SOME SURFACE CHARACTERISTICS OF HANSENULA YEASTS AS INDICATED BY SEDIMENTATION PATTERNS IN DILUTE SALINE SOLUTIONS

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This paper is concerned with the various sedimentation patterns exhibited by live yeasts in dilute aqueous saline solutions. These sedimentation patterns are very sensitive indicators of hydrophobic or hydrophilic surfaces of cells whether these are due to inherited tendencies or to growth media components. Different species or strains have characteristic and reproducible sedimentation patterns if the growth and test conditions are carefully controlled. It was of especial interest to determine whether there was a relationship between the phylogeny of the *Hansenula* yeasts as outlined by Wickerham (1951) and their sedimentation patterns.

Materials and Methods

The cells were usually grown in Wickerham's synthetic broth as stationary cultures in Dubos flasks for 48 hours at 25° C. Prior to inoculation into these flasks the cultures were activated by twice culturing them for 48 hours at 25° C in tubes of the same medium. In some experiments KNO_3 was the nitrogen source instead of $(NH_4)_2SO_4$ in the synthetic medium; in other experiments the cells were cultured in Wickerham's YM broth: yeast extract, 0.3%; malt extract, 0.3%; peptone, 0.5%; glucose 1%. The cells were removed from the growth medium by centrifugation. Several species formed a pellicle on the liquid surface. The cells forming the pellicle returned to the surface in the centrifuge tube and were removed from the other cells by decantations.

The sedimented cells were washed three times in distilled water. Washed cells were added to 10 ml of M/1500 bicarbonate buffer at pH 7.0 in a 19 mm. colorimeter tube until the optical density attained 1.5 at 620 μ in a Coleman spectrophotometer. The stock saline solution contained 192 mg. of C.P. sodium chloride dissolved in 40 ml. of M/1500 bicarbonate buffer. From this solution twofold dilutions were prepared and 1 ml. of the appropriate dilution was added serially to a set of 13x100 mm. pyrex tubes. The tubes had been cleaned in dichromate, washed with tap water at least 12 times and in distilled water 3 times. One experiment was performed in polyethylene tubes whose diameters were similar to those of the glass tubes. Then 0.2 ml. of the yeast suspension with an optical density of 1.5 was added to each of these tubes and to the control tube which contained 1 ml. of the bicarbonate buffer. The final saline concentration in the tubes varied from 16 to 4000 μg per ml.

The tubes were shaken vigorously and the cells were permitted to settle at room temperature or over night

TABLE 1.—Some Characteristics of Hansenula Yeasts (after Wickerham, 1951).

Name	NRRL Number	Ecological origin	Phylogenetic position	Ploidy	Colony appearance
H. holstii	Y 2154	*Picea* frass	primitive	H	extreme, glistening
H. capsulata	Y 1889	conifer frass	primitive	H	glist. or mucoid
H. minuta	Y 411	ferm. mushroom	primitive	H	glistening
H. sp.	Y 2167	indust. ferm.	primitive	H	glistening
	Y 2168				
H. silvicola	Y 1678	wild cherry gum	intermediate	H+D	glistening
H. angusta	Y 1798	orange juice	intermediate	H+D	glistening
*H. californica**	Y 1680	soil	intermediate	H+D	glistening
*H. saturnus**	Y 1304	soil	recently evolved	D+H	dull, glistening to mat
*H. suaveolens**	Y 1725	soil	recently evolved	D+H	mat, powdery
*H. mrakii**	Y 1364	soil	recently evolved	D+H	mat, powdery
H. wingei	Y 2340	*Picea* frass	recently evolved	D+H	glistening
H. canadensis	Y 1888	*Pinus* frass	recently evolved	D	weakly glisten ng
H. jadinii	Y 1542	human abscess	recently evolved	D	glistening
H. schneggii	Y 993	unknown	recently evolved	D	mat, powdery
H. anomala	Y 366	soil, tree sap	recently evolved	D	glistening
	Y 365	fruits	recently evolved	D	mat
	Y 1737				
H. ciferrii	Y 1031	tropical fruit	recently evolved	D	extreme mat smooth to rugose and weakly glistening

* species homothallic with saturn-shaped ascospores.

in a 4° C refrigerator. Prior to reading, the tubes were placed in a 35° C water bath or incubator for about 15 minutes, in order to minimize differences in the flowing pellet response due to the effect of temperature. That is, results obtained when a series of tubes were read at 4° C in one experiment might differ from those with the same species when the tubes were read at 37° C. The types of sedimentation patterns formed at the bottom of the tubes were: (a) flowing pellets—the cells flowed over each other within 10 seconds after the tube was tilted about 60 degrees from the vertical, (b) fixed pellets—the cells did not flow after the tube was inclined, (c) free or fixed pellet surrounded by sedimented cell clusters, and (d) a shield—the cells cover the bottom of the tube. With some strains a ring is formed. This is an intermediate pattern between the fixed pellet and shield.

