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Growth and Energetics of *Leuconostoc mesenteroides* NRRL B-1299 during Metabolism of Various Sugars and Their Consequences for Dextransucrase Production

MARGUERITE DOLS, WIDAD CHRAIBI, MAGALI REMAUD-SIMEON,* NICHOLAS D. LINDLEY, AND PIERRE F. MONSAN

Centre de Bioingénierie Gilbert Durand, Unité Mixte de Recherche du Centre National de la Recherche Scientifique 5504, Laboratoire Associé de l'Institut National de la Recherche Agronomique, Département de Génie Biochimique et Alimentaire, Institut National des Sciences Appliquées, Complexe Scientifique de Rangueil, 31 077 Toulouse Cedex, France

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The metabolic and energetic properties of *Leuconostoc mesenteroides* have been examined with the goal of better understanding the parameters which affect dextransucrase activity and hence allowing the development of strategies for improved dextransucrase production. Glucose and fructose support equivalent specific growth rates (0.6 h^{-1}) under aerobic conditions, but glucose leads to a better biomass yield in anaerobiosis. Both sugars are phosphorylated by specific hexokinases and catabolized through the heterofermentative phosphoketolase pathway. During sucrose-grown cultures, a large fraction of sucrose is converted outside the cell by dextransucrase into dextran and fructose and does not support growth. The other fraction enters the cell, where it is phosphorylated by an inducible sucrose phosphorylase and converted to glucose-6-phosphate (G-6-P) by a constitutive phosphoglucomutase and to heterofermentative products (lactate, acetate, and ethanol). Sucrose supports a higher growth rate (0.98 h^{-1}) than the monosaccharides. When fructose is not consumed simultaneously with G-1-P, the biomass yield relative to ATP is high (16.8 mol of ATP · mol of $sucrose^{-1}$), and dextransucrase production is directly proportional to growth. However, when the fructose moiety is used, a sink of energy is observed, and dextransucrase production is no longer correlated with growth. As a consequence, fructose catabolism must be avoided to improve the amount of dextransucrase synthesized.

Leuconostoc mesenteroides has been known to produce dextran from cane sugar since 1878 (35). In the 1940s, an extracellular enzyme able to catalyze this reaction was isolated and given the name dextransucrase (10, 11). This glucosyltransferase (EC 2.4.1.5) catalyzes the transfer of glucosyl residues from sucrose to dextran polymer, liberating fructose (31), and also synthesizes glucooligosaccharides in the presence of sucrose and an acceptor such as maltose (27). The dextransucrase of *L. mesenteroides* NRRL B-1299 is of interest because it is the only enzyme that produces dextran or oligosaccharides containing α(1→2) branch linkages (12, 15, 26, 27). The α(1→2)-terminated oligosaccharides are enzymatically produced at the industrial scale and are used in the cosmetics industry (22). They have also been shown to stimulate the growth of beneficial bacteria of intestinal microflora (6), and to extend their range of applications, it is important to reduce their production cost and hence to improve dextransucrase production by *L. mesenteroides* NRRL B-1299.

Numerous groups have worked to improve production of both dextran and dextransucrase (1, 2, 16, 23, 34) by *L. mesenteroides* NRRL B-512F, although few studies have been devoted to examining sucrose metabolism in order to obtain the basic information necessary to pragmatically improve fermentation strategies. Although genes coding for dextransucrase have been cloned (36), no information is available concerning the mechanism of *L. mesenteroides* dextransucrase induction by sucrose. Sucrose is the only known inducer, and enzyme production is restricted to the growth phase (2, 16, 17, 20). According to Neely and Nott (20), the glucosyl moiety of sucrose is first consumed by the bacteria, and only when sucrose is totally depleted is the accumulated fructose consumed. Various other studies mention this transitory accumulation of fructose (17, 34). Sugar transport and phosphorylation are key factors in understanding the growth behavior, but precise information is lacking in the literature, although it is generally believed that the heterofermentative lactic acid bacteria do not possess active phosphotransferase systems (28). Sucrose hydrolysis could imply either an invertase or a sucrose phosphorylase. The presence of the last enzyme seems to be dependent on the strain of *L. mesenteroides* used (9, 14).

No enzyme production occurs on a glucose-fructose medium (16, 20) or during growth on the following sugars: glucose, maltose, trehalose, melibiose, mannose, α-D-galactose, α-Me-glucoside, melezitose, xylose, raffinose, turanose, β-D-fructose, cellobiose, lactose, or β-Me-xyloside (20). Sugar metabolism is heterofermentative (8); hexose (glucose or fructose) catabolism produces equimolar quantities of lactate and acetate plus ethanol via the phosphoketolase pathway (Fig. 1). In the presence of oxygen or auxiliary substrates (e.g., fructose, citrate, and pyruvate), increased amounts of acetate are produced at the expense of ethanol. Production of acetate rather than ethanol allows the synthesis of additional ATP via acetate kinase and leads to higher specific growth rates (μs) (5, 13, 21, 24, 25, 29). Garvie (8) mentioned that many *Leuconostoc* species prefer fructose to glucose for growth. Tracey and van Rooyen (33) and Salou et al. (29) did not confirm these observations but reported a partial conversion of fructose to mannitol with *Leuconostoc oenos.*

In this paper, the metabolic behavior of *L. mesenteroides* NRRL B-1299 has been examined with various sugars (glucose, fructose, and sucrose), and particular attention has been

* Corresponding author. Phone: (33) 5 61 55 94 26. Fax: (33) 5 61 55 93 99. E-mail: remaud@insatlse.insa-tlse.fr.

