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Growth Response of *Lactobacillus brevis* to Aeration and Organic Catalysts[1]

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Under stationary and anaerobic conditions, greater cell yields of *Lactobacillus brevis* were obtained from autoclaved than from filter-sterilized glucose media. Fructose, tentatively identified as a product generated by the heating process, served as an excellent catalyst for inducing growth. The addition of micromolar quantities of pentoses or potential pentose precursors to the filter-sterilized medium was equally effective in stimulating growth. These organic catalysts were not essential for growth under aerobic conditions. Upon agitation, similar cell yields were obtained from the autoclaved and filter-sterilized media. The micromolar quantities of lactic acid produced per micromole of carbohydrate fermented appeared to be similar under aerobic and static conditions of incubation. The final concentration of acetic acid increased as the result of agitation. This increase in volatile acidity was accompanied by a significant decrease in ethyl alcohol production. The cell yield was increased nearly 50% under aerobic conditions.

Lactobacillus brevis was observed to give poor growth responses when cultured in a glucose medium sterilized by filtration. The resulting poor growth was accompanied by the formation of filamentous strands or films at the surface of the broth. These irregular growth characteristics were replaced by excellent cell yields when the identical medium was sterilized by autoclaving.

Ramsey and Lankford (8) studied the stimulatory effects of heated glucose upon the enhancement of early growth of *L. fermenti*, a heterofermentative *Lactobacillus*. These authors noted that unidentified degradation products of glucose accelerated the growth rate but did not affect the total cell yield. Similar stimulatory responses of heated media upon the growth of the lactic acid bacteria have been reported (10-12).

The purpose of the present study was to determine the nature of the stimulatory factor(s) formed as the result of autoclaving a complex medium and to compare the cell yields and fermentation products of *L. brevis* under varying conditions of aerobiosis.

MATERIALS AND METHODS

Microorganisms. L. brevis B155, *L. plantarum* B246, *Pediococcus cerevisiae* E66, *Leuconostoc mesenteroides* C33, and *Streptococcus faecalis* 8043 were obtained from the culture collection of this department.

Growth medium. The basal medium (TYE) contained (grams per liter of distilled water): Tryptone (Difco), 10.0; yeast extract (Difco), 5.0; $MnSO_4 \cdot H_2O$, 0.030; $K_2HPO_4 \cdot 3H_2O$, 1.3; and KH_2PO_4, 1.0. For growth of bacteria, 5.0-ml amounts of the TYE broth were dispensed into 16 by 150 mm tubes and were diluted with equal volumes of the carbon source to be tested. The *p*H of the final medium was 6.8. TYE medium containing 1% glucose (TGYE) was autoclaved in 10-ml samples for 10 min. The nonheated media containing the various carbon additives were sterilized by filtration through a 0.45-μ membrane filter (Millipore Corp., Bedford, Mass.) in an all-glass assembly. Anaerobiosis was maintained in a Brewer jar previously evacuated and replaced with illuminating gas three times. Cultures were agitated on a reciprocating shaker operating at 120 strokes [3 inches (7.6 cm)] per min. Stationary conditions refer to undisturbed incubation.

The inoculations into the test media were made with one loop of a 24-hr culture grown in the autoclaved TGYE medium. The cultures were incubated for 96 hr at 32 C.

Analytical methods. The amount of growth is expressed in terms of optical density at a wavelength of 660 mμ in a Bausch & Lomb Spectronic-20 colorimeter. These values were related to standard dry weight curves prepared from cells grown under conditions similar to those used in the tests. The reported results represent the average values of four determinations for each substrate level.

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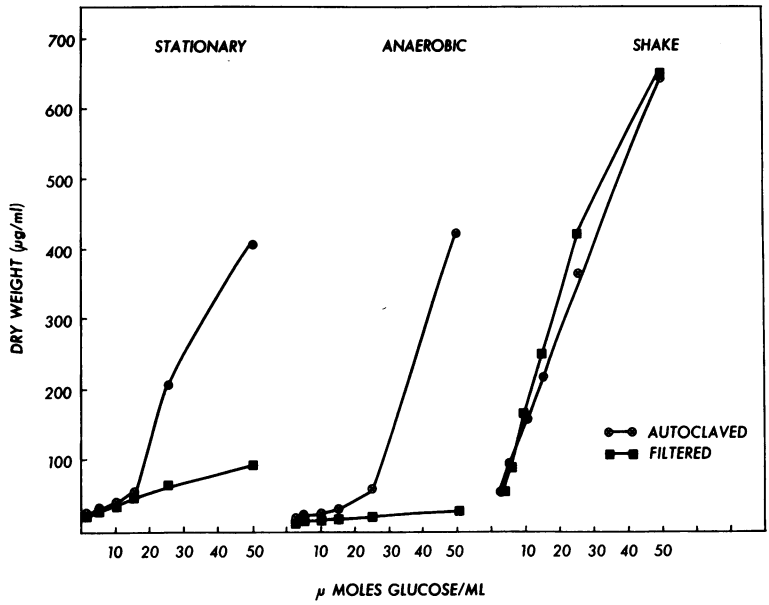