Most of the yeasts studied were of the genus *Hansenula* and were kindly provided by Dr. L. J. Wickerham of the Northern Utilization Branch of the U. S. Department of Agriculture at Peoria, Illinois. One species has not been described and will be referred to by number only. The names of these with their strain numbers and some of their characteristics are given in Table 1. Other genera and species were from the American Type Culture Collection and will be designated by their ATCC numbers.

Results

In Figure 1 the sedimentation patterns as viewed from the bottom of the tubes are graphically portrayed. The yeasts at the top of the figure have flowing or fixed pellets, those at the bottom have shields and the yeasts in the intermediate location have a combination of these pattern types. These patterns are a reflection of the relative hydrophilic or hydrophobic characteristics of the cells. That is, cells that form shields seek the interface and strongly impinge into the oil phase when examined by the technique of Mudd and Mudd (1924) against an oil-water boundary. Under these same conditions pellet-forming cells remain freely dispersed in the aqueous phase. The minimum salt concentration in the tube where a fixed pellet or shield is formed may serve as an index of the hydrophobic tendency of a given strain.

Thus, *Hansenula holstii* strain Y 2154 has flowing pellets even in 2000 μg saline and fixed pellets in higher concentrations. *Hansenula canadensis* strain Y 1888 and *H. wingei* strain Y 2340 have flowing pellets in 1000 μg/ml of saline and fixed pellets in stronger saline concentrations. *Hansenula anomala* extreme mat strain Y 1737 has shields in all the tubes, including the control. Other species in this genus have intermediate saline indices. In Figure 1 all the strains of *H. anomala* tested were grouped together although some of them may have a saline index higher than the species presented higher in the figure. Results similar to those in glass were obtained in polyethylene tubes for *H. holstii* Y 2154, *H. angusta* Y 1798, *H. anomala* Y 365 and Y 1737.

The medium in which the cells are grown influences the surface characteristics of the yeasts. Cells cultured in $(NH_4)_2SO_4$ as the nitrogen

