DISSOCIATION IN YEASTS

THESIS FOR THE DEGREE OF M. S.

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Thesis.

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By

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THESIS

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Dissociation in Yeasts

Introduction

The instability of bacterial species has been noted by various workers in the field of bacteriology since bacteria became the subject of intensive study. As early as 1877 Nägeli pointed out the possibilities of such instability. He held the view that the fission fungi represented only a single type of cell which was highly sensitive to its environment and was capable of great variability as regards its morphological and physiological characteristics. At about the same time Nägeli and his colleagues were pointing out the instability of bacterial species, another group, headed by Cohn and Koch was insisting with equal certainty on the constancy of bacterial species. Almost from the very beginning of bacteriology then one finds two widely different views on the stability of bacterial species. The more conservative views of Cohn and Koch prevailed so that there gradually was recognized for each bacterial species only one characteristic morphological and physiological type. However, from time to time different workers in bacteriology were observing variations in bacterial types too significant to be ignored. As the results of these observations were published sufficient data were accumulated to convince the most skeptical that considerable variation does exist in bacterial species as regards their morphological, physiological and immuniological characteristics. Hadley (1927) has made a most excellent and extensive review of the subject in his paper on Microbic Dissociation.

Dissociation in Yeasts

When one turns from bacteria to yeasts, one does not find the abundance of work done on dissociation in yeasts as in bacteria. In fact the whole question seems to have been neglected. However, there are certain data available which leaves no doubt but what considerable variation does exist in yeast species. Hansen (1895) has contributed the most to show that yeasts may undergo important variations some of which are permanent while others are transitory. In his work with *Saccharomyces carlsbergensis* he found that if this yeast was grown at 27°C, it produced normal cells with typical colonies. If, however, it was cultivated at 7°C, the cells were elongated forming sort of a mycelium and the colonial forms were very much different than the normal colonies. He was able to preserve this variation for six months. Variations of this nature were considered temporary.

Lepeschkin (1903) in his work with yeasts, especially *Schizosaccharomyces mellacci*, found constant variations. When a young culture of *Schizosaccharomyces mellacci* was grown in glucose yeast water, there appeared mycelial forms either with or without ascospores. He was able to maintain the mycelial forms, in pure culture indefinitely.

Guilliermond (1919) has likewise been able to obtain mycelial forms in a young culture of Schizosaccharomyces Pombe and maintain them constantly in pure culture. With Saccharomyces Ludwigii he was able to isolate and maintain in pure culture three distinct forms, a sporogenic strain, an asporogenic strain and a feebly sporogenic strain.

Linder (1909) cultivated Saccharomyces Bailii, Pichia hyalospora and Pichia farinosa for a long time on gelatin and found that they had lost their ability to produce spores. Holm according to Guilliermond (1919) has been able to do the same thing with Saccharomyces multisporus by culturing it a long time on beer wort with sucrose.

Beijerinck (1897) in growing Schizosaccharomyces octosporus on nutrient gelatin noticed three types of colonies. White colonies, the cells of which produced ascospores; light brown colonies, made up of a mixture of cells some of which were asporogenic while others were sporogenic; and brown colonies containing cells which were entirely asporogenic. The two different types of cells possessed different morphological and physiological characteristics. The sporogenic cells were elongated, liquefied gelatin and stained blue with iodine. The asporogenic cells were more oval, liquefied gelatin less readily and stained yellow with iodine. According to Guilliermond (1919) Beijerinck secured similar results with Schizosaccharomyces Pombe when cultivated on nutrient gelatin. He obtained a white colony composed of sporogenic cells and a brown colony having asporogenic cells.

It has been observed also that loss of sporulation may be accompanied by a loss of sexuality. This has been observed with Schizosaccharomyces mellacei and Saccharomyces Ludwigii.

Saito according to Guilliermond (1919) observed two types of colonies in Zygosaccharomyces mandshuricus. A transparent yellow colony containing asporagenic cells with a small amount of glycogen, the cells of which were long and sometimes formed chains. They liquefied gelatin. The other type of colony was white. Most of the cells of this type of colony were spherical, contained a large amount of glycogen, produced ascospores and did not liquefy gelatin.

It is evident from this brief review of the literature that many investigators have been able to induce or have observed morphological and colonial changes in different species of yeasts. When one turns to the physiological changes, which they have been able to induce, the results are less striking. Hansen working with Saccharomyces turbidans, a bottom yeast, was able to transform it into a top yeast by keeping it at a temperature of 5°C. On the other hand, he was not able to transform Saccharomyces validus, a typical top yeast, into a bottom yeast. In general he found that bottom yeasts could be converted into top yeasts but found that it was much more difficult to convert top yeasts into bottom yeasts. In the fermentation reactions, he was never able to entirely suppress alcoholic fermentation but was able to increase or decrease it.

Hansen was of the opinion that the composition of the medium was not an important factor in inducing variations in yeasts. He did not believe that the addition of materials such as various salts, peptone, as maltose to a solution or the use of must gelatin made any difference in inducing transformation. He was of the opinion that aeration did not make any difference in this respect. The only factor, he believed, which seemed to have any effect was temperature. He was able to induce all his variations by extremes of temperature abnormal to the yeasts.

Present Work

In trying to revive a dried up agar slant culture of Saccharomyces cerevisiae Saaz, a culture of diplococci was obtained. Upon serial transfer in malt extract broth, this diplococcic form was gradually changed back into the original form. The present work was undertaken to determine whether this was a dissociant of the yeast, comparable to dissociated forms in bacteria, or whether the formation of this form was purely accidental and if so whether it was possible to reproduce it. It raised the question also whether or not all yeasts do not under certain conditions undergo morphological, cultural and physiological changes.

Description of cultures used

The cultures used were: Saccharomyces cerevisiae Saaz, an industrial yeast, producing a bottom alcoholic fermentation. The cells are spherical and produce no scum in beer wort. The temperature limits for budding in beer

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wort are 3°C to 40°C. Ascospore formation occurs with the production of from two to four ascospores. Agar slant cultures have a smooth, white glistening appearance. For morphological, cultural and physiological characteristics of this yeast see Tables 1 and 2 and Figures 1, 4, 7 and 10.

Saccharomyces cerevisiae Froberg, a yeast having morphological and cultural characteristics similar to those of Saccharomyces cerevisiae Saaz, but producing a slightly less active alcoholic fermentation. (See Tables 1 and 2)

Saccharomyces ellipsoideus Hansen, a yeast producing a bottom fermentation. In beer wort cultures it produces either round or elliptical cells. The temperature limits for budding in beer wort are from 0.5°C to 40°C. Scum formation occurs on liquid media. The cells in the scum may be greatly elongated. Ascospore formation occurs; the ascs are ordinarily small and ellipsoidal and enclose from one to four ascospores. In about half of the cases, they germinate after having conjugated two by two (Marchaud). Agar slant cultures appear dull, white, and usually are slightly wrinkled. For detailed morphological and physiological characteristics see Tables 1 and 2 and Figure 11.

Willia anomala Saito, a spherical yeast producing a white wrinkled scum on liquid media. Agar slant cultures appear dull, white, and pebbly or wrinkled. Sporulation occurs with the production of from one to four ascospores per asc. The ascospores are shaped like a hat with a projecting edge. An alcoholic fermentation is produced. Upon carbohydrate media a fruity odor is given off due to ester formation. For

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detailed morphological and physiological characteristics see Tables 1 and 2.

Zygosaccharomyces mandshuricus Saito. Saito isolated this yeat from Chinese yeast used in making Sorgho, an alcoholic drink of Manchuria. The cells are round or oval. Agar slant cultures are white, smooth, and glistening. Ascs are formed containing from one to four ascospores. These result from an isogamic conjugation. This yeast produces an alcoholic fermentation. For detailed morphological and physiological characteristics see Tables 1 and 2.

Method

All the cultures were serially transfered in malt extract broth until upon plating all colonies presented an entire edge under low power magnification. The cultures were then single-celled, using Chamber's modification of the Barber single cell technique. After a single cell isolation had been obtained of each of the yeasts, the following methods, well known in the study of bacterial dissociation, were used to obtain the different forms of yeasts.

(a) Aging, and serial transfer in lithium chloride broth.

The lithium chloride broth used in this study had the following composition:

10 gms. peptone (Witte's)
5 gms. sodium chloride
3 gms. meat extract
2.5 gms. lithium chloride
1000 cc. distilled water.

The lithium chloride broth was adjusted to pH 7. Rapidly growing cultures of the yeasts were used. These were plated out before using to verify smooth colonial appearance. Inoculations were then made into lithium chloride broth and serial transfers into new lithium chloride broth were made every two days until growth failed to appear in the last inoculated tube. Platings were made from the lithium chloride broth every two days after each inoculation using both dextrose agar and malt extract agar. The composition of the agar media was as follows:

Dextrose agar
15 gms. agar
10 gms. peptone (Witte's)
5 gms. sodium chloride
3 gms. meat extract
10 gms. dextrose
1000 cc. distilled water
Adjusted to pH 7.0

Malt extract agar
15 gms. agar
10 gms. peptone (Witte's)
5 gms. sodium chloride
3 gms. meat extract
10 gms. dextrose
30 cc. malt extract (Trommer's)
1000 cc. distilled water
Adjusted to pH 6.0

Colonies showing variations from the normal were selected from the plates and transfers made to agar slants. Simultaneously, hanging drop preparations were made of the lithium chloride cultures and morphological studies conducted to observe the changes occurring in this medium. As a control upon lithium chloride broth, cultures of the yeasts were inoculated into malt extract broth at the same time and platings made from this at the same time as from the lithium chloride broth. Cultures of the yeasts were also allowed to age in both the lithium chloride broth and in the malt extract broth. Platings were made every two days from each of these broths to observe the influence of aging in these broths upon the yeasts.

