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Lectin-mediated Aggregation of Yeasts — Yeast Flocculation

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Introduction

Yeast flocculation describes a process whereby yeasts, essentially unicellular organisms, are gathered together into cohesive masses of thousands or even millions of cells; such aggregates are called flocs (*Figure 1*). In recent years, a greater understanding of this process has been brought about through the application of specialist knowledge from a wide variety of scientific disciplines. Physicists, working on colloidal aggregation, have demonstrated the processes by which aggregates are formed and built up. The availability of high-speed computers and their use in modelling fractal geometry have recently revolutionized our views on the formation of chaotic aggregates. Another revolution, this time in the field of molecular biology, has led to the development of very powerful techniques for the manipulation and cloning of genes, here described in their application to genes governing yeast flocculation.

A quieter, but possibly no less important, revolution has occurred in our perception of carbohydrates. Other complex biological polymers have been accepted as having sequentially ordered structures and functions derived from these structures: DNA structures functioning as genetic code; protein structures functioning as catalytic enzymes; RNA as transcription templates and even, recently, shown to have enzyme activity (Taira *et al.*, 1990). Carbohydrates have, in contrast, been regarded simply as amorphous masses, the mashed-potato of the biological scheme. Recently, detailed analysis of complex carbohydrates, has shown specific structures in polysaccharides with definite roles in cell–cell recognition and interaction phenomena.

The study of yeast flocculation has a long, even historic pedigree. Louis Pasteur (1876) made the first scientific observation on yeast flocculation which was followed around the turn of the century by many reports of flocculation in the German literature. Since the earliest reports, flocculation

Abbreviations: EDTA, ethylene-diamine-tetraacetic acid; EGTA, ethylene glycol bis(amino-ethyl ether) tetra-acetic acid; PAGE, polyacrylamide gel electrophoresis; UV, ultraviolet; YEPD, yeast extract, peptone, dextrose.

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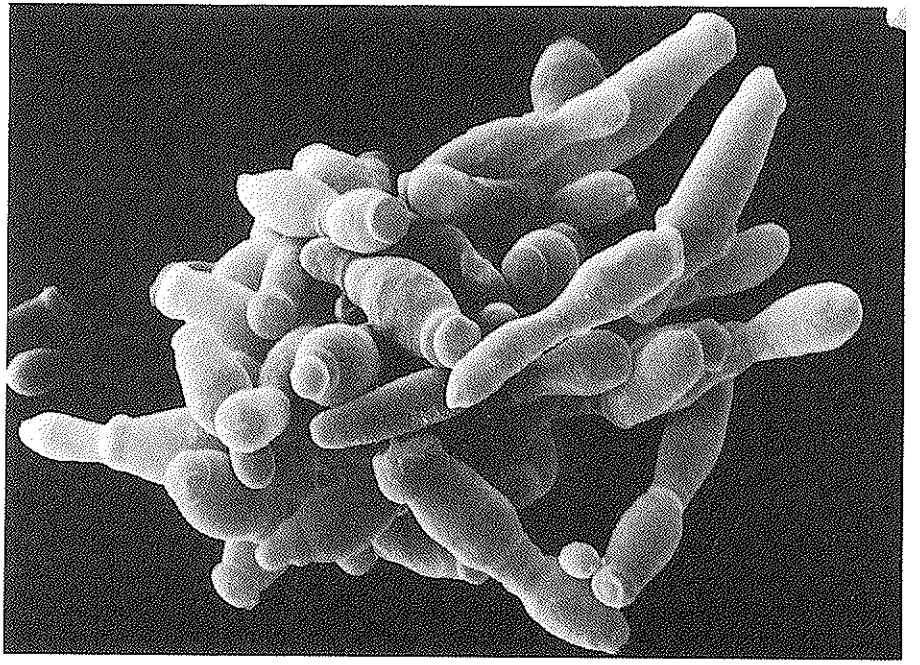

Figure 1. Scanning electron micrograph of flocculating cells of *Saccharomycodes ludwigii* NCYC 734. These skittle-shaped yeasts divide by bipolar budding and are approximately 4μm in girth.

has been intimately associated with the brewing industry. Flocculent yeasts have probably been used for centuries in beer-making, giving the final product a clear, bright appearance. As a result of this industrial — and therefore financial — interest, much of the research into yeast flocculation has concerned its role in brewing (reviews by Burns, 1937; Brohult, 1951; Jansen, 1958; Windisch, 1968; Geilenkotten and Nyns, 1971; Stewart and Russell, 1981, 1986). Because of these factors, yeast flocculation has come to be regarded as an individual, isolated curiosity of *Saccharomyces cerevisae*. In fact, flocculation has recently been recognized in a variety of yeast genera and lectin-mediated cell–cell interactions have been described in many micro-organisms. In this review, an attempt is made to view yeast flocculation, not so much in isolation, but in the context of more widespread phenomena. This present review article is not intended to be solely a reiteration of the old material, but a view from a different standpoint. It is intended to broaden, update and complement previous reviews of the fascinating subject of yeast flocculation.

Microbial and yeast aggregation phenomena

Most microbes exist predominantly as single cells, whilst 'higher' more advanced forms of life are multicellular. Clearly, the multicellular form would seem to confer a distinct advantage over unicellular organisms, in size if nothing else. However, there is much more to a discrete multicellular

organism than an aggregation of single cells. The size and complexity of multicellular organisms necessitates communication and recognition between cells. Once this has been achieved, the way is open for differentiation of cellular functions and structure and the collection of cells can behave as a discrete organism rather than as a group of individuals.

Micro-organisms that form aggregates, however, generally do not show this awareness of the aggregate-whole, as far as we can tell, and most microbial aggregates appear to behave as agglomerations of individual cells. The obvious exception to this generalization is the aggregation and communication shown by slime-mould cells (Newell, 1977).

THE THREE FORMS OF YEAST AGGREGATION

Aggregates of yeast cells can result from any one of three 'natural' causes. These are mating aggregation, chain-formation and flocculation (*Figure 2*). Mating aggregates are formed as a prelude to sexual fusion between complementary cells. This sexual process has been extensively studied in three yeast genera, *Hansenula, Saccharomyces* and *Schizosaccharomyces*, all reviewed by Calleja (1984, 1987). In the case of *Saccharomyces cerevisiae*, haploid yeast strains of complementary mating types, α and a, release short polypeptide pheromones which elicit changes in the cell walls of the opposite mating type. These changes instigate specific protein–protein bonding between cells of the complementary sexes and formation of large mixed aggregates. Fusion between complementary cells consequently results in diploid cells and ultimately haploid spores following meiosis.

Chain-formation, in contrast, will result in aggregate formation only through yeast growth. After cell division mother and daughter cells remain together, both growing and forming new buds, instead of the normal, clean separation of cells. There is theoretically no limit to this process, but in practice physical breakage of chain-formed aggregates by shear forces tends to limit them to 30–100 cells. Chain-formation in yeast is a hereditary trait (Gilliland, 1951, 1957) entirely distinct from flocculation (Eddy, 1955a). Chain-formation has been shown recently to result from disruption of a chitinase gene (Kuranda and Robbins, 1991). Bud-scars are known to be composed of chitin (Cabib and Bowers, 1971), and the chitin-rich septum formed between mother and daughter cells (Veinot-Drebot, Johnston and Singer, 1991) requires hydrolysis by chitinase prior to cell separation. Lack of cell separation also results from *CKA2* gene disruption (Padmanabha *et al.*, 1990), an essential regulatory gene coding for casein kinase II. Chain-formation can also be caused by nutrient deprivation; deficiency of magnesium ions (Jansen and Mendlik, 1951); biotin (Dunwell, Ahmad and Rose, 1961; Duffus, Levi and Manners, 1982); or inositol (Smith, 1951; Ghosh *et al.*, 1960; Lewin, 1965; Dominguez, Villanueva and Sentandreu, 1978) all causing this effect. Chain-formation in *S. cerevisiae*, which is common in top-fermenting brewery strains (Lyons and Hough, 1970a; Stratford and Assinder, 1991) would appear to be similar to the pseudo-mycelium formation found in several yeast genera, for example *Candida* or *Trichosporon*.

Extensive, branched structures are formed by growth of yeast strains in these genera, under certain nutrient conditions. These pseudomycelia show much structural similarity to the full mycelia developed by typical fungi.

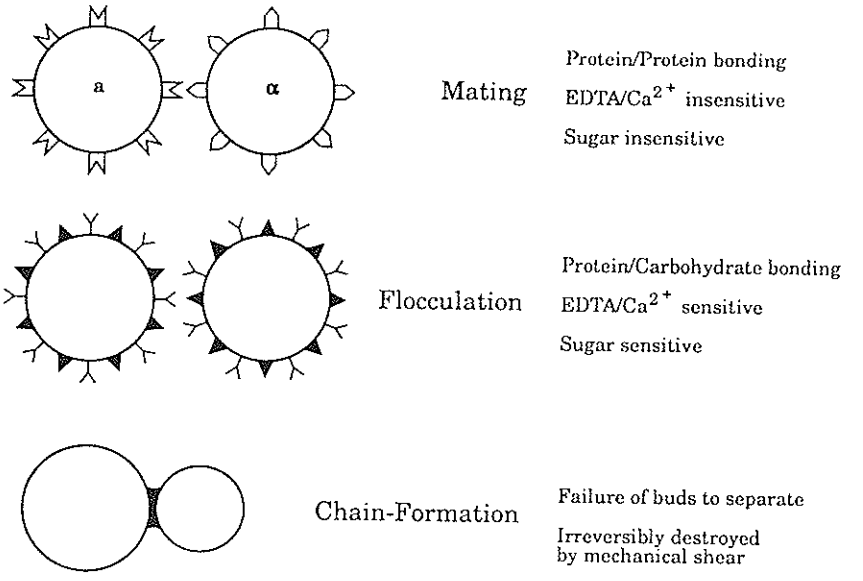

Figure 2. Summary of the characteristics of the three aggregation mechanisms in *Saccharomyces cerevisiae*. These are mating aggregation between mixed, haploid a and α strains mediated by protein–protein bonding; flocculation, a calcium-dependent protein–carbohydrate bonding between cells of single strains; and chain-formation, arising through cell division where daughter cells fail to separate from mother cells.

Flocculation in yeast appears to be an aggregation system entirely distinct from mating or chain-formation. Unlike the rigid attachment of chain-linked cells, which is irreversibly destroyed by mechanical shear (Gilliland, 1981), flocculent cells are genuinely 'sticky' and will readily reaggregate after mechanical dispersion. Adhesion between flocculent yeast strains appears to be mediated by protein (lectin)–carbohydrate bonding (Miki *et al.*, 1981, 1982; Miki, Poon and Seligy, 1982), unlike the protein–protein bonding used in sexual aggregates (Johnson *et al.*, 1988). Also unlike mating aggregation, most flocculent yeast strains are able to form aggregates from culture of single strains, no mixing of pairs of complementary strains being necessary — flocculent yeasts can do it for themselves.

THE IMPORTANCE OF BEING AGGREGATED

Calleja (1987), while discussing microbial aggregation in general, suggested the following reasons for aggregation:

> (a) a prelude to sexuality, (b) a mechanism for survival under adverse conditions, or (c) a prelude to development, morphogenesis and differentiation.

How do the three forms of yeast aggregation fit into this classification? Mating aggregation by yeast clearly falls into the first category, aggregation being a temporary state, and preluding sexual fusion; the advantages to the yeast of this being derived from the mixing of the yeast genetic pool.

Chain-formation and pseudomycelia formation by yeasts would seem to fit best into the third category as preludes to development and differentiation. However, chain-formation in particular does not appear to lead to development and it is difficult to see any advantage in this. Perhaps chain-formation is a relic of past evolution of fungi that have found advantage in the yeast form. It is also possible that survival advantage is gained from being aggregated. While chain-formation can be fortuitous as a result of genetic damage, it is too common in brewery yeast strains (Lyons and Hough, 1970a; Stratford and Assinder, 1991) for this to be entirely fortuitous and without advantage.

The reasons and advantages behind yeast flocculation, have long been debated without there being a general consensus. Flocculation appears to be entirely distinct from mating (Johnson *et al.*, 1988). While it remains possible that flocculation is a relic of a yeast-mating system, now abandoned (Stewart and Russell, 1981), this is unlikely given the absence of two sexual types in flocculation and the observed differences in cell–cell bonding between flocculation and mating aggregates.

However, it remains a possibility that, while yeast cells are in close proximity in flocs, minor exchange of genetic material could be occurring. It has been shown that transfer of genetic material may occur when bacteria adhere to yeast cells (Mazodier and Davies, 1991). No research has yet been done as to whether cytoplasmic elements, plasmids, etc. are transferred during the flocculation process.

There also appears to be a strong possibility, however, that flocculation may enhance the survival of yeast cells. This theory was first put forward by B.F. Johnson in a personal communication to Stewart and Russell (1981). This suggested that during starvation conditions, the survival of a portion of cells within flocs could be aided by death and autolysis of surrounding cells, releasing further nutrients.

In the case of *S. cerevisiae*, it is perhaps not necessary to look further than the brewery for the reason for yeast flocculation. Flocculent yeasts separate from media in the final stages of brewing and yeast flocs are then used for repitching subsequent fermentations. For centuries the brewery has acted as a powerful selective agent for flocculent yeast, and as a further aid, has acted as a fully mechanized means by which such yeasts are distributed around the countryside; not only in beer, but also on barrels, bottles and vehicles. This has occurred to such an extent that the 'wild' population of yeasts probably contains a disproportionate number of brewing strains. Thornton and Bunker (1989) reported a high incidence of flocculation in wine yeasts, although Suzzi, Romano and Zambonelli (1984), Parish and Carroll (1987) and Ciani and Rosini (1990) reported much lower levels, 6·8% of *Saccharomyces* strains.

However, artificial selection by breweries cannot contribute to flocculation of non-brewing strains. Flocculation appears to be a phenomenon widespread

throughout the yeast genera. *Table 1* shows the incidence of flocculation reported to date, together with appropriate references. It is immediately obvious that, other than brewery strains, all of these reports of flocculation in yeast date back only to the last six years. It is therefore highly probable that many more yeasts will be found to flocculate whenever the research is carried out on the less well-known strains. Given that flocculation is commonly found in the wild yeast population, what possible advantages does it confer? In a recent review of yeast selection and survival in nature (Lachance, 1990) selective factors listed included temperature, pH, water activity and toxicity by host plants, animal secretions, enzymes and antibiotics. It is likely that, by flocculating, yeast cells would be better able to survive many of these factors by sacrificing the outer layers of yeast flocs. These outer cells, alive or dead, could shield the underlying cells by absorbing toxins, enzymes or heat or by buffering pH changes, and prevent dehydration of cells within the flocs. This theory is experimentally verified by data presented in *Figure 3* (M. Stratford, unpublished results). Treatment of dispersed suspensions of flocculent yeast with ultraviolent (UV) light caused an immediate and dramatic reduction in viability, with all cells killed within 2 min. The same flocculent yeast in flocs were found to be almost indefinitely immune to UV light, a small proportion of cells surviving very prolonged UV treatment. These are believed to be those within flocs, shielded by overlying dead cells. Clearly, flocculation can confer a substantial degree of protection for yeast cells against adverse conditions. However, it must also be borne in mind that there are times when flocculation can be a disadvantage, i.e. during favourable conditions for growth. Flocculated yeasts offer a much reduced surface area for uptake of nutrients as compared with single cells, flocculation now conferring disadvantage on buried cells. This has been recently verified by Teixeira and Mota (1990), who demonstrated a lowering of sugar uptake by flocculated yeast.

To summarize, Calleja (1987) suggested three reasons for microbial aggregation, namely sexuality, development and survival. Yeast mating-aggregation is clearly a prelude to sexuality, chain-formation appears to be most closely related to development and differentiation, while flocculation may be concerned with survival in adverse conditions, although limited genetic exchange remains a possibility.

DEFINITIONS OF YEAST FLOCCULATION

There have been many and varied definitions of flocculation in the past, depending on the facts known at the time and the viewpoint of the definer. One of the best definitions was put forward by Stewart (1975) simply as 'the phenomenon wherein yeast cells adhere in clumps and sediment rapidly from the medium in which they are suspended'. This simple statement has since required modification to include top-fermenting ale yeasts, whose flocs adhere to gas bubbles and as a result do not sediment, instead rising to form a thick yeasty head on top of the fermenting medium (Stewart and Russell, 1981; Stewart *et al.*, 1990).

A good definition is one that encompasses only the subject in question and

Table 1. Incidence of flocculation in yeasts. Yeast species in which flocculation has been reported, are listed together with the sugars inhibiting flocculation where tested

Yeast	Inhibiting sugars	References
Candida utilis	–	Kalyuzhnyi, Petrushko and Novikova (1965)
Candida rugosa	–	Lee and Baerwald (1991)
Candida tropicalis	–	Kalyuzhnyi, Petrushko and Novikova (1965)
Hansenula anomala	–	Moriya *et al.* (1990) Saito *et al.* (1990)
Kluyveromyces bulgaricus	Fucose, galactose	Hussain *et al.* (1986)
Kluyveromyces marxianus	–	Bajpai and Margaritis (1986)
Pichia pastoris	–	Mbawala *et al.* (1990)
Pichia stipitis	–	Grootjen *et al.* (1991)
Saccharomyces cerevisiae Flo1 phenotype	Mannose	Stratford (1989a)
Saccharomyces cerevisiae NewFlo phenotype	Glucose, mannose	Stratford and Assinder (1991)
Saccharomyces diastaticus	–	Spencer *et al.* (1981)
Saccharomyces rouxii	–	Hartmeier, Borgmann and Voss (1990)
Saccharomyces uvarum/carlsbergenis	Glucose, mannose	Patel and Ingledew (1975) Hussain *et al.* (1986)
Saccharomycodes ludwigii	Arabinose, fucose, galactose	Stratford and Pearson (1992)
Schizosaccharomyces pombe	Galactose	Johnson *et al.* (1987) Johnson *et al.* (1988)
Zygosaccharomyces sp.	–	Suzzi and Romano (1990)

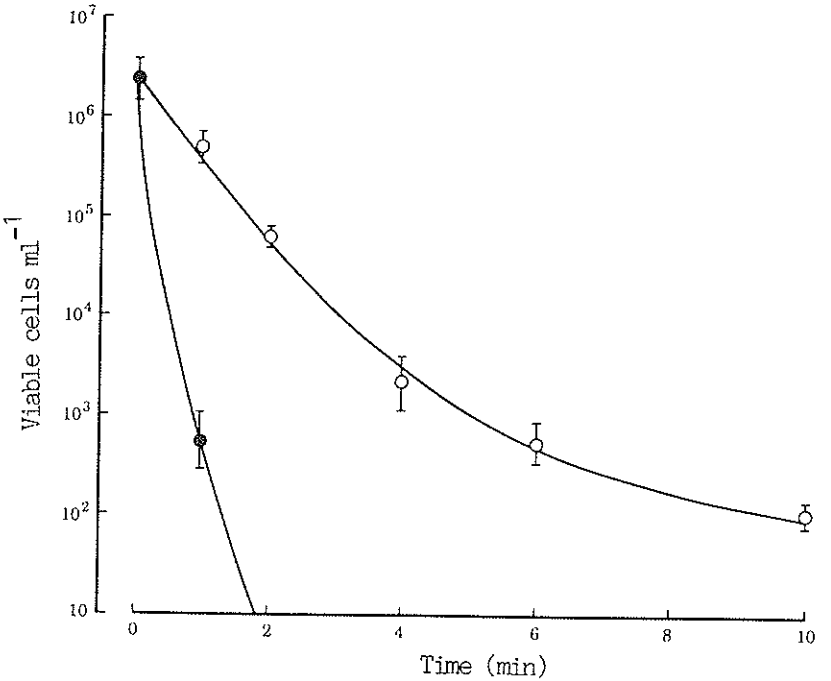

Figure 3. Death of yeast in UV light (120 μw cm^{-2}), as single cells or in flocs. Flocculent cells of *Saccharomyces cerevisiae* S646–1B were irradiated as single cells (●) in EDTA, 10 mM, or as small flocs (○) after addition of calcium chloride, 25 mM. Viability was measured by colony counting, after floc redispersal.

excludes all superficially similar phenomena. Here, three superficially similar forms of yeast aggregation are described, only one of which is flocculation. Calleja (1987), in defining cell aggregation, emphasized that this was a gathering together of cells and not merely one of being aggregated. This definition excludes cell-aggregates that are not subject to gathering, for example chain-formed aggregates formed by cell division. While accepting the general concept of gathering, there are a number of yeast strains showing constitutive flocculence (Stratford and Assinder, 1991). Normally these strains would not express the gathering process but grow as enlarging flocs, and would be excluded by the definition of Calleja (1987).

It is better, therefore, to define flocculation according to the characteristics not shared by chain-formation and mating. Chain-formed aggregates can be dissociated by mechanical shear into single cells which will not reaggregate; dissociation is irreversible, unlike flocculation or mating aggregation. Both flocculation and mating-aggregation are irreversibly lost after protease treatment (Calleja and Johnson, 1977; Eddy and Rudin, 1958a). But unlike mating aggregation, flocculation requires the presence of metallic cations and is inhibited by chelating agents such as ethylene-diamine-tetraacetic acid (EDTA) (Taylor and Orton, 1973; Johnson *et al.*, 1988). EDTA will also

cause dissociation of existing flocs, although in practice flocs with high bond strength will need vortexing in EDTA.

Due to their protein–carbohydrate bonding, yeast flocculation is also inhibited by solutions of specific sugars, unlike mating aggregation (Johnson *et al.*, 1988). The identity of inhibiting sugars varies depending on the flocculent yeast species examined. *Table 1* shows different forms of yeast flocculation that are inhibited by a variety of simple sugar types, making this less useful as a definition of flocculation.

To conclude, for the purpose of this review, yeast flocculation is defined as a reversible, non-sexual, lectin-mediated aggregation of yeast cells that may be dispersed by chelating agents, or simple sugars.

Parameters affecting yeast flocculation

Flocculation is a condition intrinsic to the cell walls of certain yeast strains at certain phases of growth. Once flocculation has been established in these cell walls it is a very stable property, able to resist acid/alkaline washing (Burns, 1937) and thermal degradation (Taylor and Orton, 1975), and it is no longer controlled by cellular metabolism since heat-killed cells show undiminished flocculence (Mill, 1964b). Metabolism is undoubtedly necessary for the development of flocculence, but once it has been established, flocculation becomes a property of flocculent cell walls that is independent of cell metabolism. This is shown by the observations that cell walls isolated from flocculent yeasts continue to show flocculence (Eddy, 1955c; Eddy and Rudin, 1958a; Masschelein and Devreux, 1957; Mill, 1966).

A very large number of papers have been published over the last hundred years on the subject of yeast flocculation. A large proportion of these, especially in the early days, can be described as 'effect' papers, entitled: 'The effect of . . . on yeast flocculation', and frequently concerning the presence or absence of various media components. In this next section, the literature is divided into three sections: (1) direct affectors of flocculation itself, e.g. inhibitors; (2) factors changing flocculation indirectly via the metabolism of yeast, e.g. nutrients modifying flocculation development; and (3) factors involved in the physical process of aggregation, often shared by other aggregation systems. Some affectors can influence flocculation in several different ways and are included in more than one section. For example, certain salts can directly inhibit flocculation, nutritionally alter the development of flocculation, and also alter surface-charge, thereby influencing the physical process of flocculation.

DIRECT AFFECTORS OF FLOCCULATION

Sugars

In the presence of certain sugars, the flocculation of yeast cells is inhibited. This was first commented upon by Lindner (1901) and inhibition by cane sugar noted by Burns (1937). Later work showed mannose and maltose to be

very effective inhibitors, sucrose and glucose less so, and galactose and fructose to be ineffective inhibitors of flocculation in *Saccharomyces cerevisiae* (Lindquist, 1953; Eddy, 1955a, b; Mill, 1964b; Fujino and Yoshida, 1976; Kamada and Murata, 1984c; Kihn, Masy and Mestdagh, 1988a). Other researchers using different strains, found flocculation to be specifically inhibited by mannose and its derivatives (Taylor and Orton, 1978; Miki *et al.*, 1982; Lipke and Hull-Pillsbury, 1984), other sugars being ineffectual. This contradiction has since been resolved, after a survey of a large number of flocculent yeasts (Stratford, 1989b). Two types of flocculent strains were found, Flo1 phenotype inhibited specifically by mannose and derivatives, and NewFlo phenotype strains inhibited by mannose, maltose, glucose and sucrose. This division of flocculent *Saccharomyces cerevisiae* strains (*Figure 4*) has been confirmed (Masy *et al.*, 1990) and extended to other flocculation characteristics (Stratford and Assinder, 1991).

