HYBRIDIZATION STUDIES INVOLVING *SACCHAROMYCES FRAGILIS* AND *ZYGOSACCHAROMYCES DOBZHANSKII*

LYNFERD J. WICKERHAM AND KERMIT A. BURTON

WITH THE TECHNICAL ASSISTANCE OF JANE A. ROBERSON

Fermentation Section, Northern Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Peoria, Illinois

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In a preceding paper (1956) the writers reported that pigmentation occurs in varying degree among strains of most species of the proposed but as yet undescribed new genus, *Dekkeromyces*. Hybrids may yield more pigment than either parent, thereby permitting easy selection of hybrids from a population consisting of both hybrids and parent species. This method of isolating hybrids requires one or both of the parents to produce a small or moderate amount of pigment, but neither parent should be strongly chromogenic.

Another procedure has been devised for separating hybrids from their parents. The parent strains may be selected without regard to the amount of pigment they produce, but they must differ from each other in the assimilation of two carbon sources.

Because this study has resulted in the first ascosporogenous yeasts known to ferment both maltose and lactose, and because the hybrids reported here may be of particular interest to geneticists, the results of this study will be presented in greater detail than was done in the previous study.

METHOD

The process depends upon biochemical means of eliminating from a mixture first one parent, then the other, leaving only the hybrids. One parent is selected which assimilates carbon source A, but not B, the other parent assimilates B but not A, and the hybrids assimilate both A and B (not all of them necessarily, but those do which survive the isolation procedure). Cells from a mixture which contains hybrids are inoculated into a flask of medium containing A as the only source of carbon. It is imperative that no organic compounds, such as those supplying assimilable carbon in yeast extract, be introduced into the basal medium. Difco's yeast nitrogen base is used plus 0.5 per cent of the desired carbohydrate. The flask is shaken until good growth is obtained, then a very minute amount of inoculum is transferred to a second flask of medium A. The inoculum consists of the cells that remain after a 3-mm loop has been dipped 4 mm deep into the culture, and then completely drained. Subsequent transfer is made in like fashion from the second to a third flask of medium A. By this time the parent which does not assimilate carbon source A has most likely been completely diluted out, leaving the parent which assimilates carbon source A and the hybrids which assimilate both A and B. Cells from the third serial flask of medium A are inoculated into the first flask of medium B, and from it to a second, and serially into a third flask of medium B. This should eliminate the parent that assimilates carbon source A, leaving only the hybrids. If, however, a few cells of the parent which does not assimilate A persist through the first three flasks in the series, and build up a large population in the second series of three flasks, it is again reduced by a final transfer in medium A. Thus the entire series of transfers is A, A, A, B, B, B, A. If no hybrids are present, growth usually fails somewhere in the series. If A and B are strongly assimilated by the respective parents, the terminal flask in the series often yields only colonies of hybrids when streaked.

Presumed hybrids are restreaked, then colonies are selected and tested for their ability to assimilate carbon compounds A and B, and any other carbon sources that are assimilated strongly by one parent but not by the other. Those assimilating both A and B, and all or nearly all the other compounds included in the tests, are hybrids.

In this report, one of the two strains hybridized is *Saccharomyces fragilis* NRRL strain

TABLE 1

Pertinent characteristics involved in hybridization of Saccharomyces fragilis and Zygosaccharomyces dobzhanskii

Species	Strains	Ascospore Shapes	Ploidy	Sex	Colonies on		Assimilation of α-glucosides				Assim. Lactose	Ferm. Lactose	Assim. Inulin
					MM Agar	MY Agar	M	Mz	α-M	T			
S. fragilis...	Y-610	Predom. reniform	Diploid	Homothallic	White	White	−	−	−	−	+	+	+
Z. dobzhanskii.....	Y-1974	Crescent	Haploid	Homothallic	V.f.p.	White	+	+	+	+	−	−	−

+, referring to assimilation, means growth at the expense of the carbon source indicated.
M, maltose; Mz, melezitose; α-M, α-methylglucoside; T, trehalose.
V. f. p., very faint pink.
+, referring to fermentation, means gas production.

