End-products, Fermentation Balances and Molar Growth Yields of Homofermentative Lactobacilli

By H. DIRAR* AND E. B. COLLINS

*Department of Food Science and Technology, University of California,
Davis, California, 95616, U.S.A.*

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SUMMARY

Y (glucose) values for *Lactobacillus plantarum*, *L. delbrueckii*, *L. casei*, *L. lactis* and *L. bulgaricus*, grown as batch cultures in defined medium, and *L. casei*, grown in complex medium, were 20.0 to 33.3. Y (galactose) values for *L. plantarum* were 25.7 to 32.5. Each species produced acetate in addition to lactate, and Y (ATP) values, corrected for the energy produced simultaneously with the formation of acetate, were 10.0 to 10.9, close to 10.5, proposed as a standard value by Bauchop & Elsdén (1960). Neither formate nor ethanol was produced in more than trace quantities. Y (glucose) values determined for *L. lactis* and *L. bulgaricus* at 40 °C were less than 50% of those determined at 37 °C. Production of lactate and acetate accounted for all of the glucose utilized at both temperatures, indicating that the production of ATP by *L. lactis* or *L. bulgaricus* at 40 °C was not coupled closely to the utilization of ATP in the synthesis of bacterial mass.

INTRODUCTION

Homofermentative lactobacilli are classic examples of organisms that employ glycolysis for energy production and ferment practically all carbohydrate to lactic acid (Elsden & Peel, 1958; Oxenburgh & Snowswell, 1965), though they do produce small amounts of acetic acid and CO₂ (Christensen, Albany & Pederson, 1958). Oxenburgh & Snowswell (1965) grew *Lactobacillus plantarum* ATCC 8014 aerobically on limiting amounts of glucose in a defined medium and determined the molar growth yield (Y) (g dry wt bacteria produced/mol glucose) as 18.8. Assuming Y (ATP) equals approximately 10.5 (Bauchop & Elsdén, 1960), this indicated that the organism had gained only 2 mol ATP/mol hexose fermented and that glycolysis was the only energy-yielding pathway used. More recently, Brown (1968) reported Y (glucose) for the same organism to be 22.5 when grown in complex medium under anaerobic conditions. Brown & VanDemark (1968) found Y (glucose) and Y (mannitol) for *L. casei* grown in complex medium under aerobic conditions to be 21.7 and 24.4, respectively.

These molar growth yield determinations were not accompanied by fermentation balances and apparently did not take into account possible losses resulting from autolysis subsequent to maximal growth (Moustafa & Collins, 1968*a*). We have determined molar growth yields and fermentation balances for homofermentative lactobacilli, giving particular attention to the precautions summarized by Stouthamer (1969) as being necessary for accuracy.

METHODS

Organisms. The species of *Lactobacillus* used were *L. plantarum* ATCC 8014, *L. casei*, *L. bulgaricus*, *L. lactis* ATCC 1235, and *L. delbrueckii* CUC-1. They were routinely subcultured

* Present address: Faculty of Agriculture, University of Khartoum, Khartoum, Sudan.

every 2 weeks as stab cultures in the basal medium with 1.5% Bacto-agar (Difco) and 2% glucose added. Inocula for experiments to determine yields were grown in test tubes containing 10 ml medium. Growth temperatures were 30 °C for *L. plantarum* and *L. casei*; 37 °C for *L. delbrueckii* and 37 °C or 40 °C for *L. lactis* and *L. bulgaricus*. Bacteria in the exponential growth phase were harvested by filtering through a Sartorius filter (pore size, 0.45 µm), washed twice with basal medium (without energy source), and suspended in 5 ml basal medium.

