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Minimal Requirements for Exponential Growth of *Lactococcus lactis*

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A minimal growth medium containing glucose, acetate, vitamins, and eight amino acids allowed for growth of *Lactococcus lactis* subsp. *lactis*, with a specific growth rate in batch culture of $\mu = 0.3\ h^{-1}$. With 19 amino acids added, the growth rate increased to $\mu = 0.7\ h^{-1}$ and the exponential growth phase proceeded until high cell concentrations were reached. We show that morpholinepropanesulfonic acid (MOPS) is a suitable buffer for *L. lactis* and may be applied in high concentrations.

Interest in the physiology of lactic acid bacteria has been stimulated by the industrial importance of these bacteria and the potential use of genetic engineering in strain optimization. For metabolic investigations, a defined growth medium for these bacteria is desirable which (i) supports growth at a reasonably high rate and (ii) allows for exponential growth over a wide range of cell concentrations. *Lactococcus lactis* has numerous growth requirements (8, 10, 13), which complicates the formulation of a suitable growth medium for these bacteria. The complex media MRS (5) and M17 (14) are extensively used but are often unsuitable for physiological studies, e.g., when a well-defined growth medium is required. Presently many investigators use a defined growth medium described by Otto et al. (11) and modified by Poolman and Konings (12) which supports the growth of *L. lactis* at reasonably high specific growth rates. Yet, this medium contains virtually all building blocks for biosynthesis of macromolecules, which complicates the study of metabolic pathways. Here we show that a combination of eight building blocks, all of which are amino acids, is sufficient for growth of derivatives of *L. lactis* subsp. *lactis* NCDO 712.

Growth on plates containing eight amino acids (BL medium). As a basis for our minimal media, we used the medium described by Neidhardt and coworkers (9), which contains the pH buffer morpholinepropanesulfonic acid (MOPS). We applied 0.19 M MOPS to the BL medium and 0.04 M MOPS to the SA medium unless otherwise stated (see Table 1 for complete medium compositions). All stock solutions were passed through Millipore filters (0.2-μm pore size). Glucose, acetate (see reference 4), and vitamins (biotin, pyridoxal, folic acid, riboflavin, niacinamide, thiamine, and calcium pantothenate) were added together with six amino acids (glutamate, histidine, isoleucine, leucine, methionine, and valine) previously shown to be essential for growth of *L. lactis* subsp. *lactis* (13).

The medium composition listed above did not allow growth of derivatives of strain NCDO 712 unless a seventh amino acid (any one of at least 5 of the remaining 14) was added to the growth medium (Table 2). Addition of asparagine together with glutamine (BL medium) stimulated growth so that colonies became visible after 2 days of incubation. To our knowledge, this is the simplest composition of amino acids presented so far that allows for growth of strains of *L. lactis* subsp. *lactis*.

Growth in batch cultures of BL medium. Overnight cultures grown in BL medium were diluted into a fresh portion of the medium (30°C) and stirred gently with magnetic bars in order to maintain a homogeneous batch culture. The growth

TABLE 1. Medium composition

Constituent	Concn (mM) or presence in medium:	
	SA	BL
L-Alanine	3.4	
L-Arginine	1.1	
L-Asparagine	0.8	0.8
L-Cysteine	0.8	
L-Glutamate	2.1	21
L-Glutamine	0.7	0.7
Glycine	2.7	
L-Histidine	0.3	0.3
L-Isoleucine	0.8	0.8
L-Leucine	0.8	1.5
L-Lysine-HCl	1.4	
L-Methionine	0.7	0.5
L-Phenylalanine	1.2	
L-Proline	2.6	
L-Serine	2.9	
L-Threonine	1.7	
L-Tryptophan	0.5	
L-Tyrosine	0.3	
L-Valine	0.9	2.6
NH_4Cl	9.5[a]	9.5
K_2SO_4	0.28[a]	0.28
KH_2PO_4	1.3[a]	1.3
Na-acetate	15	15
Glucose	50	50
MOPS	40[a]	190
Tricine	4[a]	4
$CaCl_2$	0.0005[a]	0.0005
$MgCl_2$	0.52[a]	0.52
$FeSO_4$	0.01[a]	0.01
NaCl	50[a]	50
Vitamins[b]	+	+
Micronutrients[a,c]	+	+