Y-610 (table 1), obtained in 1936 from W. D. Stovall, bearing his number 21041. It assimilates and ferments lactose but has no action on maltose or other α-glucosides, except for extremely weak assimilation of trehalose. This species is considered to be a naturally occurring hybrid (*Saccharomyces lactis* × *Zygosaccharomyces ashbyi*). The other strain is *Zygosaccharomyces dobzhanskii* strain Y-1974 which has no action on lactose but assimilates and ferments maltose, melezitose, α-methylglucoside, and trehalose. Y-610 is diploid and Y-1974 is haploid; both are homothallic, and both have reniform or crescent-shaped spores. Some ascospore isolates derived from Y-610 produce spheroidal spores almost exclusively, a characteristic presumably inherited from *S. lactis*. The stock strain and not its ascospore isolates were used in these experiments. As noted in table 1, the ability of *S. fragilis* strain Y-610 to produce pigment on the media used was nil, while the ability of *Z. dobzhanskii* was very weak. Consequently, it was anticipated the hybrids would be weakly chromogenic or non-chromogenic. These strains were selected intentionally to test the method of selecting hybrids by differences in their ability to assimilate carbon sources; chromogenic strains for the production of chromogenic hybrids are abundantly at hand.

A mixture of the two strains was serially transferred on malt extract sporulation medium (ME) to produce the hybrids. Hybrids were then isolated by serial transfer first in an α-glucoside medium, then in lactose, and again in the same α-glucoside. Maltose, melezitose, and α-methylglucoside were used, and the schedule was shortened to A, A, B, B, A, A. Cells from each of the final flasks were streaked on 6 plates of MY agar, and the plates incubated 10 days at 25 C to permit maximum differentiation of colony types.

RESULTS

Colony appearance of the hybrids obtained. Four general types of colony were discernible. The first represented parent strain Y-1974, *Z. dobzhanskii*. The colonies were white, smooth, glistening, and without tiny elevated points near the edge of the colonies. The second type was either white or only faintly pink in the center, glistening, and smooth except for tiny elevated points (E) near the edge of the colonies. The third type was characterized by isolated colonies which had faintly pink centers and radial folds or radial striations (FPR) of deeper coloration. The edges of the colonies were indented at the striations. The fourth type was usually small, rather deeply pigmented (DP) though they generally showed white sectors with age. Occasionally they appeared mat, and sometimes rugose with irregular edges. When restreaked, the resulting colonies often were as large as the other types, but they were as strongly pigmented as before.

Colonies of the parent, *Z. dobzhanskii* strain Y-1974, were found only on plates streaked from melezitose isolation medium. The FPR colonies

comprised approximately 80 per cent of the colonies from α-methylglucoside, and 5 and 2 per cent, respectively, from maltose and melezitose. The type E colonies comprised 20 per cent of the colonies from α-methylglucoside and 95 per cent of the colonies from maltose medium. The DP type was not found on the plates from the final flasks of α-methylglucoside or melezitose, and only about 0.1 per cent of the colonies from maltose were of this type. As will be shown later, type DP is composed of variants or ascoporic isolates of hybrids E and FPR. Their small numbers may be due to their poor ability to assimilate α-glucosides (table 2).

Ten colony isolates of parent strain Y-1974, 10 of E type, 8 of FPR, and 4 of DP were restreaked on malt extract-yeast extract plates. When the cultures were 3 days old, one typical colony was picked from each plate, to produce inoculum for fermentation and assimilation tests, sporulation characteristics, and cells for lyophilization. The plates were kept and observed at 11 days. Plates of parent Y-1974 colonies showed no variant colonies; the E type showed, in addition to the typical E colonies, an average of 2 per cent of DP type colonies. These arose as individual colonies, not as sectors. The FPR type also showed approximately 2 per cent of DP type colonies on the plates. Plates of the DP type isolates contained almost exclusively DP type colonies, but one plate had 2 white colonies.

