Cultivation of a facultative heterofermentative lactic acid bacteria strain in continuous culture

1. Introduction

Investigations of growth and physiology of microorganisms are most often performed with batch grown cultures. The main characteristics of a batch system are: excess of all nutrients, drastic change of population density during few generation times, drastic change of the ratio substrates/products during few generation times and (ideally) a completely homogeneous medium with no fluxes of components into or out of the system. The phenotype of an organism is highly affected by its growth medium, therefore the outcome of a batch culture is also a very specific phenotype which is not necessarily the most abundant form[1].

It is important to emphasize that batch-culture-like systems are not abundant in nature, microorganisms do not often grow under unlimited nutrient conditions at maximal rate. Characteristics of natural ecosystems are instead: limited nutrient supply, long generation times, mixed populations as well as continuous fluxes of components into and out of the system.

In 1950 Jacques Monod[2] formulated the concept of *steady-state* cultures and the cultivation of continuous cultures in laboratory *chemostats*. This technique offered a variety of new possibilities for the investigation of microbial physiology. Microorganisms can be cultivated under all kind of desired limitations. Long-term competition-, symbiosis- or mutant selection experiments can be carried out using pure cultures or mixtures of microorganisms, and above all, the growth rate of the culture can be fixed by the operator of the chemostat at all desired levels between almost zero and the maximal growth rate of the organisms.

A small scale chemostat was run in this study. As a model organism a lactic bacteria was chosen that had been enriched from a intestinal swab in rich yeast-glucose medium at 37°C. The organism was gram-positive, non-spore-forming, cocci-shaped, catalase-negative and fermented glucose to lactated in a 1:2 molar ration in batch culture.

Lactic acid bacteria are subdivided into obligatory homofermentative lactobacilli (group A), facultatively heterofermentative lactobacilli (group B) and obligately heterofermentative lactobacilli (group C)[3]. Whereas group A lactobacilli degrade hexoses almost exclusively by the *Embden-Meyerhof pathway* to lactate and cannot use pentose or gluconate, group B lactobacilli can additionally ferment pentoses via a inducible *pentose phosphoketolase* and produce acetic acid, formic acid and ethanol beside lactic acid under glucose limitation. Group C lactobacilli ferment hexoses to lactic acid, acetic acid, ethanol and CO_2 via the *phospho-gluconate pathway*.

The goal of this study was to test a small-scale chemostatic equipment for its suitability to grow microaerotolerant bacteria under steady-state conditions. Producing glucose-limited growth conditions, it was the aim to measure the pattern of fermentation products in continuous culture and compare it with the pattern obtained in batch culture.

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[1] **Jannasch, H. W.** 1974. Adv. Microbiol. Physiol. **11**:165.
[2] **Monod, J.** 1950. Annals de l'Institut Pasteur **79**:390.
[3] **Vandamme et al.** 1996. Microbiol. Rev. **60**:418-422.

2. Material and Methods

2.1 Organism and growth media

The lactic bacteria used in this study was a gram-positive, non-spore-forming, catalase-negative cocci-shaped organism that fermented glucose to lactate in a 1:2 molar ration in batch culture. It is named *ID1* in the further part of this report.

In order to get a reliable mass balance, several defined media described in the literature[4,5] were tested for their ability to support growth of *ID1*. Although the tested media contained up to 10 $g \cdot L^{-1}$ enzymatic casein hydrolysate, trace elements, 10 different vitamins, nucleic acids and 100 $mg \cdot L^{-1}$ yeast extract, the growth rate of the culture only achieved 0.12 h^{-1} compared to almost 0.40 h^{-1} obtained in rich yeast-glucose medium (1% glucose, 1% yeast extract). As a compromise between optimal growth conditions and level of media complexity the following medium composition was finally chosen: Na_2HPO_4, 30 mM; glucose, 10 mM; yeast extract, 1.8 $g \cdot L^{-1}$; NH_4Cl, 6 mM. The glucose was made up in a 100x stock solution, filter sterilized and added after autoclaving of the medium.

Batch cultures were grown in 20 mL screw cap tubes at 28°C, turbidity was measured at 600 nm in a spectrophotometer.

2.2 Chemostat culture conditions

ID1 was grown in a self-made glass fermenter with a working volume of 87 mL, the system worked with two 10 L glass bottles as medium reservoir and waste container, connected to the fermenter vessel by butyl rubber tubings. The two containers and the fermenter vessel were autoclaved separately and connected asseptically after cooling. The cooling of the reservoir medium was accelerated by flushing it with nitrogen asseptically.

