Yeast Flocculation: Influence of Nutritional Factors on Cell Wall Composition

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The walls of two strains of *Saccharomyces uvarum* (flocculent and non-flocculent) were isolated from exponential and stationary phase cells after growth in media of different Ca^{2+} and K^{+} composition. Flocculation of the walls was identical to that of intact cells. The transition from the non-flocculent to the flocculent state involved alterations of the polysaccharide, protein and mineral (Ca^{2+} and K^{+}) composition of the walls. The walls of flocculent cells had a higher mannose/glucose molar ratio, a lower percentage of protein, a lower percentage of Ca^{2+} and a higher percentage of K^{+} .

INTRODUCTION

Yeast flocculation is a phenomenon with a genetic basis (Thorne, 1951; Gilliland, 1951; Stewart *et al.*, 1976), the expression of which is more or less modulated by factors related to the environment (Gilliland, 1957; Jansen, 1958; Calleja, 1970; Stewart *et al.*, 1975*a*) and to nutritional factors (Mill, 1964*a*; Taylor & Orton, 1973, 1975; Stewart & Goring, 1976). In a previous study (Amri *et al.*, 1979*a*), we investigated the effects of vitamins, carbohydrates, amino acids and mineral elements on the flocculation of two strains of the yeast *Saccharomyces uvarum* – strain 0006, which flocculates easily and strongly, and strain 0019, which only flocculates under certain conditions. We showed that flocculation of these two strains was controlled by the $\text{Ca}^{2+}/\text{K}^{+}$ ratio in the medium. The chemical composition of the yeast cell wall changes during the development of the yeast (Bonaly *et al.*, 1971) and flocculation phenomena depend directly on the components of the wall (Masschelein *et al.*, 1963). The nature of these cell wall components and the effect that they have are the subject of contrasting theories concerning the phenomenon of yeast flocculation (Lyons & Hough, 1970, 1971; Griffin & MacWilliam, 1969; Mill, 1966).

In previous experiments on flocculation, either intact cells (Marfey *et al.*, 1977) or cell walls have been used, together with electrophoretic (Jayatissa & Rose, 1976; Fisher, 1975) or analytical techniques (Jeunehomme-Ramos *et al.*, 1964). These experiments have shown how the Ca^{2+} ions act as salt bridges between the cells when flocculation takes place (Harris, 1959; Mill, 1964*b*).

In the present study *S. uvarum* strains 0006 and 0019 have been cultivated in various media that are favourable or unfavourable for flocculation. Our aim was to study by these nutritional variations the chemical modifications that affect the cell wall and to determine their importance in flocculation.

METHODS

Micro-organisms. Two strains of *Saccharomyces uvarum* obtained from a brewery were used: SU0006 which flocculates very easily and SU0019 which flocculates moderately in liquid culture.

Culture media. The medium used was that described by Atkin *et al.* (1949) and contained (per litre): anhydrous

dextrose, 50 g; KH_2PO_4 , 0.55 g; KCl, 0.2425 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.125 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.125 g; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 2.5 mg; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 2.5 mg; citrate buffer pH 5.5 (0.4 M), 50 ml; casein hydrolysate (8%, w/v) pH 5.5, 50 ml; meso-inositol, 25 mg; calcium pantothenate, 2.5 mg; biotin, 0.025 mg; thiamin.HCl, 0.5 mg; pyridoxine, 0.5 mg. Details on the preparation of this medium and the necessary modifications for the present experiments are given in Amri *et al.* (1979a, b).

Inoculum and culture conditions. The inoculum was obtained by two successive precultures. First, cells (0.1 mg dry wt) added to 20 ml medium in a 100 ml Erlenmeyer flask were incubated at 25 °C for 24 h and stirred with a magnetic stirrer. This culture was then added to 2 l medium in a 3 l flask maintained at 22 °C and aerated by bubbling with sterilized air. After 36 h, the second culture was used to inoculate a Biolafitte fermenter, containing 16 l medium, under aseptic conditions. This culture was aerated with sterilized air at 100 l h⁻¹. Growth of the organisms was monitored by turbidity measurements at 620 nm with a Coleman Jr spectrophotometer and dry weights were determined.

Preparation of the cell walls. Two samples of cells were taken during the growth period: the first was taken during the exponential phase of growth (after 28 h) and the second during the stationary phase (54 h after inoculation). The walls were isolated at low temperature to minimize lytic enzyme activity, as recommended by Taylor & Cameron (1973), MacMurrough & Rose (1967) and Reid & Bartnicki-Garcia (1976). Drastic chemical or physical treatment was avoided to minimize damage to the walls (Taylor & Cameron, 1973).