Sporulation. The 32 isolates selected from the various isolation media were put on malt extract sporulation slants and cells were observed at 2, 5, and 14 days for characteristics of asci and ascospores. Isolates of parent strain Y-1974 varied in the number of ascospores produced at 5 days from approximately 5 to 20 per cent of the total cells. The spores were predominantly crescent in shape, and a very few bordered on reniform, but none was spheroidal. About 98 per cent of the asci consisted of 2 conjugated, previously independent cells (i.e., not mother-daughter combinations), and perhaps 2 per cent showed no conjugation, indicating the latter arose from diploid vegetative cells. The vegetative cells were small.

The FPR colony isolates produce 50 to 80 per cent ascospores at 48 hr, except 1 isolate which remained nearly asporogenous. *The spores were unlike those of either parent.* They were commonly *spheroidal, often irregular*, and none appeared to have the slender crescent shape typical of parent Y-1974. The asci were mainly healthy, 4-spored, and not conjugated. The vegetative cells were large and occasionally irregular in shape.

The colony isolates of the E type produced between 30 and 80 per cent spores at 5 days. The spores were reniform, angular, ellipsoidal, or spheroidal, but not crescent shaped, and the spores had a healthy appearance. The asci were formed exclusively or nearly exclusively from single cells, in this characteristic resembling their diploid parent, *S. fragilis*. The vegetative cells were generally large and generally were irregular in shape.

The DP type isolates were increased in number to make the results more significant. By serially restreaking the FPR and E hybrids, approximately 2 per cent of the colonies are DP type. By streaking sporulated, unheated cultures of FPR and E hybrids, up to 20 per cent DP types are obtained, presumably arising from ascospores. Such colonies may be glistening, mat, rugose in the center (figure 1), or lumpy. On restreaking, a DP type having a

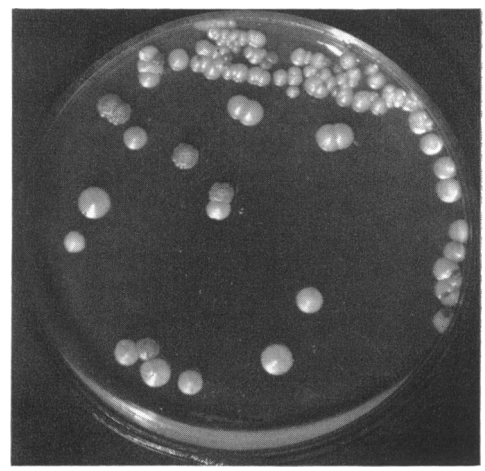

Figure 1. Colonies of hybrids of *Saccharomyces fragilis* NRRL strain Y-610 × *Zygosaccharomyces dobzhanskii* NRRL strain Y-1974. The smooth white colonies are type E hybrid, and the rugose, pink colonies are one manifestation of the DP type. The DP type colonies arose from ascospores. Plate streaked from sporulated E type hybrid, incubated 6 days at 25 C.

certain surface characteristic may give rise to DP colonies having other surface characters, and to white colonies which often are similar morphologically and biochemically to the DP type from which it arose.

Generally, ascospore isolates stemming from FPR hybrids produced large, elongated cells which neither sporulated nor conjugated. The DP isolates from the E type hybrids produced more rounded, smaller cells, and in every instance produced ascospores in cells which showed no conjugation and were therefore at least diploid before meiosis. Most of these isolates from the E type hybrids produced about 5 per cent of spores, but some produced as high as 20 per cent. Very few of the asci ruptured, they commonly contained 4 spores per ascus, and the spores were usually ellipsoidal or spheroidal, frequently irregular, and occasionally reniform.

