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THE ROLE OF FIMBRIAE IN THE FLOCCULATION OF BREWER'S YEAST

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ABSTRACT

Despite the fundamental importance of yeast flocculation in brewing, relatively little is known regarding the mechanism by which it occurs. Early investigations concentrated on the physio-chemical nature of the interactions, but increasing emphasis is being placed on the possibility of a specific biological recognition system. Consistent with this latter approach the aim of this study was to determine whether or not fimbriae play a role in flocculation of brewer's yeast and (if they were involved) to investigate the natures of the fimbriae and their interactions.

This involved the electron microscopic examination of surfaces of flocculent and non-flocculent cells in both log and stationary phases; the assessment of the effects of chemicals and enzymes on the fimbriae attached to the cell; attempts to remove and label intact fimbriae; determination of their composition and charge; and analysis of the surface proteins of flocculent and non-flocculent cells. Results show that fimbriae are found most frequently on flocculent cells (in comparison with actively growing and non-flocculent cells); that they are an integral part of the cell wall and are present, although not necessarily exposed, at all stages of the cells growth; that they are predominantly proteinaceous with mannan bases and do not appear to have a strong negative charge. Flocculated cells also exhibited slightly different antigenicity from actively growing cells and had an additional 48 000 dalton surface protein.

DECLARATION

I declare that this dissertation is my own, unaided work.
It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg.
It has not been submitted before for any degree or examination in any other University.

_____

30th day of January, 19 86.

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TABLE OF CONTENTS

Chapter		Page
1.	INTRODUCTION	1
1.1	GENERAL REVIEW	1
1.1.a	Definition of flocculation	1
1.1.b	Fimbriae and flocculation	1
1.1.c	Fimbriae	4
1.1.d	The role of flocculation in brewing	5
1.1.e	Classification of flocculation	7
1.1.f	Measurement of flocculation	8
1.2	THE MECHANISM OF FLOCCULATION	9
1.2.a	Genetics of flocculation	9
1.2.b	The yeast cell wall	12
1.2.c	Induction of flocculation	22
1.2.d	Variability of flocculation	23
1.2.e	Bond formation	25
1.2.f	Yeast metabolism	28
1.2.g	Biological recognition system	40
2.	FIMBRIAE AND FLOCCULATION	44
2.1	INTRODUCTION	44
2.2	METHODS	48
2.3	RESULTS	62
2.3.a	Verification of the presence of fimbriae on flocculated yeast cells	62

2.3.b Selection of a non-flocculent control culture..... 66

2.3.c Replication of the experiments of Day et al. (1975)..... 75

2.3.d Cells in suspension at the end of fermentation..... 81

2.3.e The effect of Concanavalin A on flocculation and on fimbriae..... 83

2.4 DISCUSSION..... 85

3. THE EFFECT OF CHEMICAL, MECHANICAL AND ENZYME TREATMENTS ON FIMBRIAE..... 96

3.1 CHEMICAL..... 96

3.1.a Methods..... 96

3.1.b Results..... 97

3.2 MECHANICAL..... 109

3.2.a Ultrasonication 110

3.2.b Centrifugation through a water-sucrose barrier..... 113

3.2.c Agitation in a waring blendor..... 114

3.3 ENZYME TREATMENT..... 114

3.3.a Methods 115

3.3.b Results 118

3.4 DISCUSSION 131

4. ANTIBODY STUDIES..... 141
4.1 INTRODUCTION 141
4.2 METHODS..... 143
4.3 RESULTS..... 153
4.3.a Antibody titre..... 153
4.3.b Labelling the antibodies 153
4.4. DISCUSSION..... 163

5. THE CARBOHYDRATE COMPOSITION OF FIMBRIAE . 168
5.1 INTRODUCTION..... 168
5.2 METHODS 168
5.3 RESULTS 174
5.3.a Labelling fimbriae with ferritin-Con A.. 174
5.3.b Determination of the nature of the carbohydrate component..... 178
5.4 DISCUSSION..... 182

6. THE CELLS WALLS OF FLOCCULENT AND NON-FLOCCULENT YEASTS..... 186
6.1 WALL PROTEINS..... 186
6.1.a Introduction..... 186
6.1.b Methods..... 188
6.1.c Results..... 190
6.2 WALL CHARGE..... 193

6.2.a Introduction..... 193
6.2.b Methods..... 193
6.2.c Results..... 194
6.3 DISCUSSION..... 198

7. DISCUSSION AND CONCLUSIONS..... 204

APPENDIX..... 222

LIST OF REFERENCES..... 237

LIST OF TABLES

Page

1. Settling of yeast cultures in Helm medium over one hour..... 67
2. Percentages of fimbriate cells in various populations..... 79
3. Correlation between flocculation and fimbriation of some non-brewery yeasts..... 80
4. Concentration of enzymes used and their buffers..... 116
5. The effect of enzyme treatment on cell flocculence... 127
6. Procedure used for serum titration..... 145
7. Procedure for the Glucose-6-phosphate dehydrogenase test to establish the presence of glucose in fimbrial extracts..... 173
8. Results of the glucose-6-phosphate-dehydrogenase test for the presence of glucose in fimbrial extracts..... 180

LIST OF FIGURES

Page

1. Diagram showing genotype and associated phenotype of yeast flocculence..... 11
2. Schematic structure of the wall of Saccharomyces.... 13
3. Active fermentations 48 hours after pitching..... 51
4. Fermentations after 120 52
5. Appendages of Escher... ...i (electron micrograph EM)..... 63
6. Fimbriae of Saccharomyces uvarum 2036 (EM)..... 64
7. Fimbriae of S.uvarum 2036 (scanning electron micrograph)..... 65
8. Clump of ...atively stained S.uvarum 2036 cells (EM) 66
9. Diagramatic representation of the settling of yeast cultures in Helm medium..... 68
10. Patterns of growth of the experi...tal and two non-flocculent cultures..... 70
11. S.uvarum 2036 in the log phase ...wth (light micrograph, LM)..... 72
12. S.uvarum 2036 in the stationary ... of growth (LM) 72
13. S.cerevisiae Y746 in the station...y phase of growth (LM)..... 73
14. Fimbriae on a flocculated ether-washed cell (EM).... 76
15. Sparse fimbriae on a log phase cell (EM)..... 77
16. Edge of a non-fimbriate stationary phase cell (EM).. 78
17. Edges of cells which had not flocculated at the end of fermentation (EM)..... 82
18. Yeast flocs formed under natural conditions (LM)... 84

19. Yeast flocc found in the presence of Con A (LM)..... 84
20. The effect of formalin on the surface of a flocculated cell (EM)..... 98
21. The effect of alcohol on a flocculated cell (EM).... 99
22. The effect of chloroform on a flocculated cell (EM). 100
23. The effect of acetic acid on a flocculated cell (EM) 101
24. The effect of urea on a flocculated cell (EM)..... 102
25. The effect of diethyl-ether on a flocculated cell (EM)..... 103
26. Water washed flocculated cells (SEM)..... 105
27. Ether washed flocculated cells (SEM)..... 106
28. Settling of S.uvarum 2036 (ether washed) in Helm medium..... 108
29. Settling of S.cerevisiae Y746 (ether washed) in Helm medium..... 108 120
30. Edge of a lipase-treated cell (EM)..... 121
31. Edge of a pronase-treated cell (EM)..... 123
32. Edge of a zymolyase-treated cell (EM)..... 124
33. Hair-like structures seen in the zymolyase extract (EM)..... 124
34. Fimbriae on the edge of a cell treated with leucine aminopeptidase (EM)..... 129
35. Settling of log phase samples treated with α-mannosidase and leucine aminopeptidase in Helm medium..... 130
36. Edge of a flocculated cell labelled with HRP-antibody conjugate (EM)..... 154
37. Edge of two flocculated HRP-labelled cells (EM)..... 155

38. Edge of HRP-antibody labelled cells postfixed with osmium tetroxide (EM)..... 156
39. Apparantly sparsely fimbriate cell labelled with ferritin goat-anti-rabbit antiserum (EM)..... 158
40. Densely fimbriate cell labelled with ferritin goat-anti-rabbit antiserum..... 159
41. FITC-labelled S.uvarum 2036 (stationary phase) (LM) 161
42. FITC-labelled S.uvarum 2036 (log phase) (LM)..... 162
43. FITC-labelled S.cerevisiae Y746 (stationary phase) (LM)..... 162
44. Edge of a fimbriate cell labelled with ferritin Con A (EM)..... 176
45. Edge of a fimbriate cell labelled with ferritin Con A (EM)..... 177
46. Standard curve for mannose: glucose ratios for the sulphuric acid-carbazole test..... 179
47. Gel scans of protein extracts from the walls of S. uvarum 2036 (log and stationary phases) and of S.cerevisiae Y746 (stationary phase)..... 191
48. Edge of a flocculated cell labelled with cationized ferritin (EM)..... 195
49. Edge of a log phase cell of S.uvarum 2036 labelled with cationized ferritin (EM)..... 196
50. Cationized ferritin labelling of two stationary phase cells of S.cerevisiae Y746..... 197
51. Diagramatic representation of a proposed mechanism for flocculation..... 219

CHAPTER 1

INTRODUCTION

1.1 GENERAL REVIEW

1.1.1 DEFINITION OF FLOCCULATION

The Latin word "flocculus" means a tuft of wool and according to Stewart (1975) the English derivative, "flocculation" describes "the phenomenon wherein yeast cells adhere together in clumps and sediment rapidly from the medium in which they are suspended".

Unlike passive aggregate formation, in which chains of cells are formed when mother and daughter cells fail to separate during exponential growth, flocculation occurs in the stationary phase. It is an active process and occurs when free cells clump as a result of the random collisions of Brownian movement and form stable aggregates.

1.1.2 FIMBRIAE AND FLOCCULATION

The presence of fimbriae on the surfaces of stationary phase flocculent yeast cells was first reported by Day, Poone and Stewart (1975). They found that cells of the yeast

Saccharomyces which had flocculated were covered with a dense mat of surface hairs or fimbriae about 0,5um long and 50-70 A in diameter, while the surfaces of log phase cells were smooth or had a few sparse fimbriae. The correlation between the ability of a culture to flocculate and the presence of fimbriae was further supported by the observation that some strains which were flocculent in wort, but not in defined medium, only possessed fimbriae in the wort medium.

The authors suggested that the fimbriae were probably the mannan-protein structures involved in flocculation and in protein secretion. Although they were not analysed, evidence provided for their being mannan-protein was twofold: Firstly, one of the major differences between the walls of flocculent and non-flocculent cells is the greater amount of mannan-protein in the flocculent cell walls (Stewart and Russell, 1981). This could be accounted for by the presence of parts of fimbriae still embedded in the walls after extraction (Day et al. 1975). Secondly, suppression of mannan-protein synthesis by cyclohexamide prevented cells from becomming flocculent (Baker and Kirsop, 1972). This inhibitor was known not to affect the synthesis of glucan fibrils (Farkas et al. 1970), but did prevent the extrusion of the fimbriae of Ustilago violaceae (Poone and Day, 1975). Considering all these effects it was concluded that, since fimbriae appeared to function in flocculation their

synthesis would also be prevented if they comprised mannoprotein (Day et al.1975).

As well as their involvement in protein secretion and flocculation they possibly also have a role in mating conjugations. Hansenula wingei cells conjugate after complementary binding of surface mannan-proteins which are specific for each mating cell. As they produce fimbriae similar to those of Saccharomyces cerevisiae it may be that these are the specific sex-agglutination factors. In spite of these three potential roles for fimbriae, it is possible that flocculation is only a secondary characteristic which the fungus uses under certain environmental conditions and which is exploited by the brewer.

In this study the cell surfaces of flocculent and non-flocculent strains of S. cerevisiae and S.uvarum examined during different stages of fermentation were found to be similar in appearance to those described by these authors. The difference in fimbriation between flocculent and non-flocculent cells was however, not as obvious as it was in the strains examined by Day et al.(1975). This study has attempted to further characterise the fimbriae and establish more firmly their role in flocculation, if such a role exists. From the results obtained it is suggested that they are an integral part of the cell wall but are exposed only in the stationary phase in flocculent strains although they

may be a feature common to most yeasts. It is also suggested that they may contribute towards reducing the repulsive negative surface charge as the cells approach the flocculent stage. Further evidence is provided for their being comprised of mannoprotein.

1.1.3 FIMBRIAE

"Fimbriae" is the Latin word for "thread" or [illegible]" and "pili" the Latin term meaning "hair" or" hair structure." The terminology used by Ottow (1975) in reference to the filamentous, non-flagellar structures of bacteria shall be used here. The term "pili" described the appendages involved in transmission of nucleic acids from one cell to another and "fimbriae" referred to all the other structures.

Fimbriae and pili differ from flagella. The latter are usually large appendages which can generally be seen under the light microscope after staining, while fimbriae and pili are very much smaller and can only be seen under the electron microscope. They also differ from flagella in being more numerous and less rigid, but thinner and straighter than the flagella. Clear cut functional differences exist as well, flagella being associated with motility of organisims and fimbriae generally having adhesive properties.

They also share a number of common features. For example they both originate from the cytoplasm and are extruded through the peptidoglycan layer of the cell wall; can arise from either the polar regions, or peritrichously; are able to be regenerated by the parent cell after being artificially removed; seem to be assembled from a pool of precursor protein.

Apart from their adhesive functions fimbriae may also operate in respiration and nutrient uptake by virtue of their increasing the cell surface area (Ottow, 1975).

Fimbriae are generally fairly easily removed from cells by mechanical agitation or by high speed centrifugation which means that they can be readily isolated for electron microscopy, x-ray diffraction and chemical analysis.

1.1.4 THE ROLE OF FLOCCULATION IN BREWING

It is of prime importance to the brewer that a palatable beer of consistent quality is produced. To this end , the constituents of his product; malt, yeast, hops and adjuncts must meet certain specifications.

When a particular yeast strain is selected for brewing purposes a number of criteria must be satisfied. It must:

have a fairly rapid rate of reproduction;

have a fairly fast rate of fermentation, this being dependant on the rate and extent of cell growth (Ryder et al. 1983);

show good attenuation of the wort,

and have the specific flocculation characteristics required for the particular beer being produced. These factors, together with the wort composition, all contribute to the flavour of the final product and the specifications for each factor will vary with the fermentation conditions used and the type of product required.

The yeast is required to ferment the wort, removing the necessary nutrients and secreting other products, and once it has fulfilled its metabolic role it must flocculate from the fermented medium, leaving a relatively yeast-free beer.

There are essentially two types of flocculation, that which occurs being strain specific. Yeast flocs may either reach a mass which is too great to remain in suspension and therefore settle to the bottom (lager yeasts) or they may rise to the surface of the medium due to adsorption of carbon dioxide bubbles (ale yeasts). Ale yeast was not used in this study.

The stage of the fermentation cycle at which flocculation occurs is particularily important. Premature flocculation may occur if the yeast is very flocculent resulting in "hung" or "stuck" fermentations in which the beer is incompletely attenuated. Such a beer would be sweeter and biologically less stable than a fully attenuated beer. While the accumulation of harsh off-flavours which is typical of yeast having had extended contact with the beer is prevented, it does not allow for the flavour modifications which would normally occur. Powdery yeasts have the reverse effect and do not completely separate from the medium. This can give rise to a variety of off flavours due largely to autolysis of the yeast cells.

It is therefore highly desirable to the brewing industry that the mechanisms controlling flocculation at all levels (biochemical, molecular and genetic) are fully understood.

If a potential role for fimbriae in flocculation is to be considered a review of the flocculation of brewer's yeast (as it is currently understood) is necessary.

1.1.5 CLASSIFICATION OF FLOCCULATION

A clasification scheme for the flocculation traits of ale and lager yeasts was devised by Stewart et al.(1975).

Five catagories of ale strains were identified which included; non-flocculent strains, co-flocculent strains, pure strains flocculent in both wort and defined medium, pure strains flocculent in defind medium only when an inducer is present and chain forming strains.

Only two catagories of lager yeasts were identified. The non-flocculent yeasts always exist as single cells and do not have the ability to form aggregates while flocculent strains all flocculate in wort and in defined medium, requiring no inducer. All flocculent strains tested flocculated as pure strains and showed less tendency to form chains than did ale strains.

These categories form only a basic classification system and considerable variation can occur within each category. For example two lager strains with totally different calcium binding capacities would both be placed in the second category eventhough one may be deflocculated by washing in distilled water, and the other may need to be washed with EDTA before it is deflocculated (Stewart and Russell,1981).

1.1.6 MEASUREMENT OF FLOCCULATION

The methods used to determine the degree of flocculation are numerous and varied, and the results obtained are generally of a qualitative rather than a quantitative nature. As such

they are highly subjective. There is very little standardization of these tests which makes it almost impossible to compare the results obtained from different laboratories. The flocculation intensity of a particular yeast strain is generally determined by monitoring the rate of sedimentation (either visually or spectrophotometrically), of a washed cell suspension in a standard medium of known pH and composition. Alternatively more accurate *in vivo* measurements of yeast concentration can be made during the course of a normal fermentation in the wort medium, although results are only obtained after a few days.

1.2 THE MECHANISM OF FLOCCULATION

1.2.1 GENETICS OF FLOCCULATION

Brewing strains are generally highly unsuitable for genetic studies since they are, most often, triploid or of greater ploidy. Many strains also sporulate poorly, have low ascospore viability and often produce sterile segregants.

Flocculence was shown by Thorne (1951), to be an inherited characteristic under the control of a multiple gene system. Although it was shown that flocculence was dominant over non-flocculence, the rate of mutation to non-flocculence was very high. The controlling genes were reported by Lewis

et.al. (1976), to comprise two dominant genes, FLO 1 and FLO 2 and one recessive gene, flo 3 of which only one needed to be present for the flocculent phenotype to be expressed, the same degree of flocculence being expressed by each gene and no additive effect resulting from the presence of more than one gene. These two dominant genes, together with another dominant gene, FLO 4, (Stewart and Russell, 1977), were later found to be allelic (Russell et al. 1980).

The recessive gene, flo 3, which conferred moderate flocculence was found in some cases to behave in a semi-dominant fashion (Lewis et al.1976). It is distinct from FLO 1 and, unlike FLO 1, is thought not to be centromere linked (Johnson and Reader, 1984). The authors consequently suggested that modifying factors, either chromosomal or cytoplasmic, may exist which could affect the intensity of flocculation. Apart from flo 3 two other recessive genes, flo 6 and flo 7, are also thought to exist (Johnson and Reader, 1984).

Another dominant flocculent gene, FLO 5, was documented by Reader, (1980). That it is not allelic with FLO 1 was shown with tetrad analysis of crosses of haploid strains carrying the FLO 1 and FLO 5 genes respectively.

The intensity of flocculation amongst yeast strains is variable, ranging from completely non-flocculent to

extremely flocculent. This phenotypic variation has been diagramatically represented in relation to the present knowledge of the genes involved (Johnson and Reader,1984).

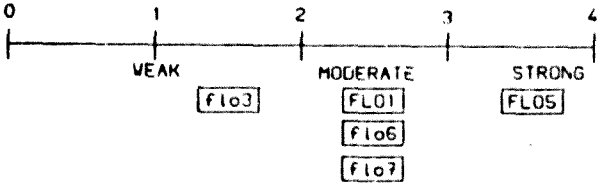


Fig.1. Genotype and associated phenotype of yeast flocculence.

While these genes may confer these phenotypes, it is nonetheless agreed that their control of a cell's flocculation characteristics may be significantly modified by a suppressor and/or modifier gene such as the fsu 1 gene which suppresses FLO 1 (Holmberg and Keilland-Brandt, 1978 ; Holmberg, 1978).

The mitochondrial genome can also be significant in determining the flocculation phenotype in some yeasts. Wilke *et al.*(1981), found that some sugar-utilizing revertants of non-flocculent petite mutants which were unable to utilize galactose and maltose were flocculent. A second class of mutants simultaneously lost their ability to

flocculate and became antibiotic resistant. Flocculence was regained and antibiotic resistance lost in the segregants of some of the crosses of these mutants together with the ability to utilize the sugars (Spencer et al. 1981, cited in Johnson and Reader, 1981). As well as being implicated in the loss of flocculence mitochondrial mutants have also been shown to enhance the flocculation intensity of a strain (Cowan et al. 1975).

1.2.2 THE YEAST CELL WALL

1.2.2.1 Structure and synthesis

The yeast cell wall is that part of the cell which is most intimately involved in flocculation. If the changes which occur in the wall at the time of flocculation are to be understood a review of its structure is necessary.

The molecular structure of the wall is extremely complex with the various components being organized in an intricate fashion in relation to one another. This high degree of organization appears to be fundamental to wall functioning. The wall comprises two layers, an outer mannan-phosphate-protein layer which is joined to an inner structural layer of glucan which lies outside the plasma membrane.

The wall comprises glucan (about 40%), mannan (about 40%),

protein (12%), chitin (1%), phosphate (1%), and lipid (1%). (Mac William 1970). The exact proportions of these components varies with the species and with the conditions under which the cells are cultivated. The major wall components are discussed in more detail.

Fig.2 shows the arrangement of the wall components in relation to one another.

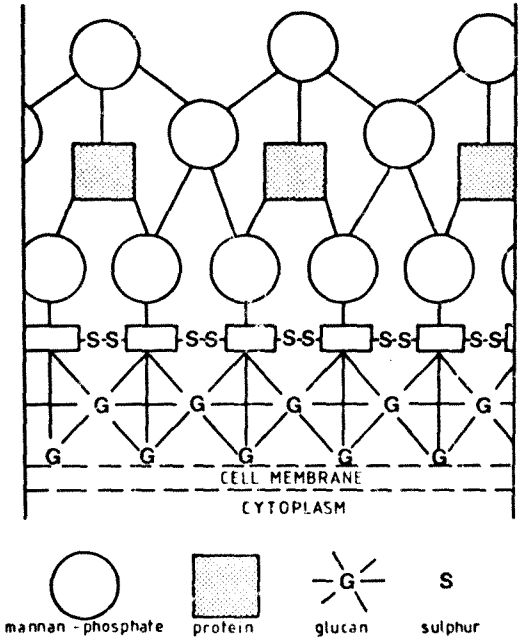


Fig.2. Schematic structure of the cell wall of Saccharomyces (from Stewart and Russell, 1981).

Mannan

Yeast mannan is a covalently linked glycoprotein complex of which the protein component comprises from five to fifty per cent of the complex by weight. The mannan structure differs even amongst different strains of S. cerevisiae.

Two forms of wall mannan exist:

1. Structural mannan which forms part of the structural component of the wall and is interspersed with glucan. This fraction contains five to ten percent protein.
2. Mannoprotein enzymes which are situated in the periplasmic space and partly in the structural layer comprise thirty to fifty percent protein and include invertase, acid phosphatase and x-glucosidase.

The unifying factor of the various mannosyl structures is the presence of a high content of D-mannose. Most mannose molecules comprise about 150 mannose units which are linked to a polypeptide chain via N-acetylglucosamine. The mannan of baker's and brewer's yeast usually comprises a long chain of x-linked units with short x-1,2 and x-1,3 linked side chains (Ballou,1974; 1976). B-mannan links have been observed in species of Hansenula, Pichia and Candida, but not in brewing yeast species (Mac William, 1970)).

It is not certain that the entire cell surface is covered with mannan, i.e. that there is no exposed glucan, since

glucan-specific antibodies could not be extracted from serum of rabbits inoculated with purified cell wall glucan fragments. Mannan is the immunodominant component of the cell wall (Ballou, 1974).

Biosynthesis of wall mannan: Mannoproteins are thought to be formed within the cell and the complexes transported through the plasmalemmma into the extracellular space. Modification of the chains, such as the addition of side chains, might occur during translocation. Blocking of the synthesis of either component prevents the secretion of the other component (Farkas et al. 1970).

Glucan

The glucan content of yeast cell walls, like the mannan content, varies between different strains of an organism and with growth conditions. It comprises two fractions, one alkali-soluble and the other alkali-insoluable.

The β,1-3 glucan, which constitutes about 85 percent of the alkali insoluble glucan is considered to form the insoluble cell envelope which gives the cell its rigidity. It is present as a continuous network of interwoven microfibrils (Kopecka et al.1974). The role of the β,1-6 glucan which makes up the other 15 percent is uncertain, but it may ensure some flexibility of the cell wall for expansion by preventing too much aggregation of the rigid, linear β,1-3

glucans.

The alkali soluble glucan constitutes about 20 percent of the dry weight of the cell wall. It is an amorphous structure with a core of β,1-3 linked units and either β,1-3 or β,1-6 linked side chains. Some mannan is present in this fraction and is secured by 1-6 linked glucose residues.

Biosynthesis of glucan

Glucan synthesis is thought to occur by enzymic breakage of glucan chains followed by extension of the chains by glucan synthetase using UDP-glucose as a precursor (Johnson, 1968)

Protein

The amount and composition of yeast cell wall protein is as variable as are the mannan and glucan contents. Values for the amount of cell wall protein are obtained by multiplying the total nitrogen by 6,25 which would cause discrepencies if the amount of non-protein nitrogen is variable. Amino acid composition is highly variable although brewer's yeast is generally high in the hydroxy amino acids, serine and threonine.

Phosphate

Phosphate is generally bound in the mannoprotein and gives the cell some of its negative charge. Phosphodiester bonds link some of the mannan side chains onto the core (Rose,

1978).

Lipid

There are significant differences in the values obtained for the amount of lipid present since some would be lost during washing and some membrane lipid would be removed during extraction. The amount of lipid in brewing yeasts is generally thought to be very low.

1.2.2.2 The cell wall and flocculation

Since the yeast cell wall is the structure most intimately involved in flocculation it is therefore also the region in which changes specifically related to floccualtion are most likely to be found. Proof that flocculence does become an intrinsic characteristic of the wall was provided by Nishihara *et al.*(1982) and Amri *et al.*(1982), who showed that the flocculation properties of the cell wall were almost identical to those of the intact cells.

A review of the available literature concerning the changes occurring in the wall during the cell cycle and differences between the walls of flocculent and non-flocculent cultures clearly illustrates that there are a number of inherent difficulties in attempting to compare wall extracts. The variability of the wall structure from strain to strain and of the same strain under different cultural conditions is

one problem. Another is the use of different techniques in different laboratories which often makes comparison of the results impossible. Where log and stationary phase cultures of the same yeast are compared it is again difficult to distinguish between changes which normally occur as the cells enter the stationary phase and those which are specific to flocculation.

