J. Inst. Brew., March–April 1997, Vol. 103, *pp.* 93–98

QUANTIFICATION OF YEAST FLOCCULATION

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Received 9 October 1996

A sedimentation method based in the Helm's flocculation test was improved. In this method, all steps were standardised: initial cell concentration, agitation, sampling and determination of settled cells. The results obtained with the sedimentation test do not differ significantly ($P = 0.05$) from those obtained with the Stratford test; besides, the precision of the two methods do not differ significantly ($P = 0.05$). The results presented clearly demonstrated that the method improved in this work is rigorous for quantification of yeast flocculation and allows the differentiation of flocculation ability of the strains.

The respective micro-flocculation tests were also compared; Stratford micro-flocculation test was selected as producing the most meaningful results.

Key Words: *Yeast, flocculation assay, quantification, sedimentation, agitation*

INTRODUCTION

Flocculation is classically defined as a reversible phenomenon wherein yeast cells aggregate spontaneously and form flocs which either sediment rapidly in the culture medium or rise to the medium's surface[44]. The flocculation characteristics of brewing yeast cells are one of the major factors when brewers select strains for beer production[48]. This property is also being used in some other industrial fermentations such as wine-making and production of fuel ethanol[48].

One of the major problems in yeast flocculation studies is the choice of a rigorous method to determine yeast flocculation. Various methods to quantify yeast flocculation have been reported in the literature. Unfortunately, there is not a consensual opinion about the flocculation method that should be used.

Notwithstanding the first method of flocculation quantification has been described by Schönfeld in 1909 (quoted by Greenshields *et al.*[17]), the sedimentation method described by Burns, in 1937[8] is the base of the most of the modern flocculation assays. This method has been refined and modified by many authors such as: Helm *et al.*[18], Mill[26,27], Greenshields *et al.*[17], Patel and Ingledew[32], Stewart[44], Miki *et al.*[24,25]. The Burn's test and its multiples versions, as well as the method described by Nielsen in 1937 (quoted by Jansen[19]), subsequently improved by Nishihara *et al.*[30] have in common the fact that the cells were removed from the growth medium, washed and then allowed to sediment in a standard medium with Ca^{2+}, at a fixed pH and defined composition. This procedure was criticised as being artificial, because these tests were usually carried out in buffer, in the absence of ethanol, with a number of cells 100–200 fold higher than the values usually obtained in the end of brewer fermentation[60]. Gilliland[15] proposed a new method in which the floccaulation measurements were performed in growth medium; this method was considered as an *in vivo* test in opposition to the tests reported above, considered as *in vitro* tests. Helm and Gilliland techniques are the basis for the most flocculation assays and are the flocculation methods adopted by the European Brewing Convention[12,13].

Beyond the sedimentation tests reported above, other methods were developed to determine yeast flocculation, based on the dispersion of the flocculated yeast's in suspension by the action of sugars[11], EDTA[43] or heat[58].

In the years 80, a more detailed analysis was performed on the influence of the initial cell concentration[22,24,25] and agitation[5,22,53,54,55,56] in the quantification of yeast flocculation. Thus, additional techniques or variants of old ones were also described. Miki *et al.*[24,25] improved a method similar to that of Woof[60]. Kihn *et al.*[21,22] and Smit *et al.*[34] developed their own methods which are a modification of the method described by Miki *et al.*[24,25]. Stratford and co-workers[53,54,55] described a completely standardised new method; in this method, an initial cell suspension consisting of entirely single cells was shaking during several hours (4–6 hours) until the equilibrium between the fraction of flocculated and free cells has been reached. A detailed revision of the methods of quantification of yeast flocculation was made by Calleja[9], Calleja and Johnson[10], and Speers *et al.*[41].

In the absence of a consensual method, many authors[40,41,48] emphasise the urgent need for standardisation of a flocculation test. For this purpose, in the last years the American Society of Brewing Chemists (ASBC) formed a subcommittee charged with the evaluation and improved the Helm assay[1,2,3,4]. Recently, Speers and Ritcey[40] proposed a prototype of an "ideal" flocculation assay.

The principal aim of this work was the improvement (throughout the standardisation of all steps) of a variant of the Helm sedimentation test. The results obtained with this standardised test were compared with those obtained by the Stratford test[51]. Additionally, two microflocculation techniques were also tested and the results compared with the respective macro-test.

