DETERMINATION OF CARBON ASSIMILATION PATTERNS OF YEASTS BY REPLICA PLATING

MOSHE SHIFRINE, H. J. PHAFF, AND A. L. DEMAIN

Department of Food Technology, University of California, Davis, California

Received for publication December 14, 1953

For the differentiation of species of yeasts, considerable value is attached to the ability of assimilating various carbon sources for growth. Beijerinck (1889) introduced for this general purpose the auxanographic plate technique which was adapted by Lodder (1934) for the study of sugar assimilation in the nonfermenting strains of the asporogenous yeasts. Diddens and Lodder (1942) expanded its use to all species (fermentative as well as nonfermentative) of the genera *Candida, Brettanomyces,* and *Trichosporon.* Lodder and Kreger-Van Rij (1952) finally applied the method to all species of the perfect and imperfect yeasts. These authors modified the synthetic mineral medium previously used by adding a trace of yeast extract or a mixture of the B vitamins to insure better growth of those yeasts possessing one or more growth factor requirements. Only glucose, galactose, sucrose, maltose, and lactose were tested by the auxanographic procedure. Okunuki (1931), who used a synthetic agar in test tubes, was the first to recommend the use of a much larger assortment of carbon sources. The amount of growth after 5 days was taken as the criterion for utilization of the carbon source. Wickerham and Burton (1948) reviewed the work of a number of other investigators and pointed out the lack of a well defined, universal and complete basal medium. Such a medium was developed by these authors and was termed yeast nitrogen base. It is used in liquid form and is supplied with 0.5 per cent of the carbon source to be tested. Turbidity readings are made after 7 and 24 days to determine the amount of growth. The number of carbon compounds used was greatly expanded, and the procedure was applied successfully in a study of yeasts belonging to the genus *Hansenula* (Wickerham, 1951).

Lederberg and Lederberg (1952) developed a method of replica plating to permit the transfer of a pattern of microbial growth from one initial agar plate to a series of others. In the procedure, sterilized velveteen is used which is stretched over the top of a cylindrical wooden block. The agar plate carrying the initial colonies is inverted onto the fabric and pressed down gently to transfer the growth to the cloth. The fabric then provides the inoculum for transfer to subsequent plates impressed in the same way. The Lederbergs recommended their method in general for all routine tests involving repetitive inoculation of many isolates on different media. Because of the extreme rapidity and simplicity of the procedure, we felt that it might be useful in the study of carbon assimilation in yeasts. When surveys of yeast floras are made, involving the handling and identification of large numbers of strains, the method should allow a great saving in time, especially when using a great number of carbon compounds. The present paper gives the results of our application of the replica plating method to the study of carbon assimilation by yeasts.

METHODS

Cultures. The yeasts used for this study were composed of 149 recent isolates from the intestinal tract of bark beetles (Shifrine, 1952) and 26 cultures from the yeast collection of the Department of Food Technology (see table 1). Of the latter group, 15 were authentic strains originally obtained from the Centraal Bureau voor Schimmelcultures, Delft, Holland, and the Northern Regional Research Laboratory, Peoria, Illinois. The new species obtained from bark beetles will be described in a separate publication. The yeasts were grown on wort agar slants for 18 hours before being transferred to the initial plate.

Initial plate. Twenty-five cultures were transferred to a plate containing either wort agar or yeast nitrogen base (Difco) to which 0.5 per cent glucose and 2 per cent agar had been added (figure 1). The pattern used is shown on the "blank" plate in figure 2. Sufficient inoculum was transferred to give pin point colonies (before

growth). Difficulties encountered in transferring "chalky" and "tough" yeasts were resolved by using even younger (12 hour) slant cultures. Then the plates were stored at room temperature (22 to 25 C) for 12 to 16 hours.

Type of agar. It was necessary to use an agar that supported as little growth as possible when mixed with yeast nitrogen base but lacking a carbon source. Five different agars were tested: bacto-agar (Difco), two experimental samples of purified Difco agars, "noble" agar (Difco), and washed agar (agar flakes, gelled, cut in cubes, and washed in distilled water for at least 3 days). Least growth was obtained with washed agar although "noble" agar also gave good results. Washing of agar before gelling was less satisfactory. For the subsequent work, the first type was used.