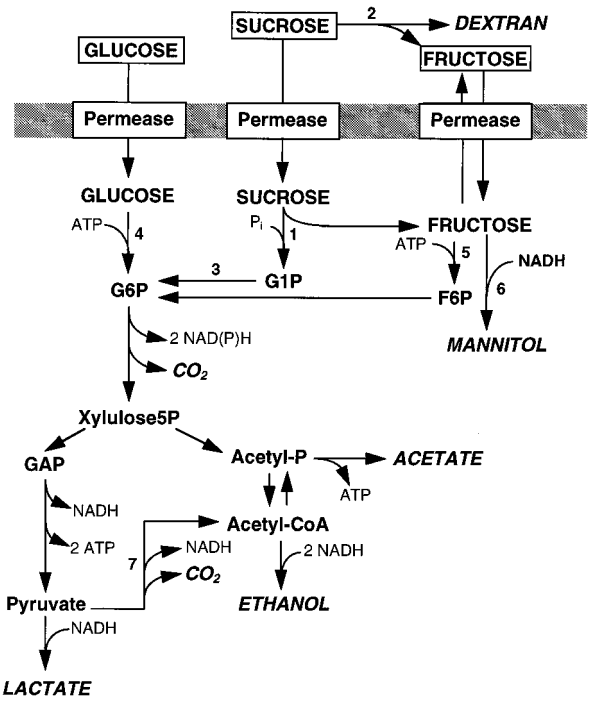

FIG. 1. Schematic representation of carbon and energy flow through the central metabolic pathways of *L. mesenteroides* during metabolism of various sugars. G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; GAP, glyceraldehyde-3-phosphate; acetyl-P, acetylphosphate; acetyl-CoA, acetyl coenzyme A; 1, sucrose phosphorylase; 2, dextransucrase; 3, phosphoglucomutase (PGM); 4, glucokinase; 5, fructokinase; 6, mannitol dehydrogenase; 7, pyruvate dehydrogenase.

paid to the energetics of cell growth and dextransucrase production in order to facilitate the development of new fermentation strategies to improve enzyme synthesis.

MATERIALS AND METHODS

Microorganism and growth conditions. *L. mesenteroides* NRRL B-1299 was used throughout this study and was stored in 18% (wt/vol) glycerol at −80°C.

The standard medium used was composed of the following: sucrose, 110 mM; yeast extract (Biomérieux), 20 g · liter^{-1}; K_2HPO_4, 20 g · liter^{-1}; $MgSO_4 \cdot 7H_2O$, 0.2 g · liter^{-1}; $MnSO_4 \cdot H_2O$, 0.01 g · liter^{-1}; NaCl, 0.01 g · liter^{-1}; $CaCl_2$, 0.02 g · liter^{-1}; and $FeSO_4 \cdot 7H_2O$, 0.01 g · liter^{-1}. The phosphate buffer pH was adjusted to 6.9 with P_i. Sucrose, yeast extract, phosphate, and salts were sterilized separately. Similar media were used for cultures with glucose or fructose, except that sucrose was replaced by 110 mM glucose or fructose or a mixture of the two sugars as specified in the text.

Shake flask cultures were incubated at 27°C on a rotary shaker (200 rpm). Batch cultures were realized in a 2-liter reactor (Sétric Génie Industriel, Toulouse, France) with a 1.5-liter working volume at a temperature of 27°C. During microaerated experiments, aeration was 0.7 liter · min^{-1} and agitation was 200 rpm. During nonaerated cultures, N_2 was added continuously to avoid CO_2 accumulation, while for well-aerated cultures, partial O_2 pressure (pO_2) was maintained at 40% of saturation value by manually increasing the air flow rate and shaking. In either case, pH was maintained at 6.7 with NaOH (7 N).

Growth rate measurements under nonaerated conditions on glucose or fructose medium were carried out with Hungate tubes as described by Cocaign-Bousquet et al. (4).

All of the cultures were inoculated (2%, vol/vol) with a 6-h culture, unless stated otherwise in the text. Fructose- and glucose-grown cells were obtained after at least three successive microaerated cultures with fructose or glucose medium.

Biomass measurements. Bacterial growth was measured by a turbidimetric method at 650 nm and calibrated against cell dry weight measurements as described by Salou et al. (29). A change of 1 U of optical density at 650 nm was equivalent to 0.4 g of dry matter · liter^{-1}. This method was not convenient for biomass measurement during cultures on sucrose medium because of the presence of dextran, which invalidated both optical density and gravimetry.

Cell numeration. Cell numeration was carried out with a graduated Thoma's cell and phase-contrast microscopy; the data were converted to cell dry weight (grams per liter) by means of a calibration graph elaborated during culture on glucose medium. A change of 109 cells · ml^{-1} was equivalent to 0.97 g of dry matter · liter^{-1}. Moreover, in the presence of sucrose, the apparent cell diameter increases because of capsule formation, but the real cell diameter decreases (3) in such a proportion that the cell volume is 40% lower than that of glucose-grown cells. As a result, the biomass concentration (grams per liter) given by the counting and the calibration graph obtained with glucose-grown cells must be multiplied by 0.6.