MATERIAL AND METHODS

Strains

The strains used in this work are listed in Table I, together with their principal characteristics. NCYC 869 is a laboratory strain, NCYC 1195 is a British ale strain and NRRL Y-265 is a strain isolated from champagne. All flocculent strains used express flocculation in YEPD media. *Saccharomyces sake* is a recognised synonym for *Saccharomyces cerevisiae*[6].

TABLE I. Principal characteristics of yeast strains used in this work

Strain	Genotype	Flocculation		Source	Reference
		Characteristics	Phenotype		
869	*MATαFLO1*	Flocculent	Flo1	NCYC	35, 38, 47, 51
1195	Unknown	Flocculent	NewFlo	NCYC	35, 38, 51
Y-265	Unknown	Flocculent	Flo1	NRRL	38, 57, 59
sake	Unknown	Non-flocculent	—	IAM	37

Note: All strains used were *Saccharomyces cerevisiae*. NCYC, National Collection of Yeast Cultures, Norwich, U.K.; NRRL, Northern Regional Research Laboratory, Agricultural Research Services, Peoria, Illinois, U.S.A.; IAM, Institute of Applied Microbiology, Tokyo, Japan.

Media and culture conditions

Yeast strains were maintained at 4°C on YEPD agar slants, containing per litre of water: Yeast extract (Difco), 10 g; Bactopeptone (Difco), 20 g; Glucose (Merck), 20 g; Agar (Difco), 20 g.

All pre-cultures were prepared inoculating a loop of yeast (from agar slant) in 100 ml of YEPD broth in 250 ml Erlenmeyer flasks. The cells were incubated at 30°C on a orbital shaker (Braun Certomat R) at 150 rpm, during 48 hours. Cultures were prepared by inoculating 1.0 litre of YEPD, in 2 litre Erlenmeyer flasks, in a ratio 1:10 (pre-culture/fresh medium). Cells were grown at 30°C, for 48 hours, on a orbital shaker at 150 rpm.

Measurement of flocculation ability

A.1. Helm sedimentation test (modified)

Flocculent cells were harvested by centrifugation (4500 G, 5 min, 4°C), washed twice in 250 mM EDTA solution, followed by washing the cells with NaCl solution (250 mM) at pH 2.0 and with NaCl solution (250 mM) at pH 4.5. Cells were finally suspended in NaCl solution (250 mM) at pH 4.5 at a final concentration near 1×10^8 cells/ml. Cell suspensions (24 ml) in NaCl solution (250 mM), at pH 4.5, were placed in a 25 ml cylinder. The suspension was adjusted to 4 mM Ca^{2+} with the addition of $CaCl_2$ solution (1.0 ml from a stock solution of 100 mM, at pH 4.5), and then agitated to promote flocculation. Unless stated otherwise, 18 inversions of the cylinder were used to promote flocculation. At defined periods of time samples were taken (200–1000 µl) from a fixed position of the cylinder (the level corresponding to 20 ml), and dispersed in NaCl solution (250 mM) at pH 2.0. Cell concentration was determined by measuring the absorbance of the suspension at 620 nm. A calibration curve (number of cells *versus* absorbance) was previously constructed for each strain.

A.2. Micro-sedimentation test

A micro-sedimentation test was performed using a spectrophotometer cuvette of 4.0 ml, with 3.0 ml of cell suspension (final volume). Cell concentration, agitation and calcium concentration were the same of the macro-test. At defined intervals of time, after flocculation has been started, samples (100–200 µl) were taken from just below the meniscus. Samples were diluted and cell concentration determined as in the macro-sedimentation test. In sampling process, the maximum sampled volume never exceeded 10% of the initial volume.

B.1. Stratford test

The flocculation test indicated in this work as *Stratford test* was originally described by Stratford and co-workers[53,54,55]. As time went on, the authors made minor modifications of the experimental protocol. The experimental procedure adopted in this work was similar to that described by Stratford and Assinder[51].

Firstly, flocculent cells were washed twice with EDTA solution (250 mM). They were then washed and suspended in deionised water at a final concentration near of 4×10^9 cells/ml. One aliquot of 1.0 ml of this suspension was added to 39.0 ml of buffer citrate (50 mM, pH 4.5) with 4 mM of $CaCl_2$, in Erlenmeyer flasks of 100 ml. The Erlenmeyer flasks were placed in a orbital shaker (Braun Biostat M) and agitated at 120 rpm. At defined intervals of time, the agitation was stopped and after 30 seconds, samples were taken (200–1000 µl) from just below the meniscus. Samples were diluted in EDTA solution (250 mM) and cell concentration determined as described above.