FIG. 1. *Effects of autoclaved and filtered glucose media on the growth of Lactobacillus brevis under various conditions of aerobiosis. Cultures incubated at 32 C for 96 hr.*

Residual glucose was determined by the anthrone reagent according to Neish (7). Glucose and fructose were identified by paper chromatography in solvent systems composed of *n*-butanol-acetic acid-water (5:4:1), *n*-butanol-pyridine-water (6:4:3), and *n*-butanol-ethyl alcohol-water (2:1:1). The spots were developed by ammoniacal silver nitrate (14) and *p*-anisidine (6). Lactic acid concentration was determined colorimetrically (1). Ethyl alcohol was determined by the microdiffusion method of Conway (2). Acetic acid was recovered from 5.0-ml samples of the fermented broths by means of a Comes-Milano microsteam distillation apparatus. The distillate was titrated to *p*H 8.3 with 0.02 N sodium hydroxide. The recovery of acetic acid standards by the above method was ±2%. The acids were identified by silicic acid chromatography (7).

RESULTS

L. brevis (B155) was found to grow poorly under stationary and anaerobic conditions in a medium sterilized by filtration (Fig. 1). Under static conditions, the maximal cell yield was 93 μg (dry weight) per ml. Under anaerobic conditions, only 55 μg (dry weight) per ml was obtained. This 40% decrease in cell yield was attributed to the low oxygen content of the medium. The total growth under each condition of incubation was affected little by increasing the substrate concentration.

The use of autoclaved medium produced more than 400 μg (dry weight) per ml or more than four times the growth provided by the filter-sterilized broths. An increase in the final cell yield was also dependent upon the concentration of glucose present at the time of autoclaving. This response to heated glucose was most apparent when the latter substrate concentration exceeded 15 μmoles/ml; the final cell yield, therefore, was dependent upon the glucose concentration and the formation of products generated during the heating process.

An increase in growth was observed when glucose was heated with phosphate buffer, yeast extract, or Tryptone. The heating of glucose independently and its subsequent addition to the other sterile constituents produced cell yields similar to those for the filtered medium. This suggested that the products which enhanced the final growth yield were formed as a result of the interaction of glucose and other constituents of the medium.

Under aerobic conditions, the filter-sterilized glucose medium supported growth equivalent to that obtained in the autoclaved medium (Fig. 1). The maximal cell yield of 655 μg (dry weight) per ml represents a 50% greater yield than that obtained under stationary conditions.

The effects of autoclaved media and agitation upon the growth of other members of the family *Lactobacilliaceae* were also studied (Table 1). *L. brevis* was the only member of the test group that showed a marked growth increment (four- to six-fold) when grown under the above conditions.

Paper chromatographic analysis of the carbohydrate composition of the filtered and autoclaved

TABLE 1. *Effects of heat-sterilized medium and agitation on the growth of various lactic acid bacteria*[a]

Bacteria	Optical density × 1,000				Acidity[b]			
	Autoclaved		Filtered		Autoclaved		Filtered	
	Static	Shake	Static	Shake	Static	Shake	Static	Shake
Lactobacillus brevis B155	1,900	2,980	442	2,510	0.26	0.35	0.04	0.28
L. plantarum B246	4,500	4,220	4,800	4,240	0.66	0.60	0.61	0.65
Leuconostoc mesenteroides C33	1,450	1,340	1,530	1,630	0.21	0.25	0.21	0.28
Pediococcus cerevisiae E66	3,380	2,690	3,220	2,750	0.30	0.25	0.30	0.28
Streptococcus faecalis 8043	1,220	1,290	1,260	1,500	0.18	0.17	0.20	0.21

[a] Incubation for 96 hr at 32 C, on Tryptone-glucose-yeast extract broth.
[b] Expressed as milliequivalents per 5.0 ml of sample.

media suggested that a second carbohydrate had been formed as the result of the heat treatment. In the basic, acidic, and neutral solvent systems, the unknown compound had R_F values identical to that of fructose. The newly generated compound gave a positive response with ammoniacal silver nitrate and produced a bright yellow color with *p*-anisidine. Similar color reactions were obtained with fructose, thus suggesting that the latter carbohydrate was produced as a result of the autoclaving step.