The malt extract broth used had the following composition:

30 cc. malt extract (Trommer's)
1 gm. K_2HPO_4
1 gm. NH_4Cl
15 cc. N/1 citric acid
1000 cc. distilled water
The reaction was pH 5

(b) Aging, and serial transfer in brilliant green medium.

The effect of brilliant green upon the yeasts was determined by using a medium of the following composition:

10 gms. peptone (Witte's)
20 gms. meat extract
7 cc. of 1% brilliant green
7 cc. of a saturated solution of picric acid.

The yeasts were transfered serially every two days in the brilliant green medium and plated after 48 hours incubation in this medium. They were also allowed to age in this medium and platings made every two days from the tubes which had aged for different lengths of time. In this way the influence of this medium on the yeasts was determined.

(c) Influence of high concentration of alcohol on yeasts.

Ethyl alcohol was sterilized by refluxing in a sterile condenser for one hour. This sterile alcohol was then added to malt extract broth to make broths containing 5,10,15,20,25,30, 40 and 50 per cent alcohol. (See Table 4) Platings and hanging drop preparations were made of the yeasts at the end of the first and second week after inoculation in the different concentrations of alcohol. Control tubes consisted of uninoculated tubes of the various concentrations of alcohol which were examined for sterility, and also cultures in alcohol-

free malt extract broth. These were examined at the same time and in the same way as the cultures in the alcohol media.

(d) Effect of dessication on the yeasts.

Actively growing malt extract broth cultures of the yeast were seeded on sterile plaster of Paris blocks and allowed to dessicate. At the end of one week hanging drop preparations and plate cultures were made from the blocks.

(e) Effect of temperature on the yeasts.

Plain broth and malt extract broth cultures of the yeasts were incubated at temperatures of 9, 17, 23, 29 and 37°C. (See Table 5) Hanging drop preparations were examined and agar plate cultures made from the two different broths at weekly intervals for a period of one month.

Definition of terms used in connection with the various forms of yests obtained in the different media.

During the course of this work certain definite colonial, morphological, cultural and physiological forms have occurred constantly in the yeasts when they were grown in the presence of certain chemicals in the different media or when they were subjected to certain physical agents as dessication and temperature. For the purpose of clarity the salient characteristics of these different forms will be described briefly.

The smooth form of a yeast is considered as one having uniform and regular morphology, which when plated on agar forms a smooth glistening colony with an entire edge under low power magnification. Agar slant cultures appear smooth and glistening.

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The physiological reactions are the same as those commonly ascribed to the species. The smooth form of the yeast is usually the normal form by which the yeast is commonly recognized although there are exceptions to this as will be noted later. The smooth form of yeast will be designated hereafter as the "S" form. (See tables 1 and 2 and Figures 1, 4, 7, 10 and 11)

The rough form of a yeast is considered as one having slender, greatly elongated or irregular shaped cells. The colonial forms are rugose, dull and when viewed under low power magnification, they have an irregular filamentous edge resembling mycelial growth in the molds. Agar slant cultures are likewise dull and rugose, often having a powdery appearance. The rough form will be referred to hereafter as the "R" form. (See Tables 1 and 2 and Figures 2, 5, 8, 10 and 12)

A third form of yeast which has been produced regularly is what we have termed a "microform" which will be designated hereafter as the "M" form. The microform of a yeast consists of either spherical or rod-shaped cells greatly reduced in size from that of the normal yeast. Colonies on agar plates usually grow slowly at first, in many cases being microscopic in size after a weeks incubation. Under low power magnification they may present either an entire edge or an irregular edge. In the later case the colonies resemble bacterial roughs. However, after they have become adapted to growing on culture media, they grow very rapidly and produce a large, flat, adherent, spreading colony. (See Figures 6, 9 and 13) Not only are the morphological and cultural characters of the M

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form of the yeast widely different from that of the S and R forms of the same yeast but also its physiological characters, since it no longer produces alcoholic fermentation with gas, but produces an acid fermentation without gas. (See Tables 1 and 2).

A fourth form of yeast cell has also been observed constantly in this work. This form has been designated as the transitional form and will be refered to hereafter as the "T" form. The "T" form has never been cultured and has been observed only under the microscope. This form of the yeast may be either oval or elongated depending upon the yeast being studied. They are practically the same size as the cells of the culture in which they appear. They appear in both S and R cultures and are characterized by being more highly refractive than the other cells and are not readily stained. If such cells are isolated and observed carefully for several hours in a moist chamber, a number of small budding forms or "gonidia" appear on the periphery of the cell. These small forms are the microforms which have just been described as the M form of the yeast. These T forms have been single celled a great many times and they have always produced the M forms and never their own kind. They are formed in all the media and under all the physical conditions used in this work. They were produced most abundantly in the malt extract broth containing the different percentages of alcohol.

They were always observed when the S and R forms of the yeast was being dissociated into the M form but were never

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observed when the M form of the yeast was being reverted to the R and S forms.

Results

Description of the various forms obtained.

As previously stated all the cultures used in this study were first reduced to what was considered the smooth or S form. After they had been reduced to this form, they were isolated by Chamber's modification of the Barber single cell technique. The cultures obtained from a single cell were then serially transferred into the various media, viz., lithium chloride broth, brilliant green broth and malt extract broth containing 5, 10, 15, 20, 25, 30, 40 and 50 per cent alcohol respectively. They were also grown in plain and malt extract broths at temperatures of 9, 17, 23, 29 and 37°C. and subjected to dessication on plaster Paris blocks. Microscopic examinations of the cultures were made every other day to determine the appearance of the different forms. The cultures were also plated on malt extract and dextrose agar every other day to check the colonial forms with the morphological observations. It was necessary to use both malt extract and dextrose agar since the M. forms did not grow readily on malt extract agar from the primary isolation. In fact it required at least five days for them to grow out on dextrose agar. However, once colonial forms had been obtained subsequent transfers grew readily on either malt extract or dextrose agar.

Since the different yeasts reacted differently to various conditions and each presented different morphological

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changes, it will be necessary to consider them separately.

Induced forms of *Saccharomyces cerevisiae* Saaz.

In lithium chloride broth the M forms of *Saccharomyces cerevisiae* Saaz began to appear in the microscopic preparations after six days aging or after being serially transferred every other day for three times. However, they were completely overgrown on the plates by the S forms. The R forms did not appear in the microscopic preparations or on the plates until the cultures had been aged in lithium chloride broth for 12 days or had been serially transferred for the same length of time. At the end of this time elongated cells averaging 14 to 20 microns in length, began to appear. The R cells produced rugose, dull, wrinkled colonies with filamentous projections extending from one to two centimeters from the colonial mass. Single cell isolations of this R form have remained stable on culture m media for six months. (See tables 1 and 2 and Figures 2, 5, and 8)

After several rapid transfers of the R forms in lithium chloride broth, or after aging in this medium for two weeks or longer M forms were obtained. It was, therefore, possible to obtain M. forms of the yeast both from the S form and the R form of the yeast by several rapid transfers in lithium chloride broth or by aging in this medium for some time. It required the same length of time for the M. forms to appear by either method. The advantage of serial transfers over the aging method was that in the former method the S or R form was gradually eliminated or greatly reduced in number. For example if cultures of the S form were used, by serial transfer the number of S forms gradually diminished and there was a corresponding increase of R and M forms.

The M forms obtained in the case of *Saccharomyces cerevisiae* Saaz were small diplococci, one micron in diameter, exactly like the culture first encountered from the dried tube of agar which had been kept 18 months. The colonies were entire and remained microscopic in size even after five days incubation. However, after this time they apparently become adjusted to the medium and formed thin, dull pebbly macroscopic colonies. Upon aging the colony turned to a pale yellow color. (See tables 1 and 2 and Figures 3, 6, 9 and 10)

The control tubes consisting of cultures of the yeast undergoing aging and being serially transferred in malt extract/broth yielded only the S form of the yeast. No changes occurred at any time.

When the S form of *Saccharomyces cerevisiae* Saaz. was serially transferred in brilliant green broth, R forms appeared within 24 hours. The S form of the culture was completely changed to the R form in this time. The morphological and cultural aspects of the R form corresponded to those obtained in lithium chloride broth which have been previously described. In this medium M. forms appeared at the end of four days aging or after the second transfer. After the third transfer in this medium subsequent transfers failed to grow and at the end of a week, it was impossible to obtain growth from any of the tubes When the concentration of the brilliant green was reduced to a point which permitted the yeast to grow indefinitely, no

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variation occurred. Under these conditions only the S form of the yeast was obtained. The M forms obtained in this medium were identical in all respects to those obtained in lithium chloride broth. Cultures of the yeast in malt extract broth, run as a control, remained in the stable S form, showing no variations.

In malt extract broth containing different percentages of alcohol no R forms of Saccharomyces cerevisiae Saaz were obtained. At the end of one week incubation at 20°C M forms of the yeast began to appear. Agar plates made from the tubes showed that the number of M forms appearing was dependent upon the amount of alcohol present up to a certain percentage. After this the concentration of the alcohol was germicidal to the yeasts. These data are given in tabular form in Table 4. The M forms were identical in all respects to those obtained in the lithium chloride and brilliant green broths. Control cultures of the yeast in alcohol-free malt extract broth remained in the stable S form.