Fermentation. The 32 isolates were tested for their ability to ferment lactose, maltose, melezitose, α-methylglucoside, and trehalose. Figures 2 through 5 show the average amounts of gas in inserts in fermentation tubes at intervals throughout 18 days of incubation. A strong fermentation is indicated by an early rise in the graph and an early return to a position near the base line. A very weak fermentation is indicated by the

Loss of α-glucosidase activity by hybrids of *Saccharomyces fragilis* strain Y-610 × *Zygosaccharomyces dobzhanskii* strain Y-1974.

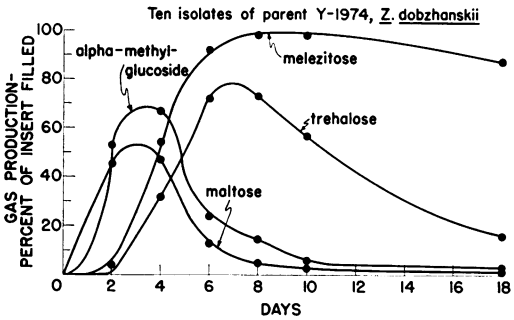

Figure 2. Average course of fermentation of α-glucosides by 10 colony isolates of parent strain Y-1974 *Z. dobzhanskii*. Lactose is neither assimilated nor fermented by this species. All four α-glucosides are fermented.

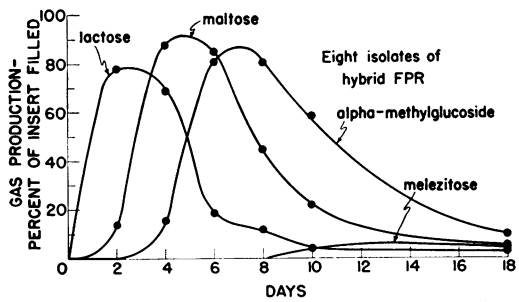

Figure 3. Average course of fermentation of eight colony isolates of hybrids of the FPR type. Melezitose is very weakly fermented, and trehalose is not fermented. Lactose is fermented by all types of hybrids.

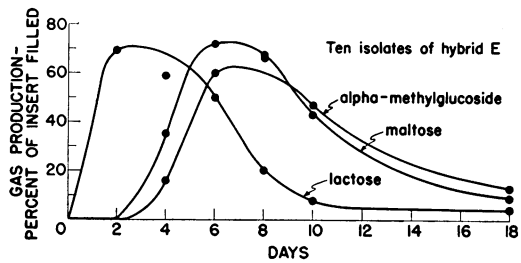

Figure 4. Average course of fermentation of 10 colony isolates of hybrids of the E type. Melezitose and trehalose are not fermented.

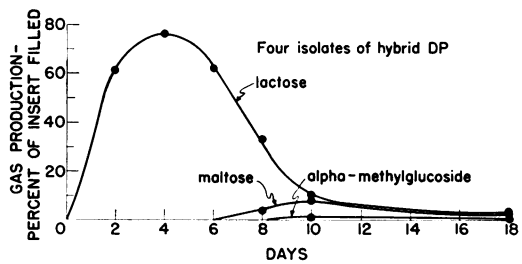

Figure 5. Average course of fermentation of four colony isolates of hybrids of the DP type, which arise as variants of the FPR and E types. α-Methylglucoside is very slightly fermented, and melezitose and trehalose are not fermented. Such hybrids continue to mutate, producing mainly variants which ferment none of the α-glucosides studied, and many which assimilate only maltose and it slowly. All variants produced by hybrids ferment lactose strongly or moderately.

lapse of several days before gas is produced in measurable quantity, and the crest remains close to the bottom of the graph. A fermentation of intermediate intensity is indicated by a high-crested curve with a gradually declining slope. The medium crest of a strong fermentation is due to the fact that within the period of 48 hr to 4 days, some isolates are rapidly filling the inserts with gas, while others have already filled the inserts and are rapidly emptying them at the termination of fermentation. Consequently, the average amount of gas for all isolates of the type will be much less than 100 per cent. In a slower fermentation, nearly all isolates may have 100 per cent gas in the inserts at one time, and this period will be increasingly longer with weaker fermentations. The very weakest fermentations, however, as stated above, are characterized by very small amounts of gas in the inserts, so their lines lie near the bottom of the graph.