Differences in cell wall composition of flocculent and non-flocculent cells

Results from determinations of the variation in carbohydrate content of log and stationary phase cells, and of flocculent and non-flocculent cultures are divergent. Three independant determinations of glucan and mannan in related flocculent (FLO 1) and non-flocculent (flo 1) strains illustrated that there was no significant variation in the amounts of these two cell wall carbohydrates (Johnson and Reader, 1984). Conversely, Stewart and Garrison (1972) recorded a much higher carbohydrate content (attributed to an increase in mannan) in the walls of co-flocculent and pure strain flocculent strains in comparison with non-flocculent strains. Mill (1966), found that the carbohydrate content of flocculent and non-flocculent cells of the same strain was similar, the only difference being an elevated level of phosphorylation of mannan at position six

in the former.

Higher levels of phosphorous in the phosphomannan layer have been noted in some laboratories (Lyons and Hough, 1970), but not in others (Stewart and Russell, 1981).

Marfey et al.(1977) compared enzyme digests of the walls of whole cells rather than using isolated walls, in order to minimize inaccuracies arising from cell wall disruption. His finding of more carbohydrate and less protein in the walls of flocculent cells agreed with previous evidence. The protein content was found to differ quantitatively as well as qualitatively, the amount of proline, lysine and arginine being considerably higher in flocculent cells. His cell wall digest also contained yeast and barley antigens in the high molecular weight fraction (40 000 -200 000 daltons), extracts from non-flocculent cells having three to ten times more barley antigens than those from flocculent cells.

Amri et al.(1982), observed that the transition from log phase to stationary phase was accompanied by changes in the carbohydrate, protein and ionic contents of the walls. The molar ratio of mannose increased while the percentage of protein decreased and the phosphorous content remained unchanged. The amino acid composition of the protein showed, in contradiction to Marfey et al.(1977), a strong

decline in the amount of proline, while the amounts of lysine and aspartic acid increased. Walls of flocculent cells had a lower calcium to potassium ion ratio than non-flocculent cells, which could alter intercellular ionic changes, and promote flocculation.

The density of carboxyl groups of proteins on the surfaces of mid log cells was consistently found to be lower than that of the stationary phase cells of the same flocculent culture (Beavan et al. 1979). The insertion of a new acidic wall protein at the onset of the stationary phase was suggested as a possibility for promoting floc formation. A number of workers have looked for different wall proteins in flocculent and non-flocculent cells, but again results have been inconsistent.

Probably the most striking difference between non-flocculent and flocculent cell walls is the presence of a dense mat of fimbriae on the surfaces of flocculent cells, but not of non-flocculent cells (Day et al. 1975). It is the involvement of these fimbriae in flocculation which is investigated in this study.

The role of surface components in flocculation

That it is the mannan-protein component which confers flocculence has been demonstrated by treatment of the cells with proteolytic enzymes (Lyons and Hough, 1970; 1971).

They removed all the mannan and much of the wall protein and phosphorous, resulting in previously flocculent cells becomming non-flocculent. Further evidence comes from the inhibition of mannan accumulation by cyclohexamide which also prevents normally flocculent cells from flocculating (Baker and Kirsop, 1972). As well as this the most powerful dispersing sugar (Eddy, 1955a), and that which has the greatest effect on the floc dissociation temperature (Taylor and Orton, 1978), is mannose, suggesting that wall mannan is specifically involved in flocculation. Masschlein (1963), reported that the intensity of flocculation was regulated by the synthesis of mannan, the period of deflocculation coinciding with the period of mannan synthesis. He suggested that the mannose acts by masking the active groups of the factor possessing the flocculence character and that it is the ratio of mannan to protein which determines the flocculent state of the cell, his experiments having shown a total amino acid increase of 30-40%.

The possible role of phosphorous in flocculation has not been completely resolved (See Chapter 1.2 e.).
That flocculence can be destroyed after treatment with protein modifying agents and proteolytic enzymes (Nishihara et al. 1977) or inhibitors of protein synthesis (Baker and Kirsop, 1972) is indicative of the importance of undamaged surface protein.

1.2.3 INDUCTION OF FLOCCULATION

Induction of flocculation is considered here to incorporate the processes involved in changing non-flocculent log phase cells into potentially flocculent stationary phase cells. The induction of flocculation potential was considered by Mill (1964a) to involve changes in the concentration of a nitrogen-containing cell wall component. Because the onset of flocculence could be delayed by the addition of certain nitrogenous compounds to the growth medium, he suggested that the stimulus responsible for the change from non-flocculence to flocculence was associated with the cell's nitrogen metabolism. The presence of a nitrogenous compound in the cell wall was proposed which maintained the cell in a non-flocculent state. If its synthesis was retarded by a reduction in the medium of the compound itself or of the materials necessary for its synthesis, its concentration in the wall would fall and the potential to flocculate would increase. The change from non-flocculence to potential flocculence would then occur whenever conditions in the growth medium favoured slower synthesis of this compound relative to the other cell wall components.

The general requirements for the induction of flocculation in brewery yeast were found to include both glucose and magnesium ions (Nishihara et al. 1976). That glucose

catabolism and energy production are necessary for induction was shown by the addition of glycolytic inhibitors and respiratory inhibitors and uncouplers, all of which prevented induction. Induction of flocculation also necessitates both cytoplasmic protein synthesis (Nishihara et al. 1976) and mitochondrial protein synthesis (Calleja et al. (1976).

1.2.4. VARIABILITY OF FLOCCULATION

1.2.4.1 Variability

Flocculation is a characteristic which varies from strain to strain and also within a strain under different growth conditions. For example, although many flocculent yeast strains are able to flocculate in both wort and in defined medium, some strains, flocculent in wort, are non-flocculent when grown in defined medium. As well as this some strains are potentially flocculent throughout their growth cycle i.e. they will form flocs in a calcium salts buffer at any stage, while others are powdery in the log phase regardless of the medium and only acquire the potential to flocculate in the stationary phase.

1.2.4.2 Instability

Flocculation is also a fairly unstable characteristic , spontaneous changes being fairly common. Powdery yeast may become more flocculent or flocculent yeast may become less

flocculent. The rate of mutation from flocculent to powdery is fairly high (Thorne, 1968) which could account for a fair amount of the instability since replication by budding does not allow for much genetic variation. The high rate of spontaneous mutation is one of the major arguments against the use of continuous culture in brewing. For example, Thorne (1968) observed that after nine months of continuous culture half the cells of a lager strain of S. uvarum had mutated.

1.2.4.3 Co-flocculation

Co-flocculation occurs when two strains which are non-flocculent alone become very flocculent when added together or fermented together, the resultant effect being the same as pure strain flocculation. Co-flocculation has not been demonstrated in lager strains, and as ale strains were not used in this study it shall be discussed only briefly. A mechanism for co-flocculation was proposed which is slightly different from that proposed for pure strain flocculation. Calcium salt bridges would be formed between the cell walls of one strain and active sites within the walls of the co-flocculent strain, and one of the groups involved would be a carboxyl group. The inducer substance which is necessary for co-flocculation would be incorporated into the wall, (as opposed to being metabolized) the steric structure of the phosphomannan-protein layer being crucial (Stewart and Garrison, 1972).

1.2.5 BOND FORMATION

The generally accepted mechanism by which flocculation is thought to occur is as follows: Divalent calcium ions form salt bridges between adjacent cells by binding to anionic groups of the cell surface components.

Two schools of thought exist regarding the type of anionic components involved in intercellular binding. One group of workers believes that the major anionic groups are the carboxyl groups of acidic peptides in the cell wall and the other group believes that bonds are formed with the phosphate groups of the phosphomannan layer.

The mechanism of binding was initially considered by Mill (1964b) who confirmed the specific requirement of flocculent cells for calcium. The salt bond formed between a calcium ion and receptor sites on adjacent cells would however be mainly ionic, and would dissociate at pH 4.6, which is within the pH range where flocculation is most intense. Another type of bond would therefore be required and as flocs have a "melting temperature" of 50-60 degrees centigrade and are dispersed by urea, these secondary bonds are probably hydrogen. These bonds would form between hydroxyl groups of the surface polysaccharides, the final complex holding the calcium complexes in a specific spacial

configuration relative to one another. The anionic groups involved were thought to be carboxyl groups.

Lyons and Hough (1970; 1971) proposed that the phospodiester linkages of the phosphomannan-protein were the main, if not the only, anionic groups associated with salt bridge formation. They initially found that the major difference between flocculent and non-flocculent strains was the elevated level of phosphorous in the former and later provided additional evidence for their theory from calculations of the ratios of bound calcium phosphate present on the cell surfaces, this being higher in flocculent than in non-flocculent strains. Enough phosphorous would be present on the surface of flocculent yeast to allow one calcium molecule to bind to two phosphorous molecules on adjacent cells, while non-flocculent strains would have insufficient surface phosphorous to enable stable salt bridges to form. Using the same strains, however, Jayatissa and Rose (1976) provided evidence for the "carboxyl group" theory rather than for the "phosphate group" theory. They cleaved the phosphodiester linkages, removing 80-90% of the phosphate, only small amounts of mannan, glucan and protein from the walls and showed that the rate of flocculation did not decrease. Flocculation did however decrease after esterification of the cells which suggested that the surface carboxyl groups are probably the ionic groups involved.

Beavan et al.(1979), provided additional evidence for the involvement of carboxyl groups in floc formation and also proposed a mechanism which could account for changes in the density of cell wall anionic groups of flocculent strains. pH electrophoretic mobility changes due to carboxyl and phosphate groups were measured in flocculent and non-flocculent S. cerevisiae strains. Electrophoretic mobility due to protein carboxyl groups of flocculent strains was greater in the stationary than in the mid-log phase, but no such changes occured in non-flocculent strains. From their results they concluded that a threshold value for carboxyl group density may exist, which, when exceeded could allow flocculation to occur.

Stabilization of these bonds may be mediated by a decrease in density of phosphodiester linkages, the degree of stabilization varying with the strain. The process could be effected by cell wall lytic enzymes.

The apparent contradiction between the evidence provided by the supporters of the carboxyl theory and those of phosphate group theory may have been resolved by Stewart et al.(1975). They suggested that the difference between cells which are deflocculated by water washing alone and those which require EDTA treatment before they are deflocculated may lie in the types of calcium-anion complexes formed. Those dispersed by

water treatment may have only calcium carboxyl complexes while the more resistant strains may have both calcium-carboxyl complexes and calcium-phospate complexes. Although most evidence advocates the calcium-carboxyl group theory the issue has not been conclusively resolved and this latter proposal may be the answer to the controversy.

1.2.6 YEAST METABOLISM

As brewer's yeast is dispersed during active growth and then flocculates during the stationary phase the transition from the dispersed to the flocculent state must involve metabolic factors. Studies on the metabolic changes and controls involved in the cells' achieving the flocculent state have been approached from two directions; the effect of the wort composition on this transition, and the changes in the metabolism of the yeast itself.

1.2.6.1 The effect of wort composition on flocculence

Promotors and inhibitors

The nitrogenous components, the carbohydrate and the ionic contents of the medium are all important in determining the time and the intensity of flocculation.

A number of laboratories have reported finding specific substances in the wort which induce or inhibit flocculation.

Although all lager strains examined were able to flocculate in both wort and in a defined medium containing glucose, ammonium salts, vitamins and ions, some flocculent ale strains required the presence of a nitrogen-containing inducer to enable them to flocculate in the defined medium (Stewart, Russell and Garrison, 1975). This suggested that a factor present in the wort, but not in the defined medium was responsible for flocculation. From the wort a peptide with a high content of acidic amino acid residues was isolated which could induce wort-flocculent strains to flocculate in defined medium. This material lost its ability to induce flocculation after hydrolysis to free amino acids. That the requirement for this peptide is very specific is unlikely since supplementation of the defined medium with either peptone or gelatin produced the same effect. The authors therefore suggested that strains which require the presence of an inducer are possibly less biosynthetically competent in their ability to produce the structures or sites necessary for flocculation than are those strains which flocculate without the inducer.

This inducer peptide possesses a number of chemical properties similar to those of the sex pheromone involved in the mating of S. cerevisiae. Sexual agglutination of the fission yeast, Schizosaccharomyces pombe which is also peptide induced, is always preceeded by flocculation (Calleja and Johnson, 1971).

A nitrogen-containing substance capable of inducing premature flocculation was purified in the Kirin Laboratory, (Morimoto et al. 1975; Fuji and Horie, 1975). This substance could also be inactivated by protease treatment and comprised α-glucan, β-glucan, araboxylan and glycoprotein, the araboxylan and protein fractions being responsible for early flocculation.

Unfermented wort has a strong dispersing effect on flocculating cells, promoting active multiplication during which they become progressively less able to flocculate in calcium salts medium (pH 4,5). The onset of flocculation was found to be controlled largely by the extent of exponential growth which in turn was determined by the amount of assimilable nitrogen (Baker and Kirsop, 1972; Mill, 1964). The addition of casein hydrolysate to fermenting wort was therefore able to delay the onset of flocculation which then occured at a greater cell density and lower specific gravity than normal. (Baker and Kirsop, 1972). Amri et al.(1979), investigated the effect of nutrients on flocculation and also concluded that the effect of amino acids in delaying flocculation was non-specific and due to their extending the period of exponential growth.

The presence of large amounts of sugar in wort also prevents flocculation and, while the effect of mannose may be to specifically prevent binding of surface groups, the effects

of other sugars is again due to the promotion of cell growth and delay of the stationary phase.

The role of metal ions and pH in flocculation

The involvement of metal ions, pH and surface charge in flocculation are closely linked to the mechanisms proposed for bond formation between cells.

That flocculating cells display a specific requirement for calcium ions is well documented. The effect of both calcium ion concentration and of ionic strength have been examined (Taylor and Orton, 1975). At the optimum ionic strength (0,05-0,005) calcium was required in only micro amounts, the transition to non-flocculence being 10^{-8}M. This minute requirement for calcium was very much less than that previously recorded. For example Mill (1964b) found that 0.2M of calcium chloride was required. It is not unlikely that reduction of surface charge, which would promote floc formation, may account for some of the calcium effects noted (Taylor and Orton, 1975).

The specificity for calcium ions was found to be lost once the cells were removed from their medium and placed in distilled water or in buffer, ie. other ions could substitute for calcium (Eddy, 1955; Nishihara et al. 1982).

In many strains other divalent ions are able to function in

intercellular bridging, although larger ions such as strontium, uranium and barium inhibit flocculation (Taylor and Orton, 1975). They may act as competitive inhibitors by competing with calcium ions for specific anionic binding sites on the cell surface, or their larger radii may prevent stabilization of the floccs by hydrogen bonds. At high concentrations monovalent ions inhibit flocc formation, their single valence preventing bridge formation. However, low concentrations of these ions are stimulating for strains which show intense flocculation in the presence of calcium. They possibly neutralize the repellant negative charges of the cells without blocking all available binding sites.

Some flocculent strains were found to bind much greater amounts of calcium than non-flocculent strains (Masschelein, 1957). Conversely, other flocculent strains have been found to adsorb an amount of calcium similar to that of some non-flocculent strains tested (Stewart et al. 1975). Here, the difference between the two strains was that the calcium was much more tightly bound to the flocculent than to the non-flocculent strains. The effect of calcium on flocculation was therefore thought to be the stereospecific manner in which it was attached to the cell rather than the actual amount which was bound. Hence, differences in the binding of calcium are probably a reflection of the differences in the surface structure of flocculent and non-flocculent cells. That calcium binding is totally dependant on cell wall

architecture was verified by the inability of mannoprotein wall extracts to bind calcium and by the poor binding capacity of protease treated cells (Stewart and Garrison, 1972).

The pH of the environment is critical since it can strongly affect the cell's response to the available calcium. This effect was studied by Porter and Macauly (1965), using top fermenting yeasts in both growth medium and in buffers. On the basis of their response to calcium and pH the strains were divided into three groups:
Group A which comprised very flocculent strains whose flocculence was more dependant on the medium than on its calcium content, only traces of calcium being required;
Group B strains which were moderately flocculent and responded only slightly to either calcium or to optimization of the pH; and
Group C strains which were potentially flocculent at higher pH levels (about pH 4,3), but which required large amounts of calcium.

Mill (1964b), also recorded more intense flocculence at higher pH values (pH4,5 - 5,5) than at lower values (around pH 2).

1.2.6.2 Yeast factors

The flocculation specific changes in yeast metabolism prior to flocculation are less well defined than are the wort parameters. Numerous metabolic changes occur when the cells enter the stationary phase and it is almost impossible to determine which changes are specific to flocculation and which are the natural consequence of other stationary phase functions. Three yeast related factors have received attention, namely glycogen metabolism, protein turnover and enzyme changes.

Glycogen Metabolism

Glycogen is a glucose polymer of α-1,4 linked chains of 10-15 glucose units joined by α-1,6 glycosidic bonds. It is the major reserve carbohydrate of brewer's yeast.

Patel and Ingeldew (1975a,b) found that a relationship exists between flocculence and glycogen levels in yeast. Increases in the acid-soluble glycogen were accompanied by increased flocculence. Studies with a strain of Saccharomyces carlsbergensis showed that when the yeast became potentially flocculent towards the end of primary fermentation, the poor availability of utilizable nitrogen promoted the synthesis of large amounts of carbohydrate. Conversely, supplementation of the wort with inorganic nitrogen caused a drop in the glycogen levels and prevented

floccculation. Although the precise involvement of glycogen in flocculence is not known they suggested that an elevated amount of glycogen would increase the amount of bound water in the cells, thus lowering the amount of free water present and promoting flocculation. Increases in bound water had previously been implicated in the promotion of flocculation (Kalynzhnyi et al. 1965, cited in Patel and Ingeldew, 1975).

Protein turnover

Stationary phase yeast cells have high rates of protein turnover (Halvorson, 1958, cited in Matile et al.1971) and since protein turnover is intimately involved in biochemical differentiation it is very likely that it is also associated with the acquisition of flocculence by stationary phase cells.

Evidence for the role of protein metabolism in flocculation is provided by the inability of cells whose protein synthesizing machinery has been blocked, to flocculate (Nishihara, et al. 1956). Differences in the protein contents (both qualitative and quantitative) of the cell walls of flocculent and non-flocculent cells also indicate that protein metabolism is involved (Marfey et al. 1977; Amri et al. 1982). For example it is possible, as proposed by Beavan et al.(1979), that only when a new acidic protein has been inserted into the cell wall that flocc-forming ability is acquired.

Enzymes

That changes occur in wall composition of log and stationary phase flocculent c. . is almost conclusive evidence for the involvement of yeast enzymes. Also, changes in the synthesis and activity of a number of enzymes have been noted as cells become flocculent, although to distinguish those enzymes which are specifically involved in flocculation from those associated with normal stationary phase functions is a formidable task.

A number of enzymes are associated with the cell walls. They are glycoproteins and include invertase, acid phosphatase, catalase and proteases. Beavan et al. (1979) tested cell extracts for changes in the activities of enzymes which could be associated with the aquisition of flocculence. The most significant differences occurred with leucine aminopeptidase and α-mannosidase whose activities both increased during the transition from log to stationary phase. Greater increase of activity occurred in flocculent than in non-flocculent cultures. Amidase activity also increased although activity was generally low throughout the growth cycle. A potential role for this enzyme could, however, not be ignored as it could generate the additional carboxyl groups produced when the cultures become flocculent. Proteinase C activity, although greater in

stationary phase than in log phase cultures did not correlate with flocculence. Increases in extracellular "mannonase" of stationary phase flocculent cultures were also detected by Lyons and Hough (1970). Mannosidase could promote flocculation by removing some of the surface anionic groups, thereby reducing the repulsive forces between cells.

When invertase and acid phosphatase were lost from the wall by cold osmotic shock, the period of maximum release of enzymes could be correlated with a loss of abiliby to flocculate (Williams and Weisman, 1973). It is therefore possible that either one or both of these enzymes is involved in flocculation, the involvement probably being structural rather than enzymic.

A role for acid phosphatase in yeast morphogenesis was postulated by Schweingruber et al.(1982). Using mutants of Schizosaccharomyces pombe, two forms of this enzyme were isolated one being active and soluble and the other inactive, structurally more complex and mainly membrane bound. The mutant which lacked the inactive form of the enzyme and was more "clumpy" was reportedly more hairy than the wild type (Walther et al.1984.

While the function of the inactive form was uncertain it was suggested that acid phosphatase functions in morphogenesis, cell clumping or regulation of growth rate, possibly in a

structural capacity.

In the light of some of the work which has been done on the yeast autolytic system the possiblity of this system being even partially associated with the cell wall changes prior to flocculation cannot be ruled out.

Yeast autolysis involves the autofermentation of stored glycogen to glucose following which irreversible cytoplasmic changes occur including granulation and proteolysis (Arnold, 1972).

Autolysis of baker's S.cerevisiae was measured using the wall enzyme, invertase. Organic solvents were found to trigger the autolytic system and as the cell wall retained its structure during autolysis, Arnold (1972) proposed that autolysis was not a random process, but involved the cleavage of specific wall polymers. Greater release of invertase was achieved after treatment with a sulfhydryl compound plus the solvent, suggesting that a polypeptide with disulfide bonds must have been cleaved to release the marker.

The enzymes responsible for autolysis were resolved into three fractions. Fractions I and II had endoglucanase activity and released 66-77% of the marker. For greater marker release fraction III was required. An active

aminopeptidase was detected in this extract and it was speculated that this may have been the component responsible for the additional release of marker enzyme.

Potentially related were the observations that cells characterized by high rates of protein turnover (such as differentiating cells) showed high activities of vacuolar aminopeptidases. Conversely, the activity in cells well adapted to their environment was very low (Matile et al. 1971). One of the aminopeptidases inceased its activity fourfold in the stationary phase. This was correlated with biochemical differentiation of the cells at this stage. Its synthesis was repressed by the presence of glutamic acid or casamino acids and induced by their absence. This effect was similar to that of nitrogenous substances on flocculation (Baker and Kirsop, 1972; Mill,1964) and is also in agreement with the observation of Beavan et al.(1979), who found an increase in the activity of leucine aminopeptidase activity when flocculent cells entered the stationary phase.

Yeasts cells produce a number of β,1-3 glucanases which are thought to function in cellular diffentiation through controlled cleavage of cell bonds. Of the six different β,1-3 glucanases only glucanase III A was dominant in the stationary phase, its activity in the cell wall increasing at this stage. A functional role for this glucanase in the

glucanase in the stationary phase is therefore likely (Rein and Fleet, 1983a,b).

1.2.7 BIOLOGICAL RECOGNITION SYSTEM

In some laboratories flocculation is now regarded as a specific biological recognition phenomenon involving the formation of protein-carbohydrate binding pairs between cells, rather than merely the formation physico-chemical bonds between adjacent cells.

The possiblity that specific complementary binding may operate in flocculation was initially suggested by Taylor and Orton (1978). They postulated that all cells possess both protein receptor sites and mannan sites which bind to one another on adjacent cells. The basis for this concept came from the effects of aromatic components, sugars and calcium on flocculation as measured by changes in the flocculation intensity and the flocc dissociation temperature. Evidence included; an absolute specificty for calcium which is not normally seen even in biological systems; the loss of flocculence when the surface protein is damaged and the specific inhibition of flocculation by mannose and α-D-mannoside over other sugars.

Amri et al.(1982) proposed that a lectin-like system might operate. They noted a number of changes in cell walls

during the transition to flocculence. A higher percentage of mannan was evident in flocculent cells and the proportions of some of the amino acids were consistantly modified in a particular pattern. This, they suggested, is proof for the existence of specific structures on the walls of flocculent cells, the structures being lectin-like in nature and promoting cell-cell binding. Features of flocculation which are similar to those of lectin binding include the protein or glycoprotein nature of lectins in which the carbohydrate component of the latter would be mannose and the fact that calcium ions and carboxyl groups are generally also associated with lectin binding.

Miki et al.(1981;1982 a,b) also considered flocculation to result from the interaction between adjacent cells of two different surface components (protein and carbohydrates) both of which are present on all cells. Electron microscopic examinations of sections of flocs showed interactions between the cell wall mannan layers of the cells. The sites of cell to cell contact were extensive, the whole cell surface apparantly being capable of binding interactions. Mannose (50mM) competitively inhibited flocculation while galatose and fructose did not, which provides evidence for the direct participation of mannose residues in cellular interactions. Miki and co-workers proposed that the x-branched mannans may act as ligands, binding specifically to lectin-like receptors which are

active at all stages of growth and which are present only on flocculent cells. In order to function the proteinaceous receptor requires the integrity of the outer wall protein and the presence of calcium. Strain specificity of flocculation would be determined by the proteinaceous receptor rather than the mannan ligand, since non-flocculent cells competed for binding sites on flocculent cells even after destruction of their surface proteins. The differences in the interactions amongst flocculent cells and between flocculent and non-flocculent cells were explained by their model for flocculation. The primary controlling gene for flocculation, FLO 1, would govern the expression of the lectin-like component either directly, if this is the gene product of FLO 1, or via a modifying protein product of FLO 1 if it is not. Binding between a flocculent and a non-flocculent cell would involve unilateral interactions, and binding between two flocculent cells would involve bilateral interactions. Floc formation would result from the interaction of each cell with a number of other ce).

They were unable to isolate the proteinaceous receptor which they proposed since the protein digests of both flocculent and non-flocculent diggests were indentical.

Such is the present state of our knowledge of flocculation. Each of the factors discussed appears to have a significant

role in the process, but the manner in which they interact to produce the final effect has yet to be illucidated.

In this study the potential role of fimbriae in the process of flocculation was investigated, and while further study would be necessary to provide irrefutable evidence for their involvement, the results presented do support the proposal that they function in flocculation.

CHAPTER 2

FIMBRIAE AND FLOCCULATION

2.1 INTRODUCTION

The finding of fimbriae on the surfaces of fungal cells is fairly recent and there is therefore a paucity of information available regarding their morphology, chemical composition and functions. Poone and Day (1975) reported the presence of fine hair-like structures on the anther smut fungus Ustilago violaceae. The classification of these structures as fimbriae was based on similarities between the morphology and development of these and of bacterial fimbriae, although this classification is as yet still speculative. The fimbriae, which vary in length from 0,5-10um with a diameter of 60-70 Å were found on log phase cells. They are flexible and some have terminal knobs like those of bacterial sex fimbriae. They can be removed by mechanical means and then regenerated, but require de novo cytoplasmic protein synthesis as opposed to bacterial fimbriae which are regenerated from previously synthesized subunits (Ottow,1975).

The point from which the U. violaceae fimbriae arise is

uncertain. Freeze etched preparations showed, on different cells, hair-like structures on the internal and external surfaces of the plasmalemma and also traversing the wall. Whether or not these structures were part of the fimbriae, wall fibrils or experimental artefacts was not confirmed, although it is likely that they arise internally. They were predominantly proteinaceous with a small amount of carbohydrate (Poone and Day, 1975).

