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# Influence of pH and initial lactate concentration on the growth of *Lactobacillus plantarum*

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**Summary.** Fermentation yields of *Lactobacillus plantarum* were measured at controlled pH between 4.0 and 8.0 and initial lactate concentrations of 0-90 g/l. Optimal growth conditions at pH 6.0 without addition of lactate gave a growth rate of 0.57 $h^{-1}$ and 20 g dry biomass/mol ATP formed ($Y_{ATP}$). The pH variations resulted in a decrease in growth rate but the effect on $Y_{ATP}$ was insignificant. The addition of lactate to the medium at 0 h resulted in linear decrease in the growth rate of the culture, and all the metabolic activities were completely inhibited at 110 g/l. The $Y_{ATP}$ and biomass/substrate yield ($Y_{x/s}$) remained fairly steady up to 33 g lactate/l, beyond which both yields decreased considerably.

## Introduction

Although the problem of plant conservation has been solved as a whole in rural temperate zones in recent years, it is still of crucial importance in tropical areas with hot, humid climates that enhance the growth of undesirable pathogenic microorganisms. Silage has proved to be an extremely useful technique for conservation. Trials carried out to improve the technique (Lindgren et al. 1988; Seale 1986) showed that massive inoculation of fodder with *Lactobacillus plantarum* improved the technique. Optimisation of the bulk production of such a bacterium is thus of considerable economic importance.

Physiological and biochemical studies have been carried out in the past decade on other lactic strains (*Streptococcus lactis* and *S. cremoris*) and have shown that lactate excretion can be bound to two protons, thus enabling extra synthesis by an ATPase of two molecules of ATP for each lactose molecule taken up under optimal culture conditions (Maloney and Hansen 1982). The process was reported to lead to a 50% energy gain for homolactic bacteria and 100% for heterolactic bacteria (Brink and Konings 1982). It also appears that the stoichiometry of proton/lactate excretion and the extra energy gain available for the bacteria is strongly dependent on the external pH and lactate conditions (Brink et al. 1985; Otto et al. 1980).

The purpose of this work was to study the effect of pH and lactate concentration on the growth of *L. plantarum*. It was thought that the kinetic study of growth and calculation of fermentation parameters, and especially $Y_{ATP}$ (dry biomass/mol ATP formed), under these different culture conditions would lead to better understanding of the physiology of this microorganism with a view to optimizing biomass production.

## Materials and methods

*Strain and maintenance.* The microorganism used was *L. plantarum* (Lactolabo, Dange Saint Romain, France). The strain was maintained at 4° C and subcultured monthly on the agar slants prepared by adding 1.4% agar to Bacto Lactobacilli MRS broth (Difco, Detroit, Mich., USA).

*Culture conditions.* The growth medium used was MRS liquid medium in which the initial pH and lactate concentrations were varied. Glucose was also added to the medium to a final concentration of 50 g/l. The fermentation studies were carried out in a 2-l bioreactor (Biolafitte, Poissy, France) at 30° C. The pH was set at the desired value with 5 M NaOH and was maintained throughout. The medium was inoculated at the 10% (v/v) level and was agitated at 200 rpm. The inoculum was grown in MRS liquid medium (Difco, without any lactate or glucose) in 250-ml erlenmeyer flasks at 30° C and an initial pH of 6.7 for 20 h.

*Analytical methods.* The biomass concentration was determined by measurement of optical density (OD) at 540 nm related to the dry weight measured after two washing and centrifugation cycles and drying at 105° C for 24 h. The lactic acid, glucose, acetic acid and ethanol contents were determined by HPLC in the supernatant using an Aminex HPX 87H column (BioRad Laboratories, Richmond, Calif., USA) with a 0.8 ml/min flow of 0.006 M $H_2SO_4$ at 65° C and with dual refractive index and UV detection.

*Calculation of the results.* Biomass/substrate yield ($Y_{x/s}$ in g/g), lactate/substrate yield ($Y_{p/s}$ in g/g) and the growth rate ($\mu$ in $h^{-1}$) were obtained by calculating, respectively, the linear regression coefficients of the biomass according to the residual glucose, lac-

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tic acid according to residual glucose and Napierian logarithm (ln) OD according to time during the exponential growth phase only. The $Y_{ATP}$ yield was calculated on the basis of production of two ATP moles per mole of glucose consumed in homolactic fermentation. The different coefficients were calculated with a confidence interval of 0.95; the standard error was about 10% in all estimates.

## Results

### *Growth kinetics and energy uncoupling of L. plantarum*

A growth rate of 0.57 $h^{-1}$, a final biomass concentration of 9.5 g/l and productivity of 1.05 g/l per hour were obtained under standard conditions. It can be seen that growth stopped at about 7 h (Fig. 1). The glucose in the medium at this stage was almost exhausted. The growth of the culture entered the stationary phase at this stage probably due to limitation of nutrients other than the carbon source, thereby leading to energy uncoupling of growth and lactic acid production. The addition of glucose to the medium at this stage did not increase the biomass concentration, but the cells functioned as resting cells, and continued to produce lactic acid until the substrate was exhausted.

