GENETICAL INVESTIGATION OF A HUNGARIAN WINE YEAST.

By Zoltán G. Bánhidi.

(From the Hungarian Biological Research Institute, Tihany, Lake Balaton.)

With 2 Tables and 10 Figures in Plate VII.

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A great development in the genetics of yeast has been made possible by operative interference in the processes of multiplication.

Satava's (1918) first observations on the heterothallia and sexual significance of the yeasts have been confirmed and elucidated by Winge's experiments using a spore-isolation technic. Winge established that Mendelian segregation is shown in the cell forms and in the giant colonies developed from the single ascospores, and that the haplo- and diplophases change regularly in yeast. Lindegren uses this method to deal principally with questions of cytoplasmatic inheritance.

These authors have studied the hereditary course of morphological and physiological characteristics, chiefly in brewers' yeast with a short culture cycle. Just for that reason it seemed timely to undertake the investigation of a wine yeast with a significantly longer culture cycle.

MATERIAL AND METHODS.

The material investigated was a very strongly sporulating strain of Hungarian wine yeast which was separated in 1937 by the Hungarian Ampelological State Institute. The one-cell cultures appear in the catalog under the name of "Balaton Füred 2" (or 750 e_1). The strain today is still completely capable of sporulation. (Ásvány, 1948.)

As culture medium an extract of brewer's malt was used in 10°Bg (Balling degrees) solution, acidified with 0.3% tartaric acid to a pH of about 5.5. The must medium was a grape must of 22° measured according to Klosterneuburg. The same were used for solid media with 12% gelatine and 2% agar-agar.

Plaster of Paris blocks were prepared for the sporulation in crystallizing cups and the SCHÖNNING type in 30 ml HANSEN bottles. The SCHÖNNING type of plaster dried out more slowly and gave greater sterility. Normal and small (WASSERMANN) test-tubes were used for the cultures; 50 ml LINDNER and 30 ml FREUDENREICH and HANSEN bottles, hollow slides, BÖTTCHER chambers and PETRI dishes.

For the inoculations I had an inoculating cabinet made which can be sterilized with steam, with a front wall movable by counterbalance. The cultures were kept at 25—28°C in a thermostat. The giant colonies inoculated on gelatine plates were kept at room temperature.

The method described by WINGE (1935,37) was used with some necessary modifications for separating the different asci, as well as for dividing the 4-spore asci and isolating the single cells.

The isolating was done with the PÉTERFI type of ZEISS micromanipulator.

As it was impossible to obtain the operating chamber described by WINGE (manufactured by the Copenhagen firm of F. C. JACOB), I made two of them, 10 mm. high and 22 mm in diamater, of glass slides enclosing two halves of a glass ring (originally for BÖTTCHER chambers) with an entrance at each side 7x7 mm by 15 mm long. The whole was attached with water glass to the object slide. I also made a 22x30 mm brick-shaped chamber of glass plates with the same entrances. To regulate the vapour content inside the chamber I put pieces of filter paper in the entrance openings with bits of cotton wool moistened with distilled water at their outer ends (F i g u r e 1.).

Originally the micromanipulator was put inside the sterilisable inoculation cabinet, but this proved to be superfluous. Rinsing the operating chamber 2—3 times with alcohol provided sufficient sterility. After each operation I sterilized the needles by washing them in suspended drops of distilled water, formol and alcohol.

Suspended drops of liquid, non-gelatinous medium were put on the fat-free cover slip. On these cover slips I also put a suspended drop containing asci suspended in sterile water. From this drop I withdrew a 4-spored ascus with a glass needle drawn out to 4—6 μ thickness over microflame and, slipping it on to a wapourized cover slip, got it into the right suspended drop of sterile medium (F i g. 3.).

Division of the ascus was carried out by LAUSTSEN's method (WINGE, 1937). Placing a glass needle of 1—3 μ thickness on it, the ascus was pressed from below with a thicker needle until it burst. (F i g 4.) With two 4—6 μ needles abruptly sharpened, one of them

pressing the ascus, the other piercing it, it was easier to open it. The isolated spores were pulled apart, into sterile culture droplets, where they formed colonies. (Fig. 5.)

For inoculating the developed colonies WINGE and LAUSTSEN at first used the LINDNER method and could inoculate only colonies which were visible to the naked eye. Later, as it must have seemed necessary to modify this technic, WINGE (1938) cut halfway through the cover lids used for the culture drops with a diamond before sterilization, in such a way that a small piece in the middle could be broken out, together with the droplet, and transferred into the culture bottle. Instead of these methods I used a micromanipulator for inoculating the colonies too. I pushed a 1 mm glass rod into one of the needle-holders of the micromanipulator. Sterilizing it in alcohol, I pulled thin bands from the end of it over microflame, the ends of which I put into the microflame, so that they formed into little glass pearls. Using the micromanipulator I dipped these pearls into the suspended drop culture, then took the glass rod in the holder from the manipulator clasp and, near a flame, broke the ends off into a small test-tube containing gelatine wort. In this way the inoculation can be effectuated rapidly and easily, as plenty of yeast cells always stick to the glass pearls. (Fig. 2).