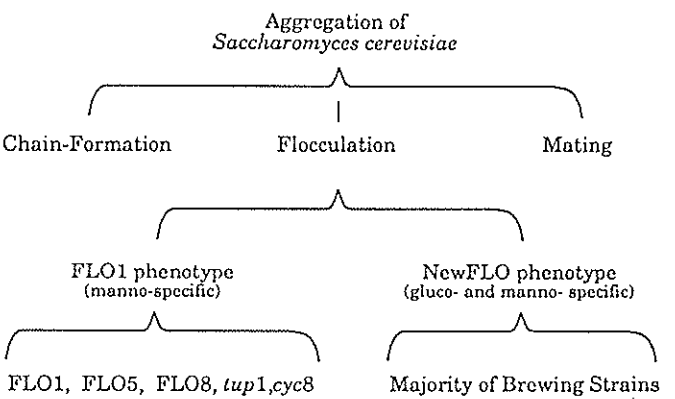

Figure 4. Position of two distinct flocculation phenotypes within aggregation of *Saccharomyces cerevisiae*. The constitutive Flo1 phenotype is consistent with a mannose-specific lectin mechanism and is found in strains containing *FLO1, FLO5* or *FLO8* genes or *tup1* or *cyc8* mutations. NewFlo phenotype flocculent strains form the majority of brewery yeasts, and flocculate in the stationary phase of growth using a gluco- and manno-specific lectin mechanism.

Flocculation of yeasts in other genera has also been reported to be inhibited by sugar (*Table 1*); the specific sugars causing inhibition vary with yeast species. For example, flocculation in *Saccharomyces uvarum* was inhibited by glucose and mannose (Hussain *et al.*, 1986), while *Kluyveromyces bulgaricus* flocculation was inhibited by fucose and galactose. The effects of sugar on flocculation have been shown not to involve cell metabolism (Brohan and McLoughlin, 1985) since flocculation of heat-killed cells was also inhibited by specific sugars (Stratford and Assinder, 1991). Lower concentrations of sugars, normally regarded as insufficient to cause inhibition, were found to be partially inhibitory when yeast flocs were subject to the stresses of agitation (Stratford, Coleman and Keenan, 1988). More detailed studies of sugar inhibition (Masy *et al.*, 1990; Stratford and Assinder, 1991) of *S. cerevisiae* showed that the NewFlo phenotype flocculation was most easily inhibited by

di- or trisaccharides with a glucose or mannose residue at the non-reducing terminal, while Flo1 phenotype flocculation was best inhibited by mannose derivatives and mannobiose.

Proteases

The first report of effects on flocculation by proteases was made by Eddy and Rudin (1958a), who found that treatment of flocculent yeast cells with papain caused an irreversible loss of flocculence. This result has since been confirmed using a variety of other proteolytic enzymes (Stewart, Russell and Garrison, 1973; White and Kidney, 1979; Nishihara, Toraya and Fukui, 1977, 1982; Miki *et al.*, 1982; Lipke and Hull-Pilsbury, 1984; Watari *et al.*, 1987), and also by chemical modification of protein components of the yeast cell surface (Nishihara, Toraya and Fukui, 1977). Modification of the yeast cell surface by lipases, DNAase, RNAase or lysozyme did not cause flocculation loss (Nishihara, Toraya and Fukui, 1982). However, flocculation loss has been reported following carbohydrate modification by α-amylase (Stewart, Russell and Garrison, 1973) and zymolyase treatment (Williams and Wiseman, 1973b). The material released from trypsin-digested cells, both flocculent and non-flocculent, has been analysed (Marfey, Sorensen and Ottesen, 1977). Flocculent cells were found to release more carbohydrate and less protein than non-flocculent cells.

The involvement of a surface protein in yeast flocculation was also suggested by the prevention of flocculation development by the protein synthesis inhibitor cycloheximide (Baker and Kirsop, 1972; Stewart, Russell and Garrison, 1973; Nishihara, Toraya and Fukui, 1976a; Lands and Graff, 1981). Addition of cycloheximide after flocculation development was ineffective. The surface protein may also be glycosylated, given that flocculation development was also arrested by tunicamycin (Stewart *et al.*, 1990). Surface proteins on flocculent yeast cells are also likely to be the antigenic determinants of antibodies to flocculent yeasts developed by Nagarajan and Umesh-Kumar (1990). Differential responses to various protease treatments have been used to distinguish between flocculation type. Hodgson, Berry and Johnston (1985) used proteases and heat to separate strains containing *FLO1* and *FLO5* genes, and two phenotypes of *Saccharomyces cerevisiae* flocculation were similarly found by Stratford and Assinder (1991). Four phenotypes of highly flocculent wine strains have also been reported (Suzzi and Romano, 1991).

Inorganic salts

Experiments to determine the effects of inorganic salts on yeast flocculation must be some of the simplest possible, and as a result probably more papers have been published on these effects than on any other aspect of flocculation. Yet no definitive consensus has been achieved, probably because there are several different effects occurring simultaneously, thereby causing apparent confusion. The tale begins before the turn of the century, when Seyffert

(1896) observed loss of flocculation due to the softness of the brewing liquor; this was regained on addition of lime. Further work on this was carried out by Hayduck and Schuckling (1908), Schonfeld (1912), Moufang (1912), Schonfeld and Schonfelder (1914), Schonfeld and Krumhaar (1918a), and is not to be confused with work on the chemical coprecipitation of non-flocculent yeasts carried out at this time (Holderer, 1901; Lange, 1901; van Laer, 1905), now described as affinity flocculation (Bentham *et al.*, 1990). Salts, chlorides and sulphates of sodium, potassium, calcium and magnesium were all shown to stimulate flocculation of washed cells (Burns, 1937; Gilliland, 1951; Jansen and Mendlik, 1951).

In general agreement with these early experiments, most researchers report the importance of calcium salts in promoting flocculation (Lindquist, 1953; Helm, Nohr and Thorne, 1953; Eddy, 1955c; Hough, 1957; Masschelein and Devreux, 1957; Harris, 1959; Mill, 1964b; Lyons and Hough, 1970a, b; Taylor and Orton, 1973, 1975; Williams and Wiseman, 1973a; Lewis and Johnston, 1974; Stewart, Russell and Garrison, 1975a; Stewart and Goring, 1976; Jayatissa and Rose, 1976; Miki *et al.*, 1982; Nishihara, Toraya and Fukui, 1982; Hussain *et al.*, 1986; Masy, Kockerols and Mestdagh, 1991). While calcium ions are generally believed to be most effective at promoting flocculation, there is a strong body of evidence indicating that flocculation is also promoted by magnesium ions and dipositive ions of transition metals, such as manganese, iron, cobalt, nickel, zinc, copper and cadmium (Jansen and Mendlik, 1951, 1953; Lindquist, 1953; Eddy, 1955c; Harris, 1959; Stewart and Garrison, 1972; Patel and Ingledew, 1975; Stewart, 1975; Stewart and Goring, 1976; Miki *et al.*, 1982; Raspor, Russell and Stewart, 1990a, b). Flocculation has also been shown in buffer alone (Nishihara, Toraya and Fukui, 1982; Stratford, 1989c). The response of flocculation to cations appears to be dependent on the strain and also on pH value (Porter and Macauley, 1965; Miki *et al.*, 1982).

Washing the ionic fractions derived from the growth medium from flocculent cells is obviously a prerequisite for determining the effects of salts. Flocculated cells of some strains may be dispersed by washing with distilled water (Jansen and Mendlik, 1951), while other strains were not (Eddy, 1955a). Strains unable to be deflocculated by water alone have been shown to be dispersed by ion chelation with EDTA (Taylor and Orton, 1973; Stewart, 1975; Stewart *et al.*, 1976). Dispersal by ion chelation was shown to be fully reversible by addition of further calcium, manganese or magnesium ions. More recently, the possibility was raised that some cations were not washed from cells, but remained attached to cell walls (Kihn, Masy and Mestdagh, 1988). Finally, it has been demonstrated (Stratford, 1989c) that intracellular, or bound, calcium ions were readily released from EDTA-washed cells when they were placed in any of a number of buffers or salt solutions. Clearly ionic leakage from within cells makes it very difficult to determine which ionic species are directly required for flocculation.

However, there is some clear evidence suggesting a specific requirement for calcium ions in flocculation. The group 2a elements (analogues of calcium) strontium and barium competitively inhibit the promotion of floccu-

lation by calcium (Taylor and Orton, 1975; Nishihara, Toraya and Fukui, 1982; Stratford, 1989c; Kuriyama, Umeda and Kobayashi, 1991). Moreover, sodium ions also competitively inhibit flocculation (Mill, 1964b; Stewart and Goring, 1976; Nishihara, Toraya and Fukui, 1982; Stratford, 1989c). The almost identical crystal ionic radii of calcium and sodium are worth noting at this point. The amount of calcium salts required to induce flocculation are extremely small (10^{-8} M; Taylor and Orton, 1975), suggesting a direct role for calcium. A final piece of evidence that magnesium at least does *not* directly induce flocculation was provided using EGTA (Stratford, 1989c), a chelating agent with a much higher affinity for calcium over magnesium. At concentrations of EGTA where all calcium was chelated, leaving magnesium ions free in solution, no flocculation occurred. This shows that magnesium ions do not induce flocculation. While this last test is only directly applicable to magnesium, it does suggest, together with the other evidence, that calcium ions are specifically required for flocculence. Flocculent cells of *Kluyveromyces bulgaricus* (Hussain *et al.*, 1986), *Saccharomycodes ludwigii* (Stratford and Pearson, 1992), *Schizosaccharomyces pombe* (Johnson *et al.*, 1988) and *Zygosaccharomyces* sp. (Suzzi and Romano, 1990) have all been shown to be dispersed by EDTA treatment and to be reflocculated again by further addition of calcium ions, suggesting that calcium requirement is a general characteristic of yeast flocculation.

In addition to this requirement for calcium ions at very low concentrations (Taylor and Orton, 1975), at high concentration a number of salts inhibit flocculation. (Calibo, Matsumura and Kataska, 1989; Kida *et al.*, 1989; Menawat and Castellon-Vogel, 1989; Castellon-Vogel and Menawat, 1990; Stratford and Brundish, 1990; Raspor, Russell and Stewart, 1990a, b). Inhibitions were caused by cations of many salts, including calcium, and effects of different cations were additive with small, highly charged ions being most effective. It has been suggested that this high salt concentration inhibition is chaotropic in nature and is the result of protein distortion caused by highly charged ions and by protein dehydration (Stratford and Brundish, 1990). The effect varied considerably with the pH value.

pH Value

It is generally accepted that flocculation of yeasts can occur across a wide range of pH values, between pH 1·5 and 9·0 (Stratford, Coleman and Keenan, 1988), with pH optima between pH 3·5 and 4·5 (St Johnston, 1953; Mill, 1964b; Williams and Wiseman, 1973b). A very low pH value causes dispersion of flocs of *S. cerevisiae* (Stratford, Coleman and Keenan, 1988; Menawat and Castellon-Vogel, 1989; Teixeira and Mota, 1990) and *S. ludwigii* (Stratford and Pearson, 1992). This effect is immediately reversible, by raising the pH value.

It was once widely held that pH value was important in determining flocculation in wort (McCandlish and Hagues, 1929; De Clerck, 1930; Malkow, Petina and Zwetkowa, 1933; Malkow, 1934; Hennig and Ay, 1938). This view has now fallen into disfavour and pH is no longer regarded as being

a determining factor in flocculation under brewery conditions (Gilliland, 1951; Jansen, 1958).

However, given more satisfactory methods of measuring flocculation that are not influenced by pH effects on yeast surface charge (Stratford and Keenan, 1988; Stratford, Coleman and Keenan, 1988), a number of yeast strains have come to light with a narrow pH window in which flocculation does occur (*Figure 5*; M. Stratford, unpublished results). These brewery ale strains, a subgrouping of the NewFlo phenotype (Stratford and Assinder, 1991), show normal low pH inhibition of flocculation but are also reversibly inhibited above pH 5–6. This limited pH flocculation explains why so many ale strains will not flocculate in defined media or YEPD media (Stratford and Assinder, 1991). The pH value in unbuffered defined media commonly falls below pH 2·0 and YEPD varies from pH 6·5–pH 5·5. Kamada and Murata (1984a) similarly reported a strain that dispersed above pH 5·5. Clearly the original reports that no flocculation in brewing liquor was found if the pH value was greater than 5·7 (McCandlish and Hagues, 1929) are to be believed after all. (He who laughs last, laughs longest.)

Temperature

Temperature does not seem to play a significant part in yeast flocculation. Although flocculent yeast cells are usually at 25°C, normal flocculation occurred in cells at temperatures down to 4°C (Stratford, Coleman and Keenan, 1988). Stewart, Russell and Goring (1975b) reported little effect of temperature on flocculation in yeast cells grown at temperatures between 15 and 32°C. However, at higher temperatures (50–60°C) flocs have been reported to be thermally dispersed (Mill, 1964b). This effect was used by Taylor and Orton (1975) to provide a measure of flocculation. Similar dissociation of flocs has been shown in *Kluyveromyces bulgaricus* (Hussain *et al.*, 1986). The temperature at which flocs 'melt' has been regarded as evidence of hydrogen bond involvement in binding flocculent cells together. It is also consistent with hydrogen bond involvement in lectin–sugar binding (Quiocho, 1986).

Ethanol

While most brewing strains of *S. cerevisiae* are able to flocculate in buffer and certain salts, a small minority of strains have been found which only flocculate in the presence of ethanol (Eddy, 1955c, Hough, 1957; Mill, 1964a; Patel and Ingledew, 1975; Amory, Rouxhet and Dufour, 1988). Only very low concentrations of ethanol are required, e.g. 0·1% (Amory, Rouxhet and Dufour, 1988). Flocculation of strains *Candida* sp. and *Kluyveromyces marxianus* has also been reported to occur only in the presence of ethanol (Shieh and Chen, 1986).

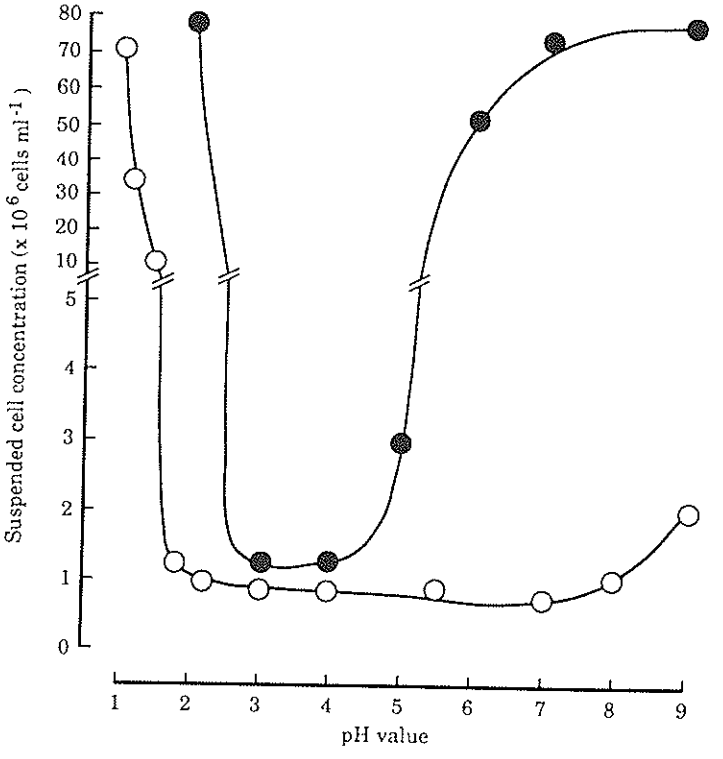

Figure 5. Effect of pH value on flocculation of *Saccharomyces cerevisiae* S646–1B (Flo1 phenotype, ○) and *Saccharomyces cerevisiae* NCYC 1190 (Narrow pH range NewFlo phenotype, ●). Yeast cells were placed in buffer containing calcium chloride, 50 mM, and agitated at 100 rpm for 4–6 h.

INDIRECT EFFECTORS OF FLOCCULATION

The development of flocculation in yeast appears to be a metabolic process involving synthesis of surface proteins which can be inhibited by cycloheximide or tunicamycin (Baker and Kirsop, 1972; Stewart, Russell and Garrison, 1973, 1990; Nishihara, Toraya and Fukui, 1976b; Lands and Graff, 1981). Clearly, any factors involved in the nutrition or general health of yeast cells are likely to have an indirect or knock-on effect on flocculation. It is difficult, therefore, to relate factors involved with the development of flocculation to definite roles in causing flocculation, or in the flocculation process itself. For example, alterations of pH value may have a direct effect on flocculation, an indirect effect via yeast health, or other effects by altering the rates of uptake of other yeast nutrients. The presence of several inorganic salts in the growth media has been associated with the ability of yeast strains to develop flocculation. Magnesium ions (Nishihara, Toraya and Fukui, 1976b) have been shown to be essential for the formation of flocculation as has the ratio of calcium to potassium ions in the media (Amri *et al.*, 1982). Phosphate was reported necessary by Eddy and Rudin (1958b), although Nishihara, Toyara and Fukui (1976b) reported poor flocculation using ammonium phosphate

rather than ammonium sulphate as a nitrogen source. Ammonia and nitrogen sources have been widely reported to be involved in flocculation. Beer worts containing high assimilable nitrogen cause delayed and poor flocculation (Devreux, 1962; Baker and Kirsop, 1972; Stewart, Russell and Goring, 1975b), an effect related to ammonium ions or basic amino acids (Mill, 1964a; Stewart, Russell and Garrison, 1973). Repression of flocculation in NewFlo phenotype brewing strains by ammonia has been reported (Stratford, 1989b). In contrast, early flocculation was reported in inositol-deficient media (Nishihara, Toyara and Fukui, 1976b), together with a requirement for glucose. Lands and Graff (1981) described flocculation in media where unsaturated fatty acids were limiting. Clearly much work has yet to be done to show which media factors are universally applicable to flocculation and which are idiosyncratic effects of individual yeast strains.

Environmental parameters, such as the pH value of the growth medium, temperature and degree of aeration have also been cited as important for flocculation expression by yeast. Low pH values of <3–4 in the growth media have been found to be antagonistic to flocculation (Porter and McCauley, 1965; Nishihara, Toraya and Fukui, 1976a). Flocculation in some yeast strains is temperature sensitive (Lund, 1951; Lindquist, 1953; Holmberg and Kielland-Brandt, 1978); for example a brewing strain flocculated when grown at low temperature but failed to at 25°C (Lund, 1951). The effects of aeration on flocculation are controversial. Most reports indicate that aeration promotes flocculation (Scriban, 1962; Miki, Poon and Seligy, 1982; Ramirez and Boudarel, 1983; Kamada and Murata, 1984a, b; Johnson *et al.*, 1987). Gilliland (1951), however, found that by aeration had no effect, while Holmberg and Kielland-Brandt (1978) and Soares, Teixeira and Mota (1991) found high aeration to inhibit flocculation. These contradictory results may reflect the different yeast strains used or variations in other culture conditions. Premature flocculation may also be caused by aeration (Piendl, 1969a, b). These results must be treated with a degree of caution since aeration is often associated with increased agitation, which has an entirely separate physical effect on flocculation (Stratford and Keenan, 1987).

There are a number of reports suggesting that mitochondrial function may be important in the expression of flocculation (Stewart *et al.*, 1976; Stewart and Russell, 1976, 1977; Holmburg, 1978; Evans *et al.*, 1980). Of 125 petite strains examined (Holmburg and Kielland-Brandt, 1978), 109 showed reduction or loss of flocculence. Carcinogens acting on mitochondria were shown to cause flocculation loss (Egilsson, Evans and Wilkie, 1979) and antibiotic-resistant mitochondrial mutants also showed flocculation loss (Spencer *et al.*, 1981). However, Nishihara, Toraya and Fukui (1976b) used chloramphenicol without effect, showing that mitochondrial function was needed for flocculation, not mitochondrial protein synthesis. The mitochondrial genes involved in flocculation were investigated by Esser, Hinrichs and Kues (1987) and Hinrichs, Stahl and Esser (1988). Simultaneous deletion of two mitochondrial sites caused flocculation loss: these were *oli1* and *oxi2*, coding for the oligomycin-sensitive ATPase and cytochrome oxidase, respectively. Mitochondria have been shown necessary for secretion of a number of proteins,

including glucoamylases, wall proteins and permeases (Gyllang and Martinson, 1972; Calleja, 1973; Spencer *et al.*, 1981; Wilkie and Nudd, 1981; Calleja *et al.*, 1986). It has been proposed that mitochondrial involvement in flocculation is mediated via a mitochondrial effect on the secretory pathway, rather than specifically on flocculation itself (Stratford, 1992b).

There have been a number of reports concerning the influence of various 'wort factors' on flocculation (Kudo, 1952, 1959; Kudo and Kujima, 1960; Morimoto *et al.*, 1975; Fujino and Yoshida, 1976; Herrera and Axcell, 1989, 1991a, b). These wort factors are generally large, complex carbohydrates, but a 'peptide inducer' from wort, peptone and gelatin has also been reported necessary for flocculation of some strains (Stewart, 1972, 1975; Stewart and Garrison, 1972; Stewart, Russell and Garrison, 1973; Stewart, Russell and Goring, 1975b). These high molecular weight compounds are unlikely to be transported into yeast cells and have been shown to adhere to cell walls (Fujino and Yoshida, 1976; Bowen and Cooke, 1989; Herrera and Axcell, 1991a, b), suggesting a direct interaction in the flocculation process.

PHYSICAL EFFECTORS OF YEAST FLOCCULATION

The physical process by which 'sticky' yeast cells adhere together in yeast flocculation is a process that has much in common with aggregation of other particles, both biological and inorganic. Much work has been done, in particular concerning the aggregation of colloidal particles, aggregates that are now recognized as fractal (reviewed by Meakin, 1988). Despite major differences in the chemical composition of particles and different forces involved in adhesion, the high degree of similarity between different aggregates and in the processes forming them has led to the concept of 'universality' of aggregates and the aggregate-forming processes (Lin *et al.*, 1989). To what extent does the aggregation of yeast cells into flocs relate to this theme of physical aggregation?

The three principal interrelated factors governing the physical process of yeast flocculation are pH value, salt concentration, and cell-wall composition. Together they determine the yeast surface charge (Brohult, 1951; Wiles, 1951; Jansen and Mendlik, 1951, 1953; Amory, Rouxhet and Dufour, 1988; Bowen and Cooke, 1989). Yeast cells have least surface charge at the isoelectric point, between pH 2 and 3·2 (Fisher, 1975; Jayatissa and Rose, 1976; Beavan *et al.*, 1979). In order for yeast cells to become bonded together in flocs, they must first make physical contact. But the yeast surface charge causes cells to remain dispersed by repulsion between like charges (Rose, 1984). In order for flocculation to occur, this like-charge repulsion must be overcome by physical agitation of the yeast suspension (Stratford and Keenan, 1987, 1988; Amory, Rouxhet and Dufour, 1988; Kihn, Masy and Mestdagh, 1988a, b; Stratford, Coleman and Keenan, 1988; Stratford, 1989a). In physical terms, this is described as orthokinetic aggregation (Tuorila, 1927; Overbeek, 1952; Yusa, 1977), as opposed to perikinetic aggregation, where particles collide due to thermal agitation — Brownian motion. Yeast cells, at 5 μm diameter, are too large to be affected by

Brownian motion. The normal size transition between Brownian motion and physical agitation has been calculated as being *circa* 1 μm (von Smoluchowski, 1917; Camp and Stein, 1943; Overbeek, 1952; O'Melia, 1972; Yusa, 1977). The effects of agitation on yeast flocculation are fully expounded by Stratford (1992b).

The process by which flocs of millions of cells are built from single cells was first suggested by the observation that the rate of flocculation was directly proportional to the square of the cell concentration (Stratford and Keenan, 1987). This is described by physical chemists as a second-order reaction (Barrow, 1973), where the rate-limiting step is the meeting of two identical particles. It was suggested (Stratford, 1992c) that flocs were built up from collisions of similar sized particles (*Figure 6*). Single cells collide to form doublets, which collide to form fours, eights, sixteens, etc., with the rate-limiting step being the initial meetings of single particles. This is termed a hierarchical model by colloidal physicists (Ball and Witten, 1984; Botet, Jullien and Kolb, 1984; Jullien, 1984; Jullien, Kolb and Botet, 1984) and describes an iterative cluster–cluster form of aggregation. It has also been termed 'Sutherland's ghost' (Witten and Cates, 1986) after the original theoretician (Sutherland, 1967).