[a] From Neidhardt et al. (9).
[b] Vitamins: 0.4 μM biotin, 10 μM pyridoxal-HCl, 2.3 μM folic acid, 2.6 μM riboflavin, 8 μM niacinamide, 3 μM thiamine-HCl, and 2 μM pantothenate.
[c] Micronutrients: 0.003 μM $(NH_4)_6(MO_7)_{24}$, 0.4 μM H_3BO_3, 0.03 μM $CoCl_2$, 0.01 μM $CuSO_4$, 0.08 μM $MnCl_2$, and 0.01 μM $ZnSO_4$.

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TABLE 2. Growth of *L. lactis* subsp. *lactis* on solid medium containing six or more amino acids

Amino acids included[a]	Incubation (no. of days)	Growth[b] of strain:		
		712	C_2	MG1363
Glu, Leu, Ile, Val, His, Met	2	−	−	−
	4	−	−	−
	7	−	−	−
	14	−	++	(+)
Glu, Leu, Ile, Val, His, Met + Asn	2	+	+	+
	4	+++	+++	+++
	7	+++	+++	+++
	14	+++	+++	+++
Glu, Leu, Ile, Val, His, Met + Gln	2	−	−	−
	4	−	−	+
	7	+	+	++
	14	++	++	+++
Glu, Leu, Ile, Val, His, Met + Asn, and Gln (=BL medium)	2	++	++	++
	4	++++	++++	++++
	7	++++	++++	++++
	14	++++	++++	++++
Glu, Leu, Ile, Val, His, Met + Ala	2	−	−	−
	4	−	−	−
	7	+	+	+
	14	+++	++	++
Glu, Leu, Ile, Val, His, Met + Arg	2	−	−	−
	4	−	−	+
	7	+	+	++
	14	++	++	++++
Glu, Leu, Ile, Val, His, Met + Thr	2	−	−	−
	4	−	+	++
	7	++	++	+++
	14	+++	+++	+++

[a] The basic medium contained glutamate, leucine, isolecine, valine, histidine, and methionine in addition to vitamins, mineral salts, and glucose at the concentrations given for BL medium (Table 1). The rest of the amino acids were added at the concentrations used in SA medium.

[b] Colony diameter: ++++, >1 mm; +++, 0.6 to 1.0 mm; ++, 0.4 to 0.6 mm; +, 0.2 to 0.4 mm; (+), <0.2 mm (visible through the microscope at ×10 magnification).

rate of MG1363, a plasmid-free derivative of strain NCDO 712 (6), was $\mu = 0.28\ h^{-1}$, and the final cell concentration obtained with BL medium gave an optical density at 450 nm (OD_{450}) of 2.3 (equal to 1.6×10^9 cells per ml). Upon dilution of the overnight culture, the exponential growth phase was reached within 30 to 60 min and lasted up to an OD_{450} of approximately 0.5, independent of the buffer concentration in the medium (Table 3). The buffer concentration was however important for high cell yields: reducing the concentration of MOPS from 0.19 to 0.04 M decreased the final cell concentration to 1 (in OD_{450} units). The growth rate in BL medium was slightly stimulated upon addition of aspartate or arginine. When arginine, serine, and tryptophan were all added together, the growth rate increased to $\mu = 0.36\ h^{-1}$ and the upper limit of the exponential growth phase was increased from 0.5 to above 1 (in OD_{450} units).

TABLE 3. Growth of *L. lactis* subsp. *lactis* MG1363 in unaerated batch cultures of BL medium or modified BL medium

Modification to BL medium	Growth rate (μ) (h^{-1})[a]	End of exponential growth (OD_{450})	Final growth yield (OD_{450})
None (=BL)	0.28	0.5	2.3
+Arg[b]	0.32	0.5	2.6
+Asp (1 mM)	0.31	0.5	2.3
+Arg, Ser, and Trp[b]	0.36	1.4	3.8
Low MOPS (0.04 M)	0.29	0.5	0.9

[a] Growth rate, μ = ln 2/doubling time (h).

