ACKNOWLEDGMENT

The authors wish to thank the management of the Colonial Sugar Refining Company, Ltd., for permission to publish this paper.

SUMMARY

"Petite colonie" mutation was shown to have taken place in reserve cultures of a strain of yeast being used in the production of alcohol from cane molasses. No reason for the change was apparent.

The "petite colonie" yeast was found to form alcohol from molasses much more slowly than did the original distillery yeast.

REFERENCES

EPHRUSSI, B. 1948 Action de l'acriflavine sur les levures. Colloq. intern. centre natl. recherche sci., **8**, Unités douées contin. genet. (Paris), 164–180.

EPHRUSSI, B. 1950 Induction par l'acriflavine d'une mutation spécifique chez la levure. Pubbl. staz. zool. Napoli, Suppl., 50–64.

EPHRUSSI, B., HOTTINGUER, H., AND CHIMÉNES, A. M. 1949 Action de l'acriflavine sur les levures. I. La mutation "petite colonie". Ann. inst. Pasteur, **76**, 351–367.

EPHRUSSI, B. AND HOTTINGUER, H. 1950 Direct demonstration of the mutagenic action of euflavin on baker's yeast. Nature, **166**, 956.

YČAS, M. 1956 A hereditary cytochrome deficiency appearing in yeast grown at an elevated temperature. Exptl. Cell Research, **10**, 746.

A Note on the Stability of Clostridia when Held in Continuous Culture[1]

R. K. FINN[2] AND J. E. NOWREY[3]

Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois

Received for publication June 19, 1958

Most of the selected strains of microorganisms used in industry are unstable, in the sense that they undergo biochemical and morphological changes on repeated transfer to fresh medium. Especially notorious in this respect are the solvent-producing clostridia (Kutzenok and Aschner, 1952) and some cultures of *Streptomyces griseus* (Williams and McCoy, 1953). The early literature on degeneration of clostridia has been cited by Perlman *et al.* (1954) in an article describing similar behavior of an asporogenous mutant of *S. griseus*. The latter workers found, for example, that *S. griseus* lost its ability to produce streptomycin after six or eight serial transfers, although it retained its ability to use glucose and to form vitamin B_{12}. Furthermore, they confirmed the widely held view that increasing the frequency of transfer brings about more rapid degeneration.

From such reports it is often inferred that continuous fermentation is impractical, the argument being that if frequent subculture is harmful then to hold a culture in active growth all the time will surely bring about rapid deterioration. To read the literature on this point is rather confusing because some writers have carelessly used the word "continuous" to describe apparatus or procedures which were in fact intermittent. The purpose of this experiment was to find out whether continuous culture causes more rapid or less rapid degeneration than serial transfer. A solvent-producing species of *Clostridium* was the only test organism used, but it may be fairly typical of other unstable microorganisms. We were particularly interested in its behavior when held continuously at a low level of growth in a rich medium, that is when held in the logarithmic growth phase under conditions similar to those in the cell propagator of a multiple-tank system. The experiments of Novick and Szilard (1950) with the "chemostat" are not comparable because growth was limited by a scarce nutrient rather than by washout of the cells.

MATERIALS AND METHODS

All of the work was done with a strain of *Clostridium saccharoacetobutylicum*[4] which has been described in the patent literature (Woodruff *et al.*, 1937). Solvent production in a molasses medium is normally about 30 per cent of the sugar fermented, and composition of the solvent is roughly 70 per cent butanol, 26.5 per cent acetone, and 2.5 per cent ethanol. The medium used here was similar to that of Kutzenok and Aschner (1952) and consisted of 0.5 per cent peptone (Difco),

[1] Presented in part at the 128th Meeting of the American Chemical Society, Minneapolis, Minnesota, September 11 to 16, 1955.

[2] Present address: Department of Chemical and Metallurgical Engineering, Cornell University, Ithaca, New York.

[3] Present address: Engineering Experiment Station, E. I. duPont de Nemours and Company, Inc., Wilmington, Delaware.

[4] Obtained from F. C. Arzberger through the courtesy of the Commercial Solvents Corporation, Terre Haute, Indiana.

0.5 per cent yeast extract (Difco), and 1.0 per cent glucose. The ingredients were not separately sterilized. Temperature was maintained at 35 C for both fermentation and growth.