The cluster–cluster method of aggregate formation is preserved within the structure of yeast flocs. When bigger clusters aggregate, the void spaces between them also get bigger. Thus floc particles become progressively less dense as they increase in size (Brohan and McLoughlin, 1984; Davis and Hunt, 1986; Logan and Wilkinson, 1991). Flocculated yeast particles are in fact a form of fractal structure. Fractal geometry is a natural description for disordered objects (Schaefer, 1989), often with sprawling, tenuous structures, that are self-similar on all scales. Fractals show the same structure patterns over and over again at different magnifications (Sander, 1987; Davey and Markx, 1990; Mandelbrot, 1990).

The proportion of void space in a fractal, in relationship to the particle size, gives a figure known as the Fractal Dimension. Solid objects with no void spaces have a fractal dimension of 3 (in Euclidean geometry). As the proportion of void space within flocs increases, the fractal dimension becomes progressively lower. Yeast flocs have been found experimentally to have a fractal dimention of 1·79 (Davis and Hunt, 1986). This indicates a very high proportion of void space within flocs and is consistent with a cluster–cluster mechanism of aggregation (Jullien, Kolb and Botet, 1984; Weitz *et al.*, 1984; Meakin, Chen and Deutch, 1985) dominated by collisions between clusters of roughly equal size (Ball and Witten, 1984; Botet, Jullien and Kolb, 1984; Jullien, Kolb and Botet, 1984; Witten and Cates, 1985).

In practical terms, it is recognized that fractal aggregates tend to be delicate, tenuous structures, and that rearrangements, restructuring and annealing of aggregates are likely to progressively increase the fractal dimension after aggregate formation (Schaefer *et al.*, 1984; Aubert and Cannell, 1986; Witten and Cates,, 1986). In real aggregates, this process is termed syneresis, the compaction and consequent exudation of water from flocs (Stevenson, 1972; Yusa, 1977). This is a process easily observed with

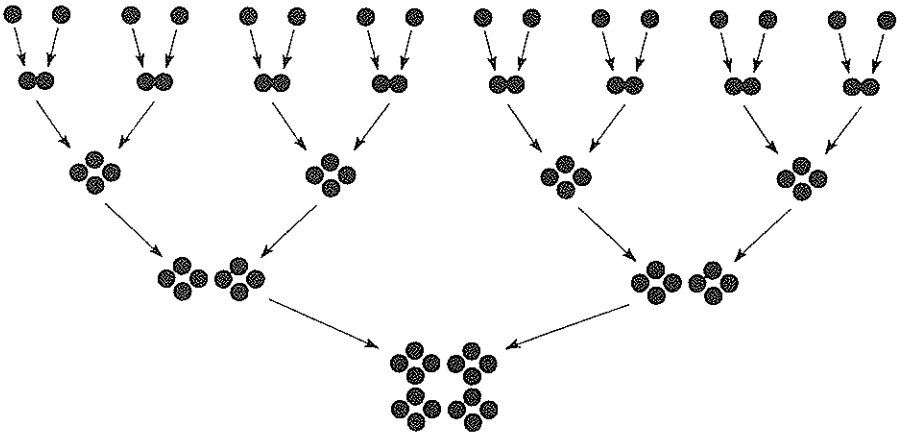

Figure 6. Hierarchical model of yeast flocculation (Sutherland's ghost). Flocs are built up through successive collisions between similar-sized particles, thus approximately doubling floc size at each stage. The rate-limiting steps are first collisions, where single cells combine to form doubles. This method of formation is reflected in the eventual cluster–cluster fractal structure of flocs.

yeast flocculation. When first formed, yeast flocs are open, tenuous structures, hence the derivation of the word 'flocculate' from the Latin *floccus*, a tuft of wool (Stewart and Russell, 1987). These tufts are rapidly and progressively compacted until floc-pellets of close-packed yeast are formed (Stratford, 1989a). This can occur as a result of either agitation or by gravity compressing the flocs. *Figure 7* shows the results of an experiment where the volume of a known amount of flocculated yeast is recorded with time. Newly-formed flocs occupy a large volume, but progressively compact while standing under gravity. Flocs agitated before standing are compressed by the agitation and consequently compact more slowly. Under prolonged agitation, flocs become sufficiently dense such that they do not compress further under gravity. Compression results in a progressive increase in the yeast-floc fractal dimension, which may account for the high values recorded by Logan and Wilkinson (1991). There is a commonly used test for yeast flocculation in British Breweries called the Burns or Helms test (Burns, 1937; Helm, Nohr and Thorne, 1953; Greenshields *et al.*, 1972), where flocculation is assessed by the volume occupied by flocs after standing for 10 min. *Figure 7* clearly demonstrates why these tests should be regarded as qualitative rather than quantitative measurements of yeast flocculation (Stratford and Keenan, 1988).

Given that yeast flocculation is an example of agitation-dependent aggregation, due to the size of particles and the surface charge, it is highly probable that aggregation or adhesion of other similar-sized particles will also be agitation-influenced (Stratford and Wilson, 1990). Almost all biological cells are far larger than 1 μm and surface charge has been implicated in aggregation of bacteria (Ofek and Beachey, 1980; Unz, 1987), erythrocytes (Jan and Chien, 1973a, b), animal cells (Aunins and Wang, 1989) and in the adhesion

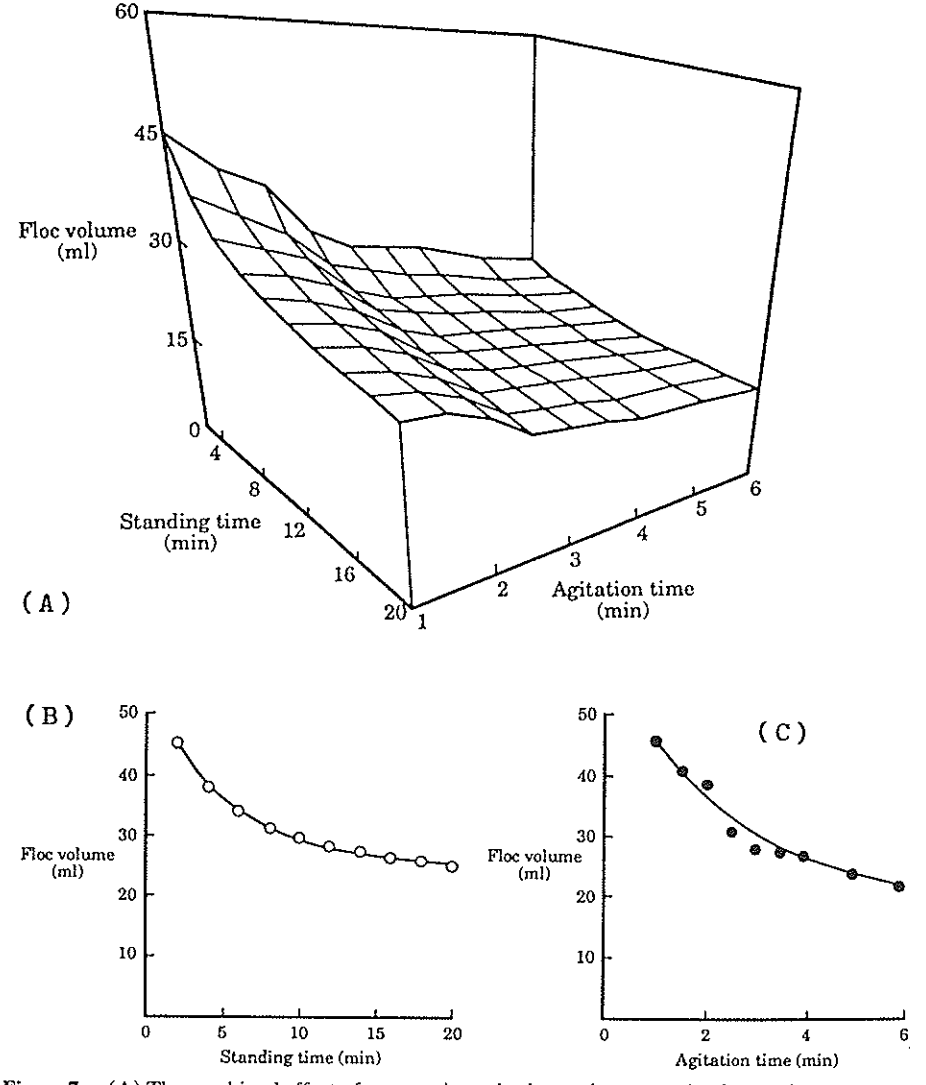

Figure 7. (A) The combined effect of progressive agitation and compression by gravity on yeast floc volume. Immediately after calcium addition to initiate flocculation, the flocs are loose, open structures, occupying a large volume that is progressively diminished by compression under gravity (B, ○), or by agitation (C, ●) which causes collisions between flocs that gradually compress their structures. After many hours agitation, flocs become close-packed and incompressible even by mild centrifugation.

of bacteria to animal cells (Heckels *et al.*, 1976; Watt and Ward, 1980; Read, 1989). It would therefore seem likely that some aspects of the 'universality' used to describe colloidal aggregations (Lin *et al.*, 1989) can also be extended to biological cell–cell interactions.

Structure and composition of the yeast cell wall

The nature of the yeast cell wall is important in flocculation. Not only are cell walls physically bonded to each other in this process, but the bonding mechanism, once established, remains a property of isolated walls (Eddy, 1955c; Masschelein and Devreux, 1957). A knowledge of the yeast cell wall structure and composition will clearly assist in determining which cell wall components participate in the bonding process.

The structure of yeast cell walls has been most fully explored in *Saccharomyces cerevisiae* and has recently been reviewed by Fleet (1991). The cell wall in *S. cerevisiae* consists largely of β-glucan and α-mannan, together with smaller proportions of chitin, protein (Griffin and MacWilliam, 1969) and possibly lipid (Fleet, 1991). Other yeasts contain different complex carbohydrates, for example galactan in *Schizosaccharomyces pombe* (Tkacz, Cybulska and Lampen, 1971; Barkai-Golan and Sharon, 1978). In *S. cerevisiae*, insoluble fibrils of β-glucan constitute an inner layer (Kopecka, Phaff and Fleet, 1974), a skeletal structure to which mannoproteins (mannan) and amorphous β-glucans are attached. Wall mannan and proteins are inextricably linked in covalent complexes. Cell wall mannan can be regarded as secreted glycoproteins, which are not released but anchored to the cell wall (Zlotnik *et al.*, 1984; Murgui, Elorza and Sentandreu, 1985). Two classes of wall mannoproteins have been recognized: wall enzymes, containing 50–70% mannan; and structural mannoproteins, containing 90% mannan (Ballou and Raschke, 1974; Frevert and Ballou, 1985). The structures of wall mannoproteins, essentially similar to secreted glycoproteins, have been extensively studied (Ballou, 1976; Cohen, Zhang and Ballou, 1982; Fleet, 1991). An inner core of 11–15 mannose residues is *N*-glycosidically linked to protein asparagine residues (*Figure 8*) via a diacetylchitobiosyl unit (Ballou, 1976). To this highly conserved inner core an outer chain of up to a further 150 mannose residues is attached (Cohen, Zhang and Ballou, 1982). Thus the bulk of cell wall mannan consists of outer-chain glycosylation. In addition, short mannan chains are directly *O*-glycosidically linked to protein serine and threonine residues. The outer-chain mannan consists of a long α-(1–6) linked backbone, to which are attached, with α-(1–2) linkage, short side-branches of 1–3 mannose residues (*Figure 8*). Phosphate groups have been reported to be present in the mannan structure (Northcote and Horne, 1952; Lindquist, 1953; Eddy, 1958b) and found to form the attachment of a small minority of mannan side-branches (Ballou, 1976).

Amorphous β-glucans are also present in the cell wall and have been implicated in the attachment of mannoproteins to the cell wall glucan fibrils (Blagoeva, Stoev and Venkov, 1991; van Rinsum, Klis and van den Ende, 1991). Chitin, linear chains of β-(1–4) linked *N*-acetyl glucosamine residues, may be a minor component of cell walls (Tronchin *et al.*, 1981), but it is an integral part of yeast bud-scars (Cabib and Bowers, 1971).

Chronology of theories of flocculation mechanism

The theories as to the mechanism by which yeasts flocculate are many and varied. In the past these theories were frequently those that were generally fashionable at the time, adjusted to fit flocculation; regrettably, the facts of flocculation were frequently adjusted to fit these fashionable theories! Our currently fashionable theory of flocculation, the lectin theory, appears to fit easily with all the known facts, yet the possibility remains that in the future this theory, too, will appear as naive and archaic as some of those listed here. Many of the early references cited here on flocculation mechanisms are abstracted from an excellent review by Jansen (1958).

The chemical precipitation theory described the flocculating action of chemical precipitates on yeast (Holderer, 1901; Lange, 1901), in this instance aluminium hydroxide, as is currently used in the purification of drinking water (TeKippe and Ham, 1971). Schonfeld and Krumhaar (1918b) were of the opinion that calcium phosphate precipitation was involved. Chemical precipitation undoubtedly removes particulate material, such as yeast (Bentham *et al.*, 1990), but is not flocculation as defined here.

The symbiotic theory of flocculation suggested the adhesion to yeasts by other organisms, usually bacteria. Barendrecht (1901) reported yeast aggregated by *Leuconostoc agglutinans*. Other lactic acid bacteria have also been shown to flocculate yeast (Malkow, 1934; Momose, Iwano and Tonoike, 1969; Yokoya and de Oliva-Neto, 1991). Flocculation of non-flocculent yeast may be caused by bacterial flocculent substances (Kurane, Takeda and Suzuki, 1986), mucillage (Beijerinck, 1908), lectins (Eshdat, Speth and Jann, 1981; Sharon *et al.*, 1981; Firon, Ofek and Sharon, 1982) or pili (Ogden and Taylor, 1991). Flocculent yeasts, on the other hand, have been shown to bind a variety of bacteria (White and Kidney, 1979; Stewart and Russell, 1987). It has, however, long been recognized that bacterial contamination is not the sole cause of yeast flocculation (Beijerinck, 1908).

The enzyme theory (Lange, 1907) suggested adhesion between yeast cells by an adsorbed layer of proteins on yeast cell walls. Non-flocculent yeasts were rich in peptidases, which dissolved this precipitate and thus prevented flocculation.

The colloidal theory of flocculation (Luers and Heusz, 1921; Geys, 1922) suggested that, like colloidal particles, yeast cells were maintained in suspension by their surface charge. Neutralization of this charge by salt addition or pH change causes rapid aggregation of colloids (Kruyt, 1952) and the importance of salts to flocculation suggested a similar mechanism. The onset of flocculation was thought to be associated with neutralization of surface charge. However, yeasts do not lose their surface charge during fermentation (Wiles, 1951; Jansen and Mendlik, 1951), and neither do they flocculate when the surface charge is artificially neutralized (Eddy and Rudin, 1958b; Fisher, 1975).

The coated yeast theory (Ranken, 1927; Ranken and Bell, 1928) involved adhesion of various materials from the medium to yeast cell walls. Addition of calcium phosphate and peptone tannins aided this process. More recently,

Bowen and Cooke (1989) have shown binding of wort components to cell walls, and wort peptide inducer has been shown to be necessary for flocculation of some strains (Stewart, Russell and Garrison, 1973, 1975). Such yeast coatings, however, cannot account for the flocculation of many yeast strains in totally synthetic media (Stratford and Assinder, 1991).

The calcium bridging theory is based largely on recognition of the importance of calcium ions in flocculation (Harris, 1959; Mill, 1964b). It was proposed that bivalent calcium ions formed bridges linking cells, supported by hydrogen bonding. Hydrogen bonding was inferred from the floc dissociation temperature (Mill, 1964b). The most probable sites for linkage by calcium bridging were thought to be carboxyl groups, present on surface proteins. Chemical modification of these groups by 1,2-epoxypropane abolished flocculation (Mill, 1964b; Jayatissa and Rose, 1976). Phosphodiester groups present in the yeast wall mannan were also suggested as sites for calcium bridging (Lyons and Hough, 1970a, b; 1971). This latter hypothesis was less likely in view of the lack of any correlation between wall phosphate content and flocculation (Griffin and MacWilliam, 1969; Jayatissa and Rose, 1976; Stewart *et al.*, 1976; Beavan *et al.*, 1979). The calcium bridging hypothesis has now been largely superseded by the lectin theory, since this latter theory is able to account for the observed sugar inhibitions of flocculation.

The lectin/lectin-like theory of flocculation was presented and fully expounded in its present form by Miki *et al.* (1981, 1982; Miki, Poon and Seligy, 1982). This proposes highly specific bonding between proteins (lectins) on flocculent cells, and the sugar residues intrinsic to the mannan which makes up the cell walls. The basis of this theory was determined, however, many years earlier. The discovery of mutual flocculation — non-flocculent strain pairs able to flocculate when mixed (Eddy, 1957; 1958a; Hough, 1957) — suggested that the flocculation bond had two non-identical parts (Eddy and Rudin, 1958a). It was also suggested from use of a protease, papain, and the observed inhibition by mannose, that these two parts were cell wall proteins and mannan (Eddy and Rudin, 1958a; Rainbow, 1966). Identification of specific inhibition by mannose (Taylor and Orton, 1978) also led to a suggestion of protein–carbohydrate bonding. The essential role of calcium ions has been suggested as one of maintaining surface lectins in an active conformation (Miki *et al.*, 1981, 1982; Miki, Poon and Seligy, 1982), as has been shown in other lectins (Sharon and Lis, 1972). It was also demonstrated (Miki *et al.*, 1981, 1982) that treatment of flocculent yeast with proteases, while irreversibly inhibiting flocculation, did not prevent these same cells from being co-flocculated by other flocculent yeasts. Protease treatment removed the lectin adhesins but not the carbohydrate receptors.

The phenomenon of heterologous flocculation, flocculation between unlike particles, is superficially complex, largely due to difficulties in terminology. Mutual flocculation (Eddy, 1957; 1958a; Hough, 1957) was first used to describe two weakly or non-flocculent strains able to flocculate strongly when mixed. This can be most easily explained by one of each strain pair having surface adhesins (lectins) and the other having receptors. This phenomenon

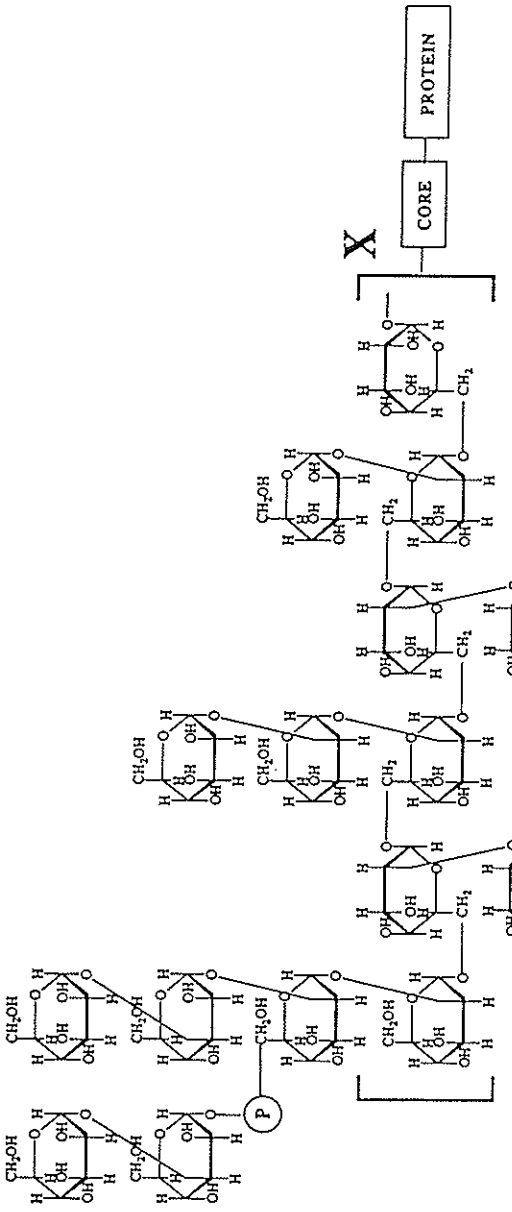

Figure 8. Schematic diagram of the *Saccharomyces cerevisiae* cell wall structural mannoproteins (Ballou and Raschke, 1974; Ballou, 1976). The major part of the carbohydrate is *N*-linked to asparagine via a conserved inner core of 12–15 mannose and two *N*-acetylglucosamine residues. The outer chain of about 150 mannose residues is formed around an α-(1–6) linked backbone to which a variety of side-branches are attached. This diagram indicates the possible types of side-branches, not their exact position or frequency.

was found to be calcium-dependent and protease-sensitive, but is here described as co-flocculation (Stewart, 1972; Stewart and Garrison, 1972; Stewart, Russell and Garrison, 1973, 1975). A related phenomenon describes the adhesion of non-flocculent yeasts to fully flocculent yeast cells. The non-flocculent particles usually possess receptors only, and flocculent yeasts adhere to them in a one-way bonding (Stratford, 1992b). This has also been called co-flocculation by Miki *et al.* (1981, 1982) and mutual flocculation by Curtis and Wenham (1958). A terminology has been proposed (Stratford, 1992b) where aggregation between pairs of non-flocculent strains is called mutual flocculation, and co-flocculation describes the adherance of non-flocculent yeast cells to flocculent yeast.

The lectin theory of yeast flocculation, of course, refers to the mechanism of yeast flocculation rather than implying that one lectin is involved. On the contrary, there is evidence that in *Saccharomyces cerevisiae* there are two distinct lectin mechanisms of flocculation: the Flo1 and NewFlo phenotypes, which involve mannospecific and gluco/mannospecific lectins, respectively (Stratford, 1989b; Masy *et al.*, 1990; Stratford and Assinder, 1991). Other lectin types, often fucose/galactose specific, have been implicated in the flocculation of other yeast genera (*Table 1*). Clearly, lectin-mediated aggregation of yeasts appears to have broader significance than merely to brewing strains.

Lectin adhesins in general and in flocculation

Lectins are a class of proteins, usually glycoproteins, that bind to specific sugars. This binding could be likened to the binding of an enzyme to a substrate without subsequent substrate modification. Lectins were originally defined (Goldstein *et al.*, 1980) as being able to agglutinate animal or plant cells and/or polysaccharides, glycolipids and glycoproteins, with at least two saccharide-binding sites. However, this definition needed modification after the discovery of extensive structural homologies between lectins and one-binding-site proteins. A lectin was redefined (Barondes, 1988) as: 'A carbohydrate binding protein other than an enzyme or antibody'.

The term lectin, coined by Boyd and Shapleigh (1954) from the Latin *legere*, to pick out, or choose, is entirely appropriate to the high specificity shown by lectins for their substrates.

Superficially similar lectins can distinguish extremely subtle variations in polysaccharides (Debray *et al.*, 1981) and the specificity shown by lectins for carbohydrates has been described as equalling or bettering that shown by antibodies for antigens (Sharon and Lis, 1972; Kabat, 1978; Doyle and Slifkin, 1989). Lectins are not antibodies, but an entirely separate group of metalloproteins (Kabat, 1978).