Concentration of the carbon source. Both from the standpoint of economy, when expensive materials are used, and when impurities are present in the compounds tested, it is desirable to use the lowest concentration still giving satisfactory readings. Six plates were replicated containing the following concentrations of glucose: 0, 0.1, 0.5, 1.0, 2.0, and 4.0 per cent. The 0.5 per cent plate gave results as good as the one with 4 per cent glucose, whereas the colonies on the plate with 0.1 per cent sugar were significantly smaller. For this reason, a 0.5 per cent concentration of the carbon source was used throughout the work, which is equivalent to 100 mg per plate of 25 yeasts.

Sterilization. All solutions were prepared in tenfold strength and sterilized by filtration. Appropriate amounts then were pipetted into sterile melted agar before pouring into petri dishes. The velveteen cloth was wrapped in kraft paper and sterilized by autoclaving.

Replication. After 12 to 16 hours, when the colonies had developed sufficiently, the plate was inverted and pressed against a sterile velveteen fabric mounted on a cylindrical wooden block. A convenient way of mounting the cloth on the wooden block is to press the fabric over the block with a sterile bottom of a petri dish. The material is held in place by stretching a rubber band over the part not covered by the petri dish. The latter also protects the cloth temporarily against infection by air borne microorganisms. In addition, working in a special inoculating room is recommended. After removal of the original plate, the cloth provides the inoculum for a series of other plates containing different carbon sources. The test plates are inverted on the block and pressed gently on the fabric. Great care should be exercised to lift the different plates in a strictly vertical direction, after stamp-

TABLE 1

Yeast species used in the replication method

GENUS AND SPECIES	NO. OF ISOLATES	SOURCE*
Saccharomyces fragilis	1	FT
S. microellipsodes	1	CBS
S. rouxii	1	CBS
S. cerevisiae	1	FT
S. pastori	13	Bark beetles
S. bisporus	1	Bark beetles
Pichia membranaefaciens	1	FT
Pichia sp., No. 52–11	3	Bark beetles
Hansenula saturnus	1	FT
H. anomala	1	FT
H. capsulata	36	Bark beetles
H. silvicola	1	Bark beetles
Debaryomyces hansenii	1	CBS
Hanseniaspora valbyensis	1	FT
Saccharomycodes ludwigii	1	CBS
Nadsonia elongata	1	CBS
Nematospora coryli	1	NRRL
Lipomyces starkeyi	1	CBS
Rhodotorula rubra	1	CBS
R. flava	1	CBS
R. glutinis	1	FT
Rhodotorula sp.	1	Bark beetles
Cryptococcus laurentii	1	CBS
C. diffluens	1	Bark beetles
Torulopsis aeria	1	CBS
T. gropengiesseri	1	NRRL
T. candida	1	FT
T. pinus	1	CBS
T. pinus var. *lactosa*	2	Bark beetles
Torulopsis sp., No. 52–85	1	Bark beetles
Kloeckera africana	1	FT
Trigonopsis variabilis	1	CBS
Candida albicans	1	FT
C. krusei	1	FT
C. parapsilosis	17	Bark beetles
C. mycoderma	3	Bark beetles
C. rugosa	1	Bark beetles
Candida sp., No. 52–54	69	Bark beetles
Trichosporon behrendii	1	CBS

* CBS—Centraal Bureau voor Schimmelcultures; NRRL—Northern Regional Research Laboratory; FT—Department of Food Technology.