The possible involvment of these wall fibrils in the conjugation of U.violaceae cells of opposite mating type was investigated (Day and Poone, 1975). Both cell types were densely fimbriate and results indicated a correlation between fimbriation and the formation of the conjugation tube. The inability to produce fimbriae was clearly associated with the inability to complete conjugation.

Fimbriae were also found on the surfaces of flocculent cells of the ascomycetous yeasts, S. cerevisiae and S. uvarum (Day et al. 1975). They differed from those of the smut fungus in a number of ways. They were short, about 0,5um, fringelike, appeared in the stationary rather than the log phase of growth and seemed to promote the flocculation of the culture, which the fimbriae of U. violaceae did not. In contrast to the smut projections, they were also relatively obscure. Despite the differences these structures were also termed fimbriae on the precedent set by the bacterial

structures regarding the use of this term. For example E.coli fimbriae vary from 0,5um of the type VII to 20um of the type F, both types being classed as fimbriae (Day et al.1975).

Their involvement in flocculation was proposed from the electron microscopic evidence that both non-flocculent strains and flocculent strains in the log or non-flocculent stage of growth were only sparsely fimbriate, while stationary phase flocculent cells were densely fimbriate. As well as this cells flocculent in wort, but not in defined medium were fimbriate after growth wort, but not after growth in defined medium. The fimbriae were thought to comprise mannan-protein.

Structures similar to those seen on flocculent cells of S.cerevisiae by Day et al. were also found on cells of an S. cerevisiae strain which had been dehydrated and subsequently rehydrated (Rapoport et al. 1983). These structures were not present unless the cells had been dehydrated. They did however differ from those of flocculent cells in some respects; firstly they were quite sparse; secondly they were longer than those of flocculent cells cells and thirdly they appeared to be branched. Whereas the branching of fimbriae of flocculent cells was attributed to possible bunch formation and intertwining during drying of the cells and fimbriae, on the electron micrographs published by Rapoport

et al. branching of the individual fibres was evident. Although the composition of the fimbriae was not investigated they were presumed to be mannan-protein. These dehydrated and rehydrated cells often aggregated readily and were not easily separated, which may have been associated with the presence of fibrils.

Electron microscopic examination of the yeasts contaminating the piping used to transfer beer from the cellar to the pub also showed fimbriae on the cells. Persistant contamination is most often caused by organisms which have adapted tc become successful colonizers of the dispense systems and consequently the interaction between the cells and the plastics of the pipes was investigated (Casson, 1984). Prior to cell attachment an organic conditioning film of long chain polysaccharides from the beer or the yeast cell walls was adsorbed onto the pipes. The cells then attached due to electrostatic forces and this attachment was secured via the production of fimbriae-like structures. These structures also functioned in intercellular binding. Electron micrographs published showed these structures to be only about 0,1um long.

Since enough confusion already exists regarding the terminology of microbial filamentous appendages, and for want of a more suitable name, the hair-like structures of brewer's yeast investigated here shall be referred to as

"fimbriae".

2.2 METHODS

2.2.1 ORGANISMS USED

Saccharomyces uvarum 2036: This flocculent yeast was obtained from the South African Breweries.

Saccharomyces cerevisiae - This top fermenting strain (ale yeast) was also obtained from the S.A. Breweries.

Saccharomyces cerevisiae Y746 was obtained from Dr.N.Ferreria of the Council for Scientific and Industrial Research, Pretoria.

Saccharomyces cerevisiae Y537 was also obtained from Dr.Ferreria.

Saccharomyces cerevisiae NCYC 1324 was obtained from the British National Council of Yeast Cultures from Dr.B. Kirsop.

The following strains came from the culture collection of the Microbiology Department of the University of the Witwatersrand: Candida shehaete, Saccharomyces cerevisiae (used for wine production) Saccharomycodes ludwigii, Schizosaccharomyces pombe.

2.2.2 MAINTENANCE OF YEAST CULTURES

Cultures were initially maintained at 4 degrees centigrade on malt agar slants, pH3,5 and sub-cultured every 2 to 3 months.

They were later transferred to and maintained on Wickerham's Stock Culture Agar (WSCA), (Appendix A) on which the control culture was more stable with regard to its flocculation properties.

2.2.3 PROPAGATION OF THE YEAST

Yeast was propagated in the medium in which it was to be fermented, which was usually wort (obtained from South African Breweries).

A loopful of inoculum was added to an Erlenmyer flask containing 50ml of sterile medium and incubated, shaking at 22 - 25 degrees centigrade overnight. A further 100ml of medium was then added to the flask which was again incubated for about 36 hours after which the yeast was harvested either by centrifugation or by allowing it to pack naturally in the cold, and then decanting the supernatant fluid.

Log phase cultures

For some experimental procedures it was considered necessary to use log phase cultures from which the flocculated inoculum had been diluted out. Continuous cultures were therefore used. 50ml of sterile wort in an Erlenmyer flask was inoculated with 0,1ml of a flocculated yeast suspension and incubated, shaking at 22 degrees centigrade overnight. Cells were spun down, fresh medium added and the culture incubated for a further eight to nine hours. The medium was replaced

with 120ml of fresh medium, incubated overnight and the cells harvested for use the following morning. Continuous log phase cultures were used for [illegible] labelling of cells, for cell wall extracts for protein and carbohydrate determinations and for examining the effect of ether washing on the performance of non-flocculent cells in an inorganic salts medium.

2.2.4 FERMENTATIONS

Fermentations were carried out in the brewery-simulated micro-fermentation system shown in Fig 3. The fermentation tubes were glass, conical-bottomed and could hold a maximum of 275ml of liquid. All fermentations were incubated stationary for 9 days at 11 degrees centigrade.
Figures 3 and 4 show the microfermentation system used.



Fig. 3. Actively fermenting yeast 48 hours after pitching.



Fig.4. Fermentations after 120 hours. Flocculated yeast is packed in the cones of the tubes. Stationary phase samples were taken from the fermentations at this stage.

2.2.4.1 **Pitching Procedure**

The yeast was pitched at a rate of 18-22 x 10^6 cells per ml. The amount of yeast present after two days of propagation was calculated from haemocytometer counts.

To 200ml of wort in a sterile one litre flask enough inoculum was added to give an estimated yeast count of 20 x 10^6 cells per ml. Haemocytometer counts were used to check this and more yeast was added if necessary. The flask was then sealed with a rubber bung and inverted 30 times to aerate. The contents of the flask were then added to the sterile fermentation vessels. This procedure was repeated for each fermentation and all fermentations were set up in duplicate.

2.2.4.2 Monitoring the Fermentations

Yeast counts were done twice daily until the cells had flocculated and thereafter were done once a day until the ninth day. Sampling was performed by inserting a sterile pipette halfway down the fermentation tube and removing 0,5ml of the fermentation. The cells were diluted one in four or one in ten towards the peak of cell counts with 10mM EDTA and shaken for 10 minutes to deflocculate them. Counts were performed using a haemocytometer.

2.2.4.3 Samples for Electron Microscopic Examination

Samples of yeast for electron microscopic examination were taken during the log phase (42-48 hours) and the stationary phase (about 120-140 hours) of the yeast growth cycle. Samples were taken from the bottom of the tubes by opening the stopcocks and collecting about 10ml of sample from each tube in small flasks. Any residual sedimented yeast was

flushed out before the log phase samples were taken and a small amount of sediment was decanted before collecting the settled stationary phase samples of the flocculent yeast.

2.2.5 **FLOCCULATION TESTS**

Flocculation potential of the cultures was determined using two methods:

Method 1

A modification of the Helm sedimentation test (Stewart,1975) routinely used by the S.A. Breweries as a measure of yeast flocculence was used for preliminary comparison of the flocculation capacities of the yeast strains as results are obtained within a short period of time.

Fermented yeast was washed twice in solution A (see Appendix A). After each wash the trub was removed from the yeast. 1g of yeast was weighed and placed in a graduated translucent centrifuge tube. The yeast was suspended in 10ml of solution D. Tubes were shaken in a waterbath at 20 degrees centigrade for 20 minutes. The cells were then remixed and allowed to stand. The levels of settling were recorded at 2,5,10,15,20,30 and 60 minutes. The critical level of settling was at 10 minutes. This procedure was later modified slightly to suit the experimental culture,

which, being very flocculent tended to form floccs during washing in solution A. Solution A was therefore only used when selecting for a non-flocculent control culture and in all subsequent tests th - was substituted for distilled water.

As this test involves the induction of floc ulation under unnatural conditions, namely in an inorganic salts medium, the ability of all strains to flocculate under natural fermentation conditions was also determined.

Method 2

Fermentations were set up and allowed to proceed normally. A cut off time of 150 hours (6,5 days), was chosen as the dividing line between a floccu'ent and non-flocculent culture. If a culture settled heavily out of suspension before this time it was considered to be unsuitable for use as a non-flocculent control, i.e. as a comparison f the flocculent experimental culture. After this time it was observed that even yeasts which showed no active clumping started to settle in a powdery fashion to the bottom of the vessels.

2.2.6 PREPARATION OF CELLS FOR ELECTRON MICROSCOPY

Cells removed from the fermentation medium were washed four times. The first wash was with distilled water, the second with 5mM EDTA, and the third and fourth washes with

distilled water. Each wash involved centrifugation at 2000g for three minutes, following which the supernatant fluid was discarded and the cells re-suspended for the next wash. The EDTA wash served to deflocculate any clumped cells. Although the log phase samples and the control samples did not require deflocculating the EDTA wash was necessary to enable valid comparisons to be made with the clumped sample.

Any chemical treatments were performed at this stage. Initially the method of Day, Poone and Stewart (1975) was followed. The cells were suspended in distilled water at a concentration of about 10^5 cells per ml. 2ml of diethyl ether were added to 1ml of suspension and the sample was shaken at 21 degrees centigrade for two hours. As only a few of the aqueous suspended cells could come into contact with the ether during this period the method was modified to ensure that all cells were ether washed. A vortex mixer was used to vigorously mix the cells with the ether until an emulsion was formed.

Samples of stationary phase cells were treated with equal volumes of the following chemicals: 50% acetic acid, acetone, 95% ethyl alcohol, choloroform, 0,5M EDTA, ethyl acetate, formalin, phosphoric acid (pH 2) and 8M urea.

Cells which were not chemically treated, i.e. water washed cells, were sometimes lysed using an ultrasonic probe on

maximum power. Five minutes of ultrasonication at maximum amplitude resulted in cell lysis but no apparant wall damage to a 5ml suspension of 10^7 cells per ml. The length of time required to cause lysis was determined by ultrasonicating the cells for periods of two minutes before spinning the cells down and testing the supernatant fluid for the presence of protein. The phenol method of Oyama and Eagle (1956) was used for the protein determination.

Protein determination

1ml of NaOH was added to 1ml of supernatant in a boiling tube. This was placed in a boiling water bath and mixed gently for about 10 minutes until all cloudiness had disappeared. Dilutions of this protein solution were then made using 1/10 N NaOH. Protein standards were prepared using bovine serum albumin (Fraction V) and these were treated exactly as for the sample. 5ml of reagent C (see Appendix A) were added to 1ml of sample, mixed and allowed to stand for 10 minutes at room temperature. To this 0,5ml of Folin-Coicelteu was added, mixed and left to stand for 30 minutes. The optical density of the samples was read at 690 nm.

Following the chemical treatments or ultrasonication the cells were washed three times in distilled water and finally suspended in distilled water at a concentration of about 10^6 cells per ml.

Mounting the cells

Using a Pasteur pipette a drop of cell suspension was placed on a formvar coated copper mesh grid.

The cells were allowed to settle for about 30 seconds before the excess water was drawn off with filter paper. The distribution of cells on each grid was checked by microscopic examination of the grid at 100x magnification. Cellular distribution could be improved if the grids were pretreated with surfactants which reduce surface tension e.g. 1% Tween 80 or 1% Triton X or Kodak photoflo (1 drop in 10ml water). Although no fimbriae were ever observed on any cells where grids had been pretreated with the Tween 80 or Triton X their use was discontinued and only photoflo was used.

Preparation of formvar coated grids

0,35% formvar films (Appendix A) were cast on glass microscope slides and floated off onto water surface. Copper grids (300 mesh) were placed on the film which was then scooped up on wire mesh and allowed to dry. Grids were stored in a dessicator.

Shadowing grids for TEM examination

Aqueous suspended yeast cells were mounted on the formvar coated grids and air dried. The grids were then shadowed using 13-20 mm of gold palladium wire at an angle of 18-22

degrees in an Edwards evaporator coating unit. As precise targeting is not possible with this unit, variation in the thickness of the deposited metal was common. Later in this study shadowing was performed in a Balzers Electron Beam Evaporation unit using carbon palladium which has a much finer grain. The unit was set at 70 mA, 1500V with a vaccuum of $3x10^{-5}$ Bars. The sample stage was set at an angle of 70 degrees (equal to 20 degrees in the Edwards unit). 2-3nm of platinum carbon was deposited on the grids. The shadowing effect obtained in this unit was reproducible.

Negative staining

Although the fimbriae were clearly defined after shadowing, only that part of the cell edge which fell between approximately 45 degrees and 135 degrees to the direction of the shadow could be examined for fimbriae. To enable valid statistical analyses to be performed, it was necessary for the whole circumference of each cell to be examined and for this reason negative staining was used. A drop of 2-3% phosphotungstic acid, pH7,0 was placed on each grid for a few seconds and then drawn off with filter paper. Problems were however experienced with this technique and the results obtained were ambiguous.

Preparation for SEM

The cells were filtered onto nucleopore filters, air dried, mounted on stubs and gold coated. They were then viewed

under a Cambridge S4 Scanning Electron Microscope.

TEM examination

Samples were examined using a Jeol Jem 100S transmission electron microscope using the second, third and fourth objectives.

The use of low light intensity minimized damage to the formvar film which could not be carbon coated to stabilize and strengthen it. Carbon coating resulted in the loss of contrast between the background and the shadow and consequently the fimbriae could barely be seen.

Determination of the number of fimbriate cells in a population

Since unidirectional shadowing of cells only allows for the examination of part of the cell edge, a statistically valid enumeration of the number of fimbriae versus non-fimbriate cells in a population was impossible. Rough estimations were therefore made by counting 25 cells on duplicate grids and repeating this for four different fermentations. Once it was possible to rotatory shadow the specimens a more realistic (although still not statistically valid) analysis was possible as the whole cell surface could then be examined.

2.2.7 THE EFFECT OF CONCANAVALIN A ON FLOCCULATION

The effect of Con A on flocculation in wort and in an inorganic salts medium was investigated.

Flocculation in an inorganic salts medium:

Both log and stationary phase cultures of the flocculent yeast, S.uvarum 2036 were tested. Deflocculated washed yeast was used. Duplicate samples were set us as follows:

Yeast	Medium
Log phase	Helm medium
Log phase	Helm medium + Con A
Stationary phase	Helm medium
Stationary phase	Helm medium + Con A

0,5g of washed yeast was added to 10ml of Helm medium. This medium was supplemented with Con A at 1mg Con A per gram of yeast cells.

Flocculation in wort:

Con A was added to laboratory scale fermentations at concentrations of 20mg, 38,5mg, 60mg, 100mg and 150mg Con A per ml. It was added after 48 hours, since high concentrations of glucose can disrupt the Con A molecule. After 48 hours of fermentation the sugar level is considerably lower than it is at pitching. Fermentations were monitored for nine days.

2.3 RESULTS

2.3.1 VERIFICATION OF THE PRESENCE OF "FIMBRIAE" ON FLOCCULATED YEAST CELLS

The first step in this investigation was to establish the presence of these hair-like structures or " fimbriae" on the surfaces of flocculated cells of brewer's yeast.

The shadowing technique with which Day et al.(1975) had identified these structures was perfected using cells of the bacterium Eschericia coli, which is known to be fimbriate.

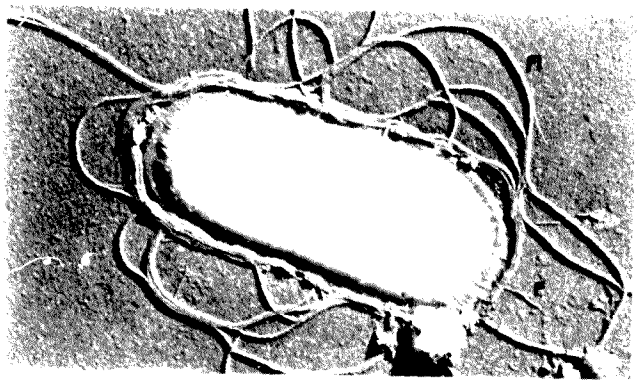


Fig.5. E.Coli cell shadowed with gold palladium at an angle of 20 degrees, using 13mm of gold palladium wire. The fimbriae are clearly visible. Magnification: 33000x (F-fimbril; Fl-flagellum)

Having established that fimbriae are well displayed by this technique fermentations were set up according to the proceedure described in Chapter 2.2.4. The flocculated yeast harvested from the bottom of the fermentation tubes (fig.4.) was treated, shadowed and examined electron microscopically for fimbriae. Fimbriae were observed on many of these cells.



Fig. 6 shows the fimbriae on a mildy ether treated, shadowed cell from a flocculated culture of S. uvarum 2036. Magnification: 94000x (F-fimbriae)

The cells were also examined under the scanning electron microscope and although fimbriae could be distinguished they were indistinct. Resolution at magnifications approaching 10 000 x was inadequate which would account for the inability to see fimbriae on most of the cells.

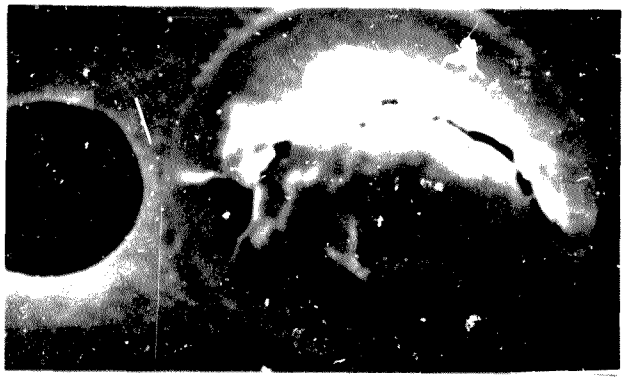


Fig. 7. Scanning electron micrograph of a flocculated yeast cell. The fimbriae are visible but indistinct. Magnification: 29000 x

Negative staining was also used to display the fimbriae. Results using this method of preparation were ambiguous due to the difficulty of distinguishing the fimbriae from natural streaking due to cell dehydration.



Fig.8 shows the edges of three negatively stained cell with fimbriae-like streaking from the cell. Magnification: 13000x

2.3.2 SELECTION OF A NON-FLOCCULENT CONTROL CULTURE

The aim of this study being to investigate the role of fimbriae in the flocculation of brewer's yeast it was necessary to find a yeast strain which would ferment wort, but which would not flocculate. The reason for this was to enable comparison of the cell walls and surface structures of the non-flocculent control culture and the flocculent experimental culture. Five cultures of *S.cerevisiae* were thererfore tested for flocculence against the flocculent experimental culture. Four of the cultures

had been classified as non-flocculent in other laboratories.

The Helm sedmentation test was carried out on cells which had been propagated in shake flasks and allowed to completely ferment the wort. The levels of setting in the Helm medium were recorded over 60 minutes.

Table 1. shows the levels of settling of the five cultures over one hour. The numbers in this table represent the level (millilitre graduations on a centrifuge tube) which the clearing zone had reached at the time of monitoring. This is represented graphically in figure 9.

Culture	*S. uvarum* 2036		*S. cerevisiae*				
Time (mins)	Stationary	log (mid)	Y 537	ale yeast	NCYC 1324	Y746 (4)*	Y746 (25)*
	1	2	3	4	5	6	7
2	7,5	9,0	9,5	9,0	10	10	10
5	3,5	7,0	9,0	6,5	10	10	10
10	2,5	5,5	8,5	3,0	9,8	10	10
15	2,0	4,5	7,5	2,0	9,6	9,8	9,7
30	2,0	3,5	7,0	1,9	9,2	9,5	9,4
60	2,0	2,0	6,2	1,7	9,0	9,1	9,0

*Note: Two cultures of the same strain were tested for

flocculence. One had been maintained at 25 degrees centigrade and the other at 4 degrees centigrade.

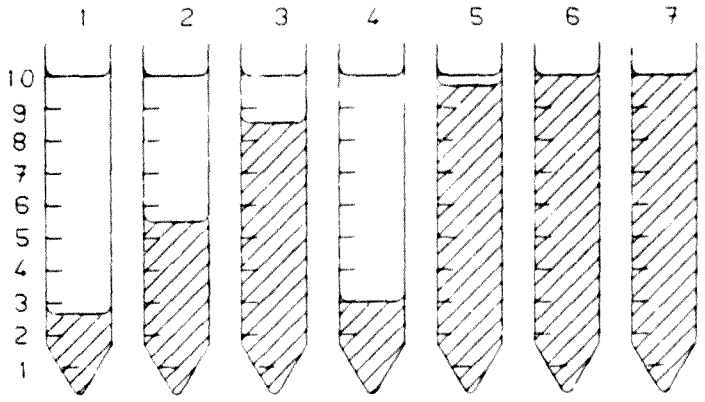


Fig.9. Diagramatic representation of the settling of the seven yeast cultures in the Helm medium after ten mirutes.

From these results S.cerevisiae NCYC 1324 and S.cerevisiae Y746 (25 degrees centigrade and 4 degrees centigrade) were considered to be non-flocculent. The mid log phase culture of the flocculent S.uvarum 2036 was found to have the potential to flocculate even at this early stage althougn under natural conditions it does not flocculate until the stationary phase. Its rate of sedimentation did however differ markedly from that of the staticnary phase culture and it did not flocculate as readily as the latter.

In order to further characterize the settling patterns of the potential control cultures they were propagated and fermented in a brewery simulated system and compared to S.uvarum 2036. Growth patterns are recorded in Fig. 10.

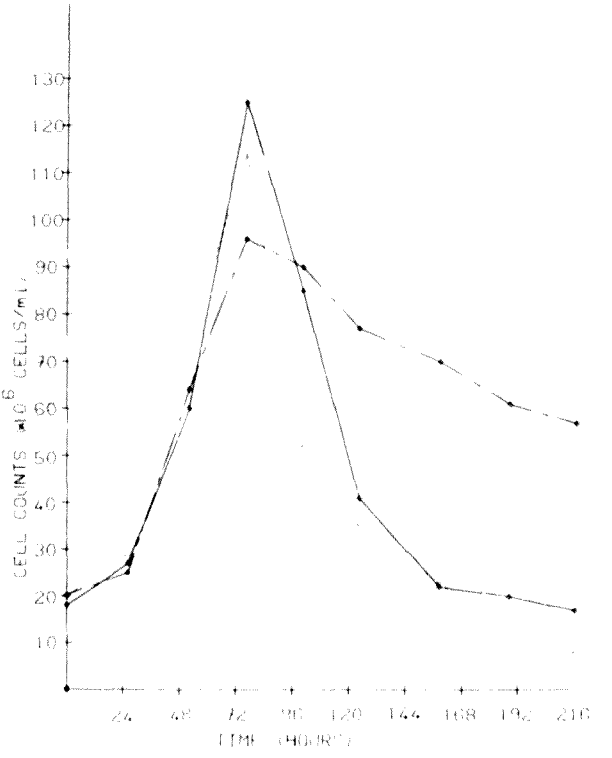


FIGURE 10: PATTERN OF GROWTH OF THE EXPERIMENTAL AND TWO NON-TOXIGENIC CULTURES.

The curve obtained for the experimental culture was typical of that of flocculent brewery yeast and correlates well with the trends shown for this yeast in the brewery laboratories, although in this micro system the peak counts were slightly higher and the lag phase often slightly longer than in the brewery system. Although S. cerevisiae NCYC 1324 appeared to be non-flocculent according to the Helm test it followed a fermentation pattern similar to that of the experimental culture, forming loose flocs and settling out of suspension after about 80 hours. S. cerevisiae Y746 did not ferment in wort as well as the other cultures did, showing a longer lag phase, a lower peak count and no floc-formation. Cells of this strain (grown in wort) were generally smaller than those of S.uvarum 2036 and S. cerevisiae NCYC 1324 and varied in shape from round to oval to elongate. Cells of the other two strains were consistently slightly oval. Some settling of the S. cerevisiae Y 746 did occur and after 14 days (320 hours) almost all the yeast had settled, but no flocs were visible. This culture was therefore selected as the control and the culture was maintained at 4 degrees centigrade.

The following three light micrographs (taken under phase contrast) show the cells of the flocculent experimental culture S.uvarum 2036 in the log phase (Fig.11) and the stationary phase (Fig.12) and the non-flocculent control strain S.cerevisiae Y746 (Stationary phase Fig 13) Magnification 2000x

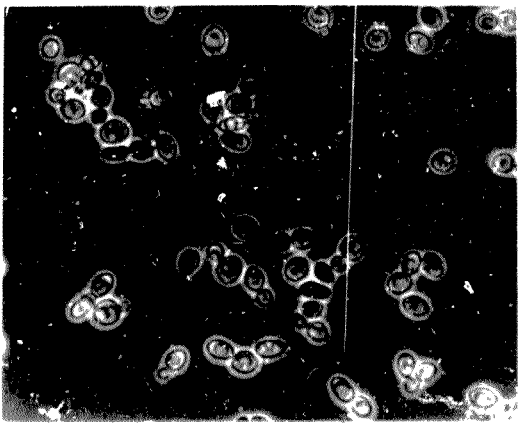


Fig.11. S.uvarum 2036 in the log phase of growth.

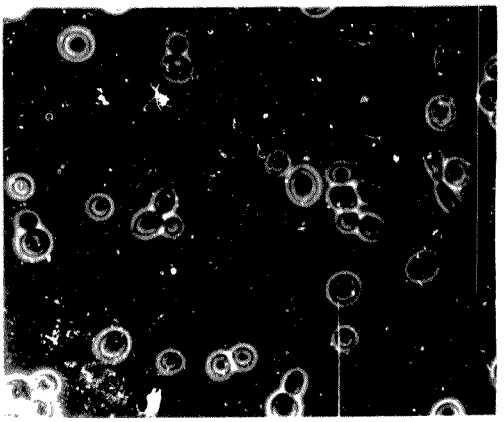


Fig.12. S.uvarum 2036 in the stationary phase of growth.