Lectins are generally multi-subunit proteins, with one saccharide-binding site per unit (Lis and Sharon, 1986), able to agglutinate cells as dimers or tetramers due to the carbohydrate binding sites at the ends of subunits (Carber, MacKenzie and Hardman, 1985). Two classes of lectins were recognized by Drickamer (1988), S-type and C-type. The more common

C-type requires metallic ions bound before active correct conformation. Those ions are calcium and manganese (Sharon and Lis, 1972). Detailed studies of concanavalin A structure have shown the Ca^{2+} and Mn^{2+} binding sites to be close to the saccharide-binding site (Blake, 1975; Einspahr *et al.*, 1988). These Ca^{2+}/Mn^{2+} binding sites are highly conserved protein sequences (Lis and Sharon, 1986). While calcium ions are clearly necessary for flocculation (Taylor and Orton, 1973, 1975; Stratford, 1989c; Kuriyama, Umeda and Kobayashi, 1991), no absolute requirement for manganese has been found. One possible explanation for this is that manganese ions are not washed off flocculent cells. It has been shown that Mn^{2+} fits before Ca^{2+} in the concanavalin A structure (Hardman and Ainsworth, 1972) with very much higher affinity (Kalb and Levitzki, 1968) and is therefore less likely to be removed. Low pH was found to inhibit concanavalin A by removal of calcium ions (McKenzie, Sawyer and Nichol, 1972) in a very similar fashion to that observed in flocculation. The high specificities of lectins for substrates were originally determined by sugar inhibitions (Goldstein, Hollerman and Smith, 1965; Smith and Goldstein, 1967), where modification of even single hydroxyl groups in sugar structures completely abolished recognition by lectins (Goldstein, Hollerman and Smith, 1965; Sharon, Firon and Ofek, 1983; Quiocho and Vyas, 1984). While lectins can be inhibited by solutions of simple sugars, it was recognized that lectins have more extended binding sites, three or more residues in length (So and Goldstein, 1968; Firon, Ofek and Sharon, 1982).

Lectins are found widespread throughout the plant and animal kingdoms in seemingly disparate and obscure roles. However, throughout their diverse application, lectins appear to play an integral part in cellular recognition and adhesion phenomena (Quiocho, 1986). They have been implicated in sperm–egg recognition, in aggregation of slime moulds, lymphocytes, marine sponges and embryonic differentiation (Phillips and Gartner, 1980; Barondes, 1981; Brandley and Schnaar, 1986). Other functions ascribed to lectins are recognition and targeting by certain toxins (van Heyningen, 1974) and mitogenic effects (Reeke *et al.*, 1974). A recent suggestion (Isla, Vattuone and Sampietro, 1991) has been made that lectins act as modifiers of enzyme activity of glycoproteins by binding to the carbohydrate residues.

In micro-organisms, however, lectins play a key role in the recognition of host cells by pathogens and subsequent attachment of pathogens to host cells (Sharon *et al.*, 1981; Lis and Sharon, 1986). This has been shown in bacteria (Firon, Ofek and Sharon, 1982; Sharon, Firon and Ofek, 1983), viruses (Markwell, 1986; Gattegno *et al.*, 1989), amoebae (Mirelman, 1987), phagocytes (Ofek and Sharon, 1988) and yeasts (Douglas, 1985). Of more than 100 micro-organisms with surface lectins described (Mirelman and Ofek, 1986), more than 90% had pathogenic potential.

How, then, does yeast flocculation fit in with these generally pathogenic and aggressive uses of lectins by micro-organisms? In a recent paper (Stratford, 1992a), it was speculated that flocculation lectins may originate from an infective agent of yeast rather than from the yeasts themselves, thus accounting for the targeting of these lectins towards the yeast cell wall, rather than a potential host cell. An association between flocculation and the

double-stranded RNA killer L virus of yeast (Stratford, 1992a) has been reported. Viral coat and spike proteins are known to have lectin activity (Markwell, 1986) and to be able to flocculate erythrocytes.

Flocculation lectins are probably highly glycosylated wall proteins, judging by the observed effect of tunicamycin (Stewart *et al.*, 1990). They may be attached to the wall by lectin–carbohydrate binding or be anchored to the wall (*Figure 9*), as has been reported for wall enzymes (Pastor, Herero and Sentandreu, 1982). Trypsin-digested flocculent *Saccharomyces cerevisiae* cells were found to release a substance, YCSS, that caused aggregation of non-flocculent yeasts (Kamada and Murata, 1984a, b, c). YCSS proved on analysis to be a mannoprotein (Kamada and Murata, 1984b) and caused aggregation that was both maltose-inhibited and calcium-dependent. This indicates that flocculation lectins are probably multimeric, since only multimeric forms of lectin would be able to bridge between cells (*Figure 9*). A similar surface protein extracted from flocculent yeast by Kijima (1964) was also found to cause aggregation.

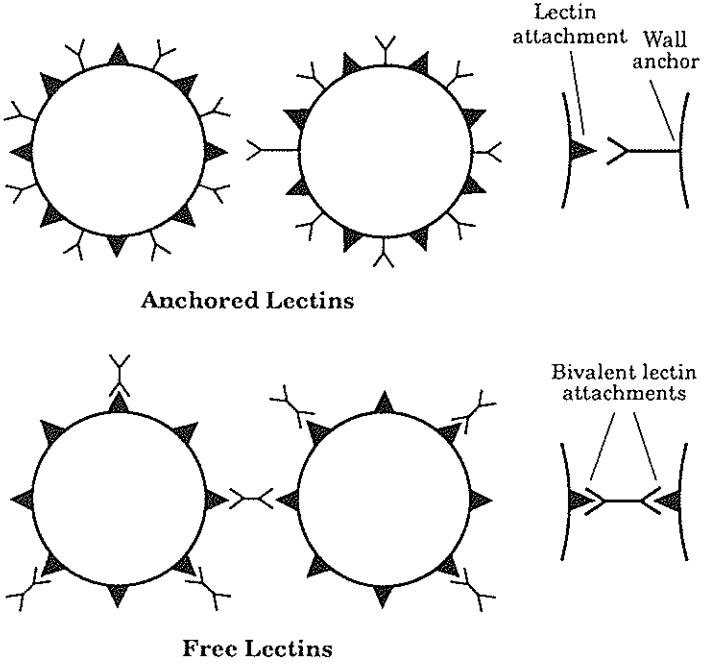

Figure 9. Two possible mechanisms of lectin bonding between yeast cells. Free bivalent lectins can be released from cells and bond to two cells by lectin action. Alternatively lectins may be anchored in the cell wall and only bond to one cell by lectin attachment.

The structural flocculation lectins of *S. cerevisiae* have not, to date, been isolated and characterized. Attempts have been made to isolate these surface proteins by comparing similar and isogenic pairs of flocculent and non-flocculent strains. A polypeptide of 13 kDa was found in alkaline extracts of flocculent cells (Holmberg, 1978). Stewart *et al.* (1976) reported a larger protein, 37 kDa, while Miki, Poon and Seligy (1982) found only minor

differences between flocculent and non-flocculent cells. Similarly, no differences were found in polyacrylamide gel electrophoresis (PAGE) patterns of cell wall proteins of *Saccharomyces uvarum* before and after expression of flocculence (Teixeira *et al.*, 1991). However, a new surface protein of M_r 13 000 was found in a non-flocculent strain, transformed to flocculence by the gene *FLO1* (Watari *et al.*, 1987).

In contrast to the flocculation lectins of *S. cerevisiae*, the lectins associated with *Kluyveromyces bulgaricus* flocculation have been isolated and characterized (Al Mahmood *et al.*, 1988, 1991). Lectins were released free into the media or after washing with galactose (Al Mahmood *et al.*, 1988; Giumelly *et al.*, 1989) and were found to be heat-stable and pronase-resistant. EDTA washing yielded two more galactose-specific lectin forms, a dimer and an octamer (Al Mahmood *et al.*, 1991), with a subunit size of 18·9 kDa. The size of the dimer (37 kDa) is therefore very similar to that associated with flocculation in *Kluyveromyces marxianus* (Teixeira *et al.*, 1989). Thus the lectins associated with flocculation in *Kluyveromyces* appear to be attached to the wall by lectin action (*Figure 9*) and are detached by sugar inhibition or EDTA chelation. In contrast, proteins associated with flocculation in *S. cerevisiae* required enzyme digestion and osmotic shocking for release (Williams and Wiseman, 1973b, Kamada and Murata, 1984a, b, c) and repeated EDTA washing did not cause any diminution of flocculation, as would be expected if lectins were released (M. Stratford, unpublished observation).

Many microbial lectins are found associated with surface spike structures, pili, fimbriae or fibrillae, in bacteria (Brinton, 1959; Pearce and Buchanan, 1980; Sharon, Firon and Ofek, 1983), yeast (Critchley and Douglas, 1987) and viruses (Markwell, 1986). Yeast flocculation has also been reported to be associated with surface fimbriae (Day *et al.*, 1975; Stewart *et al.*, 1976; Stewart and Russell, 1987), although these structures were not found by Miki *et al.* (1982). If fimbriae are present on flocculent yeasts, clearly there is a precedent for them to contain the surface lectins.

Complex carbohydrate receptors

The lectin–carbohydrate relationship has been likened to a lock and key interaction (Phillips and Gartner, 1980). With the high specificity of the lectin key, there must of course be equally sophisticated carbohydrate structures to act as the locks. For years regarded as an amorphous mush, carbohydrates are being increasingly recognized as highly complex structures of enormous diversity (Roseman, 1970; Brandley and Schnaar, 1986; Brandley, 1991). Complex carbohydrates have, like lectins, been shown to have an essential role in cell–cell recognition. Carbohydrates are in the right place — most cells have a complex carbohydrate outer shell — and have the diversity of structure necessary for cell identification and recognition (Brandley and Schnaar, 1986; Paulson, 1989). Information is continued in the complex carbohydrate structures, with lectins used to decode that information (Drickamer, 1988). Much greater structural diversity is possible in carbohydrates than in proteins,

due to their multiple linkage positions, anomers, branching and possible substitutions. This diversity can be created comparatively easily by relatively few enzymes (Roseman, 1970; Brandley, 1991). In order to be used for identification, it is necessary to envisage different types of complex carbohydrate on different cells. This is beginning to be confirmed experimentally, following the development of methods for analysis of carbohydrate structures (Brandley, 1991). In *Saccharomyces cerevisiae* cell walls, the numbers and patterns of side-chains of the wall polysaccharide mannan have been shown to be specific for different strains (Ballou and Raschke, 1974; Ballou, 1976).

Complex carbohydrate flocculation receptors have been fully defined in *S. cerevisiae*. The part of the *S. cerevisiae* wall thought most likely to contain the flocculation receptors is the α-mannan, given the observed specific inhibition of flocculation by mannose and its derivatives (Eddy and Rudin, 1958a; Taylor and Orton, 1978; Miki *et al.*, 1982). The discovery of two flocculation phenotypes in *S. cerevisiae* (Stratford, 1989a) does not alter this conclusion since both Flo1 and NewFlo phenotype flocculation were inhibited best by mannose. Further detailed sugar inhibition work (Stratford and Assinder, 1991) showed that the most likely receptors for both phenotypes were the side-branches of the outer-chain mannan, two or three residues in length (*Figure 8*). Similar studies on concanavalin A have shown that sugar inhibitions do not necessarily indicate the full, correct receptor structure (Carver, MacKenzie and Hardman, 1985).

However, flocculation receptors in *S. cerevisiae* have been further defined using cell wall mannan *mnn* mutants (Ballou and Raschke, 1974; Ballou, 1976). These mutants individually lack different parts of the α-mannan structure. It was found that co-flocculation of these strains was abolished in *mnn2* mutants, and was diminished in the *mnn5* mutant (Stratford, 1992c). *mnn2* mutants lack the mannan outer-chain side-branches, while *mnn5* mutants have reduced side-branches, only one mannose residue in length. *mnn1* mutants with two mannose-residue side-branches showed undiminished co-flocculation. These data independently confirm, as suggested by sugar inhibitions, that flocculation receptors in *S. cerevisiae* are the mannan outer-chain side-branches, two or three residues in length (*Figure 8*).

The flocculation receptors for other yeast genera have only been partially characterized by incomplete sugar inhibitions. *Saccharomycodes ludwigii* flocculation receptors are likely to have non-reducing terminal galactose, fucose or arabinose residues (Stratford and Pearson, 1992); galactose is the most likely, since this yeast co-flocculated *Schizosaccharomyces pombe*, a yeast with galactose-tipped side-branches (Horisberger, 1977). Some debate has surrounded the receptors for flocculation in *Kluyveromyces bulgaricus*. Flocculation was inhibited by galactose and fucose, but these sugars were not found in the *Kluyveromyces* cell wall (Hussain *et al.*, 1986; Giumelly *et al.*, 1989). Perhaps the receptor could be L-arabinose, a sugar similar structurally to fucose and galactose, which has been shown to be present in glycoproteins isolated from the cell wall of *K. bulgaricus* (Al Mahmood, Colin and Bonaly, 1991).

The genetics of flocculation

While flocculation has been widely reported in many yeast genera (*Table 1*), the genetic aspects of flocculation have only been studied in *Saccharomyces cerevisiae*, and in only one phenotype, Flo1 of this species. Nothing has yet been reported on the genetics of the NewFlo phenotype, probably because many of those strains will only flocculate in ill-defined media (Stratford and Assinder, 1991), thus making their study difficult.

FLOCCULATION CAUSED BY DOMINANT *FLO* GENES

The early reports that flocculation was an hereditary phenomenon (Pomper and Burkholder, 1949; Gilliland, 1951; Thorne, 1951a, b; Roman, Hawthorne and Douglas, 1951; Roman and Sands, 1953), contributed much to the demise of the colloidal theory of flocculation. However, the genetic value of some of these papers has been questioned (Calleja, 1987), since it is sometimes uncertain whether it was flocculation or chain-formation that was studied. True flocculation was found to be caused by the presence of *FLO* genes (Johnston and Lewis, 1974; Lewis and Johnston, 1974; Lewis, Johnston and Martin, 1976). Two closely linked dominant genes, *FLO1* and *FLO2*, were identified, together with the recessive *flo3* gene. The *FLO* genes and their suppressors are listed in *Table 2*. Dominant gene, *FLO4*, was discovered shortly afterwards (Stewart *et al.*, 1976; Stewart and Russell, 1977) and mapped to chromosome I, 37cM distal to *ade1*. A later reassessment of these genes showed that *FLO1, FLO2* and *FLO4* were in fact allelic, and the nomenclature was revised to combine these genes into *FLO1* (Russell *et al.*, 1980). The location of *FLO1* was confirmed by Skatrud, Kot and Helpert (1982) at 41·8 cM from *ade* 1, and by Kaback, Steensma and de Jonge (1989) at 3·8 cM from *PHO11*, and physically mapped to 24 kb from the right-hand end of chromosome I (Teunissen and Steensma, 1990). The *FLO1* gene has been isolated and cloned (Watari *et al.*, 1987, 1989, 1990, 1991a, b), enabling transformation of several non-flocculent industrial strains to a flocculent phenotype. Expression of *FLO1* was found to be suppressed in *MAT*a/*MAT*α diploids (Watari, 1990, 1991b).

FLO5 was described as a dominant gene, non-allelic with *FLO1*, conferring particularly strong flocculation (Johnston and Reader, 1982, 1983). This gene, after resisting attempts to map it for many years, recently has been shown to be also on chromosome I (Vezinhet, Blondin and Barre, 1991). *FLO1* and *FLO5* strains have been distinguished using proteases, heat and by calcium/strontium inhibition ratio (Hodgson, Berry and Johnston, 1985; Kuriyama, Umeda and Kobayashi, 1991). Recessive genes, *flo6* and *flo7*, were reported by Johnston and Reader (1982), but were considered to be possible alleles of *FLO1* that were more subject to suppression. A further dominant gene conferring flocculation, *FLO8*, was discovered by Yamashita and Fukui (1983). This showed loose linkage with *arg4* and mapped to chromosome VIII. *FLO8* was found, like *FLO1*, to be suppressed in *MAT* a/α diploids. The physiological characteristics of flocculation of *FLO1*,

FLO4, FLO5 and *FLO8* containing strains showed considerable similarity (Stratford and Assinder, 1991) consistent with constitutive expression of mannospecific lectins (Flo1 phenotype) (*Figure 4*).

Flocculation has always been regarded, especially by the brewing industry, as an unstable characteristic (Thorne, 1951b; Hough, 1957; Chester, 1963; Curtis, 1973; Stewart, 1975). Flocculation loss and instability have also been reported in genetically defined *FLO1* strains (Lewis, Johnston and Martin, 1976; Russell *et al.*, 1980; Johnston and Reader, 1982). Two genes causing suppression of flocculence have been identified (Holmberg, 1978; Holmberg and Kielland-Brandt, 1978; Stewart and Russell, 1981), and termed *fsu1*, originally *suf1*, and *fsu2*. In view of the simultaneous loss of flocculation and external enzyme secretion (Spencer *et al.*, 1981; Yamashita and Fukui, 1984), it is possible that some of the reported flocculation suppressors were acting on the yeast secretory pathway (Stratford, 1992a). The coincidence of the presence of the killer L dsRNA virus in yeast strains with the Flo1 phenotype, and its conspicuous absence in NewFlo phenotype strains, was reported by Stratford (1992a). This gave rise to the speculation that the killer L coat protein could be the structural flocculation lectin. This theory was based not only on the coincidence of Flo1 phenotype flocculation and killer L dsRNA, but also on the known lectin activity of viral coat and spike proteins (Markwell, 1986). It is also supported by the observation that *SSN6 (CYC8)* contains a TPR motif (Schultz, Marshall-Carlson and Carlson, 1990) that is also found in *SKI3*, a gene repressing replication of dsRNA viruses in yeast (Rhee, Icho and Wickner, 1989). Mutation of *SSN6/CYC8* is known to cause intense flocculation (Trumbly, 1988) of the Flo1 phenotype (Stratford and Assinder, 1991).

FLOCCULATION CAUSED BY MUTATION/HETEROLOGOUS GENE EXPRESSION

Flocculation can be caused by the presence of *FLO* genes, but in addition, flocculation can be caused by any one of a number of mutations to the *Saccharomyces cerevisiae* genome (*Table 3*). The most widely reported flocculation-causing mutations are *tup1* and *cyc8*. Both mutations have pleiotropic effects, affecting carbon catabolite repression, UV resistance, cytochrome *c* expression, mating, ARS1 stability and causing flocculation. *TUP1* and *CYC8* have been mapped onto chromosomes III and II, respectively. They have identical phenotypes and both have been cloned and sequenced (Schultz and Carlson, 1987; Trumbly, 1988; Fujita *et al.*, 1990; Williams and Trumbly, 1990). The *CYC8* and *TUP1* gene products were found to combine in a large protein complex, associated with the nucleus, that may be involved with the nuclear scaffold (Williams, Varanasi and Trumbly, 1991). The complex is certainly regulatory, possibly having a role in the inhibition of a class of transcriptional activators (Williams and Trumbly, 1990). In consequence of their many effects, *tup1* and *cyc8* mutants have been independently isolated from many non-flocculent strains, all of which have been reported to flocculate following these mutations.

Table 2. Summary of the characteristics and locations of *FLO* genes and their suppressors

Gene	Location	Dominance	Comments	References
FLO1	Chromosome I	Dominant	Suppressed in MAT a/α	Lewis and Johnston (1974)
FLO2		Dominant	Allelic with *FLO1*	Johnston and Lewis (1974) Lewis, Johnston and Martin (1976) Russell *et al.* (1980) Watari *et al.* (1989)
flo3		Recessive		Lewis and Johnston (1974)
FLO4		Dominant	Allelic with *FLO1*	Stewart *et al.* (1976) Russell *et al.*(1980) Johnston and Reader (1982)
FLO5	Chromosome I	Dominant		Vezinet, Blondin and Barre (1991)
flo6		Semi-dominant	Possible *FLO1* allele	Johnston and Reader (1982)
flo7		Semi-dominant	Possible *FLO1* allele	Johnston and Reader (1982)
FLO8	Chromosome VIII	Dominant	Suppressed in MAT a/α	Yamashita and Fukui (1983)
fsu1		Semi-dominant	*FLO4* suppressor	Holmberg (1978)
fsu2		Semi-dominant	*FLO4* suppressor	Stewart and Russell (1981)

Table 3. Mutations of the *Saccharomyces cerevisiae* genome and heterologous gene expression, giving rise to flocculation

Gene/mutation	Synonym	Characteristic selected	References
tup1	*tup1*	dTMP auxotrophy	Wickner (1974)
	flk1	Derepressed maltase	Schamhart, ten Berge and van de Poll (1975) Stark, Fugit and Mowshowitz (1980)
	umr7	UV resistance	Lemontt (1977) Lemontt, Fugit and Mackay (1980)
	cyc9	Cytochrome c expression	Rothstein and Sherman (1980)
	pd7	Abnormal mating	Manney, Jackson and Meade (1983)
	amm1	ARS1 stability	Thrash-Bingham and Fangman (1989)
	sfl2	Flocculation	Fujita *et al.* (1990)
	aer2	Heme regulation	Zhang *et al.* (1991)
	aar1	Mating regulation	Mukai, Harashima and Oshima (1991)
cyc8	*cyc8*	Cytochrome c expression	Rothstein and Sherman (1980) Trumbly (1986)
	ssn6	Derepressed invertase	Carlson *et al.* (1984) Neigeborn and Carlson (1987)
FH4C		Envelope structure	Ghosh, Montenecourt and Lampen (1973) Montenecourt, Kuo and Lampen (1973)
abs		Acid phosphatase	Hansche (1975)
wa1		Wall morphology	Lange (1979)
oxi1/oli2		Flocculation suppression	Hinrichs and Esser (1987)
sfl1		Flocculation	Fujita *et al.* (1989)
cka2		Casein kinase II	Padmanabha *et al.* (1990)
Ha-*ras*		Human Ha-*ras* p21	Clark, McGrath and Levinson (1985)
tax		HTLV-1 viral tax transactivator	Kramer *et al.* (1990)
CHS2		Chitin synthase 2 overexpression	Sudoh *et al.* (1991)

Many other mutations causing flocculation have been reported (*Table 3*), some of which, *abs, wal* and FH4C, have pleiotropic effects (Montenecourt, Kuo and Lampen, 1973; Ghosh, Montenecourt and Lampen, 1973; Hansche, 1975; Lange, 1979) and may be alleles of *tup1* or *cyc8* or others of the seven complementation groups described by Carlson *et al.* (1984) as causing flocculation. *SFL1* and *CKA2* are separate, non-allelic genes whose disruption also causes flocculation (Fujita *et al.*, 1989; Taira *et al.*, 1990; Padmanabha *et al.*, 1990).

Expression of two heterologous genes in *S. cerevisiae* has also, unexpectedly, given rise to the *FLO1*-like flocculation. These are human Ha-*ras* p21 (Clark, McGrath and Levinson, 1985) and the viral HTLV-1 *tax* transactivator (Kramer *et al.*, 1990). The Ha-*ras* gene products were localized on the inner membrane surface (Furth *et al.*, 1982), the location also of G protein which has a degree of homology with the *tup1* product, reported by Kearsey (1991) and Williams, Varanasi and Trumbly (1991). Recently over-expression of the chitin synthase 2 gene, *CHS2*, was also shown to cause flocculation in *S. cerevisiae* (Sudoh *et al.*, 1991). Clearly, there is much research still to be done concerning the network of regulators and signals involved in flocculation expression.

In view of the ease with which non-flocculent strains can flocculate by mutation (*Table 3*) and the large number of *tup1/cyc8* isolations, all of which flocculate, it is possible that most, or possibly all, strains of *S. cerevisiae* contain genes coding for flocculation lectins (Stratford, 1992a). Furthermore, the similarity between flocculation caused by *tup1* or *cyc8* mutation, and *FLO1, FLO5* and *FLO8* genes, may indicate that the *FLO* genes are also regulators, activators of an, as yet undiscovered structural lectin gene. This theory will be tested in the near future when the *FLO1* gene is fully sequenced. Preliminary results (M. Pentilla, personal communication) indicate the presence of a signal sequence, a membrane anchor and multiple repeats. If *FLO1* is a lectin gene, homology would be expected with the highly conserved regions of known lectins, the Ca^{2+} and Mn^{2+} binding sites (Lis and Sharon, 1986). On the other hand, a membrane anchor could suggest a secreted protein, in which case, if *FLO1* does code for a lectin, there must be other lectin genes present in non-flocculent cells to allow flocculation following mutation.

Onset of flocculation

While the known genetics of flocculation is exclusively the preserve of the Flo1 phenotype in *Saccharomyces cerevisiae*, the onset of flocculation is almost exclusively found in NewFlo phenotype strains. Almost all Flo1 phenotype strains are constitutively flocculent (Stratford and Assinder, 1991), therefore onset of flocculation never occurs. NewFlo phenotype strains grow and ferment as single cells and only flocculent in the stationary phase of growth. Whether flocculation occurs in the stationary phase, or whether flocculation causes the stationary phase, is a debatable point. Both statements

may be correct in different yeast strains (M. Stratford, unpublished observations).