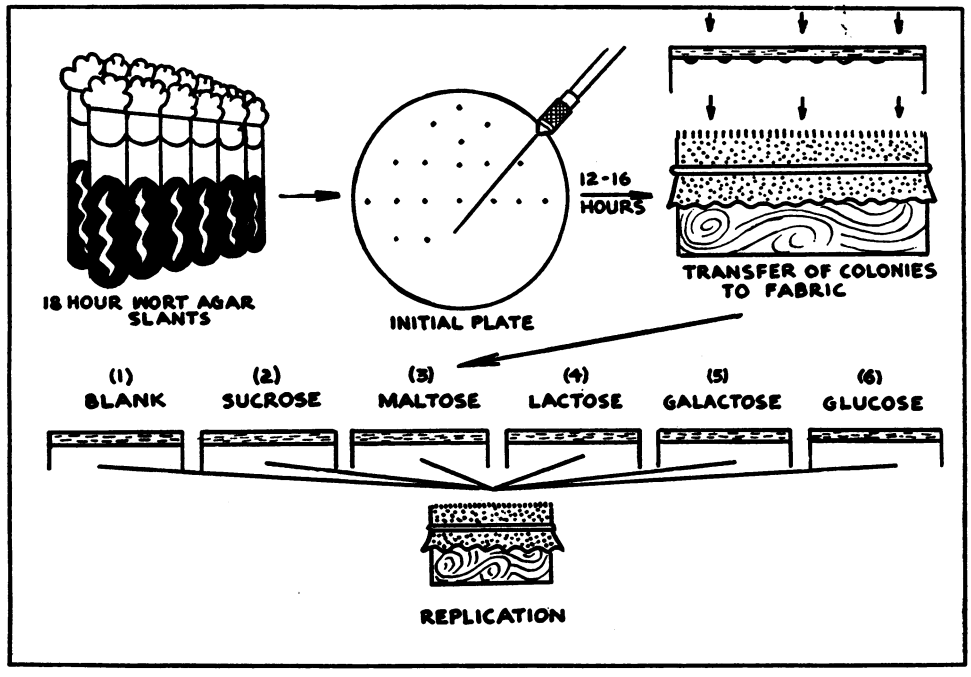

Figure 1. Illustration of the replica plating technique for the determination of sugar assimilation by yeasts.

ing, to avoid smearing of the colonies. The order of replication was as follows: blank (control plate without carbon source), sucrose, maltose, lactose, galactose, and glucose. The blank plate was impressed first to remove excess cell material. It also serves as a control to determine whether the agar was washed properly. Glucose was replicated last to determine whether sufficient growth had been transferred to the fabric to inoculate the entire series. The plates were incubated at room temperature and examined after two and six days. Occasionally observations were also made after a longer period.

Number of replications. A series of 20 glucose plates was replicated from one initial plate. Up to about the tenth plate the results were usable, but after this, smearing of the colonies became so pronounced that reading the results became difficult. Since only 5 sugars were used in this study, this presented no difficulty.

Purification of certain carbon sources. Some commercial compounds are sufficiently impure so that clear differences between positive and negative growth become obscured. The only compound used by us which was purified for the above reason was galactose. A 5 per cent solution of the sugar in distilled water was treated overnight on a mechanical shaker with a washed suspension of *Candida monosa*. This treatment was sufficient to remove the contaminating glucose, as was evidenced by paper chromatography before and after the treatment.

Reading of the results. As can be seen from figure 2, the "negative" colonies grew somewhat better on the plates containing sugar than on the control (blank). To overcome this difficulty, an unknown yeast is always compared finally with the growth on the glucose plate. Only if the growth is approximately as good on the test sugar as on glucose, is the colony considered positive. In this connection it should be pointed out that the appearance of a particular yeast colony on different sugars is not always the same. For example, it is very interesting that galactose frequently shows a strong stimulation of pseudomycelium formation (compare yeast 9 on glucose and galactose). Growth comparisons are made periodically, and the final reading is made usually after six days with the exception of the sucrose plates which are read after two days. The reason for this is explained in the next section.

