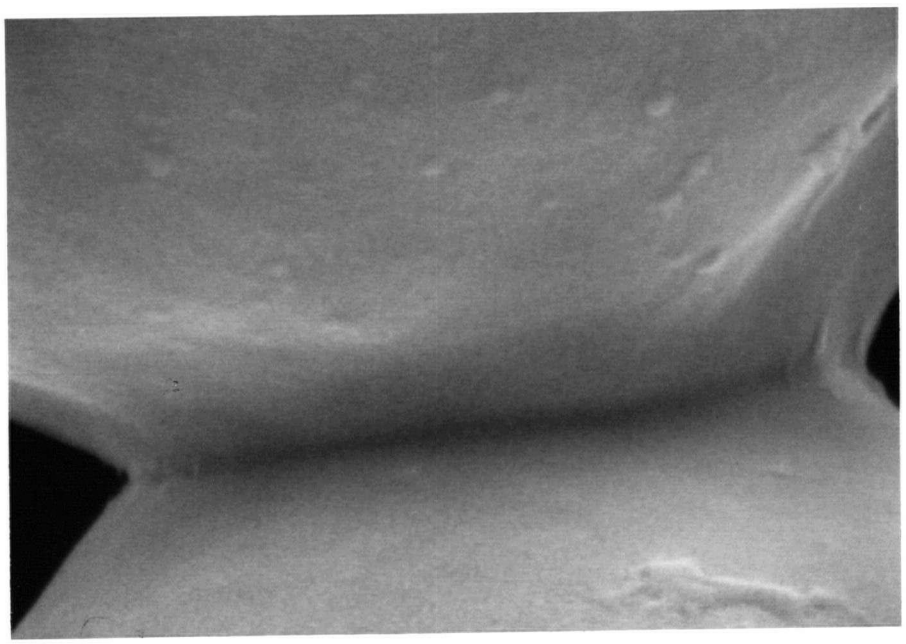

Figure IV.4. SEM micrograph of water washed flocculent ale yeast cells (bar=1μm).

non-flocculent *ale* cells (Fig. IV.3) was observed in the non-flocculent *lager* population. In the second SEM study, where the cells were suspended and washed in 5% (w /w) ethanol, few differences between cells were noticed. In the case of the flocculent ale strain, the cells exhibited reduced cell-to-cell contact areas and appeared to be covered by a latice-like network of threads as illustrated in Figure IV.5. These web-like threads may have been an artifact of cell preparation.

The pronase treated cells from strains NFL, FL and NFA displayed few of the surface features noticeable in the Figures IV.2 to IV.4. Flocculent ale cells treated with pronase are shown in Figure IV.6. These cells appear to have reduced cell-cell contacts and less of the mucus-like covering noted the FA strain in the first study. In comparing the results of the first experiment with the last two, the difference in storage times before sample preparation facilities became available (3 to 7 days) should be noted. However, as protease treatments have repeatedly been shown to reduce cell flocculation, it is not unreasonable to conclude that the mucus-like structure observed in Figure IV.2 may be important in yeast flocculation.

2. Light microscopy studies

Information regarding the aggregation number (i.e., number of cells per floc) and floc distribution indicated that the initially dispersed cells tended to flocculate into larger flocs with increased shearing time. Figure IV.7 shows such a distribution of cells in each floc versus the aggregation number for the flocculent ale strain. While the majority of the flocs had aggregation numbers of one to five, in the flocculent lager and ale strains, a few large flocs (with 20-50 cells) were noted.