While of lesser academic interest, the causes and timing of onset of flocculation are of exceptional commercial interest to the brewing industry. Flocculation can determine the degree of attenuation in brewing (Rainbow, 1966). Early or premature flocculation is one of the most common causes of 'hung' or 'stuck' fermentations (St Johnston, 1953; Stewart, 1975; Stewart *et al.*, 1976), and consequently causes considerable difficulty and expense in restarting the fermentation, and attenuating the residual sugar content.

ONSET OF FLOCCULATION: RECEPTOR OR LECTIN, SYNTHESIS OR ACTIVATION?

Assuming that the lectin theory of flocculation is correct, or at least close to the truth, the onset of flocculation must depend on the appearance of either the lectin adhesins, or the carbohydrate receptors, or both. Some years ago, there was a theory in vogue that the flocculation mechanism was masked by *too much* wall mannan (Devreux, 1962; Masschelein *et al.*, 1963; Windisch, 1968). This led in turn to much cell wall analysis which reported no correlation between mannan content and flocculation (Mill, 1966; Griffin and MacWilliam, 1969; Lyons and Hough, 1971). Despite this, a strain requiring mercaptoethanol treatment to expose mannan receptors has been reported (Nishihara and Toraya, 1987) and it has been shown that the cell wall mannan content alters during cell growth (Griffin and MacWilliam, 1969; de Nobel *et al.*, 1990a, b). However, in a comprehensive examination of a number of strains (Stratford, 1993), plenty of flocculation receptors were found at all stages of growth. Flocculation onset is therefore not likely to depend on receptor availability, but rather on the appearance of active lectins.

It has recently been found (Stratford and Carter, 1993) that onset of flocculation is more complex than was originally thought. Cycloheximide was added to yeast cultures throughout growth, to determine exactly when the proteins involved in flocculation were synthesized (*Figure 10*). It was found that flocculation lectin synthesis began early in growth, continued throughout exponential growth and stopped in stationary phase. Some hours afterwards, these flocculation lectins were 'activated' by an as yet unexplained process, that was not inhibited by the presence of cycloheximide. Pronase E treatment of yeasts in the few hours before 'activation' prevented flocculation expression. It therefore seems that lectins are synthesized and secreted to the cell wall progressively throughout growth, where they are held in an inactive form, but accessible to Pronase E. At a later time, lectins are activated and flocculation commences. It is possible that the 'activation' may involve physical uncovering of lectins using α-mannosidase (Beavan *et al.*, 1979) or proteolytic cleavage as described for killer toxins (Bussey *et al.*, 1983). Synthesis and activation of lectins at separate times was found in all 12 NewFlo phenotype strains examined; this would therefore appear to be a ubiquitous phenomenon, at least in NewFlo phenotype strains.

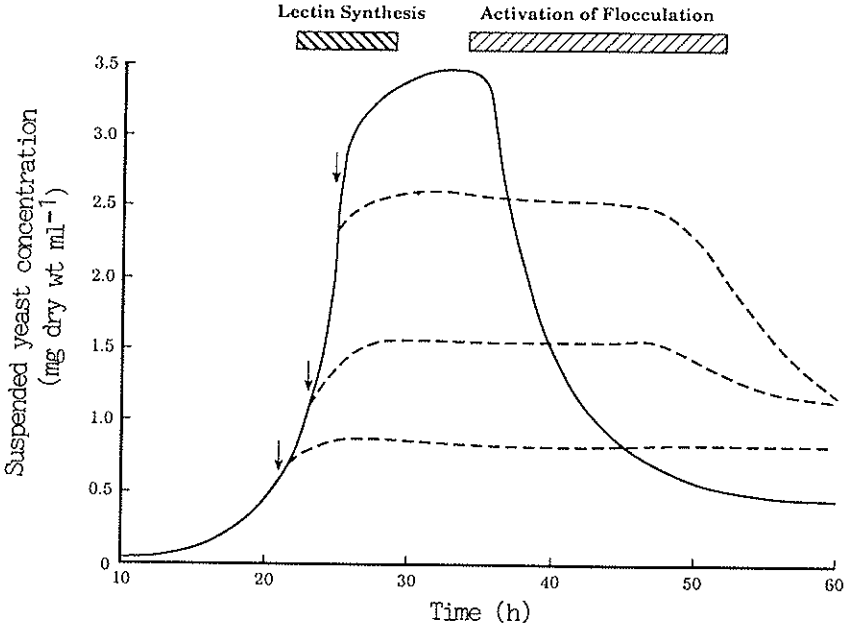

Figure 10. Growth and flocculation curve of *Saccharomyces cerevisiae* NCYC 1364. Flocculation onset occurs at about 36 h. Cycloheximide addition to different cultures at different times (arrows) stopped growth and prevented flocculation when cycloheximide was added before 21 h. This suggests that lectin synthesis begins at 21 h and continues until 28 h, in stationary phase. Some hours later, at 36 h, lectins are activated in a cycloheximide insensitive manner, thereby initiating flocculation onset (Stratford and Carter, 1993).

ENVIRONMENTAL FACTORS INFLUENCING ONSET OF FLOCCULATION

There are several controlled stages at which active surface lectins could potentially be held, pending the onset of flocculation (Stratford, 1992b). These controls are, in reverse sequence: by direct inhibition of lectins; by activation or exposure; during secretion; at the secretion process; or by transcription/translational control of the flocculation lectin genes. A number of factors influencing the onset of flocculation have been recorded, but it is not always clear at what level of control these factors operate.

The most obvious environmental factor influencing flocculation is sugar. Brewers' sweet-wort contains a large amount of maltose, a sugar strongly inhibitory to NewFlo phenotype flocculation (Stratford and Assinder, 1991). Yeast strains are unlikely to flocculate until there has been at least partial sugar depletion through fermentation (Baker and Kirsop, 1972). This is unlikely to be the whole story, since NewFlo phenotype strains show onset of flocculation when grown on non-inhibitory sugars, such as fructose (M. Stratford, unpublished observation).

The pH value is also likely to be a controlling factor in flocculation of certain strains. While most flocculent yeasts show activity across a broad pH spectrum, some strains are restricted to a pH window between 2·5 and 5·5

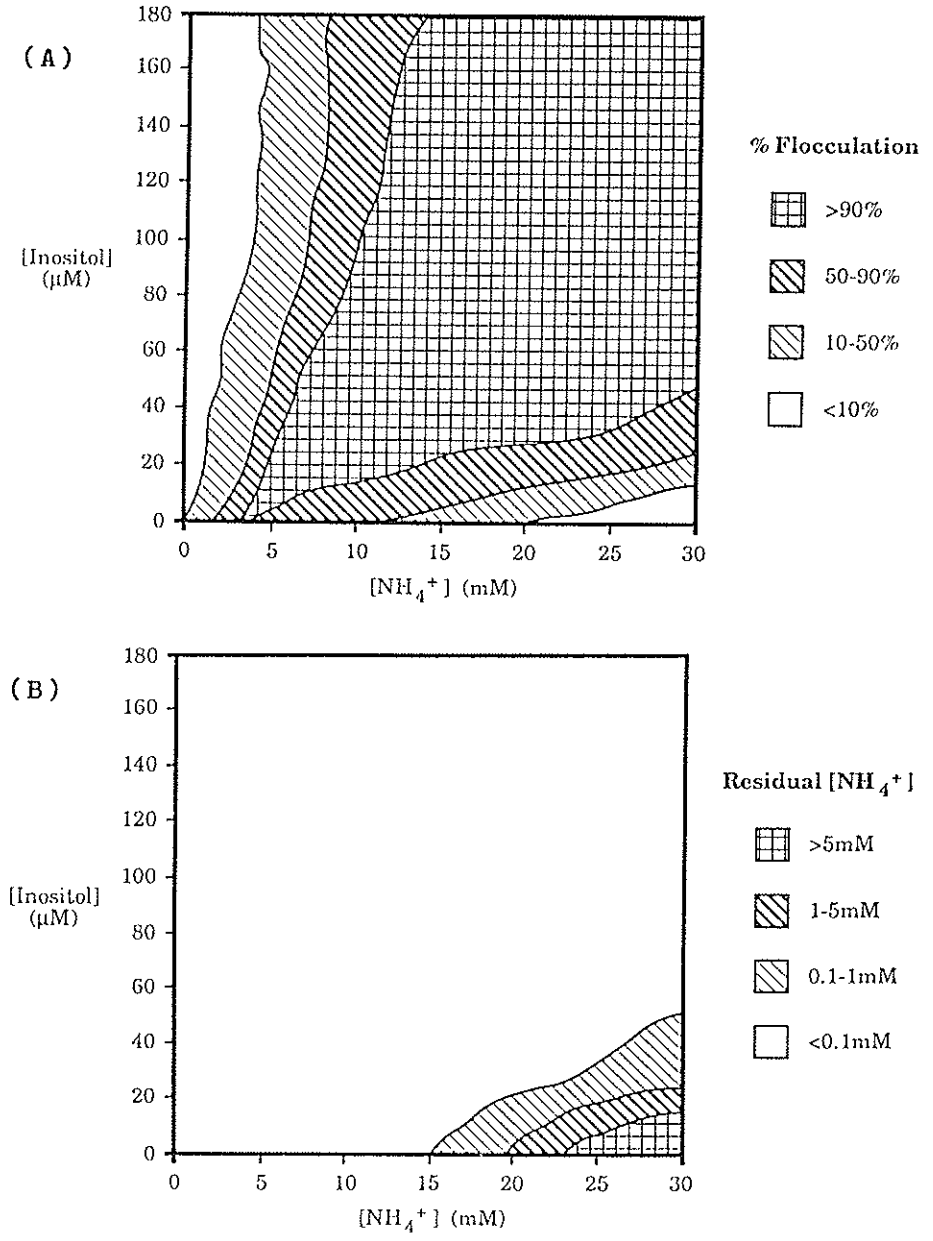

Figure 11. (A) Matrix of the effects of ammonium ion and inositol concentrations on flocculation of *Saccharomyces cerevisiae* NCYC 1364, in defined yeast base media, buffered to pH 4·0 and containing fructose, 40 g l^{-1}. Flocculation was assessed as a percentage of the cells flocculated in late stationary phase. Residual ammonium ions in the media at this stage are recorded in (B). Preliminary results suggested residual inositol present in media initially containing high inositol and low ammonium ion concentrations.

(*Figure 5*). Yeast growth usually causes a lowering of the pH value of the medium, and for pH-restricted strains no flocculation is observed if the pH is not within this window at the time of flocculation onset. In addition, flocculation has been reported to be prevented in growth media with pH values less than 3–4 (Porter and Macauley, 1965; Nishihara, Toraya and Fukui, 1976a).

The presence of assimilable nitrogen is certainly involved in development of flocculation. Assimilable nitrogen is generally limiting in brewers' wort (Gilliland, 1981). Early flocculation occurred in worts with low nitrogen contents (Gilliland, 1951), while delayed and diminished flocculation has been shown in high nitrogen worts (Devreux, 1962; Stewart, Russell and Goring, 1975; Baker and Kirsop, 1972). Stratford (1989b) reported the failure of NewFlo phenotype strains to flocculate in media containing excess ammonium ions. Ammonium salts and basic amino acids were also implicated by Mill (1964a) and Stewart, Russell and Garrison (1973).

Similarly, early flocculation has been found to occur in inositol-deficient media (Nishihara, Toraya and Fukui, 1976b; Amri *et al.*, 1982). Inositol deficiency is known to affect yeast cell walls and cause cell aggregation (Smith, 1951; Ghosh *et al.*, 1960; Challinor, Power and Tonge, 1964; Lewin, 1965; Dominguez, Villanueva and Sentandreu, 1978; Duffus, Levi and Manners, 1982) by what is usually regarded as chain-formation. However, inositol deficiency seems also involved in flocculation development. *Figure 11* shows the results of an experiment carried out in synthetic media varying in inositol and ammonium ion content, using a NewFlo phenotype flocculent yeast. True flocculation was found in most cultures but not in those containing any residual ammonium ions or inositol in the spent media. It would seem that presence of either ammonium ions or inositol prevents the development of flocculation (M. Stratford, unpublished results).

A large number of high molecular weight compounds from wort, especially wort made from Japanese six-rowed barley, have been implicated in premature flocculation in brewing (Kudo, 1952, 1959; Kudo and Kijima, 1960; Morimoto *et al.*, 1975; Fujino and Yoshida, 1976). One possible explanation for this, explored by Herrera and Axcell (1989), was that flocculation was caused by barley lectins present in the wort. One barley lectin was isolated, but it did not induce premature flocculation when added to worts. Following the discovery of a polysaccharide that induced premature flocculation, it was proposed (Herrera and Axcell, 1991a, b) that high molecular weight compounds could bridge between yeast cell walls, attached to the flocculation lectins. This theory was supported by Stratford (1992b), who argued that all of the reported compounds causing premature flocculation were complex carbohydrates, and were therefore likely to have a higher affinity for flocculation lectins than any inhibiting sugars from the medium.

The biotechnology of yeast flocculation: artificial control of flocculation onset, rate and extent

Over the last 15 years in which biotechnology has become fashionable, there has been increasing interest in yeast flocculation as a simple, natural and cheap way of separating the cell biomass from media at the end of fermentations. Separation costs can be accounted the largest single figure in the production process (Bowden *et al.*, 1987). As a result, there is considerable financial advantage to be gained by extending the flocculation property from brewery yeasts to other industrially important yeasts, and possibly to other organisms.

The objectives of artificial control of flocculation are:

1. Control over flocculation rate and extent.
2. Transfer of the flocculation property to other organisms.
3. Control of onset of flocculation.

These objectives have long been seen by some as legitimate targets to be achieved by genetic engineering and molecular biology. Others have questioned this approach. Casey (1990) described yeast flocculation as a classic example of where the use of the *FLO1* gene 'for genetic engineering purposes is severely handicapped by our lack of understanding of the chemistry and biochemistry of flocculation'. Given the possibility that *FLO1* may be regulatory, only strains containing the hidden lectin genes will be able to respond to *FLO1* stimulation to flocculate.

The ability to transfer flocculation to other yeast strains may be achieved in a variety of ways: by conventional genetic crossing (de Figueroa, de Richard and de van Broock, 1984a; Thornton, 1985; Cvetanov, Lachcev and Peceva, 1990); by protoplast fusion (Seki *et al.*, 1983; de Figueroa, de Richard and de van Broock, 1984b; Watari *et al.*, 1990); electrofusion (Urano, Nishikawa and Kamimura, 1990); as well as by transformation with cloned *FLO1* (Watari *et al.*, 1991b). In addition, flocculation induction in non-flocculent industrial strains may soon be brought about by disruption of the *tup1*, *cyc8* or *sfl1* genes, whose sequences are known, or by using ribozymic action to silence them (Taira *et al.*, 1990). Transformation by the HTLV-1 tax transactivator (Kramer *et al.*, 1990) or Human Ha-*ras* p21 (Clark, McGrath and Levinson, 1985) also causes flocculation, but is far less likely to be acceptable in industrial strains. For practical purposes, flocculation transfer would seem to be easiest by conventional mating or protoplast fusion.

Control over the onset of flocculation would seem to be the obvious advantage of the molecular biological approaches. *FLO* genes placed on suitable promotors could be activated by the addition of signal compounds. Alternatively, flocculation could be induced by the same suitable promoters used to activate either antisense plasmids to *tup1*/*cyc8*/*sfl1* or similarly targeted ribozymes. So much for the theory. In practice, these sophisticated mechanisms are of doubtful value. Flocculation onset is usually required after nutrient depletion, well into the stationary phase of growth. In the stationary phase, lectin synthesis is likely to be extremely slow and limited in volume

due to nutrient depletion, thereby leading to very slow response and limited flocculation. This view is backed up by observations of certain NewFlo phenotype strains (M. Stratford, unpublished observations) that began lectin synthesis in the stationary phase, as indicated by progressive cycloheximide addition. Flocculation in all these strains was poor. To give reasonable results, flocculation lectin synthesis must occur during growth, while there are nutrients to support it.

An alternative solution that may be worth pursuing would be to elucidate and artificially control the 'activation' mechanism of NewFlo phenotype strains. Since lectins have already been synthesized and are present in the cell wall, activation of these lectins would be expected to be considerably easier and quicker than *de novo* lectin synthesis. A second alternative would be to use pH switching on those strains shown to have narrow pH ranges for flocculation (*Figure 4*). These strains may be grown rapidly outside this pH window as single cells, and will flocculate instantly on addition of sufficient acid or alkali to shift the medium pH value within the window. The rate of response to pH change is extremely rapid and this switch to flocculation requires no metabolic input whatsoever and could be used on the metabolically inert cells in stationary phase.

The rate and extent of flocculation are dependent on many factors including bond strength, surface charge, salt concentration, pH value and cell concentration. But given a reasonable cell–cell bond strength, the easiest and most effective way of controlling rate and extent is to control the applied agitation. Extremely rapid initial rates of flocculation can be obtained by strong agitation (Stratford and Keenan, 1987) due to the agitation overcoming the charge repulsion between cells. However, strong agitation generates large shear forces which prevent flocs increasing in size, and promote cell loss from flocs (Stratford, Coleman and Keenan, 1988). By using an agitation ramping procedure (*Figure 12*), it is possible to combine the advantages of initial high rates of flocculation with the greater extent of flocculation at low agitation. This can be achieved on a batch-system, but in practical terms the best way of applying ramped agitation would be to force the yeast suspension, after activation, through a series of small tubes (*Figure 12*). These tubes could be partly constricted or obstructed to induce turbulent flow. The ramping effect would be achieved by gradually increasing the separation of the obstructions, thus lessening the turbulence. It would be necessary to avoid excessive turbulence, causing cavitation, and therefore foaming in the media. Finally, flocs may be removed in a small settling tank from the clear, supernatant medium.

Concluding remarks

In conclusion, the flocculation of yeasts can be regarded from many different points of view. Industrially, flocculation remains a commercially useful method of removing brewing yeasts from beer, while from a biochemical perspective flocculation entails calcium-dependent binding of yeast surface lectins to specific carbohydrate receptors on neighbouring cell walls. To the

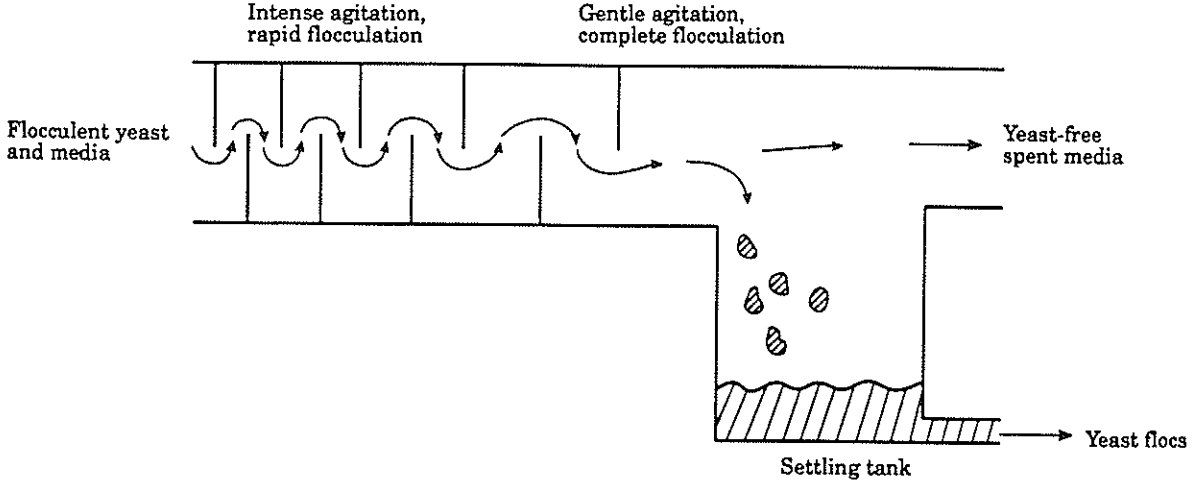

Figure 12. Agitation applied to a flocculating yeast suspension by passage through tubes, constricted by baffles to induce turbulence. By adjusting the baffles, strong initial agitation can give way to gentle mixing, enabling a ramping of agitation and initial rapid flocculation caused by strong agitation and a greater extent of flocculation by gentle agitation.

physicist, flocculation can represent the gathering of single cells into flocs; the gathering is hierarchical, and the flocs are cluster–cluster fractals. To the geneticist, this is an area where the identity and relationships between structural genes, regulators and seemingly unrelated mutations, has yet to be resolved. Physiologically, flocculation offers a unique insight into the insertion of proteins into the cell wall and the subsequent 'activation' of these proteins. Clearly flocculation has emerged from being an isolated, industrially useful property, to one of more general interest.

The reasons for yeast flocculation are a subject for debate, and probably will remain so. The most plausible theories to date concern enhanced survival of yeast in flocs, under adverse conditions. However, exchange of small pieces of genetic material between flocculated cells remains a distinct and alluring possibility.

A most striking observation from an overall view of the past 116 years of study of yeast flocculation is that while direct study of flocculation has revealed many important facts and effects, the great advances in understanding of the process have come from sideways looks from other areas of science. Most rapid progress has been gained by applying the knowledge from seemingly unrelated areas to make logical hypotheses from the known effectors of flocculation. In the future, the use of molecular biological techniques will undoubtedly resolve many of the remaining areas of doubt, especially concerning the role of *FLO* and various regulators. But, as usual in science, we may confidently predict that new enigmas will be uncovered as quickly as existing problems are solved, and that yeast flocculation will remain as unpredictable and fascinating as ever.

References

AL MAHMOOD, S., COLIN, S. AND BONALY, R. (1991). *Kluyveromyces bulgaricus* yeast lectins. Isolation of two galactose-specific lectin forms from the yeast cell wall. *J. Biol. Chem.* **266**, 20882–20887.

AL MAHMOOD, S., GIUMMELLY, P., BONALY, R., DELMOTTE, F. AND MONSIGNY, M. (1988). *Kluyveromyces bulgaricus* yeast lectins. Isolation of *N*-acetylglucosamine and galactose-specific lectins: Their relation with flocculation. *J. Biol. Chem.* **263**, 3930–3934.

AMORY, D.E., ROUXHET, P.G. AND DUFOUR, P.J. (1988). Flocculence of brewery yeasts and their surface properties: chemical composition, electrostatic charge and hydrophobicity. *J. Inst. Brewing* **94**, 79–84.

AMRI, M.M., BONALY, R., DUTEURTRE, B. AND MOLL, M. (1982). Yeast flocculation: influence of nutritional factors on cell wall composition. *J. Gen. Microbiol.* **128**, 2001–2009.

AUBERT, C. AND CANNELL, D.S. (1986). Restructuring of colloidal silica aggregates. *Phys. Rev. Lett.* **56**, 738–741.

AUNINS, J.G. AND WANG, D.I.C. (1989). Induced flocculation of animal cells in suspension culture. *Biotechnol. Bioeng.* **34**, 629–638.

BAJPAI, P. AND MARGARITIS, A. (1986). Studies on the flocculation characteristics of *Kluyveromyces marxianus*. *Biotechnol. Bioeng.* **28**, 283–287.

BAKER, D.A. AND KIRSOP, B.H. (1972). Flocculation in *Saccharomyces cerevisiae* as influenced by wort composition and by actidione. *J. Inst. Brewing* **78**, 454–458.

BALL, R.C. AND WITTEN, T.A. (1984). Particle aggregation versus cluster aggregation in high dimensions. *J. Statist. Phys.* **36**, 873–879.

BALLOU, C.E. (1976). Structure and biosynthesis of the mannan component of the yeast cell envelope. *Adv. Microbial Physiol.* **14**, 93–158.

BALLOU, C.E. AND RASCHKE, W.C. (1974). Polymorphism of the somatic antigen of yeast. *Science* **181**, 127–134.

BARENDRECHT, H.P. (1901). Die Agglutination von Hefe. *Z. Bakteriol. Parasitenkde. Infekt. Hyg., Abt. 2* **7**, 623–627.

BARKAI-GOLAN, R. AND SHARON, N. (1978). Lectins as a tool for the study of yeast cell walls. *Exp. Mycol.* **2**, 110–113.

BARONDES, S.H. (1981). Lectins: Their multiple endogenous cellular functions. *Ann. Rev. Biochem.* **50**, 207–231.

BARONDES, S.H. (1988). Bifunctional properties of lectins: lectins redefined. *Trends Biochem. Sci.* **13**, 480–482.

BARROW, G.M. (1973). *Physical Chemistry*, 3rd edition. McGraw-Hill Kogakusha Ltd., Tokyo and London.