Dextransucrase assay. Soluble and insoluble dextransucrases were separated by centrifugation (15,000 × *g*, 5 min). Soluble activity was measured in the supernatant, and insoluble activity was measured after resuspension of the cells in a 20 mM acetate buffer (pH 5.4). One unit of dextransucrase is defined as the amount of enzyme that catalyzes the formation of 1 μmol of fructose per min at 30°C in 20 mM sodium acetate buffer (pH 5.4) with 100 g of sucrose · liter, 0.05 g of $CaCl_2$ · liter^{-1}, and 1 g of NaN_3 · liter^{-1} (to avoid cell metabolism during the assay with insoluble dextransucrase). Reducing sugar production was measured as described by Remaud-Simeon et al. (27). It was confirmed that fructose was the only reducing sugar present under the experimental conditions used. The stability of the enzyme dextransucrase under the culture conditions (pH, temperature, medium) was checked. No denaturation occurred over a 12-h period, so the measured dextransucrase activity can be regarded as being proportional to the concentration of enzyme produced throughout the culture.

High-performance liquid chromatography analysis. Sucrose, glucose, lactic acid, acetic acid, and ethanol concentrations were measured by ion-exchange chromatography with an Aminex HPX87H column (Bio-Rad Chemical Division, Richmond, Calif.) at 30°C with H_2SO_4 (8.5 mM) as the eluent and at a constant flow rate of 0.5 ml · min^{-1}.

Fructose, glucose, and mannitol concentrations were measured with an Aminex HPX87K Bio-Rad column at 65°C, with K_2HPO_4 (10 mM) as the eluent and at a constant flow rate of 0.5 ml · min^{-1}. The analyses were carried out with a Hewlett-Packard 1050 series system consisting of a pump, an injector, and an HP 1047A refractometer.

The soluble dextran concentration was measured by gel permeation chromatography (Si100 Merck column) with a flow rate of 0.5 ml of ultrapure water · min^{-1}.

Insoluble dextran analysis. A total of 4.5 ml of culture medium was centrifuged (15,000 × *g*, 5 min, 4°C) and washed twice with ultrapure water. The pellet was dried, and the insoluble dextran concentration was calculated by subtraction of the cells' dry weight from the total dry weight measured. The results were reliable for total dry weights higher than 5 g · liter^{-1}. Dextran yield was calculated as dextran produced/potential dextran = dextran produced (g)/[162 × total sucrose consumed (mol)] × 100.

Enzyme activity. Enzyme activity was measured with crude cell extracts obtained by sonication. Crude extracts were prepared as follows. Cells were harvested (8,000 × *g*, 20 min, 4°C), washed twice with K_2HPO_4 (25 mM [pH 6.7]), and resuspended in Tris-tricarballylate buffer (250 mM [pH 7.4]) containing glycerol (20%) and $MgCl_2$ (5 mM). Cells were disrupted by sonication, and cell debris was removed by centrifugation at 10,000 × *g* for 10 min at 4°C. The supernatant was used for enzyme assays, and the protein concentration of the extract was determined by the method of Lowry et al. (19) with bovine serum albumin as a standard. The debris was resuspended in Tris-tricarballylate buffer and used for the assay of sucrose phosphorylase (about 50% of the activity remained linked to the membrane). Since no protein assay was possible with the debris, the activity measured was divided by the protein content of the corresponding extract to give a specific activity.

The sucrose phosphorylase activity was evaluated spectrophotometrically by measuring the appearance of NADPH at 340 nm ($\varepsilon = 6.22\ 103\ cm^{-1}\ M^{-1}$) under the following conditions: K_2HPO_4, 70 mM (pH 6.8); sucrose, 100 mM; NADP, 0.5 mM; $MgCl_2$, 5 mM; glucose-1,6-diphosphate 0.1 mM; phosphoglucomutase, 30 U · ml^{-1}; and glucose-6-P dehydrogenase, 20 U · ml^{-1}. Phosphoglucomutase activity was measured by the same method in the presence of K_2HPO_4 (70 mM [pH 6.8]), NADP (0.5 mM), $MgCl_2$ (5 mM), glucose-1-phosphate (10 mM), glucose-1,6-diphosphate (0.1 mM), and glucose-6-P dehydrogenase (20 U · ml^{-1}).

Hexokinase was measured with the following reaction mixture: Tris-HCl buffer (100 mM [pH 7.4]), $MgCl_2$ (10 mM), NADP (0.5 mM), ATP (1 mM), and glucose-6-P dehydrogenase (2 U · ml^{-1}), with glucose (10 mM) as the substrate. The fructose-phosphorylating hexokinase was measured with the same medium, except that glucose was replaced by fructose and phosphoglucose isomerase (2 U · ml^{-1}) was added. Phosphoglucose isomerase activity was also measured with the same medium, but with glucose replaced by fructose-6-phosphate (10 mM). One unit of activity was the amount of enzyme required to convert 1 nmol of substrate per min.

NADH oxidase activity was measured with the reaction mixture: Tris-HCl buffer (100 mM [pH 7.4]) and $MgCl_2$ (10 mM), with NADH (1 mM) as the substrate.

The activity of glucose and sucrose phosphotransferase of permeabilized cells was measured by the method of Dominguez and Lindley (7).