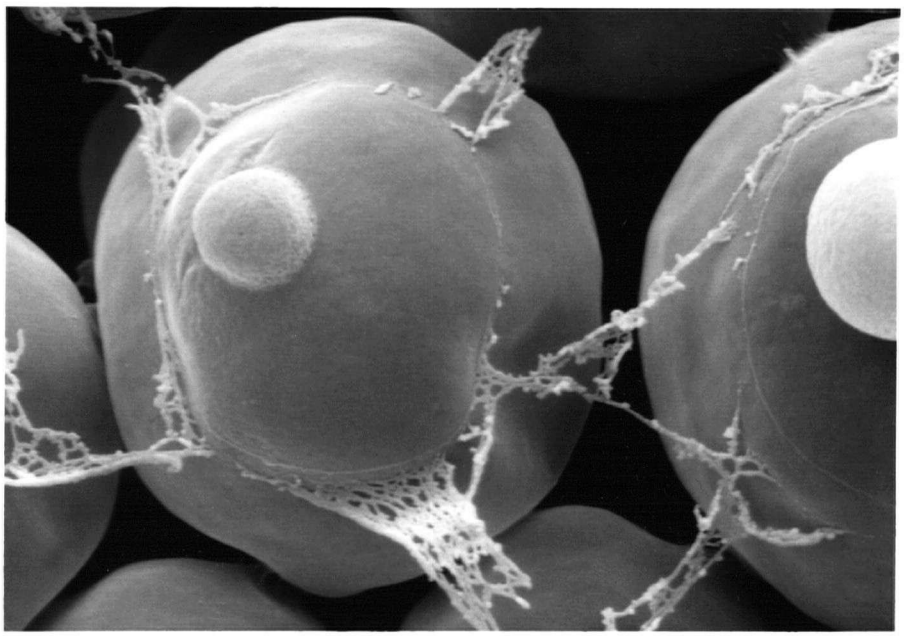

Figure IV.5. SEM micrograph of ethanol washed flocculent ale yeast cells (bar=1μm).

Figure IV.6. SEM micrograph of pronase treated flocculent ale yeast cells (bar=1μm).