The filtered glucose medium which had previously failed to support good growth served as an excellent substrate when supplemented with equimolar quantities of fructose (Fig. 2). The dry weight yields, approximately 10 μg/μmole of carbohydrate, were similar in both fructose and fructose-supplemented media.

In addition to fructose, other compounds were also tested for growth-promoting activities. Those carbon sources which were equally effective in enhancing growth were the sodium salts of gluconate, 2-ketogluconate, glucuronate, galacturonate, xylose, and arabinose.

The following compounds did not increase the final growth yield: L-sorbose, sucrose, dihydroxyacetone, polyoxyethylene sorbitan mono-oleate (Tween 80), acetaldehyde, and the sodium salts of ascorbate, glucose-6-phosphate, ketoglutarate, fumarate, glycerate, pyruvate, lactate, and acetate.

The stimulatory effects of catalytic quantities of fructose or pentoses upon the growth of *L. brevis* are shown in Fig. 3. Under static conditions, 93 μg (dry weight) was produced from 50 μmoles of available glucose. The addition of 10 μmoles of the above carbon sources per ml to the filtered glucose media increased the final cell yield nearly fivefold. A linear growth response was observed when these carbohydrates were provided at concentrations of 1 to 5 μmoles/ml.

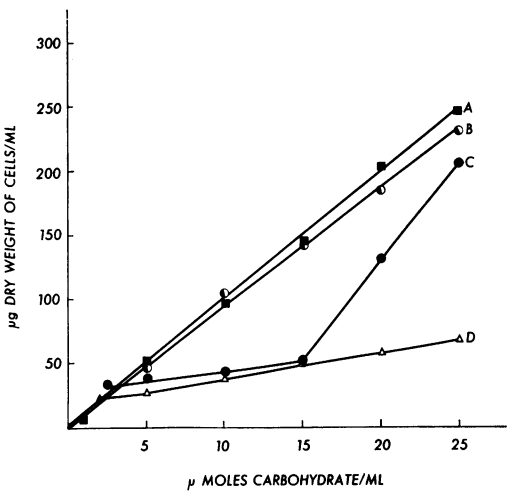

FIG. 2. *Growth response of Lactobacillus brevis to: (A) filter-sterilized fructose; (B) filter-sterilized glucose and fructose in equimolar concentrations; (C) autoclaved glucose in complex medium; and (D) filter-sterilized glucose. Cultures incubated at 32 C for 96 hr under static conditions.*

The effects of agitation and substrate concentration upon the yield of fermentation products are shown in Fig. 4. Under aerobic conditions, glucose, with the exception of the highest level, was completely utilized by *L. brevis*. Acetic acid was the major end product formed when the glucose concentration was less than 15 μmoles/ml, whereas lactic was the predominant acid formed at the higher substrate concentrations. The molar ratios of acetic to lactic acid, therefore, varied throughout the fermentation. These ratios ranged from 5:1 at the lowest glucose level to 0.45:1 at the highest concentration.

Under aerobic conditions, the molar ratios of ethyl alcohol produced from each mole of substrate fermented were also affected by the glucose

concentration (Fig. 4). Ethyl alcohol could not be detected when the glucose concentration was less than 5.0 μmoles/ml. This latter level produced 0.6 μmole of ethyl alcohol or the equivalent of 0.12 μmole of ethyl alcohol per μmole of fermentable carbohydrate. The fermentation of 48.1 μmoles of glucose produced 16.9 μmoles of ethyl alcohol or 0.35 μmole of ethyl alcohol per μmole of glucose used.

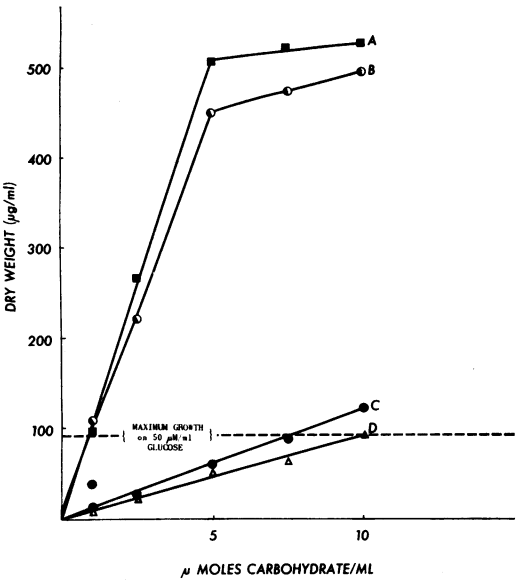

FIG. 3. *Growth response of Lactobacillus brevis to catalytic amounts of carbohydrates added to 50 μmoles of filtered glucose medium per ml. Static culture incubated at 32 C for 96 hr. (A) Xylose or arabinose; (B) fructose; (C) xylose or arabinose only; and (D) fructose only.*

