APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1979, p. 1047-1052
0099-2240/79/06-1047/06$02.00/0
Vol. 37, No. 6

Energetics of *Microbacterium thermosphactum* in Glucose-Limited Continuous Culture

BEVERLEY J. HITCHENER,[1] AUBREY F. EGAN,[1]* AND P. J. ROGERS[2]

CSIRO Division of Food Research, Meat Research Laboratory, Cannon Hill, Queensland, 4170 Australia[1] *and School of Science, Griffith University, Nathan, Queensland, 4111 Australia*[2]

Received for publication 14 March 1979

Microbacterium thermosphactum was grown at 25°C in glucose-limited continuous culture under aerobic (>120 μM oxygen) and anaerobic (<0.2 μM oxygen) conditions. The end products of the anaerobic metabolism of glucose were identified as L-lactate and ethanol. Together these compounds accounted for between 85 and 90% of the glucose utilized over the full range of growth rates studied. In addition, 4% of the glucose utilized was incorporated into cellular material. Under anaerobic conditions the molar growth yield was 40 g (dry weight) of cells per mol of glucose utilized, and the maintenance energy coefficient was 0.4 mmol of glucose utilized per g (dry weight) of cells per h. For cells grown under aerobic conditions the corresponding values were 73 g/mol and 0.2 mmol/g per h, respectively. The molar growth yield with respect to adenosine 5'-triphosphate varied with the growth rate of the culture, and the true molar growth yield with respect to adenosine 5'-triphosphate was found to be 20 g/mol of adenosine 5'-triphosphate.

Microbacterium thermosphactum is a psychrotrophic, facultative anaerobe that, together with certain lactic acid bacteria, has been implicated in the spoilage of vacuum-packaged meat (2, 18, 21). Little is known about the physiology and energetics of this organism either in batch culture or in an open system.

There is some evidence to suggest that, when microorganisms are growing on the surface of meat, growth is limited by the rate of diffusion of fermentable substrates from the underlying tissues (8, 13). When the meat is packed in plastic films of low oxygen permeability, the microorganisms may be thought of as operating in an anaerobic open system. Under these conditions the major available substrate appears to be glucose (8, 13), but whether the ultimate composition of the microflora is a result of selection on the basis of competition for glucose is not clear (8, 13, 17). This point may be clarified by studying pure and mixed cultures of spoilage organisms in glucose-limited continuous cultures under anaerobic conditions. These cultures approximate some of the conditions operative on the surfaces of packaged meat and also allow quantitation of energetic data.

In the present study, Y_{ATP}^{MAX}, the true molar growth yield with respect to adenosine 5'-triphosphate (ATP), in grams of cell dry weight per mole of ATP, has been determined for *M. thermosphactum*, together with the maintenance energy requirements of this organism growing in aerobic and anaerobic cultures under conditions of energy limitation. Y_{ATP}^{MAX} has been found to be 20 g/mol of ATP, which is almost twice the value of 10.5 ± 2.5 g/mol of ATP found for many organisms and proposed as a constant by Bauchop and Elsden (3). Our data are comparable to the data of de Vries et al. (6), who found an anomalously high Y_{ATP}^{MAX} value of 24.3 g/mol of ATP for *Lactobacillus casei*, and support the contention that observed energetic growth efficiencies may be considerably greater than first appreciated (3). In addition, we have found that *M. thermosphactum* has a very low maintenance energy requirement, which may partly explain the high Y_{ATP}^{MAX} value.

MATERIALS AND METHODS

Bacteria and culture conditions. *M. thermosphactum* ATCC 11509 was grown in a New Brunswick Bioflo C30 continuous culture apparatus fitted with dissolved oxygen and pH monitoring devices. The semisynthetic medium used was half-strength medium 56 (12) supplemented with 0.3% (wt/vol) yeast extract (Difco) and 0 to 12 mM glucose. The oxygen concentration was monitored with an autoclavable version of the Mackereth-type oxygen electrode, constructed as described by Borkowski and Johnson (4). Culture density was followed by using a Klett-Summerson colorimeter fitted with a no. 66 filter, and Klett values were converted to dry weights by a standard curve. Dry weights were determined by using 0.45-μm membrane filters (Sartorius SM 111) which had been dried to constant weight. Samples of the cultures were filtered,

and the collected cells were washed with water and then dried to constant weight.