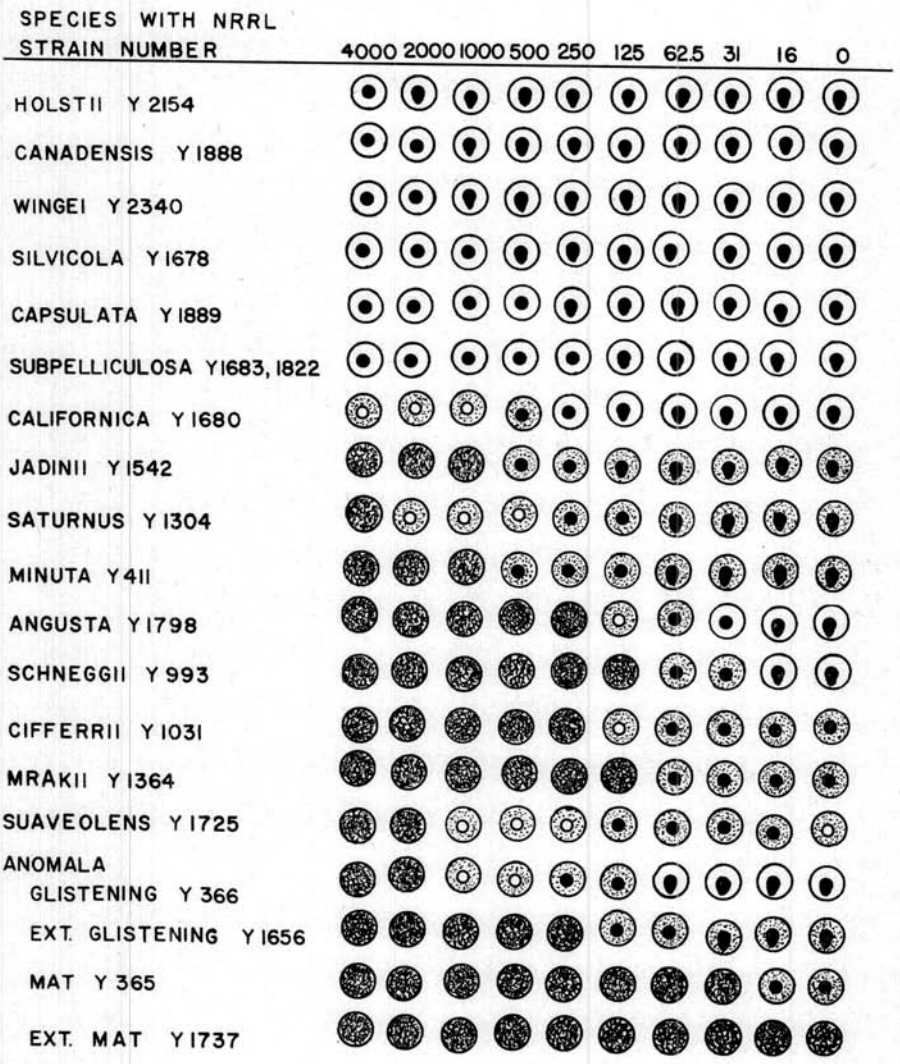

Fig. 1.—Sedimentation patterns of *Hansenula* yeasts in varying NaCl concentrations, arranged according to increasing hydrophobicity. Saline concentration in μg./ml. M/1500 bicarbonate buffer at pH 7.0.

source were always more hydrophobic than cells grown in either KNO_3 or peptone. These differences are especially prominent in *H. subpelliculosa* strain Y 1822 (Fig. 2). With one exception, *H. anomala* glistening strain Y 366, cells from populations grown in broth with peptone or KNO_3 as the nitrogen source formed the same type of pat-

INFLUENCE OF GROWTH MEDIUM
UPON SEDIMENTATION PATTERNS OF HANSENULA.
SALINE DILUTIONS ARE IN DISTILLED WATER.

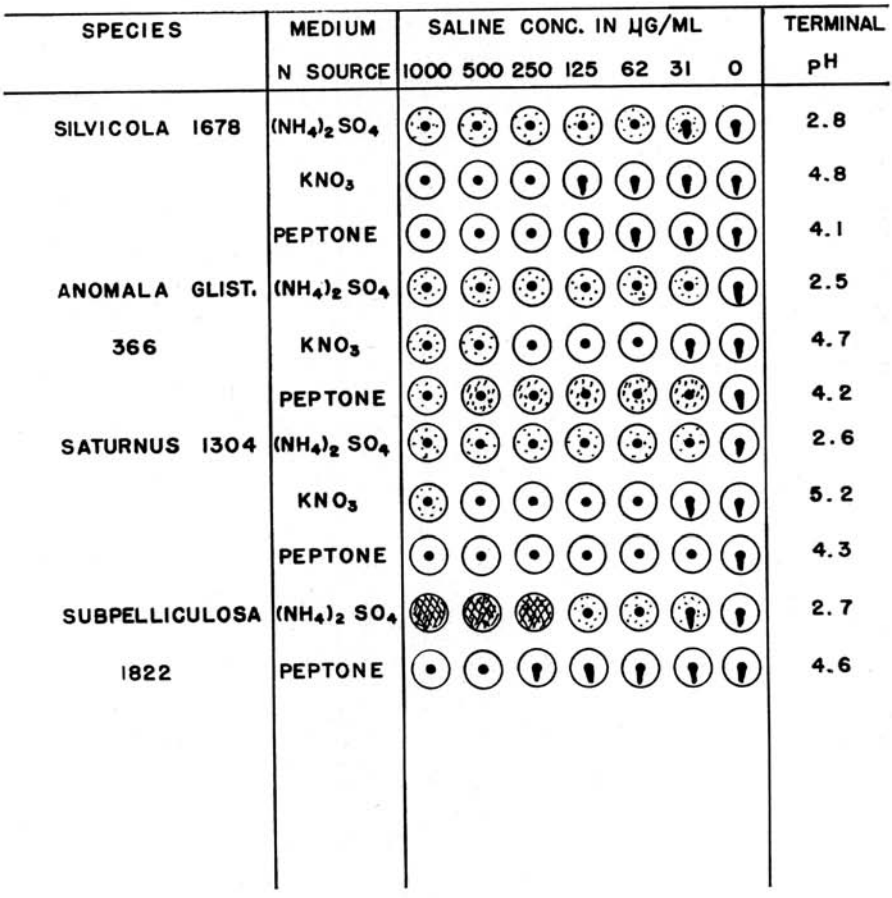

Figure 2.