[b] These amino acids were added at the concentrations contained in SA medium (Table 1).

The BL medium should be useful for the many physiological studies, in which one wishes to have a defined and simple combination of building blocks for biosynthesis of macromolecules, e.g., to obtain mutants in some biosynthetic pathway. Although not all vitamins are essential for growth of all *L. lactis* strains, some strains have such requirements (see, e.g., reference 8), and we have therefore included them in the growth media.

Batch cultures of *L. lactis* in a growth medium containing 19 amino acids (SA medium). Addition of 19 amino acids (all except aspartate) resulted in a high growth rate of *L. lactis* subsp. *lactis* ($\mu = 0.55$ to $0.73\ h^{-1}$). In this medium, 0.04 M MOPS was sufficient to sustain exponential growth up to an OD_{450} of 2, with a final cell concentration of approximately 3; this is sufficient for most purposes, and in the following studies 0.04 M was the MOPS concentration used unless otherwise stated. Table 4 documents the growth of various strains of *L. lactis* subsp. *lactis* and subsp. *cremoris* strains in batch cultures of the SA medium. For comparison, the strains were also grown in the complex M17 medium. All the strains tested grew exponentially in the SA medium supplemented with 50 mM glucose, though at a lower rate than in the complex medium. The exponential growth rate ranged from $\mu = 0.2$ to $0.73\ h^{-1}$, or between 25 and 75% of the rate obtained when the cells were grown in M17 medium.

TABLE 4. Growth of various strains of *L. lactis* in unaerated batch cultures

Strain[a]	SA medium		M17 medium	
	Growth rate (μ) (h^{-1})[b]	Final growth yield (OD_{450})	Growth rate (μ) (h^{-1})	Final growth yield (OD_{450})
L. lactis subsp. *lactis*				
MG1363	0.73 (0.03)[c]	3.0 (0.3)	1.11 (0.08)	5.7 (0.2)
MG1614	0.63 (0.04)	2.9 (0.3)	0.99 (0.03)	5.6 (0.2)
NCDO712	0.55 (0.04)	2.5 (0.3)	1.02 (0.07)	5.5 (0.5)
C2	0.63 (0.13)	2.1 (0.4)	1.02 (0.04)	5.4 (0.3)
IL1403	0.64 (0.04)	2.5 (0.1)	0.83 (0.23)	5.3
L. lactis subsp. *cremoris*				
Wg2	0.22 (0.05)	1.7 (0.8)	0.77 (0.02)	3.6 (0.3)
3107	0.45 (0.02)	1.6 (0.3)	0.59 (0.05)	3.8 (0.3)
936-1	0.44 (0.03)	2.0 (0.1)	0.50 (0.07)	3.8 (0.3)
901-1	0.41 (0.03)	2.0 (0.4)	0.48 (0.05)	3.7 (0.2)

[a] All the strains were obtained from F. V. Vogensen (The Royal Veterinary and Agricultural University of Denmark). References are as follows: NCDO712, MG1363, and MG1614 (6); C2 (7); IL1403 (3); 3107, 901-1, 936-1, and Wg2 (1).
[b] μ = ln 2/doubling time (h).
[c] Values are averages of two to five determinations; standard deviations are shown in parentheses.

Compared with the media described by other investigators (10, 12, 13), the SA medium lacks many components. To test whether the concentrations were still optimal with respect to the growth rate and growth yield of *L. lactis* subsp. *lactis* MG1363, the strain selected for physiological studies in this laboratory, we increased the concentrations of the components of the SA medium. None of the individual nutrients affected growth significantly, and we conclude that the components of the SA medium are in excess of the concentration required to support the maximum growth rate of strain MG1363.

Since strains of *L. lactis* convert a large part of their sugar substrate into lactic acid, we anticipated the buffer capacity of the medium to be important, particularly with respect to high growth yields. Indeed, as the concentration of buffer was increased from 0.02 to 0.12 M the growth yield increased, suggesting that it is the pH of the growth medium that limits the final cell density in SA medium (Fig. 1A). Using 0.12 M MOPS, we obtained the same yield as in the rich medium M17. The final pH of the medium after growth increased when higher concentrations of buffer were used.