Media. The defined medium of Henderson & Snell (1948), as modified by Oxenburgh & Snowswell (1965), was used except that salts B (MacLeod & Snell, 1947) replaced salts C unless indicated otherwise (salts C contain a higher concentration of minerals than salts B). This is referred to as basal medium. The complex medium was that of de Vries, Kapteijn, van der Beek & Stouthamer (1970) though with Difco instead of Oxoid ingredients. Media were adjusted to pH 6.8 ± 0.1 . Glucose solutions were sterilized by filtering through a micro-porous porcelain filter (no. VFA-54-03; Sela Flotronics, Springhouse, Pennsylvania, U.S.A.) and added aseptically.

Cultivation methods. Cultures were grown in 200 ml medium contained in large test tubes (40 × 290 mm) fitted with sampling assemblies which were partly immersed in a thermostatically controlled water bath. Cultures were agitated using Teflon-coated magnetic stirrers. Samples were obtained with a sterile syringe through a rubber septum in the sampling assembly. The gas drawn out of the test tube to force culture into the sampling assembly was replaced with sterile air. For anaerobic conditions, cultures were sparged continuously with nitrogen (99.99% purity) which had passed first through a sequence of alkaline pyrogallol, distilled water and two tubes of glass wool as described by Moustafa & Collins (1968*a*).

Growth and yield measurements. Growth was followed by measuring extinction, *E*, at 600 nm with a Beckman spectrophotometer, model DB, with 1 cm cuvettes. Bacterial mass was determined from a standard curve relating *E* to dry weight. *E* corresponding to 1 mg dry wt/ml for *Lactobacillus plantarum*, *L. casei*, *L. delbrueckii* and *L. lactis* (37 or 40 °C) was 4.00, 3.54, 3.79 and 3.89, respectively, and for *L. bulgaricus* 3.13 (37 °C) and 6.67 (40 °C). This relationship was linear up to at least an *E* of 0.7.

Analytical methods. Glucose was determined colorimetrically with a Glucostat (Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.) by the procedure of Chayken (1966). Lactic acid was determined by the colorimetric method of Barker & Summerson (1941), ethanol by alcohol Stat Pack (Worthington Biochemical Corporation), total volatile acids by the procedure of Neish (1950), and formate by the method of Wood & Gest (1957). Acetate was determined by subtracting formate from total volatile acids.

Radioisotope analysis. *Lactobacillus plantarum* was grown in basal medium containing 3 or 6 µmol glucose/ml plus, per ml, 0.012 or 0.024 µCi of [U-¹⁴C]glucose (sp. act. 9.6 mCi/mmol; Schwarz-Mann Laboratories, Orangeburg, New York, U.S.A.). At maximum *E*, samples of the bacteria were collected on millipore filters (Millipore Corporation, Bedford, Massachusetts, U.S.A.), washed with 40 to 50 vol. of deionized distilled water, and some were treated with chloroform, ether, methanol, and ethanol (separately and in combinations) to extract lipids. The filters were dried, and radioactivity was determined (efficiency, 75 to 80%) with a Nuclear-Chicago liquid scintillation spectrometer (Model Unilux II; Nuclear-Chicago Corporation, Des Plaines, Illinois, U.S.A.). Samples in each case were in duplicate, and radioactivity determinations were corrected for background.

Fig. 1. Influence of salts B (MacLeod & Snell, 1947) or salts C (Henderson & Snell, 1948) on growth and subsequent autolysis of *Lactobacillus plantarum* ATCC 8014. The defined medium of Henderson & Snell (1948) as modified by Oxenburgh & Snowswell (1965) contained 5 μ mol glucose/ml and salts B or salts C.

RESULTS

Growth and autolysis in defined medium

The basal medium supplemented with glucose or galactose supported good growth of each bacterium when grown in deep culture or shaken gently in an atmosphere of air. Doubling times were 2.5 to 4.0 h. The medium did not support growth of *Lactobacillus plantarum* (the only species tested) if the conditions were strictly anaerobic. (A similar finding was reported for *L. casei* by Erkama, Kauppien & Heino, 1968, and for *L. pasteurianus* by Weinfurtnner, Uhl & Ott, 1960.) However, the complex medium supported good growth of *L. plantarum* under strictly anaerobic conditions.