tern. As mentioned above, the terminal pHs in the nitrate broths were higher than, but of the same order of magnitude as, the pHs of the peptone medium; and both of these values were much higher than the terminal pH values in the synthetic broth with $(NH_4)_2SO_4$. The cells used in these experiments had been washed three times. Therefore, for a

single strain, some factor in the respective growth medium or the terminal pH produced surface differences in the cells as indicated by the different sedimentation patterns. In the peptone medium, some colloidal component may have coated the surfaces of the cells and rendered them more hydrophilic, but this could not have been a contributing factor when nitrate was the nitrogen source.

The results of some experiments to determine the similarities or differences among mating types of species are given in Figure 3. Mating type strain Y 2154, which is more hydrophilic than any other strain tested, is considerably more hydrophilic than the opposite mating type strain Y 2155. Their hybrid has the characteristics of the 2155 parent. The mating type strain Y 2167 and its counterpart strain Y 2168 and their hybrid all gave the same sedimentation patterns.

In Figure 4 are shown the patterns of some diploid strains and their haploid ascosporic isolates. For *H. angusta* strain Y 2214 and *H. subpelliculosa* strain Y 1822 the haploids are much more hydrophobic than are the diploids. There was essentially no difference between the diploids or haploids for *H. subpelliculosa* strain Y 1638 or *H. wingei* strain Y 2340.

The effect of pH upon sedimentation patterns has been determined for a few species. No differences were observed for *H. holstii* strain Y 2154 or *H. anomala* strain Y 1737 for pH values of 4.5, 5.3, 6.1, 7.3,

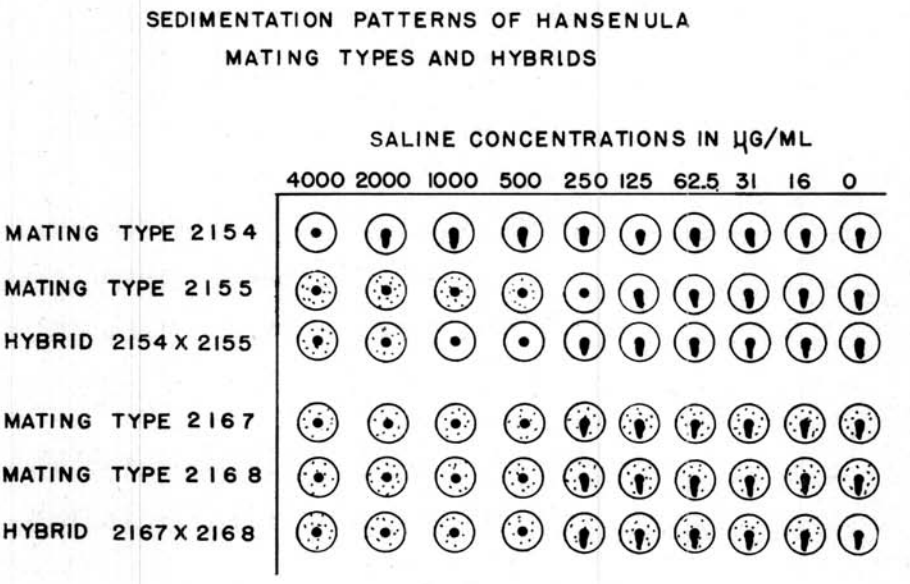

Figure 3.

and 8.0 when the buffer was M/200 phosphate. *Hansenula angusta* strain Y 1798 cells at pH 8.0 formed fixed pellets in the tubes with saline values of less than 125 μg and shields in the other tubes. The patterns for the other pH values are as given in Figure 1. The formation of shields by *H. subpelliculosa* strain Y 1683 at pH values of 4.5 and less in the absence of saline was reported upon by Chesbro and Hedrick (1957).