We analyzed how the final optical density (OD_{450}) of the culture depended on the glucose concentration in the SA medium. Below 0.015 M glucose the final cell yield decreased linearly with decreasing glucose concentration (Fig. 1B). This introduces the possibility of substrate runout experiments and well-defined starvation experiments; with the rich medium M17, this was not possible since this medium supports the growth of *L. lactis*, without addition of sugar, until a cell concentration of approximately 0.8 (in OD_{450} units).

We also tried to exchange the MOPS buffer in SA medium with phosphate. This resulted in a lower growth rate of strain MG1363 at a high buffer concentration compared with that in the medium containing MOPS (Fig. 2). These results are in line with the results of Neidhardt and coworkers (9), who showed that enterobacteria also grew faster with MOPS as the buffer compared with phosphate. Perhaps phosphate causes partial dissipation of the proton gradient across the

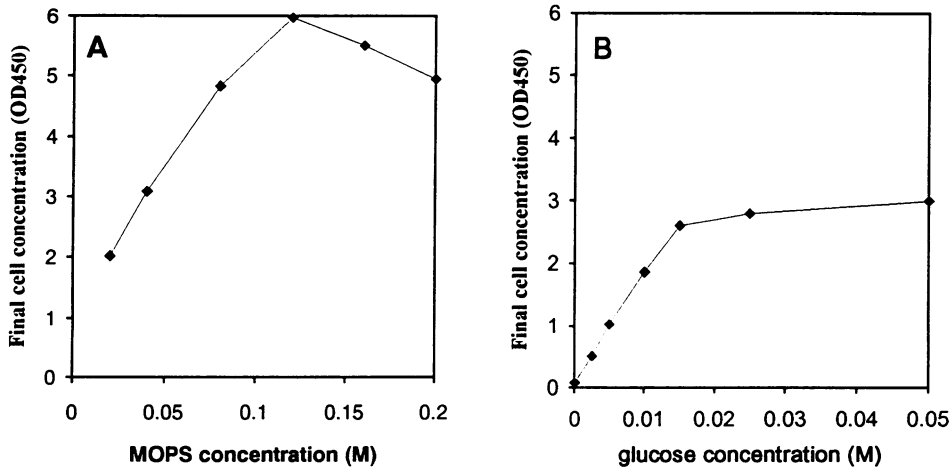

FIG. 1. Dependence of final growth yield on the concentrations of buffer and sugar in the SA medium for strain MG1363. (A) Dependence of yield on the buffer (MOPS) concentration at a constant concentration of sugar (50 mM glucose). (B) Dependence of yield on the glucose concentration at a constant concentration of buffer (0.04 M MOPS).

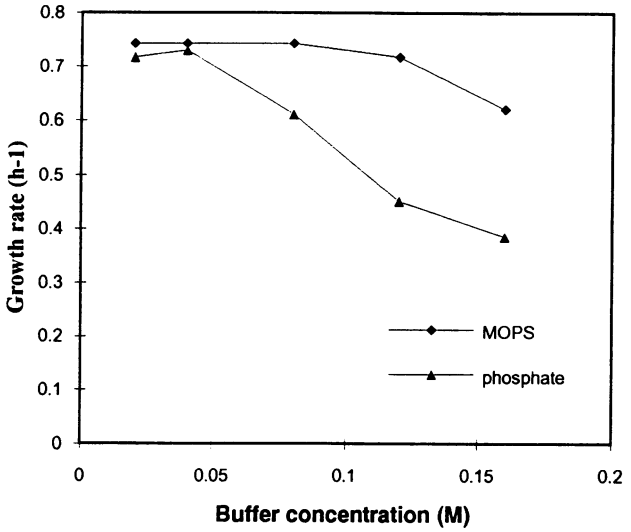

FIG. 2. Relationship between exponential growth rate and buffer concentration in SA medium with either MOPS or phosphate as the pH buffer.

cytoplasmic membrane. The beneficial effect of MOPS compared with phosphate may also be a result of MOPS being transported into the cells and playing a role in the osmoregulation of *L. lactis*, as has previously been shown for *Escherichia coli* (2).

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