Anaerobic cultures were maintained by continuously sparging the culture vessel with sterile high-purity nitrogen containing 5% carbon dioxide and less than 1 μl of oxygen (Commonwealth Industrial Gases, Brisbane, Australia) per liter. The medium in the nutrient reservoir was continuously sparged with sterile high-purity nitrogen. No dissolved oxygen was detected in the medium in the nutrient reservoir or in the cultures of cells growing anaerobically. This indicates that the oxygen concentration was less than 0.2 μM (equivalent to 0.1% air saturation) (15). Aerobic cultures were maintained by sparging the culture vessel with sterile air so that the steady-state oxygen concentration was always greater than 50% of the value determined for air-saturated culture medium (>120 μM) (15).

The pH values of the media were 6.5 ± 0.1 and 6.7 ± 0.1 for the anaerobic and aerobic cultures, respectively.

Analytical procedures. L-Lactate, glucose, and ethanol were determined in cell-free culture filtrates by using assay kits supplied by Calbiochem. Concentrations were obtained by referring to standard curves prepared by using appropriate concentrations of standards in aqueous solution, after subtraction of medium blanks. Total lactate was estimated chemically by the method of Barker and Summerson (1).

The proportion of glucose being incorporated into cellular material was estimated by following the incorporation of radioactivity into trichloroacetic acid-insoluble material after the addition of D-[*U*-^{14}C]glucose (Amersham; 317 mCi/mmol) to the chemostat cultures. The radioactive glucose was injected directly into the culture vessel of the chemostat to produce a final [^{14}C]glucose concentration of 0.2 μCi/ml. Samples (5 ml) were taken at various times until two complete medium volume changes had occurred, and from each sample two 1-ml volumes were immediately transferred into 1-ml portions of ice-cold 10% trichloroacetic acid. The precipitated samples were then left for at least 30 min at 0°C before being collected on membrane filters (0.45-μm pore diameter; Sartorius SM 111). The filters were washed twice with 2 ml of 5% trichloroacetic acid, dried, placed in 5 ml of toluene scintillation fluid, and counted in a Packard Tri-Carb liquid scintillation counter. The radioactivity incorporated was expressed as a percentage of the total radioactivity per milliliter of culture at each time point. The experiment was carried out four times at dilution rates between 0.1 and 0.2 h^{-1}.

RESULTS

Anaerobic cultures. Preliminary experiments carried out at dilution rates between 0.10 and 0.20 h^{-1} showed that cell growth was limited by glucose availability up to a medium glucose concentration of almost 7 mM. Cell yield was then determined for a series of cultures with varying glucose concentrations and at a constant dilution rate of 0.10 h^{-1}. As Fig. 1 shows, there was some growth in medium containing yeast extract alone, and cell yield increased in proportion to glucose utilized, up to a glucose concentration of at least 6 mM. The slope of the straight line so obtained gave an observed cell yield (Y_{glu}) of 37 g (dry weight) of cells per mol of glucose utilized. A glucose concentration of 3.7 mM was chosen for all further experiments, and Fig. 2 shows the data obtained for a series of anaerobic cultures plotted as a function of dilution rate. The cell yield was maximal at dilution rates between about 0.10 and 0.25 h^{-1} and declined at higher or lower values (Fig. 2A). In the absence of added glucose (but with 0.3% yeast extract in the medium), the yield was essentially independent of dilution rate (Fig. 2B). By subtraction, the net yield of cells due to glucose metabolism alone was determined (Fig. 2C) and was maximal at exponential growth rates between 0.10 and 0.22 h^{-1}. Y_{glu} values were calculated and are shown in Fig. 2D. Cell yield was dependent upon growth rate, and to determine the "true" cell yield and maintenance coefficient the equation of Pirt (14) was used. This equation relates these values as follows: $\mu/Y_{glu} = \mu/Y_{glu}^{MAX} + m_s$, where, for cells growing with glucose as the limiting substrate, Y_{glu}^{MAX} is the true cell yield, μ is the exponential growth rate (dilution rate), and m_s is the maintenance coefficient, defined as the rate of substrate uptake in the absence of growth. When μ/Y_{glu} is plotted against μ, the slope of a straight line plot so obtained is $1/Y_{glu}^{MAX}$, and the y-axis intercept gives m_s. The rate of glucose consumed (μ/Y_{glu}; moles of glucose per gram of dry weight per hour) has been calculated for each value of the dilution rate by using the data in Fig. 2D and has been replotted as a function of μ (Fig. 3). A straight-line relationship was found; Y_{glu}^{MAX} was calculated to be 46 g (dry weight) of cells per mol of glucose, and