For inoculating cultures developed in suspended drops the gelatinized wort seemed more reliable.

EXPERIMENTS AND OBSERVATIONS.

In the wine yeast investigated, "Balaton Füred 2" (750 e_1), in general 1, 2, 3 and 4 spored asci were formed. In the 3-spored the spores were in one row or in triangles; in the 4-spored asci they were in rhombic or tetrahedral forms. In some cases the spores were arranged irregularly and fourfold and 6-spored asci could also be found arranged in one row.

I took the 4-spored asci under investigation, supposing them to have the greatest degree of genetical segregation. 32 4-spored asci were separated from the mother strain with the micromanipulator into individual drops of wort. 28 of the separated asci germinated and formed colonies. With the exception of a single case it could always be observed that in germaniting two normal "spore zygotes" (WINGE, 1940) of neighbouring spores formed inside the ascus, and thus heterozygote-diploid cells arose from them.

Together with the others I also separated one 4-spored ascus arranged in one row, but its spores did not form either spore zygotes or cell zygotes, but immediately, supposedly by direct diploidization, grew cells corresponding in size to the mothers (Fig. 6.).

The 4-spored asci were cut in the way described in the chapter on method and on putting the isolated spores into individual sterile droplets, some of them germinated. From 36 spores isolated from 9 (4-spored) asci 22 colonies developed.

TABLE I.

Germination of spores isolated from the 4-spored asci.

No. of asci	Characteristics	Germinated spores singly	Germinated spores total	Remarks
1	4-spored	1	1	
3	„ „	2	6	
2	„ „	2+1	4+2	1—1 small colony died.
1	„ „	3	3	
2	„ „	4	8	
9 asci	36 spores		22 colonies	

The cells of 4 haploid cultures deriving from one ascus had smaller, and round shapes in the beginning, but by the time they developed their 3—6th cell, zygote formation could be observed (Fig. 7.), and in the cultures inoculated with the developed colonies, cells resembling the mother were principally visible, as in these four cases.

In one of the 3 colonies developed from the 4-spore 750 e_1 divided ascus an abnormal form developed consisting of 8—10 elongated, tube-shaped cells 14—18 μ in length and 10 μ wide. As the colony developed the abnormal form stopped multiplying and though removed by micromanipulator and put into an individual droplet, it could not be made to bud (Fig. 8.).

Seeking for possible genetical segregation and observing degeneration, I investigated ring-formation, the shapes of the giant colonies and capacity for sporulaton.

The rings appeared on the sides of the FREUDENREICH flask very characteristically. The original strain in any case on a liquid medium

forms an easily distinguishable ring on the sides of the flask, independent of whether it is taken from a solid or a liquid medium. Contrary to this, in the third week, when rings can well be seen in the mother strain, nothing is as yet apparent in the daughter strains; only in the fifth week in three one-spore cultures very faint traces appear on the sides of the FREUDENREICH flask (Fig. 10.).

LINDEGREN (1943d) recommends a new method for the hybridization of yeast by using the copulation of haplophasic cultures in a liquid medium. In making the inoculation from the haplophasic cultures according to the possibilites for combination without repetition of a second class (a+b; a+c; a+d; b+c; b+d; c+d) I found no copulation during several days of microscopic observation, nor greater differences in the shape and size of the cell than in the heterozygote diploid and homozygote cultures. Ring-formation in the homozygotes was likewise much reduced.

The giant colonies which the strains formed in the Petri dish or gelatinous must showed no morphological differences. The giant colonies of all four homozygote strains were identical in size and shape with the mother colonies of the same age. Their shape was characteristically crater-like and slightly rough on top. They were 10—12 mm in diameter on the 23rd day. (Fig. 9.).

On investigating sporulating capacity it was noticed that the one isolated "single-ascospore", from which I had already made inoculations, dried out in the hollow slide and, in drying, all the 4 single ascospore cultures had formed regular 1, 2, 3 and 4 spore asci.

The inoculated single ascospore cultures were sporulated on plaster blocks and separated like the mother strain into wort drops. The germinating capacity of these asci differed nowise from that of the mother strain.

TABLE II.

Germination of 4-spored asci.