B.2. Micro-flocculation test

As in the case of the sedimentation test, a micro flocculation technique similar to the technique described by Stratford and Assinder[51] was used. Cell suspensions were placed in citrate buffer (50 mM, pH 4.5) containing $CaCl_2$ (4 mM), in test tubes of 15 mm diameter and 50 mm of high, at a final concentration near of 1×10^8 cells/ml. The final volume of the suspension was 2.0 ml. The tubes were sealed, stirred vigorously in a vortex, for 10 s, and agitated, in a horizontal position during 4 hours, on a orbital shaker (Braun Biostat M) at 100 rpm. After agitation, the tubes were allowed to stand undisturbed for 30 s, in a vertical position, after which samples (100–200 µl) were taken from just below the meniscus, and dispersed in EDTA solution (250 mM). The cell concentration was determined spectrophotometrically at 620 nm, as it was described above.

All measurements of yeast flocculation were performed at laboratory temperature (20°C).

Statistical treatment

The means of the results, of each strain, of the macro- and micro-sedimentation test, such as the macro- and micro-test of Stratford, were subject to *one-way analysis of variance* (One-Way ANOVA[28]). When the analysis of variance lead to a rejection of the null hypothesis, at the level of significance used, the *least significant difference*[28] was used to test the significant differences between pairs of means.

The precision of the results obtained with the flocculation techniques reported above were compared using the *F*-test[28].

RESULTS

Studies of a variant of Helm sedimentation test

(A) Effect of the agitation in the settling profile of the strains

In Figure 1, a typical sedimentation profile of the three strains in study can be observed. The settling profile of the three strains is similar. After two minutes of settling, more than 90% of the cells have been settled. In the first minute occurs the settling of the larger flocs followed by the smaller flocs. After the third and fifth minute of settling period, respectively, the percentage of settled cells is practically constant for the strain NRRL Y-265 as well as for the strains NCYC 1195 and NCYC 869 (Fig. 1).

The inversion of the cylinder seems to be a very efficient process in the promotion of collisions between the cells. Only one inversion produces nearly the sedimentation of 40% of the cells (Fig. 2). The percentage of settled cells increases highly with the initial number of inversions; subsequent inversions produce a little increase in the fraction of settled cells. The percentage of settled cells is nearly constant for more than 12

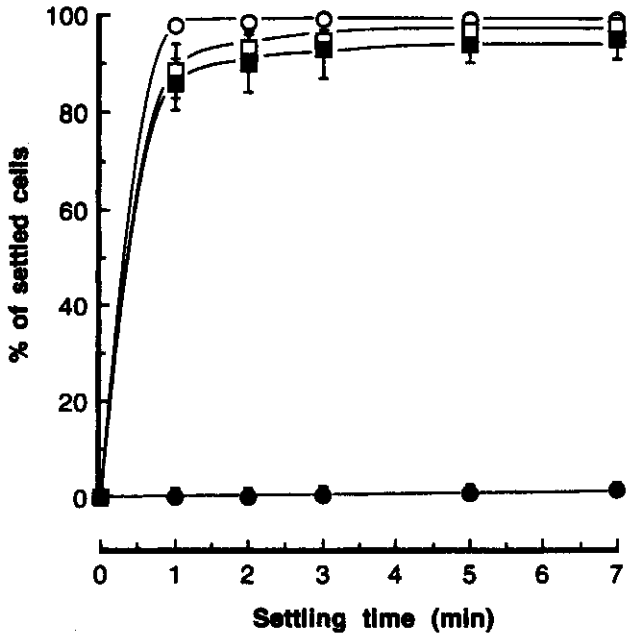

FIG. 1. Settling profiles of NRRL Y-265 (○), NCYC 869 (■), NCYC 1195 (□) and *Sach. sake* (●). 1×10^8 cells/ml were suspended in NaCl solution (250 mM), at pH 4.5. After the addition of Ca^{2+} (at a final concentration of 4 mM), flocculation was initiated by inversion of the cylinder (18 inversions). For more details see Materials and Methods. Each point represents the mean of two determinations, which were made from three independent cultures for each strain. The results are presented with 95% confidence limits ($n = 6$).

