A STUDY OF VARIATION IN A CHROMOGENIC ASPOROGENOUS YEAST

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Expressed or implied, in recent publications dealing with mutation-like variations in bacteria, may be found the idea that the occurrence of such variations supports a theory of complex life cycles in bacteria, life cycles similar to those presented by higher fungi. It is assumed that mutation-like variations result from hybridization, or that they represent fixed "cyclostages" of an elaborate cycle. In the one case the occurrence of sexual reproduction is a necessary assumption; in the other, it appears highly probable. It would seem well worth while to compare the variations observed in bacteria with those which occur in higher microorganisms, and to determine to what extent variations in these higher organisms are dependent upon sexual reproduction and the occurrence of complex life cycles. It is known that yeasts, molds, and green algae may exhibit in artificial cultures more or less permanent modifications or variations. These are easily observed in giant colonies either as aberrant sectors or as secondary colonies. The occurrence of sexual reproduction in yeasts and molds may be readily detected by microscopic examination. It seems to us that the appearance of mutation-like variations in cultures of yeasts and molds in which sexual reproduction is known not to occur, has a very important bearing upon theories of life cycles in bacteria. It has recently been observed that such mutations may appear in cultures of smut fungi derived from a single sporidium, known to be haploid (Hanna, 1929; Christensen, 1931). Here, undoubtedly, no sexual reproduction could occur.

Temporary modifications and more permanent variations in
yeasts have been observed by a number of authors. The occurrence of sectors and of secondary colonies may be observed in a number of published photographs of giant colonies of yeasts. References to older literature on variations in yeasts may be found in the publications of Pringsheim (1910) and Guilliermond (1920). Beijerinck (1912) has studied variations in *Schizosaccharomyces octosporus*, particularly with regard to spore formation, yet no very extensive investigations have appeared.

The present paper deals with variations in a yeast, *Torula pulcherrima*. This yeast was discovered by Lindner (1887). It has been extensively studied by Beijerinck (1918). The same yeast was rediscovered by Grosbüsch (1914) and renamed *Torula rubefaciens*. We have carefully compared a subculture of this strain received from Dr. Grosbüsch with a strain of *Torula pulcherrima* from Dr. Tanner’s collection, and can detect no differences in morphological or cultural characters. The work here reported was carried on with the strain obtained from Dr. Grosbüsch. It has been subcultured in this laboratory for a number of years.

*Torula pulcherrima* occurs naturally on various fruits, especially grapes, and has also been recovered from the nectar of flowers and from insects. It is an oval, budding yeast of medium size (3 to 7μ). It is recorded as non-sporogenous by Lindner, Beijerinck and Grosbüsch, and we, ourselves, have made a careful search for spores a number of times on plaster blocks, Gorodkowa’s medium, and McKelvey’s plaster-carrot agar, without success. We have also been constantly on the lookout for spores in these researches. We believe that under the conditions of artificial cultivation this yeast does not form spores. The researches of Guilliermond have clearly established the sexual character of spore formation in some yeasts. The spores of yeasts are ascospores, and such ascospores constitute the sole evidence of conjugation in yeasts. We feel warranted, therefore, in concluding that an absence of spores indicates an absence of sexual reproduction. In cultures several days old the cells develop fat globules which may reach an enormous size, almost completely filling the cells. Beijerinck (1912) noted a mutant ("Saccharomyces
pulcherrimus-secondarius”) which failed to develop such fat globules.

In artificial cultures this yeast forms a striking maroon-colored pigment. This pigment is quite different from the coral pink or red pigment produced by the chromogenic yeasts so frequently encountered in cultures from dairy products and other substrates. The pigment of the common red yeasts is a carotinoid pigment soluble in fat solvents. The pigment of Torula pulcherrima is a water soluble pigment. It has been studied by Beijerinck (1918) who believed it to be an anthocyan. It may be extracted with alkalies, which also decolorize, but the color returns upon neutralization of the alkali with acid. Beijerinck claimed that the pigment is secreted as a colorless chromogen which becomes colored only in the presence of iron salts and oxygen.

