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ETHANOL-INDUCED CELL AGGREGATION (FLOCCULATION) AND ITS PHYSIOLOGICAL BACKGROUND IN *SCHIZOSACCHAROMYCES POMBE* RIVE 4-2-1*

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Cell aggregation (flocculation) of the yeast *Schizosaccharomyces pombe* strain RIVE 4-2-1 developed in glucose-containing medium, but only in the presence of ethanol. Cell surface proteins which participated in cell to cell interactions were characterised by the susceptibility of flocculation to different proteolytic enzymes, heat treatment, denaturing and thiol compounds and by the inhibition of flocculation by sugars and derivatives. It was shown that a galactose-specific lectin was involved in this new type of flocculation.

Keywords: Schizosaccharomyces pombe – flocculation – ethanol – lectin

INTRODUCTION

Flocculation, the typical non-sexual aggregation of cells, is a relatively widespread phenomenon among different yeast species. Much more is known about the flocculation of brewer's yeasts and of laboratory strains of *Saccharomyces cerevisiae* (for a review see [12]). The flocculation of cells generally develops during the late exponential or early stationary growth phase, but also occurs exceptionally at the beginning of the exponential phase, as in case of *Kluyveromyces bulgaricus* [4].

Flocculation is under genetic control [18], but cultural and nutritional conditions also play an important role in the expression of the flocculation phenotype [10]. The involvement of lectin or lectin-type proteins and carbohydrate receptors of the cell wall in flocculation was first demonstrated in *Saccharomyces cerevisiae* [7] and later in *Kluyveromyces bulgaricus* [4], *Schizosaccharomyces pombe* [5], *Saccharomycodes ludwigii* [14] and in *Zygosaccharomyces* strains [15]. Bivalent cations, preferably Ca^{2+}, are a prerequisite for flocculation, their role being the reversible activation of lectins by ensuring the proper conformation [9, 16]. Flocculation is reversibly lost by chelating Ca^{2+} with EDTA or by adding specific sugars, which compete with cell wall receptors for lectin binding [4, 8], or by mild heat treatment [17].

*Dedicated to Professor Lajos Ferenczy on the occasion of his 70th birthday.

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The flocculation constituents, especially lectins, can be well defined by the structures of simple sugars, which specifically inhibit flocculation. In some strains of *Saccharomyces cerevisiae,* mannose-specific lectins govern flocculation by interacting with the outer-chain mannan side-branches of the cell wall [13]. In a flocculent strain of *Kluyveromyces bulgaricus,* galactose- and N-acetyl glucosamine-specific lectins were isolated [1], while in *Zygosaccharomyces fermentati* a mannose-specific lectin was also involved in flocculation [15].

Lectin-mediated flocculation in *Schiz. pombe* L972 and L975 strains developed only when the cells were cultured under carbon- or nitrogen-limitation. Deflocculation occurred on addition of 50% galactose or 10 mM EDTA and the flocs were metastable in water [5].

The aims of this study were to determine the physiological conditions which influence the expression of the flocculent phenotype of *Schiz. pombe* strain RIVE 4-2-1 and to characterise the cell surface components playing roles in the cell to cell interaction.

MATERIALS AND METHODS

Organism and media

Schiz. pombe RIVE strain 4-2-1 was obtained from the Culture Collection of Yeasts, Bratislava, Slovakia. Shaken cultures were propagated in YEPD broth (0.5% yeast extract, 0.5% peptone, 1% glucose), at 180 rpm at 25 °C for 24 hours.

Preparation of deflocculated cell suspension

Shaken cultures were prepared in YEPD broth supplemented with 6% (v/v) ethanol. 50 mM EDTA-Na_2 (pH 8) was added and the homogeneous cell suspension was centrifuged (2500×*g*, 10 min). Sedimented cells were washed twice with deionized water and the absorbance of the suspension (A_{400nm}) was adjusted to 1.5.

Determination of flocculation index

EDTA-deflocculated cells were suspended in deionized water at a concentration of A_{400nm} 1.5. Two ml aliquots were distributed into test tubes and a series of twices dilutions was made with solutions of different salts, as indicated in Table 1. After mixing, the suspensions were allowed to sediment for 5 minutes and the absorbance (A_{400nm}) was measured with Novaspec II spectrophotometer (Pharmacia). As a control, a deflocculated cell suspension of the same volume was applied and measured in the same way as the sample. The flocculation index (FI) was calculated according to the equation $FI = 1 - (A_{sample}/A_{control})$. The FI values theoretically vary between 0

and 1. Our strain had FI values of 0.5–0.6 in case of the best flocculation, while a value of 0.1 or less was taken as virtually complete deflocculation.