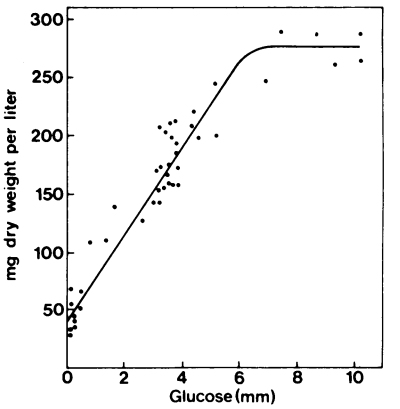

FIG. 1. *Anaerobic growth yield of M. thermosphactum in continuous culture at a dilution rate of 0.1 h^{-1} as a function of glucose concentration.*

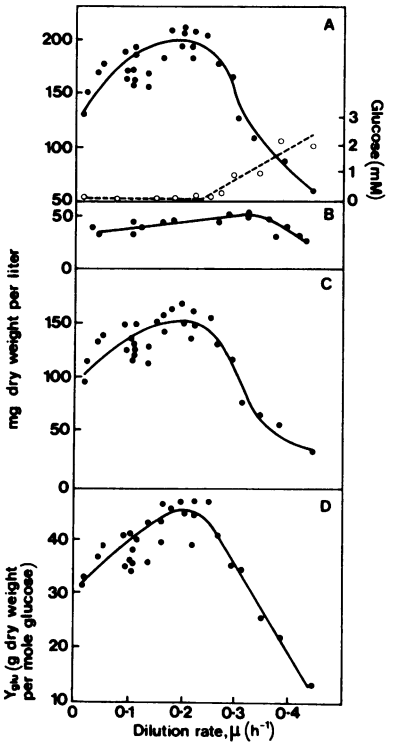

FIG. 2. *Growth yields of M. thermosphactum in anaerobic glucose-limited continuous cultures as a function of dilution rate. (A) Input medium contained 3.7 mM glucose and 0.3% yeast extract. Symbols: ●, dry weight; ○, glucose concentration. (B) Input medium contained 0.3% yeast extract only. (C) Calculated net dry weight of cells due to the metabolism of glucose. (D) Calculated net yield (Y_{glu}) in grams dry weight of cells per mole of glucose.*

m_s was calculated to be 0.4 mmol of glucose per g (dry weight) per h.

The end products of the anaerobic metabolism of glucose were identified and estimated initially in cultures of cells with an exponential growth rate of 0.15 h^{-1}. As expected, L-lactate was the major end product, and 68% of the glucose utilized was converted to this compound. When a sensitive enzymatic assay (D-lactate dehydrogenase coupled with glutamate pyruvate transaminase) was used, no D-lactate was detected. Chemical determinations of total lactate concentration agreed well with the value for L-lactate determined enzymatically.

By using gas chromatography, ethanol was identified as a major end product, but no volatile fatty acids or acetoin could be detected in culture filtrates. Pyruvate was not an end product. Further enzymic estimations showed that ethanol accounted for 19% of the glucose utilized.

The concentrations of L-lactate and ethanol were determined in a series of culture filtrates obtained from cultures grown over a range of dilution rates from 0.05 to 0.30 h^{-1}. In all cases L-lactate was the major end product, but significant concentrations of ethanol were also always present (approximate ratio, 3:1). These compounds between them accounted for between 85 and 90% of the glucose utilized in all cases. The experiments with [^{14}C]glucose showed that an average of 4% (range, 3.4 to 4.3%) of the glucose utilized was incorporated into cellular material. This means that 7.2 g of glucose was incorporated for each mol utilized. Assuming that the carbon content of the cells was 50% of the dry weight, and since glucose is 40% carbon by weight, then 5.8 g (dry weight) of cells is obtained from glucose carbon for each mol of glucose utilized. Thus, the corrected value for Y_{glu}^{MAX} is 40 g (dry weight) of cells per mol of glucose utilized.

In a typical experiment 91% of the glucose consumed was accounted for 87% as end products and 4% by incorporation. Assuming a maximum value of 2 mol of ATP produced per mol of glucose consumed, then 1.82 mol of ATP was produced per mol of glucose utilized, and Y_{ATP}^{MAX}, corrected for the energy of maintenance, was 22 g/mol. Assuming that the 9% of glucose which is unaccounted for also yields 2 mol of ATP per mol, then Y_{ATP}^{MAX} becomes 20 g/mol.