BEAVAN, M.J., BELK, D.M., STEWART, G.G. AND ROSE, A.H. (1979). Changes in electrophoretic mobility and lytic enzyme activity associated with development of flocculating ability in *Saccharomyces cerevisiae*. *Can. J. Microbiol.* **25**, 888–895.

BEIJERINCK, M.W. (1908). Die Erscheinung der Flockenbildung oder Agglutination bei Alcoholhefen. *Z. Bakteriol. Parasitenkde Infect., Abt. 2* **20**, 641–650.

BENTHAM, A.C., BONNERJEA, J., ORSBORN, C.B., WARD, P.N. AND HOARE, M. (1990). The separation of affinity flocculated yeast cell debris using a pilot-plant scale scroll decanter centrifuge. *Biotechnol. Bioeng.* **36**, 397–401.

BLAGOEVA, J., STOEV, G. AND VENKOV, P. (1991). Glucan structure in a fragile mutant of *Saccharomyces cerevisiae*. *Yeast* **7**, 455–461.

BLAKE, C.C.F. (1975). Structure of a lectin. Crystallographic results. *Nature* **255**, 278.

BOTET, R., JULLIEN, R. AND KOLB, M. (1984). Hierarchical model for irreversible kinetic cluster formation. *J. Phys. A, Math. Nucl. Gen.* **17**, L75–L79.

BOWDEN, C.P., LEAVER, G., MELLING, J., NORTON, M.G. AND WHITTINGTON, P.N. (1987). Recent and novel developments in the recovery of cells from fermentation broths. In *Separations for Biotechnology* (M.S. Verrall and M.J. Hudson, Eds), pp. 49–61. Ellis Horwood, Chichester.

BOWEN, W.R. AND COOKE, R.J. (1989). Studies of *Saccharomyces cerevisiae* during fermentation: an *in vivo* electrokinetic investigation. *Biotechnol. Bioeng.* **33**, 706–715.

BOYD, W.C. AND SHAPLEIGH, E. (1954). Specific precipitating activity of plant agglutinins (Lectins). *Science* **119**, 419.

BRANDLEY, B.K. (1991). Carbohydrates as mediators of cell adhesion. *Glyconews* **1**, 2–4.

BRANDLEY, B.K. AND SCHNAAR, R.L. (1986). Cell-surface carbohydrate in cell recognition and response. *J. Leukocyte Biol.* **40**, 97–111.

BRINTON, C.C. (1959). Non-flagellar appendages of bacteria. *Nature* **183**, 782–786.

BROHAN, B. AND MCLOUGHLIN, A.J. (1984). Characterization of the physical properties of yeast flocs. *Appl. Microbiol. Biotechnol.* **20**, 16–22.

BROHAN, B. AND MCLOUGHLIN, A.J. (1985). The effect of physiological state on the flocculation of different yeast types. *Irish J. Food Sci. Technol.* **9**, 43–52.

BROHULT, S. (1951). General report: yeast flocculation and primary fermentation. *Proc. Eur. Brewery Convention Congr.*, Brighton, pp. 3–9.

BURNS, J.A. (1937). Yeast flocculation. *J. Inst. Brewing* **43**, 31–43.

BUSSEY, H., SAVILLE, D., GREENE, D., TIPPER, D.J. AND BOSTIAN, K.A. (1983). Secretion of *Saccharomyces cerevisiae* killer toxin: processing of the glycosylated precursor. *Mol. Cell. Biol.* **3**, 1362–1370.

CABIB, E. AND BOWERS, B. (1971). Chitin and yeast budding. Localization of chitin in yeast bud scars. *J. Biol. Chem.* **246**, 152–159.

CALIBO, R.L., MATSUMURA, M. AND KATAOKA, H. (1989). Continuous ethanol fermentation of concentrated sugar solutions coupled with membrane distillation using a PTFE module. *J. Ferment. Bioeng.* **67**, 40–45.

CALLEJA, G.B. (1973). Role of mitochondria in the sex-directed flocculation of a fission yeast. *Arch. Biochem. Biophys.* **154**, 382–386.

CALLEJA, G.B. (1984). *Microbial Aggregation*. CRC Press, Boca Raton, FL.

CALLEJA, G.B. (1987). Cell aggregation. In *The Yeasts, volume 2*, 2nd edition (A.H. Rose and J.S. Harrison, Eds), pp. 165–238. Academic Press, London and New York.

CALLEJA, G.B. AND JOHNSON, B.F. (1977). A comparison of quantitative methods for measuring yeast flocculation. *Can. J. Microbiol.* **23**, 68–74.

CALLEJA, G.B., NASIM, A., LUSENA, C.V. AND LEVY-RICK, S. (1986). Excretion of yeast amylases: requirement for oxygen and mitochondrial function. *Biochem. Int.* **12**, 81–87.

CAMP, T.R. AND STEIN, P.C. (1943). Velocity gradients and internal work in fluid motion. *J. Boston Soc. Civil Eng.* **30**, 219–237.

CARLSON, M., OSMOND, B.C., NEIGEBORN, L. AND BOTSTEIN, D. (1984). A suppressor of *SNF1* mutations causes constitutive high-level invertase synthesis in yeast. *Genetics* **107**, 19–32.

CARVER, J.P., MACKENZIE, A.E. AND HARDMAN, K.D. (1985). Molecular model for the complex between concanavalin A and biantennary-complex class glycopeptide. *Biopolymers* **24**, 49–63.

CASEY, G.P. (1990). Yeast selection in brewing. In *Yeast Strain Selection* (C.J. Panchal, Ed.), pp. 65–111. Marcel Dekker, New York.

CASTELLON-VOGEL, M.A. AND MENAWAT, A.S. (1990). A method to disperse aggregates of a flocculent yeast for photometric analysis. *Biotechnol. Progr.* **6**, 135–141.

CHALLINOR, S.W., POWER, D.M. AND TONGE, R.J. (1964). Effects of inositol-deficiency on yeast with particular reference to chemical composition of the cell and of the cell wall. *Nature* **203**, 250–251.

CHESTER, V.E. (1963). A study of changes in flocculation in a single cell culture of a strain of *Saccharomyces cerevisiae*. *Proc. Roy. Soc., Ser. B* **157**, 223–233.

CIANI, M. AND ROSINI, G. (1990). Selection of strains of *Saccharomyces cerevisiae* of 'Sagrantino' D.O.C. for their wine-making properties: preliminary results. *Ital. J. Food Sci.* **2**, 165–172.

CLARK, S.G., MCGRATH, J.P. AND LEVINSON, A.D. (1985). Expression of normal and activated human Ha-*ras* cDNAs in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **5**, 2746–2752.

COHEN, R.E., ZHANG, W. AND BALLOU, C.E. (1982). Effects of mannoprotein mutations on *Saccharomyces cerevisiae* core oligosaccharide structure. *J. Biol. Chem.* **257**, 5730–5737.

CRITCHLEY, I.A. AND DOUGLAS, L.J. (1987). Isolation and partial characterization of an adhesin from *Candida albicans*. *J. Gen. Microbiol.* **133**, 629–636.

CURTIS, A.S.G. (1973). Cell adhesion. *Progr. Biophys. Mol. Biol.* **27**, 317–386.

CURTIS, N.S. AND WENHAM, S. (1958). Some observations on the declining attenuative power of brewery yeast. *J. Inst. Brewing* **64**, 421–428.

CVETANOV, O., LACHCEV, K. AND PECEVA, M. (1990). Improvement of hybrid yeast strains with better flocculating properties for white wine-making. *Kvasny Prumysl* **36**, 360–363.

DAVEY, C.L. AND MARKX, G.H. (1990). An introduction to fractal geometry. *Binary* **2**, 145–152.

DAVIS, R.H. AND HUNT, T.P. (1986). Modelling and measurement of yeast flocculation. *Biotechnol. Progr.* **2**, 91–97.

DAY, A.W., POON, N.H. AND STEWART, G.G. (1975). Fungal fimbriae. III. The effect on flocculation in *Saccharomyces*. *Can. J. Microbiol.* **21**, 558–564.

DE CLERCK, J. (1930). Surface tension and its role in the Brewery. *Bulletin de l'Association des Anciens Eleves de l'Ecole Superieure de Brasserie de l'Université de Louvain* **30**, 127 (Abstract 1931 *J. Inst. Brewing* **37**, 126).

DE FIGUEROA, L.I., DE RICHARD, M.F. AND DE VAN BROOCK, M.R. (1984a).

Transfer of the flocculation property to the baker's yeast *Saccharomyces cerevisiae* by conventional genetic manipulation. *Biotechnol. Lett.* **6**, 171–176.

DE FIGUEROA, L.I., DE RICHARD, M.F. AND DE VAN BROOCK, M.R. (1984b). Use of the somatic fusion method to introduce the flocculation property into *Saccharomyces diastaticus*. *Biotechnol. Lett.* **6**, 587–592.

DE NOBEL, J.G., KLIS, F.M., MUNNIK, T., PRIEM, J. AND VAN DEN ENDE, H. (1990a). An assay of relative cell wall porosity in *Saccharomyces cerevisiae*, *Kluyveromyces lactis* and *Schizosaccharomyces pombe*. *Yeast* **6**, 483–490.

DE NOBEL, J.G., KLIS, F.M., PRIEM, J., MUNNIK, T. AND VAN DEN ENDE, H. (1990b). The glucanase-soluble mannoproteins limit cell wall porosity in *Saccharomyces cerevisiae*. *Yeast* **6**, 491–499.

DEBRAY, H., DECOUT, D., STRECKER, G., SPIK, G. AND MONTREUIL, J. (1981). specificity of twelve lectins towards oligosaccharides and glycopeptides related to N-glycosylproteins. *Eur. J. Biochem.* **117**, 41–55.

DEVREUX, A. (1962). Definition, characteristics, causes and mechanism of (yeast) degeneration. *Colloque sur les levures, Nancy* 45–60. (Abst. 1963 *J. Inst. Brewing* **69**, 266).

DOMINGUEZ, A., VILLANUEVA, J.R. AND SENTANDREU, R. (1978). Inositol deficiency in *Saccharomyces cerevisiae* NCYC 86. *Antonie van Leeuwenhoek* **44**, 25–34.

DOUGLAS, L.J. (1985). Adhesion of pathogenic *Candida species to host surfaces*. *Microbiol. Sci.* **2**, 243–247.

DOYLE, R.J. AND SLIFKIN, M. (1989). Applications of lectins in microbiology. *Am. Soc. Microbiol. News* **55**, 655–658.

DRICKAMER, K. (1988). Two distinct classes of carbohydrate-recognition domains in animal lectins. *J. Biol. Chem.* **263**, 9557–9560.

DUFFUS, J.H., LEVI, C. AND MANNERS, D.J. (1982). Yeast cell-wall glucans. *Adv. Microbial Physiol.* **23**, 151–181.

DUNWELL, J.L., AHMAD, F. AND ROSE, A.H. (1961). Changes in the polysaccharide composition of yeast resulting from biotin deficiency. *Biochim. Biophys. Acta* **51**, 604–607.

EDDY, A.A. (1955a). Flocculation characteristics of yeasts I. Comparative survey of various strains of *Saccharomyces cerevisiae*. *J. Inst. Brewing* **61**, 307–312.

EDDY, A.A. (1955b). Flocculation characteristics of yeast II. Sugars as dispersing agents. *J. Inst. Brewing* **61**, 313–317.

EDDY, A.A. (1955c). Flocculation characteristics of yeasts III. General role of flocculating agents and special characteristics of a yeast flocculated by alcohol. *J. Inst. Brewing* **61**, 318–320.

EDDY, A.A. (1957). Some chemical aspects of yeast growth. *Chem. Ind.*, 26 January, p. 99.

EDDY, A.A.(1958a). Composite nature of the flocculation process of top and bottom strains of *Saccharomyces*. *J. Inst. Brewing* **64**, 143–151.

EDDY, A.A.(1958b). The structure of the yeast cell wall. II. Degradative studies with enzymes. *Proc. Roy. Soc., Ser. B* **149**, 425–440.

EDDY, A.A. AND RUDIN, A.D. (1958a). Part of the yeast surface apparently involved in flocculation. *J. Inst. Brewing* **64**, 19–21.

EDDY, A.A. AND RUDIN, A.D. (1958b). Comparison of the respective electrophoretic and flocculation characteristics of different strains of *Saccharomyces*. *J. Inst. Brewing* **64**, 139–142.

EGILSSON, V., EVANS, I.H. AND WILKIE, D. (1979). Toxic and mutagenic effects of carcinogens on the mitochondria of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **174**, 39–46.

EINSPAHR, H., PARKS, E.H., PHILLIPS, S.R. AND SUDDATH, F.L. (1988). Crystal structure studies of legume lectins. In *Lectins, Biology, Biochemistry, Clinical Biochemistry*, volume 6, Proceedings of the 9th Lectin Meeting, Cambridge, July

1987 (T.C. Bog-Hansen and D.L.J. Freed, Eds), pp. 245–263. Sigma Chemical Company, St Louis, MO.

ESHDAT, Y., SPETH, V. AND JANN, K. (1981). Participation of pili and cell wall adhesin in the yeast agglutination activity of *Escherichia coli*. *Infect. Immun.* **34**, 980–986.

ESSER, K., HINRICHS, J. AND KUES, U. (1987). Genetic control of flocculation of yeast with respect to application in biotechnology. In *Flocculation in Biotechnology and Separation Systems* (Y.A. Attia, Ed.), pp. 383–398. Elsevier, Amsterdam.

EVANS, I.H., DIALA, E.S., EARL, A. AND WILKIE, D. (1980). Mitochondrial control of cell surface characteristics in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **602**, 201–206.

FIRON, N., OFEK, I. AND SHARON, N. (1982). Interaction of mannose-containing oligosaccharides with the fimbrial lectin of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **105**, 1426–1432.

FISHER, D.J. (1975). Flocculation — some observations on the surface charges of yeast cells. *J. Inst. Brewing* **81**, 107–110.

FLEET, G.H. (1991). The yeast cell wall. In *The Yeasts*, volume 4, 2nd edition (A.H. Rose and J.S. Harrison, Eds), pp. 199–277. Academic Press, London.

FREVERT, J. AND BALLOU, C.E. (1985). *Saccharomyces cerevisiae* structural cell wall mannoprotein. *Biochemistry* **24**, 753–759.

FUJINO, S. AND YOSHIDA, T. (1976). Premature flocculation of yeast induced by some wort constituents. *Rep. Res. Lab. Kirin Brewery Co. Ltd* **19**, 45–53.

FUJITA, A., KIKUCHI, Y., KUHARA, Y., MATSUMOTO, S. AND KOBAYASHI, H. (1989). Domains of the SFL1 proteins of yeasts are homologous to Myc oncoproteins or yeast heat-shock transcription factor. *Gene* **85**, 321–328.

FUJITA, A., MATSUMOTO, S., KUHARA, S., MISUMI, Y. AND KOBAYASHI, H. (1990). Cloning of the yeast *SFL2* gene: its disruption results in pleiotropic phenotypes characteristic for *tup1* mutants. *Gene* **89**, 93–99.

FURTH, M.E., DAVIS, L.J., FLEURDELYS, B. AND SCOLNICK, E.M. (1982). Monoclonal antibodies to the p21 products of the transforming gene of Harvey sarcoma virus and of the cellular *ras* gene family. *J. Virol.* **43**, 294–304.

GATTEGNO, L., CLERGET-RASSAIN, B., SADEGHI, H., FENOUILLET, E., GLUCKMAN, J.C. AND BAHRAOUL, E. (1989). Has the major envelope glycoprotein of HIV-1 a lectin-like activity? In *Vth International Conference on AIDS. The Scientific and Social Challenge*, Montreal (R.A. Morisset, Ed.), p. 670.

GEILENKOTTEN, I. AND NYNS, E.J. (1971). The biochemistry of yeast flocculence. *Brewers Digest* **46**, 64–70.

GEYS, K. (1922). Flocculation of yeast and the influence of pure, culture thereon. *Zeitschrift fur das gesamte Brauwesen* **45**, 51–53; 57–61 (Abstract 1922 *J. Fed. Inst. Brewing* **28**, 479–480).

GHOSH, A., CHARALAMPOUS, Y., SISON, Y. AND BORER, R. (1960). Metabolic functions of myo-inositol I. Cytological and chemical alterations in yeast resulting from inositol deficiency. *J. Biol. Chem.* **235**, 2522–2528.

GHOSH, B.K., MONTENECOURT, B. AND LAMPEN, J.O. (1973). Abnormal cell envelope ultrastructure of a *Saccharomyces* mutant with invertase formation resistant to hexoses. *J. Bacteriol.* **116**, 1412–1420.

GILLILAND, R.B. (1951). The flocculation characteristics of brewing yeasts during fermentation. *Proc. Eur. Brewing Convention Congr.*, Brighton, pp. 35–58.

GILLILAND, R.B. (1957). The flocculation of brewing yeast. *Wallerstein Lab. Commun.* **20**, 41–70.

GILLILAND, R.B. (1981). Brewing yeast. In *Brewing Science*, volume 2 (J.R.A. Pollock, Ed.), pp. 1–60. Academic Press, London.

GIUMELLY, P., SEPARI, F., SUPTIJAH, P. AND BONALY, R. (1989). Cell wall receptors of the lectins produced by *Kluyveromyces bulgaricus*. *Yeast* **5**, S309–S311.

GOLDSTEIN, I.J., HOLLERMAN, C.E. AND SMITH, E.E. (1965). Protein–carbohydrate

interaction. II. Inhibition studies on the interaction of concanavalin A with polysaccharides. *Biochemistry* **4**, 876–883.

GOLDSTEIN, I.J., HUGHES, R.C., MONSIGNY, M., OSAWA, T. AND SHARON, N. (1980). What should be called a lectin? *Nature* **285**, 66.

GREENSHIELDS, R.N., YATES, J., SHARP, P. AND DAVIES, T.M.C. (1972). Studies in tower continuous fermentation. 1. Methods for the characterization of flocculent yeasts. *J. Inst. Brewing* **78**, 236–242.

GRIFFIN, S.R. AND MACWILLIAM, I.C. (1969). Variation of cell wall content in flocculent and non-flocculent yeast strains. *J. Inst. Brewing* **75**, 355–358.

GROOTJEN, D.R.J., VLEESENBEEK, R., WINDMEIJER, M.G.A., VAN DER LANS, R.G.J.M. AND LUYBEN, K.CH.A.M. (1991). A flocculating strain of *Pichia stipitis* for the conversion of glucose/xylose mixtures. *Enzyme Microbiol. Technol.* **13**, 734–739.

GYLLANG, H. AND MARTINSON, E. (1972). Properties of some spontaneous mutants from brewer's yeast with increased sedimentation rate. *Proc. Eur. Brewing Convention Congr.*, Estoril, pp. 265–271.

HANSCHE, P.E. (1975). Gene duplication as a mechanism of genetic adaption in *Saccharomyces cerevisiae*. *Genetics* **79**, 661–674.

HARMAN, K.D. AND AINSWORTH, C.F. (1972). Structure of concanavalin A at 2·4–Å resolution. *Biochemistry* **11**, 4910–4919.

HARRIS, J.O. (1959). Possible mechanism of yeast flocculation. *J. Inst. Brewing* **65**, 5–6.

HARTMEIER, W., BORGMANN, R. AND VOSS, S. (1990). Continuous production of glycerol with a flocculent strain of *Saccharomyces rouxii*. *Food Biotechnol.* **4**, 481.

HAYDUCK, F. AND SCHUCKLING, K. (1908). Significance of lime salts in brewing water on yeast and fermentation. *Wochenschrift fur Brauereien* **25**, 241–244; 253–258; 265–270; 277–281; 297–300 (Abstract 1908 *J. Fed. Inst. Brewing* **14**, 489–490).

HECKELS, J.E., BLACKETT, B., EVERSON, J.S. AND WARD, M.E. (1976). The influence of surface charge on the attachment of *Neisseria gonorrhoeae* to human cells. *J. Gen. Microbiol.* **96**, 359–364.

HELM, E., NOHR, B. AND THORNE, R.S.W. (1953). The measurement of yeast flocculence and its significance in brewing. *Wallerstein Lab. Commun.* **16**, 315–326.

HENNIG, K. AND AY, H. (1938). Die kataphoretische untersuchung der wein- und brauereihefen. *Biochem. Z.* **299**, 123–132.

HERRERA, V.E. AND AXCELL, B.D. (1989). The influence of barley lectins on yeast flocculation. *Am. Soc. Brewing Chem. J.* **47**, 29–34.

HERRERA, V.E. AND AXCELL, B.C. (1991a). Induction of premature yeast flocculation by a polysaccharide fraction isolated from malt husk. *J. Inst. Brewing* **97**, 359–366.

HERRERA, V.E. AND AXCELL, B.C. (1991b). Studies on the binding between yeast and a malt polysaccharide that induces heavy yeast flocculation. *J. Inst. Brewing* **97**, 367–373.

HINRICHS, J. AND ESSER, K. (1987). Influence of mitochondrial DNA on flocculation in *Saccharomyces cerevisiae*. *International Botanical Congress Abstracts. Genetics and Plant Breeding* **17**, 179.

HINRICHS, J., STAHL, U. AND ESSER, K. (1988). Flocculation in *Saccharomyces cerevisiae* and mitochondrial structure. *Appl. Microbiol. Biotechnol.* **29**, 48–54.

HODGSON, J.A., BERRY, D.R. AND JOHNSTON, J.R. (1985). Discrimination by heat and proteinase treatments between flocculent phenotypes conferred on *Saccharomyces cerevisiae* by the genes *FLO1* and *FLO5*. *J. Gen. Microbiol.* **131**, 3219–3227.

HOLDERER, H. (1901). *Jahrbuch-Versuchs-und Lehranstalt für Brauerei, Berlin* **4**, 306.

HOLMBERG, S. (1978). Isolation and characterization of a polypeptide absent from

non-flocculent mutants of *Saccharomyces cerevisiae*. *Carlsberg Res. Commun.* **43**, 401–413.

HOLMBERG, S. AND KIELLAND-BRANDT, M.C. (1978). A mutant of *Saccharomyces cerevisiae* temperature sensitive for flocculation. Influence of oxygen and respiratory deficiency on flocculence. *Carlsberg Res. Commun.* **43**, 37–47.

HORISBERGER, M. (1977). Polysaccharides immobilized in polyacrylamide gel as high-capacity adsorbents for *Bandeiraea simplicifolia* lectin and concanavalin A. *Carbohydr. Res.* **530**, 231–237.

HOUGH, J.S. (1957). Characterizing the principal components of pitching yeasts. *J. Inst. Brewing* **63**, 483–487.

HUSSAIN, T., SALHI, O., LEMATRE, J., CHARPENTIER, C. AND BONALY, R. (1986). Comparative studies of flocculation and deflocculation of *Saccharomyces uvarum* and *Kluyveromyces bulgaricus*. *Appl. Microbiol. Biotechnol.* **23**, 269–273.

ISLA, M.A., VATTUONE, M.A. AND SAMPIETRO, A.R. (1991). Proteinaceous inhibitor from *Solanum tuberosum* invertase. *Phytochemistry* **30**, 739–743.

JAN, K.M. AND CHIEN, S. (1973a). Role of surface electric charge in red blood cell interactions. *J. Gen. Physiol.* **61**, 638–654.

JAN, K.M. AND CHIEN, S. (1973b). Influence of the ionic composition of fluid medium on red blood cell aggregation. *J. Gen. Physiol.* **61**, 655–668.

JANSEN, H.E. (1958). Flocculation of yeasts. In *The Chemistry and Biology of Yeasts* (A.H. Cook, Ed.), pp. 635–667. Academic Press, New York.

JANSEN, H.E. AND MENDLIK, F. (1951). A study on yeast flocculation. *Proc. Eur. Brewing Convention Congr.*, Brighton, pp. 59–83.

JANSEN, H.E. AND MENDLIK, F. (1953). A study on yeast flocculation II. *Proc. Eur. Brewing Convention Congr.*, Nice, pp. 143–154.

JAYATISSA, P.M. AND ROSE, A.H. (1976). Role of wall phosphomannan in flocculation of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **96**, 165–174.

JOHNSON, B.F., WALKER, T., MIYATA, M., MIYATA, H. AND CALLEJA, G.B. (1987). Sexual co-flocculation and asexual self-flocculation of heterothallic fission-yeast cells (*Schizosaccharomyces pombe*). *Can. J. Microbiol.* **33**, 684–688.