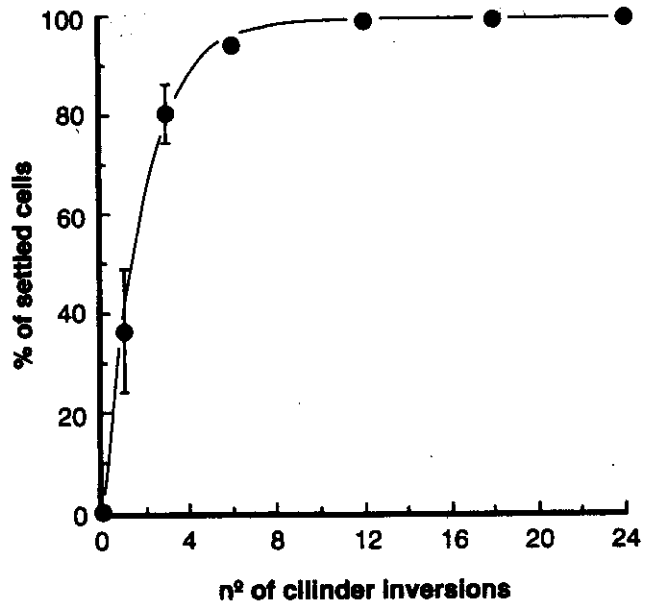

FIG. 2. Effect of the mechanical agitation in the promotion of flocculation. 1×10^8 cells/ml of NRRL Y-265 were suspended in NaCl solution (250 mM), at pH 4.5 in the presence of 4 mM of Ca^{2+}. Each point represents the mean of two determinations, which were made from three independent cultures. The results are presented with 95% confidence limits ($n = 6$).

inversions. Similar results were obtained with the strains NCYC 1195 and NCYC 869 (data not shown).

(B) Effect of the original cell concentration in the percentage of settled cells

The original cell concentration affects the percentage of settled cells. For low cell concentration, the flocculation does not occur; more concentrated cell suspensions produce a typical settling profile (Fig. 3). Above 4×10^7 cells/ml, the concentration of cells in suspension was practically constant for the three strains studied in this work (Fig. 4).

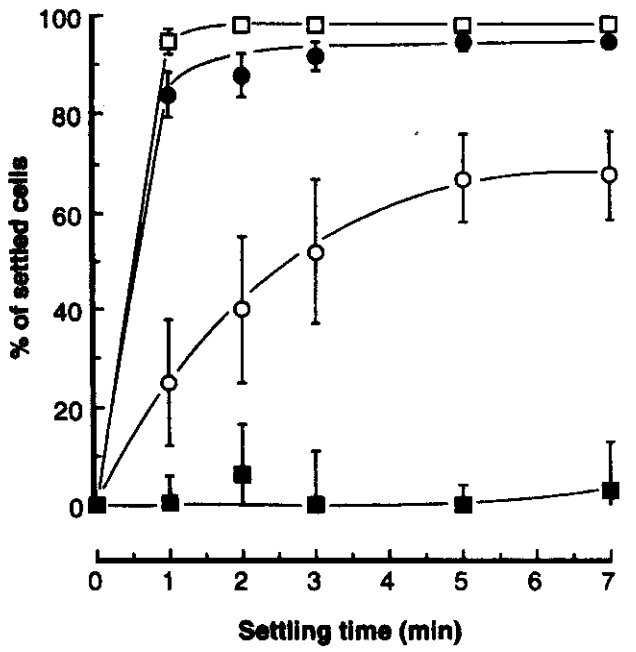

FIG. 3. Effect of the initial cell concentration in the settling profile. (■) 0.18×10^7, (○) 0.98×10^7, (●) 3.9×10^7, (□) 16×10^7 cells/ml of NRRL Y-265 were suspended in NaCl solution (250 mM) at pH 4.5 in the presence of 4 mM of Ca^{2+}. Flocculation was initiated by inversion of the cylinder (18 inversions). Each point represents the mean of two determinations, which were made from three independent cultures. The results are presented with 95% confidence limits ($n = 6$).

