CHAPTER II

Studies on flocculation characteristics of the flocculent yeast strain *Saccharomyces cerevisiae* NCIM 3528

SUMMARY

S. cerevisiae NCIM 3528, a highly flocculent strain which constitutively flocculates in a calcium dependent manner was characterised with respect to its flocculation. Flocculation of the strain showed exclusive mannose specificity with methyl α-mannoside being a potent inhibitor of flocculation. The strain could flocculate well in broad pH range of 3-10. Flocculation was sensitive to the temperatures above 60°C as well as to the action of broad specificity proteases such as pronase E and proteinase K but relatively resistant to chymotrypsin. Tunicamycin did not inhibit flocculence of this strain. The strain showed most of the flocculation characteristics similar to the Flo1 phenotype.

INTRODUCTION

Aggregation of cells is a widespread phenomenon found among prokaryotes and eukaryotes, among bacteria, yeasts, cellular slime moulds, filamentous fungi, algae and protozoa [1]. Microbial aggregation has been exploited for long time in various applications such as biological wastewater treatment, alcoholic fermentations, antibiotic fermentation and enhanced sedimentation to aid in cell recovery or retention [2].

Different mechanisms of aggregations have been described. Aggregation in *E. coli* has been proposed to take place due to pili-pili interaction whereas that in *Zooglea ramigera* occurs due to excessively produced exopolysaccharide [2]. Amongst yeasts such as *Hansenula wingei*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae* and *Pichia amenthionera* sex-directed aggregation is very common which involves glycoprotein recognition and is mating type specific [1]. Flocculation of the cells observed in brewer's yeasts is a reversible, asexual aggregation of the cells, usually developed in the late exponential phase or stationary phase and proposed to take place due to carbohydrate-protein interactions [3,4].

Flocculation is a complex phenomenon affected by various physiological, environmental and genetic factors. Knowledge of flocculation characteristics of a brewing yeast strain could be of paramount importance to the brewer, since the time and the environmental conditions under which flocculation is expressed can greatly affect the performance of the strain during the fermentation. Flocculation has been described in many yeast genera and the flocculation characteristics of most of them have been reported. In general, all flocculation systems are Ca^{2+} dependent and sensitive to proteases and sugars. However, flocculation in some wine strains of *S. cerevisiae* and *Kloeckera apiculata* as well as some strains of *Zygosaccharomyces fermentatii* has been shown to be protease resistant [5,6,7]. Sugar insensitive flocculation also has been reported in some strains of *S. cerevisiae* [8].

The highly flocculent strain of *S. cerevisiae* NCIM 3528 used in the present investigation is a bottom fermenting brewer's yeast and had been shown previously to flocculate constitutively in a calcium dependent manner [9]. In order to understand the molecular mechanism underlying flocculation, further characterisation of the strain with respect to its flocculation behaviour was necessary. In this chapter, the data on flocculation characteristics of *S. cerevisiae* NCIM 3528 has been presented.

MATERIALS AND METHODS

MATERIALS

Yeast strain and culture conditions

Yeast strain used in the present study was a highly flocculent *Saccharomyces cerevisiae* NCIM 3528, obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. It was maintained at 4°C on MGYP agar slants containing (g/L): malt extract, 3.0; glucose, 20.0; yeast extract, 3.0; peptone, 5.0 and agar, 20.0. The yeast cells were grown in Erlenmeyer flasks (1 lit. capacity) containing 300 ml liquid MGYP medium, at 30°C with shaking (200 rpm).

Various monosaccharides, disaccharides, proteases and tunicamycin were obtained from Sigma (USA). All other chemicals used were of analytical grade.

METHODS

Measurement of flocculence

S. cerevisiae NCIM 3528 cells were grown in MGYP medium, harvested after 24 h and washed twice with double distilled water. After deflocculation using 100 mM EDTA (pH 7.4), cell density was adjusted spectrophotometrically (A_{600}=1.5). EDTA was removed by pelleting the cells. The pellet was washed twice with double distilled water followed by suspension in the flocculation buffer (50 mM sodium acetate buffer, pH 4.5, containing 5 mM $CaCl_2$). 5 ml suspension, in 25 ml conical flasks, was incubated on a rotary shaker (150 rpm)

for 1 h at room temperature (25°C) and then allowed to stand for 1 min. The samples (0.1 ml) were removed from just below the meniscus, dispersed completely in 100 mM EDTA and the free cell concentrations were determined spectrophotometrically.