When the S forms of Saccharomyces cerevisiae Saaz were placed in malt extract and nutrient broth and incubated at temperatures of 9, 17, 23, 29 and 37°C., various changes in the yeast form occurred. At the higher temperatures, 29 and 37°C., the R forms began to appear at the end of two weeks. At the end of four weeks at 37°C. both the S and R forms had disappeared and only the M form was present. At 29°C. at the end of four weeks all three forms were present. At the lower temperatures the S form remained stable and there was no evidence of either the R or M forms. (See Table 5)

On plaster Paris blocks Saccharomyces cerevisiae Saaz, when examined at the end of week showed no R forms but both S and M forms were obtained.

With single cell isolations from a typical culture of Saccharomyces cerevisiae, Saaz which was considered as the S form of the culture, it was possible to convert it into two other distinct forms by serially transferring it in lithium chloride broth, or brilliant green broth or by allowing it to age in these media., or by aging in different percentages alcohol. Similar results were obtained by aging at temperatures of 29 and 37°C or by dessication on plaster Paris blocks. The morphological and colonial appearance of the R cultures were considerably different than the S cultures. (See Tables 1 and 2 and Figures 1 to 10) The fermentation reactions of the R form was the same as that of the S form. However, the M forms produced were radically different from those of either the S or R forms. They were much smaller, different in shape, produced a different type of colony and possessed entirely different fermentation reactions. The S and R forms fermented the same sugars with the production of alcohol and carbon dioxide while the M forms produced no alcoholic fermentation but an acid fermentation. Besides causing an acid instead of an alcoholic fermentation, the M form fermented all the sugars that the S and R forms fermented except raffinose and in addition caused an acid fermentation in lactose, glycerol, mannitol and dextrin. (See Table 1). The M form of this

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yeast was asporogenic, while both the S and R forms were sporogenic. (See Table 2) Thermol death point determinations on the S, R and M forms of Saccharomyces cerevisiae Saaz revealed that the M form was more resistant to heat than either the S or R form. The M form had a thermol death point of 56°C., while the S and R forms run at the same time and under the same conditions had a thermol death point of 52°C.

Reversion of M and R forms to S forms

After the M forms had been obtained, they were single celled and two different cultures of single cell isolations were serially transferred in malt extract broth every ether day for a period of two weeks. At the end of this time both of the cultures from the single cell isolation gave tetrad forms, 88, the diameter of which were 3 to 3.5 microns. Upon transferring the tetrad forms for two weeks longer in malt extract broth, one of the cultures gave three different colonial forms. One of the colonial forms obtained was an S form which produced smooth pink colonies. The cells were spherical and were from 5 to 6 microns in diameter. When these pink colonies were transferred to a dextrose or malt agar slant, they produced an abundant pink growth which after four or five days became mucoid in character and run off the slant to the bottom of the tube where it collected in a large viscous mass. Subsequent transfers on agar slants behaved similarly. This mucoid form was serially transferred for three weeks in malt extract broth at the end of which time, it had completely reverted to the normal S form.

Another colonial form obtained from this culture was a white wrinkled powdery R form similar to those obtained from lithium chloride broth and the other methods used in converting the S forms to the R forms. The cells were 4 x 12 microns. After 16 days serial transferring of this R form, it reverted to the normal S form. The third colonial form obtained from this single cell culture was an R form which produced dull black wrinkled colonies. The size of the cells of the black form was 4 x 12 microns. After transferring serially for 22 days in malt extract broth, they reverted to normal S forms.

The other culture obtained from a single cell isolation was transferred at the same time and under the same conditions in malt extract broth for a period of six weeks. At the end of this time it reverted to the normal S type without producing the pink S form or the white R form or black R form. However, as previously stated, it produced the tetrad forms at the end of two weeks the same as the other single cell culture.

Summary

In the case of <u>Saccharomyces</u> <u>cerevisiae</u> Saaz, it was possible to convert single cell isolations of the normal S form by means of chemicals added to suitable media and by physical influences into typical R and M forms having certain morphological, cultural and physiological characteristics. These forms were obtained in stable form. In the case of the R form, cultures were obtained which have remained stable for

a period of six months and in the case of the M forms cultures were obtained which have remained stable for a period of ten months.

It has likewise been possible to make single cell isolations of the R and M forms and by serially transferring these single cell cultures in malt extract broth to change them back into the normal S form. The reversion process may take place in an orderly manner or several intermediates may be formed as pink mucoid forms or black rough forms both of which, upon continued transfer, revert to the normal S form.

Induced forms of Saccharomyces cerevisiae Froberg

In lithium chloride broth the R forms of Saccharomyces cerevisiae Froberg began to appear in the microscopic preparations and on the plates made from the lithium chloride broth after fourteen days aging in the broth, or after being serially transferred in the broth for the same period of time. The R cells were long slender rods 3 x 14 microns. Macroscopically they produced dull, rugose, wrinkled colonies the edges of which under low magnification were filamentous and extended about one centimeter from the colonial mass. Single cell isolations of this R form reverted to the normal S form at the end of three weeks aging on agar. No stable R form was obtained.

After continued aging or serial transfer of the S form in lithium chloride broth for a period of twenty-two days M forms were obtained. M forms were also obtained by aging or serial transfer of the R form in lithium chloride

broth for a period of two weeks.

The M forms obtained from Saccharomyces cerevisiae Froberg were small rods which measured 1 x 1.5 microns. At the end of three days on agar plates, the colonies were macroscopic in size and had an irregular edge, the colony resembling the colonies formed by R forms of bacteria. After five days incubation at room temperature on an agar plate, the M form gave a thin, dull gray, adherent, spreading colony. Upon subsequent transfer to sterile agar plates, colonies were produced which spread over the entire surface of the plate in one weeks time. Cultures of the S form in malt extract broth run under the same conditions and in the same manner as the lithium chloride tubes, remained stable.

When the S form of Saccharomyces cerevisiae Froberg was aged in or serially transferred in brilliant green broth, R forms appeared within 24 hours. The morphological and cultural aspects of the R form corresponded to those obtained from lithium chloride broth. Single cell isolations of this R form likewise reverted to the normal S form at the end of three weeks on agar slants exactly as had the R forms obtained from lithium chloride broth. In brilliant green broth M forms appeared after six days aging or after three serial transfers. After the fourth transfer in this medium, subsequent transfers failed to grow and at the end of ten days it was impossible to obtain growth from any of the tubes. The M forms obtained in this medium were identical to those obtained in lithium chloride broth;control tubes remained in the stable S form.

In malt extract broth containing different percentages of alcohol, no R forms were obtained of Saccharomyces cerevisiae Froberg. However, M forms appeared at the end of one week incubation at 20°C. The percentages of S and M colonies developing on agar plates made from the tubes revealed that the number of M forms present in the media was directly dependent on the concentration of the alcohol in the media. (See Table 4) The M forms obtained in these alcohol media were identical to those obtained in the lithium chloride and brilliant green broths. Control cultures in alcohol-free malt extract broth remained stable in the S form.

When the S form of Saccharomyces cerevisiae Froberg was inoculated in malt extract and nutrient broth and incubated at temperatures of 9, 17, 23, 29 and 37°C., R forms were obtained only at a temperature of 37°C. At this temperature, R forms began to appear at the end of two weeks and at the end of four weeks both S and R forms were present in about the same proportion. No M forms were obtained at any time. At the lower temperatures the S form remained stable with no appearance of either R or M forms in the tubes.

On plaster Paris blocks Saccharomyces cerevisiae Froberg produced no R forms. When examined at the end of one week, both S and M forms were obtained.

By the methods outlined above, a typical culture of Saccharomyces cerevisiae Froberg obtained from a single cell isolation considered as the S form was converted into two other distinct forms, the R and M forms. The morphological and cultural characteristics of the R form were greatly different from those of the S form. However the R form possessed the same fermentation reactions as the S form. (See Table 1) The M forms obtained differed greatly from either the S or R forms. They were much smaller, different in shape, being short rods, had a different colonial formation and entirely different fermentation reactions. Whereas, the S and R forms produced an alcoholic fermentation with gas, the M forms produced an acid fermentation. The M form had lost the ability to ferment maltose, galactose, and raffinose but had retained the ability to ferment sucrose, dextrose and levulose. The S and R forms fermented all of these sugars. In addition to this the M form gave an acid fermentation in mannitol, while the S and R forms did not ferment mannitol. The M form of this yeast also differed from the S and R form in that it was asporogenic and liquefied gelatin. The S and R forms were both sporogenic and did not liquefy gelatin at the end of four weeks. (See Table 2)

Reversion of R and M Forms to S forms

Typical cultures of the R and M forms were obtained in single cell isolations. The resulting R and M cultures from these single cell isolations were planted in malt extract broth and serially transferred every other day. At the end

of ten days the R form had completely reverted to the normal S form. Cultures of the R form on agar slants also reverted to the S form at the end of three weeks. After being serially transferred in malt extract broth for 24 days, the M form reverted to the normal S form. This reversion was abrupt, no intermediate forms appearing.

Induced forms of _Saccharomyces_ _ellipsoideus_

In lithium chloride broth R forms of _Saccharomyces ellipsoideus_ began to appear after six days aging of the S form in this broth, or after serial transfer of the S form in this broth every other day for a period of six days. The R cells were long slender rods from two to six microns in diameter and averaging sixteen microns in length. They produced dull, rugose, wrinkled colonies the edges of which when examined under lower power magnification showed very fine, slender filamentous chains of cells extending from three to five centimeters from the colonialmass. (See Figure 12) Single cell isolations of this R form have remained stable on agar slants for five months.