Strain	Phase	Asci isolated	No. of colonies formed.
730	Heterozygote diploid	32	28
^{Z}III	Homozygote diploid	28	26
^{Z}III	„	25	23
^{Z}III	„	28	25
^{Z}III	„	27	27

In the giant colonies of the 750 e_1 strain and its homozygote derivatives mutation-sectors also occurred but, surprisingly, those with concave sides and bulging ends showed greater vitality than the original strain.

DISCUSSION.

The first observations on the germination of yeast spores which dealt with cell forms developing from the spore were made by ŠATAVA (1918) in reduced cell forms, or "dwarf cultures", which manifestly correspond to the haplophase. He distinguished three types. In the first are formed spores with a diminished capacity for germination, in the second and third sporulation does not occur. WINGE (1937), investigating changes in a *Saccharomyces ellipsoideus* generation by spore-isolation, found that one spore deriving from a 4-spore ascus germinated with small round cells and on plaster blocks did not form spores, but on plaster and carrot was capable of conjugation, while the "dumb-bell shaped" zygotes sporulated directly like the *Zygosaccharomyces*. The second and third spores germinate with stretched diploid cells and can sporulate. The fourth spore germinates with small round cells, incapable of sporulation; these cannot be made to diploidize and so can be compared to Torula.

LINDEGREN (1943c) confirmed the observations of WINGE and LAUSTSEN in the case of *Saccharomyces cerevisiae*. He states definitely that if in the haplophase there is, exceptionally, sporulation, the viability of such spores is much lower than that of spores obtained from the original diplophase. In another work LINDEGREN (1944a) states that "the asci from single ascospore cultures generally contain only one or two spores, and their viability is much diminished". (p. 315).

From all this we can see that the authors speak, as of general principles, of a decrease, or a partial loss of sporulating capacity in the progeny of sister spores originating from one ascus in the case of homozygote diploid and haploid successors. Contrary to their findings, in the wine yeast examined there was no such decrease in viability in the first generation in any of the cultures developed from the four sister spores; they behaved with complete uniformity.

In a later work (1940) WINGE publishes exceptions to the above general rule. One is *Saccharomyces validus*, a yeast of low fermentation causing faults in beer, the other is *Zygosaccharomyces Priorianus*,

isolated from honey, in which, however, direct diploidization was not observed.

The subject of the present investigations is a true wine yeast which cannot be identified with any of the foregoing. The cell has elliptical characteristics at germination and, as can be seen (Fig. 6.), direct diploidization (nuclear fission) could also be observed. Otherwise true wine yeasts are in general considered to be forms of *Saccharomyces ellipsoideus* (HANSEN), *Saccharomyces cerevisiae* var. *ellipsoideus* (HANSEN) DEKKER.

WINGE (1937) finds that the giant colonies of 4-spored cultures deriving from one ascus were not pairwise identical and that in general morphological segregation could be observed. The completely identical colonies of the four haploid progeny of the "Balaton Füred 2" (750 e_1) wine yeast, as well as the identical capacities of the viable spores, forces the conclusion that the wine yeast investigated is completely homozygote and thus exempt from the general consequences of inbreeding degeneration. But the absence or decrease in ring formation nevertheless argues against its being completely homozygote.

In respect to differences in ring formation, there are no data to be found in the literature on yeast genetics, nor does the mechanism of ring formation seem cleared up. KRUIS and ŠATAVA (1918) established from the formation of membrane that in the reduced (haploid) phase the membrane-formation disappears, or appears only in small islets. Hence in this field a similarity is shown between the behaviour of the membrane and the ring.

LINDEGREN (1943c) sees as explanation of the disappearance of the membrane, i. e., the purity of the supernatant fluid, that the haploid cells stick together much better, producing a four-leaf-clover effect, and thus form rough colonies on the gelatine, as opposed to the heterozygote diploids which form smooth colonies and membranes. We are obliged to extend this explanation to ring formation and can consider its absence in the same culture a consequence of the haplophase, apparent in the wine yeast at present investigated. In this case the absence of the ring does not argue against the yeast's being homozygote.

SUMMARY.

Dividing a well sporulating Hungarian wine yeast into four haploid strains, by micromanipulator, segregaton between the mother and daughter strains is not shown in sporulation, germinating capacity and

giant colonies, which shows that the strain investigated is homozygote and that the four haploid sisters in the first generation are free from the inbreeding degeneration generally observed in the *Saccharomyces* species.

Absence of rings as characteristic of the haploid phase is discussed, compared with the absence of membrane, and explained by the haploid cells' sticking together in a "four leaf clover" effect.

In connection with the description of the technic of spore isolation comparatively simple methods were given for inoculating micro-colonies.