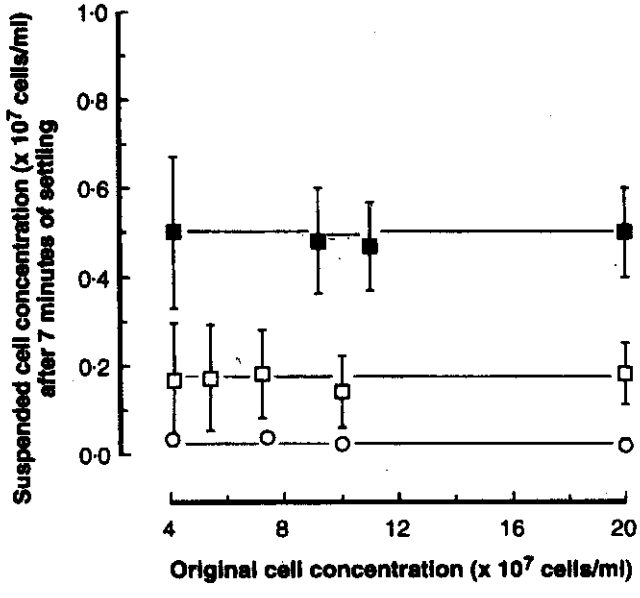

FIG. 4. Effect of the initial cell concentration in the concentration of cells remained in suspension. Yeast cells of NRRL Y-265 (○), NCYC 869 (■), NCYC 1195 (□), were suspended in NaCl solution (250 mM), at pH 4.5. After the addition of Ca^{2+} (at a final concentration of 4 mM), flocculation was initiated by inversion of the cylinder (18 inversions). Each point represents the mean of two determinations, which were made from three independent cultures, for each strain. The results are presented with 95% confidence limits ($n = 6$).

(C) Effect of the presence of NaCl in the settling profile of the strains

In the presence of Ca^{2+} (4 mM) at pH 4.5, the settling profile of the strain NRRL Y-265 was not affected by the presence of Na^+ in a range of concentration up to 430 mM (Fig. 5). Similar results were obtained with the strains NCYC 1195 and NCYC 869 (data not shown).

The variation of pH value during the sedimentation test was a maximum of 0.1 units. Sedimentation profile and the percentage of settled cells were identical when the flocculation was measured in buffer citrate (50 mM at pH 4.5) with 250 mM of NaCl or in a NaCl solution (250 mM) at pH 4.5, adjusted with HCl (data not shown). By this fact, the buffer was not included in the flocculation solution.

Comparison between the sedimentation test and the Stratford test

Figure 6 shows the progress of flocculation of the strains studied in this work evaluated by Stratford test. After 1 hour of agitation, the percentage of settled cells is practically constant for the three strains, but is strain dependent.

The mean percentage of settled cells, after 7 minutes of settling period, in the experimental conditions used in this work and the mean percentage of flocculated cells, after 4 hours, in the Stratford test (Table II) do not differ significantly for the strains NRRL Y-265 and NCYC 1195 ($P = 0.05$) and NCYC 869 ($P = 0.01$). Besides, the precision of the two methods (Table III) does not differ significantly ($P = 0.05$).

Utilisation of a micro-flocculation technique

The utilisation of a micro-flocculation technique is useful when it is necessary to determine the flocculation ability of a suspension with a small number of cells[35] or in the evaluation of the effect of an expensive reagent in the flocculation, such as exotic sugars.

TABLE II. Comparison between sedimentation test and Stratford test

	Sedimentation test (% of settled cells after 7 minutes)		Stratford test (% of flocculated cells after 4 hours)	
	Macro-test	Micro-test	Macro-test	Micro-test
NRRL Y-265	$99.4^{a} \pm 0.3$	$97.3^{b} \pm 0.5$	$99.8^{a} \pm 0.1$	$99.7^{a} \pm 0.2$
NCYC 869	$94.6^{c} \pm 2.5$	$96.2^{c**} \pm 0.7$	$92.2^{d**} \pm 1.4$	$94.3^{c} \pm 1.2$
NCYC 1195	$98.3^{e} \pm 0.9$	$98.8^{e} \pm 0.6$	$99.2^{e} \pm 0.6$	$99.0^{e} \pm 0.4$

Note: The initial cell concentration of all tests were 1×10^8 cells/ml. In the sedimentation test, cells were suspended in NaCl 250 mM (at pH 4.5), in the presence of $CaCl_2$ (4 mM); flocculation was promoted by the inversion of the cylinder (18 inversions). In Stratford test, cells were suspended in citrate buffer (50 mM, at pH 4.5) with 4 mM of $CaCl_2$; the cell suspensions were agitated in an orbital shaker at 120 rpm. The micro-methods were performed in similar conditions to the macro-methods (for more details see Material and Methods).