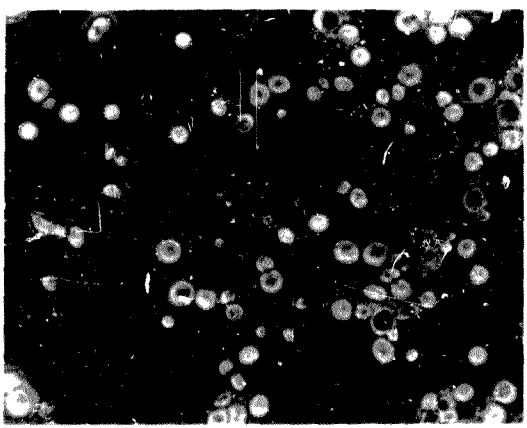


Fig.13. S.cerevisiae Y746 in the stationary phase of growth.

A number of budding cells are apparent in this stationary phase sample. S.cerevisiae Y746 started a second budding phase after cell numbers had already peaked and begun to decline (about 120 hours).

2.3.2.1 Change of the flocculence character of the control culture

The fermentation pattern of the control culture remained constant over eight months after which it appeared to adapt to the wort medium, displaying shorter lag periods, larger, more regularly shaped cells, higher peak counts and sedimented after about 90 hours. Although large flocs of yeast similar to those of the experimental culture were not

seen some small clumps were apparent and the culture settled completely in Helm medium after 20 minutes. Repeated fermentations confirmed this change in the culture and it was therefore deemed unsuitable for continued use as a contol. Attempts were made to reselect a non-flocculent strain from the altered strain by streaking cells remaining in suspension after flocculation onto malt agar plates, propagating individual colonies of varying sizes in wort and then testing them for flocculence. All colonies were also tested for respiratory sufficiency using the TTC overlay technique (Appendix A) since petite mutants have been reported amongst cells with altered flocculation characteristics (Johnson and Reader, 1984). All colonies tested were respiratory sufficient and were still flocculent in wort. Since repeated attempts at reselecting a non-flocculent colony were unsuccessful it was concluded that the culture had undergone a genetic change in its flocculence character and a new culture was obtained.

The new culture displayed flocculation characteristics almost identical to those of the original control culture and was therefore used for all subsequent experiments. It did however differ from the original culture in being better able to ferment wort, showing a shorter lag period and having consistently round to slightly oval cells.

2.3.3 REPLICATION OF EXPERIMENTS OF DAY ET AL.(1975)

Having established that flocculated cells of S.uvarum 2036 were fimbriate and having selected a non-flocculent control culture, cells of the control and experimental cultures were examined for the presence of fimbriae in both the log phase and the stationary phase. Fimbriae were readily visible after ether treatment of the cells, but were more difficult to find on water washed cells, i.e. many water washed cells appeared to be devoid of fimbriae.

The following observations were made:

Experimental culture (S.uvarum 2036)

Stationary phase i.e. flocculated cells: Many of these cells were covered with a dense mat of fimbriae. After ether treatment a greater proportion of cells appeared to be fimbriate.



Fig. 14 Shows the fimbriae on a flocculated ether washed cell. Magnification: 54000x

Log phase cells: A few log phase cells were covered with fimbriae and some were sparsely fimbriate. However, the proportion of fimbriate cells was much lower than that of the stationary phase cells.

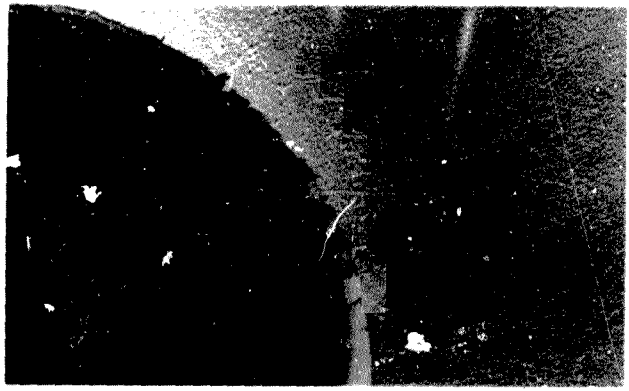


Fig. 15 Shows sparse fimbriae on a log phase cell.
Magnification: 42000x

Control culture (S.cerevisiae Y746)

The cells of this culture closely resembled those of the log phase experimental culture with regard to fimbriae, but again the proportion of fimbriate cells was increased when the cells were washed in diethyl ether. Differences between log and stationary phase cells of this culture were insignificant.



Fig. 16 Shows the edge of a non-fimbriate stationary phase cell of the control culture. Magnification: 40000x

The correlation between fimbriae and flocculation which was observed by Day et al.(1975) therefore also existed with these strains.

2.3.3.1 Proportions of fimbriate cells in each population

The proportion of fimbriate cells in various yeast cell populations was determined after both ether washing and water washing. These values are presented in Table 2 as percentages.

Table 2. Percentages of fimbriate cells in each population.

Strain / Treatment	S. uvarum 2036		S. cerevisiae Y746	
	log	stationary	log	stationary
ether washed	>90%	>90%	>90%	>90%
water washed	25-35%	60%	25%	30%
negative stain	inconsistent		inconsistent	

When the non-flocculent S.cerevisiae Y746 became flocculent the percentage of fimbriate cells in stationary phase populations increased from about 30% to 80%.

2.3.3.2 Correlation between flocculation and fimbriation of some non-brewery yeasts

An attempt was made to further correlate the presence of fimbriae with the degree of flocculation using some non-brewery strains which were fermented in both wort and in synthetic medium.

Table 3. is a summary of the results obtained. The degree of flocculation and the proportion of fimbriate cells in each population has been recorded on a subjective scale and all observations are relative to those routinely recorded for the experimental (S. uvarum 2036) and control (S. cerevisiae

Y 746) cultures.

Table 3. Correlation between flocculation and fimbriation of some non-brewery yeasts

Organism	Flocculence		Fimbriation	
	Wort	synthetic medium	wort	synthetic medium
S. uvarum 2036	strong	strong	+++	+++
S. cerevisiae Y746	none	none	+	+
S. cerevisiae (wine yeast)	slight	slight	+	+
S. cerevisiae (ale yeast)	strong	strong	+++	+++
Schizosaccharomyces pombe	strong	strong	+++	+++
Saccharomycodes ludwigii	slight	slight	++	++
Candida shehatae	strong	strong	++++	++++
S.cerevisiae NCYC 1324	slight	slight	++	++

From this table it can be seen that there is a very strong correlation between the intensity of flocculation and the number of fimbriate cells of each population. The strains tested showed the same flocculation characteristics in both brewery wort and synthetic medium.

2.3.4 CELLS IN SUSPENSION AT THE END OF FERMENTATION

At the completion of all fermentations ie. after nine days when the wort was fully attenuated, some cells still remained suspended in the beer. As these cells did not flocculate with the rest of the population the question of whether or not they had lost their potential to flocculate arose. The flocculation potential and the surface structure of the cells was therefore investigated.

Duplicate nine day fermentations were centrifuged and the cells harvested. Due to the exceptionally low cell yield the standard flocculation test could not be performed. Some cells were water washed, mounted and shadowed for EM examination. The bulk of the samples were added to 75ml of fresh wort and incubated shaking at 25 degrees centigrade overnight in order to obtain large enough inocula for pitching new fermentations. These were allowed to run for nine days and were compared with control fermentations for which the inocula had been raised from a slope culture.

Results

Fermentations: These proceeded normally, there being no difference in the time of flocculation in comparison with the control fermentations.

Some flocculated cells were harvested for EM examination.

Electron microscopy: The cells which had not flocculated were fimbriate and the proportion of fimbriate cells was the same as that of normally flocculent cells. The only difference was that, despite having been washed, the cells had a large amount of adsorbed material (possibly lipid or protein) on their walls. On some cells this was also attached to the fimbriae.



Fig.17 Shows the edges of cells which had not flocculated at the end of a fermentation. Magnification: 20000x

The flocculated cells which were harvested from the fermentations which had been pitched with the non-flocculated cells of a previous fermentation were apparently no different from the control cells. These cells were very clean in

comparison with the cells from which they had been produced.

2.3.5 THE EFFECT OF CONCANAVALIN A ON FLOCCULATION AND ON FIMBRIAE

It was noted by Miki et al.1981, that under certain conditions, the lectin Concanavalin A could delay flocculation. Since it had been shown to have an effect on flocculation it was added to fermentations of Saccharomyces uvarum 2036 in order to determine whether or not it also effected fimbriae.

The results of Miki et al.(1981) could not be repeated and Con A was found to have no effect on the time of flocculation in a natural (wort) system, but strongly promoted settling of both log and stationary phase cells in an inorganic salts medium. Differences in the flocs themselves were however apparent, those formed in the presence of Con A being much larger, but less dense than those formed under natural conditions (Figures 18 and 19).

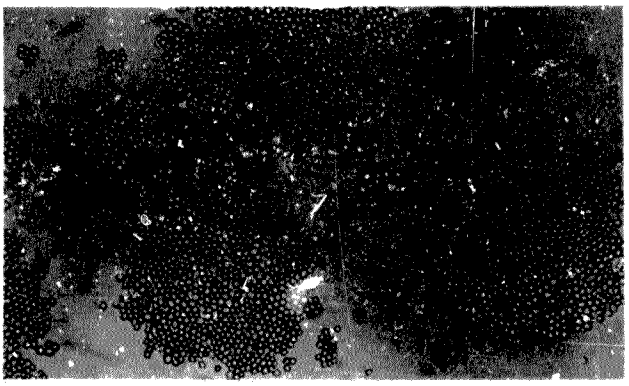


Fig.18. Shows flocs of yeast from a fermentation run under normal conditions and to which Con A had not been added. Magnification 500x



Fig.19 shows a typical floc from a fermentation to which Con A (400 ug/ml) had been added. This floc is much larger

than those from normal fermentations. The greater density of the flocs formed in the presence of Con A is not apparant from the micrographs as the flocs were flattened into two dimensional structures when coverslips were placed over them. Magnification 500x

When cells from fermented wort to which Con A had been added were examined under the electron microscope they were found to be no different from cells from normal fermentations. Both the proportion of fimbriate cells and the length of the fimbriae were the same as untreated samples.

2.4 DISCUSSION

2.4.1 ELECTRON MICROSCOPIC TECHNIQUES

Shadowing

The resolution of a shadowed sample depends upon the average size of the crystals which are formed on electron bombardment and is about twice this value. Finer granulation and therefore higher resolution is obtained with metals with the highest melting points (Henderson *et al.*1972; Bradley,1965).Yeast cells were shadowed with gold palladium which has a large grain size in comparison with the tungsten oxide used by Day *et al.*(1975). While resolution achieved with the gold palladium would therefore

be lower than that achieved with tungsten oxide, this latter metal has the disadvantage of yielding inconsistent results (Henderson et al. 1972; Bradley,1965). High resolution microscopy was possible when, towards the end of the study a Balzers Electron Beam Evaporation unit was used. This allowed both for precise targeting of the shadowing material (the finer grained carbon palladium was used) and for deposition of specified thickness of metal. Rotary shadowing was also possible which enabled examination of the whole cell circumference.

Negative staining

In some cases negative staining worked very well, but in others it was impossible to distinguish between streaking due to shrinkage of the cells (or drying of the stain) and the fimbriae. Poone and Day (1975) recorded similar difficulties in distinguishing negatively stained fimbriae from surface "slime" which was drawn out in strands.

As negative staining involves the application of chemicals to the yeast, there was also the risk of damage to the fimbriae.

Scanning electron microscopy

While it was possible to see fimbriae on some cells under SEM, resolution was poor. This was probably largely due to the inability to fix the samples adequately as results from this and other studies have shown yeast fimbriae to be

easily damaged by chemical treatment. Poone and Day (1975) found that, amongst others, fixatives such as formaldehyde and gluteraldehyde damaged the fimbriae of Ustilago violaceae. Likewise the fimbriae of Schizosaccharomyces pombe were seldom seen after the cells had been subjected to critical point drying, dehydration, or chemical fixation Walther et al. (1984).

While it is acknowledged that air drying is not ideal, the apparent susceptibility of fimbriae to damage by chemical fixation made this the method of choice.

2.4.2 SELECTION OF A NON-FLOCCULENT CONTROL CULTURE

As the only taxonomical difference between S.cerevisiae and S. uvarum is the ability of latter to use the sugar melibiose (Lodder,1970) a non-flocculent strain of S.cerevisiae was considered to be a suitable control culture for comparison with the flocculent experimental culture, S.uvarum 2036.

Testing for non-flocculence

Numerous proceedures have been used for the measurement of a culture's flocculation capacity (Calleja and Johnson 1976; Miki et al.1982 a,b) most of which rely on the performance of the culture in an inorganic salts medium. While such methods allow for rapid determination of flocculation

potential, they do not necessarily always provide a true indication of floc formation under natural conditions. This was demonstrated by S.cerevisiae NCYC 1324 which was non-flocculent when tested in an inorganic system, but flocculent when fermented in a brewery simulated system. Consequently, when testing strains for flocculence a modified version of the Helm sedimentation test was used as a preliminary test and results were always confirmed using laboratory scale microfermenations.

Change of flocculence of the control culture

The acquisition of the flocculence character by the non-flocculent S.cerevisiae provides some of the most convincing evidence in support of a role for fimbriae in flocculation. While non-flocculent, only about 30% of stationary phase cells were fimbriate. However, once the culture became flocculent this value increased to about 80%. There is thus a direct correlation between the presence of fimbriae and the ability to flocculate.

2.4.3 REPLICATION OF THE EXPERIMENTS OF DAY ET AL.(1975)

Although the results presented Table 2 do show a correlation between flocculation and a high proportion of densely fimbriate cells, the apparently very clear cut differences between flocculent and non-flocculent cells noted by Day et al.(1975) were not evident. In the early stages of this

study the trends noted by these authors for water washed or gently ether washed cells were confirmed. However, if the cells were washed in ether by vortex mixing these differences were no longer apparent and almost all cells, regardless of their growth phase or intrinsic flocculation potential were found to be fimbriate.

Whilst this may argue against fimbriae playing a role in flocculation, ether washing is nonetheless an unnatural process and under natural conditions a correlation between flocculation and fimbriae was evident. The appearance of fimbriae after ether treatment may however mean that they are present throughout the cell's life cycle, although they are not exposed, and raised the question of whether or not they are true fimbriae or an integral part of the cell wall.

Proportions of fimbriate cells in each population

Statistically accurate enumeration of the numbers of fimbriate and non-fimbriate cells is not possible for two reasons:

1. Unidirectional shadowing only allows for about half of each cell to be examined and if movement of the cells during drying had occurred in the same direction as the shadow fimbriae would not have been visible on most cells.
2. Rotational shadowing would have overcome the first problem were it not that the cells formed clumps during drying allowing only the outer cells to be viewed. This

clumping, which was followed under the light microscope (100x Magnification) was passive and occurred as the water evaporated from the grids ie. it was not flocculation.

Counts were nonetheless performed and do give a rough idea of the proportion of fimbriate cells in each population.

Ether treatment:
Although the significant variability in counts for the number of fimbriate cells in gently ether washed samples was eliminated by the use of vigorous ether washing this produced fimbriae on all cells. The effect of ether treatment is considered further in Chapter 3.

Fimbriae on stationary phase cells of S. uvarum 2036:
Although a high proportion of these cells was fimbriate upto 40% were devoid of fimbriae. It is therefore likely (if fimbriae do function in flocculation) that fimbriate cells bind to smooth cells rather than to other fimbriate cells.

Fimbriae on log phase cells of S.uvarum 2036:
That fimbriae were found on some log phase cells was to be expected as, when a new fermentation is set up, the inoculum comprises stationary phase (flocculated) cells from a previous generation. Many of these cells would therefore be fimbriate. The proportion of fimbriate cells in a log phase population would be a function of the time elapsed since exponential growth had begun. With reference to figure 10

this means that if a sample had been taken at 32 hours (when the cell number had doubled since pitching) one would have expected to find half as many fimbriate cells as would have been present in the stationary phase inoculum. As log phase samples were generally taken after 42-48 hours (i.e. when cell numbers were about 50-60 x10^6 cells/ml) the proportion of fimbriate cells contributed by the inoculum would be about 20-30% which is the average amount of fimbriate cells found in these mid log phase populations.

If this is the case it could explain the observation by Baker and Kirsop (1972) that a culture retains the ability to flocculate in an organic salts buffer for a while after being pitched into fresh wort, and that this ability is gradually lost as the cells multiply. If fimbriae are involved it may be that the culture becomes less flocculent as the fimbriate inoculum is diluted out and that the potential to flocculate is only regained once a critical minimum density of fimbriate cells is again attained.

Fimbriae on the control cells:

Although it has not yet been established whether fimbriae are a feature common to all yeast species or a flocculation specific feature, the apparant contradiction between the results presented here and those reported by Day et al. (1975) needs to be considered. The non-flocculent cultures studied by the original authors were either devoid of fimbriae or sparsely fimbriate. Conversely, about 30% of

the cells of the non-flocculent culture used here were always fimbriate.

Since S.cerevisiae Y 746 showed that it does have the potential to become flocculent, it may, under normal conditions (non-flocculent) be flocculation incompentent due to the lack of another requirement for flocculation. Flocculation is controlled by a number of genes and possibly also by environmental factors (Johnson and Reader,1984). As well as this flocculation intensity is variable, the spectrum ranging from totally non-flocculent to highly flocculent It is therefore not unlikely that this strain has all the biochemical machinery for flocculence (including the ability to produce fimbriae), with the exception of one factor such as the ability to synthesize a particular enzyme.

The potential of this strain to become flocculent may have some bearing on the discrepancies between the results of this study and those reported by Day et al. (1975).

Correlation between the presence of fimbriae and the degree of flocculation of some non-brewery yeasts.

Using the original experimental system the theory that fimbriae were associated with the flocculation process seemed to hold for the cultures investigated. Greater justification for continuing to investigate this potential role was then obtained when a variety of yeast species were

tested for flocculence (in a natural system) and examined for the presence of fimbriae. With the exception of the ale yeast all cultures were non-brewery strains and they too showed a similar trend, the more intensely flocculent cultures having a greater proportion of fimbriate cells than the non-flocculent strains.

Some of the strains examined by Day et al. (1975) flocculated in wort, but not in defined medium and these cultures were found to be fimbriate only after growth in wort. Unfortunately the strains used here showed identical flocculation characteristics in wort and in synthetic medium so that the correlation could not be extended any further.

2.4.4 CELLS REMAINING IN SUSPENSION AT THE END OF FERMENTATION

Although the cells which remained suspended after the completion of the fermentation did not participate in flocculation they were fimbriate. The adsorbed substances on the cells were not removed by the washing procedures which successfully cleaned flocculated cells and these may have blocked the active sites and prevented flocculation. There was apparently no genetic alteration of their flocculent natures as they produced cells which fermented and flocculated normally. The adsorbed material on the non-flocculated cells may also account for the observation of Ryder et al. (1983) that "flocculated yeast cells show a

higher fermentation capacity than those in suspension".

2.4.5 THE EFFECT OF CONCANAVALIN A ON FLOCCULATION

Results obtained from this study were strikingly different from those reported by Miki *et al.* (1981), who found that Con A inhibited flocculation when it was bound to yeast cells prior to flocculation being initiated. In this study Con A promoted heavy flocculation of both log and stationary phase cells of *S.uvarum* 2036. The reason for these differences may lie in the differences between the buffers used. In this study the Helm medium (pH 4,5) was used, while in the experiments of Miki *et al.* (1981) phosphate buffered saline to which calcium chloride, magnesium chloride and manganese chloride had been added was used. Although the pH of this buffer was not specified it is likely (being PBS) that it was around neutrality. According to McKenzie *et al.* (1972) Con A exists as a stable monomer at pH4,5 - pH5,6 but that above this it begins to dimerize, the extent of the reaction increasing with increasing pH. Dimerization could change the number of available binding sites and/or alter the magnitude of the binding constants. It is therefore possible if their experiments were performed above pH5,6 that the inhibition of flocculation reported by Miki *et al.* (1981) may have been due to the altered properties of the Con A molecules, in particular the formation of large aggregates which could have prevented intimate cell to cell contact.

Because Con A caused strong agglutination of log phase cells and because the flocs formed were quite different from those formed naturally, it was concluded that the agglutination caused by Con A is quite different from normal flocculation. If this is the case, then even if differences in the fimbriae had been noted, they would probably not have been related to flocculation.

CHAPTER 3

THE EFFECTS OF CHEMICAL, MECHANICAL AND ENZYME TREATMENTS ON FIMBRIAE AND FLOCCULATION

3.1 CHEMICAL

Flocculated cells of the experimental culture were treated with various chemicals for two reasons.

1. To enable the fimbriae to be seen more clearly under the electron microscope.
2. To establish, in part, the biochemical nature of the fimbriae.

3.1.1 METHODS

The following chemicals were used: acetic acid, acetone, alcohol, chloroform, diethyl ether, EDTA, ethyl acetate, formalin, phosphoric acid, and urea (8M).Cells were treated following the procedure in Chapter 2.2 .

A fatty acid test was performed on ether extracts. The ether was evaporated and the residue was dissolved in a drop

of benzene. A drop of a saturated solution of Rhodamine B in benzene was added to this and mixed. Two drops of a 4% solution of uranyl acetate was added and mixed. The benzene layer turned pink-red in the presence of fatty acids and would fluoresce orange under ultraviolet light.

The presence of protein was tested for, using the method described in Chapter 2.2.

3.1.2 RESULTS

Cells treated with either acetone or formalin were generally very dehydrated, often appearing almost angular under the TEM. Very few fimbriate cells were observed.

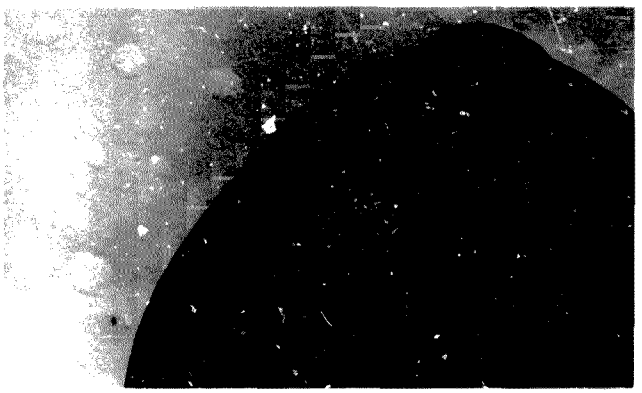


Fig. 20. This electron micrograph illustrates the effect of formalin treatment. The cell edge is typically well defined and devoid of fimbriae. Magnification: 28000x

Alcohol had a similar dehydrating effect, but this was less marked than that of acetone and formalin. More fimbriate cells were seen, but many of these were sparsely rather than densely fimbriate.

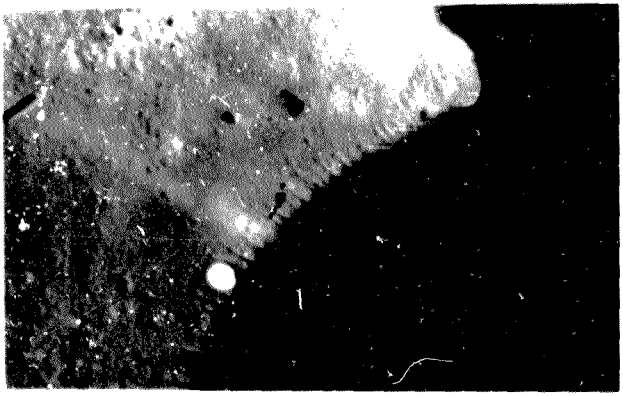


Fig.21. Fimbriae can be clearly seen on the edge of this alcohol-washed cell, but they are not as numerous as those on water washed cells (particularily towards the bottom of the micrograph). Magnification : 45000x

The edges of cholorform treated cells such as the cell in figure 22 were very clean and the fimbriae very well displayed. Not all cells were fimbriate.

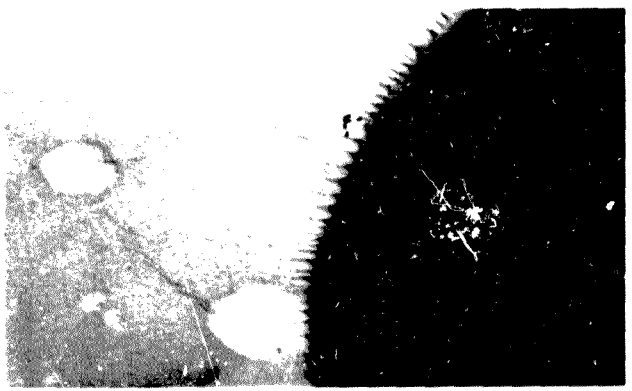


Fig.22. Edge of a chloroform treated cell.
Magnification: 30000x

Cells washed with ethyl acetate cells also had cleaner surfaces than those of water washed cells, but the proportion displaying fimbriae was similar.

About 95% of cells treated with acetic acid were fimbriate. No cellular dehydration was evident. Phosphoric acid also displayed the fimbriae well although the proportion of fimbriate cells was less than 95%. Cells were usually much cleaner than water washed cells.

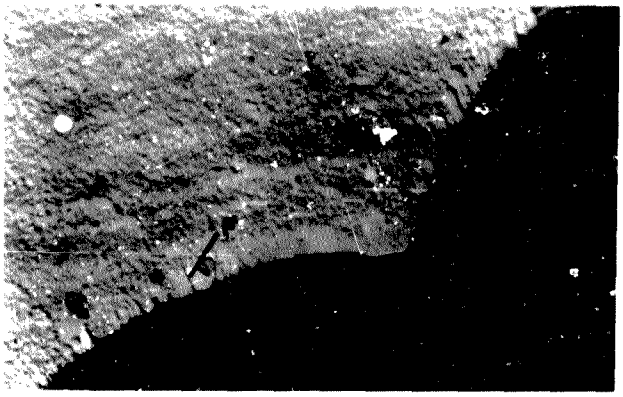


Fig.23 shows the edges of two acetic acid washed cells with fimbriae clearly visible on the lower cell. Magnification: 30000x

EDTA washing slightly increased the number of fimbriate cells presumably due to the cells having been efrectively

deflocculated and therefore better distributed on the grids.

No clearly defined fimbriae were observed on urea treated cells which were covered with the small electron dense bodies (DB) seen in figure 24.



Fig.24. Flocculated cell treated with urea.
Magnification: 43000x

At the electron microscopic level a significant difference was apparent between aqueous suspended cells which had been gently shaken with an equal volume of diethyl ether for an hour and cells which had been vortexed with the ether to form an emulsion during the one hour incubation period.

The mildly treated populations were comprised of both fimbriate and of non-fimbriate cells and the differences

between flocculent and non-flocculent populations observed with water washed cells were confirmed. In contrast, those populations which had been vortexed in the ether all showed nearly 100% or densely fimbriate cells, regardless of whether the populations were flocculent or non-flocculent.