Each sample was cooled to 4 °C and centrifuged at 2500 g for 10 min. The sediments were washed three times with cold distilled water (4 °C) and resuspended in water (2–3 g dry wt per 10 ml). The cells were broken in a 70 ml flask in a MSK Braun homogenizer with 10 g glass beads (0.5 mm diam.) by treatment for 3 min while being cooled with a stream of liquid CO₂. The beads were separated by decanting and the supernatant containing the cell walls and any unbroken cells was retained and supplemented with the washings of the glass beads. The suspension was then centrifuged at 2500 g for 10 min at 4 °C. The absence of unbroken cells from the supernatant was established by microscopy of methylene blue-stained preparations (Chattaway *et al.*, 1968). (If more than 5% of unbroken organisms were present the sample was subjected to further homogenization.) The supernatant was decanted and stirred for 5 h in 0.01 M-Na₂HPO₄ (pH 8.0) at 10 °C to improve the dissolution of proteins. The wall pellets were washed 20 times with distilled water by centrifugation at 4 °C, the speed being progressively diminished from 16000 to 1400 g. Cleanliness was checked by phase contrast and electron microscopy. After processing, the walls were lyophilized and stored in the dark.

Quantitative analysis. To determine the proportion of neutral sugars, 10 mg walls were hydrolysed with HCl as recommended by Spick *et al.* (1969). The total amount of carbohydrate was determined by the Rimington (1940) technique with orcinol. The Elson–Morgan method, as adapted by Ghuysen *et al.* (1966), was used to assay hexosamines. Molar ratios of neutral sugars were determined by partition chromatography using Whatman 3MM paper with pyridine/ethyl acetate/water (1:2:2, by vol.) as solvent, according to Date (1958). Total nitrogen was determined using the Kjeldahl technique, as modified by Dumazert & Marcellet (1937). Amino acid analysis was by ion-exchange chromatography using a Technicon autoanalyser according to the standard procedure described by Piez & Morris (1960). The composition was established after hydrolysis with 6 M-HCl: to obtain the best liberation, a serial hydrolysis from 4 to 24 h was performed to estimate the cleavage of peptide bonds. Phosphorus was determined by using ammonium phosphomolybdate (Allen, 1940) after sulphoperchloric mineralization of the walls. Ca, Mg, Fe, Na and K ions were determined by atomic absorption using a Perkin Elmer 306 spectrophotometer, according to Govindaraju (1975).

Technique for measuring the degree of flocculation. The degree of flocculation was measured using the techniques of Gilliland (1957) and Stewart *et al.* (1975b), as described by Amri *et al.* (1979a).

RESULTS

Wall flocculation and total chemical composition

Walls were obtained from cells sampled during the exponential and stationary phases of growth in media with different Ca²⁺/K⁺ ratios. The quantities obtained, their degree of flocculation and their total chemical composition are summarized in Table 1. The percentage of wall was between 7 and 29%, which is in accordance with the results of Griffin & MacWilliam (1969). Hexosamines accounted for 0.10–0.67% of the dry weight of the walls, results similar to those reported by MacWilliam (1970). Nitrogen in the walls comprised between 1.50 and 4.76%. High proportions of nitrogen have also been found (Lyons & Hough, 1970) in walls of *S. cerevisiae* and presumably correspond to a high content of amino acids.

Modification of the carbohydrate composition of the walls

The association of mannose units together with phosphorus and proteins produces complexes of a phospho-peptidomannan type located on the peripheral part of the walls, whereas the

Table 2. *Content of glucan and mannan, amino acid nitrogen and phosphate in cell walls from flocculent and non-flocculent S. uvarum strains grown in different media*

For details of growth media, see Table 1. Amino acid nitrogen and phosphate contents are expressed as percentage of dry weight of walls. Results are the mean of two (†) or four (‡) determinations where indicated.

	<i>S. uvarum</i> 0006										<i>S. uvarum</i> 0019			
	0.034/2.72		0.034/0		0.034/3.16		0/2.72		0.034/2.72 inositol only		0.034/2.72 no inositol		0.034/2.72	
Ca ²⁺ /K ⁺ ratio in growth medium	G	S	G	S	G	S	G	S	G	S	G	S	G	S
Growth stage														
Degree of flocculation	0	4	0	5	0	0	0	0	0	0	0	4	0	4
Mannose/Glucose (molar ratio)†	0.59	0.86	0.76	0.81	0.84	0.68	2.90	0.77	0.67	0.60	0.43	1.09	0.57	0.47
Amino acid nitrogen†	1.70	1.62	3.09	1.72	1.56	1.50	2.16	3.32	1.80	2.58	2.50	2.06	1.64	1.15
Phosphate†	0.07	0.09	0.27	0.09	0.10	0.08	0.15	0.01	0.06	0.07	0.12	0.06	0.07	0.05

Table 3. Amino acid composition of cell walls from flocculent and non-flocculent *S. uvarum* strains grown in different media

For details of growth media, see Table 1. Walls were hydrolysed with 6 M-HCl. This procedure destroyed tryptophan, partially destroyed cysteine and deaminated asparagine and glutamine; these last two amino acids were then determined with aspartic acid and glutamic acid. Results are expressed as $\mu\text{mol (mg N)}^{-1}$ (100 mg yeast cell wall) $^{-1}$ and are the mean of two determinations.