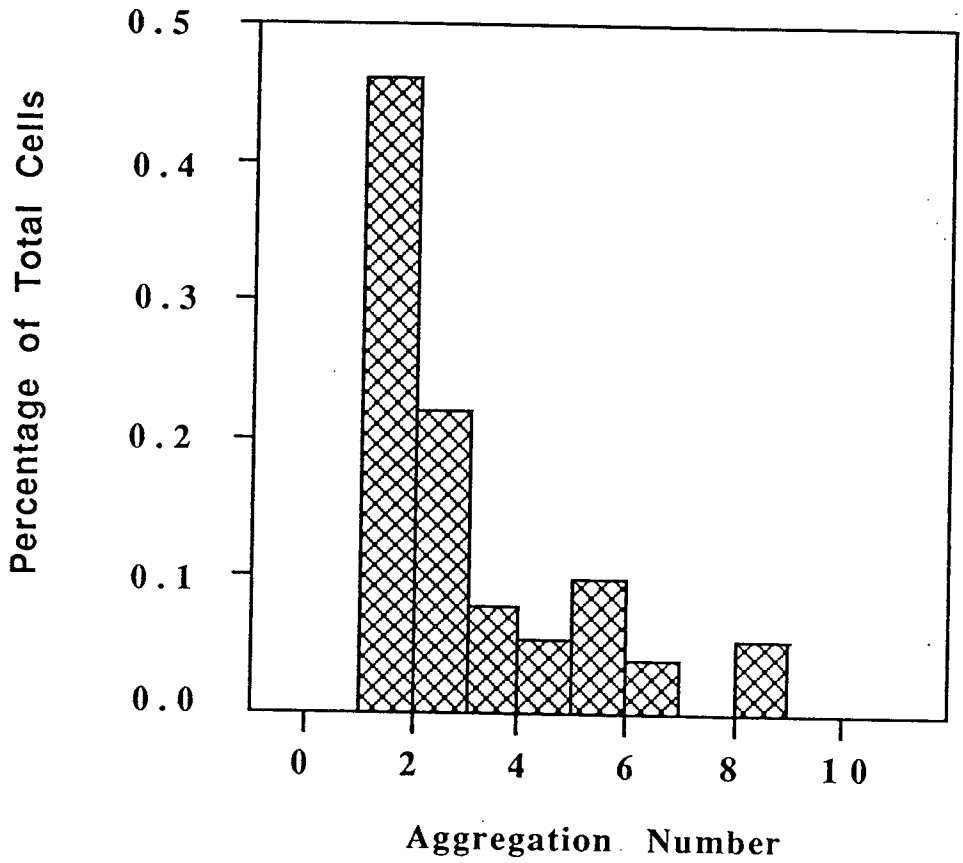

Figure IV.7. Cell distribution with floc aggregation number of flocculent ale yeast sheared in decarbonated ale after 20 min of shear (@15 ^{o}C, 2.5 x 10^{7} cells/mL and 200 s^{-1})

As very few large flocs were observed using this haemocytometric technique, no attempt was made to evaluate their fractal nature. In general, the larger flocs appeared to be tightly packed and essentially spherical.

Without more information on the floc size-aggregation numbers measured here it was difficult to draw any quantitative conclusions. The method was extremely tedious and it would have been difficult to obtain more light micrographs at each measurement time without introducing unacceptable error into these orthokinetic flocculation experiments.

D. Conclusions

While research presented in this study should be considered preliminary, a few tentative conclusions may be drawn. First, based on SEM examination of the four industrial strains it appeared that a number of distinct structures mediated cell-to-cell flocculation. Further study is needed to confirm that these are, in fact, different structures. Examination of these four strains also indicated that intra-strain differences occurred. Pronase treated yeast cells exhibited reduced cell-to-cell contacts.

Light microscopic examination of yeast flocs after shearing at 200 s^{-1} demonstrated that floc size increased with time. The majority of flocs were of an aggregation number of less than five; however, larger flocs (estimated at 20-50 cells/floc) were occasionally observed.

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CHAPTER V

THE CELL FLOC MODEL

A. Introduction

In the preceding chapters of this thesis a number of physical properties of yeast flocculation have been reviewed and investigated. In this final chapter, the value of the cell floc model in explaining the rheological and colloidal data reported herein will be examined. Also, suggestions for further research will be discussed in light of the findings of this study.

B. The Model

The cell floc model, a variation of Hunter's elastic floc model, stipulates that the Bingham yield stress is a function of the energy of collision of single cells in a laminar shear field:

$$\sigma_y = 3\ \alpha_o\ \varphi_c^2\ (E_r)\ /\ \pi^2\ r^3 \quad (\dot{\gamma} > \dot{\gamma}_c) \qquad (V.I)$$

where σ_y is the Bingham yield stress, α_o is the capture coefficient, φ_c is the cell volume, E_r is the energy required to separate doublet flocs, r is the cell radius, $\dot{\gamma}$ is the shear rate and $\dot{\gamma}_c$ is a critical shear rate above which only singlet and doublet flocs occur in Bingham flow (Speers, Durance and Tung, 1989; Chapter II). This model was constructed after consideration of rheological data, and it is now appropriate to re-examine it in light of additional colloidal data collected in Chapters III and IV of this report.

Of the four strains examined in decarbonated beer (for which the most complete data exists), the flocculent ale yeast strain exhibited the largest yield stress and flocculation tendency. Table V.1 summarizes the rheological and colloidal properties of this strain suspended in decarbonated ale.

According to the cell floc model, the critical shear rate (γ_c) represents the minimum rate of shear at which doublet flocs are separated in the shear field. Using values for the rate of shear at which doublet flocs separate, researchers have estimated the force required to separate doublet flocs (Tha and Goldsmith, 1986a, b). The equation employed in these estimates is based on the analysis of the rotation of two connected spheres in a laminar shear field:

$$F_r = \eta\, r^2\, \dot{\gamma}_c\, C \qquad \text{(V.II)}$$

where F_r is the maximum value of the separation force, η is the viscosity of the suspending media and C is constant. This constant C is dependent on the distance between the two spheres and has been calculated to be; 1) 19.33 in the case of sphered red blood cells connected by a polymer bridge of 20 nm (Tha and Goldsmith 1986a) and, 2) values ranging from 8.4 (Curtis and Hocking, 1970) to 38.45 (Goren, 1971) in the case of two spheres directly connected to one another.