### *Effect of pH*

The growth rates calculated from fermentation kinetics at different pH values are shown in Fig. 2. The optimum pH was close to 6.0 and limit values of 3.4 and 8.8 were estimated. The fermentation parameters obtained for each pH are shown in Table 1. The biomass ($Y_{x/s}$), lactate ($Y_{p/s}$) and $Y_{ATP}$ yields were very high and fairly even throughout a large pH range (4.0–8.0). Thus, the data indicated that pH essentially affects the growth rate but not the overall yield.

### *Effect of lactate*

The inhibitory effect of lactate on *L. plantarum* growth was studied by comparing fermentation kinetics at different initial lactate concentrations within the range 0–90 g/l. The fermentation parameters are summarised in Table 2. The data on $Y_{ATP}$ and the growth rates calculated from the fermentation kinetics at different initial lactate concentrations are shown in Fig. 3. The data showed a linear relationship between the growth rate and the initial lactate concentration over a broad concentration range studied. Thus a critical concentration was estimated at 110 g/l at which the growth rate became zero. The $Y_{x/s}$, $Y_{ATP}$ yields and final biomass obtained remained fairly constant up to a concentration of 33 g/l and then decreased rapidly at higher concentrations (Table 2).

It was observed that during this work *L. plantarum* remained homofermentative at all times since no ace-

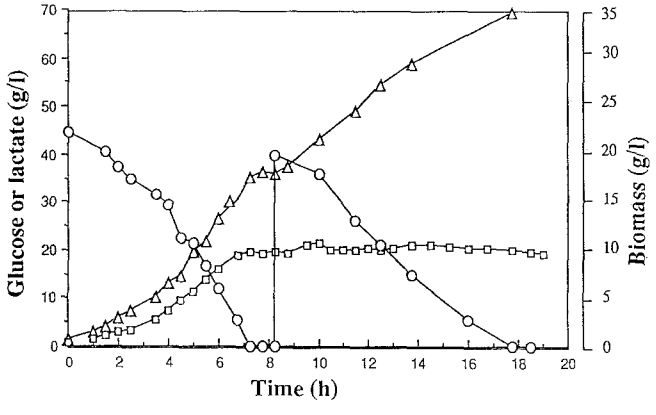


**Fig. 1.** The growth and energy uncoupling of *Lactobacillus plantarum* on MRS medium with addition of 40 g glucose/l after 8 h at 30° C and pH 6: ○, glucose; △, lactic acid; □, biomass

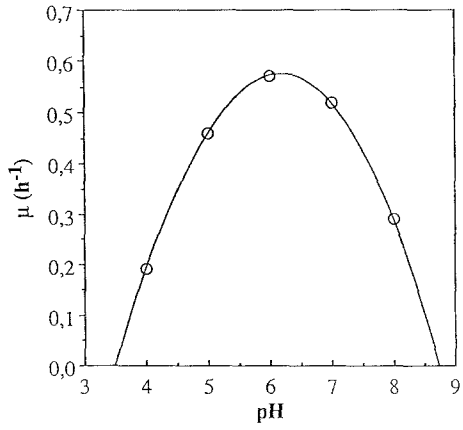


**Fig. 2.** Influence of pH on the growth rate (μ) of *L. plantarum* at 30° C

**Table 1.** Fermentation parameters of *Lactobacillus plantarum* cultivated at different pH settings

| pH | μ | $Y_{x/s}$ | $Y_{p/s}$ | $Y_{ATP}$ | Productivity |
|---|---|---|---|---|---|
| 4.0 | 0.19 | 0.27 | 0.74 | 24.3 | 0.29 |
| 5.0 | 0.46 | 0.25 | 0.70 | 22.9 | 0.77 |
| 6.0 | 0.57 | 0.22 | 0.75 | 19.8 | 1.05 |
| 7.0 | 0.52 | 0.26 | 0.81 | 23.2 | 1.04 |
| 8.0 | 0.29 | 0.18 | 0.81 | 16.2 | 0.52 |

Temperature 30° C, no lactate addition: μ, growth rate; $Y_{x/s}$, biomass/substrate yield; $Y_{p/s}$, lactate/substrate yield; $Y_{ATP}$, dry biomass per mole ATP formed

**Table 2.** Fermentation parameters of *L. plantarum* cultivated at different initial lactate concentrations

| Lactate (g/l) | μ | $Y_{x/s}$ | $Y_{p/s}$ | $Y_{ATP}$ | Productivity |
|---|---|---|---|---|---|
| 0 | 0.57 | 0.22 | 0.75 | 19.8 | 1.05 |
| 12 | 0.55 | 0.22 | 0.73 | 19.8 | 1.01 |
| 24 | 0.49 | 0.24 | 0.75 | 21.9 | 0.80 |
| 33 | 0.41 | 0.22 | 0.74 | 19.8 | 0.58 |
| 48 | 0.37 | 0.19 | 0.61 | 16.7 | 0.45 |
| 90 | 0.06 | 0.08 | 0.65 | 7.1 | 0.09 |