Comparison of the fimbriae from the gently and vigorously washed populations showed that vigorously washed cells had much longer fimbriae than gently washed cells.

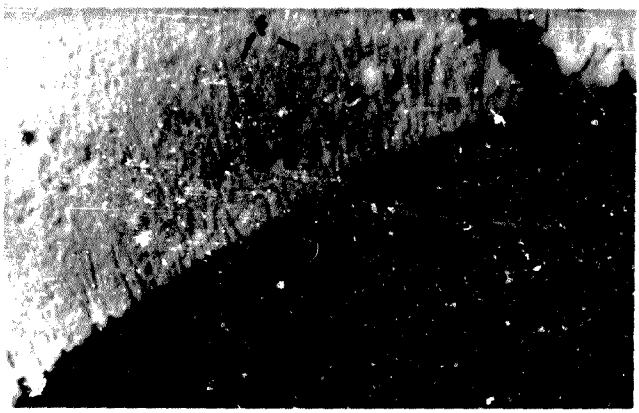


Fig.25 shows the edge of a vigorously ether washed cell with fimbriae of 1um long. The fimbriae of mildly washed cells were the same length as those of water washed cells, i.e. around 0,5 um. Magnification: 52000x

The effect of diethyl ether on the cells of S.uvarum 2056: Due to these differences the effect of ether on the yeast cells was investigated. The ether extracts of log and stationary phase cells were examined for the presence of fatty acids and protein and the aqueous extracts for the presence of protein. All tests were positive meaning that both fatty acids and protein had been removed from the cells when they were vortexed with ether. The presence of protein in excess of 1200ug /ml in both log and stationary phase extracts suggested that extensive cell leakage had occurred. Wall rupture was not apparant under the TEM, but comparison of water washed and ether washed cells under SEM showed that the ether treated cells had completely collapsed which was not the case with water washed cells. See figures 26 and 27.

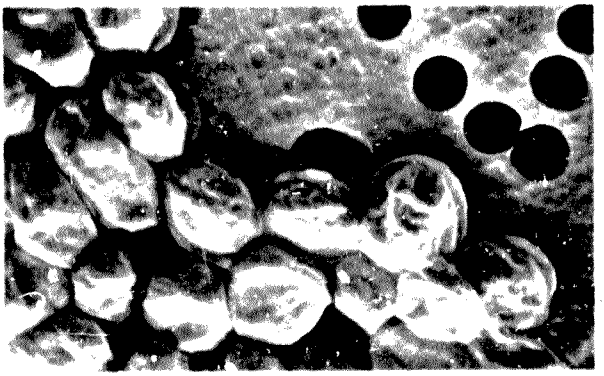


Fig. 26 shows a clump of water washed cells under SEM. These cells collapsed partially under vacuum as would be expected since they had not been fixed.
Magnification: 5200x



Fig.27 shows a clump of ether washed cells which, in contrast to the water washed cells, had completely collapsed and appeared as a lattice of cell walls lacking cellular contents. Magnification 5500x

The effect of ether washing on the flocculence of various cell populations in the Helm flocculation medium was investigated.

Method: Log and stationary phase cells of the control and experimental cultures were harvested and washed. Duplicate samples of each were suspended in distilled water at a concentration of E540=2,6. One sample of each duplicate was concentrated, treated with diethyl ether, washed and resuspended in the original volume of Helm's medium. The other duplicate was pelleted and resuspended in Helm's medium. Samples were shaken at 25 degrees centigrade for 10

minutes, resuspended and their settling monitored. The ease with which the flocculated cells could be deflocculated after flocculation in the Helm medium was also determined.

Results

All ether treated samples displayed exactly the same settling patterns as their water washed counterparts.

The ether and water washed samples of both log and stationary phase S.uvarum 2036 sedimented at the same rate. This was unexpected as in previous experiments stationary phase cells had settled faster than log cells. After two minutes large flocs began to form and a clearing zone became visible just below the miniscus. By five minutes all samples had completely settled.

The water washed S. cerevisiae Y 746 log and stationary cultures both remained in suspension as did the ether washed samples of these cultures. Slight settling of the cells was evident after 30 minutes, but no flocs formed. figures 28 and 29 show the settling of the 8 samples after 10 minutes.

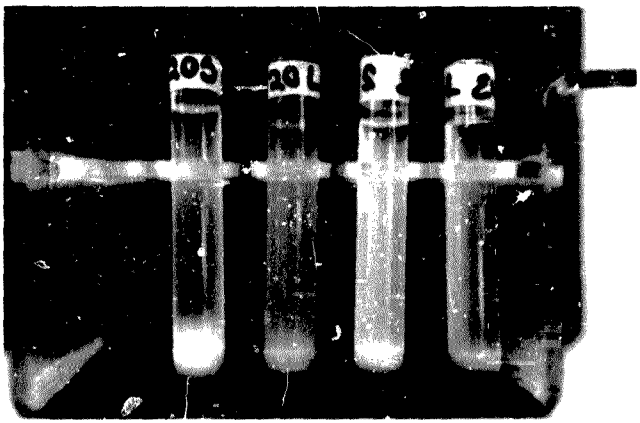


Fig. 28 shows the settling of S. uvarum 2036. From the left the samples are, stationary phase and water-washed; log phase and water washed; stationary phase and ether washed; log phase and ether washed.

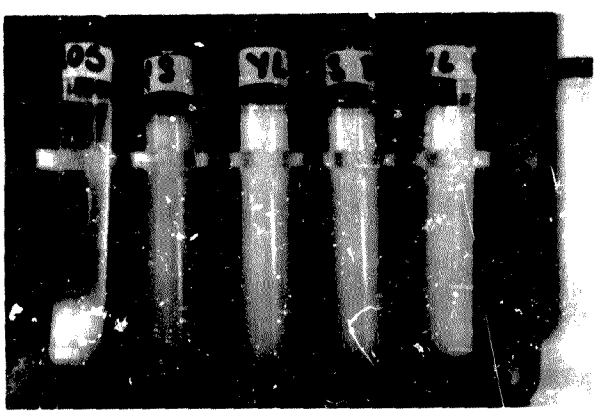


Fig. 29 shows the settling of the control culture S.cerevisiae Y746 in comparison with that of S. uvarum 2036. From the left the samples are: S. uvarum 2036; S.cerevisiae,

ether washed.

Thus although ether treatment did induce fimbriation in intrinsically non-flocculent populations it did not cause them to flocculate.

In order to establish the ease with which flocculated cells could be resuspended the Helm medium was decanted and replaced with distilled water. Both the ether and the water-washed log phase cells were easily suspended by inverting the tubes a couple of times. However the stationary phase cells were firmly packed and required vortexing for about 45 seconds to resuspend them. The samples were washed three times in distilled water and while only one wash was required to completely deflocculate log cells, three washes were insufficient to deflocculate either of the stationary phase cultures. Only after vortexing in 10 mM EDTA were the flocs broken up.

3.2 MECHANICAL

The aim of the mechanical treatments was the removal of the fimbriae. This would be the first step in the isolation of these structures.

The following proceedures were used as they were found by Day et al. (1975) to be effective in removing the fimbriae of

The following proceedures were used as they were found by Day et al (1975) to be effective in removing the fimbriae of Ustilago violaceae; ultrasonication, centrifugation through a water-sucrose barrier, agitation in a Waring Blendor.

3.2.1 ULTRASONICATION

Probe ultrasonication (Heat Systems-Ultrasonics Inc. N.Y.) was performed using a 50% pulse, but all the times given have been converted into continuous i.e. 100% ultrasonication. Cells were kept on ice at all times.

2,5 minutes with a microprobe at maximum power (120 watts, 14khz) effectively deflocculated clumped yeast cells. After five minutes extensive cell lysis occured as shown by the appearance of large amounts of protein in the suspending medium in comparison with the 2,5 minute sample. TEM examination of the cell [illegible] not show wall breakage, implying that lysis due to ultrasonication may have been the result of the cell membrane becoming 'leaky'.

Electron microscopic examination of shadowed cells did however show that after five minutes of treatment the cells were still fimbriate. The fimbriae were the same length as those of water washed rather than ether washed cells. No cell damage was apparent. When cells ultrasonicated for five minutes were subjected to the Helm test they

flocculated heavily i.e. ultrasonication did not result in the loss of flocculation potential.

Loss of flocculence after prolonged ultrasonication

Since prolonged ultrasonication generally has a highly disruptive effect on the cell walls it seemed logical that if sonicated for long enough fimbriate cells would eventually lose their fimbriae. Flocculated cells were therefore subjected to probe ultrasonication and examined at regular intervals for the presence of fimbriae and the ability to regain flocculence.

Flocculated cells from a static fermentation were deflocculated with EDTA and washed. They were then ultrasonicated for five minutes, washed and tested for flocculence in Helm medium (Appendix A). Control samples were not ultrasonicated. Treatment was repeated four times for periods of seven minutes each. Cells were then washed and tested for flocculence. Some cells were mounted for electron microscopic examination.

Results

Flocculation tests: The tendency of the ultrasonicated cells to regain flocculence was reduced after each successive treatment. The following changes were noted:

1. The time taken to settle in the Helm medium was longer after each successive treatment.

2. The size of the flocs appeared to become progressively smaller.
3. The settling line was less distinct after each treatment i.e. increasingly more cells remained in suspension and did not participate in flocculation.
4. After the last treatment i.e. a total of 33 minutes of ultrasonication the cells were rendered completely non-flocculent.

TEM examination: Fimbriae were seen on cells after each treatment. However, after each treatment, progressively more cells were visibly disrupted and the proportion of fimbriate cells reduced. After 26 minutes the proportion of fimbriate cells was only slightly less than the proportion of non-fimbriate cells.

After 33 minutes of ultrasonication the amount of fimbriate cells had been reduced to less than 5% and almost all the cells were visibly damaged.
The supernatants obtained after 19 minutes, 26 minutes and 33 minutes of ultrasonication were examined for the presence of detached fimbriae using the shadowing technique and immuno-serological electron microscopy (ISEM) (See Chapter 4.2). None were found.

3.2.2 CENTRIFUGATION THROUGH A WATER-SUCROSE BARRIER

Day et al.(1975) reported successful removal of the fimbriae of U.violaceae after centrifuging aqueous suspended cells through 40 % sucrose, the fimbriae being left at the water-sucrose interface. A similar proceedure was therefore followed using flocculated S.uvarum 2036 cells in an attempt to isolate intact fimbriae.

Method

Two samples were used. Cells of one sample were vigorously ether washed to expose the fimbriae maximally and cells of the other were water washed in case the ether treatment acted as a fixation process which would prevent easy detachment of the fimbriae. 5ml of aqueous suspended cells were overlaid on 10ml of 40% sucrose and centrifuged at 2000 x g for five minutes to pellet the cells. The water-sucrose interface was harvested, and dialysed extensively against distilled water and then concentrated against polyethylene glycol (molecular weight 20000 daltons). The solution was mounted and shadowed for TEM examination.
Pelleted cells were also examined for fimbriae.

Results

Fimbriae were not seen on grids prepared from either the water washed or the ether washed samples using rotational

shadowing, unidirectional shadowing or ISEM. Shadowed preparations of the cells which had been pelleted showed numerous densely fimbriate cells.

3.2.3 AGITATION IN A WARING BLENDOR

This was the most successful method used by Day et al.(1975) for removal of the Ustilago violaceae fimbriae.

Method

Water washed and ether washed flocculated cells were placed in the blendor and agitated for five minutes after which the cells were pelleted by gentle centrifugation. The supernatant fluid (30ml) was harvested, concentrated to 2ml and examined for intact fimbriae. Cells were then subjected to a further five minutes of agitation and the supernatant again examined.

Results

Fimbriae were not found in any of the samples.

3.3 ENZYME TREATMENT

The flocculent yeast culture was treated with a variety of enzymes in order to see their effect on both the fimbriae and on the flocculation capacity of the yeast. Electron

microscopic examination of the enzymically treated cells would also contribute to our knowledge of: (i) the composition of the fimbriae as the physical effect of each enzyme on the fimbriae could be observed directly, and (ii) the importance of various surface components in flocculation.

3.3.1 Methods

Flocculated water washed cells were treated with the following enzymes; acid phosphatase, α-amylase, cellulase, β-glucuronidase, leucine aminopeptidase, lipase, α-mannosidase, pronase and zymolyase.

Treated cells were examined electron microscopically and were tested for flocculence in fermented, clarified wort supplemented with 80ppm calcium in the form of $CaCl_2$ and in an inorganic salts medium. Where fimbriae had been removed from cells the reaction buffers of the samples were examined for the presence of fimbriae.

Mid log phase cells were treated with leucine aminopeptidase and α-mannosidase since Beavan et al. (1979) had found that the transition of a culture to the floccculent phase was accompanied by an increase in the activity of these two enzymes. The ability of the cells to flocculate in Helm medium was then compared with that of water washed mid-log phase cells.

The ability of pronase-treated stationary phase cells to grow was determined by repitching them into wort. They were incubated at 25 degrees centigrade for 24 hours and examined for growth. They were then incubated for a further 48 hours, whereafter they were tested for flocculence in the Helm medium.

The buffers for the enzyme reactions are listed in Appendix D. Enzyme reactions were all carried out at 25 degrees centigrade for two hours in an orbital shelter. Where larger amounts of yeast than those specified in Table 4 were required for flocculation tests the quantities were scaled up proportionately.

Table 4: Concentrations of enzymes used and their buffers

Enzyme	Buffer See App.D	Amount of enzyme	Amount of cells
acid phosphatase	citrate pH 5,6	20ul/ml (6 unit ml)	10^9 cells/ml
α-amylase	Sodium phosphate pH6,9	0,15mg/ml	10^9 cells/ml

cellulase	protoplast	2 mg/ml	10^9 cells/ml
β-glucuron-idase	protoplast	75 ul/ml 1ml=95400 Fleishman units	10^9 cells/ml
leucine amino-peptidase	Trie Magnesium chloride	20 ul/ml 1ml=50000 units	10^9 cells/ml
Lipase	PBS, pH 7,2	3 ul/ml	0,15g / 5 ml buffer
α-mann-osidase	citrate pH 4,15	35ug/ml	10^9 cells/ ml
pronase	Tris-HCI pH 6,8	2 mg/ml	0,25g in 10ml buffer
zymolyase	protoplast	200ug/ml	0,15g / 5ml buffer

Acid phosphatase,α-amylase, leucine aminopeptidase, α-mannosidase, pronase and lipase were obtained from Boehringer Mannheim Pty Ltd.; cellulase from BDH Chemicals, England and zymolyase from Kirin Laboratories, Yokohama and β-glucuronidase from the Sigma Chemical Corporation. See Appendix E for notes on the enzymes used.

The extracts of the pronase and zymolyase treated cells were examined for the presence of fimbriae. The reaction mixtures were centrifuged to remove the cells, dialysed against glass distilled water for three days at 4 degrees centigrade and concentrated by dialysis against polyethylene glycol (molecular weight 20000 daltons). The extracts were mounted on copper grids for electron microscopic examination. Both the shadowing and the ISEM techniques were used.

3.3.2 Results

3.3.2.1 TEM examination of the enzyme treated cells showed the following:

Acid phosphatase: This enzyme had no apparant effect on either the fimbriae or on the cell walls since in two

experiments no difference could be seen between water washed and acid phosphatase treated cells. However in a third experiment the surface charge of many of the cells seemed to have changed. These cells immediately migrated to the bars of the copper mesh grids on which the cells were mounted. No fimbriae could be seen on these cells. A few cells did not move to the edge of the grid squares and these cells were generally fimbriate.

α-amylase: Again, no differences could be detected between water-washed and α-amylase treated cells.

Cellulase: No effect was observed on the cells or the fimbriae.

β-glucuronidase: The walls of some cells treated with this enzyme were degraded and on these cells no fimbriae were seen. Cells not obviously degraded looked no different from water-washed cells. The fimbriae of these intact cells showed no sign of having been damaged and were the same length as water-washed fimbriae. The lack of extensive wall damage by the β-glucuronidase was probably the omission of mercaptoethanol from the reaction buffer, since, when this was included under identical conditions, protoplasts were formed (Gaphthal, personal communication). The addition of this chemical would have made it impossible to establish whether or not fimbrial damage was the result of the enzyme

or the chemical.

Leucine aminopeptidase: This enzyme had no apparent effect on the cell wall or the fimbriae.

Lipase: The major difference between lipase treated cells and water-washed cells was the greater number of treated cells which had fimbriae. These fimbriae resembled ether-washed cells in that they often formed a more dense mat around the cell edges than was seen on water-washed cells and the fimbriae were frequently longer than those of water-washed cells. As expected these cells were very clean.

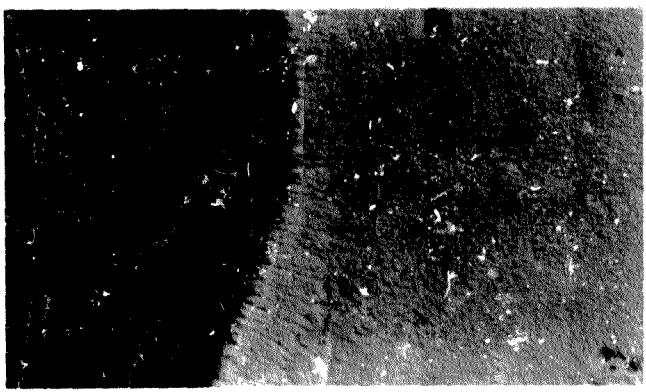


Fig.30. Edge of a cell which had been treated with lipase. Fimbriae are well displayed. Magnification: 30000x

α-mannosidase: Most cells examined were no different from water washed cells. Some cells did however seem to have shorter fimbriae than water washed cells, indicative of the possible loss of the end portion of the fimbriae.

Pronase: Cells treated with pronase under the conditions specified (Chapter 3.3) were devoid of fimbriae. Reduction in the amount of pronase used, in the reaction time, or an increase in the density of the cell suspension resulted in only partial removal of the fimbriae. The short, stubby structures seen on many of these cells were presumably partially damaged fimbriae.

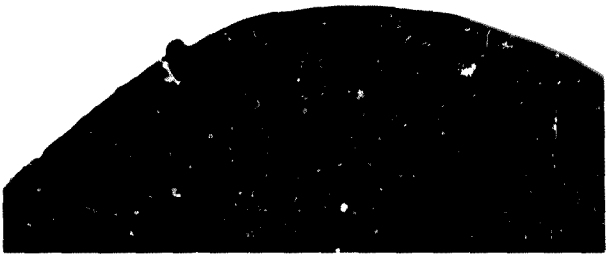


Fig. 31. Shows a pronase treated cell. All fimbriae have been removed. Magnification: 53000x

The ability of pronase-treated cells to grow and multiply Pronase-treated, defimbriated cells which were repitched into fresh wort were found to have retained the ability to grow and multiply despite having lost both their fimbriae and their ability to flocculate. The repitched cells also regained the ability to flocculate after 30 hours.

Zymolyase: This enzyme caused extensive wall damage and in order to see any effect on the fimbriae it had to be used at very low concentrations. At low concentrations cells at various stages of degradation were found. Fimbriae were occasionally found on cells with partially degraded walls. No fimbriae were seen on extensively damaged cells. Short hair-like structures were found in one sample of zymolyase treated cells. These were about 0,25um long and resembled short fimbriae (Fig.33). One of the partially degraded cells examined possessed short fimbriae and it is possible that the zymolyase cleaved the fimbriae at a point halfway down their lengths and that the structures seen in Fig.33 are the top portion of the fimbriae.

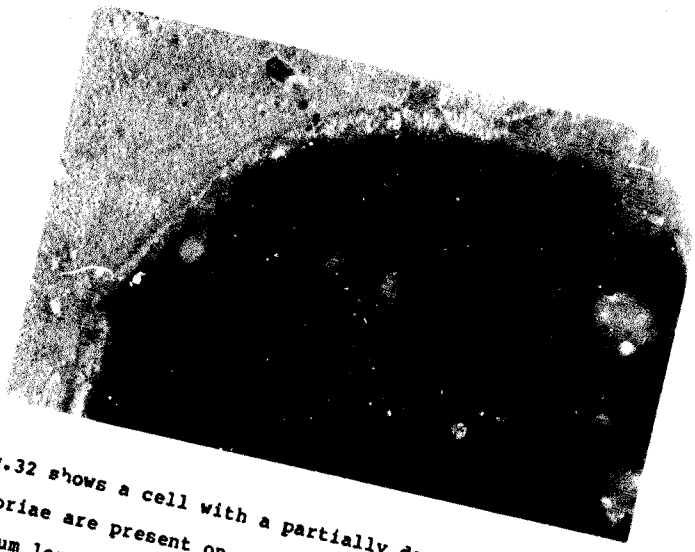


Fig.32 shows a cell with a partially degraded wall. Short fimbriae are present on part of the wall. These are about 0,25um long which is half the length of fimbriae of untreated cells of S.uvarum 2036. Magnification 16000x

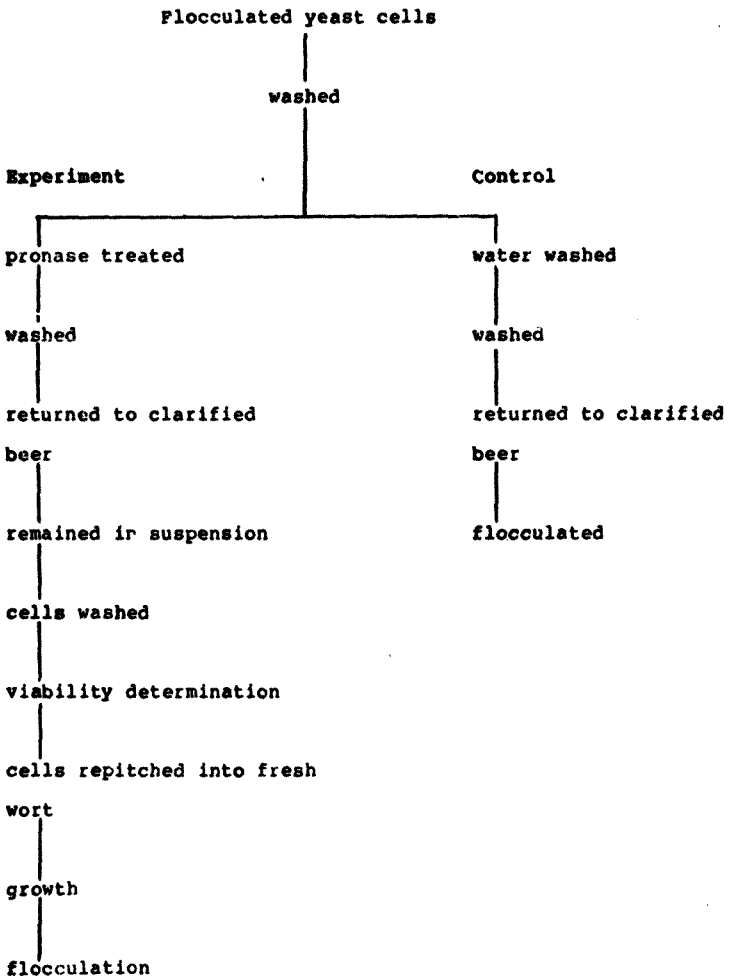


Fig.33 shows hair-like structures (up to 0,25um long) which may be the terminal portion of the fimbriae. Magnification: 8000x

3.3.2.2 Flocculence of enzyme treated cells in clarified "beer"

It was desirable to see whether or not cells which had been enzyme-treated were still able to flocculate in their own fermented medium i.e. the beer from which they had been removed (in a flocculated state). This test was initially performed on only the pronase treated cells since it was known that this treatment removed fimbriae from the cells. The viability of the defimbriated cells was determined using methylene blue. Washed pronase treated cells were also tested for their ability to grow and multiply.

Procedure and Results



The clarified ''beer'' was supplemented with calcium since without it even the control sample (i.e. not pronase treated) was unable to flocculate. It seemed likely that when the cells were deflocculated with EDTA the adsorbed calcium was chelated and too little remained in the fermented product to support flocculation. 80ppm is the amount of calcium normally present in wort.

On addition to the clarified ''beer'' the control cells immediately formed large flocs and settled to the bottom of the tube. The pronase treated cells remained in suspension and even the addition of more calcium did not induce flocc formation.

This test was repeated on cells treated with the other enzymes and the settling monitored over 10 minutes, at 30 minutes and at one hour. Flocculation characteristics were confirmed using the Helm sedimentation test. The control consisted of water-washed cells which were also incubated (shaking at 25 degress centigrade) for two hours and then added to wort supplemented with calcium.

Results

The effects of the enzymes on flocculence are recorded in Table 5.

Table 5. The effect of enzyme treatment on cell flocculence

Cell treatment	Flocculence
Control i.e.untreated	Cells flocculated completely within two minutes.
α-amylase	Settling and floc formation were identical to the control.
acid phosphatase	Flocculation was completed in just under two minutes and flocs were much larger than those of the control.
β-glucuronidase	Flocculation occurred over four minutes. The settling line was not very clear i.e. some yeast remained in suspension and did not settle, even over 60 minutes.
lipase	Flocculation was completed in just under two minutes. Cells packed more firmly than the control cells.

leucine aminopeptidase	Flocculence was unchanged, settling having occured within two minutes.
α-mannosidase	Flocculence occurred within about 30 seconds. No yeast remained in suspension and the flocs were much larger than those of the control.
pronase	Flocculation did not occur and only slight settling (due to gravity) occurred over 60 minutes.
zymolyase	No flocculation occurred.

3.3.2.3 The effect of α-mannosidase and leucine aminopeptidase (LAP) on log phase cells.

Log phase cells of the flocculent culture were treated with α-mannosidase and LAP and then examined for the presence of fimbriae and tested for the ability to flocculate.

Results

Electron microscopy

The fimbriae of log phase cells which had been treated with α-mannosidase and leucine aminopeptidase appeared to be no

differen from those of the untreated cells.

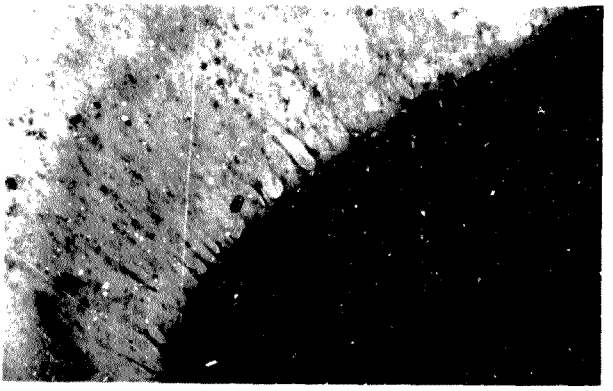


Fig.34. Fimbriae are present around the edge of this LAP treated log phase cell. The slightly different appearance of these fimbriae in comparison with those in figures 6 and 12 is due to the cell having been suspended in a salts medium. Magnification: 57000x

Flocculence

The ability of these cells to flocculate in both fermented medium and in Helm medium was tested. Settling was compared with that of an untreated mid log phase sample. Within seconds of being added to the Helm medium large flocs began to form in the α-mannosidase treated samples. The LAP treated sample also flocculated, but more slowly than the α-mannosidase-treated cells. The control sample did settle,

but at a much slower rate than either of the other samples.