Time course of growth and flocculence of *S. cerevisiae* NCIM 3528

The cells were grown in MGYP medium for 16 h at 30°C and harvested by centrifugation (10000 rpm, 4°C, 10 min). Cells were washed with sterile, 100 mM EDTA to ensure complete deflocculation. EDTA was removed by washing the cells with distilled water. 50 ml MGYP medium in each of the 250 ml capacity Erlenmeyer flasks was inoculated with 1 ml deflocculated cell suspension. Flasks were incubated at 30°C with an agitation speed of 150 rpm in an incubator shaker. Flasks were removed at fixed time intervals, and total cell density was measured after complete deflocculation with EDTA by taking cell counts. EDTA was removed by centrifugation, cells were washed twice with double distilled water and flocculence of the cells was measured as mentioned above.

Effect of sugars and sugar binding proteins on flocculation

Effect of sugars on flocculation was checked according to Masy *et al.* [8] with some modifications. Freshly grown cells were washed with distilled water by centrifugation, the cell density was adjusted spectrophotometrically after deflocculation with 100 mM EDTA (pH 7.4). After removal of EDTA by centrifugation, the cells were washed with double distilled water and suspended in 5 ml of flocculation buffer (50 mM acetate buffer, pH 4.5 and 5 mM $CaCl_2$) containing different sugars (1 M) in 25 ml conical flasks. The cells were incubated in presence of sugars for 1 h, at 25°C on a rotary shaker. After completion of flocculation, flocculation level of the cells was measured as mentioned above.

Effect of mannose specific lectin viz. concanavalin A and its succinylated form, Succinyl con A (final concentration 2 mg/ml) on flocculation was checked in a similar manner. Succinylation of con A was carried out by the method of Gunther *et al.* [10].

Effect of tunicamycin on flocculation

The stock solution of tunicamycin (1 mg/ml) was prepared in NaOH (0.01 M) and the solution was filter sterilised. Different concentrations of tunicamycin were added to 10 ml of the growth medium (MGYP) and the tubes were inoculated with 0.05 ml of deflocculated cell suspension of *S. cerevisiae* NCIM 3528. Inoculum was prepared by growing the cells in MGYP medium for 16 h at 30°C. The cells were harvested by centrifugation (10000 rpm, 4°C, 10 min) and washed with sterile, 0.5 M EDTA solution to ensure complete deflocculation. EDTA was removed by washing the cells with distilled water and this deflocculated suspension was used as an inoculum. The inoculated medium containing tunicamycin was incubated for 48 h at 30°C and growth and flocculation level of the cells was monitored.

Effect of proteases on flocculation

Effect of proteases on flocculation was checked by the method of Hodgson *et al.* [11] with some modifications. Freshly grown *S. cerevisiae* NCIM 3528 cells were washed with distilled water by centrifugation and the cell density was adjusted to 2.5 after deflocculating the cells with 100 mM EDTA (pH 7.4). EDTA was removed by centrifugation, the pellet was washed twice with distilled water and the cells were suspended in enzyme solution and incubated at 30°C under gentle agitation. At specific intervals, the reaction was stopped by adding 1 M acetate buffer (pH 4.5), the protease solution was removed by centrifugation in a microcentrifuge (1 min, 4°C), and the pellet of cells was resuspended in the flocculation buffer to measure the flocculence. Protease treatments were performed in sodium phosphate buffer (50 mM, pH 7.5) containing 50 mM

EDTA and different proteases viz. proteinase K, pronase, trypsin and chymotrypsin at the concentration of 100 μg/ml.

Effect of temperature and pH on flocculation

Effect of temperature on flocculation was carried out according to Hodgson *et al.* [11] with slight modifications. Cells of *S. cerevisiae* NCIM 3528 were deflocculated with EDTA and the cell density was adjusted (A_{600} = 1.5). After removal of EDTA by centrifugation the cells were suspended in 5 ml of acetate buffer (50 mM, pH 4.5) containing 5 mM $CaCl_2$ and incubated at various temperatures (8-70°C) for 60 min in a shaker water bath, after which the percentage of flocculating cells was measured.

Effect of pH on flocculation was checked by suspending the cells in 5 ml of universal buffer (A_{600} = 1.5) in the pH range 3-10, containing 5 mM $CaCl_2$. The suspension was taken in 25 ml conical flasks and agitated for 60 min on a rotary shaker (150 rpm) at 25°C and flocculation level of the cells was measured.