After achieving maximum growth in basal medium supplemented with growth-limiting amounts of glucose, each species exhibited the lytic phenomenon reported for species of *Streptococcus* by Moustafa & Collins (1968*a, b*). Studies with *Lactobacillus plantarum* revealed that autolysis did not occur if galactose replaced glucose in the medium. Autolysis increased considerably if salts C (Henderson & Snell, 1948) were supplied in place of salts B (Fig. 1).

Molar growth yields

Yields, end-products and the calculated $Y(\text{ATP})$ values for the five species of *Lactobacillus* are given in Table 1. $Y(\text{glucose})$ values varied from 20.0 to 33.3, but $Y(\text{ATP})$ for each of the organisms, when corrected for acetate production, was very close to the value of 10.5 recorded by Bauchop & Elsdon (1960). The constancy of $Y(\text{ATP})$ was not altered by substituting complex medium for the basal medium, anaerobic conditions for the usual

Table 1. Molar growth yields and end-products produced from glucose or galactose by homofermentative lactobacilli

Species	Growth temperature (°C)	Substrate concentration (μmol/ml)	End-products*/mol substrate			Y(sub- strate) (g dry wt/mol)	ATP/mol substrate† (mol)	Y(ATP) (g dry wt/mol)
			Lactate (mol)	Acetate (mol)	Total* (mol)			
Glucose								
<i>Lactobacillus plantarum</i>	30	3 to 15	1·67	0·34	2·01	25·7	2·35	10·9
<i>L. delbrueckii</i>	37	3 to 18	1·66	0·35	2·01	23·6	2·36	10·0
<i>L. lactis</i>	37	2 to 5	1·57	0·47	2·04	25·0	2·51	10·0
<i>L. lactis</i>	37	6 to 15	1·97	trace	1·97	20·0	1·97	10·2
<i>L. bulgaricus</i>	37	4 to 14	1·95	0·10	2·05	22·0	2·15	10·2
<i>L. casei</i>	30	3 to 18	1·85	0·18	2·03	23·4	2·21	10·6
<i>L. casei</i> ‡	30	1 to 5	0·80	1·23	2·03	33·3	3·26	10·2
Galactose								
<i>L. plantarum</i>	30	6 to 16	1·75	0·38	2·13	25·7	2·51	10·2
<i>L. plantarum</i>	30	1 to 5	1·22	0·88	2·10	30·0	2·98	10·1
<i>L. plantarum</i> ‡ §	30	1 to 5	0·97	1·00	1·97	32·5	2·97	10·9

* Not including CO₂.

† Mol lactate per mol substrate + 2 (mol acetate per mol substrate).

‡ Complex medium. The defined basal medium was used for other tests.

§ Strictly anaerobic conditions.

deep-culture conditions, or galactose for glucose. *Y*(glucose) for *Lactobacillus casei* was appreciably greater if complex medium replaced the defined basal medium, but more acetate was produced in the complex medium, and the *Y*(ATP) values corrected for energy produced during the production of acetate were essentially the same. Results for ethanol and formate are not included in Table 1, because each was rarely detected and, then, only in trace amounts.

Incorporation of glucose into bacterial materials

Results with *Lactobacillus plantarum* showed that glucose was used primarily as a source of energy and that the yields were not invalidated by the incorporation of glucose into bacterial components. The organism was grown in the basal medium containing 3 or 6 μmol glucose/ml plus [U-¹⁴C]glucose (0.012 or 0.024 μCi/ml). When growth ceased, the bacteria were washed and the lipids extracted (see Methods). Only 2.9% of the metabolized glucose was incorporated into the bacteria (1.1% in the lipids and 1.8% otherwise assimilated). This is similar to findings for species of *Streptococcus* (Bauchop & Elsdén, 1960; Harvey & Collins, 1963).