For fermentation kinetic analysis, the μs were calculated from the cell mass profiles. As generally assumed for lactic acid bacteria, carbon and nitrogen

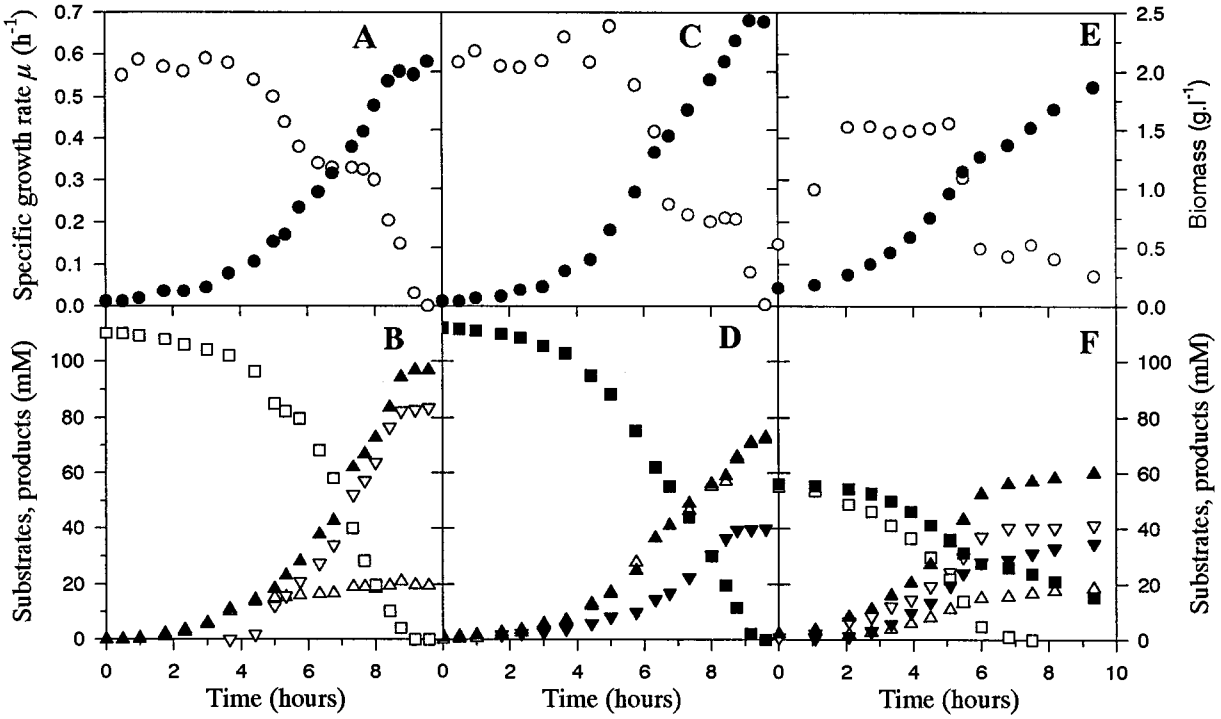

FIG. 2. Batch fermentation profile for the growth of *L. mesenteroides* under microaerated conditions (oxygen limitation after 4 h, $pO_2 = 0\%$). (A and B) Glucose (110 mM). (C and D) Fructose (110 mM). (E and F) Equimolar quantities (55 mM) of glucose and fructose. ●, biomass; ○, μ; □, glucose; ■, fructose; ▲, lactate; △, acetate; ▽, ethanol; ▼, mannitol.

constituents of cell biomass and growth factors were considered to be supplied by the yeast extract component of the broth.

Kinetic and energetic analyses. The amount and rates of ATP synthesized by substrate-level phosphorylation reactions were calculated from the stoichiometric balance equations and the flux estimations based on rates of substrate consumption and product accumulation, respectively, according to the metabolic pathways shown in Fig. 1.

RESULTS AND DISCUSSION

Growth of *L. mesenteroides* on various sugars. (i) Glucose. During growth of *L. mesenteroides* NRRL B-1299 on glucose with microaeration, a μ of 0.58 h^{-1} was reached in the early stages of growth (Fig. 2A), irrespective of the initial glucose concentration, and a classical heterolactic fermentation pattern of equimolar production of lactate and acetate was observed with the following stoichiometry: 1 glucose + 1 O_2 → 0.92 lactate + 0.95 acetate + 1 CO_2.

The CO_2 value was estimated assuming heterolactic fermentation of glucose. Coenzyme regeneration, which would normally be expected to generate ethanol as an end product rather than acetate, was attained because of the direct oxidation of NAD(P)H by an oxidase activity measured in cell extracts (results not shown). After approximately 4 h of culture, μ fell to 0.33 h^{-1}, coincident with a shift in product profile to include significant ethanol production, no doubt due to oxygen limitation (dissolved oxygen saturation fell to zero [Fig. 2B]). Under such conditions, the following stoichiometry was observed: 1 glucose → 0.93 lactate + 0.96 ethanol + 1 CO_2.

When adequate aeration was maintained throughout the culture, no significant production of ethanol occurred and higher biomass concentrations were reached. Under all conditions, the specific rate of substrate consumption and the biomass yield relative to ATP (Y_{ATP}) remained constant at approximately 21 mmol of glucose · g of biomass^{-1} · h^{-1} and 16.5 g of biomass · mol of ATP^{-1} until glucose depletion occurred (Table 1).

Carbon balance estimations based upon stoichiometric coefficients indicated that approximately 5% of the consumed glucose could not be accounted for, although such equations did not take into account the possible contribution of glucose to biomass. This has previously been shown to account for less than 5% of biomass in other *Leuconostoc* species (30). A similar shortfall in carbon balance has also been reported for *L. oenos* during glucose metabolism (18).