All the data correspond to the mean of 6 determinations, made from three different cultures, for each strain; standard deviation were calculated. The means followed by the same letter were not significantly different ($P = 0.05$) (One-Way ANOVA). In the case of the strain NCYC 869, the means were not significantly different ($P = 0.01$), except those indicated with a double asterisk (**).

The mean results obtained with Stratford's micro-test does not differ significantly with those obtained with the respective macro-test (Table II), for the strains NRRL Y-265 and NCYC 1195 ($P = 0.05$) and NCYC 869 ($P = 0.01$). The precision of both techniques also does not differ significantly ($P = 0.05$), as it is shown in Table III.

The mean values obtained on the quantification of flocculation of the strains NCYC 1195 and NCYC 869, by the macro- and micro-technique of sedimentation do not differ significantly ($P = 0.05$); however, in the case of the strain NRRL Y-265, the results obtained by the macro- and micro-test was significantly different ($P = 0.05$) (Table II). The precision of macro- and micro-techniques of sedimentation does not differ significantly ($P = 0.05$), as can be seen in Table III.

Discussion

One of the questions usually raised in the evaluation of flocculation is how to distinguish between a true flocculation event and sedimentation over time. According to Calleja and Johnson[10], the number 10 constitutes the barrier between the free cells ($N \leq 10$) and the flocs ($N > 10$). Since the rate of sedimentation of free cells[50] in water is 5–15 mm/h, after 7 minutes (standard time of settling used in this work), free cells fall 0.5–2 mm, while the flocs ($N > 10$), particularly macro-flocs composed by many millions of cells, obviously fall considerably faster. A typical settling profile of flocculent and nonflocculent cells can be seen in Figure 1.

In the original Helm sedimentation test[18], like as in the Helm method described in *EBC Microbiologica*[13] and in the version included in Methods of Analysis of ASBC[3], the quantification of flocculation was made through the determination of sediment volume, in a conical centrifuge tube. However, with this procedure it was only possible to distinguish between flocculent and nonflocculent cells. In the sedimentation test performed in this work, previously used by the authors in other works[36,37,38], a quantitative measurement of cell settling during the test, using a spectrophotometric measurement, was done; at defined intervals of time, sample was taken at defined level of the cylinder (20 cm^3) and deflocculated (to minimise errors in spectrophotometric reading) and the concentration of cells in suspension determined spectrophotometrically. With this procedure, the visual comparison of sediment volume was replaced by a objective measurement of flocculation; by this way the flocculation ability of the strains was determined and distinguished rigorously.

After stopping the mechanical agitation, gross-flocs settles, remaining in suspension a very low cell concentration; the collisions between these cells can be due to Brownian motions (perikinetic aggregation) or due to collisions during the settling of cells or micro-flocs (so-called ballistic aggregation[42]). The percentage of settled cells is nearly constant after the third minute (NRRL Y-265) or the fifth minute (NCYC 869 and NCYC 1195) of settling time (Fig. 1). By this way, the percentage of settled cells (or in suspension), after seven minutes, can be used as a measure of the flocculation ability of the strains.

In the presence of a suitable concentration of Ca^{2+} and without mechanical agitation, dispersed flocculent cells were

TABLE III. Comparison of the precision of the results obtained with the flocculation techniques studied in this work. The figures in the table correspond to *F*-values

	Macro-test of sedimentation *versus* Macro-test of Stratford	Test of sedimentation: macro-test *versus* micro-test	Test of Stratford: macro-test *versus* micro-test	Micro-test of sedimentation *versus* Micro-test of Stratford
NRRL Y-265	6.04	2.29	2.74	5.05
NCYC 869	3.29	1.26	1.28	2.97
NCYC 1195	1.80	2.09	2.48	2.13

Note: Critical value of $F_{0.05\,[5.5]} = 7.146$ (two-tailed test).