As may be observed in Figure 5, some species of yeasts in other genera do not exhibit any such responses to differences in saline concentration. *Schizosaccharomyces versatilis* ATCC strain 9987 and *Saccharomyces cerevisiae* ATCC strain 7754 formed fixed pellets in all saline concentrations and in the control. *Candida utilis* ATCC strain 9950 had flowing pellets in tubes with saline levels of 250 μg per/ml, and less and fixed pellets or a ring in the higher saline concentrations. Satellites were formed in tubes of a saline level of 125 μg per/ml and above. *Saccharomyces carlsbergensis* ATCC strain 9080 produced flowing pellets up through 125 μg per/ml of saline and fixed pellets in the stronger saline concentrations. *Kloeckera brevis* ATCC strain 9774 had flowing pellets in tubes with 62 μg per/ml and less of saline while the other tubes had pellets surrounded by cell clusters with satellite formation.

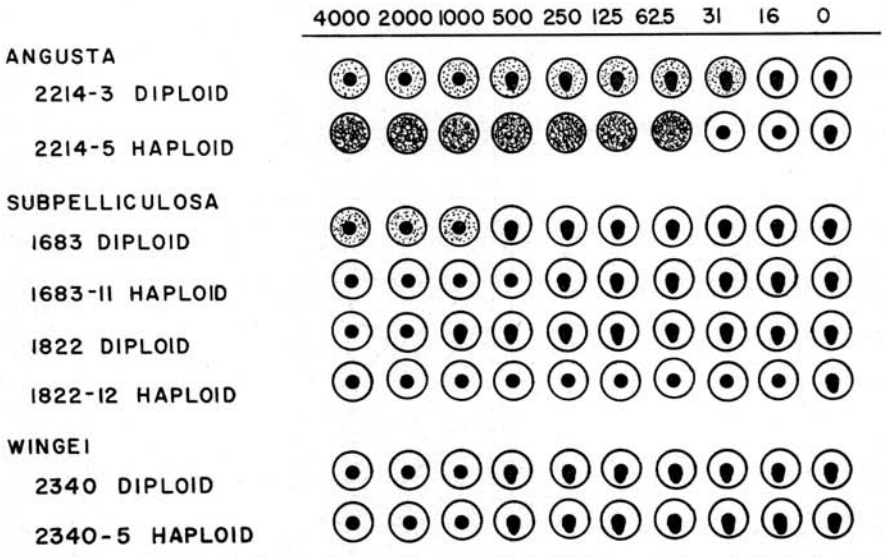

Fig. 4.—Sedimentation patterns of some diploid *Hansenula* and their haploid ascosporic isolates. Saline concentration in μg./ml. M/1500 bicarbonate buffer at pH 7.0.

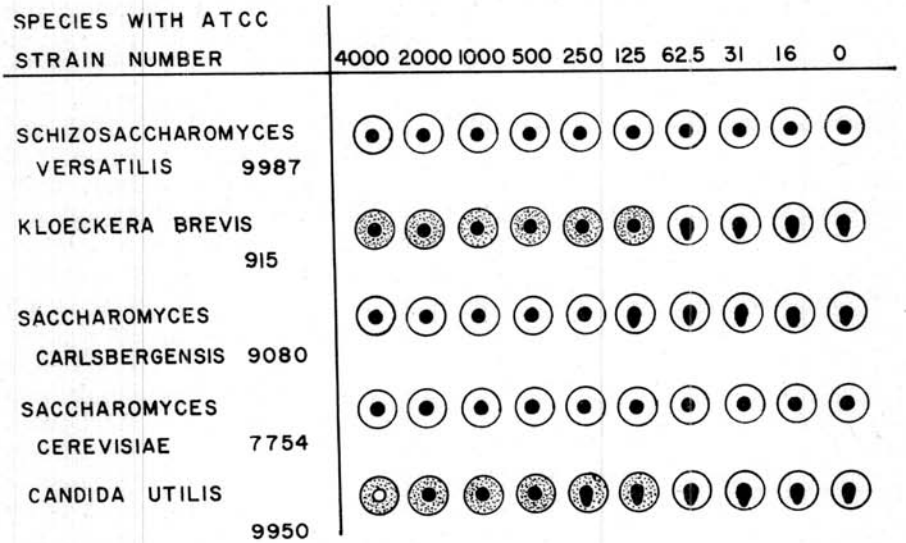

Fig. 5.—Sedimentation patterns of some other yeasts. Saline concentration in μg./ml. M/1500 bicarbonate buffer at pH 7.0.

Discussion

The sedimentation patterns have a high degree of reproducibility either when considering results obtained from one experiment to another or multiple series of the same experiment, provided that the growth and experimental conditions are identical. For example, in one series the sedimentation patterns for *H. angusta* strain Y 1798, a species that produces all the types of sedimentation patterns, were found to be identical in ten replicate tubes of each saline concentration.