Effect of metal ions on flocculation

Effect of metal ions on flocculation was carried out according to Stratford [12] with slight alterations. *S. cerevisiae* NCIM 3528 cells were deflocculated with EDTA, the cell density was adjusted and EDTA was removed by centrifugation. The cells were suspended in 5 ml of 10 mM Tris-HCl, pH 7.2 (A_{600} = 1.5) containing different metal ions (5 mM) and incubated on a rotary shaker (150 rpm) for 60 min at 25°C after which the flocculation level was determined.

Scanning electron microscopy

Samples for electron microscopy studies were pre-treated as suggested by Hanschke and Schauer [13]. Freshly grown stationary phase cells, harvested by centrifugation and washed twice with distilled water were deflocculated with EDTA (100 mM, pH 7.4) and the cell density was adjusted to 5×10^6 cells per

ml. The cells were fixed for 2.5 h at room temperature in 3 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) with 1 mM $CaCl_2$ and 1 mM $MnCl_2$ and were kept in the fixative overnight at 4°C. After being washed four times for 10 min each with 0.1 M cacodylate buffer (pH 7.4) the cells were post fixed in tannic acid (1% in 0.05 M cacodylate buffer, pH 7.4), then washed four times for 1.5 h in 0.5% uranyl acetate (pH 4.8) and washed twice with double distilled water. Then the specimens were dehydrated in a graded ethanol series (10, 20, 30, ...,90, 96%, 10 min each, overnight 70% ethanol only) followed by three changes in 100% dehydrated ethanol (15 min each). All manipulations except overnight storages, were done at room temperature. After pretreatment the cells were mounted on the standard specimen mounting stubs and air dried. The samples were coated with a thin layer of gold in a sputter coater, Bio-Rad, UK, to prevent the charging of the specimen. The micrographs of the sample with 20KV EHT and 25 pA beam current were recorded by 35 mm camera attached on the high resolution recording unit. The scanning electron microscope used was S-120, Cambridge Instruments, UK.

RESULTS

Time course of flocculation

Constitutive flocculation of *S. cerevisiae* NCIM 3528 was confirmed by checking flocculence of the cells during growth. Eighty percent of the cells were flocculent even in the early log phase and the percentage of flocculating cells increased to 98% as the cells approached late exponential phase (Fig. II.1). Floc size of the cells (visually examined), also was found to increase in the late exponential phase as compared to that in the lag phase. Slightly higher percentage of the flocculent cells was observed at 2 h compared to the cells at 4 and 8 h. This can be attributed to the majority of the parental cells coming from the inoculum which dominate the cell population at 2 h of growth.

Effect of sugars and sugar binding proteins on flocculation

Sugar sensitivity of flocculation of *S. cerevisiae* NCIM 3528 was checked by incubating the cells with various sugars (1 M) for 60 min at 25°C on a rotary shaker, and the free cell concentration was determined spectrophotometrically. In presence of mannose almost 86% of the cells were found to be deflocculated but all other monosaccharides tested had hardly any effect on flocculation. Methyl α-mannoside also could prevent flocculation as efficiently as mannose (Table II.1). However, other derivatives of mannose viz. mannosamine (100 mM) and *p*-nitrophenyl α-mannopyranose (10 mM) appeared ineffective as compared to that of methyl α- mannoside. Effect of lowering the concentration of mannose and methyl α- mannoside on flocculation was checked (Fig. II.2). At lower concentrations, methyl α- mannoside was a better inhibitor of flocculation than mannose deflocculating 80% of the cells even at the concentration of 600 mM. The mannose specific lectin concanavalin A and its dimeric derivative succinyl con A also could prevent flocculation completely (Table II.1).

Effect of tunicamycin on flocculation

Effect of tunicamycin on flocculation of the cells of strain NCIM 3528 was checked by adding different concentrations of tunicamycin in the growth medium and checking for growth and flocculation of the cells. With the increasing concentration of tunicamycin progressive decrease in the cell number was obtained. Growth of the cells was completely inhibited at the concentration of 6 μg/ml. Flocculation of the cells however, remained unaffected at all concentrations of tunicamycin tested, including those which are close to the growth inhibitory one i.e. 4 μg/ml (Fig. II.3).