After further aging or serial transfer of the R form in lithium chloride broth, M forms appeared at the end of twelve days. The M forms obtained from _Saccharomyces ellipsoideus_ were small rods 1 x 1.5 microns. On agar plates, the colonies remained microscopic in size growing very slowly for the first five days. However, after this time they developed more rapidly and after two weeks formed a dull gray, thin, adherent, spreading colony, 5 to 7 cm. in

diameter, with an irregular edge. (See Figure 13) Agar slant cultures had a thin spreading growth and produced a dark greenish discoloration of the medium. S cultures in malt extract broth, run under the same conditions and in the same manner as the lithium chloride cultures remained stable and contained nothing but S cells.

When the S form of Saccharomyces ellipsoideus was aged or serially transferred in brilliant green broth, R forms were obtained at the end of two days. Morphological and cultural aspects of this R form were identical with those obtained from lithium chloride broth. In the brilliant green broth M forms appeared at the end of four days aging or after the second serial transfer. After the fourth serial transfer, subsequent transfers failed to grow and at the end of eight days it was impossible to obtain growth from any of the tubes. This M form was similar in every detail to the M forms obtained from lithium chloride broth. Control cultures in malt extract broth remained stable in the S form. No other forms were obtained from the tubes.

In malt extract broth containing different percentages of alcohol no R forms were obtained of Saccharomyces ellipsoideus. At the end of one weeks incubation at 20°C., M forms began to appear in certain tubes (See Table 4) depending upon the concentration of alcohol. The M forms obtained were similar to those obtained in the other two media. Control cultures in alcohol-free malt extract broth did not produce the M form, but were stable and remained in the S form.

When the S form of Saccharomyces ellipsoideus was

planted into malt extract and nutrient broth and incubated at temperatures of 9, 17, 23, 29 and 37°C., changes occurred in the form of the yeast only at the two higher temperatures. At the end of two weeks at 37°C, only the R form of the yeast was present. At the end of the third week M forms began to appear and at the end of the fourth week were present in large numbers. At 29°C. R forms began to appear at the end of two weeks and at the end of four weeks both S and R forms were present. At this temperature the S forms were more stable than at 37°C since they persisted for a much longer time. No M forms occurred at this temperature. At the lower temperatures, the S form remained stable; there was no appearance of either the R or M forms. (See Table 5)

On plaster Paris blocks Saccharomyces ellipsoideus produced no R forms. M forms were obtained upon examination of the blocks at the end of one week.

By the methods outlined above, a typical culture of Saccharomyces ellipsoideus, from a single cell isolation, considered as the S form, gave rise to two other distinct forms, the R and M forms. The R form differed from the S form in morphological and cultural characteristics but possessed the same fermentation reactions. The M forms differed greatly from either the S or R form. They were much smaller, being short rods, had a different colonial formation, and different fermentation reactions. Whereas, the S and R forms produced an alcoholic fermentation with gas, the M form produced an acid fermentation. The M form fermented the same sugars as the S and R forms with the

exception of maltose, and in addition caused an acid fermentation in mannitol, glyceral, and levulose. (See Table 1). The M form of this yeast liquefied gelatin at the end of one week, while the S and R forms did not liquefy gelatin. The M form of this yeast differed from the S and R forms in another respect. Both the S and R forms were sporogenic; however, the M form was asporogenic. (See Table 2)

Reversion of the R and M forms to the S form.

Typical stable cultures of the R and M forms were obtained in single cell isolations. The resulting R and M cultures from these isolations were planted in malt extract broth and serially transferred every two days. At the end of 28 days the R form had completely reverted to the S form. In the case of the M form, reversion to the S form took place after 34 days of serial transfer in malt extract broth. This reversion of the M to the S form was abrupt, no intermediate forms appeared.

Induced forms of Willia anomala

In the case of Willia anomala the culture that we had at the start was an R form. After three weeks serial transfer in malt extract broth, this R form gave smooth typical S form colonies. This S form was single celled and has since remained as a stable S form.

When the S form of Willia anomala was planted into lithium chloride broth and allowed to age, or was serially transferred in the broth, R forms appeared at the end of four days. No S forms were present in the cultures, the conversion to the R form had been complete in this time. The R cells

were from 6-8 x 14-20 microns. The R cells produced white, rugose, wrinkled, powdery colonies the edges of which were filamentous. Under low power magnification the filaments were visible as long chains of the R forms. The filaments were relatively large and extended one centimeter from the colonial mass. Single cell isolations of this R form have remained stable on agar slants for four months.

After continued aging or serial transfer of this R form in lithium chloride broth, at the end of ten days M forms appeared. The M forms obtained from Willia anomala were small diplococci measuring from 1-1.5 microns in diameter. The colonies on agar plates grew very slowly, and were microscopic in size, for the first five days after plating. After this time they grew more rapidly forming dull light gray, spreading, adherent colonies having an irregular edge. Upon agar slants, a darkening of the medium occurred.

The S form run as a control in malt extract broth remained stable and did not produce either the R or M form.

When the S form of Willia anomala was aged or serially transferred in brilliant green broth, one hundred per cent conversion to the R form occurred in 24 hours. Upon further aging or serial transferring of the R form in this medium, M forms were obtained at the end of four days. After the fifth transfer subsequent transfers failed to grow and at the end of one week it was impossible to obtain growth from any of the tubes. The R and M forms of Willia anomala obtained in this medium were identical to those obtained in

lithium chloride broth. Control cultures in malt extract broth remained in the stable S form, showing no variation.

When the S form of Willia anomala was planted into malt extract and nutrient broth and these cultures incubated at temperatures of 9, 17, 23, 29 and 37°C., changes in the form of the yeast occurred only at the two higher temperatures. At 37°C. R forms appeared at the end of the first week and at the end of the third week the culture was one hundred per cent R. At the end of the fourth week M forms began to appear; both R and M forms being present. At 29°C R forms appeared at the end of the second week and at the end of the fourth week both S and R forms were present. The presence of the S form at the end of four weeks at this temperature indicates the influence of temperature on dissociation since at 37°C all the S forms had disappeared in a much shorter time. No M forms were obtained at this temperature. At the lower temperatures the S form remained stable; neither R nor M forms appeared in any of the tubes.

On plaster Paris blocks Willia anomala produced no R forms. Upon examination of the blocks at the end of one week M forms were obtained.

By the methods outlined above, a culture of Willia anomala, from a single cell isolation, considered as the S form, gave rise to two other forms, the R and M forms. The R form differed culturally and morphologically from the S form but possessed the same fermentation reactions. The M forms differed radically from the S and R form. They were

small diplococci, had a different colonial formation and fermentation reactions. The S and R forms produced an alcoholic fermentation with gas. The M form produced an acid fermentation without gas. The M form fermented the same sugars as the S and R forms with the exception of raffinose, and in addition caused an acid fermentation in maltose. Moreover, the M form of this yeast liquefied gelatin rapidly, while the S and R forms did not liquefy gelatin. Furthermore, the M form of this yeast differed from the S and R forms in that it was an asporogenic form, while both the S and R forms were sporogenic. (See Table 2)

Reversion of the R and M forms to the S form

Typical R and M forms were obtained in single cell isolations. The resulting cultures were planted into malt extract broth and serially transferred in this medium every other day. At the end of three weeks the R form had been converted back to the S form. In the case of the M form, after serial transfer in this medium for 34 days, the S form was obtained. This reversion was abrupt, no intermediate forms were obtained.

Induced forms of Zygosaccharomyces mandshuricus

When the S form of Zygosaccharomyces mandshuricus was aged or serially transferred in lithium chloride broth R forms appeared in six days. The R cells were many shaped averaging from 6-9 microns wide and 10-20 microns long. Many bizarre forms occurred. The R cells formed dull, wrinkled colonies with a frilly edge. Single cell isolations of this

R form reverted to the normal S form on agar slants at the end of two or three weeks aging.

Upon further transfer of the R form of the culture in lithium chloride broth, M forms appeared at the end of fourteen days. The M forms obtained from Zygosaccharomyces mandshuricus were small rods from 1.2 to 1.5 microns in length. The colonies grew slowly at first on agar plates and remained microscopic in size for five days after plating. At the end of this time they became adapted to growth on dextrose agar and produced colonies about one centimeter in diameter at the end of two weeks. The M form produced dull smooth colonies with an entire edge. The colonies were bright orange in color. Agar slants likewise were bright orange in color. This M form was very adherent to the agar. Control cultures in malt extract broth, run at the same time and under the same conditions as the lithium chloride cultures, yielded only the S form, no variations occurring at any time.

When the S form of Zygosaccharomyces mandshuricus was aged or serially transferred in brilliant green broth, no R forms were obtained. M forms were obtained in this medium at the end of six days aging or by serial transfer. The M form obtained in this medium was identical to those obtained in lithium chloride broth. (See Table 4) Control cultures in malt extract broth showed no change; the S form remained stable in this medium.

When the S form of Zygosaccharomyces mandshuricus was planted into malt extract and nutrient broth and incubated at 9, 17, 23, 29 and 37°C., changes occurred in the form of the yeast at the two higher temperatures. At 37°C., R forms were obtained at the end of three weeks incubation. At the end of four weeks all three forms, the S, R, and M forms, were present. At 29°C., R forms appeared at the end of the fourth week, but no M form of this yeast was produced at this temperature. At the lower temperatures the S form remained stable, neither the R nor M forms appeared. (See Table 5)

On plaster Paris blocks Zygosaccharomyces mandshuricus produced no R forms. Upon examination of the blocks at the end of one week M forms were obtained.