Since it is possible that the doublet yeast cells are connected by a polymer bridge and since the value calculated by Tha and Goldsmith, 19.33, lies between that calculated by Curtis and Hocking (1970) and Goren (1971), 19.33 was somewhat arbitrarily assigned to the constant C. By substitution of values of 80 s^{-1}, 1.15 mPa·s (estimated from a 5% (w/w) ethanol-water solution at 15 ^{o}C [Weast, 1984]) and 7.1 μm (the average cell diameter) into Equation V.II one obtains a separation force of 2.2 x 10^{-11} N.

Table V.1. Rheological and orthokinetic properties of the flocculent ale yeast suspended in decarbonated ale.

Suspension colloidal or rheological property	Value	Cell concentration (cells/mL)
Yield stress (Pa)	3.8	2.5×10^9
Critical shear rate (s^{-1})	80	2.5×10^9
Capture coefficient	1.0×10^{-3}	2.5×10^7
Zeta potential (mV)	-2.5	1.0×10^6
Cell diameter		
major axis* (μm)	7.3	-
minor axis* (μm)	7.0	-

*Cells assumed to be prolate spheroids (formed by rotating an ellipse about its major axis).

This value agrees with the results of Tha and Goldsmith (1986b) who studied the separation of antibody agglutinated, sphered red blood cells as well as Mage et al. (1986) who measured the force to remove newly attached mouse macrophage-like cells from glass. It is noteworthy that in employing Equation V.II one assumes that the yeast doublets are prefect spheres rather than prolate spheroids.

This estimate of the force necessary to separate connected cells is similar to estimates of the strength of a single antibody bond, 4×10^{-11} N (Bell, 1978). It is reasonable to conclude, as have the research teams led by Goldsmith and Mage, cited above, that doublet cells are bound by antibodies (or in the case of yeast cells, lectin-like structures).

In Chapter II it was hypothesized that differences in yield stress values measured in suspensions of the four yeast strains was due to differences in the energy required to separate the doublet yeast cells. Using the values in Table V.1 and Equation V.I an estimate of the energy term of the order of 10^{-11} J was calculated. This value is very much larger than that which can be attributed to either the energy of separation of lectin-like bonds of 6×10^{-20} J, (Bell, 1978) or by DLVO-type interaction energies. Thus, it would appear that the magnitude of the energy term in the cell floc model is not due to separation energies, but rather, as postulated by Hunter, the result of the energy of deformation as doublet cells collide. Based on the findings of this study, it is hypothesized that the Bingham yield stress is a function of the orthokinetic capture coefficient, the cell volume fraction, the deformation energy (E_d) and the size of the yeast cell:

$$\sigma_y = 3\,\alpha_o\,\varphi_c^2\,E_d / \pi^2 r^3 \quad (\dot{\gamma} > \dot{\gamma}_c) \qquad \text{(V.III)}$$

The use of Equation V.III requires a number of assumptions as outlined in Chapter II.

In order to confirm the utility of this model further studies are required. In the concluding section of this report a number of suggestions will be made for further research aimed at validation of the cell floc model.

C. Suggestions For Further Research

While this research has developed and indicated that the cell floc model is a valid semi-empirical relationship to explain the flow behaviour of brewing yeast suspensions, a number of further studies are required in order to support the model. Three of the most important future objectives might be; 1) development of a direct measurement technique to monitor floc size and aggregation number while yeast cells are undergoing laminar shear, 2) confirmation that the onset of doublet cell breakup coincides with the critical shear rate, and finally, 3) investigation of the extent and number of lectin-like binding sites, measurement of orthokinetic capture coefficients, and correlation of these data with theoretical estimates of orthokinetic capture coefficients (Bell, 1981).

The apparently hydrophobic nature of ale yeast cells, noted in rheological investigations in Chapter II is also interesting. Studies investigating the nature of this hydrophobic interaction are presently ongoing and involve the simultaneous examination of the temperature dependence of yeast suspension viscosity and measurement of cell wall surface hydrophobicity.