Aerobic cultures. As in the anaerobic experiments, a limiting concentration of 3.7 mM glucose was used. In Fig. 4 the data obtained for a series of aerobic cultures have been plotted as a function of dilution rate. Cell yield was relatively constant over a range of dilution rates from about 0.05 to 0.30 h^{-1} (Fig. 4A), but at higher dilution rates the cell yield declined rapidly and there was a concomitant increase in the steady-

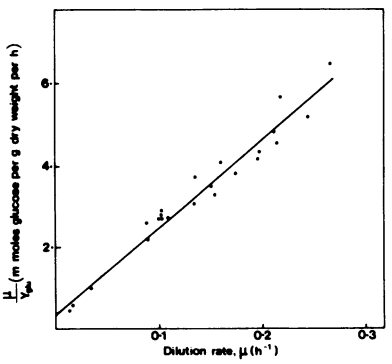

FIG. 3. *Effect of specific growth rate on the rate of glucose consumption in anaerobic, glucose-limited, continuous cultures of M. thermosphactum.*

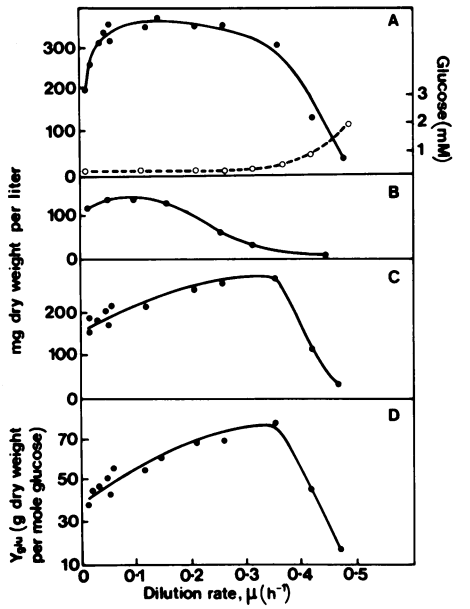

FIG. 4. *Growth yields of M. thermosphactum in aerobic, glucose-limited continuous cultures as a function of dilution rate. (A) Input medium contained 3.7 mM glucose and 0.3% yeast extract. Symbols: ●, dry weight; ○, glucose concentration. (B) Input medium contained 0.3% yeast extract only. (C) Calculated net dry weight of cells due to the metabolism of glucose. (D) Calculated net yield (Y_{glu}) in grams dry weight of cells per mole of glucose.*

state concentrations of glucose and lactate. Extrapolation of this data gives a maximum growth rate equivalent to a generation time of about 1.4 h.

The cell yields in media lacking added glucose (but containing 0.3% yeast extract) were relatively high at dilution rates up to 0.15 h^{-1} and thereafter declined steadily (Fig. 4B). Figure 4C shows the net yield of cells due to glucose metabolism, which was obtained by subtracting the cell yield without glucose from the cell yield with glucose. Cell yield was maximal at an exponential growth rate of 0.35 h^{-1}. Y values were also determined (Fig. 4D).

Using the data in Fig. 4D, μ/Y_{glu} was calculated, and when plotted as a function of μ (up to values of $\mu = 0.25$ h^{-1}), a straight line was obtained. From this graph Y_{glu}^{MAX} was found to be 73 g (dry weight) of cells per mol of glucose, and m_s was found to be 0.2 mmol of glucose per g (dry weight) of cells per h.

DISCUSSION

In glucose-limited cultures of *M. thermosphactum* L-(+)-lactate was the major end product of glucose catabolism. In addition, although not previously reported as a metabolic end product, ethanol was also detected in cell-free filtrates. Since no volatile fatty acids appeared as end products, there are only three possible pathways for glucose catabolism: (i) the Embden-Meyerhof glycolytic pathway, in which, after the conversion of glucose to 2 mol of pyruvate, this latter compound is metabolized to lactate and/or ethanol plus CO_2 and 2 mol of ATP is produced per mol of glucose metabolized; (ii) the hexose monophosphate pathway (phosphoketolase pathway), in which glucose is converted via 6-phosphogluconate and pentose phosphate to equimolar amounts of lactate, ethanol, and CO_2 and 1 mol of ATP is produced per mol of glucose metabolized; and (iii) the Entner-Doudoroff pathway, in which glucose is converted via 6-phophogluconate and 2-keto-3-deoxy-6-phosphogluconate to 2 mol of pyruvate and 1 mol of ATP is produced per mol of glucose metabolized.