Under stationary conditions of growth, glucose in autoclaved medium was only partially utilized. Inconsistent utilization of the substrate occurred when the glucose level was less than 15 μmoles/ml (Fig. 4). At this latter concentration, 5.1 μmoles or only 34% of the available glucose was fermented. When the glucose concentration exceeded 15 μmoles/ml, nearly 80% of the available glucose was utilized.

Under stationary conditions, acetic acid was the minor end product. Only 7.9 μmoles of acetic acid was recovered from 41.1 μmoles of fermented glucose. Under these conditions, the volatile acid fraction was only 60% of the amount observed under aerobic cultivation. The ratio of acetic to lactic acid approached unity when the substrate concentration was less than 5 μmoles/ml. Increased glucose concentrations further reduced this ratio to 1:5. This decrease in total volatile acidity was accompanied by a significant increase in ethyl alcohol formation; therefore, the more anaerobic conditions of incubation increased the ethyl alcohol values nearly 50%.

The analytical results of the filtered medium fermented under stationary and anaerobic conditions are not included because of the poor growth obtained under these conditions.

DISCUSSION

Carbohydrates, when heated in complex media, can undergo innumerable changes. Nonenzymatic browning reactions have been extensively studied

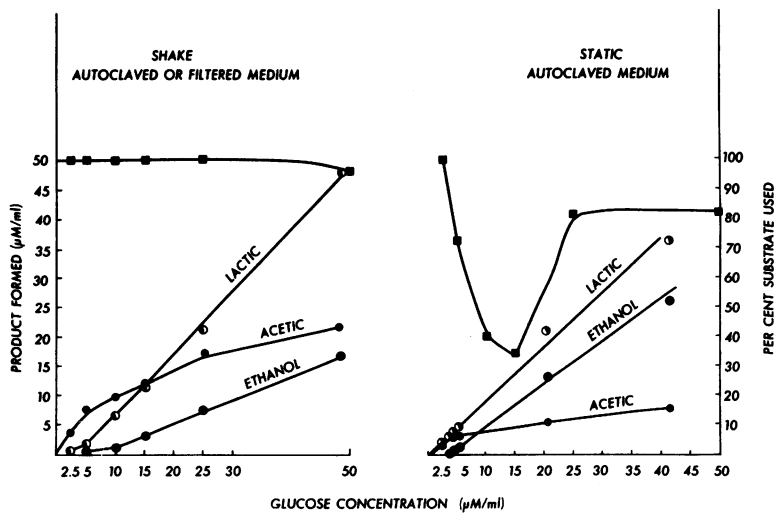

FIG. 4. *Effect of agitation upon the product yield of Lactobacillus brevis. Symbol: ■, percentage of substrate used. Cultures incubated at 32 C for 96 hr.*

by such investigators as Davis (3) and Richards (9). The formation of fructose or similar analogues in a heated glucose medium is therefore an understandable result. When *L. brevis* is incubated under anaerobic and stationary conditions in an autoclaved medium, the maximal growth is not derived from glucose exlcusively, but is due in part to the newly generated fructose. The meager cell yield and low acid production in a filter-sterilized medium may lead to an erroneous interpretation of the glucose fermentative capacities of *L. brevis*.

The initiation of growth and the final cell yields may be related to the availability of organic hydrogen acceptors. L-Sorbose, a sugar similar to fructose, will not serve as a hydrogen acceptor and is not fermented by *L. brevis*. However, the reduction of fructose to mannitol by a dehydrogenase isolated from *L. brevis* has been described by Horeker et al. (5). Hexuronic and hexonic acids, potential pentose precursors, may possibly serve as hydrogen acceptors. Starr and co-workers (13) observed that cell extracts of *Erwinia* and *Aerobacter* reduced D-galacturonic and D-glucuronic acids to the corresponding L-hexonic acids. The reduction of sodium gluconate and 2-ketogluconate by cell extracts of *L. brevis* has been reported by Eltz and VanDemark (4), who also reported that aldopentoses undergo isomerization to aldoketoses. The latter ketoses or the phosphorylated products of cleavage may also serve as hydrogen acceptors in the growth system. It is of interest that the addition of nonphosphorylated trioses, namely, dihydroxyacetone, glycerate, and pyruvate, to the medium did not enhance growth.