Ca ²⁺ /K ⁺ ratio in growth medium	<i>S. uvarum</i> 0006										<i>S. uvarum</i> 0019					
	0-034/2-72		0-034/0		0-034/3-12		0/2-72		inositol only		0-034/2-72 no inositol		0-034/2-72		0-102/2-72	
Growth stage	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S
Degree of flocculation																
Asp	0	4											0	0	0	4
Thr	3-20	6-81	6-37	6-96	7-57	7-29	8-06	5-13	6-88	7-30	7-59	8-42	7-65	8-32	7-81	8-78
Ser	7-55	7-05	5-41	6-15	8-48	9-66	7-16	6-63	5-68	5-74	6-42	8-53	13-90	16-90	7-74	9-45
Glu	6-50	7-50	6-21	6-45	9-38	7-71	4-29	7-15	6-80	5-49	6-13	7-34	9-32	11-25	6-66	5-77
Pro	10-64	10-95	8-99	10-60	11-80	8-37	10-87	11-22	10-47	9-80	11-85	11-70	8-91	9-97	9-21	13-75
Gly	13-67	2-00	7-43	1-00	3-00	6-66	2-00	8-23	5-87	6-67	7-39	1-50	7-46	8-40	8-00	1-50
Ala	7-36	8-70	8-26	9-91	8-98	7-40	8-78	6-39	9-79	10-90	8-21	7-49	6-74	7-38	9-98	9-37
Val	11-15	10-35	10-20	12-12	11-55	11-10	10-58	11-76	10-47	11-67	11-38	9-44	11-15	10-78	8-25	12-78
Met	7-40	5-50	8-79	6-67	9-76	6-88	8-51	7-08	9-11	7-67	7-74	7-32	7-13	6-20	9-98	7-39
Ile	0-87	2-66	1-35	1-44	1-22	2-02	1-01	1-87	1-33	1-02	1-20	1-68	1-51	2-04	1-52	1-07
Leu	5-89	8-24	6-54	6-00	6-87	5-02	7-89	4-66	5-54	5-46	5-77	6-53	3-76	4-35	5-28	4-86
Tyr	7-03	8-70	8-41	8-91	8-08	5-32	9-40	4-94	7-64	8-00	7-41	7-30	6-06	3-51	6-40	6-09
Phe	2-30	2-88	1-86	2-84	6-02	1-98	2-20	4-91	2-62	2-65	1-64	4-15	3-32	3-20	1-79	4-19
Lys	3-70	5-54	5-45	5-05	2-56	3-16	4-70	5-22	3-72	5-65	4-80	4-33	4-15	2-00	4-44	3-72
His	5-85	6-21	6-54	7-27	6-93	3-80	6-64	3-52	5-03	4-41	5-79	6-11	4-67	3-94	5-71	5-01
Arg	2-07	1-85	2-05	2-70	2-57	1-41	2-10	1-68	1-87	1-86	1-81	2-26	1-56	1-57	2-12	1-27
	4-46	4-98	5-07	5-46	6-90	11-40	5-64	9-40	6-69	5-09	4-47	4-70	2-23	2-17	4-56	4-01

carbohydrates are situated in the internal zones. Hexoses represented 40–72% of the peripheral constituents, and the analysis of the molar ratios of mannose to glucose (the only neutral hexoses present) therefore showed one of the structural alterations of the wall when the yeasts changed from a non-flocculent to a flocculent state. Table 2 clearly shows that when the conditions of the medium were favourable to flocculation, the mannose/glucose molar ratio was consistently higher in the walls of flocculent cells. However, the variations of the total percentage of amino acids and the percentage of phosphorus did not correlate with the variations of the mannose/glucose ratio. Only a slight decrease in the percentage of amino acids was observed when there was flocculation of the cells. Table 2 also shows that with strain 0006 an inositol deficiency in the culture medium did not result in a decrease in the mannose/glucose molar ratio – as is the case for *S. cerevisiae* (Ghosh *et al.*, 1969) – but produced the opposite reaction, i.e. an increase in the mannose/glucose ratio and flocculation of the cells.