(ii) Fructose. The μs observed for *L. mesenteroides* on fructose were similar to those obtained on glucose under aerated conditions (μ = 0.60 h^{-1}), although no stimulation of growth rate as observed for *L. oenos* (29) was seen, and μs were significantly lower when growth medium became oxygen depleted (μ = 0.19 h^{-1} [Fig. 2C]). Fructose metabolism involved two distinct pathways: a fraction of fructose was reduced to mannitol associated with the oxidation of NADH, while the remainder was metabolized via the same phosphoketolase pathway that operates during glucose fermentation. The fraction of fructose converted to mannitol varied relative to the aeration strategy, being highest (approximately 65% of fructose consumption) under anaerobic conditions, although some mannitol production was observed even when the cultures were aerated. The fructose metabolized via the phosphoketolase pathway was always converted to equimolar quantities of lactate, acetate, and CO_2 (Fig. 2D); no ethanol production occurred even under anaerobic conditions. Thus, during aerated cultures, the experimental stoichiometry was 1 fructose + 0.58 O_2 → 0.28 mannitol + 0.71 lactate + 0.72 acetate + 0.72 CO_2, while under oxygen-limited growth, higher mannitol production was seen: 1 fructose → 0.53 mannitol + 0.56 lactate + 0.57 acetate + 0.57 CO_2.

TABLE 1. Maximal specific rates and energetic biomass yield obtained during batch cultivation of *L. mesenteroides* on various sugars under different aeration conditions

Sugar used	Aeration	Maximal uptake of glucose or global sucrose uptake (mmol · h^{-1} · g of biomass^{-1})[a]	Specific rate of fructose consumption (mmol of fructose · h^{-1} · g of biomass^{-1})[b]	Mannitol production (mmol · h^{-1} · g of biomass^{-1})	Y_{ATP} (g of biomass · mol of ATP^{-1})[c]	μ_{max} (h^{-1})
Glucose	Aerated	20			16.8	0.58
	O_2 limited	23			16.8	0.33
	Nonaerated	ND[d]			16.5	0.30
Fructose	Aerated		25	7	16.5	0.59
	O_2 limited		12	6.8	16.8	0.19
	Nonaerated		ND	ND	17.4	0.29
Glucose-fructose[e]	Aerated	19.9	6.5	6.3	15.4	0.60
	Nonaerated	17	12.5	12.5	17.8	0.42
Sucrose	Aerated[f]					
	1	71 (19.5)	−70.5	0	16.9	0.98
	2	33 (14.3)	−26.1 (5.9)	1	10.7	0.40

[a] For global sucrose uptake, sucrose was converted into glucose-1-phosphate and fructose plus sucrose converted to dextran and fructose by dextransucrase. The values in parentheses represent the flux of sucrose catabolized through the phosphoketolase pathway (millimoles of sucrose per hour per gram of biomass).
[b] Negative values indicate an accumulation of fructose in the medium during cultivation on sucrose medium. The value in parentheses represents the fructose flux (converted into glucose-6-phosphate) calculated from the carbon balance.
[c] Y_{ATP} was calculated from the global stoichiometric coefficients.
[d] ND, not determined.
[e] Glucose and fructose in equimolar quantities (55 mM) in a culture inoculated with fructose-grown cells.
[f] The numbers 1 and 2 represent the nature of the substrate catabolized (1, sucrose; 2, sucrose plus fructose).

The totality of the consumed fructose was recovered (99% carbon recovery), indicating that unlike the glucose cultures, no additional products were being synthesized.

(iii) Glucose-fructose mixtures. In the presence of equimolar concentrations of both glucose and fructose, the μ measured in the presence of oxygen was the same as that obtained on each sugar alone (μ = 0.6 h^{-1} [Table 1]). Glucose and fructose were consumed simultaneously by the bacteria, although glucose, as reported for *L. oenos* (29), was consumed more rapidly. Indeed the specific rate of glucose consumption (19.9 mmol of glucose · g of biomass^{-1} · h^{-1}) was similar to that seen on glucose alone. The fermentation end products during this period of growth were a mixture of mannitol, acetate, and lactate (Fig. 2F), and there was a direct correlation between the quantity of fructose consumed and the mannitol produced according to the following stoichiometry: 1 glucose + 0.32 fructose + 0.84 O_2 → 0.97 lactate + 0.98 acetate + 0.32 mannitol + 1 CO_2. When glucose had been fully depleted, metabolism of fructose took place in a manner similar to that observed with growth on fructose alone (see above).

Similar results were obtained if glucose-grown cells were used as the inoculum, although the capacity to metabolize fructose to mannitol was always somewhat lower under such conditions (results not shown).

(iv) Sucrose. The growth of *L. mesenteroides* in sucrose medium was considerably more rapid (μ = 0.98 h^{-1}) under aerated conditions than for other sugars. However, the final biomass concentration was somewhat lower (1.75 g · $liter^{-1}$) than that obtained on either glucose or fructose under the same conditions, since a significant part of the sucrose was converted to dextran (Fig. 3A). This carbon loss accounted for 62% of the glucose moiety of sucrose and led to a final dextran concentration of 10.9 g · $liter^{-1}$ (Fig. 3B). Dextransucrase activity accumulated throughout the culture, to reach 5 U · ml^{-1}; 90% of the enzyme was linked to the cells, as previously described (26, 27, 32).