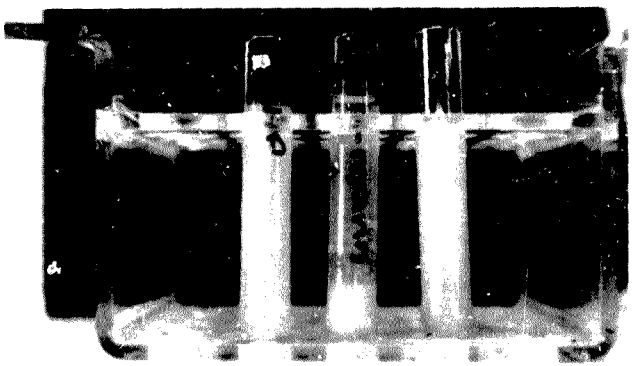


Fig.35 Shows the settling in the Helm medium (as opposed to beer) after one minute. Settling in the beer does not show up well in photographs due to the brown pigment and therefore lack of contrast. Significant settling of the α-mannosidase treated cells had already occurred after one minute.

3.3.2.4 Examination of the enzyme extracts for the presence of fimbriae.

The enzyme extracts of the pronase treated and the zymolyase treated cells were examined for the presence of fimbriae using the shadowing and ISEM (see Chapter 4) techniques. Fimbriae were not found in either extract.

3.4 DISCUSSION

3.4.1 CHEMICAL TREATMENTS

The effects of some of the chemicals used to wash the cells were similar to those observed by Poone and Day (1975) on the fimbriae of Ustilago violaceae. However these authors did find the fimbriae of this fungus to be different from those of brewer's yeast and it is therefore not surprising that the effects of some chemicals on Saccharomyces uvarum 2036 were quite different from their effects on the Ustilago violaceae fimbriae. Glacial acetic acid, which fragmented the fungal fimbriae into pieces about 0,1um long, enhanced the visualization of the fimbriae of S.uvarum 2036 considerably. Phosphoric acid, with which brewery yeast is often treated prior to repitching, also displayed the

fimbriae well and appeared to have "cleaned" the cells. The reason for investigating the effect of phosphoric acid is that it deflocculates the yeast and the likelihood of its removing the fimbriae was considered. As this did not occur the deflocculation action of the acid may have been due to interference with receptor sites or the ionic charges associated with intercellular binding.

The U. violaceae fimbriae were sometimes better displayed after acetone treatment while fimbriae were generally absent from the S. uvarum 2036 cells which were dehydrated and often distorted after acetone treatment. Chloroform and ether treatment often made the fimbriae of both cell types more easily visible and no fimbriae were visible on either type after urea or formaldehyde treatment. Electron dense bodies presumed to be contracted fimbriae were seen at the U.violaceae cell edges after urea or formaldehyde washing. On S. uvarum 2036 electron dense bodies were seen after urea treament, but were presumed to be urea deposits.

Ether treatment

As ether washing appeared to cause the appearance of fimbriae on cells which had been previously devoid of fimbriae its effect was further investigated.

At the electron microscopic level the fimbriae of ether washed cells resembled those of water washed cells except

that in some cases they were slightly longer. This may have been due to the removal of part of the cell wall. The fatty acids in the ether extract may have arisen from lipid adsorbed onto the cell surface during fermentation, but since large amounts of protein were also extracted and SEM showed that the cells had been lysed it is possible that the fatty acids arose from membrane damage.

The effect of ether treatment on log phase cells is proof that the fimbriae are not produced in the stationary phase, but are present throughout the cell's life cycle. That there is a difference between the amount of fimbriate cells in log phase and stationary phase populations suggests that an event occurs during the transition to flocculence which exposes fimbriae which were previously "hidden".

The effect of ether on the yeast surface is explained in the light of the work of Kamada and Murata (1984). They showed that the surfaces of lager yeast increased in hydrophobicity between the dispersed and flocculent phases. Loss of hydrophobicity at the flocculent stage resulted in a loss of flocculence. The steric structure of surface protein was also shown to be of considerable importance in flocculation, hydrophobic interactions playing a significant role in this tertiary structure.

As fimbriae are largely proteinaceous (Day et al.1975; this

study Chapter 6) hydrophobic interactions probably exist between the non polar groups within fimbriae. When cells are transferred from an inorganic, aqueous environment to an organic environment (ether) the hydrophobic interactions would be destroyed which would, in turn, disrupt the tertiary structure of the proteinaceous fimbriae. Consequently, had the fimbriae been lying adpressed to the cell surface in "bundles" they may then have been able to radiate freely outwards from the cell. The possibility of fimbriae being embedded in cell walls and radiating outwards is unlikely since the wall is about 0,1um wide and the fimbriae are about 0,5um long.

That the naturally produced fimbriae are shorter than those which are artificially produced may be because, during natural exposure they are not completely unravelled, but are completely unravelled by ether treatment. Alternatively, the ether may remove a substance (possibly lipid) which embeds the lower portion of the fimbriae under natural conditions.

If fimbriae are an integral part of the cell wall prior to being exposed, this could account for the greater sensitivity of flocculent (as opposed to non-flocculent) cells to the enzyme B-glucuronidase (Day et al. 1975) While the difference was attributed to differences in the distribution of mannan it could also be that while lying

adpressed to the surface (as would occur in non-flocculent cells) they offer greater protection to the cells than they would once exposed and radiating away from them (as would occur in flocculent cells.)

Flocculation of cells with ether-induced fimbriae

The flocculation characteristics of the ether washed cells in the Helm sedimentation test were exactly the same as their water washed counterparts. This means that even though ether washing exposed fimbriae on non-fimbriate cells these fimbriae were unable to function in flocculation. If therefore, fimbriae do function in flocculation one of two processes must occur; either they are modified prior to the onset of flocculation (for example by the addition or loss of a protein), or receptor sites to which they can bind and which are not present (or are masked) on log phase cells must be elaborated on adjacent cells.

Sedimentation rates of S.uvarum 2036 log and stationary phase cells

In previous tests and in most subsequent tests log phase cells of the flocculent culture did not sediment at the same rate as the stationary phase cells. The log phase culture used for this test must have been in the late rather than mid log phase. It was later confirmed that late log cells, while not yet flocculent in wort, sediment at a similar rate to stationary phase cells in organic salts medium.

Intercellular binding is however, not as strong, as shown by the ease with which the late log cells were deflocculated.

The variable performance of the log phase cells in the Helm medium (compare with Table 1) indicates the importance of sampling log phase cells in the mid rather than the late log phase. While this was easily accomplished in static fermentations it could not be accurately judged in continuous fermentations. The cultures used for the ether tests (where identical settling rates were recorded for both log and stationary phase cultures) were presumably late log phase cultures. This conclusion is based on the observation that they had not yet become flocculent in wort and in the Helm medium showed a markedly different intensity of flocculation from the stationary phase cells (as measured by the ease with which the cells were resuspended).

3.4.2 MECHANICAL TREATMENT

The fimbriae of S.uvarum 2036 appeared to be highly resistant to removal by mechanical means. None of the methods (despite repeated trials) with which Day et al. (1975) had successfully removed the fimbriae of U. Violaceae removed the yeast fimbriae. Prolonged ultrasonication was found to be effective in reducing flocculation potential and at the same time reducing the number of fimbriate cells in

the population which suggests some correlation between the presence of fimoriae and the ability to flocculate. The inability to isolate intact fimbriae suggested that they had not been removed intact from the cells, but that they had been fragmented.

3.4.3 ENZYME TREATMENT

The only enzyme used which effectively removed the fimbriae without doing any electron microscopically detectable damage to the cell was pronase, which is proof that fimbriae are, at least in part, proteinaceous. This is in agreement with previous results for the removal of fimbriae from brewer's yeast (Day et al.1975).

Failure to detect fimbriae in the pronase extract indicates that they are not removed from the cells intact and are probably extensively damaged. Zymolyase effectively removed the fimbriae, but only after severe wall damage. It was presumed that the underlying glucan layer had been digested thus releasing the surface mannoprotein layer. Had this occurred, the fimbriae would have been released intact, but in only one extract were structures bearing any resemblance to fimbriae found (fig.33). Since the sum of the lengths of the fimbriae in figures 32 and 33 is 0,5um there is a possibility of these structures being the terminal portions

of the fimbriae. Further than this their resemblance to fimbriae is questionable as they appear to be thicker and more rigid than fimbriae. As the only reference to the successful isolation of fimbriae is an abstract (Poone et al. 1978) it is not known whether the fimbriae isolated resemble the structures in figure 33 or the fimbriae attached to the cells. That the β-glucuronidase and cellulase did not damage the fimbriae suggests that they do not comprise β-glucan or include β-1,4 glucoside linkages. The inability of α-mannosidase to remove the fimbriae was probably due to this enzyme being an exoenzyme which cleaves only terminal non-reducing α-D-mannose units. That some fimbriae appeared to be shorter than normal may have been due to slight contamination of the α-mannosidase with N-acetylglucosaminidase (NAG-ase). The mannose chains of glycoproteins are attached to the protein component via NAG and NAG-ase was present in the α-mannosidase used, albeit at a concentration of less than 1% (Manufacturer's specifications). The α-mannosidase from Jack bean, was found previously to have little effect on yeast mannan (Ballou, 1976).

As LAP is also an exoenzyme the absence of electron microscopically visible changes to the cell wall and fimbriae were expected.

As acid phosphatase cleaves phospho-mono-ester bonds and the

predominant cell surface linkages are phosphodiester it was not unexpected that no visible surface effects were observed.

Effect of enzyme treatment on flocculence

The flocculating ability of cells treated with zymolyase and pronase was totally destroyed, while those treated with β-glucuronidase showed a diminished ability to flocculate. As fimbriae were not damaged by the β-glucuronidase suggests that the poor flocculence could not be directly linked to fimbrial damage. Wall damage by zymolyase was also too great for a correlation to be drawn between the lack of fimbriae and non-flocculence. The inability of pronase-treated cells to flocculate could perhaps be linked to the loss of fimbriae. Eventhough pronase may have damaged surface components other than the fimbriae three features of pronase treated cells support this view. Firstly, the only electron microscopically visible damage to the cells was the loss of fimbriae. Secondly, cell viability only decreased by about 2%. Thirdly, although both fimbriae and the ability to flocculate were lost, the cells retained the ability to grow and multiply.

The stronger flocculation observed after lipase treatment was probably due to the lipase having "cleaned" the cells. This could have exposed masked binding sites, allowing them to participate in intercellular binding. As the fimbriae

were better displayed than those of untreated cells, these binding sites may be fimbrial groups.

The promotion of flocculation by α-mannosidase cannot be explained on the basis of electron microscopy. The possibility does exist that one of the changes occuring during the transition to flocculence may be the removal of terminal mannose units which exposes binding sites.

CHAPTER 4

ANTIBODY STUDIES

4.1 INTRODUCTION

The serological patterns of yeasts are variable, which is indicative of differences in their surface components. Mannan is the predominant antigenic component and it has been suggested that the various yeast mannans may confer the species-specific information necessary for the organization of the cell wall and for cell-cell recognition (Ballou, 1974), an observation which could be of particular significance to flocculation if indeed a specific biological recognition system does operate.

It was shown that the structural and immunological properties of flocculent and non-flocculent yeast were very similar although isolated mannan from the non-flocculent strains did precipitate more antibody than did the flocculent mannan (Cawley and Ballou, 1972).

The ability of fimbrial antiserum of Ustilago violaceae to agglutinate a variety of yeasts including Saccharomyces

cerevisiae was tested (Gardiner et al.1982). Results correlated well with electron microscopic examinations as the cells which were agglutinated were also fimbriate. It was concluded that a family of fimbrial proteins exists which has been conserved to a large extent amongst smut fungi, basidiomycetous and ascomycetous yeasts.

Antibodies against fimbriae were raised to facilitate more extensive research into the nature of the fimbriae than would have been possible with less specific techniques.

Yeast fimbriae are not easily visible, even when attached to the cells. Their small size makes fimbriae even more difficult to find when detached from the cell (particularly if low concentrations are present) and they could be confused with tiny folds in the formvar or even dust particles. By labelling the fimbriae with specific antibodies they would be easily distinguished from other structures.

Apart from positively identifying the fimbriae, the specific antibodies would also allow for the comparison of the antigenicity of the fimbriae with the rest of the cell surface, with log phase cells and with non-flocculent cultures. This would indicate;
i) whether or not fimbriae-specific components exist, ii) whether or not fimbrial carbohydrate and/or protein

is present on the cells throughout their lifecycles;

iii) whether or not their biochemical composition is shared by fimbriae of non-flocculent strains.

4.2. METHODS

4.2.1 ANTISERUM PRODUCTION

4.2.1.1 Preparation of the inoculum

As it had not been possible to remove intact fimbriae from the cells it was necessary to inject the rabbits with whole fimbriate cells. Cells of S.uvarum 2036 which had been through a normal fermentation in brewery wort and which had flocculated were harvested and washed four times in distilled water. They were then vortexed in diethyl ether over a period of one hour (to expose the maximum amount of fimbrial antigen) and thoroughly washed in distilled water. Cells were suspended in distilled water at about 10^9 cells/ml. This suspension was used for intravenous inoculation of the rabbit. Subcutaneous inoculation was performed on another rabbit and for this the cell suspension was emulsified with 1ml of incomplete Freund's adjuvant.

4.2.1.2 Raising the antiserum

Prior to inoculation of the rabbits, a 5ml blood sample was taken from each animal and the serum used to test for non-specific agglutination of flocculated yeast cells. This serum was later used as a control when titrating the antiserum.

Antiserum was raised in New Zealand white rabbits. The first animal was inoculated according to the procedure of Dawes et al.(1983). 0,5 ml of yeast emulsion was injected subcutaneously into each of four sites (one above each limb). After six weeks the rabbit was given booster injections using the same procedure. Nine days later the antiserum titre was checked by titration and on the tenth day the animal was bled. The second animal was inoculated intravenously with 0,8ml of aqueous yeast cell suspension. Booster injections were given at seven and fourteen days and the animal was bled on day 21 after titration of the serum.

4.2.1.3 Serum titration

Flocculated yeast cells were washed and deflocculated by suspending them in an EDTA-Isoton solution (1,85 g EDTA per litre of Isoton, pH 8,1) and then shaking them for ten minutes. Cells were then washed twice in distilled water and suspended in phosphate buffered saline at a concentration of 10^8 cells per ml. A round-bottomed

microtitre tray was used for the tritration which followed the following regime.

Table 6: Procedure used for antiserum titration.

WELL	1	2	3	4	5	6	7	8	9	10
PBS (ml)	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.5	0.025	0.1
ANTISERUM (ml)	0.05	←	DOUBLING DILUTIONS					→	0.05	—	—
YEAST CELLS (ml)	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	—
ANTISERUM DILUTION	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/2	—	—

The tray was incubated at 37 degrees centigrade for one hour and then examined for agglutination under an inverted microscope.

4.2.1.4 Purification of the IgG.

The purification method of Horejski and Semanta (1956) was followed.

The blood was allowed to clot in the cold for two hours after which the serum was decanted. The pH was adjusted to 8,0-8,5 with 0,1% NaOH and diluted with physiological saline, adding half the serum volume. 4% Rivanol was slowly added to the serum and vortexed after each addition

to precipitate other serum proteins. The amount of Rivanol added was 3,5 times the original volume of the serum. This was centrifuged at 10 000 x g for ten minutes and the supernatant fluid retained. The Rivanol was then precipitated by addding half the volume of the serum and Rivanol mixture and centrifuged at 15000 x g for 20 minutes. The supernatant was decanted and its volume measured accurately. To this two thirds of its volume of saturated ammonium sulfate was gradually added, mixing constantly, and allowed to stand at four degrees centigrade for 30 minutes. The solution was centrifuged at 10 000 x g for ten minutes and the supernatant fluid discarded. The precipitate was redissolved in 15ml of physiological saline, to which 10 ml of saturated ammonium sulfate was gradually added and again centrifuged at 10 000 x g for ten miniutes. This was repeated twice more. The final preciptate was dissolved in 10ml physiological saline and dialysed against glass distilled water for three days at four degrees centigrade. The antiserum was divided into 2 ml aliquots and frozen at -20 degrees centigrade.

4.2.1.5 Removal of some of the non-specific antibodies

Since whole cells were used to raise the antiserum a considerable portion of the antibodies raised would have been specific for cell surface antigens other than the fimbriae. As it was desirable that the antiserum be as specific for the fimbriae as was possible, it was absorbed

out using non-fimbriate cells. Smooth yeast cells were prepared by treating flocculated yeast cells with pronase (Chapter 3.3.1) and then washing them thoroughly to remove the debris. These "smooth" cells were then added to the purified IgG at a concentration of 10^{10} cells per ml, incubated for one hour at 37 degrees centigrade and then overnight at four degrees centigrade. Cells were separated from the IgG by centrifuging at 2500 x g for three minutes and then harvesting the supernatant.

The antiserum was again titrated against flocculated cells to ensure that all the anti-fimbrial activity had not been absorbed out. This could have occurred if all the fimbrial antigens were common with other cell surface antigens.

4.2.2 PRODUCTION OF ANTISERUM - HORSERADISH PEROXIDASE (HRP) CONJUGATES

4.2.2.1 Conjugation of antiserum with HRP

Some of the prepared antiserum was then conjugated with HRP (molecular weight 40 000 daltons). A modification of the two-step method was used (Avrameas and Ternynck, 1971). 20mg of HRP (Sigma Chem. Corp.) was dissolved in 0,50ml of 0.1 M bicarbonate buffer containing 2,5 % gluteraldehyde (see Appendix B) and incubated for two hours at room temperature. This was passed through a 60cm x 0,9 cm

Sephadex G25 column eluted with physiological saline containing 0,05 % NaN_3. This would remove HRP which had not been activated by the gluteraldyde. The activated fractions were pooled and concentrated by dialysis against polyethylene glycol to 20mg/ml using E 1% 403nm = 18.

2,5 mg IgG (E 1% 280 = 14,3) was dissolved in 1ml of physiological saline and added to 1ml of HRP plus 0,1ml 1M carbonate buffer (see Appendix B). After two days of incubation at four degrees centigrade 0,2M lysine was added to stop the reaction and left for two hours. This was then dialysed against physiological saline for three days and concentrated. Unconjugated antibody was removed by filtration through a 75cm x 2,5cm Sephacryl S200 column. Elution was monitored at 403 nm and the peak obtained was checked for the presence of protein at 280 nm, before being concentrated.

4.2.2.2 Determination of the purity of the IgG - peroxidase conjugate (see Appendix B)

In order to ensure against the blocking of specific labelling by unbound HRP, immunoelectrophoresis was carried out. The central well was filled with 10-20 ul of conjugate and electrophoresis was allowed to proceed for 60 minutes. The position of the enzyme, the antibody and the conjugate were determined by flooding the slide with 0,02 M guaicol and 0,1 % hydrogen peroxide for one minute, washing

the slide well with PBS and observing the appearance of a brown colour after a few minutes. The absence of separated bands towards the anode and cathode and the presence of stain around the well confirmed the purity of the conjugate.

4.2.2.3 Labelling the cells

Yeast cells from a normal fermentation were harvested at the desired stage (either log or stationary phase) and washed well in distilled water. They were suspended at a concentration of about 10^6 cells /ml. 0,25ml of suspension was added to a similar volume of conjugate and incubated shaking for two hours at 25 degrees centigrade. The cells were then thoroughly washed (five times) in glass distilled water to remove the excess conjugate.

4.2.2.4 Modification of conjugate

The peroxidase was stained with diaminobenzidine (BDH Chemicals, England) in 0.01 % hydrogen peroxide for five minutes at room temperature and the cells were then thoroughly washed.

4.2.3 FLUORESCENT ANTIBODY LABELLING

4.2.3.1 Preparation of conjugate

IgG was purified and absorbed out using pronase treated cells. The concentration of the antiserum was determined

from $E^{1\%}_{280nm}$ for IgG = 14,3 and adjusted to 1mg per ml with borate buffer, pH 9,3 (Appendix B). 5ml of antibody solution was dialysed overnight at four degrees centigrade against the borate buffer. This buffer was then replaced by 3mg fluorescein isothiocyanate (FITC) disolved in 100 ml borate buffer and again dialysed overnight at four degrees centigrade in the dark. The unbound flourochrome was removed by replacing the fluorescent solution with 0,15M sodium phosphate buffer, pH 7,4. Dialysis was allowed to proceed for five days, the buffer being changed twice daily. Dialysis was complete when the dialysis solvent no longer fluoresced under an ultraviolet lamp. The fluorescent conjugate was kept in a silicone coated glass container at four degrees centigrade in the dark.

4.2.3.2 Labelling the cells

The following cell types were thoroughly washed in glass distilled water and suspended in 0,1m phosphate buffer (pH7,4) at a concentration of 10^9 cells per ml: Saccharomyces uvarum 2036 (log phase from continuous culture), Saccharomyces uvarum 2036 (stationary phase), Saccharomyces uvarum 2036 (stationary phase; ether washed), Saccharomyces cerevisiae Y746 (stationary phase).

Fluorescent antibody was added to each sample (one volume of cells and one volume of antibody suspension) and incubated at 25 degrees centigrade for 45 minutes. Cells

were washed gently in phosphate buffer (four times) and mounted in 30% glycerol for viewing under ultraviolet light. Autofluoresence of the yeast cells was checked for.

4.2.3.3 Adsorption of the antibody using log phase cells

0,5 ml of the whole FITC labelled antiserum was added to 10^9 packed yeast cells (log phase from a continuous culture) and incubated, shaking for 30 minutes. The cells were spun down and disgarded and the supernatant fluid was added to another 10^9 cells. This procedure was repeated until the log phase cells no longer fluoresced under ultraviolet light. The supernatant was then added to 10^4 stationary phase cells of S.uvarum 2036 and incubated shaking for 45 minutes. The cells were rinsed and viewed under ultraviolet light.

4.2.4 IMMUNOSEROLOGICAL ELECTRON MICROSCOPY (ISEM)

This technique is based on the premise that proteins adsorb strongly onto the surfaces of the support films used for electron microscopy. It was performed according to the method of Leseman (1982).

Carbon coated formvar grids were treated with anti-yeast antiserum of varying dilutions. Serum dilutions of 1:250 to 1:2000 were used, as dilutions below 1:500 (of the particular antiserum tested) had been found to inhibit

trapping of virus particles (Leseman, 1982). Dilutions were made in 0,1M phosphate buffer pH7,0. The grids were floated film side down on droplets of antiserum on parafilm. They were incubated for 25 minutes at 28 degrees centigrade. Excess, unadsorbed antiserum was removed by washing the grids with 20 to 30 drops of buffer from a Pasteur pipette following which they were drained on filter paper. Grids were subsequently floated on antigen suspension for 30 minutes at 28 degrees centigrade and then carefully rinsed.

4.2.5 FERRITIN LABELLED GOAT-ANTI-RABBIT IgG

Ferritin labelled goat-anti-rabbit IgG (Sterilab) was used to label the rabbit IgG raised against the cell surfaces of flocculated, fimbriate yeast. Binding was most readily seen on cells which had been lysed by ultrasonication, presumably because these cells seemed to have lost their three dimensional structure under the EM vacuum and the cells lay adpressed to the formvar film. Water washed, ultrasonicated cells were therefore incubated with the "absorbed out" rabbit-anti-yeast IgG, shaking for 90 minutes at 25 degrees centigrade. The cells were gently washed and suspended in 0,1M sodium phosphate buffer, pH6,8. To this an equal volume of ferritin labelled goat-anti-rabbit IgG was added and allowed to react for a further 90 minutes. (Dilutions of the ferritin labelled

antibody in excess of two times resulted in poor labelling). Cells were gently washed, mounted on grids, air dried and viewed under the TEM.

4.3 RESULTS

4.3.1 ANTIBODY TITRE

Titration of the antiserum showed the antibody concentration to be 1/64. After being absorbed out with pronase treated stationary phase cells ie. non-fimbriate cells, the titre fell to 1/6.

4.3.2 LABELLING THE ANTIBODIES

Four methods of labelling were used, namely peroxidase, fluorescein isothiocyanate (FITC), ferritin labelled goat-anti-rabbit antiserum and ISEM.

4.3.2.1 Labelling with horseradish peroxidase

The success of the peroxidase conjugates was limited due to the poor visibility of unstained DAB-peroxidase conjugates under TEM and due to the effect of osmium tetroxide on the fimbriae. Only where large deposits of conjugate occurred i.e. when cells were incubated with a high concentration of conjugate and presumably where the peroxidase to antibody ratio exceeded 1:1 were labelled fimbriae to be found. Very few labelled cells were seen.

Figures 36 and 37 show a flocculated cell of S.uvarum 2036 with label approximately 0,5 um from the cell edge. Fine streaks are visible between the label and the cells and are interpreted as being fimbriae. Distinct fimbriae would not be seen as the cells were not shadowed.

Cells were not post-fixed with OsO_4.

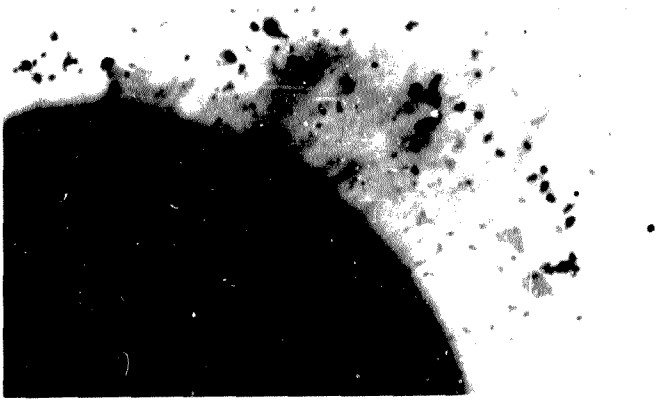


Fig. 36. Edge of a flocculated cell labelled with the HRP-antibody conjugate. Magnification: 46000x



Fig. 37. Portion of two flocculated, labelled cells with label extending 0,5um from the cell edges. Magnification: 29000x.