The production of ethanol as found in the present study requires the concomitant formation of a one-carbon fragment. Since no formate was produced, it appears likely that CO_2 is an end product and that the organism is behaving as a heterofermentative strain under glucose-limited conditions. This agrees with the earlier finding that *M. thermosphactum* produces acid and gas from the fermentation of glucose (10). However, irrespective of the pathway (or combination of pathways) operating in glucose-limited cultures, the maximum yield of ATP produced per mole of glucose metabolized under anaerobic conditions must be 2 mol. If all of the glucose utilized yielded this amount of ATP, then a minimum value for Y_{ATP}^{MAX} of 20 g/mol can be calculated. If either the hexose monophosphate pathway or the Entner-Doudoroff pathway were also operative, the net gain of ATP per mole of glucose metabolized would decline, and consequently the apparent Y_{ATP}^{MAX} would increase. Even the minimum Y_{ATP}^{MAX} value of 20 g/mol is considerably higher than values commonly obtained for microorganisms and also is almost twice the value of 10.5 ± 2.5 g/mol proposed by Bauchop and Elsden (3). A number of reports of high Y_{ATP} values have previously appeared, but because of lack of precise knowledge of metabolic pathways or unsatisfactory carbon recoveries, these values were considered to be of questionable validity (7). More recently, a value of 24.3 g/mol was reported for *L. casei* (6), and Stouthamer and Bettenhaussen (20) argued that this study was of an organism with known metabolic pathways and that satisfactory carbon recovery data was presented. Our carbon balance studies for *M. thermosphactum* showed

that 91% of the carbon consumed as glucose could be accounted for. This is comparable to the data presented for *L. casei* (6), and it seems unlikely that the 9% of the carbon which is unaccounted for could yield enough ATP to reduce the value to that of Bauchop and Elsden (3). If we assume a Y_{ATP} of 13.0 g/mol (upper limit of the Bauchop and Elsden range), it can be calculated that the remaining 9% of the glucose would have to yield 14 mol of ATP per mol of glucose. In determining the cell yield a correction was applied for the yield of cells obtained in the absence of glucose (yield due to yeast extract). This correction would be inaccurate if, in the presence of glucose, cells were able to utilize for energy additional components of the yeast extract, since in this case an anomolously high cell yield would result. However, since a straight-line relationship existed between glucose utilized and cell yield (Fig. 1), it appears unlikely that additional energy was in fact being obtained.

Stouthamer (19) has discussed the effect of growth rate and maintenance energy on Y_{ATP}, and the extremely low maintenance energy coefficient found in the present study may explain the high Y_{ATP}^{MAX} value found. Stouthamer and Bettenhausen (20) derived a series of theoretical curves relating Y_{ATP} and μ at various values of m_e (the maintenance energy expressed in terms of ATP consumed; moles of ATP per gram of dry weight of cells per hour). Assuming that *M. thermosphactum* produces 2 mol of ATP per mol of glucose utilized, then the m_e value is 0.8 mmol/g per h. The theoretical Y_{ATP} values predicted from the calculations of Stouthamer and Bettenhausen (20) would range from about 19 g/mol at $\mu = 0.1\ h^{-1}$ to 23 g/mol at $\mu = 0.25\ h^{-1}$. These values are in good agreement with the data shown in Fig. 2D.

There was an increase in the growth yield when cells of *M. thermosphactum* were grown in the presence of excess oxygen, and this is in agreement with results found for other facultative anaerobes (5, 9). If it is assumed that Y_{ATP} is a constant for both aerobically and anaerobically grown cells, then the additional ATP obtained from respiration is 1.65 mol/mol of glucose. Aerobically, glucose is quantitatively converted to acetoin (L. E. Brownlie, M.Sc. thesis, University of Sydney, Sydney, Australia, 1969), and this reaction involves the overall transfer of 4[H]. Thus, if 1.65 mol of ATP is obtained per 2[O] we can project a P/O ratio of 0.82. This is considerably less than the value of 3 reported by Meyer and Jones and determined from H^+/O studies (11). However, a number of reports suggest that optimum P/O ratios are not found when the metabolism of whole cells is studied (16).

ACKNOWLEDGMENTS

This work was supported in part by the Australian Meat Research Committee.

We are grateful to R. F. Adams and P. L. Conway for gas chromatographic analyses.