By the methods outlined above, a culture of Zygosaccharomyces mandshuricus, from a single cell isolation, considered as the S form, gave rise to two other forms, the R and M forms. The R form differed morphologically and culturally from the S form but possessed the same fermentation reactions. The M form differed greatly from the S and R forms, being much smaller, and presented different cultural appearances. The S and R forms produced an alcoholic fermentation with gas. The M form had very feeble fermentative powers, produced no alcoholic fermentation, but a very slight acid fermentation. The M form fermented all the sugars that the S and R forms did with the exception of sucrose, and in addition produced an acid fermentation in raffinose. The S and R forms did not liquefy gelatin in four weeks while the M form completely, liquefied gelatin inside of a week. Furthermore, the M form

of this yeast was an asporogenic form, while the S and R forms both formed spores.

Reversion of the R and M forms to the S form

Typical R and M forms were obtained in single cell isolations. The resulting cultures were planted into malt extract broth and serially transferred every other day. At the end of two days the R form had completely reverted to the normal S form. In the case of the M form, after six days of serial transfer in malt extract broth the M form had reverted to the S form. The change was abrupt no intermediate forms of the yeast occurred.

Mechanism of the changing of an S form to an R or M form

During the course of studying the various morphological, cultural and physiological changes which took place in the different yeasts as they were transferred serially or aged in the various media or subjected to the different physical agents, it appeared that the changes occurred in an orderly manner. Starting with the smooth form of the yeast with typical morphological, cultural and physiological characteristics, there was a gradual change to the R form. The cells gradually changed to an elongated form with all degrees of elongation intervening between the normal S form of cell and the stable R form of cell which has been described previously and the characteristics of which are given in tables 1 and 2. Likewise, intermediate colonial forms were obtained varying in degree of roughness between the normal S type and the

stable R type of colony which has been described earlier in the paper.

In the reversion of the R form to the S form the reverse process occurred. The cells gradually lost their elongated character and the colonial forms likewise gradually lost their wrinkled, rugose appearance and their filamentous edge until a complete transformation to the S form had occurred.

In the transformation of an S form to an M form two methods of change occurred. During the process of transformation cells ranging in size from the normal S form or from the typical R form to the M form occurred in the medium. Apparently, there was a gradual diminution in the size of the cells until the stable M form was reached. This method of change from the S to the M form was very common in Saccharomyces ellipsoideus when grown in lithium chloride broth, or when grown at a high temperature. This type of change was less prevalent in the other yeasts studied. It was also less prevalent in Saccharomyces ellipsoideus when this yeast was grown in any of the other media.

The second type of change from an S form to an M form was of an altogether different character. Normal sized S or R cells became highly refractile and became transformed into another form of the yeast which has been described earlier in the paper as the transitional form and which has been designated as the "T" form. These highly refractile T cells did not reproduce by normal budding as did the S and R cells, but by an entirely different process. A multitude of minute buds appeared on the periphery of the cell. These minute

buds, upon becoming detached from the cell, were the M form of the yeast. Numerous single cell isolations of these T cells which were covered with minute buds always gave rise to pure cultures of the M form. This type of transformation of an S form to an M form was observed in all the yeasts studied in all the different media and under all the physical conditions employed. This type of change was prevalent in all the liquid media used, and was especially so in the alcohol media where this was the only type of transformation that occurred.

Apparently then, the transformation of an S form to an M form may be either a gradual process accomplished by a graded diminution in the size of the cells, or may be an abrupt process accomplished by the formation of an intermediate T cell.

In the reversion of an M form to an S form the transition was either gradual with the formation of various intermediates as was the case with the M form of *Saccharomyces cerevisiae* Saaz, as described earlier in the paper, or was abrupt, giving no intermediate forms, as was the case with the M forms of all the other yeasts studied. The T form was never encountered during the reversion process.

Relationship between scum formation and the different forms.

Scum formation has always been used as one of the criteria or characteristics for the identification of yeasts. In view of this fact a series of experiments was set up to study the relationship between scum formation and the various

forms of the different yeasts. For this study malt extract broth and nutrient broth media were used. Single cell isolations of the three forms, S, R, and M, of each of the five different yeasts were inoculated into each of these media and incubated at temperatures of 9, 17, 23, and 29 and 37°C. for a period of three months. There was no further change at the end of four weeks so the data in Table 6 are given only over a period of four weeks.

The results of these studies are given in detail in Table 6 and only a general discussion of the results will be given here. Scum formation occurred in all the R forms of all the yeasts studied regardless of the temperature. The S form only produced scum at the two higher temperatures. It was at these same two temperatures which our previous studies showed dissociation of the S form occurred. Plates made from the S cultures when scum formation was noted at these temperatures showed the presence of R forms of the yeast.

In the case of Zygosaccharomyces mandschuricus both the R and M forms produced scum at all temperatures. At the two higher temperatures scum formation occurred in the S form of this yeast as in the other yeasts but here again when cultures showing scum formation were plated and examined microscopically, they all showed the presence of the R forms of the yeast.

As a result of these studies, it may be concluded that scum formation in yeasts occurs when the R form of the culture is present or in some cases when the M form is

present.

Discussion

It has been possible to take single cell isolations of a pure culture of five species of yeasts and by subjecting them to the influence of certain chemical and physical agents to produce certain definite morphological, cultural and physiological variations. The new forms of the yeasts differ wholly or partially from the form from which they were obtained. In changing from the original to the induced forms, they followed a certain definite sequence of changes which could be reproduced repeatedly and in an orderly manner. The various chemical and physical agents acted in essentially the same manner and differed only in the degree and in the rapidity with which they caused the changes. The various forms when once obtained were in most cases stable and have been kept for several months with little or no tendency to revert to the original forms from which they were obtained.

It has been possible also by certain definite procedures outlined to convert the induced forms back to the original forms which when obtained had all of the characteristics of the original form.

The production of these various forms serves to clarify several points in connection with yeasts which have remained obscure or for which no lucid explanation has been given. Scum formation has long been used as one of the characteristics used in the identification of yeasts. Hansen in his numerous and valuable researches on yeasts observed in many species of yeasts such as <u>Saccharomyces validus</u>,

Saccharomyces intermedius, *Saccharomyces pastorianus* and *Saccharomyces ellipsoideus* scum formation at the higher temperatures in a much shorter time than at the lower temperatures. For example, in the case of *Saccharomyces ellipsoideus* he made the following observation on the ability of this species of yeast to form scum at the following temperatures:

At 38°C. no formation of scum.

33-34°C	scum	formation	complete	at	the	end	of	8-12	days
26-28°C	"	"	"	"	"	"	"	9-16	"
20-22°C	"	"	"	"	"	"	"	10-17	"
13-15°C	"	"	"	"	"	"	"	15-30	"
6-7°C	"	"	"	"	"	"	"	2-3	Months

5°C No formation of scum

In the light of the work reported here (See Table 6) and from Hansen's own observations, it is obvious that when this yeast was placed at the various temperatures, the tendency to scum formation was due to the dissociation of the S form of *Saccharomyces ellipsoideus* to the R form. Scum formation took place in a shorter time at the higher temperatures than at the lower temperatures because the yeast dissociated more rapidly at these temperatures than at the lower temperatures. On the other hand the R form of the yeast formed scum at all temperatures while the S and M forms showed no scum formation at any of the temperatures. The one exception to this was the S form at 37°C where scum was produced due to the dissociation of this form into the R form.

Under industrial conditions as in the manufacture

of beer, a yeast which may always have given good results may suddenly give a beer with evident defects. Likewise in the manufacture of industrial alcohol certain strains of yeasts which have been giving satisfactory yields may suddenly fail to produce satisfactory yields. In fact in some cases they fail to produce any alcohol.

A microscopic examination of the culture from the vats reveals small diplococci. In the past these have been regarded as bacterial contamination. In the light of the present work it would appear that what has happened in such cases is that the yeasts have been transformed in the presence of the alcohol into the M form. The M form as the results here show are produced very abruptly from the S form in the presence of alcohol within one week. Under the conditions found in an industrial alcohol plant where the yeasts were manufacturing their own alcohol the changes doubtless would be less abrupt.

Guilliermond states "Besides morphological variations, one may also observe physiological variations. A yeast may, for example, under certain conditions, induce more or less active fermentations in the same way that a certain bacteria, Bacillus anthracis, for instance, may be made avirulent, among the yeasts it is impossible to suppress the fermenting function. One may decrease it or even increase it but never entirely blot it out."

By "fermenting function" it is assumed that alcoholic fermentation is referred to. This work would indicate that

it is possible to entirely suppress the ability of a yeast to produce alcohol by converting it into the M form. No M forms of any of the yeasts reported in this paper or any that are now being studied have produced an alcoholic fermentation. They produce an acid fermentation without the presence of gas.

Gelatin liquefaction is another instance where there is a fundamental difference between the various forms. The S and R forms do not liquefy gelatin while the M forms do. There is one exception to this in the case of the M form of Saccharomyces cerevisiae Saaz. None of the three forms of this yeast liquefied gelatin. It is obvious then that some of the aberrant results obtained in gelatin liquefaction may be explained on the basis of dissociation.

Summary and conclusion.

Dissociation was induced in cultures from single cell isolations of five different yeasts: viz., Saccharomyces cerevisiae Saaz, Saccharomyces cerevisiae, Froberg, Saccharomyces ellipsoideus, Willia anomala, and Zygosaccharomyces mandshuricus. Certain definite forms of these yeasts appeared constantly and have been designated as S, R, M and T forms. The R, M and T forms of the yeasts were induced from the normal S form by aging or serial transfer of the S form in the following media; lithium chloride broth, brilliant green broth, and by aging them in broths containing high concentrations of alcohol. They were

also induced by dessication and by abnormal temperatures. These forms have been described in detail in the paper.