A vessel for continuous culture was constructed from a 35 by 200 mm test tube. The provision for adding sterile medium was essentially as described by Finn and Wilson (1954), and an effluent port of 8-mm glass tubing was sealed into the propagator 1½ in. from the bottom, although broth plus cells were finally discharged through an adjustable overflow leg. Flow in and out of the apparatus was at a rate of 1.70 ml per min, and since the hold-up of broth was 77 ml, the average retention time for the culture was about 45 min.

In a laboratory apparatus of this type, a troublesome growth of microorganisms on the walls is commonly encountered. For example, the broth in the propagator may be only slightly turbid, indicating that the organisms are in an early phase of the growth cycle, yet the amount of bacterial substance clinging to the walls may be sufficient to cause a high concentration of metabolic products. This situation would not be so serious in a large tank, where the ratio of volume to surface is higher, but it had to be overcome in the laboratory work reported here. The only successful solution devised was to scour the walls with small glass particles (⅛ in. glass helices crushed in a mortar). These were filled into the propagator to a depth of about ¾ in. and were stirred at moderate speed by a large paddle. The latter consisted of a single blade, 1 in. by 1¼ in., cut from 1/16-in. Teflon sheet.

The degeneration experiment was carried out as follows. A culture was brought up in the continuous propagator, and when gassing was sufficient to insure that anaerobic conditions could be maintained (about 3 hr after inoculation), the flow of sterile nutrient was started. A portion of the culture used in starting the propagator was also used to inoculate flasks for serial transfer experiments. A serial transfer consisted in removing 5 ml of a 24-hr culture to 120 ml of fresh medium in a 125-ml flask. Every other day samples were removed from the flow apparatus and used to inoculate flasks for batch fermentations. Analyses on batch fermentations were made at the end of 60 hr, and the cultures in serial transfer were also analyzed after 60 hr.

Procedures for determining glucose (anthrone reagent) and acetone (alkaline salicaldehyde) were those recommended by Neish (1952). Partition chromatography as a method of analysis for butanol and ethanol became too tedious for routine use, and equally good results were obtained with a rapid colorimetric method (Reid and Truelove, 1952) which uses ceric ion in nitric acid. Butanol and ethanol gave approximately the same color on a weight basis, and acetone did not interfere. Apparently this convenient way of analyzing for alcohols has not been used much in fermentation studies.

Growth was followed by measuring the optical absorbance of washed cell suspensions at 655 mμ and also by scattered-light measurements (Coleman Nepho-Colorimeter Model 9).[5]

Results and Discussion

The glucose utilization, pH, and solvent production at 60 hr were chosen as criteria for "degeneration"; Kutzenok and Aschner (1952) have shown that these characteristics reflect other deep-seated changes in morphology and serology. Figure 1 compares the behavior of *C. saccharoacetobutylicum* when subcultured intermittently and continuously. The continuous propagator was operated for 14 days, after which a final sample was taken and subsequently transferred to fresh medium at 24-hr intervals for another 4 days.

The contrast between continuous and intermittent culture is immediately evident from figure 1. After 4 serial transfers there were signs of severe degeneration (in other experiments it was occasionally possible to get 5 or 6 passages), but the continuously-grown cells retained their ability to use glucose and to form solvents. The comparison is even more striking if expressed in terms of cell generations instead of days; whereas only 19 generations occurred in 4 serial transfers,[6] there were about 650 cell generations in the

[5] Coleman Instruments, Inc., Maywood, Illinois.

[6] Calculated from the formula $n/n_0 = 2^g$ and from the fact that n/n_0 was 125 ml per 5 ml (allowing no loss in viability). The number of generations, g, was 4.65 per transfer. For continuous culture the observed generation time of 31 min was used to make the estimate.

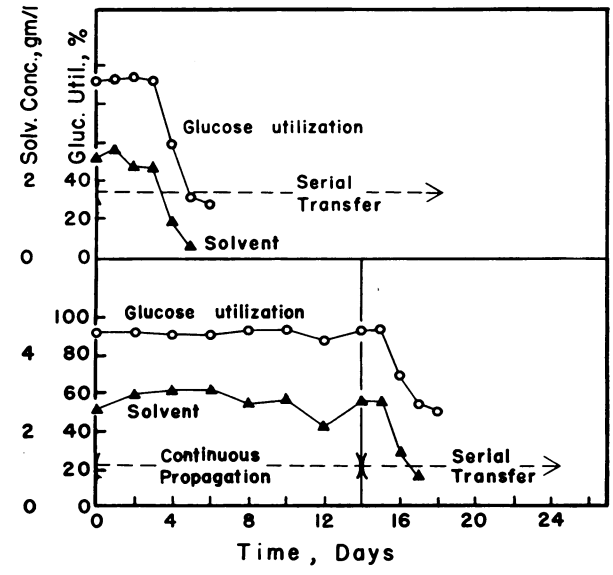

Figure 1. A comparison of serial transfer and continuous propagation (followed by several transfers) for a strain of *Clostridium saccharoacetobutylicium*.