During the first period of growth, the sucrose taken up by

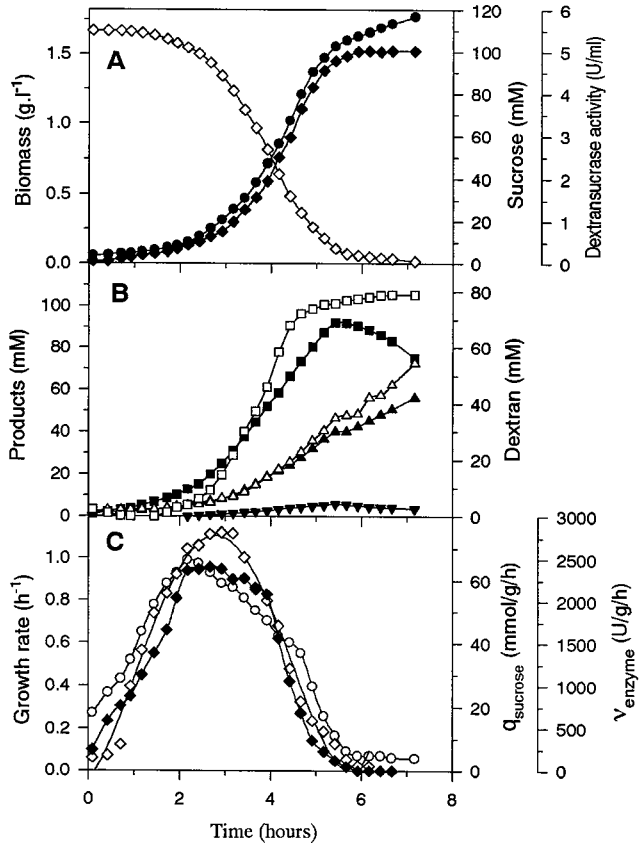

FIG. 3. Batch fermentation profile for the growth of *L. mesenteroides* with sucrose (110 mM) under aerobic conditions (pO_2 maintained at a value higher than 40% of air saturation). (A) ◇, sucrose; ●, biomass; ◆, dextransucrase activity. (B) ■, fructose; ▲, lactate (millimolar); △, acetate; ▼, mannitol; □, dextran (millimoles of glucosyl residues per liter). (C) ○, μ; ◇, specific rate of sucrose consumption; ◆, specific rate of dextransucrase production.

the cells and metabolized via the phosphoketolase pathway can be summarized by the stoichiometry 1 sucrose + 1 O_2 → 1 fructose + 1 lactate + 1 acetate + 1 CO_2.

During this period, only the glucose moiety was consumed and transformed to an equimolar mixture of lactate and acetate, with no trace of either ethanol or mannitol being produced. The fructose moiety accumulated in the culture broth together with that associated with the synthesis of dextran. This accumulation of fructose suggests that *L. mesenteroides* probably possesses an efflux mechanism for fructose such as that postulated to be operating during sucrose metabolism by *Corynebacterium glutamicum* (7). Although the overall sucrose consumption rate was extremely high (71 mmol of sucrose · g of biomass^{-1} · h^{-1}), much of this was due to dextran synthesis, and the actual flux through the phosphoketolase pathway was similar to that seen on glucose alone (i.e., 19.5 mmol of glucose · g of biomass^{-1} · h^{-1}), and a Y_{ATP} of 16.9 g of biomass · mol of ATP^{-1} was calculated. Some of the fructose liberated intracellularly (approximately 43%) was consumed together with the glucosyl moiety (Table 1), the remainder accumulating extracellularly. The specific rate of fructose consumption was similar to that seen during aerobic growth on glucose-fructose mixtures (i.e., 5.9 mmol of fructose · g of biomass^{-1} · h^{-1}) and was sufficient to maintain the flux through the phosphoketolase pathway despite a decrease in the rate of glucose metabolism (14.3 mmol of glucose · g of biomass^{-1} · h^{-1}). The following stoichiometry was observed: 1 sucrose + 1.64 O_2 → 0.52 fructose + 0.05 mannitol + 1.22 lactate + 1.62 acetate + 1.83 CO_2.

Under such conditions, the Y_{ATP} was very low: 10.7 g of biomass · mol of ATP^{-1}. Moreover, the amount of acetate and CO_2 produced no longer corresponds to a strict heterolactic fermentation and can only be explained by some flux of pyruvate towards acetate via the pyruvate dehydrogenase complex corresponding to 2.8 mmol of pyruvate · g of biomass^{-1} · h^{-1}. This was never observed in anaerobically grown cultures, since pyruvate dehydrogenase is inactive during the anaerobic growth of many microorganisms.

Once sucrose was depleted, the accumulated fructose was metabolized with a considerable decrease in μ. However, unlike during growth on fructose alone, the rate of mannitol production was extremely low (1 mmol of mannitol · g of biomass^{-1} · h^{-1}), and coenzyme equilibria were maintained predominantly by direct oxidation of reduced coenzymes via the NADH oxidase-peroxidase systems.

Under anaerobic conditions, growth on sucrose was somewhat less rapid (μ = 0.78 h^{-1}) and dextran production was somewhat lower despite a similar production of dextran sucrase (final activity, 5.2 U/ml). The maximum rate of sucrose consumption was slightly lower than during aerobic growth (i.e., 60 mmol of sucrose · g of biomass^{-1} · h^{-1}), although the flux of hexose into the phosphoketolase pathway (19.8 mmol of hexose · g of biomass^{-1} · h^{-1}) and Y_{ATP} (16.8 g of biomass · mol of ATP^{-1}) were comparable with the maximum flux observed for any of the conditions used in this study. The fermentation pattern was different, with an initial phase of sucrose metabolism in which only the glucose moiety was consumed but in which only lactate and ethanol were produced. This period of accelerating growth was followed by a period in which fructose consumption occurred at rates slightly higher than that liberated by intracellular sucrose cleavage, necessitating some fructose uptake from the broth. During this period, all of the fructose consumed was reduced to mannitol, and the following stoichiometry was observed: 1 sucrose + 0.09 fructose → 1.09 mannitol + 0.97 lactate + 0.52 acetate + 0.48 ethanol + 1 CO_2. As in the aerated cultures, fructose was consumed once sucrose was depleted, with production of lactate and acetate as well as mannitol.