The temperature of the chemostatic culture was maintained at 28°C, the culture was agitated by a magnetically driven impeller and surface of fermenter and medium reservoir gassed with nitrogen. Medium flow was controlled by a peristaltic pump (GILSON). The inoculum (1% v/v) for the chemostat was a static batch culture. The culture was assumed to be in steady-state when at least 5 volumes had passed through the chemostat. pH of the culture was measured in steady-state and found not to drop below 6.5. In order to test the effectiveness of the sterility precautions the system was run without inoculation of bacteria for 72 h, no growth was monitored during this time. Moreover, no growth of organisms was observed in the autoclaved reservoir medium during the whole period of the experiment (18 d) demonstrating the sterility of the medium used.

2.3 Analytical procedures

Turbidity of the cultures was measured at 600 nm in a spectrophotometer. 3 mL samples were taken from batch- and continuous culture periodically and acidified immediately with 30 µL of foaming hydrochloric acid in order to prevent further metabolization. Samples were spinned 5 min at 13K and 20 µL of it were injected into a HPLC connected to a refractory index detector (WATERS). The mobile phase was 0.2% phosphoric acid (0.5 $mL \cdot min^{-1}$ flux) and the signals were integrated. Standard injections of ribose, acetate, formate, ethanol and lactate were made and found to be detected linearly within the range of concentrations measured in this study.

2.4 Calculations

Determination of Gibbs free energy of fermentation reactions ΔG_0 under defined conditions were made using the EXCEL spreadsheet TERMODYN written by Damgaard[6]

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[4] **Harty, D. W. S. et al.**1988. J. Appl. Bacteriol. **65**: 143-152.
[5] **Kemp, C. W. et al.**1983. Appl. Environ. Microbiol. **45**: 1277-1282.
[6] **Damgaard, L.** 1996. MBL course in microbial diverstity.

3. Results and Discussion

3.1 Fermentation products and growth yield in batch culture

Growth of *ID1* and production of lactate was monitored in batch culture containing 10 mM of glucose as defined and 1.8 gL^{-1} yeast extract as undefined sources of carbon (figure 1).

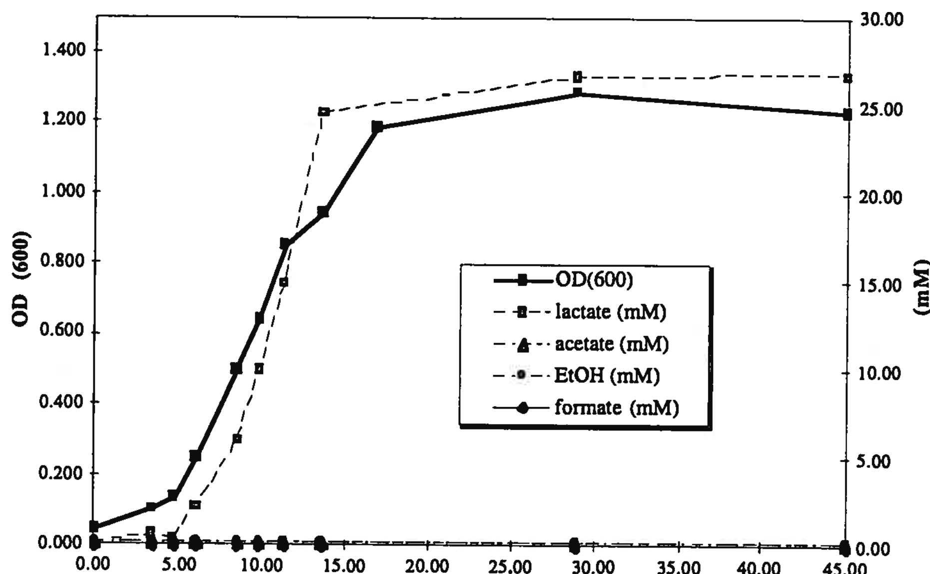

figure 1: Batch growth of lactic acid bacteria *ID1* at 28°C in semidefined medium containing 10 mM glucose and 1.8 g/L yeast extract as carbon sources.

No formation of fermentation products other than lactic acid was detected, where the detection limit for acetate, formate and ethanol was 0.5 mM each. The final optical density of the culture was 1.28 at 600 nm. A linear fit of the exponential part of the growth curve in a half-log-scale plot showed a maximal growth rate μmax of 0.27 h^{-1} (curve fit not shown).

Batch cultivation of *ID1* same medium using 10 mM ribose instead of glucose resulted in the formation of 16.5 mM lactate but also detectable amounts of acetate, ethanol and formate (figure 2).