Influence of temperature on bacterial yields

Before determining the molar growth yields for *Lactobacillus lactis* and *L. bulgaricus*, we determined *Y*(glucose) values for these organisms at 40 °C at, or near, their optimum growth temperature (*Bergey's Manual of Determinative Bacteriology*, 1957), and found them to be 9.7 (*L. lactis*) and 9.4 (*L. bulgaricus*). *Lactobacillus lactis* at 40 °C produced, per μmol glucose, 1.58 μmol lactate and 0.47 μmol acetate, and the *Y*(ATP) value, calculated from these amounts, was 3.9. *Lactobacillus bulgaricus* produced, per μmol glucose, 1.35 μmol lactate and 0.70 μmol acetate, giving a *Y*(ATP) value of 3.4. As the species produced higher yields

at 37 °C, 40 °C must have been outside the critical temperature range wherein the production of ATP is closely coupled to its utilization in the synthesis of bacterial mass (Senez, 1962; Forrest, 1967).

DISCUSSION

Each of the five species of *Lactobacillus* studied autolysed after attaining maximum growth on limiting amounts of glucose in the defined medium. *Lactobacillum plantarum*, however, did not lyse if galactose replaced glucose, suggesting that this species, similar to certain streptococci (Moustafa & Collins, 1968*b*), has difficulty synthesizing galactosamine from glucose. Autolysis was more severe if salts C (Henderson & Snell, 1948) replaced salts B (MacLeod & Snell, 1947). Thus, the low yield which Oxenburgh & Snowswell (1965) obtained for this strain of *L. plantarum* on limiting glucose medium is explained: they used the same medium as we used but with salts C and, furthermore, determined bacterial yields after the cultures had been incubated for 48 h rather than at maximum extinction.

Our calculations of $Y(\text{ATP})$ values assumed that lactate was produced by the Embden-Meyerhof-Parnas (EMP) pathway with the simultaneous production of 1 net mol ATP/mol lactate and that acetate was produced only with the simultaneous production of 2 net mol ATP/mol acetate, one during the formation of pyruvate by the EMP pathway and one during the conversion of pyruvate to acetate. Buyze, van den Hamer & de Haan (1957) found enzymes of the pentose cycle absent from *Lactobacillus lactis* and *L. delbrueckii*, and likewise Machida, Mizushima & Kilahara (1963) found the pentose cycle inoperative in *L. plantarum* during fermentation of hexose. Since only trace amounts of formate were produced by the lactobacilli, we conclude that acetate was produced from pyruvate either by the oxidation system reported for *L. delbrueckii* (Hager, Geller & Lipmann, 1954) or the system that requires lipoic acid (O'Kane & Gunsalus, 1948; O'Kane, 1950), each of which yields 1 mol ATP/mol acetate.

The $Y(\text{glucose})$ values for *Lactobacillus lactis* and *L. bulgaricus* determined at 40 °C were less than 50% of those determined at 37 °C, though, judging from the amounts of lactate and acetate produced, *L. lactis* produced as much ATP per mol of glucose at 40 °C as it did at 37 °C, and *L. bulgaricus* actually produced more ATP at 40 °C. This is another example of inefficient coupling of anabolic and catabolic processes (Gunsalus & Shuster, 1961; Forrest & Walker, 1965; Cole, Wimpenny & Hughes, 1966). Senez (1962) suggested that maximum efficiency might occur only at temperatures within a restricted range, and Forrest (1967) found yields of *Streptococcus faecalis* and *Zymomonas mobilis* to drop at temperatures outside critical ranges. The ATP produced by *L. lactis* and *L. bulgaricus* at 40 °C and not utilized in the synthesis of bacterial mass must have been hydrolysed by an adenosine triphosphatase system (Gunsalus & Shuster, 1961), since the production of lactate and acetate accounted for all of the glucose utilized at both temperatures.

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