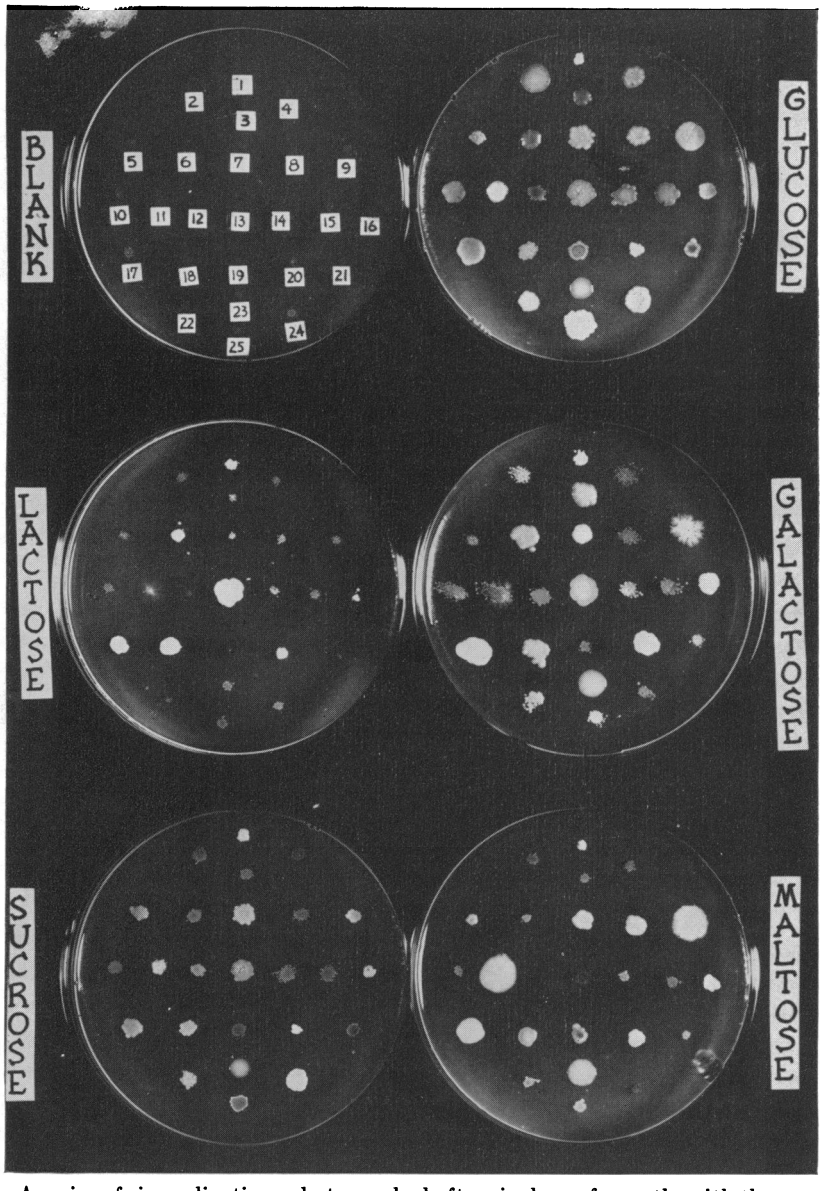

Figure 2. A series of six replications photographed after six days of growth, with the exception of the sucrose plate, which is two days old. The yeasts referred to by numbers and interpretation of the results are listed in table 2.

RESULTS AND DISCUSSION

The 175 yeasts used to test the reliability of the replica plating technique represent 16 genera, 38 species, and one variety. These are listed in table 1. In all cases the growth on replicate plates was compared with that obtained by the auxanographic technique. Complete agreement was found between the two methods. One set of replica plates is shown in figure 2. Our interpretation of the results is reported in table 2.

Sucrose is the only carbon source that presented some difficulty. On this sugar, all the colonies seemed positive on six day examination. However, the reading after two days showed distinct differences in sucrose assimilating ability. This can be seen in figure 3 where plate A

TABLE 2

Description of the yeasts and interpretation of the replication results depicted in figure 2

YEASTS	READING OF THE RESULTS IN FIGURE 2					
No. and Name	Blank	Lactose	Sucrose	Maltose	Galactose	Glucose
1. *T. aeria*	−	+	+	+	+	+
2. *S. pastori*	−	−	−	−	−	+
3. *T. pinus* var. *lactosa*	−	−*	−	−	+	+
4. *S. pastori*	−	−	−	−	−	+
5. *C. diffluens*	−	−	+	+	−	+
6. *T. pinus* var. *lactosa*	−	±*	−	−	+	+
7. *S. cerevisiae*	−	−	+	+	+	+
8. *H. capsulata*	−	−	−	+	−	+
9. *C. sylvicola*	−	−	+	+	+	+
10. *S. bisporus*	−	−	−	−	−	+
11. *C. parapsilosis*	−	−	+	+	−	+
12. *T. gropengiesseri*	−	−	+	−	+	+
13. *S. fragilis*	−	+	+	−	+	+
14. *S. pastori*	−	−	−	−	−	+
15. *S. pastori*	−	−	−	−	−	+
16. *C. parapsilosis*	−	weak	+	+	+	+
17. *T. candida*	−	+	+	+	+	+
18. *C. laurentii*	−	+	+	+	+	+
19. *K. africana*	−	−	−	+	−	+
20. *C. parapsilosis*	−	+	+	+	+	+
21. *S. ludwigii*	−	−	−	−	−	+
22. *S. microellipsodes*	−	−	+	−	+	+
23. *L. starkeyi*	−	−	+	+	+	+
24. *H. saturnus*	−	−	+	−	−	+
25. *P. membranaefaciens*	−	−	−	−	−	+