The colony isolates of parent *Z. dobzhanskii* (figure 2), strain Y-1974, fermented all four of the α-glucosides; the fermentations of maltose and α-methylglucoside were strong but the fermentations of melezitose and trehalose were weaker. The hybrids of FPR type (figure 3) did not ferment trehalose, and melezitose only very weakly. The E type hybrids (figure 4) fermented neither trehalose nor melezitose, though maltose and α-methylglucoside were fermented with moderate intensity by both types.

Only 1 of the 4 DP isolates (figure 5) fermented any α-glucoside. This one required 6 days to get a fermentation of maltose started, and relatively little gas was produced. It fermented α-methylglucoside to a detectable extent but did not ferment melezitose or trehalose. The other 3 isolates of DP type fermented none of these sugars, though they did assimilate them (table 2). Of 25 additional DP isolates from various sources, only 2 fermented maltose strongly and most did not ferment it at all.

All isolates of hybrid types FPR, E, and DP fermented lactose strongly or moderately.

Carbon assimilation. The 32 isolates were inoculated very lightly into carbon assimilation media containing separately lactose, maltose, melezitose, α-methylglucoside, and trehalose. All isolates of all the types, except parent strain Y-1974, assimilated lactose (table 2). None of the isolates of parent strain Y-1974 produced any growth in lactose throughout the 20-day period of the experiment. α-Glucosides were assimilated somewhat more slowly by E and FPR isolates than by parent strain Y-1974.

The first four DP isolates studied assimilated α-glucosides weakly. Eleven of the DP isolates stemming from ascospores of the FPR hybrids assimilated inulin, lactose, and maltose rapidly, melezitose and α-methylglucoside less rapidly, and trehalose slowly, with all carbohydrates yielding dense growth at 20 days of incubation. Eight DP colonies stemming from E type hybrids were similarly tested, with six of them strongly assimilating inulin, lactose, and maltose, but having no action whatever on melezitose, α-methylglucoside, or trehalose throughout the 20-day period of incubation. Cells from the maltose tubes were incapable of assimilating these three α-glucosides, thus indicating the specificity of the enzyme concerned. The other

TABLE 2

Comparative ability of different types of hybrids of Saccharomyces fragilis strain Y-610 × Zygosaccharomyces dobzhanskii strain Y-1974 to assimilate lactose, maltose, melezitose, α-methylglucoside, and trehalose

Reactions given by majority of isolates.

Colony Type	3 Days					6 Days					20 Days				
	L	M	Mz	α-M	T	L	M	Mz	α-M	T	L	M	Mz	α-M	T
Hybrid FPR....	3+	2+	+	2+	+	3+	3+	2+	3+	2+	3+	3+	3+	3+	3+
Hybrid E.....	3+	3+	+	±	±	3+	3+	2+	2+	+	3+	3+	3+	3+	3+
DP, from FPR..	2+	2+	+	+	−	3+	3+	2+	2+	+	3+	3+	3+	3+	3+
DP, from E.....	2+	2+	−	−	−	3+	3+	−	−	−	3+	3+	−	−	−
Strain Y-1974...	−	3+	2+	2+	+	−	3+	2+	3+	2+	−	3+	3+	3+	3+
Strain Y-610....	3+	−	−	−	−	3+	−	−	−	−	3+	−	−	−	−

3+ = abundant growth, 2+ = moderate growth, + = scant growth, ± = trace.
− = no growth.

two DP isolates assimilated melezitose, α-methylglucoside, and trehalose but acted on these compounds more slowly than did the DP isolates from the FPR type of hybrid.