The peroxidase - DAB conjugate is rendered electron dense by post fixation with osmium tetroxide (Kurstak et al. 1977). Three methods of OsO_4 fixation were carried out.

i) Fixation of mounted cells by vapourization, using 2% and 10% OsO_4 for two minutes and for five minutes.

ii) Fixation of mounted cells by floating grids on 1% OsO_4 for varying time periods, followed by thorough washing.

iii) Fixation of aqueous suspended cells for 20 minutes following the method of (Kuhlmann, 1977).

Methods ii) and iii) were performed on both unfixed and formalin-fixed cells.

Cells subjected to the first treatment appeared to be no different from untreated cells. All preparations using methods ii) and iii), with one exception, showed well defined, fixed cells with completely smooth surfaces and therefore no fimbriae. The one minute of post fixation of ether washed, but not formalin fixed cells produced the effect seen in figure 38.

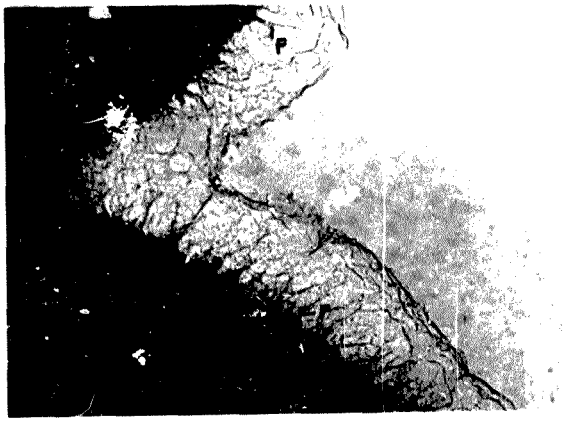


Fig. 38 shows part of the edges of two yeast cells post fixed with osmium tetroxide for one minute.
Magnification: 80 000 x

The short period of post-fixation with OsO_4 presumably allowed for incomplete fixation and under the electron beam the DAB-OsO_4 complexes broke down, the area around the

cells becomming translucent and enabling the fimbriae to be seen. The fimbriae seen here are about half the length of those normally seen i.e. a about 0,25um and are largely degraded. Examination of the outer edges of these fimbriae [illegible] network of fine fimbriae-like structures forming a "seal" around the fimbriae. The fimbriae, which are largely proteinaceous (Chapter 3.3.) were probably disrupted by the OsO_4, which although buffered, is nonetheless a strong acid. As the exposure to the OsO_4 was short only partial degradation of the fimbriae occurred, the disrupted fragments forming a seal around the edges of the remaining parts of the fimbriae.

Due to the effects of the osmium tetroxide on the fimbriae other labelling techniques had to be used.

4.3.2.2 Immunosurological Electron Microscopy (ISEM). The ISEM technique of was performed on fimbriate cells, on zymolyase cell wall extracts and on the extracts obtained from the mechanical procedures for the isolation of fimbriae.

Negatively stained antibody was easily detected on the grids, but fimbriae were not seen. Antibody was visible around the edges of the fimbriae of intact cells, but this was indistinct and was not seen along the length of the fimbriae.

4.3.2.3 Ferritin labelled goat-anti-rabbit antiserum

The reasons for using this antiserum were twofold: Firstly to see where the rabbit anti-yeast antibody was binding and secondly to establish a technique by which isolated fimbriae could be labelled and identified.

Figures 39 and 40 show the edges of flocculated cells of S.uvarum 2036 which had been labelled with rabbit-anti-yeast antiserum and then counter labelled with ferritin conjugated goat-anti-rabbit antiserum.



Fig. 39. Fimbriae appear to be present on the edge of this cell and are labelled along their lengths. The dense mat of fimbriae seen on most flocculated cells was not apparant. Magnification: 53000x

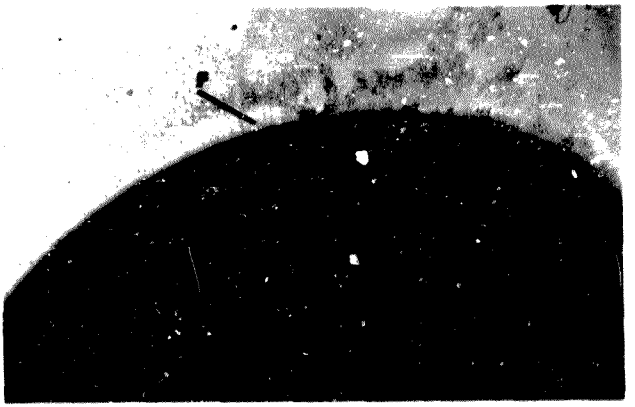


Fig. 40. A dense mat of fimbriae seem to be present around the edge of this cell, but are not labelled along their lengths. Label is only present at the ends of the fimbriae. Magnification: 28000x

Control cells (incubated with goat-anti-rabbit antiserum only) showed very little labelling and the binding seen in figures 39 and 40 is therefore likely to be specific binding of the goat-anti-rabbit antiserum to the rabbit-anti-yeast antibodies with which the cells had been incubated. No differences in labelling of the fimbriae were apparant when log and stationary phase cells were compared, although log cells were generally only labelled the cell surface indicating the absence of fimbriae.

4.3.2.4 Labelling with FITC

FITC - antibody conjugates were prepared since the antibody could then be used at the light microscope level. Using fluorescent antibodies similarities and differences between fimbriate and non-fimbriate cells (both flocculent and non-flocculent) could be established, since the cells did not autofluoresce.

As shown in figures 41 to 43 all three samples showed fluorescence, indicating common surface antigens. Fluorescence of the flocculated sample (S. uvarum 2036) was "stronger" than that of the other samples. The ether washed S.uvarum 2036 could not be distinguished from the water washed sample using the FITC label despite the antiserum having been raised to ether treated cells.

When the fluorescent antibody was adsorbed out with log phase cells until these no longer fluoresced, stationary phase (flocculated) S. uvarum 2036 incubated with the absorbed out antiserum exhibited very weak fluorescence.

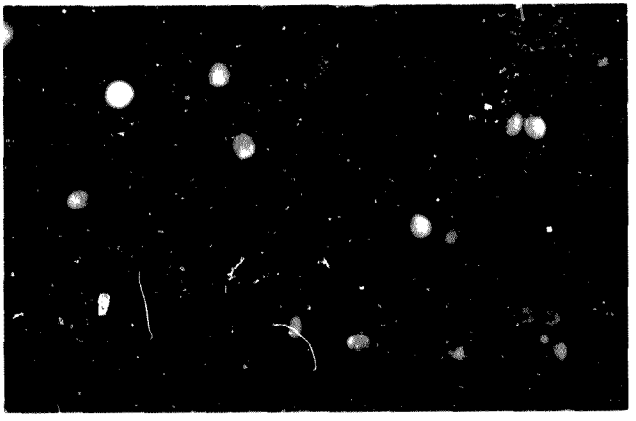


Fig.41. FITC-antibody labelled S.uvarum 2036 (flocculated sample): Comparison with a light micrograph of the same field showed that all cells fluoresced. Some cells fluoresced more intensely than others. The reason for this is not known, but is unrelated to the incubation time. Magnification: 1250x

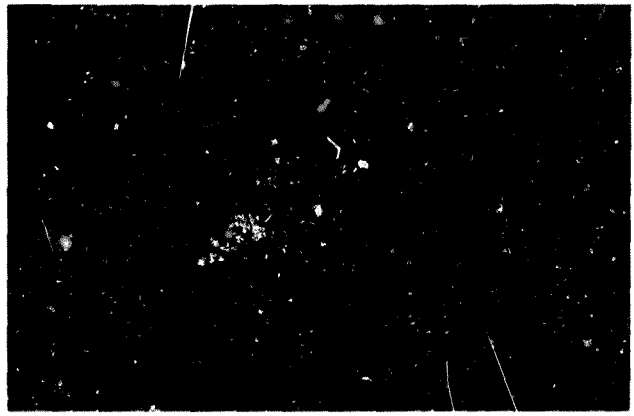


Fig.42. S.uvarum 2036 (log phase): Fluorescence was not as intense as that of the flocculated cells. Magnification: 1250x



Fig.43. S.cerevisiae Y746 (Stationary phase): Fluorescence was very similar to that of log phase S.uvarum 2036. A considerable amount of common antigenicity is therefore indicated. Magnification:1250x

4.4 DISCUSSION

4.4.1 ANTISERUM PRODUCTION

Production of antiserum to purified fimbriae would have been far more desirable than the method used here. Unfortunately, due to the inability to isolate intact fimbriae, antiserum had to be raised against whole cells. Antibodies to all wall components would therefore have been produced. The method used to absorb out some of the non-fimbrial antibodies was also not ideal since much of the surface mannan would have been removed by the pronase treatment. As most of the terminal groups (which were probably common to both the flocculent and non-flocculent cells) would have been lost, the antibodies to these would not have been removed from the serum. Despite the drawbacks of this method the antiserum titre did drop from 1/64 prior to absorption to 1/6 after absorption, indicating that the "fimbriae-less" cells did nonetheless remove many of the antibodies.

4.4.2 LABELLING THE CELLS WITH THE ANTISERUM

4.4.2.1 HRP-antibody labelling

Although the yeast used in this study does have endogenous peroxidase activity this should not have interfered with

the results obtained using HRP-antibody conjugates, the reason being that the conjugates were to be used to detect isolated i.e. detached fimbriae. However, before this could be done it was necessary to assertain (on intact cells) whether or not the conjugates did label the fimbriae.

The coloured substrate of HRP, namely diamino-benzidine is not readily detected at the electron microscopic level (Kurstak et al.1977) and therefore if used for EM examination it must be counterstained with osmium tetroxide. The osmium tetroxide appeared to both fix the cells and to disrupt the fimbriae (figure 38). It would not therefore have been possible to detect isolated fimbriae using the HRP-antibody conjugates and other labelling techniques had to be used.

4.4.2.2 ISEM

This technique was developed to enable the detection of low concentrations of virus particles at the EM level as well as to obtain information on the antigen relatedness of various viruses. Because of its sensitivity it was used in this study to detect fimbriae in the extracts from the procedures used to remove them, since, had they been removed intact they would almost certainly been present in low concentrations.

Failure to detect fimbriae in any of the extracts may therefore indicate that none were present. However, as the effect of PTA on fimbriae was later found to be inconsistent and possibly damaging this may have had some bearing on the inability to identify fimbriae using ISEM.

That individual fimbriae on intact cells did not show up clearly may have been due to their being too dense and therefore the interstices between them too small to allow for adequate contrast between antibody molecules and negative stain of adjacent fimbriae.

4.4.2.3 Labelling with ferritin labelled goat-anti-rabbit antiserum

The lack of labelling along the lengths of the fimbriae in figure 40 may have been due to the presence of a dense mat of fimbriae. The interstices between them may also have been too small to allow free penetration by the ferritin-labelled antiserum, since (as shown in figure 39) binding was able to occur when the fimbriae were not too close together. The technique was therefore inadequate for labelling attached fimbriae, but may be suitable for labelling and detecting isolated fimbriae.

4.4.2.4 Labelling with fluorescent antibodies

Due to the problems experienced with the EM-antibody techniques, fluorescent labelling was used in order to get

an indication of the antigenic properties of flocculent (fimbriate) and non-flocculent (smooth) cells.

All cell samples which were incubated with the FITC antibody conjugates fluoresced confirming that common antigens are shared by all of them. The stronger fluorescence exhibited by the flocculated S. uvarum 2036 cells was expected since it was against these cells that the antiserum was raised. However, the fluorescence of these cells was stronger than that of the non-flocculated (log phase) cells of the same strain which indicated that the surface antigenicity did change between the log and stationary phases. This was confirmed by absorbing out the antiserum with log phase cells until these no longer fluoresced and then incubating the residual FITC-conjugates with flocculated cells. These cells did fluoresce, albeit very weakly, indicating the presence of an antigen(s) on the surface of flocculated yeast which was not present on log phase cells. The possibility of this additional antigen(s) being flocculation-specific and perhaps even a fimbrial component is not remote. The stronger fluorescence of the stationary phase cells in comparison with the others may have been contributed to by this additional antigen(s).

That ether treated cells did not fluoresce more brightly than the water washed cells suggests that ether washing, while it exposes fimbriae, does not significantly change

the surface antigenicity. (The fluorescent technique is probably not sensitive enough to detect minor changes.) This confirms earlier results that exposure of the fimbriae is not sufficient to induce flocculation and that the modification of the cell surface (presumably of the fimbriae) is also required. Had the ether treated log phase cells exhibited stronger fluorescence than water washed log cells this would have indicated that the treatment did expose an additional antigen(s) and that the inability of these cells to flocculate would have been difficult to account for.

It had been hoped that fluorescent labelling would have shown distinct differences between fimbriate and non-fimbriate cells, some cells fluorescing strongly and some not. While the intensity of fluorescence of cells within each population did differ this could not be correlated with the percentage of fimbriate cells as determined by the shadowing technique.

While these results do indicate the presence of a flocculation related antigen, the limitations of the experiment must be realized. The use of antibodies not specific for fimbrial antigens of flocculent cells and of a light microscopic technique for the illucidation of an electron microscopic problem, mean that the results are to some extent subjective.

CHAPTER 5

THE CARBOHYDRATE COMPOSITION OF THE FIMBRIAE

5.1 INTRODUCTION

It was established with enzyme treatments that the fimbriae are, at least in part, proteinaceous. It is very likely that they are glycoprotein since the outer cell wall is composed of protein and mannan and since mannoprotein structures are thought to be involved in flocculation (Day et al. 1975). A number of techniques were used to confirm this and to show the nature of the carbohydrate component.

5.2 METHODS

5.2.1 LABELLING CELLS WITH FERRITIN CONCANAVALIN A.

Water washed and ether washed cells were suspended in 0,05M sodium phosphate buffer, pH 6,8 to which the ferritin Con A (Sigma Chemical Co,St.Louis) was added at a concentration of 2,5 mg per ml. The reaction was allowed to proceed for 30 minutes at 25 degrees centigrade and the cells were then

washed in four changes of buffer and finally in distilled water. Washed cells were mounted on formvar coated grids and viewed at 80kV on the JEM 100S electron microscope.

Alternatively, the cells were mounted prior to labelling and allowed to dry. The grids were then floated face down on a drop of ferritin Con A for five minutes and washed by floating on eight changes of fresh buffer and then on distilled water (Nicholson and Singer, 1971).

5.2.2 DETERMINATION OF THE NATURE OF THE CARBOHYDRATE COMPONENT

Since the only method to successfully remove the fimbriae was treatment with pronase, this enzyme was used to obtain a fimbrial extract, the carbohydrate composition of which could then be analysed.

Thoroughly washed cells from both log and stationary phase cultures of the flocculent and non-flocculent cultures were suspended in 6ml of Tris buffer plus pronase (see Table 4) at an optical density of E 540nm = 2,60. This same solution of Tris-pronase was used later as a blank against which the samples were read. Pronase digestion was allowed to proceed for one hour at 30 degrees centigrade in an orbital shaker (100 rpm). Cells were spun down and the supernatant fluid either frozen at -20 degrees centigrade

or analysed immediately.

The presence of mannan in the extracts was expected since digestion of the protein components of the mannoprotein surface molecules would also release a considerable amount of mannan. However, the presence in the extract of the other major wall carbohydrate, namely glucose, would have raised the possibility of the fimbriae being partially glucose, while its absence would have suggested that the carbohydrate component was mannan.

Two methods were used in an attempt to establish the presence or absence of glucose.

5.2.2.1 Sulphuric acid - carbazole differential extinction method

A modification of the test described by Mc Murrough and Rose (1967) was used. At the wavelengths 475mu and 535mu the relative amounts of absorption of different sugars are signifcantly different and the ratios of their extinction values at these wavelengths would be characteristic for each sugar (Gurin and Hood, 1939). Consequently this test allows for the determination of the relative proportions of glucose and mannose in a mixture and subsequently of the exact amounts of the sugars present. All determinations were performed in duplicate. To 0,5ml of sample in a

boiling tube was added exactly 5ml of sulphuric acid diluted with water in the proportion of eight parts of sulphuric acid to one part of water. Each sample was vortexed and cooled. 0,3 ml of carbazole reagent was then added and the mixture again vortexed. The carbazole reagent comprised 0,5% w/v carbazole which was first sublimed to remove impurities. This sublimate was disolved in 95% v/v ethanol. Samples were covered with foil caps and placed in a vigorously boiling water bath for exactly ten minutes. They were then cooled and their extinctions read in a spectrophotometer in a 1cm cuvette. Extinctions were read at 535mu and 435mu since these are the wavelengths at which glucose and mannose respectively give maximum extinction peaks. A standard curve was obtained from solutions containing various ratios of the two sugars and these were performed in triplicate for four different sugar concentrations. The ratio of E535/E435 was plotted for each standard and the ratios of mannose to glucose read off the curve from the sample values of E535/E435. Using standard solutions of each sugar it should then have been possible to calculate the exact amount of each sugar in the samples from the value for E535.

5.2.2.2 Glucose - 6 - Phosphate dehydrogenase (G-6-P D) Test

Fimbriae were removed from 6ml of cells suspended at a

concentration of E 540nm = 2,70 by treatment with pronase (see Table 4). Cells were then removed by centrifugation and the supernatant fluid was tested for the presence of glucose using G-6-P dehydrogenase. The proceedure followed was that given in the Boehringer Mannheim catalogue (Food Analysis), but the individual enzymes rather than the test kit were used. Glucose was converted to glucose-6-phosphate by the addition of hexokinase and ATP according to the reaction:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{G-6-P} + \text{ADP}$$

In the second part of the reaction glucose-6-phosphate was converted to gluconate-6-phosphate with the reduction of NADP to NADPH + H according to the reaction:

$$\text{G-6-P} + \text{NADP} \xrightarrow{\text{(G-6-P-D)}} \text{gluconate-6-P} + \text{NADPH} + \text{H}.$$

The amount of NADPH produced was then measured. Separate samples of the fimbrial extract were also treated with β-glucuronidase (Sigma Corporation) and α-amylase (Boehringer Mannheim) prior to conversion to G-6-P. As the pronase used to remove the fimbriae from the cells would probably have destroyed the other enzymes this was inactivated by placing the samples in a boiling waterbath for 45 minutes. The glucuronidase and α-amylase were also inactivated before testing for glucose. Reactions were

carried out in serological test tubes to which the following were added:

Table 7. Glucose-6-phosphate test to establish the presence of glucose on fimbrial extracts.

SOLUTION	REAGENT BLANK	ENZYME BLANK	SAMPLE
Solution 1 (see Appendix A)	1,0ml	1,0 ml	1,0ml
Sample	-	-	0,1ml
Inactivated Enzyme solution	-	0,1 ml	-
Glass-distilled water	2,0ml	1,9ml	1,9ml
The tubes were vortexed and the absorbance read after three minutes. The enzymes were then added and mixed.			
Hexokinase	21ul	21ul	21ul
G-6-P-D	21ul	21ul	21ul
After 15 minutes the E340 was read and the amount of glucose calculated from formula 1.			

The experiment was repeated using 1ml of sample rather than 0,1 ml and the amount of glass-distilled water added was proportionally reduced.

Formula 1

$$c = \frac{V \times \text{molecular weight} \times A}{E \times d \times v \times 1000}$$

where: c = glucose concentration
V = final volume (ml)
E = absorption coefficience of NADPH at 340 nm = 6.3 ($l \times mmol \times cm$)
d = light path (cm)
v = sample volume (ml)

This test was again repeated on extracts from cells which had been ether washed prior to pronase treatment in order to expose the maximum number of fimbriae.

5.3 RESULTS

5.3.1 LABELLING FIMBRIAE WITH FERRITIN CON A

The ability of lectins to bind tightly and reversibly to cell surfaces without entering the cells makes them effective surface probes. Due to their specific sugar binding capacities they are frequently used as probes to determine both the nature of cell surface carbohydrates and their localization.

The lectin concanavalin A has an affinity for both glucose and mannose residues. When attached to the electron dense iron compound, ferritin, the sites at which the Con A has bound can be seen under TEM. Thus it could be established whether or not the fimbriae did contain carbohydrate, depending on whether or not they were labelled by the ferritin-Con A.

Fimbriate, flocculated cells of S. uvarum 2036 were treated with ferritin-Con A and examined under the transmission electron microscope. Figures 44 and 45 from two different preparations are typical of the types of labelling seen.



Fig. 44. The ferritin label around the cell radiates outwards in fimbriae-like streaks and beneath some of this label, faint streaking, resembling fimbriae can be seen. Magnification: 32000x

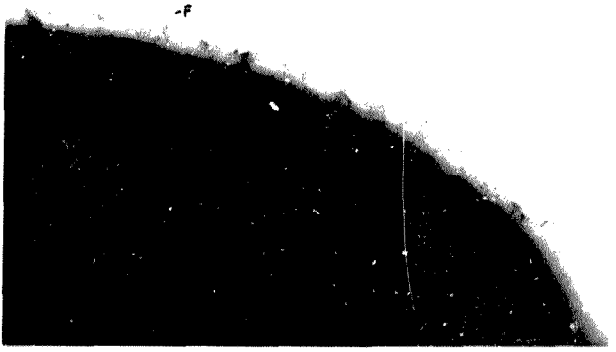


Fig. 45. This type of labelling was seen more frequently. There is less background stain and, while the fimbriae-like streaks are still clearly visible they are not as long as those seen in figure 44. Magnification: 42000x

These results do show that carbohydrate is present, along the lower portion of the fimbriae, but are ambiguous re the terminal portion. From figure 44 it could be concluded that the terminal portion is carbohydrate due to the presence of a heavy label 0,5 um from the cell edge. However this effect could also have been caused by cell shrinkage during drying, the band of label being the original position of the cell edge. From the more effectively washed preparation i.e. where less background label is apparant (figure 45) it could be concluded that

the terminal portion of the fimbriae is neither glucan nor mannan since labelling extends less than 0,5 um from the cell edge. The possibility cannot however be ruled out that some terminal label may have been lost during washing.

5.3.2 DETERMINATION OF THE NATURE OF THE CARBOHYDRATE COMPON NT

Having confirmed that the fimbriae are glycoprotein the possiblity of their being gluc- nan was investigated using two methods. The first method used was the sulfuric acid carbazole test used by Mc Murrough and Rose (1967) for determining changes in the glucan and mannan contents of yeast cell walls Fimbriae were removed from both log and stationary phase lls of S.uvarum 2036 using pronase. The mannan:glucan ratio of the extract was analysed. Results from this test were considered to be invalid since, despite accounting for the added enzyme, the ratios from nine different determinations were all negative as shown in figure 46.

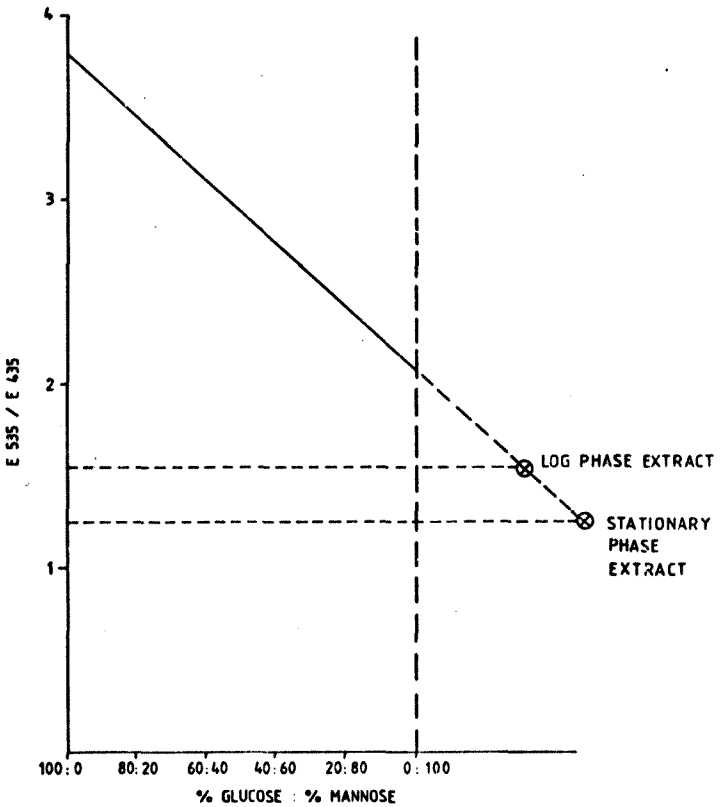


FIGURE 46 : STANDARD CURVE FOR GLUCOSE : MANNOSE RATIOS FOR THE SULPHURIC ACID - CARBAZOLE TEST.

It was assumed that interference in the reaction was caused by another component/s present in the pronase extracts. That the value for the pronase blank was slightly variable and since DNA has been reported to give significant values for E 535/E 435 (McMurrough and Rose, 1967). DNA contamination from either the pronase or from cell lysis (although this was less than 2%) may have been responsible for these results.

The other method employed was a glucose-6-phosphate dehydrogenase (G-6-PD) test. All samples were read against appropriate blanks. The following results were obtained.

Table 8. Results of the G-6-P-D test for the presence of glucose in fimbrial extracts.

Sample	A 340nm *	ug glucose/ml
Pronase only		
S. cerevisiae 1**	0,010	0,869
S. cerevisiae 2***	0,005	0,435
S. uvarum 1	0,004	0,348
S. uvarum 2	0,004	0,348

continued.....

Pronase and β-D-glucuronidase		
S. cerevisiae 1	0,002	0,174
S. cerevisiae 2	0,005	0,435
S. uvarum 1	0,010	0,869
S. uvarum 2	0,009	0,782
Pronase and α-amylase		
S. cerevisiae 1	0,005	0,435
S. cerevisiae 2	0,009	0,782
S. uvarum 1	0,003	0,261
S. uvarum 2	0,002	0,174
Glucose control		
10 ug/ml	0,107	9,32
100 ug/ml	1,053	95,10

Note: * Average of 3 determinations.

* * Sample 1 = log phase sample

* * * Sample 2 = stationary phase sample

The results of this test are below the sensitivity limit of the test which is about 3ug/ml. Had the major carbohydrate component of the fimbriae been glucose, results well within

the sensitivity limits of this test would have been expected. These results, in conjunction with the results of the enzyme degradation tests suggests that mannose is probably the primary fimbrial carbohydrate.

5.4 DISCUSSION

Is any polysaccharide present in the fimbriae?