LITERATURE CITED

1. **Barker, S. B., and W. H. Summerson.** 1941. The colorimetric determination of lactic acid in biological material. J. Biol. Chem. **138**:535-554.
2. **Barlow, J., and A. G. Kitchell.** 1966. A note on the spoilage of prepacked lamb chops by *Microbacterium thermosphactum*. J. Appl. Bacteriol. **29**:185-188.
3. **Bauchop, T., and S. R. Elsden.** 1960. The growth of microorganisms in relation to their energy supply. J. Gen. Microbiol. **23**:457-469.
4. **Borkowski, J. D., and M. J. Johnson.** 1967. Long lived steam-sterilizable membrane probes for dissolved oxygen measurement. Biotechnol. Bioeng. **9**:635-639.
5. **Buchanan, B. B., and L. Pine.** 1967. Path of glucose breakdown and cell yields of a facultative anaerobe, *Actinomyces naeslundii*. J. Gen. Microbiol. **46**:225-236.
6. **de Vries, W., W. M. C. Kapteijn, E. G. van der Beek, and A. H. Stouthamer.** 1970. Molar growth yields and fermentation balances of *Lactobacillus casei* L3 in batch cultures and continuous cultures. J. Gen. Microbiol. **63**:333-345.
7. **Forrest, W. W., and D. J. Walker.** 1971. The generation and utilization of energy during growth. Adv. Microb. Physiol. **5**:213-274.
8. **Gill, C. O.** 1976. Substrate limitation of bacterial growth at meat surfaces. J. Appl. Bacteriol. **41**:401-410.
9. **Hadjipetrou, L. P., J. P. Gerrits, F. A. G. Teulings, and A. H. Stouthamer.** 1964. Relation between energy production and growth of *Aerobacter aerogenes*. J. Gen. Microbiol. **36**:139-150.
10. **McLean, R. A., and W. L. Sulzbacher.** 1953. *Microbacterium thermosphactum*, spec nov; a nonheat resistant bacterium from fresh pork sausage. J. Bacteriol. **65**: 428-433.
11. **Meyer, D. J., and C. W. Jones.** 1973. Oxidative phosphorylation in bacteria which contain different cytochrome oxidases. Eur. J. Biochem. **36**:144-151.
12. **Monod, J., G. Cohen-Bazire, and M. Cohn.** 1951. Sur la biosynthèse de la β-galactosidase (lactase) chez *Escherichia coli*. La spécificité de l'induction. Biochim. Biophys. Acta **7**:585-599.
13. **Newton, K. G., and C. O. Gill.** 1978. The development of the anaerobic spoilage flora of meat stored at chill temperatures. J. Appl. Bacteriol. **44**:91-95.
14. **Pirt, S. J.** 1965. The maintenance energy of bacteria in growing cultures. Proc. R. Soc. London Ser. B **163**:224-231.
15. **Rogers, P. J., and P. R. Stewart.** 1973. Respiratory development in *Saccharomyces cerevisiae* grown at controlled oxygen tension. J. Bacteriol. **115**:88-97.
16. **Rogers, P. J., and P. R. Stewart.** 1974. Energetic efficiency and maintenance energy characteristics of *Saccharomyces cerevisiae* (wild type and petite) and *Candida parapsilosis* grown aerobically and micro-aerobically in continuous culture. Arch. Microbiol. **99**:25-46.
17. **Roth, L. A., and D. S. Clark.** 1975. Effect of lactobacilli and carbon dioxide on the growth of *Microbacterium thermosphactum* on fresh beef. Can. J. Microbiol. **21**: 629-632.
18. **Seideman, S. C., C. Vanderzant, M. O. Hanna, Z. L. Carpenter, and G. C. Smith.** 1976. Effect of various types of vacuum packages and length of storage on the

microbial flora of wholesale and retail cuts of beef. J. Milk Food Technol. **39**:745–753.

19. **Stouthamer, A. H.** 1977. Energetic aspects of the growth of microorganisms, p. 285–315. *In* B. A. Haddock and W. A. Hamilton (ed.), Microbial energetics. Society for General Microbiology, 27th Symposium. Cambridge University Press, Cambridge.
20. **Stouthamer, A. H., and C. Bettenhaussen.** 1973. Utilization of energy for growth and maintenance in continuous and batch cultures of microorganisms. A re-evaluation of the method for the determination of ATP production by measuring molar growth yields. Biochim. Biophys. Acta **301**:53–70.
21. **Sutherland, J. P., J. T. Patterson, and J. G. Murray.** 1975. Changes in the microbiology of vacuum-packaged beef. J. Appl. Bacteriol. **39**:227–237.