Temperature 30° C, pH 6

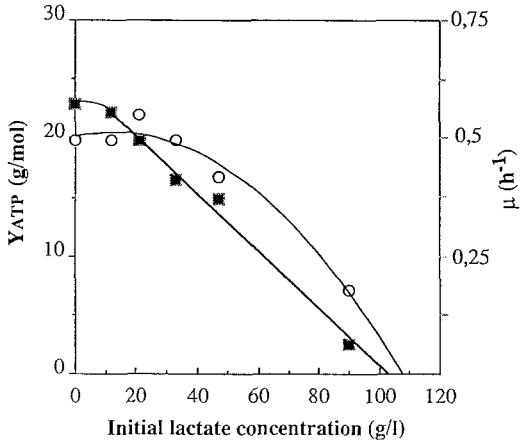


**Fig. 3.** Influence of initial lactate concentration on the growth rate and the dry biomass per mole ATP formed ($Y_{ATP}$) of *L. plantarum* at 30° C and pH 6: ■, μ; ○, $Y_{ATP}$

tate or ethanol production was observed in HPLC analysis.

## Discussion

The maximum theoretical $Y_{ATP}$ of Gram-positive anaerobic bacteria was estimated by Belaïch (1986) at 21–28 g/mol. In the present study, performed on the basis of two ATP moles produced per mole of glucose consumed, the maximum *L. plantarum* $Y_{ATP}$ reached up to $24.3 \pm 2.7$ g/mol, and the average calculated under non-limiting conditions was $21.4 \pm 2.2$, which is very close to the maximum theoretical yield and close to twice the generally reported value of 10.5 g/mol for anaerobic bacteria (Beauchop and Elsden 1960). This is in agreement with Kemp et al. (1989) who obtained a $Y_{x/s}$ of 0.27 g/g, i.e. a $Y_{ATP}$ of 24.3 g/mol, but is much higher than the 9.3 g/mol calculated from the results of Oxenburg and Snoswell (1965). The main differences with the latter work are pH regulation, which can have considerable importance, and also the method used for calculation of $Y_{ATP}$, which takes into account in the present case only the exponential growth phase and not the overall growth phase.

The hypothesis of Brink and Konings (1982), based on the existence of a proton pump system enabling the ATPase synthesis of an extra mole of ATP in *S. cremoris*, may account for the high $Y_{ATP}$ yields observed in *L. plantarum*. However, the results reported here do not allow confirmation of such a system and complementary studies would be necessary.

Study of the pH influence indicates the necessity of keeping the pH at 6.0 to ensure maximum biomass productivity. This result is similar to those of Kemp et al. (1989), who found an optimum pH of *L. plantarum* of 5.5 to 6.5. In addition, it was observed in the present work that the pH had a considerable effect on the growth rate but had little influence on $Y_{x/s}$, $Y_{p/s}$ and $Y_{ATP}$. This confirms the observations of the influence of pH on the growth of *S. cremoris* made by Bibal et al. (1988) who studied the variation of these parameters in a smaller pH range (5.6–7.5).

The metabolism of the strain remained homofermentative under the conditions studied, in contrast to the reports of Rhee and Pack (1980). The latter author observed a change from homofermentative to heterofermentative metabolism in *L. bulgaricus* according to pH. Murphy et al. (1985) showed a heterofermentative metabolism in *L. plantarum* when oxygen was present in the medium. The transition from homolactic to heterolactic metabolism is probably related to specific conditions that were not observed during our research.

The addition of lactate also reduced the growth rate and lowered the $Y_{x/s}$, $Y_{p/s}$ and $Y_{ATP}$ when added at concentrations higher than 33 g/l. It should be noted here that with 90 g lactate/l, the sum of $Y_{x/s}$ and $Y_{p/s}$ yields was $0.73 \pm 0.04$ g/g, which would tend to indicate that under extreme conditions of lactate concentration (1 mol), nearly 25% of the substrate is used to ensure cell maintenance. A concentration of 110 g/l can be considered the maximum tolerated by *L. plantarum*. Bibal et al. (1988) observed a decrease in $Y_{x/s}$ in *S. cremoris* as soon as 12 g/l of lactate was added and estimated the critical concentration to be 70 g/l. It thus appeared that *L. plantarum* was more tolerant to lactic acid than *S. cremoris*. It should be mentioned that sodium hydroxide was used to regulate the pH in this work whereas Bibal et al. (1988) used ammonium hydroxide; the salts (sodium or ammonium lactate) may have a different effect on the behaviour of microorganisms. This hypothesis should be verified and completed using calcium hydroxide.

With regard to massive biomass production, the fermentation yields were very high, but the biomass concentration was limited since when the substrate concentration exceeds 50 g/l, the bacteria switch to a non-proliferation state and quantitatively convert glucose into lactic acid. The optimization of the levels of the other nutrient components of the medium should make it possible to increase the final concentration.

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