As it had not been possible to obtain a pure preparation of isolated fimbriae, the detection of polysaccharide within fimbriae was made more difficult than it would otherwise have been. The sensitivity of fungal fimbriae to chemical treatment (Poone and Day, 1975; this study, Chapter 3) also made it undesirable to use carbohydrate- specific stains, although these have been used in other studies. Casson (1984) in his study on the adherence of yeast to beer dispense pipes used ruthenium red, alcian blue and silver methenamine and from his results concluded that the fimbriae on his cells were either polysaccharide or glycoprotein. However, as these fimbriae were embedded in a polysaccharide film, coating of the fimbriae with this polysaccharide could have occurred. Ferritin labelled Con A was the "stain" of choice for this study as Con A binds to both major wall sugars, namely glucose and mannose and the possibility of physical damage to the cell wall by the lectin would be remote.

Interpretation of the results was nonetheless not easy as a certain amount of cellular dehydration generally occurs in air dried preparations. That the basal portion of the fimbriae comprises some polysaccharide is clearly shown in figures 44 and 45, but some doubt still exists as to the nature of the terminal portion. As the tip of the fimbriae would most probably be that part of the structure involved in initial receptor recognition and binding it is of considerable importance to establish its nature. Since the staining was generally not very heavy the fimbriae are presumably predominantly protein rather than polysaccharide.

Is the carbohydrate component glucan or mannan?
The cell walls of brewer's yeast comprise essentially two layers, an inner glucan layer and an outer mannoprotein layer. In none of the literature reviewed has reference been made to the presence of glucan on the exterior surface of the wall. There is however no direct evidence that fimbriae do not comprise glucan since it is not known from which layer they arise, and because they have not been widely acknowledged in the literature (they are probably lost during the preparation of walls for analysis).

The fimbriae of U. violaceae were found not to include

polysaccharide as they did not stain with ruthenium red or PTA although the possibility of stabilization with carbohydrate was not excluded (Day et al. 1975). That this carbohydrate could be glucan was ruled out by the finding that fimbriae were not extruded in the presence of cyclohexamide (Poone and Day, 1975) which suppresses manno-protein synthesis, but which has no effect on the production of glucan fibrils (Baker and Kirsop, 1972). Day et al. 1975, also suggested that the yeast fimbriae comprise manno-protein, included in their evidence being the increased wall mannan content of floccu[illegible]pposed to non-flocculent strains (Stewart et al. 1[illegible] A later report (Gardiner, 1981) referred to an abstract (Poone et al.1978) in which it was stated that chemical analysis of the fimbriae of S. cerevisiae had found them to be protein and carbohydrate, probably mannan.

Thus, while correlative evidence does exist to suggest that yeast fimbriae do contain mannan it would nonetheless have been short sighted to merely assume that no glucan was present. The presence of glucan in the fimbriae was therefore tested for.

Using the sulfuric acid - carbazole test originally described by Dische (1930), (cited in Gurin & Hood, 1939) and modified for yeast wall analysis by McMurrough and Rose (1967), it should have been possible to determine the ratios

of glucose to mannose in the fimbrial extracts and hence the variation of these ratios between different extracts. However, repeated determinations produced results which had a glucose to mannose ratio exceeding 0:100 ! The log phase extract did have a consistently higher ratio than the stationary phase extract, but this value was still not on the standard curve.

Results of the glucose - 6 - phosphate dehydrogenase test indicated that the predominant polysaccharide was almost certainly not glucan. Thus, by a process of elimination it was established that the carbohydrate component was mannan. This is in agreement with the results of Poone et al. (1978). Very small quantities of glucose were found, the largest amount being 0,869ug/ml, which is extremely low. Assay instructions state that the amount of glucose in the cuvette should be within the range of 3-80ug/ml.

Therefore, while it is probably quite safe to conclude that mannan is the major fimbrial polysaccharide, the possibility of some glucan linkages cannot be disregarded.

CHAPTER 6

THE CELL WALLS OF FLOCCULENT AND NON-FLOCCULENT YEASTS

6.1 WALL PROTEINS

6.1.1 INTRODUCTION

Although treating cells with proteolytic enzymes does not prove that proteins are involved in flocculation (other components are also removed), Nishihara et al. (1977);(1982) showed that cells can also be deflocculated by treatment with protein modifying agents. They concluded that a number of functional groups of the amino acid side chains of cell wall proteins such as disulfide bridges, carboxyl and phosphate groups, amino groups, the imidazole groups of histidine and phenolic hydroxyl groups of tyrosine are probably all essential for flocculation. Some of these are probably buried within the proteins as they were only modified in the presence of 8M urea. They could therefore play an important role in maintaining the steric structures of some of these proteins, the alteration of which would prevent floc formation.

Proteins from the cell wall extracts of flocculent and non-flocculent cells were separated by gel electrophoresis to establish whether or not different proteins are present in the walls of the S.uvarum 2036 when in the non-flocculent and flocculent phases.

Different wall proteins in flocculent and non-flocculent cells have been looked for in a number of laboratories, but results are inconsistent. Miki et al.(1982b) found neither proteins which were specific to flocculation, nor any which could be correlated with the induction of flocculation in any of their strains. Holmberg (1978), using a genetically characterized flocculent strain of S. cerevisiae which was mutated for non-flocculence, showed the existence of a 12 900 dalton protein in the flocculent culture that was absent from the non-flocculent culture. This protein was thought to be a subunit of a much larger multimeric protein. Stewart (1981) found a 37 000 dalton protein in extracts of flocculent cells which could not be found in non-flocculent extracts.

It was therefore thought necessary to establish whether or not a flocculation specific protein/s could be identified in the wall extracts of the fimbriate, flocculent strain used here.

6.1.2 METHODS

Alkaline extracts were obtained from the cell walls of log and stationary phase cells of the flocculent strain S.uvarum 2036 and of stationary phase cells of the non-flocculent S.cerevisiae Y 746. These extracts were then run electrophoretically on polyacrylamide gels.

6.1.2.1 Cell extracts

Alkaline extracts were obtained by following the method of Holmberg (1978) with slight modifications. Cells were harvested from fermentations, washed four times in distilled water to remove protein absorbed from the wort and then resuspended in 5ml distilled water at an optical density at 540 nm of 2,6. To each sample 0,1ml of 1M NaOH was added and the suspension shaken for 60 minutes at 30 degrees centigrade. The cells were pelleted and the supernatant neutralized by the dropwise addition of 0,5 M HCl. The samples were put on ice, following which 5,3 ml of ice cold 12,5 % TCA was added. The samples were left at four degrees centigrade overnight and the protein precipitates harvested the next morning by centrifugation.

Pellets were solubilized in 0,2ml to 0,5ml of splitting solution (Appendix C) depending on the size of the pellet and were placed in a boiling water bath in a fume hood for five minutes. The protein concentration of each sample was

determined using the Bio-Rad Protein Assay kit (Bio-Rad Labs., Munich) and bovine serum albumin (fraction V) standards.

6.1.2.2 Polyacrylamide gel electrophoresis

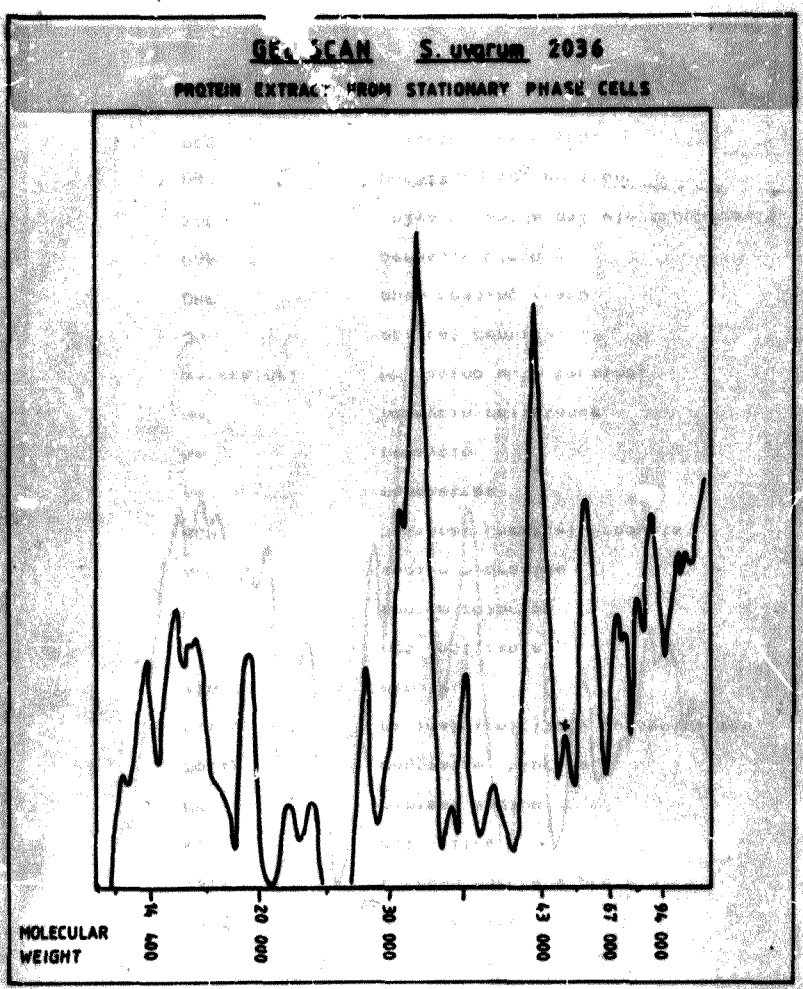
12% polyacrylamide gels (Appendix C) were poured the day before electrophoresis. The electrophoresis bath was assembled with bath buffer (Appendix C) covering the electrodes and the gels preelectrophoresed overnight with a current of 15 mA per gel. 50-100 ug of protein solution was carefully layered into each well. A sample containing low molecular weight markers (see Appendix C) to which bromothymol blue (BTB) had been added was placed in the first well of each gel. Gels were electrophoresed with a current of 40 mA per gel until the BTB marker reached the bottom of the gel.

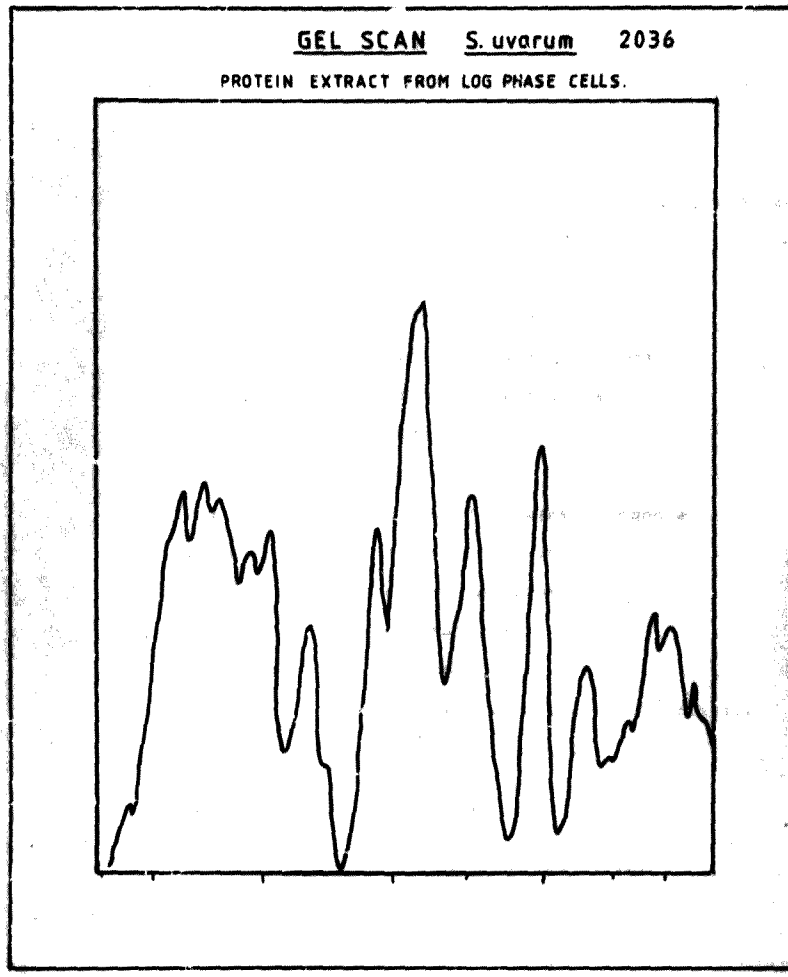
Upon completion of electrophoresis the gels were removed from their cassettes and fixed in 15 % TCA for two hours. Staining was carried out overnight at 37 degrees centigrade in covered glass troughs following which gels were destained (Appendix C) for two to three hours (or until the protein bands could be clearly distinguished from the background). Gels were then scanned using a laser densitometer (LKB 2202 Ultroscan) and the protein peaks of different samples compared.

6.1.3 RESULTS

Numerous electrophoretic runs had to be performed to obtain gels which could be scanned. All tracks stained heavily making detection of the protein bands, which were very close together extremely difficult. In order to reduce this streaking effect, ammonium sulfate was also used to precipitate the protein and an attempt was made to remove some of the mannan from the glycoprotein. Neither α-mannosidase treatment followed by dialysis of the extract nor ammonium sulfate precipitation produced clearer results than TCA precipitation. The scans presented came from TCA precipitated samples.

With continuous repetition gels from two experiments (both run in duplicate) were obtained which were adequate for scanning. The results all corresponded and the best scans from each extract are presented.


GE. SCAN S. uvarum 2036
PROTEIN EXTRACT FROM STATIONARY PHASE CELLS
MOLECULAR WEIGHT
14 400
20 000
30 000
43 000
67 000
94 000


GEL SCAN S. uvarum 2036
PROTEIN EXTRACT FROM LOG PHASE CELLS.

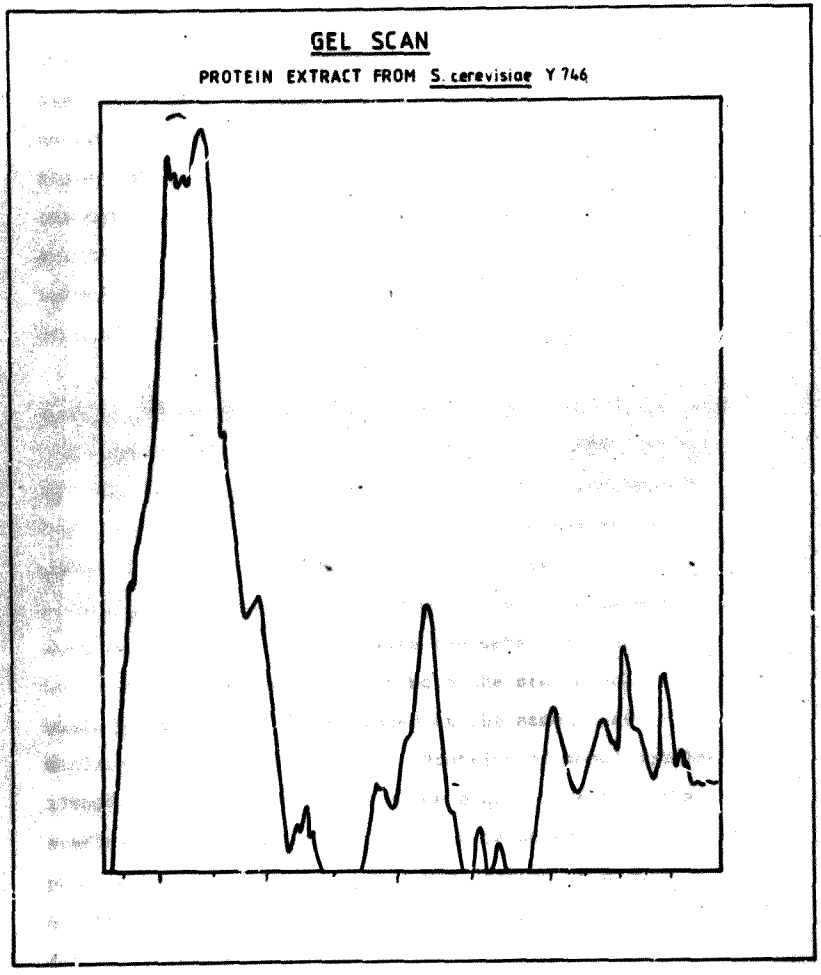


Fig. 47. Gel scans of the protein extracts from walls of S.uvarum 2036 (log and stationary phases) and of S.cerevisiae Y746 (stationary phase).

From the gel scans presented in figure 47 it can be seen that the protein composition of the walls of log and stationary phase S.uvarum 2036 is very similar. However, an extra protein is apparant in the wall extract of the flocculent (stationary phase) cells which is absent from the non-floculent (log phase) cell extract. This protein was also absent from the extract of stationary phase cells of the non-flocculent control culture. The molecular weight of the protein is about 48000 daltons.

Clearly, there are quantitative differences in the protein component of the walls of log and stationary phase cells, although there is very little overall difference between the protein species. The glycoproteins of log phase extracts were generally poorly resolved and no electrophoretic runs of the log phase non- flocculent culture were obtained which were adequate for scanning. Consequently, when extracts of both the stationary and log phase S.uvarum 2036 were scanned at the same sensitivity, differences appeared to exist. Proteins of about 13000d, 27000d, 48000d and 68000d which were all present in the stationary phase extract appeared to be absent from the log phase extract. When this track was scanned at a much greater sensitivity all proteins, with the exception of the 48000d protein were identified.

6.2 WALL CHARGE

6.2.1 INTRODUCTION

The walls of yeast cells are generally negatively charged (Jansen and Mendlik, 1951; cited in Geilenkotten and Nyns, 1971) which results in overall repulsion between adjacent cells and is one of the factors which contribute to keeping fermenting cells in suspension. A number of electrophoretic mobility studies have been carried out to determine cell charge and variation in this charge throughout the growth cycle.

In the light of this information it was decided to see whether or not such a change in surface charge occurred in S. uvarum 2036 and if it did whether or not it could be correlated with the appearance or presence of fimbriae. To this end cells were treated with cationized ferritin which has a high affinity for negative charges and is visible under an electron beam.

6.2.2 METHODS

Labelling cells with cationized ferritin

Cells were labelled according to the procedure used by Berdicevsky (Pers.comm.) with minor changes. Washed cells were rinsed in cacodylate buffer, pH 6,8 and then suspended

in 250 ug per ml of cationized ferritin (Sigma Corporation) in cacodylate buffer. Cells were incubated with gentle shaking at 28 degrees centigrade for 30 minutes. They were then washed three times in buffer, mounted and air dried for electron microscopic examination. Alternatively, air dried preparations were labelled by floating the grids face down on the ferritin solution and then washed by floating on several changes of buffer.

6.2.3 RESULTS

Cells were treated with the cationized ferritin. Of the two methods used, that in which the cells were incubated with the label prior to being mounted gave less background stain than did labelling by floating mounted grids on a ferritin solution. Cells were therefore labelled prior to being mounted.

From the electron micrographs of log and stationary phase S. uvarum 2036 cells it can be seen that the log phase cells are much more heavily labelled with ferritin than are the stationary phase (flocculated) cells.

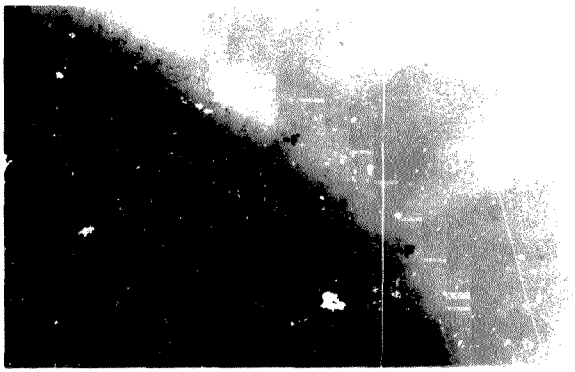


Fig.48. Electron micrograph of the edge of a flocculated yeast cell showing the ferritin label on the cell surface. The regions of the cell edges labelled, F, are thought to be the bases of the fimbriae and appear to have quite a strong negative charge in comparison with the rest of the fimbrial structure. The fimbriae could not be clearly seen as the ferritin label did not radiate out from the cells suggesting that they are not strongly negatively charged along their lengths. Magnification: 72000x

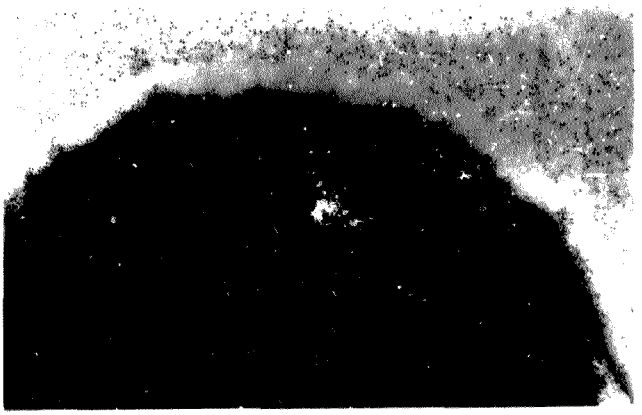


Fig. 49. Electron micrograph of a log cell of S. uvarum 2036. The ferritin label around the cell edges is much heavier than that observed around the edges of flocculated cells. Most cells showed similar labelling, although a few cells were labelled in a fashion similar to that of the flocculent culture. Magnification: 60000x

The label on the non-flocculent control culture was usually very light even on cells labelled by floating mounted grids on the ferritin solution (which generally gave a heavier label than did labelling prior to mounting). Labelling was generally comparable with the flocculated S.uvarum rather than the heavily labelled log phase cells. Some S.cerevisiae cells did bind more label than the flocculent

cells, suggesting a greater negative surface charge, but most cells were more faintly labelled than flocculated cells. Figure 50 shows a cell of S. cerevisiae Y746 labelled with cationized ferritin. Magnification:47 000x

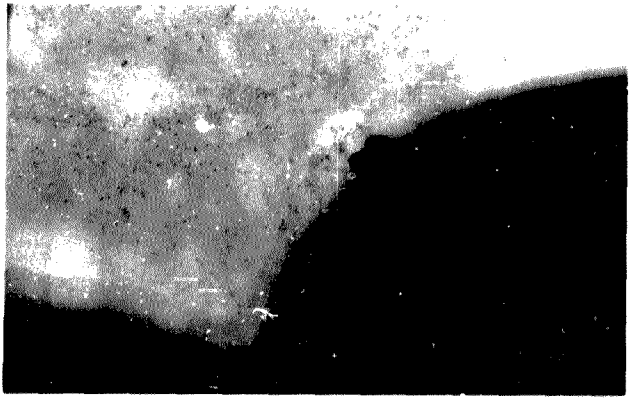


Fig.50 Part of the edges of two stationary phase cells of S.cerevisiae Y746. Ferritin labelling is very slight. Magnification: 47000x

6.3 DISCUSSION

6.3.1 WALL PROTEINS

The difficulty experienced in obtaining adequate separation of polypeptides could perhaps be accounted for by two factors. Firstly, the wall extracts contained numerous protein species and secondly, the protein was present as mannoprotein, the mannan component of which seemed to be cause streaking and hence poor resolution. According to Ballou (1976) yeast wall mannoproteins resolve poorly on gels largely due to the attached mannan.

From a genetically characterized flocculent strain, Holmberg (1978) isolated a 129000 polypeptide. It was absent from a mutant which was non-flocculent due to an unlinked suppressor of FLO 4, namely fsu 2. He also observed this difference in other mutants and showed the polypeptide to be located externally. Under certain conditions his mutant was flocculent and when flocculent was found to possess this protein. In contrast to these results the strain used in this study, which is flocculent only at a certain stage of growth was found to possess this protein, even in the non-flocculent state. It was however found to be much less prominent than the protein of stationary phase cells, since although similar amounts of

protein were layered into each well of the polyacrylamide gel, very sensitive scanning was necessary to show it up on the log phase track in comparison with the stationary phase track. This protein was not found in the extract of the non-flocculent strain.

Also using sodium hydroxide extraction (which irreversibly deflocculated the cells and removed the fimbriae) Stewart (1981) noted the presence of 37000d polypeptide from flocculent cells which was absent from non-flocculent cultures. In this study a polypeptide of similar molecular weight was found to be prominent in extracts from both log and stationary phase cultures of S. uvarum 2036, but not in control culture extracts. The possibility exists that this and the 29000d polypeptide are common to strains which are potentially flocculent even in th non-flocculent phase, but are not present in inherently non-flocculent strains. However, while they may be flocculation associated proteins it is unlikely that either of them is responsible for the transition from non-flocculence (logarithmic growth) to flocculence of a flocculent culture.

Fimbriae were shown earlier in this study to be present on both flocculent and non-flocculent cells. They were also shown to be largely proteinaceous, surface protein having frequently been implicated in flocculation (Baker and Kirsop, 1972, Nishihara et al.1977). It is therefore not

unlikely that they function in flocculation, and if they do, differences in protein composition between the log phase fimbriae and the stationary phase fimbriae would be expected to be slight. The presence of a relatively minor protein in stationary phase as opposed to log phase cell extracts may therefore be significant. That it is not merely a normal stationary phase protein which is unrelated to flocculation is confirmed by its absence from stationary phase non-flocculent cells.

The two adjacent proteins (41000d and 58000d) are prominent in both log and stationary phase extracts of the flocculent culture and it is suggested that rather than a new protein being synthesized, one of these adjacent proteins may be modified at the onset of flocculence.

The presence of this additional protein should be seen in conjuction with the slightly different antigenicity of flocculent and non-flocculent cells of the same culture observed with flourescent antibodies. A difference in the protein compostion would correspond to slightly different antigenicity.

While it has been established that cytoplasmic protein synthesis is necessary for the transition to flocculence (Baker and Kirsop, 1972 ; Nishihara et al. 1976) the nature of the involved proteins and their roles has not been

established. A number of possibilities exist. The proteins may be enzymes which function in enzymic or structural capacities or they may be structural components or both of these. The importance of structural protein was confirmed by the destruction of flocculation capacity by proteases or protein modifying agents (Nishihara et al. 1982; Day et al.1975; this study). There is little consensus regarding the existence of flocculation specific proteins, results from this study having shown the presence of yet another flocculation related protein. It is possible, perhaps, that a complement of flocculation associated proteins exist, all of which must be present for flocculation to occur. The lack of consensus may be due to the use of different flocculent strains for protein analysis, the protein which is absent from the log phase populations being strain specific.

It is also possible that the 48 000 dalton protein identified in this study is not a structural protein, but is part of a flocculation related enzyme complex. As the transition to flocculence involves cellular differentiation, enzymes would also be involved. A further possibility is that it is an enzyme or part of an enzyme complex which has a structural role during flocculation. Acid phosphatase, for example, has been implicated in flocculation and is thought to have a structural role (Williams and Weisman, 1973; Schweingruber et al. 1984).

Author Graham Lynne Theres ├¿
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