The ability of *ID1* to grow on ribose indicates the presence of a *pentose phosphoketolase* and determines *ID1* as a facultatively heterofermentative organism. The maximal growth rate with ribose was 0.25 h^{-1}, not significantly different from the growth rate on glucose. Gluconate on the other hand was found not to allow growth of *ID1* indicating the lack of the *phosphogluconate pathway*.

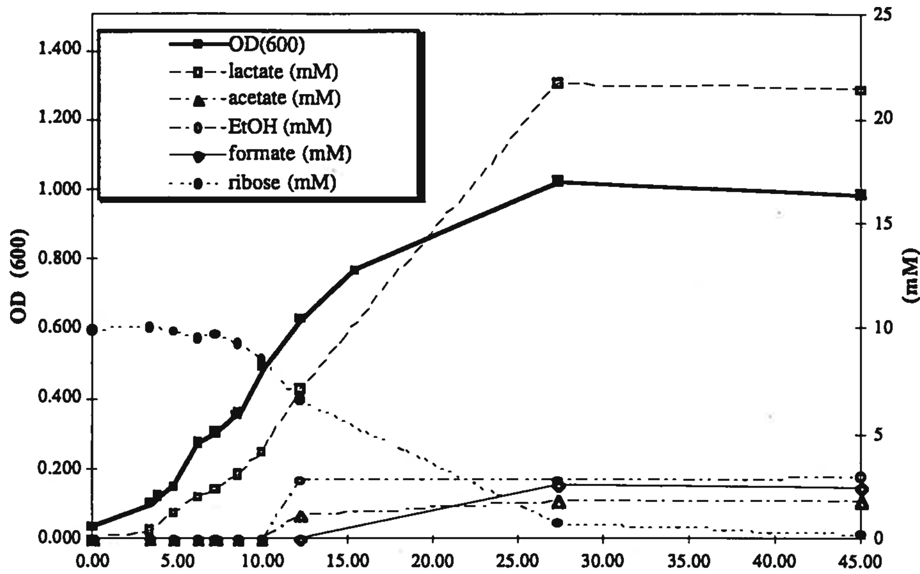

figure 2: Batch growth of lactic acid bacteria *ID1* at 28°C in semidefined medium containing 10 mM ribose and 1.8 g/L yeast extract as carbon sources.

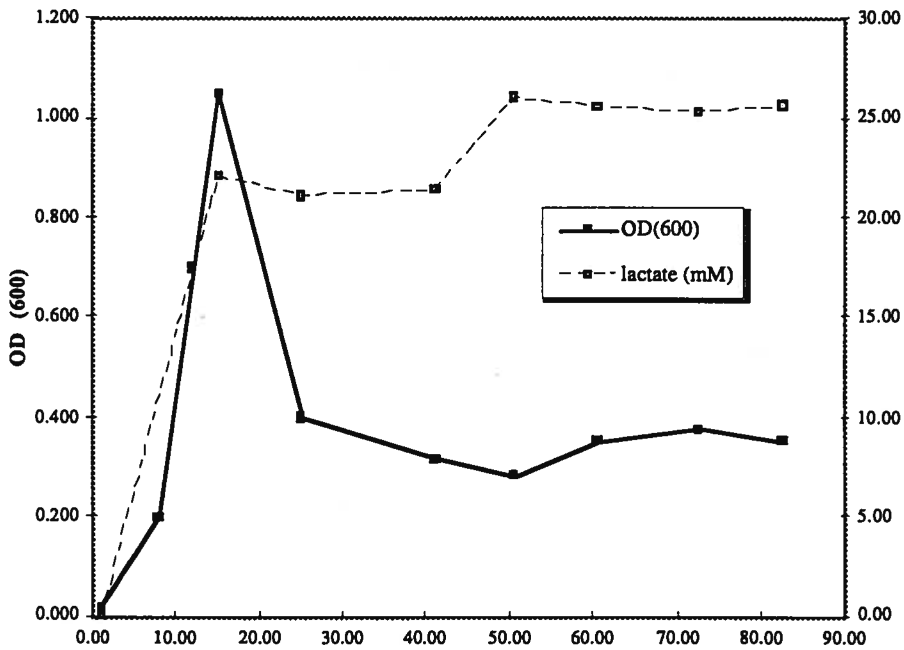

figure 3: Growth of lactic acid bacteria *ID1* in fermenter at 28°C in semidefined medium containing 10 mM ribose and 1.8 g/L yeast extract as carbon sources. Peak of OD (600) indicates switch from batch- to continuous culture ($D=0.09h^{-1}$)

3.2 Fermentation products and growth yields in continuous culture

The fermenter was inoculated with 1% (v/v) of a batch grown culture of *ID1*. The system was run as a batch culture for 15 h. As the optical density (600 nm) of the culture had achieved 1.0, the medium flow was started with a initial dilution rate of 0.09 h^{-1}. Against the expectations, the optical density of the culture decreased during 50 h to a minimal value of 0.282 before it stabilized at 0.350 in a steady-state (figure 3).