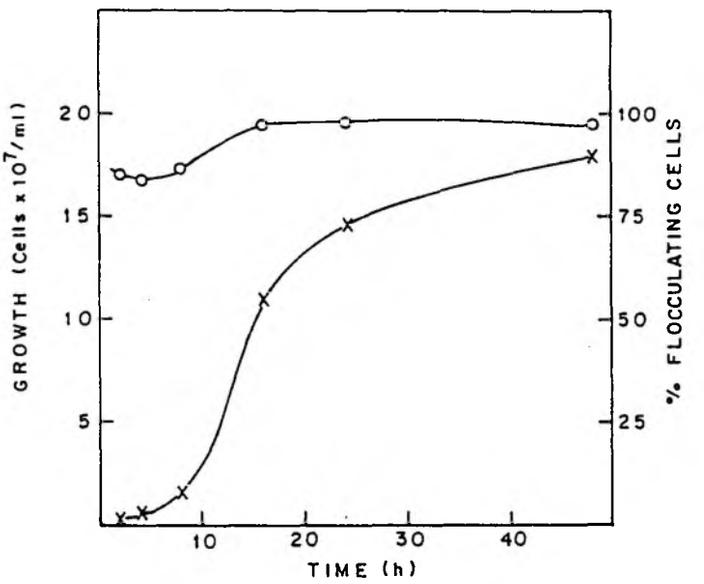

Fig.II. 1 Time course of flocculation of *S. cerevisiae* NCIM 3528

The cells were grown in MGYP medium for different time intervals and checked for the increase in cell number (×) as well as flocculence (o).

Table II.1

Effect of monosaccharides on flocculation of *S. cerevisiae* NCIM 3528

Monosaccharide	% free cells
Glucose	10
Galactose	15
Mannose	86
Methyl α- mannoside	87
***Mannosamine**	30
****p*-nitrophenyl α-mannoside**	28
Xylose	19
Lyxose	23
Ribose	14
Arabinose	11
Fucose	22
Rhamnose	18
Concanavalin A	89
Succinyl con A	85

* - concentration of the sugar - 100 mM

** - concentration of the sugar - 10 mM

All other sugars were used at the concentration of 1 M

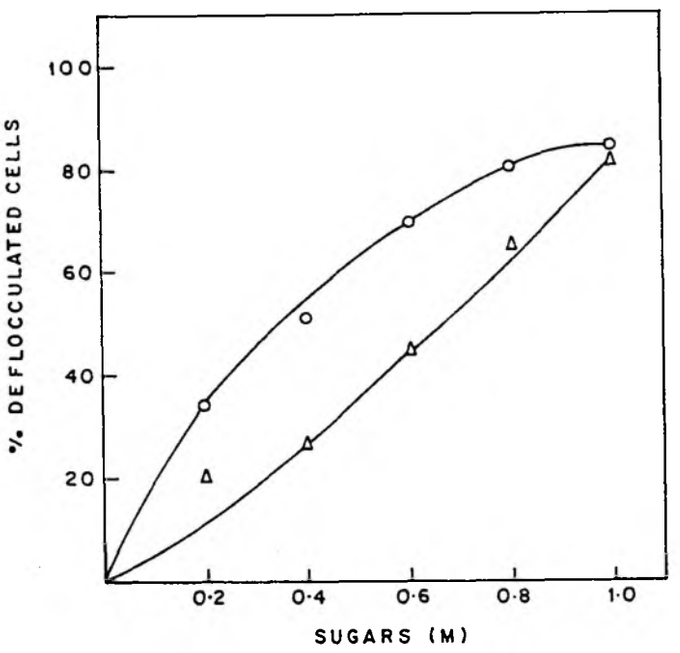

Fig. II. 2 Effect of different concentrations of mannose (Δ) and methyl α-mannoside (o) on flocculation of *S. cerevisiae* NCIM 3528

The cells were incubated in flocculation buffer (50 mM acetate buffer, pH 4.5; 5 mM $CaCl_2$) containing various concentrations of these sugars for 1 h. Flocculation of the cells was measured as described in **Materials and Methods**.