JOHNSON, B.F., WALKER, T., CALLEJA, G.B. AND SELIGY, V.L. (1988). Sexual co-flocculation and asexual self-flocculation in budding and fission yeasts: experimental establishment of a fundamental difference. *Can. J. Microbiol.* **34**, 1105–1107.

JOHNSTON, J.R. AND LEWIS, C.W. (1974). Genetic analysis of flocculation in *Saccharomyces cerevisiae* and tetrad analysis of commercial brewing and baking strains. In *Second Int. Symp. on the Genetics of Industrial Microorganisms* (K.D. MacDonald, Ed.), pp. 339–355. Academic Press, London.

JOHNSTON, J.R. AND READER, H.P. (1982). Genes conferring and suppressing flocculation in brewing and laboratory strains of *Saccharomyces*. *Proc. 11th Int. Conf. on Yeast Genetics and Molecular Biology*, Montpelier, p. 124.

JOHNSTON, J.R. AND READER, H.P. (1983). Genetic control of flocculation. In *Yeast Genetics, Fundamental and Applied Aspects* (J.F.T. Spencer, D.M. Spencer and A.R.W. Smith, Eds), pp. 205–224. Springer-Verlag, New York, Berlin, Heidelberg and Tokyo.

JULLIEN, R. (1984). Transparency effects in cluster–cluster aggregation with linear trajectories. *J. Phys. A: Math. Nucl. Gen.* **17**, L771–L776.

JULLIEN, R., KOLB, M. AND BOTET, R. (1984). Aggregation by kinetic clustering of clusters in dimensions d>2. *J. Phys. Lett.* **45**, L211–L216.

KABACK, D.B., STEENSMA, H.Y. AND DE JONGE, P. (1989). Enhanced meiotic recombination on the smallest chromosome of *Saccharomyces cerevisiae*. *Proc. Nat. Acad. Sci. USA* **86**, 3694–3698.

KABAT, E.A. (1978). Dimensions and specificities of recognition sites on lectins and antibodies. *J. Supramol. Struct.* **8**, 79–88.

KALB, A.J. AND LEVITZKI, A. (1968). Metal-binding sites of concanavalin A and their role in the binding of α-methyl D-glucopyranoside. *Biochem. J.* **109**, 669–672.

KALYUZHNYI, M.Y., PETRUSHKO, G.M. AND NOVIKOVA, G.P. (1965). Flocculation of *Candida utilis* and *Candida tropicalis* yeast cells and its relation to flotation. *Microbiology* **34**, 800–805.

KAMADA, K. AND MURATA, M. (1984a). On the mechanism of brewer's yeast flocculation. *Agric. Biol. Chem.* **48**, 2423–2433.

KAMADA, K. AND MURATA, M. (1984b). Flocculating activity on the cell surface of brewer's yeast. *Nippon Nogeikagaku Kaishi* **58**, 977–982.

KAMADA, K. AND MURATA, M. (1984c). Effect of environmental condition on flocculation-dispersion of brewer's yeast. *Nippon Nogeikagaku Kaishi* **58**, 983–990.

KEARSEY, S. (1991). The *SFL2 (tup1?)* protein of *Saccharomyces cerevisiae* contains a repeating motif homologous to beta subunits of G protein. *Gene* **98**, 147–148.

KIDA, K., YAMADAKI, M., ASANO, S.I., NAKATA, T. AND SONODA, Y. (1989). The effect of aeration on stability of continuous ethanol fermentation by a flocculating yeast. *J. Ferment. Bioeng.* **68**, 107–111.

KIHN, J.C., MASY, C.L. AND MESTDAGH, M.M. (1988). Yeast flocculation: competition between nonspecific repulsion and specific bonding in cell adhesion. *Can. J. Microbiol.* **34**, 773–778.

KIHN, J.C., MASY, C.L., MESTDAGH, M.M. AND ROUXHET, P.G. (1988). Yeast flocculation: factors affecting the measurement of flocculence. *Can. J. Microbiol.* **34**, 779–781.

KIJIMA, M. (1964). Comparative biochemical studies of alcoholic fermentation with special reference to the flocculation phenomena in yeast cells. III. Cell surface substances of some varieties of brewery yeast. *Rep. Res. Lab. Kirin Brewery Co. Ltd* 7, 7–14.

KOPECKA, M., PHAFF, H.J. AND FLEET, G.H. (1974). Demonstration of a fibrillar component in the cell wall of the yeast *Saccharomyces cerevisiae* and its chemical nature. *J. Cell Biol.* **62**, 66–76.

KRAMER, R.A., TOMCHAK, L., RUBEN, S.M. AND ROSEN, C.A. (1990). Expression of the HTLV-1 Tax transactivator in yeast: correlation between phenotypic alterations and Tax function in higher eukaryotes. *Aids Res. Human Retroviruses* **6**, 1305–1309.

KRUYT, H.R. (1952). General Introduction. In *Colloid Science*, volume I: *Irreversible Systems* (H.R. Kruyt, Ed.), pp. 1–57. Elsevier, Amsterdam, Houston, New York and London.

KUDO, S. (1952). On a polysaccharide as a new factor stimulating the flocculation of yeast in the primary fermentation of beer. *Proc. Japan Acad.* **28**, 93–96.

KUDO, S. (1959). Flockung der brauerei-hefe. Über anomale bruchbildung der hefe bei der hauptgarung. *Rep. Res. Lab. Kirin Brewery Co. Ltd* **2**, 11–19.

KUDO, S. AND KIJIMA, M. (1960). Studies on yeast flocculation. Identification of a yeast-flocculating agent 'Barmigen' and yeast flocculation caused by the related substances. *Rep. Res. Lab. Kirin Brewery Co. Ltd* **3**, 33–37.

KURANDA, M.J. AND ROBBINS, P.W. (1991). Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **266**, 19 758–19 767.

KURANE, R., TAKEDA, K. AND SUZUKI, T. (1986). Screening for and characteristics of microbial flocculants. *Agric. Biol. Chem.* **50**, 2301–2307.

KURIYAMA, H., UMEDA, I. AND KOBAYASHI, H. (1991). Role of cations in the flocculation of *Saccharomyces cerevisiae* and discrimination of the corresponding proteins. *Can. J. Microbiol.* **37**, 397–403.

LACHANCE, M.A. (1990). Yeast selection in nature. In *Yeast Strain Selection*, Bioprocess Technology Series 8 (C.J. Panchal, Ed.), pp. 21–41. Marcel Dekker, New York.

LANDS, W.E.M. AND GRAFF, G. (1981). Effects of fatty acid structure on yeast cell physiology. In *Current Developments in Yeast Research. Advances in Biotechnology* (G.G. Stewart and I. Russell, Eds), pp. 331–343. Pergamon Press, Toronto.

LANGE, H. (1901). *Jahrbuch-Versuchs-und Lehranstalt für Brauerei, Berlin* **4**, 19.

LANGE, H. (1907). Physiological conditions of yeast. *Wochenschrift für Brauereien* **24**, 417–421; 433–436; 445–449; 457–463; 474–476; 489–491; 505–515; 521–524 (Abstract 1908 *J. Fed. Inst. Brewing* **14**, 110–113).

LANGE, P. (1979). Pleiotropic effect of two cell wall mutants on the activity of some cell wall enzymes in the yeast *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **173**, 211–212.

LEE, K.Y. AND BAERWALD, G. (1991). Kinetic behaviour of *Candida rugosa* in the batch fermentation of sugar beet stillage; temperature dependence of growth and flocculation characteristics. *Biotechnol. Lett.* **13**, 595–598.

LEMONTT, J.F. (1977). Pathways of ultraviolet mutability in *Saccharomyces cerevisiae*. III. Genetic analysis and properties of mutants resistant to ultraviolet-induced forward mutation. *Mutation Res.* **43**, 179–204.

LEMONTT, J.F., FUGIT, D.R. AND MACKAY, V.L. (1980). Pleiotropic mutations at the *tup1* locus that affect the expression of mating-type-dependent functions in *Saccharomyces cerevisiae*. *Genetics* **94**, 899–920.

LEWIN, L.M. (1965). Effects of meso-inositol deficiency on some important biological and chemical characteristics of yeast. *J. Gen. Microbiol.* **41**, 215–224.

LEWIS, C.W. AND JOHNSTON, J.R. (1974). Genes controlling flocculation in yeast. *Proc. Soc. Gen. Microbiol.* **1**, 73.

LEWIS, C.W., JOHNSTON, J.R. AND MARTIN, P.A. (1976). The genetics of yeast flocculation. *J. Inst. Brewing* **82**, 158–160.

LIN, M.Y., LINDSAY, H.M., WEITZ, D.A., BALL, R.C. AND MEAKIN, P. (1989). Universality in colloid aggregation. *Nature* **339**, 360–362.

LINDNER, P. (1901). *Jahrbuch-Versuchs-und Lehranstalt für Brauerei, Berlin* **4**, 309–310.

LINDQUIST, W. (1953). On the mechanism of yeast flocculation. *J. Inst. Brewing* **59**, 59–61.

LIPKE, P.N. AND HULL-PILLSBURY, C. (1984). Flocculation of *Saccharomyces cerevisiae tup1* mutants. *J. Bacteriol.* **159**, 797–799.

LIS, H. AND SHARON, N. (1986). Lectins as molecules and as tools. *Ann. Rev. Biochem.* **55**, 35–67.

LOGAN, B.E. AND WILKINSON, D.B. (1991). Fractal dimensions and porosities of *Zoogloea ramigera* and *Saccharomyces cerevisiae* aggregates. *Biotechnol. Bioeng.* **38**, 389–396.

LUERS, H. AND HEUSZ, R. (1921). *Zeitschrift für das gesamte Brauwesen* **44**, 18 (Abstract Jansen, 1958).

LUND, A. (1951). Flocculation as a racial character in bottom fermentation yeast. *Proc. Eur. Brewing Convention Congr.*, Brighton, pp. 10–19.

LYONS, T.P. AND HOUGH, J.S. (1970a). The role of yeast cell walls in brewing. *Brewers Digest* **45**, 52–60.

LYONS, T.P. AND HOUGH, J.S. (1970b). Flocculation of brewer's yeast. *J. Inst. Brewing* **76**, 564–571.

LYONS, T.P. AND HOUGH, J.S. (1971). Further evidence for the Cross-Bridging hypothesis for flocculation of brewer's yeast. *J. Inst. Brewing* **77**, 300–305.

MALKOW, A. (1934). Studien uber flockenbildung (agglutination). Der hefe. II. Mitteilung. *Z. Bakteriol. Parasitenkde. Infekt., Abt. 2* **90**, 212–217.

MALKOW, A., PETINA, A. AND ZWETKOWA, N. (1933). Studien uber flockenbildung (Agglutination). der hefe. *Z. Bakteriol. Parasitenkde. Infekt., Abt. 2* **88**, 193–200.

MANDELBROT, B.B. (1990). Fractals—a geometry of nature. *New Scientist* **127** (1734), 38–43.

MANNEY, T.R., JACKSON, P. AND MEADE, J. (1983). Two temperature-sensitive mutants of *Saccharomyces cerevisiae* with altered expression of mating-type functions. *J. Cell Biol.* **96**, 1592–1600.

MARFEY, P., SORENSEN, S.B. AND OTTESEN, M. (1977). Studies on yeast flocculation. Comparison of enzymatic digests of flocculent and non-flocculent cells of *Saccharomyces carlsbergensis*. *Carlsberg Res. Commun.* **42**, 353–367.

MARKWELL, M.A.K. (1986). Viruses as hemagglutinins and lectins. In *Microbial Lectins and Agglutinins: Properties and Biological Activity* (D. Mirelman, Ed.), pp. 21–53. John Wiley & Sons, New York, Chichester, Brisbane, Toronto and Singapore.

MASSCHELEIN, C. AND DEVREUX, A. (1957). Floculence et structure de la paroi cellulaire de la levure. *Proc. Eur. Brewing Convention Congr.*, Copenhagen, pp. 194–211.

MASSCHELEIN, C.A., JEUNEHOMME-RAMOS, C., CASTIAU, C. AND DEVREUX, A. (1963). Mechanism of phenotypic variations in the flocculence character of yeast. *J. Inst. Brewing* **69**, 332–338.

MASY, C.L., KOCKEROLS, M. AND MESTDAGH, M.M. (1991). Calcium activity versus 'calcium threshold' as the key factor in the induction of yeast flocculation in simulated industrial fermentations. *Can. J. Microbiol.* **37**, 295–303.

MASY, C.L., HENQUINET, A., SCOMAN, I., MESTDAGH, M.M. AND COLSON, A.M. (1990). Study of protein receptors on the cell surface of *Saccharomyces cerevisiae* strains of the type *FLO1* and New FLO. *Yeast* **6**, s149.

MAZODIER, P. AND DAVIES, J. (1991). Gene transfer between distantly related bacteria. *Ann. Rev. Genetics* **25**, 147–171.

MBAWALA, A., AL MAHMOOD, S., LOPPINET, V. AND BONALY, R. (1990). Acetolysis and ^{4}H NMR studies on mannans isolated from very flocculent and weakly flocculent cells of *Pichia pastoris* IFP 206. *J. Gen. Microbiol.* **136**, 1279–1284.

MCCANDLISH, D. AND HAGUES, G. (1929). Some practical advantages to be derived from the neutralisation of brewing liquor. *J. Inst. Brewing* **35**, 61–68.

MCKENZIE, G.H., SAWYER, W.H. AND NICHOL, L.W. (1972). The molecular weight and stability of concanavalin A. *Biochim. Biophys. Acta* **263**, 283–293.

MEAKIN, P. (1988). Fractal aggregates. *Adv. Colloid Interface Sci.* **28**, 249–331.

MEAKIN, P., CHEN, Z.Y. AND DEUTCH, J.M. (1985). The translational friction coefficient and time dependent cluster size distribution of three dimentional cluster–cluster aggregation. *J. Chem. Phys.* **82**, 3786–3789.

MENAWAT, A.S. AND CASTELLON-VOGEL, M. (1989). A method to disperse aggregates of a flocculent yeast for photometric analysis. *198th American Chemical Society National Meeting*, Paper Abstract No. MBTD 184.

MIKI, B.L.A., POON, N.H. AND SELIGY, V.L. (1982). Repression and induction of flocculation interactions in *Saccharomyces cerevisiae*. *J. Bacteriol.* **150**, 890–899.

MIKI, B.L.A., POON, N.H., JAMES, A.P. AND SELIGY, V.L. (1981). Flocculation in *Saccharomyces cerevisiae*: Mechanism of cell–cell interactions. In *Current Developments in Yeast Research* (G.G. Stewart and I. Russell, Eds), pp. 165–170. Pergamon Press, Toronto.

MIKI, B.L.A., POON, N.H., JAMES, A.P. AND SELIGY, V.L. (1982). Possible mechanism for flocculation interactions governed by gene *FLO1* in *Saccharomyces cerevisiae*. *J. Bacteriol.* **150**, 878–889.

MILL, P.J. (1964a). The effect of nitrogenous substances on the time of flocculation of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **350**, 53–60.

MILL, P.J. (1964b). The nature of the interactions between flocculent cells in the flocculation of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **35**, 61–68.

MILL, P.J. (1966). Phosphomannans and other components of flocculent and non-flocculent walls of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **44**, 329–341.

MIRELMAN, D. (1987). Ameba–bacterium relationship in amebiasis. *Microbiol. Rev.* **51**, 272–284.

MIRELMAN, D. AND OFEK, I. (1986). Introduction to microbial lectins and agglutinins. In *Microbial Lectins and Agglutinins: Properties and Biological Activity* (D. Mirelman, Ed.), pp. 1–19. John Wiley & Sons, New York, Chichester, Brisbane, Toronto and Singapore.

MOMOSE, H., IWANO, K. AND TONOIKE, R. (1969). Studies on the aggregation of yeast caused by *Lactobacilli*. IV. Force responsible for aggregation. *J. Gen. Appl. Microbiol.* **15**, 19–26.

MONTENECOURT, B.S., KUO, S.C. AND LAMPEN, J.O. (1973). *Saccharomyces* mutants with invertase formation resistant to repression by hexoses. *J. Bacteriol.* **114**, 233–238.

MORIMOTO, K., SHIMAZU, T., FUJII, T. AND HORIE, Y. (1975). Some substances in malt inducing early flocculation of yeast. I. Preliminary investigation on high molecular weight substances in malt and wort. *Rep. Res. Lab. Kirin Brewery Co. Ltd* **18**, 63–74.

MORIYA, K., IEFUJI, H., SHIMOI, H., SATO, S.I. AND TADENUMA, M. (1990). Treatment of distillery wastewater discharged from beet molasses-spirit production using yeast. *J. Ferment. Bioeng.* **69**, 138–140.

MOUFANG, E. (1912). Influence of diminished mashing on the mashing process. *Wochenscrift für Brauereien* **29**, 721–723 (Abstract 1913 *J. Fed. Inst. Brewing* **19**, 244).

MUKAI, Y., HARASHIMA, S. AND OSHIMA, Y. (1991). AAR1/TUP1 protein, with a structure similar to that of the β subunit of G proteins, is required for a1–α2 and α2 repression in cell type control of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **11**, 3773–3779.

MURGUI, A., ELORZA, V. AND SENTANDREU, R. (1985). Effect of papulacandin B and calcafluor white on the incorporation of mannoproteins in the wall of *Candida albicans* blastospores. *Biochim. Biophys. Acta* **841**, 215–222.

NAGARAJAN, L. AND UMESH-KUMAR, S. (1990). Antigenic studies on flocculating brewer's yeast *Saccharomyces cerevisiae* NCYC 227. *J. Gen. Microbiol.* **136**, 1747–1751.

NEIGEBORN, L. AND CARLSON, M. (1987). Mutations causing constitutive invertase synthesis in yeast: Genetic interactions with *snf* mutations. *Genetics* **115**, 247–253.

NEWELL, P.C. (1977). How cells communicate: the system used by slime moulds. *Endeavour (N.S.)* **1**, 63–68.

NISHIHARA, H. AND TORAYA, T. (1987). Essential roles of cell surface protein and carbohydrate components in flocculation of a brewer's yeast. *Agric. Biol. Chem.* **51**, 2721–2726.

NISHIHARA, H., TORAYA, T. AND FUKUI, S. (1976a). Factors affecting flocculation of brewer's yeast. *J. Ferment. Technol.* **54**, 351–355.

NISHIHARA, H., TORAYA, T. AND FUKUI, S. (1976b). Induction of floc-forming ability in brewer's yeast. *J. Ferment. Technol.* **54**, 356–360.

NISHIHARA, H., TORAYA, T. AND FUKUI, S. (1977). Effect of chemical modification of cell surface components of a brewer's yeast on the floc-forming ability. *Arch. Microbiol.* **115**, 19–23.

NISHIHARA, H., TORAYA, T. AND FUKUI, S. (1982). Flocculation of cell walls of brewer's yeast and effects of metal ions, protein denaturants and enzyme treatments. *Arch. Microbiol.* **131**, 112–115.

NORTHCOTE, D.H. AND HORNE, R.W. (1952). The chemical composition and structure of the yeast cell wall. *Biochem. J.* **51**, 232–236.

OFEK, I. AND BEACHEY, E.H. (1980). General concepts and principles of bacterial adherance in animals and man. In *Bacterial Adherence, Receptors and Recognition*, Series B, volume 6 (E.H. Beachey, Ed.), pp. 3–29. Chapman and Hall, London and New York.

OFEK, I. AND SHARON, N. (1988). Lectinophagocytosis: a molecular mechanism of recognition between cell surface sugars and lectins in the phagocytosis of bacteria. *Infect. Immun.* **56**, 539–547.

OGDEN, K.L. AND TAYLOR, A.L. (1991). Genetic control of flocculation in *Escherichia coli*. *J. Ind. Microbiol.* **7**, 279–286.

O'MELIA, C.R. (1972). Coagulation and flocculation. In *Physiochemical Processes for Water Quality Control* (W.J. Weber, Ed.), pp. 61–109. Wiley-Interscience, New York, London, Sydney and Toronto.

OVERBEEK, J.TH.G. (1952). Kinetics of flocculation. In *Colloid Science*, volume 1:

Irreversible Systems (H.R. Kruyt, Ed.), pp. 278–301. Elsevier, Amsterdam, Houston, New York and London.

PADMANABHA, R., CHEN-WU, J.L.-P., HANNA, D.E. AND GLOVER, C.V.C. (1990). Isolation, sequencing, and disruption of the yeast *CKA2* gene: casein kinase II is essential for viability in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**, 4089–4099.

PARISH, M.E. AND CARROLL, D.E. (1987). Fermentation characteristics of *Saccharomyces cerevisiae* isolates from *Vitis rotundifolia* grapes and musts. *Am. J. Enol. Viticulture* **38**, 45–48.

PASTEUR, L. (1876). *Etudes sur la Biere*, p. 196. Gauthier-Villars, Paris.

PASTOR, F.I.J., HERERO, E. AND SENTANDREU, R. (1982). Metabolism of *Saccharomyces cerevisiae* envelope mannoproteins. *Arch. Microbiol.* **132**, 144–148.

PATEL, G.B. AND INGLEDEW, W.M. (1975). A flocculation test for moderately flocculent brewing yeast. *J. Inst. Brewing* **81**, 123–126.

PAULSON, J.C. (1989). Glycoproteins: what are the sugar chains for? *Trends Biochem. Sci.* **14**, 272–275.

PEARCE, W.A. AND BUCHANAN, T.M. (1980). Structure and cell membrane-binding properties of bacterial fimbriae. In *Bacterial Adherence, Receptors and Recognition*, Series B, volume 6 (E.H. Beachey, Ed.), pp. 289–344. Chapman and Hall, London and New York.

PHILLIPS, D.R. AND GARTNER, T.K. (1980). Cell recognition systems in eukaryotic cells. In *Bacterial Adherence, Receptors and Recognition*, Series B, volume 6 (E.H. Beachey, Ed.), pp. 399–438. Chapman and Hall, London and New York.

PIENDL, A. (1969a). Brauereitechnologische Faktoren und Bruchbildung der Hefen. *Proc. Eur. Brewing Convention Congr.*, Stockholm **12**, 381–395.

PIENDL, A. (1969b). Brauereitechnologische Faktoren und Bruchbildung der Hefen. *Tageszeitung für Brauerei* **85**, 553–554.

POMPER, S. AND BURKHOLDER, P.R. (1949). Studies on the biochemical genetics of yeast. *Proc. Nat. Acad. Sci. USA* **35**, 456–464.

PORTER, A.M. AND MACAULAY, R.J. (1965). Studies on flocculation I. A relationship between the pH and calcium content of the growth medium. *J. Inst. Brewing* **71**, 175–179.

QUIOCHO, F.A. (1986). Carbohydrate-binding proteins: tertiary structures and protein-sugar interactions. *Ann. Rev. Biochem.* **55**, 287–315.

QUIOCHO, F.A. AND VYAS, N.K. (1984). Novel stereospecificity of the L-arabinose-binding protein. *Nature* **310**, 381–386.

RAINBOW, C. (1966). Flocculation of brewer's yeast. *Process Biochem.* **1**, 489–492.

RAMIREZ, A. AND BOUDAREL, M.J. (1983). Continuous production of ethanol on beet juice by a flocculant strain of *Saccharomyces cerevisiae*. *Biotechnol. Lett.* **5**, 659–664.

RANKEN, C. (1927). The surface of yeast as a factor in fermentation. *J. Inst. Brewing* **33**, 76–84.

RANKEN, C. AND BELL, J.R. (1928). The surface of yeast as a factor in fermentation. II. *J. Inst. Brewing* **34**, 265–274.

RASPOR, P., RUSSELL, I. AND STEWART, G.G. (1990a). Impact of the mineral ratio on flocculation in the yeast *Saccharomyces*. *Food Biotechnol. NY* **4**, 486.

RASPOR, P., RUSSELL, I. AND STEWART, G.G. (1990b). An update of zinc ion as an effector of flocculation in brewer's yeast strains. *J. Inst. Brewing* **96**, 303–305.

READ, R. (1989). Bacteria with a sticky touch. *New Scientist* **124** (1688), 38–41.

REEKE, G.N., BECKER, J.W., CUNNINGHAM, B.A., GUNTHER, G.R., WANG, J.L. AND EDELMAN, G.M. (1974). Relationships between the structure and activities on concanavalin A. *Ann. NY Acad. Sci.* **234**, 369–382.