The salient characteristics of the various forms are as follows: The S form of a yeast is the normal form described in the literature for most species and which possesses the morphological, cultural, and physiological properties usually ascribed to the species. All the species reported here were sporogenic. The R form of a yeast consists of greatly elongated cells which form dull, rugose, wrinkled colonies having a filamentous edge when viewed under low power magnification. This form likewise produces ascospores. The physiological properties of an R form are the same as those of an S form. Studies of scum formation at various temperatures revealed that the R form is a scum producer at all temperatures allowing growth; whereas, the S form produces scum only at those temperatures favorable for dissociation.

The M form of a yeast consists of cells greatly reduced in size from that of the S and R forms. These cells are asporogenic, produce an acid instead of an alcoholic fermentation and differ somewhat from the S and R forms in the sugars fermented. Upon the initial isolation, they grow very slowly on culture media producing colonies microscopic in size at the end of a weeks incubation. Upon becoming adapted to growth on culture media the M form produces a dull, thin, spreading colony.

A fourth type, the T form, is a transitional form of the yeast between the S or R form and the M form. It consists of highly refractile cells which produce the M form by the formation of a multitude of minute buds on the periphery of the cell. This form has never been cultured.

In the case of Saccharomyces cerevisiae Saaz, intermediate chromogenic forms have also been obtained: a pink S form which became mucoid in character upon being aged on an agar slant, and a black R form. Chromogenesis also occurred in one form of Zygosaccharomyces mandshuricus. The M form of this yeast produced bright orange colored colonies.

Single cell isolations of the R and M forms of the yeasts have been reverted to the normal S form by the use of a suitable technique.

Morphological, cultural, and physiological studies were conducted on these induced forms of yeasts. From the results obtained, an attempt has been made to clarify some of the obscure points hitherto noted in the behavior of yeasts.

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Table 1 showing the fermentation reactions of the various forms – of yeasts.

Culture	Form of yeast	Arbinose	Dextrose	Levulose	Galactose	Sucrose	Maltose	Lactose	Raffinose	Glycerol	Mannitol	Dextrin
S. cerevisiae Saaz	*S	–	0	0	0	0	0	–	0	–	–	–
	*R	–	0	0	0	0	0	–	0	–	–	–
	*M	–	3+	2+	2+	3+	4+	3+	–	3+	+	+
S. cerevisiae Froberg	S	–	0	0	0	0	0	–	0	–	–	–
	R	–	0	0	0	0	0	–	0	–	–	–
	M	–	2+	2+	–	4+	–	–	–	–	+	–
S. ellipsoideus	S	–	0	–	–	0	0	–	–	–	–	–
	R	–	0	–	–	0	0	–	–	–	–	–
	M	–	3+	+	–	4+	–	–	–	2+	+	–
Willia anomala	S	–	0	0	–	0	–	–	0	●	–	–
	R	–	0	0	–	0	–	–	0	●	–	–
	M	–	2+	2+	–	4+	3+	–	–	2+	–	–
Z. mandshuricus	S	–	0	0	–	0	–	–	–	●	–	–
	R	–	0	0	–	0	–	–	–	●	–	–
	M	–	+	4+	–	–	–	–	+	+	–	–

0 = alcohol and CO_2; ● = acid and gas; + = acid, figures indicate intensity of reaction
– = no action.
*S = smooth form
*R = rough form
*M = micro form

Table 2. Showing size of cells, gelatin liquefaction, alcohol production, and sporulation of the different forms of the yeasts studied.

Culture	Size of cells in microns			Gelatin liquefaction at end of four weeks			Alcohol production after four weeks in cider			Size of ascospores in microns.		
	S	R	M	S	R	M	S	R	M	S	R	M
S. cerevisiae Saaz	6	4x14-20	1	-	-	-	5.3%	4.8%	-	3.5-4	3.5-4	-
S. cerevisiae Froberg	6.5	3x14	1x1.5	-	-	+	4.2%	4.0%	-	3.2-3.5	3-4.5	-
S. ellipsoideus	5x8	2-6x16	1x1.5	-	-	+	6.2%	4.7%	-	2.5x3.5	2.8-4	-
W. anomala	5	6-8 x 14-20	1-1.5	-	-	+	4.2%	2.8%	-	3-4	2-2.5	-
Z. Mandshuricus	6.5-9.5	10-20	1.2x1.5	-	-	+	3.4%	3.2%	-	3.5x4.5	3.5-4	-

+ = present - = absent

S = smooth form; R = rough form; M = microform.

Table 3. Showing influence of aging in lithium chloride on the dissociation of the S form of the different yeasts.

Culture	First appearance in days R	M
S. cerevisiae Saaz	12	6
S. cerevisiae Froberg	14	22
S. ellipsoideus	6	12
W. anomala	4	10
Z. mandshuricus	6	14

W. anomala - naturally rough - required 3 weeks rapid transfer in malt broth to get smooth.

Table 4. Showing influence of alcohol on dissociation of the S form of the yeasts.

Culture	5	10	15	20	25	30	40	50
	At end of one week incubation at 20°C. Percentage of alcohol							
S. cerevisiae Froberg	5% M	10% M	20% M	50% M	100% M	Few cells all M	Very few cells all M	No cells present
S. cerevisiae Saaz	No M	No M	5% M	25% M	30% M	Few cells all M	Very few cells all M	no cells present
S. ellipsoideus	10% S 85% R 5% M	50% R 50% M	100% M	100% M	100% M	Few cells all M	No cells present	No cells present
	Two weeks incubation at 20°C							
S. cerevisiae Froberg	100% S	100% M	100% M	no cells present	No cells present	No cells present	No cells present	No cells Present
S. cerevisiae	100% S	100% S	50% S 50% M	100% m	No cells present	No cells present	No cells present	No cells present
S. ellipsoideus	10% S 90% R	50% R 50% M	10% R 90% M	No cells present	No cells present	No cells present	No cells present	No cells present

S = smooth form of yeast; R = rough form of yeast; M = microform of yeast.

Per cent refers to the number of cells of each form observed based on the total number of cells present.

Table 5. Showing effect in malt extract broth and nutrient broth of temperature on dissociation in yeasts.

Culture	one week S	one week R	one week M	Two weeks S	Two weeks R	Two weeks M	Three weeks S	Three weeks R	Three weeks M	Four weeks S	Four weeks R	Four weeks M
37°C												
S. cerevisiae Saaz	+	-	-	+	+	-	+	+	+	-	-	+
S. cerevisiae Froberg	+	-	-	+	+	-	+	+	-	+	+	-
S. ellipsoideus	+	-	-	-	+	-	-	+	+	-	+	+
W. anomala	+	+	-	+	+	-	-	+	-	-	+	+
Z. mandshuricus	+	-	-	+	-	-	+	+	-	+	+	+
29°C.												
S. cerevisiae Saaz	+	-	-	+	+	-	+	+	-	+	+	+
S. cerevisiae Froberg	+	-	-	+	-	-	+	-	-	+	-	-
S. ellipsoideus	+	-	-	+	+	-	+	+	-	+	+	-
W. anomala	+	-	-	+	+	-	+	+	-	+	+	-
Z. mandshuricus	+	-	-	+	-	-	+	-	-	+	+	-
23°C												
S. cerevisiae Saaz	+	-	-	+	-	-	+	-	-	+	-	-
S. cerevisiae Froberg	+	-	-	+	-	-	+	-	-	+	-	-
S. ellipsoideus	+	-	-	+	-	-	+	-	-	+	-	-
W. anomala	+	-	-	+	-	-	+	-	-	+	-	-
Z. mandshuricus	+	-	-	+	-	-	+	-	-	+	-	-
17°C												
S. cerevisiae Saaz	+	-	-	+	-	-	+	-	-	+	-	-
S. cerevisiae Froberg	+	-	-	+	-	-	+	-	-	+	-	-
S. ellipsoideus	+	-	-	+	-	-	+	-	-	+	-	-
W. anomala	+	-	-	+	-	-	+	-	-	+	-	-
Z. mandshuricus	+	-	-	+	-	-	+	-	-	+	-	-

Table 5 Con't.

	9°C											
	one week			Two weeks			Three weeks			Four weeks		
Culture	S	R	M	S	R	M	S	R	M	S	R	M
S. cerevisiae Saaz	+	-	-	+	-	-	+	-	-	+	-	-
S. cerevisiae Froberg	+	-	-	+	-	-	+	-	-	+	-	-
S. ellipsoideus	+	-	-	+	-	-	+	-	-	+	-	-
W. anomala	+	-	-	+	-	-	+	-	-	+	-	-
Z. mandshuricus	+	-	-	+	-	-	+	-	-	+	-	-

+ = present   - = absent

S = smooth form; R = rough form; M = microform

Table 6. Showing the effect of temperature on scum formation of the various forms of yeasts.