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APPENDIX I

ESTIMATION OF THE PSEUDO-CAPTURE COEFFICIENT

A. Derivation

As single cells are sheared in laminar flow, the combined number of singlets and doublets at any time (early in the reaction), N_t, has been shown by Swift and Friedlander (1964) to be :

Given:

$$N_t = N_o \, e^{-4\alpha_o \phi_o \dot{\gamma} / \pi \, t} \qquad (A.1)$$

where N_o is the original number of single cells α_o is the orthokinetic capture coefficient, φ_o is the initial volume fraction, $\dot{\gamma}$ is the shear rate and t is time. Since the breakdown of doublets to single cells in a shear field can also be assumed to occur, the above expression can be modified to include an equilibrium term, N_e:

$$N_t = N_o \, e^{[-4\alpha_o \phi_o \dot{\gamma} / \pi] \, t} + N_e \qquad (A.2)$$

In the case where a population of singlets is sheared to form a mixture of doublets and singlets, the formation of doublets can be shown to be equal to:

$$D_t = D_e - D_e \, e^{[-4\alpha_o \phi_o \dot{\gamma} / \pi] \, t} \qquad (A.3)$$

where D_t is the number of doublets at any time and D_e is the equilibrium number of doublets formed. Also, if one assumes the increase in the number of doublets is directly related to the increase in the energy required for flow (either for viscous flow or doublet collision and separation) then Equation A.3 can be expressed as:

$$\eta_t = (\eta_o - \eta_e)e^{[-4\alpha_o\phi_o\dot{\gamma}/\pi]\,t} + \eta_e \qquad (A.4)$$

where η_o is the initial viscosity due to singlets and η_e is the equilibrium viscosity. Where changes in the viscosity over time are known (from experiments such as those described in this report), approximations of α_o, the orthokinetic capture coefficient may be estimated when the rate constant of doublet breakdown is much smaller than the rate constant of doublet formation.

Use of this semi-empirical derivation should be employed with caution in view of the many assumptions made in its derivation. These calculated orthokinetic capture coefficients have been termed *pseudo*-capture coefficients in recognition of these concerns.

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APPENDIX II

RHEOMETRIC DATA CORRECTION

A. Introduction

When undertaking viscometric evaluations of suspensions, care must be taken not only in gathering the experimental data, but also in the calculation of corresponding shear rate and viscosity values. This caveat is especially important when evaluating coaxial cylinder viscometric data. Errors are commonly made by assuming that Newtonian flow occurs between coaxial cylinders when in fact a non-Newtonian fluid is being sheared within the gap. Aside from consideration of slippage in the region of the cup boundary, which was discussed in Chapter II, one must also: (1) consider the effect of non-Newtonian flow on calculated Newtonian shear rates and (2) ensure that shear stresses within the gap are sufficient to cause laminar flow.

B. Shear Rate Correction

While the Krieger (1968) non-Newtonian shear rate correction for power-law fluid measurement is relatively well known, one can also calculate shear rates when either Bingham or Casson fluids are sheared within the gap. Use of these corrections have been detailed by Speers (1984) and Tung and Speers (1986) as shown below, and are based on work by Reiner and Riwlin (1927) and Hanks (1983).

Given[1]:

- the Bingham flow equation (Bingham, 1922):

$$\sigma = \sigma_y + \eta_\infty \dot{\gamma}_B \tag{A.5}$$

– the Bingham coaxial cylinder flow equation (Reiner and Riwlin, 1927):

$$\dot{\gamma}_B = [(\sigma - \sigma_y)\,\Omega] / \{(\sigma / 2)\,[(r_c^2 - r_b^2) / r_c^2] - \sigma_y \ln (r_c / r_b)\} \tag{A.6}$$