* See text.

represents the earlier reading and plate C shows the same plate after six days. That this effect is due to interaction between the colonies was proven by setting up a replication experiment with only the ten colonies which appeared negative after two days. When these colonies were replicated in the absence of sucrose assimilating yeasts, no significant increase in growth occurred from the second to the sixth day (plates B and D, respectively). For this reason, it is advisable to read sucrose plates after two days and to re-replicate the "negative" colonies alone on a fresh plate. The final observation is then made after six days.

Because of the phenomenon of interaction between colonies grown on sucrose, the sucrose plate shown in figure 2 was photographed after two days, whereas all other plates were six days old. The colonies on the sucrose plate actually should be compared to a two day old glucose plate. Since the glucose plate shown in the same figure is 6 days old, it is not strictly a fair comparison. However, only two slow growing positive colonies, numbers 9 and 21, show a significantly smaller amount of growth on sucrose than on glucose. They would be considered positive, however, if a two day glucose plate had been used for comparison. Another approach to reduce interaction is to use a smaller number of colonies per plate.

In order to determine whether the observed interaction was due to exocellular invertase production or to the secretion of metabolic products, the sucrose positive yeasts were grown for 6 days in liquid yeast nitrogen base containing 0.5 per cent of sucrose. The cells were removed then by centrifugation. The supernatant liquids were tested for invertase activity, which was negative in all cases. It was found, however, that several culture fluids contained variable, but appreciable, quantities of reducing sugars. Examples of such organisms are *Toru-*