propagator. Some changes, however, were taking place within the propagator because at the end of 2 weeks the continuous culture could sustain only a single batchwise transfer. Furthermore, a downward trend in final pH from the 6th to the 12th day indicated a decreasing ability to reduce acids to solvents. These changes may have been due in part to operating difficulties with the propagator which allowed high levels of growth on occasion.

The results for intermittent subculture were similar to those of Kutzenok and Aschner (1952), although their organism, a strain of *Clostridium butylicum*, could withstand 7 to 9 passages before the sudden onset of degeneration. The explanation was offered that mutants arise which have enhanced acid-producing abilities but which do not have the necessary enzymes for the reduction of acids. Because a low pH in turn favors growth of the mutants, degeneration becomes autocatalytic. Such an explanation may be an oversimplified one, but it helps to account for many of the observations.

The growth medium used for the present study was not a favorable one because clostridia were so unstable in it. Nevertheless it was chosen deliberately in order to provide as severe a test as possible of continuous propagation. In other experiments (Nowrey, 1955) the inoculum medium of Perlman (1948) was used, which contained added salts and which had more buffering action. In this medium, 13 to 15 successive transfers were required for degeneration. After continuous propagation for 10 days in Perlman's medium the cells failed to produce solvents unless calcium carbonate was added. Then, however, normal batch fermentation was obtained, with good yield of solvent.

It was not really an unexpected result for the continuously-grown cells to be more stable. If microorganisms are kept in a constant instead of a fluctuating environment, no adaptive changes in their enzymatic make-up can occur. With regard to mutants, only two types need cause concern as possessing selective advantage in the propagator; those with enhanced ability to cling to the walls, and those with an intrinsically higher growth rate in the medium chosen. The former type can be eliminated by mechanical devices, and the latter type can be controlled by proper choice of constituents in the medium. To devise a suitable broth for continuous fermentation may not be easy, but at least the depletion of nutrients present initially can be discounted as a factor favoring selection if growth is always kept somewhere in the exponential phase. There is no reason, of course, to avoid the development of rapid-growing strains, provided they can supply the desired fermentation product in high yield. There was in fact some evidence that the generation time of our strain of *C. saccharoacetobutylicum* decreased during its continuous propagation. Some day perhaps the screening of cultures for rapid growth may be as commonplace as screening for a combination of biological activity and low pigment production.

Experiments reported here do not conflict with the observation that changes may occur more rapidly when the frequency of intermittent subculture is increased. If mutants arise which are less sensitive to toxic cell products or which can continue to multiply at a lower nutrilite concentration, and if such mutants can compete favorably with the original type when transferred to fresh medium, then their relative proportion will be boosted with each successive transfer. The rate of degeneration will be greater as the "environmental stress" becomes more frequent, (that is, more frequent subculture). On the other hand, if the intervals between transfer become short enough, the culture does not proceed far into the phase of declining growth rate and the *intensity* of the repeated stress is lessened. As an illustration, one can cite the early experience of the Wisconsin group (Williams, 1936, Phelps, 1941). By transferring clostridia at 12-hr intervals instead of at 24-hr intervals it was possible to carry corn-mash cultures through 90 or more transfers instead of 40 to 50 transfers; continuous propagation was being approached. Nevertheless it is impractical to simulate