-and:

$$\Omega = (\dot{\gamma}_N / 2) / [(r_c^2 - r_b^2) / r_c^2] \tag{A.7}$$

- by combining and rearranging Equations A.5, A.6 & A.7:

$$\sigma = \sigma_y [\ln (r_c / r_b)\,(2 r_c^2 / (r_c^2 - r_b^2))] + \eta_\infty \dot{\gamma}_N \tag{A.8}$$

Given:

- the Casson equation (Casson, 1959):

$$\sigma^{1/2} = \sigma_y^{1/2} + (\eta_\infty \dot{\gamma}_C)^{1/2} \tag{A.9}$$

-the Casson coaxial cylinder flow equation (Hanks, 1983):

$$\sigma - 4 (\sigma\,\sigma_y)^{1/2} + \sigma_y \ln (r_c^2 / r_b^2) + 4 (r_b / r_c) (\sigma\,\sigma_y)^{1/2} = (r_b^2 / r_c^2)\,\sigma_y + \eta_\infty (1 - r_b^2 / r_c^2)\,\dot{\gamma}_N \tag{A.10}$$

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[1]A complete explanation of symbols used in this appendix is listed in section D.

- then by setting:

$$a = (2 - 2\, r_b / r_c) \; / (1 - r_b^2 / r_c^2) \qquad (A.11)$$

- and:

$$b = [\, \ln (r_b^2 / r_c^2)] \; / \, [1 - (r_b^2 / r_c^2)\,] \qquad (A.12)$$

- then Equation A.10 can be shown to take the quadratic form:

$$\sigma^{1/2} - 2a\, (\sigma\sigma_y)^{1/2} + b\, \sigma_y = (\eta_\infty \dot{\gamma}_N) \qquad (A.13)$$

- or since a^2 is very nearly equal to b (<0.0011 @ $r_b / r_c = 0.85$)

$$\sigma^{1/2} = a\, \sigma_y^{1/2} + (\eta_\infty \dot{\gamma}_N)^{1/2} \qquad (A.13)$$

C. Plug Flow Correction

To ensure that laminar flow occurs within the entire coaxial cylinder gap, one must confirm that the shear stress acting on the cup boundary is greater than the yield stress of the material. This can be easily checked by use of the flow equations of Reiner and Riwlin (1927) and Hanks (1983):

- to maintain complete laminar flow within the gap:

$$\sigma_y < M / (2\, \pi\, r_c^2\, h) \qquad (A.14)$$

- then for a *Bingham fluid* a critical shear rate can be defined where:

$$\sigma_y = M / (2\, \pi\, r_c^2 h) \qquad (A.15)$$

- by substitution and rearrangements of Equations A.5, A.6 & A.7:

$$\dot{\gamma}_{Bc} = (\sigma_y/\eta_\infty)\{(r_c^2/r_b^2) - [(2\,r_c^2/(r_c^2 - r_b^2)]\ln(r_c/r_b)\} \quad (A.16)$$

- for a *Casson fluid* a critical shear rate can also be defined (Hanks, 1983) where:

$$\sigma_y = \sigma_c\,(r_b^2/r_c^2) \quad (A.17)$$

- by rearrangement and substitution of Equations A.11 & A.17:

$$\dot{\gamma}_{Cc} = (\sigma_c/\eta_\infty)\,[(r_c/r_b) - a]^2 \quad (A.18)$$

D. Literature Cited

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E. Symbols Used

r_b = cup radius,

r_c = bob radius,

$\dot{\gamma}_B$ = Bingham shear rate,

$\dot{\gamma}_C$ = Casson shear rate,

$\dot{\gamma}_N$ = Newtonian shear rate,

η_∞ = infinite shear viscosity,

σ = shear stress,

σ_y = yield stress,

Ω = angular velocity,

h =effective bob height,

M = torque acting on bob,

$\dot{\gamma}_{Bc}$= critical (Bingham) shear rate (below which plug flow occurs),

$\dot{\gamma}_{Cc}$ = critical (Casson) shear rate below which plug flow occurs),

σ_c = critical shear stress (below which plug flow occurs).