unable to form flocs and sediment, as the nonflocculent cells (Fig. 1). These results are in agreement with the data available in the literature, which report the absolute need of a minimum mechanical energy to promote the flocculation[5,22,54,56]. This can be explained by the fact that walls of yeast cells, at pH above the isoelectric point (between pH 2 and 3.2) show a negative charge[14,20], which provokes the repulsion between the cells and difficult cell–cell contact[33]. The repulsion between charges of the same signal acts as an effective barrier to cellular aggregation, impairing the approach between cells and the possibility of establishing a flocculent binding. According to many authors, mechanical agitation promotes an increase in the kinetic energy of the cells necessary to overcome repulsion between cells[50,54,56]. Neither in the original Helm's test[18] nor in the protocol of *EBC Microbiologica*[13] and the version included in Methods of Analysis of ASBC[3], the agitation was rigorously controlled. Until now, this subject was not also analysed by the ASBC subcommittee on yeast flocculation or in recent works about Helm sedimentation test[7,16]. The Figure 2 shows that one inversion of the cylinder is enough to promote flocculation; this demonstrates that the inversion of the cylinder is a very powerful process to promote efficient collisions of the cells. Between 12 and 24 inversions of the cylinder, the percentage of settled cells, after seven minutes, is similar and independent of the number of inversions. These results clearly demonstrated that when quantification of flocculation is performed, using a sedimentation method, it is absolutely necessary to report the agitation.

With a mechanical agitation consisting of 12–18 inversions of the cylinder and initial cell concentration higher than 4×10^7 cells/ml, all the flocculent strains used in this work showed a typical sedimentation profile and the number of cells in suspension after the settling period was practically constant; however, the number of cells in suspension was strain dependent (Fig. 4).

The induction of flocculation by the presence of divalent ions is strain dependent[46] and influenced by pH value[24]. Due to the numerous works available in the literature (some of them for the strains used in this work[49,52]) about the influence of the pH and the presence of calcium ions in the flocculation, these effects were not studied. The calcium concentration used (4 mM) and the value of pH (pH 4.5) correspond to the values of the original Helm test[18].

In our protocol a washing step with EDTA was included, to ensure floc dispersion. In the case of highly flocculent strains, as in the case of strain NRRL Y-265, at pH 4.0, it was only necessary micro amounts of Ca^{2+} (10^{-6} M) to promote flocculation[58]. By this fact, cells of this strain dispersed by washing with EDTA, start to flocculate few minutes after being washed and suspended in deionised water, probably due to release of calcium intracellular. To avoid this problem the dispersion of highly flocculent cells was ensured by washing the cells with EDTA solution and then wash and suspending the cells in NaCl solution (250 mM at pH 4.5).

The effect of salts in yeast flocculation has been deeply studied[8,21,23,24,31,39,45,46,49,52,57,58]. The influence of the ions in the flocculation is very complex and is subject to various interdependent variables, such as: type of the cation and its concentration, yeast strain, pH and ionic strength of the solution[52]. Since Na^+ can competitively inhibit flocculation[24,27,31,46,49], the effect of this ion in the settling profile of various strains was studied. As can be seen in Figure 5, at pH 4.5 and with the simultaneous presence of Ca^{2+} (4 mM), the presence of Na^+ (250 mM) does not affect the sedimentation profile. This result is in agreement with the data available in the literature, which describe that the flocculation promoted by Ca^{2+} at pH 4.0 was not antagonised by Na^+ ions in 100-fold excess over Ca^{2+} ions[24]. It is important to point out that the increase of density and ionic strength due to the dissolution of NaCl (in the range of values tested) did not affect the flocculation of the strains used in this work (Fig. 5). Curiously, Nishihara *et al.*[29] reported that Ca^{2+} shows an increasing

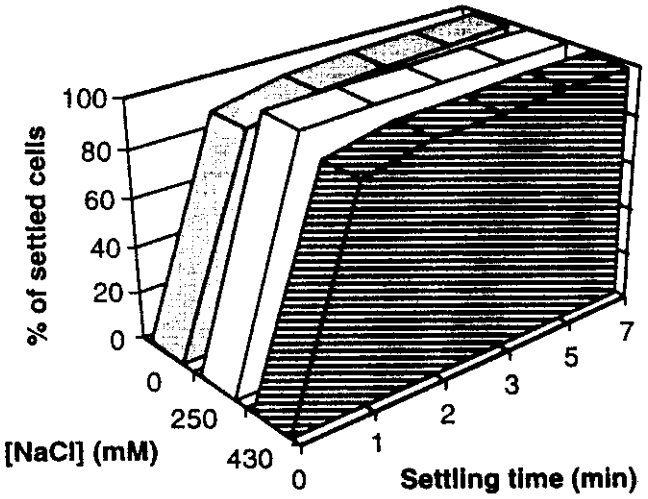

FIG. 5. Effect of sodium chloride on flocculation of the strain NRRL Y-265. 1×10^8 cells/ml were suspended at pH 4.5 in the presence of 4 mM of Ca^{2+}. Flocculation was initiated by inversion of the cylinder (18 inversions). This is a typical example of an experiment repeated three times.