TABLE 2. Intracellular enzymatic activities implicated in substrate phosphorylation reactions during exponential growth on various sugars

Enzyme	Enzyme activity (mmol of NADP · g of protein^{-1} · h^{-1}) in cells grown on:		
	Glucose	Fructose	Sucrose
Fructokinase	2.5	19	3.5
Glucokinase	38.2	51.5	22
Phosphoglucose isomerase	1.2	6.1	0.8
Phosphoglucomutase	7.0	ND[a]	23.1
Sucrose phosphorylase	0.3	0	40.2

[a] ND, not determined.

Enzymes responsible for sugar phosphorylation. Different possibilities exist to explain the uptake and phosphorylation reactions yielding hexose phosphates, so an enzymatic analysis was undertaken. Since no extracellular glucose could be detected, the extracellular invertase activity was assumed to be very low. No activity could be detected for phosphoenolpyruvate-dependent phosphotransferase activity for any of the sugars used in this study. However, significant activities of both glucokinase and fructokinase were detected, as were an inducible intracellular sucrose phosphorylase and a constitutive phosphoglucomutase (Table 2). It is therefore postulated that all three sugars are taken up by permeases and that sucrose is then concomitantly phosphorylated and hydrolyzed by sucrose phosphorylase to yield glucose-1-phosphate and fructose intracellularly. Glucose-1-phosphate is then converted by phosphoglucomutase to glucose-6-phosphate, which enters the phosphoketolase pathway. The sucrose phosphorylase reaction employs P_i and not a high-energy phosphate bond and therefore offers a significant energy gain for the cell (see below).

Growth energetics. The manner in which the growth behavior of *L. mesenteroides* relative to ATP production has been quantified with stoichiometric and kinetic data is given in Table 1. When these values are examined, it may be seen that the growth rate was directly proportional to the rate at which ATP can be synthesized (Fig. 4), indicating that growth was always energy limited. The yield of cell biomass, excluding capsular dextran, relative to ATP (Y_{ATP}) was fairly constant at approximately 16.5 g of cells · mol of ATP^{-1}. Since the flux through the phosphoketolase seems to be limited to a value of approximately 21 mmol of hexose · g of biomass^{-1} · h^{-1}, the growth rate will depend directly on the efficiency with which each sugar can be fermented. Under the conditions used here, sucrose represents the sugar able to support the best growth, since less expenditure of ATP is necessary to activate (phosphorylate) the sugar molecule. The decrease in Y_{ATP} seen towards the end of cultures on sucrose coincided with the use of fructose, but the reason for this decrease in apparent growth efficiency cannot be explained by the catabolic pathways, since such values were not seen during growth on fructose alone.

Kinetics of dextransucrase production during growth on sucrose. The production of dextransucrase was always associated with the use of sucrose, but dextransucrase and sucrose phosphorylase were not coinduced by their common substrate. Furthermore, as can be seen in Fig. 3C, the rate at which the enzyme was produced was directly related to the growth rate, with a maximum rate of enzyme production of 2,400 U · h^{-1} · g of biomass^{-1}. This correlation did not hold towards the end of the period of sucrose consumption, when significant

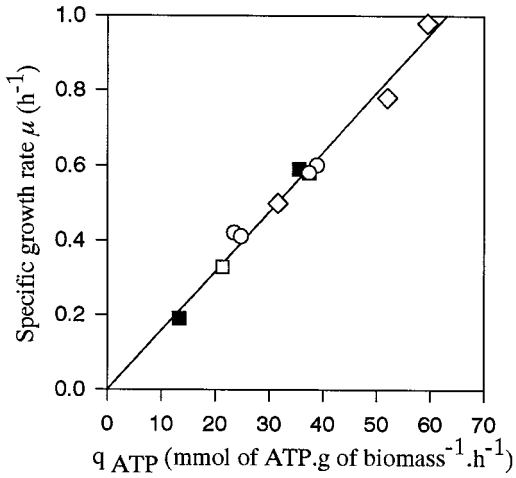

FIG. 4. Correlation between growth rate (μ) and the rate of energy synthesis (q_{ATP}) during batch cultivation under various aeration conditions on the following sugars: □, glucose (110 mM); ■, fructose (110 mM); ○, glucose plus fructose in equimolar quantities (55 mM); and ◇, sucrose (110 mM).

consumption of fructose by the phosphoketolase pathway was observed (Fig. 5). When growth on sucrose was initiated with a glucose-grown inoculum, results were similar to those shown in Fig. 3. As observed above, the correlation between growth rate and dextransucrase production was only valid during the period of sucrose metabolism in which no fructose was catabolized by the phosphoketolase pathway. In fact, during the first 2 h, fructose was consumed simultaneously with sucrose via the phosphoketolase pathway (Fig. 6B), and dextransucrase production was very slow (Fig. 6A and C). After that period, fructose catabolism via the phosphoketolase pathway stopped and mannitol accumulated, concomitant with an accelerated production of dextransucrase. Thus, while sucrose phosphorylase was rapidly induced, the high levels of dextransucrase activity normally seen during sucrose exploitation were not seen in the initial stages of the fermentation. It thus appears that while sucrose is necessary for induction of dextransucrase, fructose metabolism, when it involves significant feeding of carbon into the phosphoketolase pathway, is sufficient to block expression. The rate of enzyme synthesis is directly correlated to growth rate and hence to biochemical energy status. Low production of dextransucrase was always associated with an apparently low efficiency of cell synthesis ($Y_{ATP} < 11$ g of biomass · mol of ATP^{-1}). Thus, when energy production is high, growth will be proceeding at maximal rates, since the maximum flux through the phosphoketolase pathway is attained, and hence no further increase in either substrate throughput or growth is possible.