Flocculation at different pH values

Buffers described by Stratford were used [11]. At pH's below 2.0 HCl was used at pH's above 9.0, NaOH was applied.

Measurement of calcium content of cell suspensions

Cells were sedimented by centrifugation and the Ca^{2+}-content of the supernatant was determined with an atomic absorption spectrophotometer of type 902 (GBC Ltd.).

Enzyme treatment of flocculent cells

Flocculent cells were treated with cell wall lytic enzymes, proteases and carbohydrases according to Hodgson et al. [2]. The following enzymes were used: *Trichoderma* lysing enzyme 200 μg ml^{-1}, *Basidiomycetes* driselase 200 μg ml^{-1}, *Arthrobacter* lyticase 10 μg ml^{-1}, β-glucuronidase 200 μg ml^{-1}, proteinase K 200 μg ml^{-1}, pronase E 200 μg ml^{-1}, trypsin 100 μg ml^{-1}, α-chymotrypsin 100 μg ml^{-1}, β-glucosidase 200 μg ml^{-1} and α- or β-galactosidase 200 μg ml^{-1}. All enzymes were purchased from Sigma.

The flocculation grade was determined visually after treatment for 15, 30, 75 or 120 minutes at 37 °C.

Sugar and lectin inhibition tests

To determine the sugar specificity of flocculation, different sugars and derivatives were added together with 50 mM $CaCl_2$ to the homogeneous cell suspension in the following concentrations: 1200 mM, 750 mM, 500 mM, 250 mM, 100 mM, 50 mM, 10 mM and 5 mM. The mannose-specific lectin concanavalin A and the galactose-specific lectin abrin (both from Sigma) were added to the Ca^{2+}-reflocculated cell suspensions (A_{400} = 0.5) at concentrations of 4, 2, 1, 0.5 and 0.25 mg ml^{-1}.

RESULTS

Effects of culturing conditions on expression of flocculation phenotype

Floc formation of *Schiz. pombe* strain RIVE 4-2-1 in YEPD medium was observed in static cultures, under a low oxygen content (fermentative conditions) when the cells entered the stationary phase of growth. The cells remained dispersed, however,

when the cultures were aerated by shaking and the cells produced no ethanol. The direct role of ethanol and not the mode of glucose catabolism (i.e. by respiration or by fermentation) in the induction of flocculation was evident, because shaken (respiring) cultures flocculated readily and strongly when more than 4% (v/v) of ethanol was added to the culture medium. The optimal concentration of ethanol was between 6% and 8% (data not shown). The effect of ethanol was limited to the very early stage of growth, because strong flocculation developed only when ethanol was present at the time of inoculation. The flocculability decreased if ethanol was supplemented after the cells started multiplying, but was without effect when the cells entered the exponential phase of growth (data not shown). Flocculation started, however, only in the late exponential growth phase and was completed by the beginning of the stationary phase, independently of whether it was induced at the start or later. The addition of ethanol to a stationary culture was completely ineffective even during a longer (e.g. one day) incubation period.

The hydrogen ion concentration of the culture medium was indifferent as concerns flocculation, because the cells were able to propagate and flocculate equally well in YEPD – ethanol broth at the pH range between 3.0 and 7.0.

Roles of monovalent and bivalent cations in flocculation

Sedimented flocs of cells remained fairly aggregated after several washings with deionized water, but immediate deflocculation took place on the addition of EDTA-Na_2 in the concentration range of 10–50 mM. Flocculation was restored by suspend-

Table 1
Flocculation-inducing effects of bivalent and monovalent cations on *Schizosaccharomyces pombe* RIVE 4-2-1

Salt	Concentration				
	0.01 M	0.05 M	0.10 M	0.15 M	0.20 M
$CaCl_2$	0.51 ± 0.06[a]	0.53 ± 0.07	0.42 ± 0.12	0.45 ± 0.13	0.37 ± 0.12
$MgCl_2$	0.50 ± 0.14	0.51 ± 0.06	0.52 ± 0.04	0.56 ± 0.11	0.49 ± 0.07
$CoCl_2$	0.42 ± 0.14	0.44 ± 0.03	0.38 ± 0.07	0.33 ± 0.06	0.35 ± 0.12
$CuSO_4$	0.21 ± 0.11	0.03 ± 0.03	0.02 ± 0.01	0.09 ± 0.04	0.11 ± 0.03
$MnCl_2$	0.28 ± 0.03	0.05 ± 0.01	0.07 ± 0.01	0.08 ± 0.06	0.12 ± 0.01
$ZnCl_2$	0.54 ± 0.12	0.55 ± 0.05	0.45 ± 0.06	0.35 ± 0.10	0.30 ± 0.12
KCl	0.49 ± 0.06	0.50 ± 0.10	0.49 ± 0.08	0.42 ± 0.05	0.40 ± 0.06
NaCl	0.45 ± 0.03	0.41 ± 0.06	0.39 ± 0.08	0.50 ± 0.05	0.45 ± 0.05

[a] The degree of flocculation was determined as the flocculation index (FI), as defined in the Materials and methods.