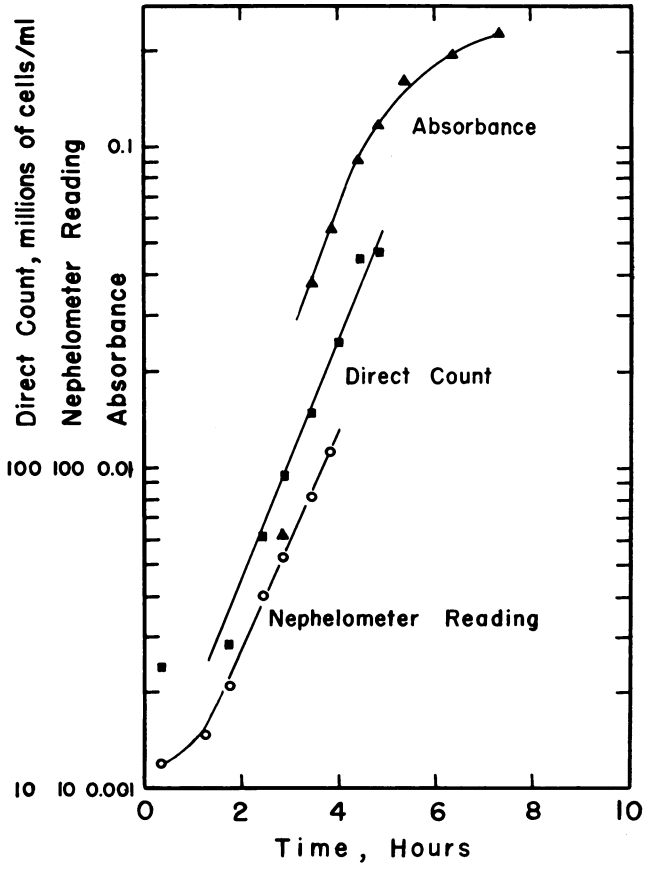

Figure 2. Brevity of the logarithmic phase is illustrated in this comparison of methods for following growth. (Medium of Kutzenok and Aschner but with 5 per cent glucose.)

the continuous propagator by a series of rapid transfers because the logarithmic phase is generally so short. Figure 2 shows that, with the medium of Kutzenok and Aschner as used here, the culture passed beyond its logarithmic growth phase even before reliable readings could be taken of its optical absorbance.

Finally it should be emphasized that each fermentation process has its unique problems of culture stability. Where heterokaryosis is involved (Haas *et al.*, 1956) it may be exceedingly difficult to adjust the growth medium so as to maintain the desired strain. Any deeper understanding of variation must, of course, rest on the sort of nutritional studies which have been carried out so extensively for *Brucella* species; this note merely points out that stability in continuous culture cannot be predicted from the behavior of an organism on repeated transfer.

Summary

Cells of a strain of *Clostridium saccharoacetobutylicum* showed less tendency to degenerate when held in logarithmic growth in a continuous propagator than when serially transferred in flasks at 24-hr intervals. This means that the usual laboratory subculturing is not a reliable guide to the feasibility of continuous fermentation. Broad generalizations about stability should be interpreted cautiously until other microorganisms are tested.

References

Finn, R. K. and Wilson, R. E. 1954 Population dynamics of a continuous propagator for microorganisms. J. Agr. Food Chem., **2,** 66–69.

Haas, F. L., Puglisi, T. A., Moses, A. J., and Lein, J. 1956 Heterokaryosis as a cause of culture rundown in *Penicillium*. Appl. Microbiol., **4,** 187–195.

Kutzenok, A. and Aschner, M. 1952 Degenerative processes in a strain of *Clostridium butylicum*. J. Bacteriol., **64,** 829–836.

Neish, A. C. 1952 *Analytical methods for bacterial fermentation.* National Research Council, Ottawa, Canada.

Novick, A. and Szilard, L. 1950 Experiments with the chemostat on spontaneous mutations of bacteria. Proc. Natl. Acad. Sci. U. S., **36,** 708–719.

Nowrey, J. E. 1955 Multi-tank continuous fermentation. Ph.D. Thesis, University of Illinois.

Perlman, D. 1948 Desthiobiotin and σ-heterobiotin as growth factors for "normal" and "degenerate" strains of *Clostridia*. Arch. Biochem. **16,** 79–85.

Perlman, D., Greenfield, R. B., Jr., and O'Brien, E. 1954 Degeneration of a *Streptomyces griseus* mutant on repeated transfer. Appl. Microbiol., **2,** 199–202.

Phelps, A. S. 1941 Studies upon butyric anaerobes with special reference to solvent formation. Ph.D. Thesis, University of Wisconsin.

Reid, V. S. and Truelove, R. K. 1952 The colorimetric determination of alcohols. Analyst, **77,** 325–328.

Williams, A. M. and McCoy, E. 1953 Degeneration and regeneration of *Streptomyces griseus*. Appl. Microbiol., **1,** 307–313.

Williams, F. T. 1936 The effect of repeated transfers on certain spore-forming bacteria. Ph.D. Thesis, University of Wisconsin.

Woodruff, J. C., Stiles, H. R., and Legg, D. A. 1937 Process of producing butyl alcohol. U. S. Patent 2,089,522.