Beijerinck and Grosbüsich have both noted a variability in the production of this pigment. It may be present in some media and absent in others, due to the presence or absence of iron, according to Beijerinck. The cells alone may be colored, or the pigment may also diffuse into the medium; or, the growth may be white while the medium just beneath is red. These variations appear to depend largely upon the composition of the medium. But we have noted marked variations in successive cultures upon the same medium, or indeed in different parts of a single colony. It is clear, then, that pigment production may undergo variations independent of the environment, due to intrinsic causes. In giant colonies these color variants appear usually as sectors, occasionally as secondary colonies.

This yeast is a particularly favorable subject for an investigation of micbic variation, because its color variations are striking and easily detected by naked eye observation; because the cells are relatively large and may easily be isolated singly to start pure line cultures; and because we believe that the element of sexual reproduction may be excluded. The variations in pigmentation appear to be of the same character as those observed in Serratia marcescens and designated as “mutations” by Beijerinck (1912), and in various bacteria by Nirula (1928), who called these variations “saltations.” Our opinion that these vari-
ations in the yeast *Torula pulcherrima* are of the same general character as those exhibited by bacteria was confirmed during the course of the investigation by the separation of smooth and rough variants.

All cultures were grown on 1.5 per cent agar containing 1 per cent Difco peptone and 5 per cent Difco glucose. The reaction was not adjusted. For single cell isolations this agar was filtered through paper. All cultures were started from single cells isolated as follows: The yeasts were suspended in the above agar, melted and cooled to 45°C. From this suspension three loop-dilutions were prepared with tubes of the same medium. A loop-ful of the last dilution was placed on a flamed coverslip which was then inverted over a sterile moist-chamber slide sealed with melted paraffin. The preparation was examined with the medium power objective, and if a single cell was found with no others near it, the slide was clamped in position and the microscope set aside. A dozen or more such preparations may be made and examined in a few hours. After 48 hours incubation at room temperature, the slides were re-examined. If the cell had grown, a minute colony just visible to the naked eye was apparent. The position of the colony may be marked, while the slide is still on the microscope, with a fine pen point and India ink. The neighborhood of the colony was now examined carefully for the presence of other microcolonies, and if none were found, the coverslip was carefully removed with flamed forceps and quickly inverted into a sterile Petri dish. The marked colony was next transferred to a glucose agar slant with an inoculating wire. If one makes a careful search both before and after incubation, focussing through all layers of the agar, to make certain that no other cells lie near enough to the isolated cell to allow contamination, this procedure is perfectly reliable, and much simpler than micromanipulator techniques. The drop of agar was spread over an area about 15 mm. in diameter, and not more than 3 or 4 microcolonies developed on those coverslips from which subcultures were made.

From the agar slant cultures giant colonies were grown upon the same glucose agar, in narrow mouth 44-x-100-mm. bottles.
CHROMOGENIC ASPOROGENOUS YEAST

These bottles contained 100 cc. of agar, which about half filled them. Transfers from the slant were made with a straight wire which was just allowed to touch the middle of the agar surface. The bottles were incubated at room temperature from four to six weeks. At first the bottles were sealed with paraffin after two weeks incubation, but we soon noted what had also been observed by Beijerinck, that the color began to fade due to an absorption of the oxygen. Therefore the bottles were left unsealed, but were kept in a moist chamber to prevent evaporation of the agar.

After the colonies had developed sufficiently, they were examined carefully, and from the various differentiated parts of the colony—sectors, secondary papillae, rough portions, etc.—a loopful was removed and suspended in sterile media. From these suspensions single cell isolations were again made to start new giant colonies. The bottle was next cut at the agar level, and the colony photographed. After this the various parts of the colony were examined microscopically for morphologic variations in the cells.

From the original stock culture four single cells were isolated and grown into giant colonies. From various parts of these giant colonies single cells were again isolated and grown into a second "generation" of giant colonies. This process was carried on through four "generations." This is hardly sufficient to warrant any conclusions regarding the stability of the types isolated. It has, however, required nearly a year to complete these observations. The labor increases in geometrical proportion. The variants encountered multiply with each "generation." It is obviously impossible to carry on such a series of observations indefinitely. We believe that the results attained warrant a progress report at this time.