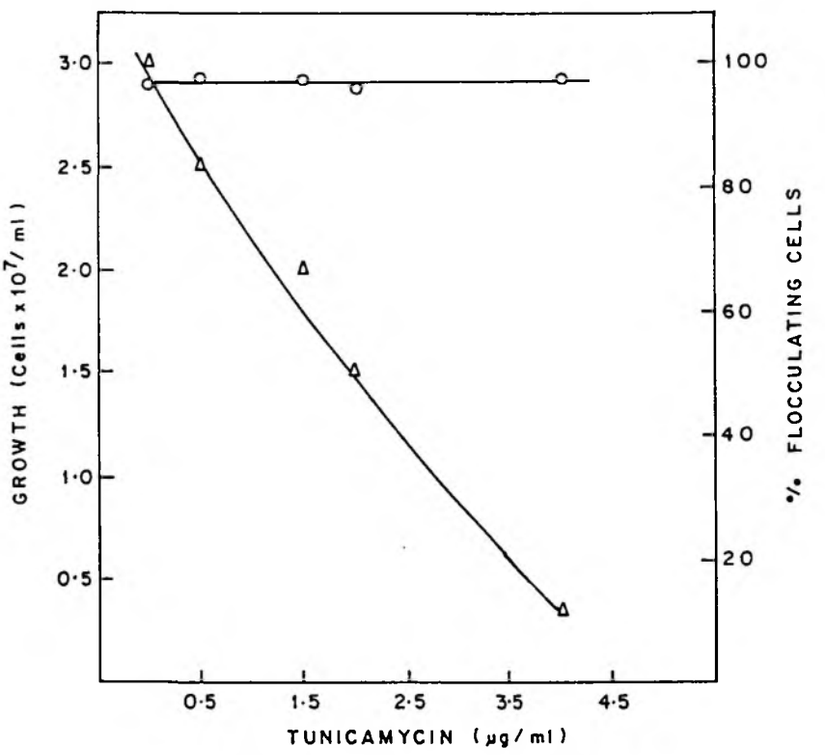

Fig. II. 3 Effect of tunicamycin on flocculation of *S. cerevisiae* NCIM 3528

The cells were grown in MGYP medium containing various concentrations of tunicamycin. The cells were harvested at the stationary phase and increase in the number of cells (Δ) and their flocculation level (o) was determined.

Effect of proteases on flocculation

Susceptibility of flocculation to different proteases was checked by incubating the flocculent cells with proteases for different time intervals. When incubated in presence of broad specificity proteases such as proteinase K and pronase E, the cells lost their flocculence completely within 30 min (Fig. II. 4). Trypsin was found to be less effective as only 60% cells lost the flocculence in 30 min, however within one hour complete loss of flocculation was observed. Flocculation of cells was found to be relatively resistant to the action of chymotrypsin as compared to other proteases. Within first 60 min of incubation with chymotrypsin, 80% of the cells remained flocculent and after three hours only 40% of the cells lost their flocculence.

Effect of temperature and pH on flocculation

S. cerevisiae NCIM 3528 cells were incubated at different temperatures for one hour and change in the flocculence was observed. Flocculation of the cells was found to be stable till 50°C but cells started losing their flocculence at the temperatures above 50°C and complete deflocculation of the cells was observed at 70°C (Fig. II.5a).

Ability of NCIM 3528 cells to flocculate at different pHs was judged by incubating the cells in universal buffer of different pHs and it was observed that the cells could flocculate efficiently at all pHs in the pH range 3-10. Slightly higher flocculation at acidic pHs was noticed (Fig. II. 5b).

Effect of metal ions on flocculation

Effect of different metal ions on flocculation was checked by incubating the cells in presence of various metal ions (Table II.). Though almost 80% of the cells could flocculate in Tris-HCl buffer (10 mM, pH 7.2) in absence of the metal ions, addition of Ca^{2+} and Zn^{2+} (5 mM) increased the percentage of flocculating cells to 98%. Na^{+} and Mg^{2+} ions did not affect the flocculation levels whereas, Ba^{2+}, Mn^{2+}, Fe^{3+} and Al^{3+} caused almost complete deflocculation.

Electron microscopic studies

The cells of *S. cerevisiae* NCIM 3528 were observed under scanning electron microscope for the presence of surface structures. Fig. II.6 shows flocculent cells with smooth surface. The bud scars present on the cell surface are clearly visible. No hair like appendages or fimbrial structures could be seen on the surface of the cells.