The glucose however was metabolized almost quantitatively to lactate during this stabilization period. The yield of biomass per mol of fermented glucose had therefore dropped by 60-70%.

It is unclear what affected this loss. Stirring and the absolute absence of oxygen in the fermenter vessel were shown not to inhibit growth (data not shown). It is possible that ID1 requires a high amount of maintenance energy at low growth rates.

The alteration of the substrate/product ratio in the chemostat could make the fermentation metabolism energetically less favorable compared to batch cultures.

3.3 Fermentation products and growth yields at different dilution rates

In contrast to batch grown cells, the chemostat culture produced detectable amounts of acetate, formate and ethanol beside lactate. Table 1 shows fermentation products and growth yield at different steady-states.

table 1: Effect of dilution rate on the growth yield and metabolic end products of lactic bacteria *ID1*

	product formed (mol/mol glucose)				
dilution rate (/h)	lactate	formate	acetate	ethanol	growth yield (OD600)
batch	1.80	nd	nd	nd	1.288
0.09	1.72	0.11	0.08	0.19	0.380
0.15	1.74	0.11	0.12	0.14	0.613
0.26	1.75	0.06	0.06	0.16	0.636
0.37	1.46	nd	0.03	0.09	*0.569

*wash out

Unlike in earlier studies[7,8] the production of acetate, formate and ethanol was rather low at all dilution rates. However, a slight tendency of the process becoming more heterofermentative with decreasing dilution rate could be observed. At a dilution rate of 0.37 h^{-1} the culture was washed out as expected from the maximal growth rate of the organism determined from batch culture.

3.4 Calculation of Gibbs free energy

A theoretic approach of investigating microbial physiology is the calculation of the maximal energy gain that organisms can get from a particular metabolic reaction. The free Gibbs energy ΔG is a negative value in cases of energetically favorable reaction, where as processes of positive free Gibbs energy are not supposed to take place in nature. Table 2 shows the

[7] **Harty, D. W. S. et al.**1988. J. Appl. Bacteriol. **65**: 143-152.
[8] **Kemp, C. W. et al.**1983. Appl. Environ. Microbiol. **45**: 1277-1282.

stochiometry of end products formed during growth of *Streptococcus salivarius* strain HB on glucose in continuous culture at different dilution rates[9]. Entirely homofermentative metabolization of glucose to lactate and homoacetic fermentation are given as further examples. The EXCEL spread sheet TERMODYN was used to calculate the free Gibbs energy according to the given stochiometry. As an approach the concentration of glucose was assumed to be 10 mM and the concentrations of all endproducts 1 mM.

table 2: Stochiometry of glucose fermentation of *Streptococcus salivarius* and calculation of Gibbs free energy

	product formed (mol/mol glucose)					
dilution rate (/h)	formate	acetate	lactate	ethanol	growth yield (g/mol)	Gibbs free energy ΔG (kJ/mol)
batch	0.245	0.134	1.670	0.131	48.4	- 213.45
0.10	1.787	0.844	0.116	0.855	42.1	- 397.26
0.22	1.753	0.864	0.232	0.834	45.8	- 389.26
0.72	1.678	0.819	0.230	0.804	46.5	- 359.57
1.10	1.524	0.744	0.347	0.667	46.2	-342.52
homofermentative	0	3	0	0		- 219.74
homoacetic	0	0	2	0		- 350.83

The ΔG varies significantly among the described fermentation stochiometries. Heterofermentative growth appears to be energetically much more favorable than homofermentative growth. Growth under glucose limiting conditions enhances the formation of fermentation products other than lactic acid and moreover, *Streptococcus salivarius* seems to optimize the energy gain with decreasing dilution rates.

4. Conclusions

The used small scale chemostatic equipment has been shown to be suitable for studies of microaerotolerant bacteria under substrate limiting conditions. The system is easy to establish and to maintain and allows long term experiments with little time cost. The system revealed a bacterial phenotype different from the one observed in batch culture. The fermenter offers many possibilities of manipulating the growth conditions and growth rates of microbial cultures.

[9] **Harty, D. W. S. et al.**1988. J. Appl. Bacteriol. **65**: 143-152.