Culture	one week malt broth	one week plain broth	two weeks malt broth	two weeks plain broth	three weeks malt broth	three weeks plain broth	four weeks malt broth	four weeks plain broth
37°C.								
S. cerevisiae Saaz								
S	-	-	-	++	+	++	++	++
R	++	++	++	++	++	++	++	++
M	-	-	-	-	-	-	-	-
S. cerevisiae Froberg								
S	-	-	-	-	-	++	-	++
R	++	++	++	++	++	++	++	++
M	-	-	-	-	-	-	-	-
S. ellipsoideus								
S	-	-	-	-	-	++	-	++
R	++	++	++	++	++	++	++	++
M	-	-	-	-	-	-	-	-
W. anomala								
S	-	++	+	++	++	++	++	++
R	++	++	++	++	++	++	++	++
M	-	-	-	-	-	-	-	-
Z. mandshuricus								
S	-	-	-	-	-	++	-	++
R	-	-	-	++	-	++	-	++
M	-	++	-	++	-	++	-	++
29°C								
S. cerevisiae Saaz								
S	-	+	-	++	+	++	-	++
R	++	++	++	++	++	++	++	++
M	-	-	-	-	-	-	-	-
S. cerevisiae Froberg								
S	-	-	-	-	-	-	-	-
R	-	++	+	++	++	++	++	++
M	-	-	-	-	-	-	-	-
S. ellipsoideus								
S	-	-	-	-	-	++	-	++
R	++	++	++	++	++	++	++	++
M	-	-	-	-	-	-	-	-
W. anomala								
S	-	-	-	++	+	++	++	++
R	++	++	++	++	++	++	++	++
M	-	-	-	-	-	-	-	-
Z. mandshuricus								
S	-	-	-	-	-	+	-	++
R	-	-	-	+	-	++	-	++
M	-	++	-	++	-	++	-	++

Table 6 Con't.

Culture	One week malt broth	One week plain broth	Two weeks malt broth	Two weeks plain broth	Three weeks malt broth	Three weeks plain broth	Four weeks malt broth	Four weeks plain broth
23°C								
S. cerevisiae Saaz								
S	-	-	-	-	+	-	+	-
R	++	++	++	++	++	++	++	++
M	[illegible]	-	-	-	-	-	-	-
S. cerevisiae Froberg								
S	-	-	-	-	-	-	-	-
R	+	++	+	++	++	++	++	++
M	-	-	-	-	-	-	-	-
S. ellipsoideus								
S	-	-	-	-	-	-	-	-
R	-	-	-	+	-	++	+	++
M	-	-	-	-	-	-	-	-
W. anomala								
S	-	-	+	-	+	-	+	-
R	++	++	++	++	++	++	++	++
M	-	-	-	-	-	-	-	-
Z. mandshuricus								
S	-	-	-	-	-	-	-	-
R	-	-	-	+	+	++	+	++
M	-	++	-	++	-	++	-	++
17°C								
S. cerevisiae Saaz								
S	-	-	-	+	-	+	-	+
R	++	++	++	++	++	++	++	++
M	-	-	-	-	-	-	-	-
S. cerevisiae Froberg								
S	-	-	-	-	+	-	+	-
R	+	++	++	++	++	++	++	++
M	-	-	-	-	-	-	-	-
S. ellipsoideus								
S	-	-	-	-	+	-	++	+
R	+	++	++	++	++	++	++	++
M	-	-	-	-	-	-	-	-
W. anomala								
S	-	-	+	-	+	-	+	+
R	++	++	++	++	++	++	++	++
M	-	-	-	-	-	-	-	-
Z. mandshuricus								
S	-	-	-	-	-	-	-	-
R	-	-	-	-	-	-	-	-
M	-	++	-	++	-	++	-	++

Table 6 Con't.

9°C.

Culture	One Week		Two Weeks		Three Weeks		Four weeks	
	malt broth	plain broth	malt broth	plain broth	malt broth	plain broth	malt broth	plain broth
<u>S. cerevisiae</u> Saaz								
S	-	-	-	-	+	+	+	+
R	+	+	++	++	++	++	++	++
M	-	-	-	-	-	-	-	-
<u>S. cerevisiae</u> Froberg								
S	-	-	-	-	-	-	-	-
R	+	+	++	++	++	++	++	++
M	-	-	-	-	-	-	-	-
<u>S. ellipsoideus</u>								
S	-	-	-	-	-	-	-	-
R	-	-	-	-	-	-	-	-
M	-	-	-	-	-	-	-	-
<u>W. anomala</u>								
S	-	-	-	-	-	+	-	+
R	+	+	++	++	++	++	++	++
M	-	-	-	-	-	-	-	-
<u>Z. mandshuricus</u>								
S	-	-	-	-	-	-	-	-
R	-	-	-	-	-	-	-	-
M	-	-	-	-	+	+	++	++

++ = scum　　+ = ring　　- = negative

S = smooth form; R = rough form; M = Microform

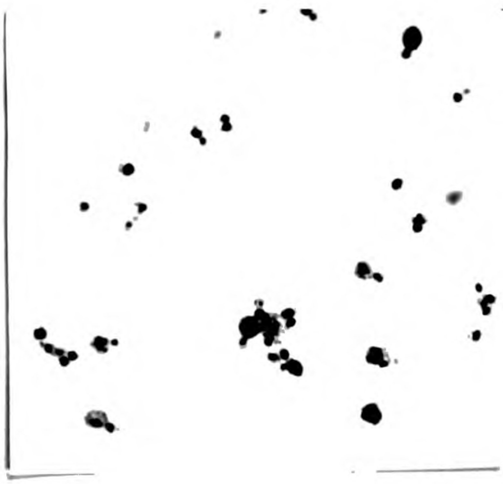

Fig. 1. Photomicrograph of S form of Saccharomyces cerevisiae Saaz. (450X)

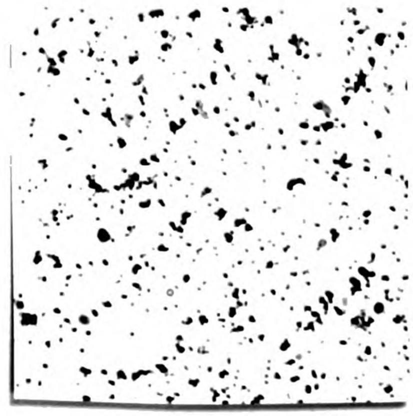

Fig. 3. Photomicrograph of M form of Saccharomyces cerevisiae Saaz. (450X)

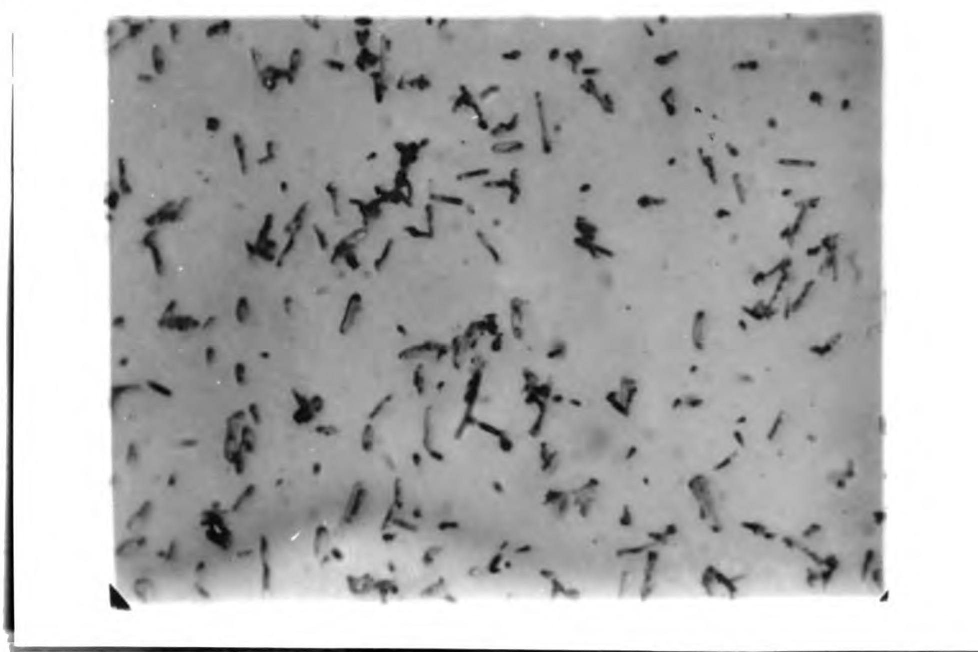

Fig. 2. Photomicrograph of R form of Saccharomyces cerevisiae Saaz. (450X)

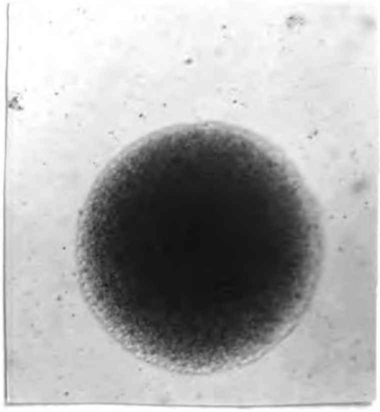

Fig. 4. Photomicrograph of colony of S form of *Saccharomyces cerevisiae* Saaz. (100X)

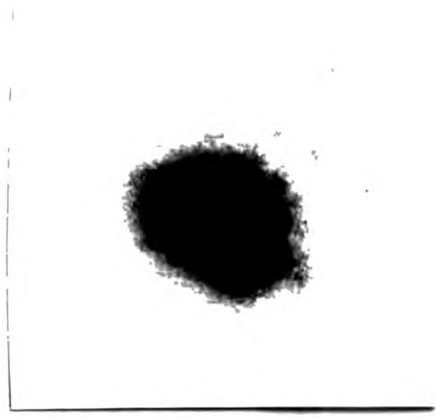

Fig. 5. Photomicrograph of colony of R form of *Saccharomyces cerevisiae* Saaz. (100X)

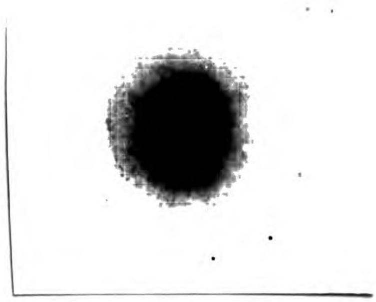

Fig. 5a. Photomicrograph of colony of a form intermediate between the S and R forms of *Saccharomyces cerevisiae* Saaz. (100X)