RHEE, S.-K., ICHO, T. AND WICKNER, R.B. (1989). Structural and nuclear localization signal of the SKI3 antiviral protein of *Saccharomyces cerevisiae*. *Yeast* **5**, 149–158.

ROMAN, H. AND SANDS, S.M. (1953). Heterogeneity of clones of *Saccharomyces* derived from haploid ascospores. *Proc. Nat. Acad. Sci. USA* **39**, 171–179.

ROMAN, H., HAWTHORNE, D.C. AND DOUGLAS, H.C. (1951). Polyploidy in yeast and its bearing on the occurrence of irregular genetic ratios. *Proc. Nat. Acad. Sci. USA* **37**, 79–84.

ROSE, A.H. (1984). Physiology of cell aggregation: Flocculation of *Saccharomyces cerevisiae* as a model system. In *Microbial Adhesion and Aggregation, Life Science Report No. 31*, Dahlem Konferenzen (K.C. Marshall, Ed.), pp., 323–335. Springer-Verlag, Berlin, Heidelberg, New York and Tokyo.

ROSEMAN, S. (1970). The synthesis of complex carbohydrates by multiglycosyltransferase systems and their potential function in intercellular adhesion. *Chem. Phys. Lipids* **5**, 270–297.

ROTHSTEIN, R.J. AND SHERMAN, F. (1980). Genes affecting the expression of cytochrome c in yeast: Genetic mapping and genetic interactions. *Genetics* **94**, 871–889.

RUSSELL, I., STEWART, G.G., READER, H.P., JOHNSTON, J.R. AND MARTIN, P.A. (1980). Revised nomenclature of genes that control yeast flocculation. *J. Inst. Brewing* **86**, 120–121.

SAITO, K., SATO, S.I., SHIMOI, H., IEFUJI, H. AND TADENUMA, M. (1990). Flocculation mechanism of *Hansenula anomala* J224. *Agric. Biol. Chem.* **54**, 1425–1432.

SANDER, L.M. (1987). Fractal growth. *Scient. Am.* **265**, 82–88.

SCHAEFER, D.W. (1989). Polymers, fractals and ceramic materials. *Science* **243**, 1023–1027.

SCHAEFER, D.W., MARTIN, J.E., WILTZIUS, P. AND CANNELL, D.S. (1984). Fractal geometry of colloidal aggregates. *Phys. Rev. Lett.* **52**, 2371–2374.

SCHAMHART, D.H.J., TEN BERGE, A.M.A. AND VAN DE POLL, K.W. (1975). Isolation of a catabolite repression mutant of yeast as a revertant of a strain that is maltose negative in the respiratory-deficient state. *J. Bacteriol.* **121**, 747–752.

SCHONFELD, F. (1912). Chemical composition of yeast in relation to its behaviour in fermentation. *Wochenschrift für Brauereien* **29**, 393–396 (Abstract 1912 *J. Fed. Inst. Brewing* **18**, 701).

SCHONFELD, F. AND KRUMHAAR, H. (1918a). Causes of the flocculent and powdery forms of yeast. *Wochenschrift für Brauereien* **35**, 302–304 (Abstract 1919 *J. Fed. Inst. Brewing* **25**, 183).

SCHONFELD, F. AND KRUMHAAR, H. (1918b). Flocculent and powdery forms of yeast—their causes. *Wochenschrift für Brauereien* **35**, 342–343 (Abstract 1919 *J. Fed. Inst. Brewing* **25**, 184).

SCHONFELD, F. AND SCHONFELDER, G. (1914). *Wochenschrift für Brauereien* **31**, 245 (Abstract Jansen, 1958).

SCHULTZ, J. AND CARLSON, M. (1987). Molecular analysis of *SSN6*, a gene functionally related to the *SNF1* protein kinase of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7**, 3637–3645.

SCHULTZ, J., MARSHALL-CARLSON, L. AND CARLSON, M. (1990). The *N*-terminal TPR region is the functional domain of SSN6, a nuclear phosphoprotein of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**, 4744–4756.

SCRIBAN, R. (1962). L'aeration du mout et son ensemencement. *Brasserie* **17**, 125–133.

SEKI, T., MYOGA, S., LIMTONG, S., UEDONO, S., KUMNUANTA, J. AND TAGUCHI, H. (1983). Genetic construction of yeast strains for high ethanol production. *Biotechnol. Lett.* **5**, 351–356.

SEYFFERT, H. (1896). Remarks on pure culture yeasts and their nourishment. *Zeitschrift für das gesamte Brauwesen* **19**, 318–322 (Abstract 1896 *J. Fed. Inst. Brewing* **2**, 589).

SHARON, N. AND LIS, H. (1972). Lectins: cell-agglutinating and sugar-specific proteins. *Science* **177**, 949–959.

SHARON, N., ESHDAT, Y., SILVERBLATT, F.J. AND OFEK, I. (1981). Bacterial adherence to surface sugars. *Ciba Found. Symp. Ser.* **80**, 119–141.

SHARON, N., FIRON, N. AND OFEK, I. (1983). Oligomannoside units of cell surface

glycoproteins as receptors for bacteria. *Pure Appl. Chem.* **55**, 671–675.

SHIEH, W. AND CHEN, L. (1986). Effect of innoculum on yeast cell aggregation. *Appl. Microbiol. Biotechnol.* **25**, 232–237.

SKATRUD, P.L., KOT, E.J. AND HELBERT, J.R. (1982). Genes governing the fermentation of maltose and flocculation in a brewer's yeast. *Am. Soc. Brewing Chem. J.* **40**, 52–55.

SMITH, E.E. AND GOLDSTEIN, I.J. (1967). Protein-carbohydrate interaction. V. Further inhibition studies directed toward defining the stereochemical requirements of the reactive sites of concanavalin A. *Arch. Biochem. Biophys.* **121**, 88–95.

SMITH, R.H. (1951). A study of the role of inositol in the nutrition of *Nematospora gossypii* and *Saccharomyces carlsbergensis*. *J. Gen. Microbiol.* **5**, 772–780.

SO, L.L. AND GOLDSTEIN, I.J. (1968). Protein–carbohydrate interaction. XIII. The interaction of concanavalin A with α-mannans from a variety of microorganisms. *J. Biol. Chem.* **243**, 2003–2007.

SOARES, E.V., TEIXEIRA, J.A. AND MOTA, M. (1991). Influence of aeration and glucose concentration in the flocculation of *Saccharomyces cerevisiae*. *Biotechnol. Lett.* **13**, 207–212.

SPENCER, J.F.T., MILLER, R., SPENCER, D.M. AND WILKIE, D. (1981). Flocculation and starch utilization in some antibiotic-resistant mitochondrial mutants of *Saccharomyces diastaticus*. In *Current Developments in Yeast Research, Advances in Biotechnology* (G.G. Stewart and I. Russell, Eds), pp. 33–39. Pergamon Press, Toronto.

ST JOHNSTON, J.H. (1953). The termination of primary fermentation. 1. Experimental data obtained from flocculation and fermentation tests. *Wallerstein Lab. Commun.* **16**, 39–69.

STARK, H.C., FUGIT, D. AND MOWSHOWITZ, D.B. (1980). Pleiotropic properties of a yeast mutant insensitive to catabolite repression. *Genetics* **94**, 921–928.

STEVENSON, D.G. (1972). Flocculation and floc behaviour. *J. Inst. Water Eng., Lond.* **26**, 155–169.

STEWART, G.G. (1972). Co-flocculation of brewer's yeast. *Master Brewers Assoc. Am. Techn. Q.* **9**, 25.

STEWART, G.G. (1975). Yeast flocculation. Practical implications and experimental findings. *Brewers Digest* **50**, 42–56.

STEWART, G.G. AND GARRISON, I.F. (1972). Some observations of co-flocculation in *Saccharomyces cerevisiae*. *Proc. Am. Soc. Brewing Chem.*, pp. 118–131.

STEWART, G.G. AND GORING, T.E. (1976). Effect of some monovalent and divalent ions on the flocculation of brewer's yeast strains. *J. Inst. Brewing* **82**, 341–342.

STEWART, G.G. AND RUSSELL, I. (1976). Biochemical and genetic studies on a flocculent haploid yeast strain. *Proc. 5th Int. Ferment. Symp.*, Berlin, p. 187.

STEWART, G.G. AND RUSSELL, I. (1977). The identification, characterization and mapping of a gene for flocculation in *Saccharomyces* sp. *Can. J. Microbiol.* **23**, 441–447.

STEWART, G.G. AND RUSSELL, I. (1981). Yeast flocculation. In *Brewing Science*, volume 2 (J.R.A. Pollock, Ed.), pp. 61–92. Academic Press, London.

STEWART, G.G. AND RUSSELL, I. (1986). Centenary review. One hundred years of yeast research and development in the brewing industry. *J. Inst. Brewing* **92**, 537–558.

STEWART, G.G. AND RUSSELL, I. (1987). The relevance of the flocculation properties of yeast in today's brewing industry. In *European Brewery Convention Monograph—XII*, E.B.C. Symposium on brewer's yeast, Vuoranta (Helsinki), Finland. November 1986, pp. 53–70. Carl Brauwelt-Verlag, Nurnberg.

STEWART, G.G., RUSSELL, I. AND GARRISON, I.F. (1973). Further studies on flocculation and co-flocculation in brewer's yeast strains. *Proc. Am. Soc. Brewing Chem.*, pp. 100–106.

STEWART, G.G., RUSSELL, I. AND GARRISON, I.F. (1975). Some considerations of the

flocculation characteristics of ale and lager yeast strains. *J. Inst. Brewing* **81**, 248–257.

STEWART, G.G., RUSSELL, I. AND GORING, T. (1975). Nature–nurture anomalies—further studies in yeast flocculation. *Proc. Am. Soc. Brewing Chem.* **33**, 137–147.

STEWART, G.G., GARRISON, I.F., GORING, T.E., MELEG, M., PIPASTS, P. AND RUSSELL, I. (1976). Biochemical and genetic studies on yeast flocculation. *Kemia-Kemi* **3**, 465–479.

STEWART, G.G., CRUMPLEN, R., D'AMORE, T., DOWHANICK, T. AND RUSSELL, I. (1990). The genetic improvement of brewing and other ethanol producing yeasts. In *Fermentation Technologies. Industrial Applications* (P.K. Yu, Ed.), pp. 221–232. Elsevier, London and New York.

STRATFORD, M. (1989a). Yeast flocculation: the influence of agitation. *Yeast* **5**, s97–s102.

STRATFORD, M. (1989b). Evidence for two mechanisms of flocculation in *Saccharomyces cerevisiae*. *Yeast* **5**, s441–s445.

STRATFORD, M. (1989c). Yeast flocculation: calcium specificity. *Yeast* **5**, 487–496.

STRATFORD, M. (1992a). Yeast flocculation: reconciliation of physiological and genetic viewpoints. *Yeast* **8**, 25–38.

STRATFORD, M. (1992b). Yeast flocculation: a new perspective. *Adv. Microbial Physiol.* **33**, 1–72.

STRATFORD, M. (1992c). Yeast flocculation: receptor definition by *mnn* mutants and concanavalin A. *Yeast* **8**, 635–645.

STRATFORD, M. (1993). Yeast flocculation: flocculation onset and receptor availability. *Yeast* **9** (in press).

STRATFORD, M. AND ASSINDER, S. (1991). Yeast flocculation: Flo1 and NewFlo phenotypes and receptor structure. *Yeast* **7**, 559–574.

STRATFORD, M. AND BRUNDISH, H.M. (1990). Yeast flocculation: cationic inhibition. *Yeast* **6**, 77–86.

STRATFORD, M. AND CARTER, A.T. (1993). Yeast flocculation: Lectin synthesis and activation. *Yeast* **9** (in press).

STRATFORD, M. AND KEENAN, M.H.J. (1987). Yeast flocculation: kinetics and collision theory. *Yeast* **3**, 201–206.

STRATFORD, M. AND KEENAN, M.H.J. (1988). Yeast flocculation: quantification. *Yeast* **4**, 107–115.

STRATFORD, M. AND PEARSON, B.M. (1992). Lectin-mediated flocculation of the yeast *Saccharomycodes ludwigii* NCYC 734. *Lett. Appl. Microbiol.* **14**, 214–216.

STRATFORD, M. AND WILSON, P.D.G. (1990). Agitation effects on microbial cell–cell interactions. *Lett. Appl. Microbiol.* **11**, 1–6.

STRATFORD, M., COLEMAN, H.P. AND KEENAN, M.H.J. (1988). Yeast flocculation: a dynamic equilibrium. *Yeast* **4**, 199–208.

SUDOH, M., SHIMADA, H., ARISAWA, M., YANO, K. AND TAKAGI, M. (1991). Yeast flocculation mediated by over-expression of the Chitin Synthase 2 gene in *Saccharomyces cerevisiae*. *Agric. Biol. Chem.* **55**, 2901–2903.

SUTHERLAND, D.N. (1967). A theoretical model of floc structure. *J. Colloid Interface Sci.* **25**, 373–380.

SUZZI, G. AND ROMANO, P. (1990). Flocculazione in *Zygosaccharomyces*. *Industrie delle Bevande* **19**, 306–308.

SUZZI, G. AND ROMANO, P. (1991). Flocculent phenotypes in wine yeasts. *Lett. Appl. Microbiol.* **13**, 7–10.

SUZZI, G., ROMANO, P. AND ZAMBONELLI, C. (1984). Flocculation of wine yeasts: frequency, differences and stability of the character. *Can. J. Microbiol.* **30**, 36–39.

TAIRA, K., ODA, M., SHINSHI, H., MAEDA, H. AND FURUKAWA, K. (1990). Construction of a novel artificial-ribozyme-releasing plasmid. *Protein Eng.* **3**, 733–737.

TAYLOR, N.W. AND ORTON, W.L. (1973). Effect of alkaline-earth metal salts on flocculence in *Saccharomyces cerevisiae*. *J. Inst. Brewing* **79**, 294–297.

TAYLOR, N.W. AND ORTON, W.L. (1975). Calcium in flocculence of *Saccharomyces cerevisiae*. *J. Inst. Brewing* **81**, 53–57.

TAYLOR, N.W. AND ORTON, W.L. (1978). Aromatic compounds and sugars in flocculation of *Saccharomyces cerevisiae*. *J. Inst. Brewing* **84**, 113–114.

TEIXEIRA, J.A. AND MOTA, M. (1990). Experimental assessment of internal diffusion limitations in yeast flocs. *Chem. Eng. J.* **43**, B13–B17.

TEIXEIRA, J., GONCALVES, M.H., GAMA, F.M., MORADAS-FERREIRA, P. AND MOTA, M. (1989). Partial characterization of cell wall from a flocculent strain of *Kluyveromyces marxianus*. *Biotechnol. Lett.* **11**, 579–582.

TEIXEIRA, J.M., TEIXEIRA, J.A., MOTA, M., GUERRA, B., MACHADO-CRUZ, J.M. AND SA ALMEIDA, A.M. (1991). The influence of cell wall composition of a brewer's flocculent lager yeast on sedimentation during successive industrial fermentations. *Proc. Eur. Brewing Convention Congr.*, Lisbon, pp. 241–256.

TEKIPPE, R.J. AND HAM, R.K. (1971). Velocity-gradient paths in coagulation. *J. Am. Water Works Assoc.* **63**, 439–448.

TEUNISSEN, A.W.R.H. AND STEENSMA, H.Y. (1990). Physical localization of the *FLO1* gene of *Saccharomyces cerevisiae*. *Yeast* **6**, s35.

THORNE, R.S.W. (1951a). The genetics of flocculence in *Saccharomyces cerevisiae*. *Compte rendu des Travaux du Laboratoire de Carlsberg, Serie Physiologique* **25**, 101–140.

THORNE, R.S.W. (1951b). Some aspects of yeast flocculence. *Proc. Eur. Brewing Convention Congr.*, Brighton, pp. 21–34.

THORNTON, R.J. (1985). The introduction of flocculation into a homothallic wine yeast. A practical example of the modification of winemaking properties by the use of genetic techniques. *Am. J. Enol. Viticulture* **36**, 47–49.

THORNTON, R.J. AND BUNKER, A. (1989). Characterization of wine yeasts for genetically modifiable properties. *J. Inst. Brewing* **95**, 181–184.

THRASH-BINGHAM, C. AND FANGMAN, W.L. (1989). A yeast mutation that stabilizes a plasmid bearing a mutated *ARS1* element. *Mol. Cell. Biol.* **9**, 809–816.

TKACZ, J.S., CYBULSKA, E.B. AND LAMPEN, J.O. (1971). Specific staining of wall mannan of yeast cells with fluorescein-conjugated concanavalin A. *J. Bacteriol.* **105**, 1–5.

TRONCHIN, G., POULAIN, D., HERBAUT, J. AND BIGUET, J. (1981). Localization of chitin in the cell wall of *Candida albicans* by means of wheat germ agglutinin. Fluorescence and ultrastructural studies. *Eur. J. Cell Biol.* **26**, 121–128.

TRUMBLY, R.J. (1986). Isolation of *Saccharomyces cerevisiae* mutants constitutive for invertase synthesis. *J. Bacteriol.* **166**, 1123–1127.

TRUMBLY, R.J. (1988). Cloning and characterization of the *CYC8* gene mediating glucose repression in yeast. *Gene* **73**, 97–111.

TUORILA, P. (1927). Über orthokinetische und perikinetische koagulation. *Kolloid-chemische Beihefte* **24**, 1–122.

UNZ, R.F. (1987). Aspects of bioflocculation: An overview. In *Flocculation in Biotechnology and Separation Systems* (Y.A. Attia, Ed.), pp. 351–368. Elsevier, Amsterdam.

URANO, N., NISHIKAWA, N. AND KAMIMURA, M. (1990). Electrofusion of a brewer's yeast, construction of fusants between a brewer's yeast and *Saccharomyces cerevisiae* (*FLO5*). *Proc. Convention Inst. Brewing (Austral. Section)* **21**, 154–157.

VAN HEYNINGEN, W.E. (1974). Gangliosides as membrane receptors for tetanus toxin, cholera toxin and serotonin. *Nature* **249**, 415–417.

VAN LAER, H. (1905). Certain coagulation phenomena produced by borates: agglutination of yeast. *Bulletin-Societé Chimique de Belgique* **19**, 31–47 (Abstract 1905 *J. Fed. Inst. Brewing* **11**, 573–574).

VAN RINSUM, J., KLIS, F.M. AND VAN DEN ENDE, H. (1991). Cell wall glucomannoproteins of *Saccharomyces cerevisiae mnn9*. *Yeast* **7**, 717–726.

VEINOT-DREBOT, L.M., JOHNSTON, G.C. AND SINGER, R.A. (1991). The *RSF1* gene

regulates septum formation in *Saccharomyces cerevisiae*. *J. Bacteriol.* **173**, 2556–2561.

VEZINHET, F., BLONDIN, B. AND BARRE, P. (1991). Mapping of the *FLO5* gene of *Saccharomyces cerevisiae* by transfer of a chromosome during cytoduction. *Biotechnol. Lett.* **13**, 47–52.

VON SMOLUCHOWSKI, M. (1917). Versuch einer mathematischen theorie der koagulations-kinetiek kolloid losungen. *Z. für Physik. Chem.* **92**, 129–168.

WATARI, J., TAKATA, Y., NISHIKAWA, N. AND KAMADA, K. (1987). Cloning of a gene controlling yeast flocculence. *Proc. Eur. Brewing Convention Congr.*, Madrid, pp. 537–543.

WATARI, J., TAKATA, Y., OGAWA, M., NISHIKAWA, N. AND KAMIMURA, M. (1989). Molecular cloning of a flocculation gene in *Saccharomyces cerevisiae*. *Agric. Biol. Chem.* **53**, 901–903.

WATARI, J., KUDO, M., NISHIKAWA, N. AND KAMIMURA, M. (1990). Construction of flocculent yeast cells (*Saccharomyces cerevisiae*) by mating or protoplast fusion using a yeast cell containing the flocculation gene *FLO5*. *Agric. Biol. Chem.* **54**, 1677–1681.

WATARI. J., TAKATA, Y., MURAKAMI, J. AND KOSHINO, S. (1991a). Breeding of flocculent brewer's yeast by genetic engineering. *Proc. Eur. Brewing Convention Congr.*, Lisbon, pp. 297–304.

WATARI, J., TAKATA, Y., OGAWA, M., MURAKAMI, J. AND KOSHINO, S. (1991b). Breeding of flocculent industrial *Saccharomyces cerevisiae* strains by introducing the flocculation gene *FLO1*. *Agric. Biol. Chem.* **55**, 1547–1552.

WATT, P.J. AND WARD, M.E. (1980). Adherance of *Neisseria gonorrhoeae* and other *Neisseria* species to mammalian cells. In *Bacterial Adherence, Receptors and Recognition*, Series B, volume 6 (E.H. Beachey, Ed.), pp. 251–288. Chapman and Hall, London and New York.

WEITZ, D.A., HUANG, J.S., LIN, M.Y. AND SUNG, J. (1984). Dynamics of diffusion-limited kinetic aggregation. *Phys. Rev. Lett.* **53**, 1657–1660.

WHITE, F.H. AND KIDNEY, E. (1979). The influence of yeast strain on beer spoilage bacteria. *Proc. Eur. Brewing Convention Congr.*, Berlin, pp. 801–815.

WICKNER, R.B. (1974). Mutants of *Saccharomyces cerevisiae* that incorporate deoxythymidine-5'-monophosphate into deoxyribonucleic acid *in vivo*. *J. Bacteriol.* **117**, 252–260.

WILES, A.E. (1951). The action of finings and its relation to the electrokinetic properties of the yeast cell. *Proc. Eur. Brewing Convention Congr.*, Brighton, pp. 84–97.

WILKIE, D. AND NUDD, R.C. (1981). Aspects of mitochondrial control of cell surface characteristics in *Saccharomyces cerevisiae*. In *Current Developments in Yeast Research, Advances in Biotechnology* (G.G. Stewart and I. Russell, Eds), pp. 345–349. Pergamon Press, Toronto.

WILLIAMS, F.E. AND TRUMBLY, R.J. (1990). Characterization of *tup1*, a mediator of glucose repression in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**, 6500–6511.

WILLIAMS, F.E., VARANASI, U. AND TRUMBLY, R.J. (1991). The CYC8 and TUP1 proteins involved in glucose repression in *Saccharomyces cerevisiae* are associated in a protein complex. *Mol. Cell. Biol.* **11**, 3307–3316.

WILLIAMS, N.J. AND WISEMAN, A. (1973a). Enzyme-kinetic analogy for yeast flocculation data. *Biochem. Soc. Trans.* **1**, 291–292.

WILLIAMS, N.J. AND WISEMAN, A. (1973b). Loss of yeast flocculence after release of extracellular invertase (β-fructofuranosidase) and acid phosphatase by a modified osmotic shock procedure that maintains viability. *Biochem. Soc. Trans.* **1**, 1301–1303.

WINDISCH, S. (1968). Flocculation of brewing yeasts, a review. *Brewers Digest* **43**, 62–66.

WITTEN, T.A. AND CATES, M.E. (1986). Tenuous structures from disorderly growth processes. *Science* **232**, 1607–1612.

YAMASHITA, I. AND FUKUI, S. (1983). Mating signals control expression of both starch fermentation genes and a novel flocculation gene *FLO8* in the yeast *Saccharomyces*. *Agric. Biol. Chem.* **47**, 2889–2896.

YAMASHITA, I. AND FUKUI, S. (1984). Isolation of glucoamylase-non-producing mutants in the yeast *Saccharomyces diastaticus*. *Agric. Biol. Chem.* **48**, 131–135.

YOKOYA, F. AND OLIVA-NETO, P. (1991). Caracteristicas da floculacao de leveduras por *Lactobacillus* fermentum. *Revista de Microbiologia, Sao Paulo* **22**, 12–16.

YUSA, M. (1977). Mechanisms of pelleting flocculation. *Int. J. Mineral Process.* **4**, 293–305.

ZHANG, M., ROSENBLUM-VOS, L.S., LOWRY, C.V., BOAKYE, K.A. AND ZITOMER, R.S. (1991). A yeast protein with homology to the β-subunit of G proteins is involved in control of heme-regulated and catabolite-repressed genes. *Gene* **97**, 153–161.

ZLOTNIK, H., FERNANDEZ, M.P., BOWERS, B. AND CABIB, E. (1984). *Saccharomyces cerevisiae* mannoproteins form an external cell wall layer that determines wall porosity. *J. Bacteriol.* **159**, 1018–1026.