White colonies arising from DP type colonies had the same reactions, even to idiosyncrasies, as possessed by the DP isolates from which they arose. Insufficient study has been made of the correlation of assimilation pattern and differences in surface structure of the DP colonies, but interesting correlations are believed to exist.

Incidentally, it is of interest to note that type FPR grew more rapidly in α-methylglucoside than did types E and DP, thus explaining the prevalence (80 per cent) of this form in the final isolation flask of this medium, while FPR colonies comprised only 5 and 2 per cent of the colonies from maltose and melezitose flasks, respectively. Although the DP type seems to arise by a relatively slow but constant mutation from vegetative cells, and more rapidly by sporulation of the E and FPR types, its initial slow growth prevents its occurrence in large numbers in mixtures with other hybrid types, and precludes pigment formation as an indicator that hybridization has occurred. It is evident, of course, that the types of hybrids isolated by a combination of carbon compounds will be influenced by the compounds employed.

Thus, all the hybrids yet obtained from the cross *S. fragilis* × *Z. dobzhanskii* ferment lactose strongly, but there is much variation in the degree to which different types of hybrids attack α-glucosides. Although one parent ferments all four α-glucosides rather strongly and the other does not attack them at all, the most active hybrids ferment only maltose and α-methylglucoside strongly, and melezitose weakly. The weakest ferment none of the α-glucosides and assimilate only maltose, having no action on melezitose, α-glucoside, or trehalose.

DISCUSSION

This study has provided a simple laboratory procedure for producing hybrids and two procedures for isolation of hybrids from mass matings of their parents. Of these two, the production and isolation of pigmented hybrids probably will prove to be more generally applicable. The methods are designed specifically for the new genus to be proposed under the name *Dekkeromyces*, which will contain, in addition to several other species, those now known as *S. lactis*, *S. fragilis*, and *Saccharomyces marxianus*.

This paper presents an illustration of a biochemical means of separating hybrids from parents through differences in their ability to assimilate two carbon sources. First one parent, then the other, is diluted out of the mixture by serially transferring them in media in which they cannot grow but which will support growth of the hybrids. One of the parents selected was *S. fragilis*, itself believed to be a naturally occurring hybrid (*S. lactis* × *Z. ashbyi*) which assimilates and ferments lactose. The other parent selected was *Z. dobzhanskii* which assimilates and ferments maltose. The hybrids obtained ferment both these sugars.

The hybrids resemble the diploid parent, *S. fragilis*, more strongly than they do the haploid parent, *Z. dobzhanskii*. Most of the hybrids are diploid in the vegetative state and their cells are large as are those of *S. fragilis*.

S. fragilis ferments lactose strongly, but *Z. dobzhanskii* has no action on it whatsoever; *S. fragilis* has no action on the α-glucosides maltose, α-methylglucoside, or melezitose, and almost none on trehalose, but *Z. dobzhanskii* assimilates and ferments all four. The intensity of fermentation decreases in the above order with maltose being the most strongly fermented. The strains used in this study produce very weakly-pigmented hybrids which ferment the α-glucosides less actively than the parent strain of *Z. dobzhanskii*. The fermentation of melezitose is very weak, and no discernible fermentation of trehalose occurs. White hybrids are also produced. They ferment maltose and α-methylglucoside less intensely than do the faintly pigmented hybrids, and neither trehalose nor melezitose is fermented. The faintly pigmented and white hybrids yield densely pigmented variants and ascospore isolates, and these in turn yield white variants. Only a very few of the pink and white variants ferment maltose, still fewer produce any gas from α-methylglucoside, and none ferments melezitose or trehalose. Most of the variants are incapable of fermenting any of the four α-glucosides, and they vary downward in activity to those which assimilate slowly only maltose. In contrast to the many levels of α-glucosidase activity, all hybrids and variants ferment lactose strongly or moderately.