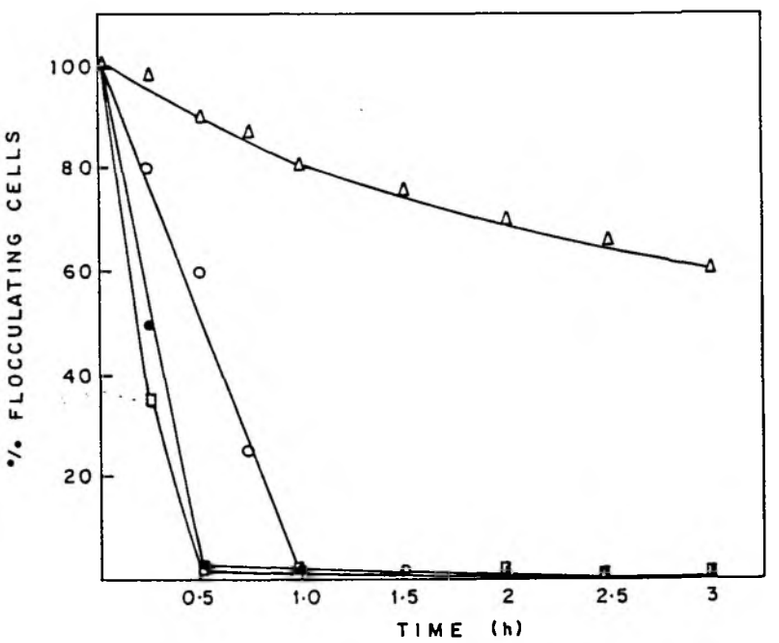

Fig. II. 4 Effect of different proteases on flocculation of *S. cerevisiae* NCIM 3528

The cells were incubated in presence of pronase (●), proteinase K (□) trypsin (o) and chymotrypsin (Δ) at the concentration of 100 μg/ml for different time intervals. The protease treatments were performed in sodium phosphate buffer (50 mM, pH 7.5) containing 50 mM EDTA. Flocculation of the protease treated cells was measured as described in **Materials and Methods**.

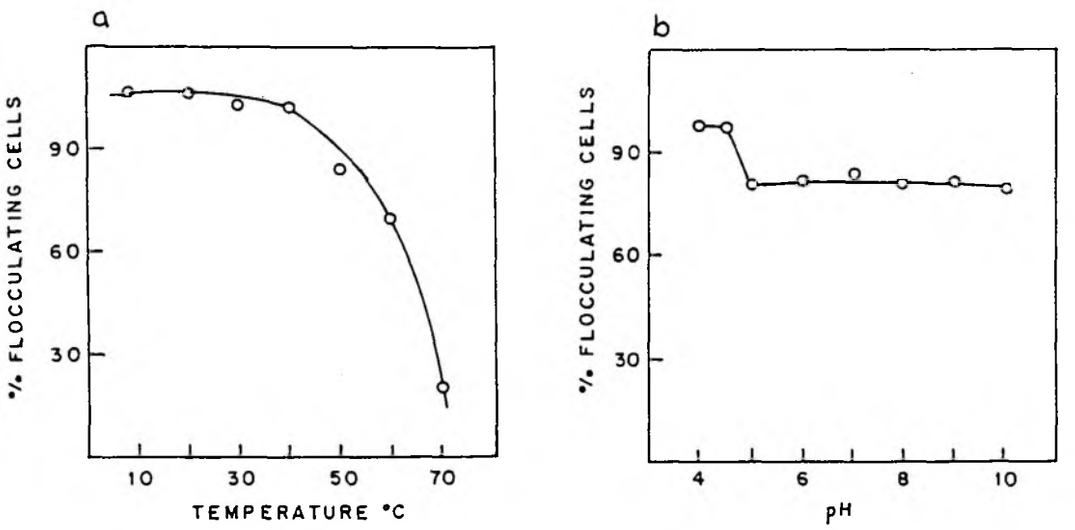

Fig. II.5a Effect of temperature on flocculation of *S. cerevisiae* NCIM 3528.
The cell suspension in flocculation buffer (50 mM acetate buffer, pH 4.5 containing 5 mM $CaCl_2$) was incubated at various temperatures for 1 h and flocculation level of the cells was measured as described in **Materials and Methods**.

Fig. II. 5b Effect of pH on flocculation of *S. cerevisiae* NCIM 3528
The cells were incubated in the universal buffer adjusted to different pH values for 1 h and the flocculation level was measured as described in **Materials and Methods**.

Table II. 2

Effect of metal ions and EDTA on flocculation of *S. cerevisiae* NCIM 3528.

Metal ion	% flocculating cells
Ca^{2+}	98
Mn^{2+}	26
Mg^{2+}	84
Zn^{2+}	98
Co^{2+}	61
Ba^{2+}	12
Na^{+}	82
Al^{3+}	11
Fe^{3+}	32
EDTA	4
*No metal ions	81

All the metal ions were used in the form of chloride salts.