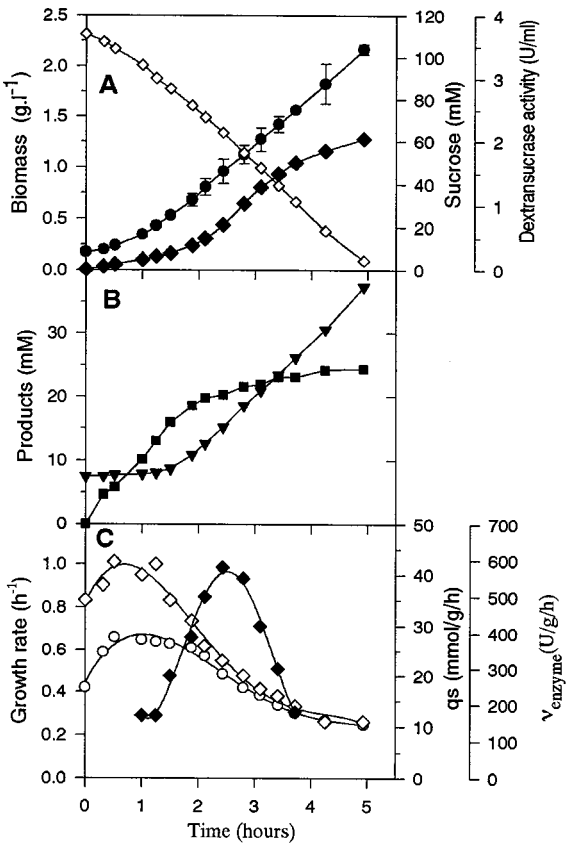

FIG. 6. Batch fermentation profile for the growth of *L. mesenteroides* with sucrose under aerobic conditions; the medium was inoculated with fructose-grown cells. (A) ◇, sucrose; ●, biomass; ◆, dextransucrase activity. (B) ■, amount of fructose catabolized via the phosphoketolase pathway since the beginning of the culture; ▼, mannitol. (C) ○, μ; ◇, specific rate of sucrose consumption; ◆, specific rate of dextransucrase production (v_{enzyme}).

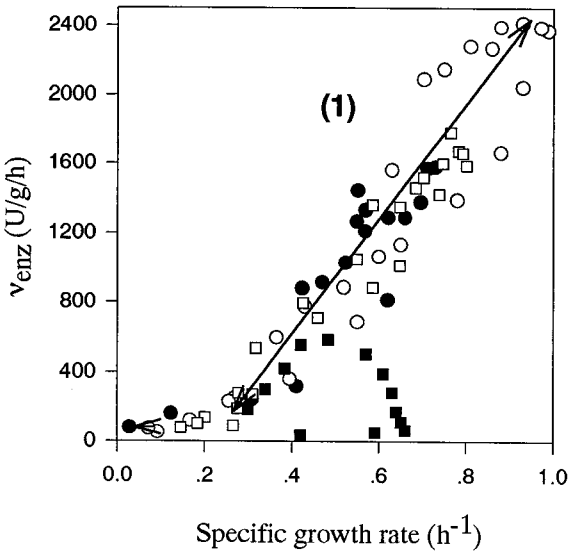

FIG. 5. Correlation between growth (μ) and dextransucrase production (v_{enz}) during aerobic (○) and anaerobic (●) growth on sucrose, aerobic growth on sucrose with a glucose-grown inoculum (□), and aerobic growth on sucrose with a fructose-grown inoculum (■). 1, correlation between growth and dextransucrase production during a period in which no fructose was catabolized via the phosphoketolase pathway.

The data obtained in this study enable some degree of optimization of enzyme production to be envisaged. Active catabolism of fructose via the phosphoketolase pathway clearly has a negative effect on dextransucrase synthesis. Therefore, the state of the inoculum will be of prime importance, since cells grown either on fructose or taken from the late stage of growth on sucrose and hence actively metabolizing fructose will delay the onset of enzyme synthesis. This probably explains why Remaud-Simeon et al. (27) obtained significantly smaller amounts of enzyme with the same strain on an identical medium, since overnight cultures were used as inocula. Further-

more, growth conditions facilitating high energy production will favor rapid enzyme synthesis, although final enzyme yields relative to sucrose will not necessarily be much altered, since higher substrate conversion to dextran will occur because of the presence of the enzyme.

Aeration will also play an important role, since higher rates of ATP production are obtained under aerobic conditions, although later in the culture, such aerobic conditions will favor fructose consumption via the phosphoketolase pathway, with a drastic effect on enzyme synthesis. This period is delayed under anaerobic conditions, since fructose is transformed mostly to mannitol, which appears not to affect synthesis of dextransucrase. Therefore, optimization of the aeration profile or obtaining mutants defective in fructokinase activity, thereby blocking fructose catabolism, may improve enzyme production.

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