The cultures derived from the four original single cell isolations have been designated A, B, C, and D. Second generation colonies from these are denoted by numbers, as A1, A2, A3, etc.; third generation colonies by small letters, as A1a, A1b, etc.; and fourth generation colonies by small letters from the opposite end of the alphabet, as A1ax, A1ay, etc. From this system the "genealogy" of any colony may be readily determined. Thus the
colony D1cz was derived from some portion of the colony D1c, which in turn originated from a cell isolated from some part of D1, which came from D.

Variations appeared in the giant colonies most frequently as sectors. These sectors were white, or all possible shades of maroon. The illustrations of colonies A1 and B3c show striking white sectors on predominantly red colonies; B1a shows a dark red smooth sector on a lighter red rough colony. Sometimes sectors are so numerous and intergrade to such a degree that analysis is nearly impossible, as in A3a and C3d. Sectors intergrade with lobes, broad expanses of growth of a variant character extending from one side of the colony. A white lobe is shown in B. Such lobes may extend so as eventually almost completely to surround the colony, forming a border. Variation was also made apparent in a differentiation of the central and peripheral portion of the colonies. The central portion may appear rough, the peripheral portion smooth, as in B2. Colony D1cz shows a central red rough portion and a peripheral smooth white zone. This differentiation does not appear until the colony has attained a certain degree of development. Thus a pure white colony may after a time develop red pigment in the center, and this red pigment will then gradually spread toward the periphery, as may be seen in D1. This we have designated secondary growth. Variations may also appear as secondary papillae, of the same sort as those exhibited by the Neisser-Massini "mutant" of the colon bacillus. Red secondary papillae on a white colony are shown in C1b.

A study of the microscopic appearance of the yeast cells in these various colonies did not yield any significant facts save in the case of rough variants. For the most part the cells appeared as they usually do with old cultures of Torulae,—round to oval, and varying markedly in size. Many of them contain a fat globule. An occasional very large cell with a thick wall presented the appearance of the "dauerzellen" or characteristic resting forms of yeasts. An occasional cell was also found to be elongated and cylindrical in form, and such cells attached end to end formed short filaments of "sprossmycel," the rudimentary mycelium found so frequently in old cultures of yeasts. But in rough colonies, or in rough por-
tions of a colony, this rudimentary mycelium reached a much greater development, and here we could find occasional short, sometimes branched, filaments of true, cylindrical, septate mycelium. It is very clear that the wrinkled tenacious growth of rough colonies is definitely associated with, and probably due to, the production of rudimentary mycelium: while the "normal" smooth, pasty growth is associated with free round or oval cells. No morphologic differentiation could be established between red and white variants.

![Photomicrograph of a Wet Preparation from the Rough Center of Colony B4, Showing Rudimentary Mycelium and Yeast Cells](image)

The "dauerzell" is seen in the lower right corner.

The individual characters of the sixty giant colonies, and the sequence of the variations encountered, are indicated in the following tabulation:

A—From stock culture; smooth red with one red and one white sector; round, oval cells.
A1—From red sector of A; red with two white sectors; round, oval cells.
A2—From center of A; smooth red with rough center, light red sectors; round, oval, and elongated cells.
A3—From margin of A; rough red center, white and light red sectors; round, oval cells.
Fig. 2. Representative Giant Colonies, Showing Variations of Torula pulcherrima
A1a—From white sector of A1; smooth white with red secondary growth; round, oval cells.
A1b—From white sector of A1; smooth white; round, oval cells.
A1c—From white sector of A1; smooth white with red secondary papillae; round, oval cells.
A1ax—From margin of A1a; smooth white, red secondary papillae; round, oval cells.
A1cx—From margin of A1c; smooth white with red center; round, oval cells.
A1cy—From center of A1c; smooth white with red sector; round, oval cells.
A1cz—From red papillae of A1c; red rough with white margin; elongated cells.
A2a—From the edge of A2; smooth red; round, oval, and elongated cells.
A2b—From the center of A2; smooth red with rough center; round cells and mycelium.
A3a—From pink sector of A3; smooth red with white sectors and white edge; round, oval cells.
A3b—From white sector of A3; smooth white; round, oval cells.
A3c—From edge of A3; smooth red with white sector and lobe; round, oval cells.
A3d—From center of A3; smooth red with rough center; slight mycelium.
A3ax—From margin of A3a; smooth red; round, oval cells.
A3ay—From red center growth of A3a; red and white mixture; round, oval cells.
B—From stock culture; smooth red with white lobe and sector; round, oval cells.
B1—From white lobe of B; rough red center, smooth red sector, and white sector; elongated cells.
B2—From white sector of B; red with rough center; elongated cells.
B3—From red sector of B; smooth red with rough center and white sector; elongated cells and mycelium.
B4—From center of B; smooth red with rough center and white sector; oval cells and mycelium.
B1a—From pink sector of B1; red with rough center, smooth red sector, light red lobe; round and oval cells.
B1b—From white sector of B1; smooth white; round and oval cells.
B1c—From rough center of B1; red rough; mycelium very abundant.
B2a—From rough center of B2; red with rough center; mycelium abundant.
B3a—From rough center of B3; smooth red with rough center and white sector; mycelium abundant.
B3b—From smooth edge of B3; smooth red with rough center; elongated cells and mycelium.
B3c—From pink sector of B3; red with white sectors; elongated cells and mycelium.
B4b—From white sector of B4; smooth red with rough center and white sector; elongated cells.
C—From stock culture; smooth red with rough center and two white sectors, secondary papillae; round and oval cells.
C1—From white sector of C; smooth white; round and oval cells.
C2—From white sector of C; smooth white; round and oval cells.
C3—From secondary papillae of C; smooth red with rough center, light red sector, and white sectors; round and oval cells.
C1a—From center of C1; smooth white with red secondary papillae; round and oval cells.
C1b—From the edge of C1; smooth white with red secondary papillae; round and oval cells.
C2a—From the white center of C2; red and white with white sectors, central white papillae; round and oval cells.
C1ax—From white margin of C1a; smooth white; round and oval cells.
C1ay—From red secondary growth of C1a; smooth red; elongated cells.
C1az—From red rough growth of C1a; smooth red with rough center and white sector; elongated cells and mycelium.
C1bx—From white margin of C1b; smooth white with red secondary papillae; round and oval cells.
C1by—From secondary papillae of C1b; smooth red; round and oval cells.
C3a—From center of C3; smooth red with light red lobe; elongated and oval cells.
C3c—From edge of C3; smooth red; round and oval cells.
C3d—From white sector of C3; pink with red border; round and oval cells.
D—From stock culture; smooth red with white lobe; round and oval cells.
D1—From white lobe of D; white with red secondary growth in center; round and oval cells.
D2—From red margin of D; smooth red; round and oval cells.
D1a—From red center of D1; smooth red with rough center; mycelium and elongated cells.
D1b—From rough red center of D1; white with red secondary papillae, rough center; elongated cells and mycelium.
D1c—From margin of D1; smooth white; round and oval cells.
D1bx—From white margin of D1b; smooth white; round and oval cells.
D1by—From red center of D1b; white with red papillae and red rough center; elongated cells.
D1cx—From white margin of D1c; smooth white; round and oval cells.
D1cy—From secondary papillae of D1c; white with red secondary papillae; round and oval cells.
D1cz—From red center of D1c; smooth white with red rough center; mycelium and elongated cells.
D2b—From red edge of D2; smooth red; round and oval cells.
D2c—From smooth red sector of D2; smooth red; round and oval cells, and a few elongated cells.

It is very evident from the above that this yeast is tending to split along two different lines,—into red and white, and into smooth and rough, races. It was possible to obtain entirely smooth red colonies (as B3b), entirely smooth white (C2), and entirely rough red colonies (B1c, A1cz). So far a rough white colony has not been obtained. The colony D1b is nearly all white, and appears rough, but this apparent roughness is due to the formation of large numbers of secondary papillae, not to the formation of the tenacious wrinkled growth characteristic of the rough red colonies. It is noteworthy that red secondary papillae on white colonies usually gave rise to solid red rough colonies. There is, however, no clear evidence of linkage between the two pairs of characters. Color and texture may vary independently.