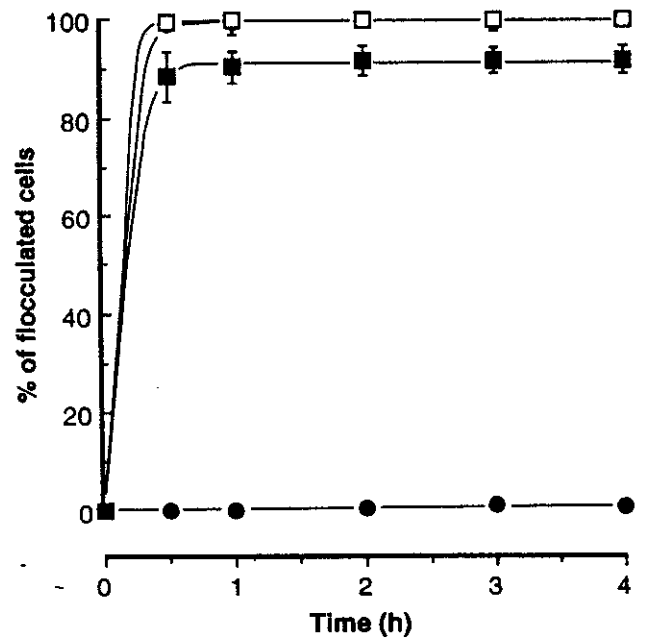

FIG. 6. Evolution of flocculation measured by Stratford test. Yeast cells (1×10^8 cells/ml) of NRRL Y-265 (○), NCYC 869 (■), NCYC 1195 (□), *Saccb. sake* (●), were suspended in citrate buffer (50 mM, pH 4.5) with 4 mM of Ca^{2+}, in Erlenmeyer flasks of 100 ml. The flasks were placed in an orbital shaker and agitated at 120 rpm. Each point represents the mean of two determinations, which were made from three independent cultures for each strain. The results are presented with 95% confidence limits (n = 6).

efficacy in the promotion of flocculation with the simultaneous presence of NaCl 150 mM, comparatively to pure water.

The evolution of flocculation measured by Stratford test showed that, after one hour, the percentage of flocculated cells were very close to the results obtained after 4 hours (Fig. 6). These data are in agreement with those obtained by Stratford; the author[49] reports that the measurements effectuated after one hour are very close to the equilibrium.

The results obtained clearly demonstrated that for the strains used in this work, the values of settled cells obtained by the sedimentation test improved were absolutely comparable with those obtained by the Stratford test (Table II). Besides, the

precision of the two methods does not differ significantly ($P = 0.05$) (Table III).

The comparison of the results obtained with the micro-flocculation technique showed that the micro-test of Stratford produced better results than the micro-sedimentation technique (Table II); the results obtained with the micro-sedimentation technique were significantly different ($P = 0.05$ or $P = 0.01$) from those obtained with the macro-technique, in the case of the strain NRRL Y-265.

In conclusion, the percentage of settled cells or in suspension after the settling period (7 minutes) can be used as a measure of the extension of flocculation and consequently in the evaluation of the flocculation ability; this parameter is immediately intelligible and allows a direct comparison of flocculation intensity of different strains. The sedimentation test with all steps standardised, as it was made in this work (initial cell concentration, agitation, sampling and quantification of cell suspension by spectrophotometry), is absolutely adequate to the rigorously quantification of yeast flocculation and allows the differentiation of flocculation ability of the various strains. The sedimentation test improved is very simple and has the advantage (comparatively to Stratford test) of being faster. The results presented in this work demonstrated that the sedimentation test improved rather than an empirical method, is a rigorous and a objective method for quantification yeast flocculation.

Acknowledgements. This work was supported by Junta Nacional de Investigação Científica e Tecnológica (JNICT). E.V.S. was financially supported, during experimental work, by a grant from JNICT (BD/BIC/M/52/90-IF).

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