It should be emphasized that this sedimentation response to the low saline concentrations used in this study is a very sensitive tool for detecting slight differences in the surface characteristics of cells. Hodge and Metcalfe (1958) in their studies of the flocculation of *Pasteurella tularensis* with methylcellulose found that at least 5% sodium chloride was required in the stock solution of the colloid when stored at 4° C to prevent hydration of the methylcellulose. This colloid-saline mixture was diluted 1:9 with a culture of the bacteria. With saline percentages of 5 or higher no flocculation of the cells occurred but with lesser saline concentrations the colloid had become sufficiently hydrated to cause aggregation and rapid settling of the cells. At 5% or higher saline concentrations the combined number of sodium and chloride ions had a greater affinity for the neighboring solvent water molecules than did the colloid particles, hence the colloid could not become sufficiently hydrated to cause aggregation of the bacterial cells by hydrogen bonding.

In the present study, varying amounts of saline have been employed to determine the relative degrees of hydration of the yeast cells under the particular conditions of the experiment. In many instances saline concentrations of only a few micrograms per ml were required to demonstrate differences in the hydrophilic tendencies of different species or strains of yeasts or within the same strain when grown in media with differing components.

The cellular surfaces of strains that form shields in low saline levels contain a preponderance of nonpolar groups, such as—CH_3 and —C_6H_5. Such cells have weak suspension stability and the addition of small amounts of saline removes the protective water envelope, thus the cells are forced out of suspension by surface tension factors.

Cells with hydrophilic surfaces have a preponderance of exposed polar groups, such as —OH, —NH_2, —COO—, —OPO_3—, or NH_2. Such cells are more easily hydrated than cells that have more hydrophobic surfaces (Lamanna and Mallette, 1959). Phosphomannans (Slodki, Wickerham and Cadmus, 1960) and other surface polysaccharides are probably major contributing factors to this affinity for adjacent water molecules.

In an attempt to discern some of the reasons for the types of sedimentation, cells from the various tubes were resuspended and examined in a hemacytometer to determine the degree of cellular aggregation. The number of cellular units (single cells, cells with one to four buds or four to five cells in linear arrangement) varied with the size of the individual cells, but the range was 80 to 225 x 10^4 per/ml for the yeasts without aggregation. Two species had a large number of clumps. *Hansenula ciferrii* strain Y 1031 had about 8 x 10^4 clumps per/ml and 20 x 10^4 unclumped cellular units per/ml. *H. anomala* extreme mat strain Y 1737 had 14 x 10^4 clumps and 45 x 10^4 unclumped cell units per/ml. The aggregations of strain Y 1737 were growth clusters of many cells, but those of *H. ciferrii* were not growth clusters but were definite agglutinated clumps. Although many of the *ciferrii* cells were aggregated, shields were formed only in the tubes with 4000 and 2000 μg of saline, fixed pellets were present in tubes with 125 μg or less of saline and between these two extremes were intermediate types. As mentioned previously, *H. anomala* strain Y 1737 produced shields in all the tubes including the control.

Two more observations are pertinent to this situation. *Hansenula anomala* strain Y 365, which formed shields in all saline tubes, except the control, had no clumps in any of the tubes. Furthermore, in none of the yeast suspensions studied were there more clumps in the suspensions with the higher saline concentrations than in the suspensions with no saline. Therefore, aggregation of cells is not responsible for the shields or cell clusters in tubes with different amounts of saline.

The cell cluster formation or the scattered deposition of cells around a flowing or fixed pellet can best be explained by the fact that there are differences in hydrophobic tendencies among cells of a population. Ferlin and Hedrick (1958) noted that some

cells of the population of *H. schneggii* strain Y 993 adsorbed and were clumped with methylene blue plus acid, while other cells were clumped with ferric chloride and some cells were not clumped by either reagent.

In experiments with flat-bottomed tubes the cellular suspension that gave pellets in tubes with curved bottoms produced a uniform settling. Therefore pellet formation is due to the gravitational gradient of the curved surface of the tubes. Cells in fixed pellets have less affinity for water than cells in flowing pellets; hence, those cells in contact with glass or plastic, especially those at the margin of the pellets, are more firmly adherent to the surface and are fixed. Suspensions that produce shields represent an extension of this hypothesis in that the saline ions successfully compete for the adjacent solvent molecules, and cells leave the water phase and form shields regardless of whether the tubes have a flat or curved bottom.