It is, of course, of prime importance to determine to what extent these variations are transmitted through subcultures, and to what extent the different variants may stabilize as distinct races. These questions may not be answered from the data here presented. There is evident a tendency for the variants to produce for a time cultures of the same type. But there is also evident a tendency to continuous instability, leading on the one hand to the development of more and more new variants, on the other to a reversion to ancestral forms.

For example, colony C developed two white sectors. Subcul-
tures from both of these (C1 and C2) grew into pure white, entirely smooth colonies. A subculture from the white center of C2 yielded a mixed red and white colony with prominent white sectors. Subcultures were made from the white center and the white periphery of C1. Both yielded white smooth colonies which later developed red secondary papillae (C1a and C1b). Subcultures from the white margin of C1a again yielded a pure white smooth colony (C1ax), but subcultures from the red secondary papillae yielded in one case a pure smooth red colony (C1ay) and in another case a smooth red colony with a rough center and one white sector (C1az).

The central portion of colony A was somewhat elevated and a little irregular in contour, but not distinctly rough. A subculture from this portion gave a pure red colony (A2), rough in the center and smooth at the periphery. A subculture from the smooth peripheral portion (A2a) was entirely smooth; while from the rough central portion there was obtained again a colony (A2b) with a rough center and smooth periphery.

We cannot find in these observations anything to support a theory of sex or life cycles as responsible for the observed variations. If sexual reproduction occurs, it would be ruled out by single cell isolation if the single cell were unisexual, or haploid. If this cell is bisexual, it should give rise to ascospores. While the presence of spores in yeasts is not necessarily proof of conjugation, their absence may be considered evidence of an absence of sex. The morphologic variations encountered are of the sort which may be expected in an aging culture, not of the degree to be found in a life cycle; they represent a cytomorphosis rather than a cyclogenesis. The transformation from free cells to rudimentary mycelium is a mere change of growth-form characteristic of many lower fungi, and quite independent of life cycles.

While unable to state the fundamental causes of these mutation-like variations, there are apparently two factors involved. One of these is the artificial selection practiced throughout the experiment. Each subculture is started from some chosen differentiated part of the colony. And again, in isolating single cells from the chosen part of the colony, a selection is made, blindly, it is true,
but nevertheless a selection. It is obvious that such a procedure, often repeated, will bring to light an inherent tendency to variation which might be entirely masked if the culture were carried through a series of generations by simple mass transfers. The other factor is intrinsic in the culture itself. It is noteworthy that the degree of variation observable in a giant colony progresses steadily with the age of the culture. This has been observed repeatedly, also, in studies of variation in bacteria. It may well be due to the action of some product of metabolism upon the cells, inducing either a temporary modification of the organism, or a permanent alteration of genes, resulting in transmissible variations.

The fact that such variations appear more frequently in giant colonies than in ordinary mass cultures may be due to the establishment of a metabolic gradient from the oldest central part of the colony to the youngest peripheral zone. Such a gradient would explain the differentiation of a colony with a rough (or red) central portion, and a smooth (or white) peripheral portion. It would readily explain the peculiar symmetrical pattern of such a colony as D1b. It would not, however, account for sectors and secondary papillae, which appear to be entirely fortuitous or accidental phenomena. It is probable that we have to deal here with two distinct categories of phenomena: a differentiation of the colony, analogous to the differentiation of the cells of higher organisms into tissues and organs, and probably determined by a radial metabolic gradient; and sudden, mutation-like changes occurring in any part of the colony, analogous to bud-sports in higher plants. Variations in the first category should not be permanent, while those of the second type should be more or less so. But a clear separation of these two types of variation will prove a difficult task.

SUMMARY

Variations in a chromogenic, asporogenous yeast, *Torula pulcherrima*, have been observed in giant colonies started from single-cell isolations.
Red and white, smooth and rough variants were encountered. These variations occur independently. Rough forms are associated with the formation of rudimentary mycelium. Although the observed variations showed a tendency to breed true, no completely stable variants were obtained.

REFERENCES

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