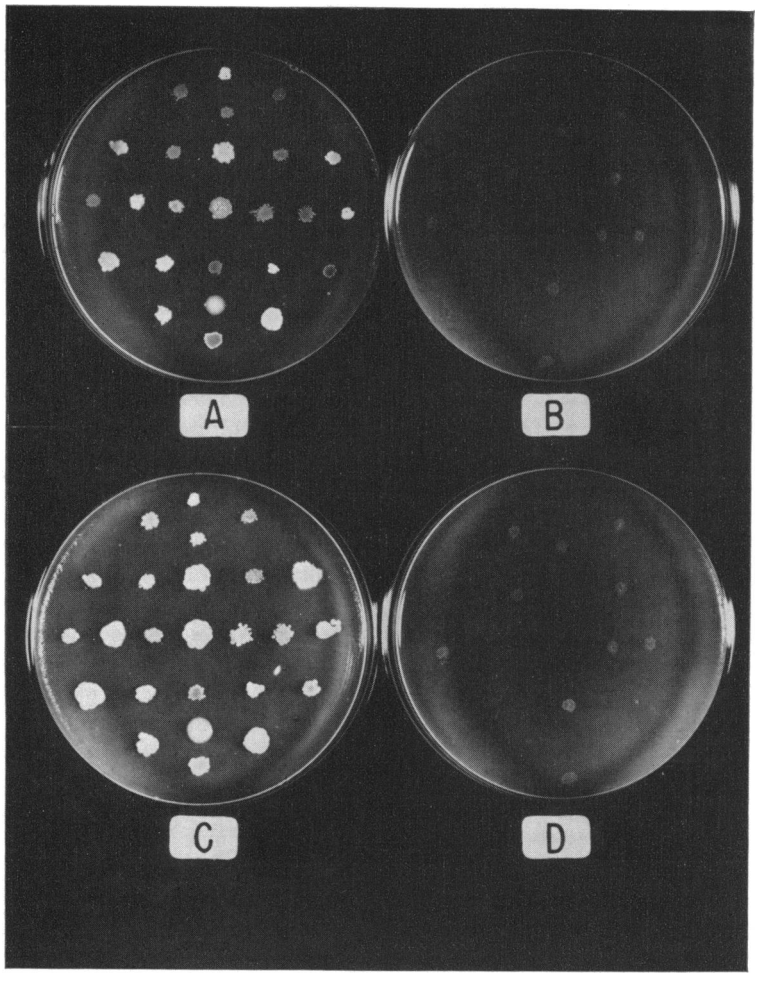

Figure 3. Interference phenomenon between sucrose positive and sucrose negative yeasts. Replication on sucrose plates of the same yeasts shown in figure 2. Plates A and C contain all 25 yeasts, and B and D, only the sucrose negatives ones. Plates A and B are two days old, and C and D are six days old.

lopsis aeria, *Cryptococcus diffluens*, *Torulopsis gropengiesseri*, and *Torulopsis candida*. Other sucrose positive yeasts such as *Saccharomyces cerevisiae* and *Saccharomyces fragilis* showed no reducing sugar in the centrifuged culture liquid. Therefore, the conclusion seems justified that the first group of yeasts can hydrolyze sucrose at a faster rate than it can utilize one or both of the resultant hexoses. When the latter diffuse through the agar, sucrose negative colonies nearby can utilize them for growth.

Other difficulties in reading the results were rarely experienced. We encountered a few yeasts, usually characterized by chalky, dull colonies, which appeared to produce a greater than average amount of growth on a blank plate or sugars which they cannot utilize as judged by the auxanographic procedure or the liquid medium technique. However, in comparing the growth to a glucose plate, there is little doubt in diagnosing a negative result. The replica plating technique has certain advantages over other methods of testing carbon assimilation. For example, with the auxanographic procedure, Lodder and Kreger-Van Rij (1952) encountered difficulties with slow growing yeasts and certain carbon compounds. One of these yeasts, *Lipomyces starkeyi*, was used in the series depicted in figure 2. As can be seen, this organism (no. 23 in figure 2) gave clear-cut results with the

replication procedure. Similarly we have noted that for certain strictly oxidative, nonpellicle forming yeasts, such as some species of *Cryptococcus*, growth is very slow in the liquid medium of Wickerham, and unless the cultures are allowed to grow for a long period of time, the results may be inconclusive. Growth is distinct in 6 days with the replication procedure as is illustrated by *C. diffluens* (no. 5 in figure 2). It might be added that the conditions for growth of such aerobic yeasts on the surface of a plate are undoubtedly more favorable than the more anaerobic conditions existing in a liquid test tube culture.

Problems may arise with yeasts requiring a period of adaptation towards certain sugars, especially when this period is long. An example of this is found in our isolates of *Torulopsis pinus* var. *lactosa*. This variety resembles the description of the type species (Lodder and Kreger-Van Rij, 1952) except that it utilizes galactose weakly and lactose only after an initial lag period of no visible growth. This period varies between the two strains, the assimilation method used, and other as yet unknown factors. Using the auxanographic method, it is found that after three to four days for strain no. 6 (table 2) a type of growth results consisting of numerous individual colonies in the area of lactose deposition. On the same plate with glucose a dense evenly opaque area is formed in one day. The significance of this observation is that apparently only a very small percentage of the cells embedded in the agar can acquire the ability to grow with lactose as a carbon source. If the results are read only after one or two days as is recommended by Lodder and Kreger-Van Rij, the lactose phenomenon would not be observed. With the liquid medium no growth occurs during four or five days, after which the organism suddenly begins to multiply and in about 12 days reaches a 3+ turbidity according to Wickerham's scale. This procedure gives no information about the percentage of cells able to grow with lactose. With the replication procedure the imprint on the lactose plate remains negative for 5 to 12 days and then suddenly develops an outgrowth which continues to grow rapidly. The lag period was found to vary from experiment to experiment. In figure 2 the colony began to grow after 5 days and is, therefore, somewhat smaller than that on the glucose plate. Strain no. 3 behaves similarly except that the lag is longer on all media. The results appear to be negative, therefore, after a six day examination (figure 2). After 12 days, however, the colony is as large as the one on glucose. Therefore, it may be desirable to keep replication plates longer than 6 days in order to detect such delayed positive strains. The suddenness of the growth after an initial lag is the most striking visual difference between this phenomenon and the gradual secondary growth at the expense of metabolic products of neighboring colonies.