A few observations have been made on the effect of storage of cells in the bicarbonate buffer at 4° C. Some species, namely *H. californica* strain Y 1680, *H. holstii* strain Y 2154, and *H. minuta* strain Y 411, became more hydrophobic during a six weeks storage period. For example in Y 1680 the saline index for flowing pellets was reduced from 125 μg saline per/ml. to less than 16 μg per/ml; that of Y 2154 from 2000 to 125 μg per/ml. and Y 411 formed shields in all the tubes including the control. There was no change in the pattern on storage for some yeasts tested, namely *H. angusta* strain 1798, *H. anomala* strain Y 366, and *H. sp.* Y 2167, Y 2168 or their hybrid.

From the preceding presentation it is apparent that there is a general but not a strict relationship between sedimentation patterns and the phylogenetic origin of yeasts in the genus *Hansenula*. The most hydrophobic yeasts are exclusively diploid, some of the most hydrophilic are exclusively haploid and two entirely haploid species, *H. minuta* strain Y 411 and *H. sp.* strains Y 2167 and Y 2168, are intermediate in hydrophobicity. The species *H. canadensis* strain Y 1888, a diploid, and *H. wingei* strain Y 2340, mostly diploid, although with a more recent evolutionary history, have become physiologically less active as a consequence of their association with bark beetles of coniferous trees (Wickerham, 1956). This is reflected in their sedimentation patterns which indicates that they have about the same proportion of surface polar groups as the primitive haploid *H. holstii* strain 2154 and *H. capsulata* strain Y 1889. *Hansenula silvicola* strain Y 1678, mostly haploid and intermediate in phylogeny, but isolated from gums of wild cherry trees, is just as hydrophilic as some of the primitive yeasts whose habitat is the conifer trees.

It is interesting to speculate why forms whose natural habitats are the conifer trees or cherry gum should be more hydrophilic. One suggestion offered by Wickerham (1951) is that the capsular material formed by these species may be a protective agent for some toxic factor present in the gummy exudate of the trees, the protective but restrictive environment of these yeasts.

Another explanation based on the present study is that the hydrophilic surfaces would enable the cells to compete more favorably with their environmental colloids for water and enable the cells to live separately rather than flocculated as would be true if their surfaces were more hydrophobic. Evidence obtained by Marilyn Kvetkas (personal communication) indicates that when yeasts are flocculated in a culture medium, there is a cessation of growth among the cells. Thus, only species whose cells were hydrophilic could exist in such an environment.

As mentioned earlier with reference to Figure 5, cells of *Schizosaccharomyces versatilis* ATCC strain 9987 and *Saccharomyces cerevisiae* ATCC strain 7754 had only fixed pellets that were the same as in the control irrespective of the saline concentrations. This is in decided contrast to the performance of any of the *Hansenula* yeasts. The result obtained with the *Schizosaccharomyces* was totally unexpected as this species forms a mycelium in addition to arthrospores. *Candida utilis* ATCC strain 9950, *Kloeckera brevis* ATCC strain 9774, and *Saccharomyces carlsbergensis* ATCC strain 9080 gave sedimentation patterns typical of the intermediate group of *Hansenula.*

Hansenula is remarkable in that the various species exhibit such a wide range of hydrophilic and hydrophobic tendencies. In general, the more hydrophilic species are physiologically less active. These require one or more vitamins (Furutani, Betz, and Hedrick, 1953) and ferment either none of the sugars or only one or two sugars and most of them do not produce esters (Wickerham, 1951). The yeasts that are more hydrophobic ferment several sugars, do not require added vitamins and many of them produce esters. In fact, the species of the genus present a variety of forms with different phylogenetic origins and with a variety of other attributes that should make them of interest for further work in comparative genetical, biochemical and biophysical investigations.

Summary

The sedimentation patterns of flowing pellets, fixed pellets, and shields formed by the settling of cells in varying concentrations of saline have been shown to be very sensitive indicators of differences in surface characteristics of yeast cells. The different species of *Hansenula* yeasts form an array of sedimentation patterns from ones that are free flowing in most of the tubes, suggesting very hydrophilic tendencies, to patterns that are shields in all the tubes, indicating extreme hydrophobic characteristics. These patterns and their associated surface attributes have been discussed in relation to phylogeny within the genus *Hansenula.*

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