Values are means of the results of three independent experiments (±S.D.).

ing the cells in salt solutions containing bivalent (Ca^{2+}, Mg^{2+}, Zn^{2+} or Co^{2+}) and monovalent (K^+ or Na^+) cations, but Mn^{2+} and Cu^{2+} proved to be ineffective (Table 1). This reinduced flocculation was reversed again on the addition of EDTA-Na_2, even in those cases when reflocculation was achieved with K^+ and Na^+. This suggested that these monovalent cations probably did not have a direct role in flocculation. Their effect could have been caused by the triggering of a set of events which finally led to the excretion of bivalent cations (probably Ca^{2+}) which activated flocculation, similarly, as occurred in *Saccharomyces cerevisiae* [11]. To prove this, the concentration of Ca^{2+} excreted by the cells in the presence of KCl or NaCl was determined. The amount of Ca^{2+} released into the medium after 15 minutes of treatment was 0.11 and 0.17 mM in cases of 100 mM KCl and NaCl, respectively. The Ca^{2+} concentration of the suspending medium was further increased when cell wall bound Ca^{2+} was released by the addition of 50 mM EDTA-Na_2. The values were 0.17 and 0.21 mM Ca^{2+} in KCl and NaCl solutions, respectively.

Effects of pH on floc formation

The effects of pH values between 1 and 12 on deflocculation and the inhibition of reflocculation were monitored. The flocs remained intact and dispersed cells reflocculated readily on the addition of Ca^{2+} in the very wide pH range from 2 to 12, while only the pH below 2 inhibited reflocculation and caused a slow breaking-down of the flocs.

Effects of enzymes, urea and sulphydryl compounds on flocculation

Cell surface components thought to be involved in the cell to cell interactions were characterised by treating dispersed cells for a short time or by prolonged exposure to protein-denaturing agents (SDS and urea), sulphydryl compounds (2-mercaptoethanol and dithiothreitol), cell wall lytic enzyme complexes (lysing enzyme, lyticase and driselase), proteases (pronase E, proteinase K, trypsin and α-chymotrypsin) and carbohydrases (β-glucuronidase, β-glucosidase and α- or β-galactosidase). The floc forming ability of the cells was lost on treatment for 15 minutes in all cases except SDS, thiol compounds and carbohydrases, even after exposure for 2 hours.

Heat stability of flocculation

The effects of heat on deflocculation were studied by gradually heating Ca^{2+}-reflocculated cells until the T_F value was reached, at which point the flocs were completely deflocculated [17]. In case of RIVE 4-2-1, the T_F value proved to be 55 °C. At this temperature, deflocculation was proved to be reversible by allowing suspensions to cool down to room temperature. On further elevation of temperature, however, floc-

Table 2
Effect of heat treatment on flocculation of *Schizosaccharomyces pombe* RIVE 4-2-1

Duration of heat treatment (min)	Medium of heat treatment[a]	Temperature				
		60 °C	65 °C	70 °C	75 °C	80 °C
30	$CaCl_2$	0.43 ± 0.08[b]	0.33 ± 0.10	0.10 ± 0.05	0.09 ± 0.01	0.04 ± 0.01
	D.W.	0.36 ± 0.09	0.46 ± 0.09	0.41 ± 0.05	0.51 ± 0.03	0.08 ± 0.02
60	$CaCl_2$	0.55 ± 0.06	0.26 ± 0.03	0.11 ± 0.03	0.08 ± 0.01	0.01 ± 0.01
	D.W.	0.48 ± 0.08	0.28 ± 0.03	0.37 ± 0.06	0.40 ± 0.05	0.16 ± 0.03
90	$CaCl_2$	0.40 ± 0.07	0.25 ± 0.04	0.08 ± 0.02	0.07 ± 0.03	0.01 ± 0.01
	D.W.	0.37 ± 0.06	0.29 ± 0.07	0.33 ± 0.06	0.36 ± 0.03	0.03 ± 0.01
120	$CaCl_2$	0.42 ± 0.08	0.21 ± 0.02	0.09 ± 0.01	0.10 ± 0.02	0.02 ± 0.02
	D.W.	0.49 ± 0.10	0.33 ± 0.09	0.36 ± 0.08	0.35 ± 0.06	0.04 ± 0.02

[a] Cells were suspended in 50 mM $CaCl_2$ ($CaCl_2$) or deionized water (D.W.) at a concentration of A_{400nm}= 0.75.