* The cells of *S. cerevisiae* NCIM 3528 were incubated in Tris-HCl (10 mM, pH 7.2) buffer without metal ions.

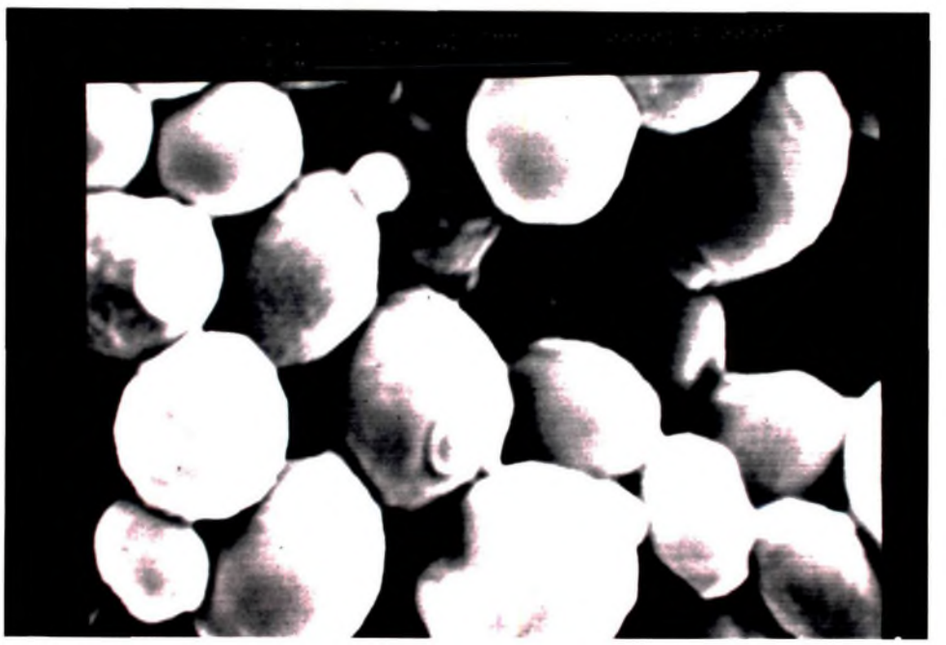

Fig. II. 6 Scanning electron micrograph of highly flocculent *S. cerevisiae* NCIM 3528 cells

The cells were fixed in 3 % glutaraldehyde and dehydrated with graded ethanol series. The cells were observed using scanning electron microscope, S-120, Cambridge Instruments, UK. Micrographs of gold coated samples with 16 KV EHT and 25 pA beam current were recorded by 35 mm camera attached to high resolution recording unit.

DISCUSSION

It has now been accepted that yeast flocculation is the result of a network formed among lectin-like glycoproteins present on the cell surface and mannan. Based on this theory two distinct phenotypes of the flocculent strains have been described, mannose sensitive Flo1 phenotype and glucose/ mannose sensitive NewFlo phenotype [14,15]. Flo1 phenotype is exhibited by the strains with a defined genetic make up harbouring any of the genes *FLO1*, *FLO5*, *FLO8* and these strains express the flocculation constitutively. NewFlo phenotype, which is a characteristic of many brewing strains, is however expressed only at the late exponential phase or stationary phase and the genetic constitution of these strains is not defined. These two phenotypes also differ with respect to their sensitivity to proteases, low pH and high salt concentration [15].

The flocculent strain *S. cerevisiae* NCIM 3528 used in the present investigation has been isolated from brewery and genotype of the strain is not known. However, constitutive expression and mannose specificity of flocculation observed in this strain indicates that it belongs to the Flo1 group. This strain flocculates equally well at all pHs tested in the pH range 3-10 which is also a characteristic of Flo1 strains [16]. Flocculation of the strain seems to be more sensitive to the inhibition by methyl α-mannoside than mannose and other mannose derivatives. Masy *et al.* [8] in a detailed sugar inhibition studies reported mannosamine inhibiting flocculation of the NewFlo strains in a concentration range of 100-500 mM, whereas flocculation of the strains belonging to the Flo1 group (and especially those containing *FLO1* gene) was found to be insensitive to it. Flocculation of Flo1 strains was shown to be inhibited by *p*-nitrophenyl α-mannoside in the concentration range of 10-50 mM [8]. In case of the strain NCIM 3528, flocculation was not inhibited by mannosamine (100 mM) similar to that of the Flo1 strains, however *p*-nitrophenyl α-mannoside (10 mM) also could not inhibit flocculation, but the

concentration of *p*-nitrophenyl α-mannoside used might be lower than the inhibitory concentration required.