It should be borne in mind that, if the strains used in the hybridization of *S. fragilis* and

Z. dobzhanskii had been selected for their ability to produce pigment instead of their lack of pigmentation, the relationship of pigmentation to fermentative ability would have been different. It might also be pointed out that hybrids have been produced by mating strains of an α-glucoside-fermenting species with a chromogenic, α-glucoside-assimilating strain of *S. lactis*, to produce a rather strongly pigmented hybrid which ferments lactose, and which, by the stronger complement of α-glucosidases from the two parents, ferments α-glucosides more strongly than the hybrids described in this paper.

It is of interest to note that in the preceding paper weakly pigmented parents produced pink hybrids which yielded many white ascosporic isolates. In the present paper, the reverse is approximately true; nearly colorless parents produced white or very faintly pink hybrids which yielded almost exclusively pink ascospore isolates. Inheritance of pigmentation might prove to be an interesting genetic study.

It has been rather common practice for geneticists to study inheritance of carbohydrases by fermentation tests. If a sugar were not fermented, it was assumed the specific gene was not active in the hybrid. This is not true in *Dekkeromyces*, for a gene or genes may be present which acts so weakly that a gaseous fermentation does not occur, but growth is permitted as the sugar becomes available through slow enzymic action. Thus, assimilation tests, as used in this study, are a more sensitive indicator of the presence of certain genes than are fermentation tests, though for maximum information, both tests should be used.

An interesting and unexpected finding is the fact that the hybrids produce ascospores which are quite irregular, but tend more toward spheroidal than crescentic in shape. This is strange, for parent *Z. dobzhanskii* produces crescent spores, and *S. fragilis* produces generally reniform spores with only a few spheroidal spores. The rather infrequent spheroidal spores of the presumed natural hybrid *S. fragilis*, as well as the much more numerous spheroidal spores of the hybrids reported here, are thought to be caused by a gene for round spores contributed by their presumed parent and grandparent, respectively, *S. lactis*.

The primary reason for writing this and the preceding paper is to inform geneticists regarding the yeasts which will constitute the new genus *Dekkeromyces*. It is hoped these species may prove useful in genetic studies. Parent strains, hybrids, variants, and ascospore isolates have been lyophilized in small numbers and are available to investigators interested in doing experimental work with them. Two detailed manuscripts covering the work reported in these papers are available for loan to interested persons. The main reason for hybridizing species of the genus and briefly analyzing the hybrids was to enable the writer to set the limits of the genus and of its species. These results will be reported when the genus is monographed.

SUMMARY

In a preceding paper procedures were presented for the production of hybrids and for their differentiation from the parent strains by their greater ability to produce pigment. In the present paper, a second procedure for isolation of hybrids is presented. Parents are selected so that their hybrids will differ sufficiently in the ability to assimilate carbohydrates to permit isolation on this basis. The mixture of parents and hybrids is serially transferred in a medium containing a carbon source which one parent cannot utilize and is thus eliminated from the mixture; then the remaining parent is eliminated by serial transfer in a second medium containing a different carbon source which it cannot use. The hybrids, however, can grow at the expense of the carbon sources used in both media, and thus survive their parents.

The two species selected to illustrate this procedure are *Saccharomyces fragilis* which assimilates and ferments lactose, and *Zygosaccharomyces dobzhanskii* which assimilates and ferments maltose. The hybrids ferment both, and are the first ascosporogenous yeasts known to have this property. They ferment α-glucosides less actively than *Z. dobzhanskii*. The hybrids give rise to variants and ascosporic cultures which vary downward by slight degrees in their ability to ferment and assimilate α-glucosides to those which assimilate only maltose, and it slowly.

REFERENCE

WICKERHAM, L. J. AND BURTON, K. A. 1955 Hybridization studies involving *Saccharomyces lactis* and *Zygosaccharomyces ashbyi*. J. Bacteriol., **71**, 290–295.