[b] The degree of flocculation was determined as the flocculation index (FI), as defined in the Materials and methods.

Values are means of the results of three independent experiments (± S.D.)

culation ceased to be reversible at 70 °C. The floc-forming ability of the cells was tested by heat treatment in deionized water or in 50 mM $CaCl_2$. The data presented in Table 2 clearly indicate that 50 mM $CaCl_2$ as a heat-treating medium made the cells more sensitive to the denaturing effect of heat than did deionized water. Prolongation of the treatment from 30 to 120 minutes was without effect in both cases.

Flocculation inhibitory effects of sugars and derivatives

The results of the above experiments strongly suggested that lectin-like cell surface proteins were involved in cell aggregation in the case of *Schiz. pombe* strain RIVE 4-2-1. The sugar specificity of lectins was determined by testing the flocculation inhibitory effects of various sugars and sugar derivatives in the presence of Ca^{2+} (Table 3). Although most of them caused slight or complete inhibition of the flocculation at high (1.2 M) concentration, only D-galactose, 2-deoxy-D-galactose and disaccharides bearing β-linked D-galactose in a terminal, non-reducing position (lactose and o-nitrophenyl-β-D-galactoside) maintained this ability through a long series of dilutions, indicating the presence of galactose-specific lectin-like proteins in the cell surface. Compounds containing α-linked D-galactose at the terminal, non-reducing end had a reduced inhibitory effect, e.g. p-nitrophenyl-α-D-galactoside, or they were completely ineffective, e.g. melibiose and raffinose.

Table 3
Flocculation inhibitory effects of sugars and derivatives on *Schizosaccharomyces pombe* RIVE 4-2-1

Carbohydrate	Concentration (mM)
Fructose	–[b]
D-Mannose	–
D-Sorbitol	–
D-Mannitol	–
D-Glucose	1200[a]
2-Deoxy-D-glucose	–
Maltose	–
Saccharose	–
D-Galactose	100
2-Deoxy-D-galactose	250
N-Acetyl-D-galactosamine	–
Lactose	50
o-Nitrophenyl-β-D-galactoside	50
p-Nitrophenyl-α-D-galactoside	250
Melibiose	–
Raffinose	–

Cells were suspended in 50 mM $CaCl_2$, at a concentration of $A_{400} = 0.75$.

[a] Values are the minimal concentrations required for complete deflocculation of the cells, based on the repetition of three independent experiments.

[b] –: No deflocculation even at the highest concentration (1200 mM) applied.

Effects of exogenous lectins on flocculation

The flocculation inhibitory effects of the manno-specific lectin concanavalin A and of the galactose-specific lectin, abrin on the ethanol-grown cells were monitored. As expected, concanavalin A had no effect, but abrin inhibited flocculation in the concentration range of 1–4 mg ml^{-1}. In the latter case, the inhibitory effect was probably a consequence of the competition of abrin with the endogenous cell-surface lectins of the cells.

DISCUSSION

A new cell aggregation (flocculation) phenotype, which developed only in the presence of ethanol, was discovered in *Schiz. pombe* strain RIVE 4-2-1. For definition and discrimination of the surface lectins, the susceptibility of flocculation to different proteolytic enzymes, heat treatment, denaturing and thiol compounds [2, 9] proved very useful in this case, too. Further typing of the cell surface lectin by sugar

specificity proved not only that the terminal monosaccharide and the mode of glycosidic linkage (i.e. α or β) determines the binding of carbohydrate to the lectin, but also that the attached part of the terminal monosaccharide can modify or alter the binding. While the flocculation of strain RIVE 4-2-1, governed by a galactose-specific lectin, was inhibited by both p-nitrophenyl-β-D-galactoside and o-nitrophenyl-α-D-galactoside, their disaccharide analogues, lactose and melibiose, respectively, had different deflocculating effects: lactose gave rise to inhibition, but melibiose did not. The lack of an inhibitory effect of the natural α-galactosides melibiose and raffinose was surprising because the *Schiz. pombe* cell wall contains terminal, non-reducing α-galactoside residues in its phosphogalactomannan outer cell wall layer [3].

What can the role of ethanol be in the flocculation of this strain? One possibility is that it modifies the structure of the flocculation receptor by altering the galactomannan side-branches, similarly as methanol acts in a weakly flocculent strain of *Pichia pastoris* [6]. It is also possible that chemical modification concerns the lectin itself by activating the molecules for binding to the carbohydrate moieties. It cannot be excluded, however, that specific metabolic pathways are induced by ethanol, which directly or indirectly lead to cell aggregation. To elucidate this, further work is needed.

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