The requirement for catalytic amounts of external carbon sources suggests that, once active growth has been initiated, hydrogen acceptors are generated as the result of the catabolic processes. In the event that organic hydrogen acceptors are unavailable to the microorganism, oxygen may serve as the terminal acceptor and initiate growth. The unusual growth characteristics such as the formation of surface films and strands may be attributed to the insufficient oxygen content of freshly prepared media. The effects of microaerophilic conditions upon the growth of lactic acid bacteria have also been reported by Whittenbury (15) and G. Buyze (Ph.D. Thesis, Univ. of Utrecht, Utrecht, Netherlands, 1955).

The micromolar quantities of lactic acid produced per micromole of carbohydrate fermented appear to be similar under aerobic and stationary conditions of incubation. Greater yields of acetic acid are produced under aerobic conditions. The increase in volatile acidity apparently arises at the expense of alcohol production; therefore, the variations in the acid ratios are due to the formation of acetic acid.

The reason for the increased cell yield of *L. brevis* under aerobic conditions is not understood. This observation may be analogous to the presently unexplained high growth yield of *Leuconostoc* as described by Wood (16). In the latter case, the observed growth yields were much greater than the anticipated values, thus suggesting that additional energy-yielding mechanisms were operative.

This study also demonstrates the importance of selecting the proper hexose for obtaining maximal growth and biochemical differentiation of heterofermentative lactic acid bacteria. Filtered glucose, when supplied as the sole carbon source, is an inadequate substrate under stationary and anaerobic conditions. Under similar conditions, fructose serves as an excellent hexose for growth. Thus, the incorporation of fructose into the medium may serve as an aid in propagating and isolating these microorganisms from natural products.

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. CONWAY, E. J. 1950. Microdiffusion analysis and volumetric error. Crosby, Lockwood and Son, London.
3. DAVIS, J. G., AND J. J. ROGERS. 1939. The effect of sterilization upon sugars. Zentr. Bakteriol. Parasitenk. Abt. II **101**:102–110.
4. ELTZ, R. W., AND P. J. VANDEMARK. 1960. Fructose dissimilation by *Lactobacillus brevis*. J. Bacteriol. **79**:763–776.
5. HORECKER, B. L., G. MARTINEZ, AND H. A. BARKER. 1963. A specific mannitol dehydrogenase from *Lactobacillus brevis*. J. Biol. Chem. **238**:1598–1603.
6. HOUGH, L., J. K. N. JONES, AND W. H. WADMAN. 1950. Quantitative analysis of mixtures of sugars by the method of partition chromatography. J. Chem. Soc., p. 1702.
7. NEISH, A. C. 1952. Analytical methods for bacterial fermentations. Natl. Res. Council Can. Bull. 46-8-3.
8. RAMSEY, H. H., AND C. E. LANKFORD. 1956. Stimulation of growth initiation by heat degradation products of glucose. J. Bacteriol. **72**:511–518.
9. RICHARDS, E. L. 1956. Non-enzymic browning: The reaction between *D*-glucose and glycine in the "dry" state. Biochem. J. **64**:639–644.
10. ROGERS, D., T. E. KING, AND V. H. CHELDELIN. 1953. Growth stimulation of *Lactobacillus gayonii* by N-D-glucosylglycine. Proc. Soc. Exptl. Biol. Med. **82**:140–144.
11. SMILEY, K. L., C. F. NIVEN, JR., AND J. M. SHERMAN. 1943. The nutrition of Streptococcus salivarius. J. Bacteriol. **45**:445–454.

12. SNELL, N., AND J. C. LEWIS. 1953. Nutritional studies with Lactobacillus fermenti. J. Bacteriol. **65**:671–677.
13. STARR, M. P., J. DE LEY, AND W. W. KILGORE. 1957. Catabolism of hexuronic acids by *Erwinia* and *Aerobacter*. Science **125**:929.
14. TREVELYAN, W. E., D. P. PROCTOR, AND J. S. HARRISON. 1950. Detection of sugars on paper chromatograms. Nature **166**:444–445.
15. WHITTENBURY, R. 1963. The use of soft agar in the study of conditions affecting the utilization of fermentable substrates by lactic acid bacteria. J. Gen. Microbiol. **32**:375–384.
16. WOOD, W. A. 1962. Fermentation of carbohydrates and related compounds, p. 99. *In* I. C. Gunsalus and R. Y. Stanier [ed.], The bacteria, vol. 2. Academic Press, Inc., New York.