Wickerham's procedure, using the liquid medium, is the most reliable reference method. No interaction occurs, and since the test is done in a pure culture, it can be observed for a considerable period of time.

The greatest advantages of the replica plating procedure are its relative simplicity, the enormous saving in time (even when certain doubtful results have to be rechecked by one of the other procedures), and the saving in carbon source when expensive compounds are used. Actually only 4 mg would be required per yeast since 100 mg are used for each plate of 25 yeasts. These advantages are realized particularly when large numbers of yeasts are being identified and the number of carbon sources is increased as suggested by Wickerham. The replication procedure enables a quick survey of new compounds to determine their suitability for differential purposes. Two prerequisites are required of each compound used in the procedure. First, the purity must be sufficient so that no false positive results are obtained at the expense of contaminating compounds. Secondly, one must make certain that no interaction occurs due to accumulation and secretion of intermediates in the metabolism of a compound.

The replicate plating procedure also serves well to check the production of starch-like compounds. Growth on yeast nitrogen base plus glucose lowers the pH sufficiently so that "starch" formation takes place by appropriate yeasts. Lodder and Kreger-Van Rij used this property to differentiate the genera *Cryptococcus* (positive) and *Torulopsis* (negative). By flooding the glucose plate with dilute Lugol solution, a blue to purplish color in or surrounding a colony is easily detected.

ACKNOWLEDGMENTS

The authors are grateful to Mr. John E. Schade of the Department of Food Technology

for technical assistance and to Difco Laboratories for supplying various samples of purified agar.

SUMMARY

The replica plating technique, developed by the Lederbergs for the detection of biochemical mutants in bacteria, has been applied to the study of carbon assimilation by yeasts. Six to ten successive replications by the velveteen block method can be made of a plate containing 25 different yeast colonies. The procedure, which is described in detail, affords a tremendous saving in time when large numbers of yeasts have to be identified, and it economizes in the use of expensive carbon sources. One hundred and seventy-five isolates of yeast were tested by the new technique using the five common sugars. The results were checked by the auxanographic plate technique and in some cases by the liquid medium of Wickerham. In general, excellent agreement between the three methods was found. Advantages and disadvantages of the new procedure have been discussed.

REFERENCES

BEIJERINCK, M. W. 1889 L'auxanographie, ou la méthode de l'hydro diffusion dans la gélatine appliquée aux recherches microbiologique. Arch. néerland. sci., **23**, 367–372.

DIDDENS, H. A., AND LODDER, J. 1942 *Die Hefesammlung des "Centraalbureau voor Schimmelcultures" II Teil. Die Anascosporogenen Hefen, Zweite Hälfte.* North Holland Publishing Co., Amsterdam.

LEDERBERG, J., AND LEDERBERG, E. M. 1952 Replica plating and indirect selection of bacterial mutants. J. Bact., **63**, 399–406.

LODDER, J. 1934 Die Hefesammlung des "Centraalbureau voor Schimmelcultures" II Teil, Die Anaskosporogenen Hefen, Erste Hälfte. Koninkl. Akad. Wetenschappen Amsterdam, Afdeel. Natuurkunde, 2 Sectie, verhandel., **32**, 1–256.

LODDER, J., AND KREGER-VAN RIJ, N. J. W. 1952 *The yeasts. A taxonomic study.* North Holland Publishing Co., Amsterdam.

OKUNUKI, K. 1931 Beitrage zur Kenntnis der rosafarbigen Sproszpilze. Japan. J. Botany, **5**, 285–322.

SHIFRINE, M. 1952 The association of yeasts with certain bark beetles. M.S. Thesis, University of California.

WICKERHAM, L. J. 1951 Taxonomy of yeasts. 1. Techniques of classification. 2. A classification of the genus *Hansenula*. U. S. Dept. Agri. Tech. Bull. no. 1029, 1–56.

WICKERHAM, L. J., AND BURTON, K. A. 1948 Carbon assimilation tests for the classification of yeasts. J. Bact., **56**, 363–371.