Amino acid content of the walls

Many workers have postulated that flocculation is determined by peripheral anionic charges on the cell wall, and particularly by the carboxylic groups of amino acids. When flocculation occurred, we observed a slight decrease in the percentage of amino acids which might reflect a decrease in all or only some amino acids. The amino acid composition of the walls was therefore established after a 14 h hydrolysis with 6 M-HCl. Table 3 gives the molar percentages of the wall amino acids corresponding to different conditions of culture. During flocculation, the proline content of both strains fell considerably, whereas the percentages of lysine and aspartic acid increased, especially in strain 0006.

Variations in the cation content of the walls

The influence on flocculation of the cations Ca^{2+} and Mg^{2+} and of K^{+} in the medium has been studied by Taylor & Orton (1973, 1975). However, no information was available on the variations of these ions in the cell wall itself, in relation to the age of the cells, the culture medium and flocculation. Table 4 shows that when the yeasts passed from the exponential phase to the stationary phase, the wall contents of Mg^{2+} and Ca^{2+} decreased and the content of K^{+} increased. No such characteristic changes in Na^{+} and Fe^{2+} contents were observed. It is also interesting to note that when there was a deficiency of K^{+} in the medium the cells concentrated this cation in their walls. Flocculent cell walls always had a lower content of Ca^{2+} than non-flocculent walls.

DISCUSSION

According to Masschelein *et al.* (1963), the mannoproteins of yeast walls play a fundamental role in flocculation, the walls of flocculent strains having more mannans than those of non-flocculent strains. However, this view was criticized by Griffin & MacWilliam (1969), who failed to demonstrate any differences in the amounts of these polymers between flocculent and non-flocculent yeasts.

Lyons & Hough (1970) found that the walls of the flocculent strains NCYC 1005 and NCYC 1063 contained a greater amount of phosphorus than did walls of the non-flocculent strain NCYC 1004, and they suggested that, during flocculation, salt bridges are formed composed of Ca^{2+} between the phosphate groups of the peripheral phosphomannoproteins of the cells. However, Stewart *et al.* (1975a), Mill (1964b) and Jayatissa & Rose (1976) reported experiments which proved that carboxylic acid groups are more important than phosphate groups for the establishment of salt bridges with Ca^{2+} . Even though all these experiments showed that ionic groupings have a definite action in connection with flocculation, there have been no previous analyses of the alterations to the wall which occur when yeast changes from a non-flocculent to a flocculent state.

Our study of the walls of two strains of *S. uvarum*, one flocculent (0006) and the other less flocculent (0019), during the exponential and stationary phases of growth shows that two modifications of the wall composition are related to the culture medium and flocculation. These

modifications – one concerning the concentrations of Ca^{2+} and K^+ , the other concerning the levels of peptides and mannan in the walls – take place in the same manner whenever the growth medium favours cell flocculation. Therefore, it appears that in the absence of inositol, or when there is a high $\text{Ca}^{2+}/\text{K}^+$ ratio in the medium, the wall compositions of strains 0006 and 0019 are propitious for the establishment of cross-bridges between the cells.

Significant changes in the concentrations of Ca^{2+} and K^+ in the walls always occur at the point of flocculation of the two strains. Ca^{2+} is always higher in the walls of the non-flocculent cells, the percentage of K^+ being less important, whereas flocculent walls have less Ca^{2+} and more K^+ . Wuytack & Gillet (1978) observed that the ionic alterations of *Nitella flexilis* were dependent on the percentage of Ca^{2+} in the walls of these organisms. Jayatissa & Rose (1976) hypothesized that in flocculent organisms there is competition between the repulsion forces generated by the anionic groups and the attractive forces. They also suggested that the attraction of one cell to another is probably intensified by hydrogen bonding between mannose residues. Our results support this theory. Indeed, as a high $\text{Ca}^{2+}/\text{K}^+$ ratio in the walls is not favourable for flocculation, whereas a low $\text{Ca}^{2+}/\text{K}^+$ ratio appears to favour this phenomenon, the levels of these cations may influence the attractive forces.

The second modification concerns the architecture of the walls. The percentage of mannose is higher in the walls of flocculent yeasts, whereas there are no such alterations with respect to phosphorus. Although the total protein or peptide does not alter as a result of flocculation, the percentages of some amino acids, e.g. proline, aspartic acid and lysine, are always modified in a certain way. These modifications provide evidence for the formation of specific structures on the cell wall when yeast flocculation takes place. These structures may correspond to lectin-like compounds which permit cell–cell interactions. Lectins are generally glycoproteins in which mannosyl units constitute the carbohydrate moiety. Their mechanisms of action are not entirely clear but Ca^{2+} ions and carboxylic groups are often involved in binding. It is possible that at an appropriate stage of growth the flocculent yeasts are able to elaborate such lectin compounds on their cell surface and then flocculate.

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