S. cerevisiae NCIM 3528 readily got deflocculated in presence of broad specificity proteases, pronase E and proteinase K which was in contrast to the behaviour of Flo1 group strains reported previously. Flo1 strains have been shown to be resistant to the action of proteinase K for at least first 30 min of incubation [15]. Hodgson *et al.* [11] have described two different phenotypes of flocculent strains within a Flo1 group, on the basis of their sensitivity to proteases and high temperatures. The strain having *FLO5* gene exhibited sensitivity of flocculation to high temperatures and resistance to the action of chymotrypsin whereas those harbouring *FLO1* gene are relatively resistant to deflocculation at high temperatures but sensitive to proteases including chymotrypsin. Flocculation of the strain NCIM 3528 was found to be sensitive to high temperatures and relatively resistant to the action of specific proteases such as chymotrypsin and to some extent trypsin, similar to that of the *FLO5* strains. Sensitivity of flocculation to high temperatures has been reported previously in number of other flocculating strains as well [17,18].

Around 80% of the cells of *S. cerevisiae* NCIM 3528 flocculated only in buffer in absence of any added metal ions, but complete removal of metal ions using EDTA caused reversible deflocculation of the cells indicating that trace amounts of metal ions are enough to cause flocculation in this strain. It has been reported previously that very low concentration of Ca^{2+} (10^{-8} to 7.2×10^{-6}) is required to induce flocculation [19,20]. Metal ions are known to affect flocculation in a pH dependent manner. At neutral or high pH values, besides calcium, low concentration of the other metal ions such as Mg^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+} are reported to be equally effective in inducing flocculation [1]. Flocculation of the strain NCIM 3528 also was enhanced in presence of Zn^{2+} and Mg^{2+} although Co^{2+} caused deflocculation to some extent whereas Al^{3+}, Fe^{3+}, Mn^{2+} and Ba^{2+} ions caused almost complete deflocculation. Deflocculation of the flocculent cells in presence of Mn^{2+}, Ba^{2+}, Sr^{2+} has been reported previously. These ions

antagonise the effect of Ca^{2+} ions most probably by competing for the same site on the cell walls [21].

Tunicamycin is a fungal antibiotic, which specifically inhibits synthesis of dolichol-pp-*N*-acetyl glucosamine, the first step in *N*-glycosylation of proteins. The flocculation of *S. cerevisiae* NCIM 3528 remained unaffected when the cells were grown in the presence of tunicamycin even at the concentration closer to the inhibitory one. Contradictory reports on the effect of this antibiotic on flocculation are available in the literature. Deflocculation of the cells when grown in presence of tunicamycin has been reported [22]. Recently however, Siero *et al.* [23] reported no effect of tunicamycin on flocculation of some of the industrial as well as laboratory strains. These authors related differential sensitivity of flocculation to proteases with the differential glycosylation of the proteins involved in flocculation.

Fine structural studies of the cell surface of flocculent cells of various yeast genera by electron microscopy has revealed different flocculation specific structures on the cell surface. Flocculent cells of *S. cerevisiae* showed presence of hair like appendages [24] when observed by transmission electron microscope. Such fibrillar structures were also reported in flocculent cells of *Schizosaccharomyces pombe* [25,26]. Thick mucilaginous coat has been reported to be present on the flocculent cells of *Kloeckera apiculata* and *Zygosaccharomyces fermentatii* as well [5,6,7]. However many authors failed to detect presence of pili on the flocculent cell surface of *S. cerevisiae* using scanning electron microscope [4,26,27-29]. According to Swoden and Walker, [25] the loss of flocculum material, if there is any, is due to it being sensitive to drying procedures. The failure to detect any fibrillar or mucilaginous structures on surface of *S. cerevisiae* NCIM 3528 cells could be partly due to this.

In conclusion, the constitutively and highly flocculent *S. cerevisiae* NCIM 3528 when characterised for flocculation exhibited characteristics close to Flo1 phenotype described by Stratford [14]. In addition, its strong flocculation,

sensitivity of flocculation to high temperatures and relative resistance to chymotrypsin indicates the possibility that it could be a *FLO5* strain.

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