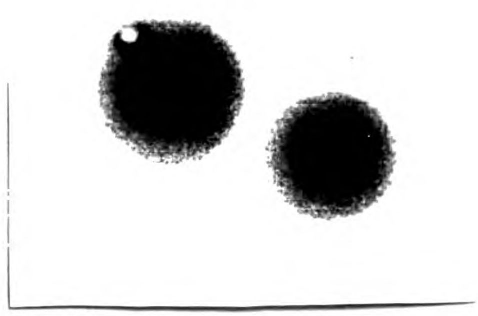

Fig. 6. Photomicrograph of colonies of M form of *Saccharomyces cerevisiae* Saaz. (100X)

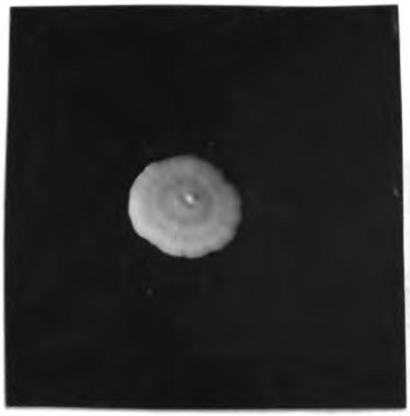

Fig. 7. Photograph of colony of S form of Saccharomyces cerevisiae Saaz. Actual size.

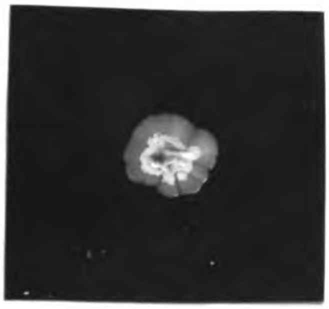

Fig. 8. Photograph of colony of R form of Saccharomyces cerevisiae Saaz. Actual size.

Fig. 9. Photograph of colony of M form of Saccharomyces cerevisiae Saaz. Actual size.

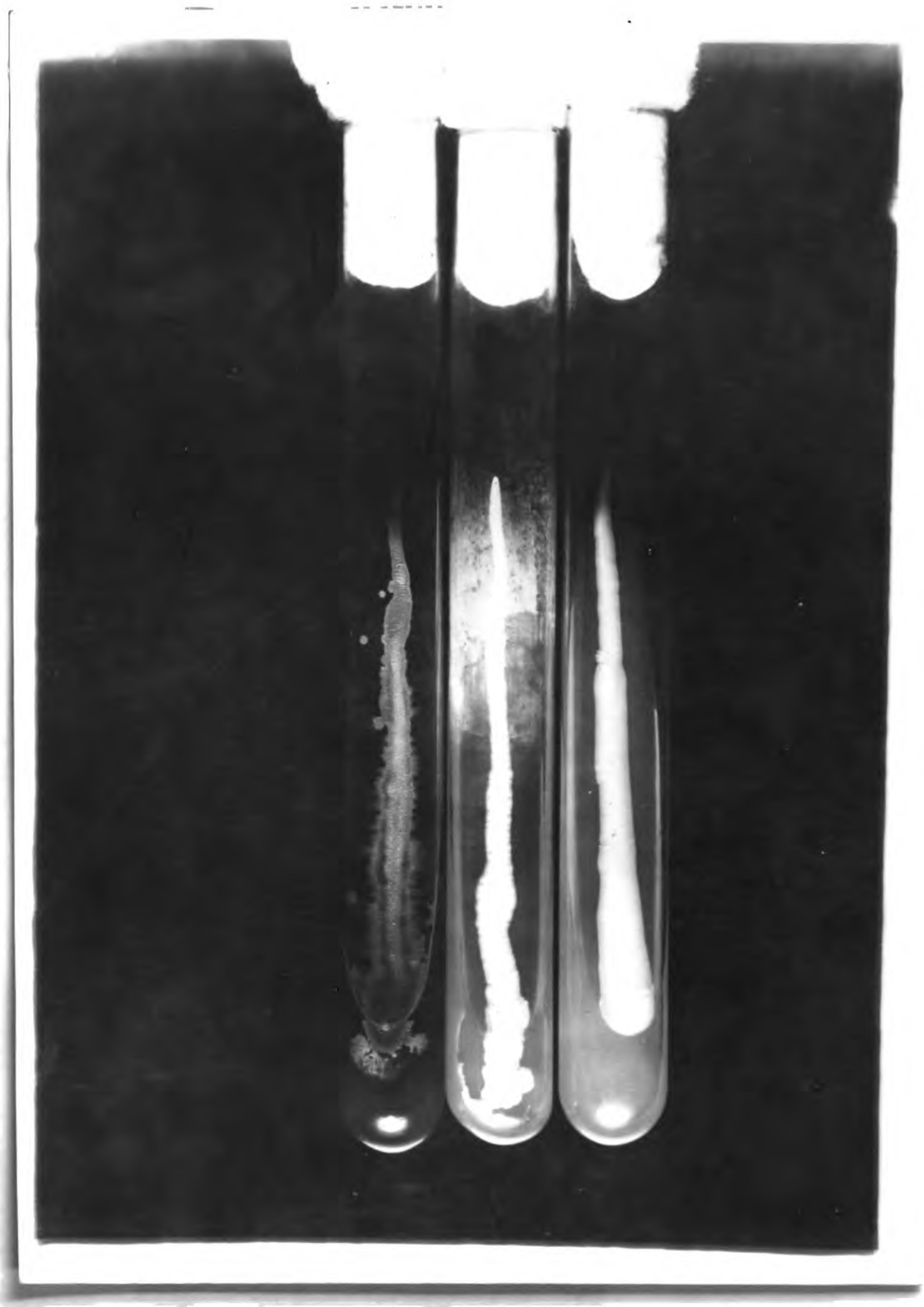

Fig. 10. Photograph of slant cultures (from left to right) of the M, R and S forms of Saccharomyces cerevisiae Saaz.

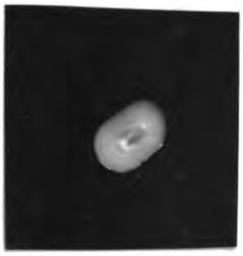

Fig. 11. Photograph of colony of S form of Saccharomyces ellipsoideus. Actual size.

Fig. 12. Photograph of colony of R form of Saccharomyces ellipsoideus. Actual size.

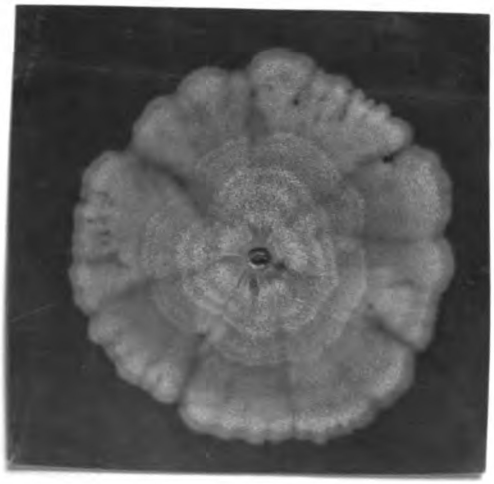

Fig. 13. Photograph of colony of M form of Saccharomyces ellipsoideus. Actual size.

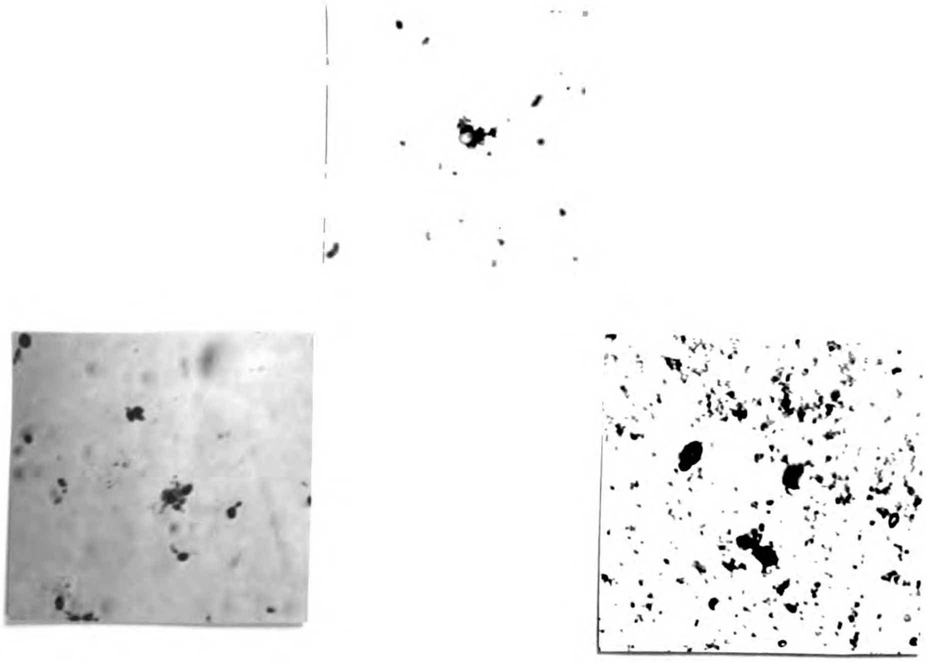

Fig. 14. Photomicrographs showing transitional forms of Saccharomyces cerevisiae Saaz. (450X)

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