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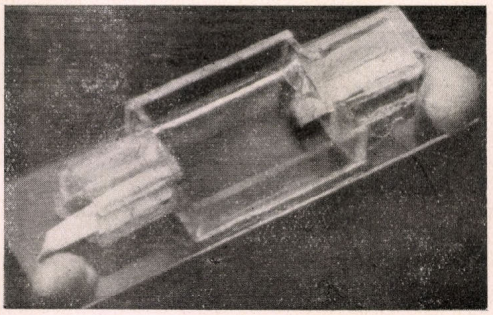
EXPLANATION OF THE FIGURES ON PLATE VII.

Figure 1. Micro-operating chamber, mounted.
Figure 2. Glass pearl used for inoculating micro-cultures, with cells cohering.
Figure 3. One separated 4-spored ascus and the two operating needles.
Figure 4. Two spores isolated and two being cut.
Figure 5. Micro-colony originating from one isolated spore, in the inoculating opening of the wort droplet.
Figure 6. Four-spored ascus with direct diploid germination.
Figure 7. Cell-zygote formation in a one-spore culture.
Figure 8. Abnormal cell forms, which afterwards died.
Figure 9. Giant colonies of the mother strain (in the center) and dauohter strains.
Figure 10. The mother strain forms ring in a liquid medium; this does not appear in the daughter strains.



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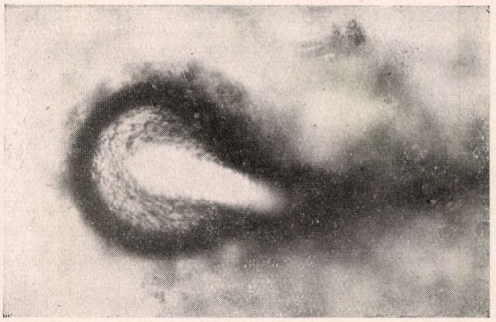

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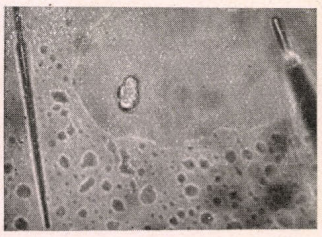

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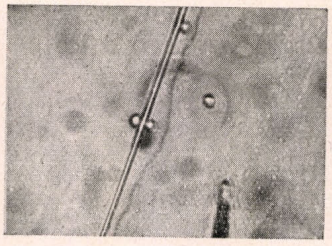

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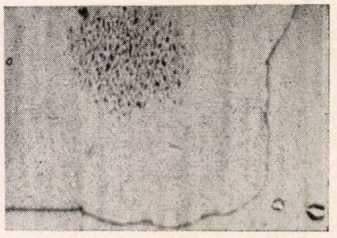

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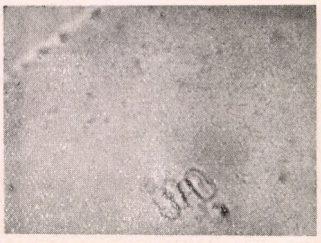

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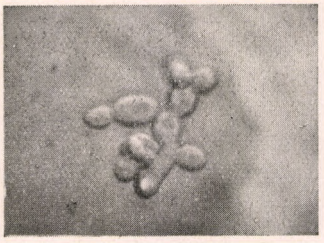

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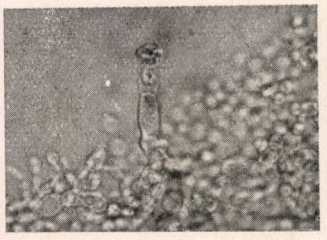

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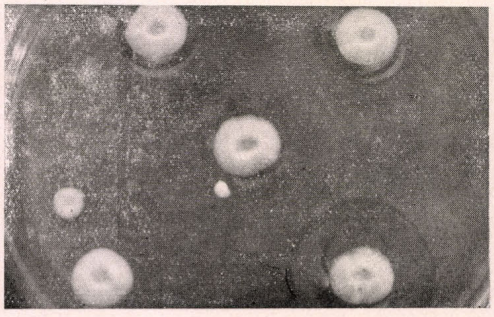

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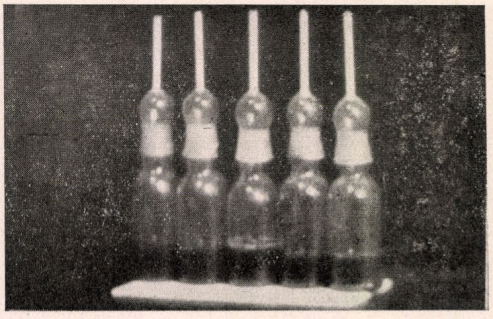

Figure 10.