INFLUENCE OF MEDIUM COMPOSITION, pH AND TEMPERATURE ON THE GROWTH AND VIABILITY OF *Lactobacillus acidophilus*

P. TAILLANDIER*, F. GILIS, F. RAMON PORTUGAL, P. LAFORCE[1], P. STREHAIANO

Laboratoire de Génie Chimique, CNRS UMR 5503,
ENSIGC, 18 Chemin de la Loge, 31 078 Toulouse Cedex, France

[1] Lallemand S.A., BP 4412, 31405 Toulouse Cedex, France

ABSTRACT

The *Lactobacillus acidophilus* growth was investigated to find the optimal concentrations of 2 nitrogen sources (thought to be in excess) and 2 organic acids. A Plackett and Burman experimental design was used allowing identification of the more important factors with very few experiments. In the studied range, all the factors had a linear effect and a great influence on the final viability. 20 g/L of each nitrogen sources, 3 g sodium-citrate/L and 5 g sodium acetate/L are necessary. pH, temperature and glucose had poor influence.

INTRODUCTION

Lactic acid bacteria are heterotrophic for many amino acids and are generally very demanding nutritionally. Traditionally, these bacteria are cultivated on the medium proposed by de Man, Rogosa and Sharpe, well known as MRS (De Man *et al.*, 1960; Champagne and Gardner, 1990). MRS is a very rich medium in which some nutrients may be supplied in excess.

The aim of this work was to study the conditions of growth of*Lactobacillus acidophilus*. *Lactobacillus spp.* are widely used in many food industries, particularly in fermented milk elaboration (Loones, 1994) and have particularly complex nutritional requirements for amino acids and vitamins (Kandler and Weiss, 1986).The initial culture medium was a modified MRS. The factors thought to be important were components of the medium (sugar, two nitrogen sources and two organic acids) and factors such as pH and temperature. To determine rapidly the importance of these factors we chose a Plackett and Burman design. This kind of design allows the study of the effect of 7 factors with only 12 experiments (Haaland, 1989). The responses studied were the maximal specific growth rate (μmax), the maximal viable biomass obtained (Xmax) and the productivity in viable

biomass (Px) since these criteria are very important when the bacteria is cultivated for commercial purpose (starter cultures).

MATERIAL AND METHODS

Organism and inocula preparation. *Lactobacillus acidophilus* was obtained from Lallemand S.A. (Toulouse, France). The strain was grown in De Man, Rogosa and Sharp (MRS) medium (Biokar). For preparation of inocula, MRS broth (100 ml) was inoculated with 4 ml of frozen bacterial suspension and incubated for a period of 7 hours in a 250 mL flask at 37°C with magnetic agitation at 250 rpm. The fermentors were inoculated with 3% (v/v) of this culture.

Culture medium. The strain was usually grown on a standard medium similar to MRS medium but with different concentrations of some components. Its composition was the following in g/L of water: glucose, 50; peptone E2, (Organotechnie) 12.5, Sodium-citrate, 2; Sodium-acetate, 3; yeast extract, 12.5; K_2HPO_4, 2; $Mg(SO_4)_2$, 0.2; $Mn(SO_4)$, 0.05; Tween 80, 1.08. According to the different experiments of the experimental design the concentration of the following components was modified: glucose, peptone, yeast extract, sodium-acetate, sodium-citrate.

Culture conditions. Batch cultures were performed in 2 L fermenters (Sétric Génie-Industriel, Toulouse). The volume of the medium in the fermenter was 1.6 L. Agitation rate was 400 rpm, aeration rate was 0.2 vvm. In standard conditions the temperature was regulated at 33.5 °C and the pH was regulated at 6 by addition of ammoniac 5M. The broth without glucose and $MgSO_4$ was sterilised in the fermentor 15 min at 120 °C, glucose and $MgSO_4$ were autoclaved separately and then added to the fermentor.

Analytical determination. Biomass was estimated gravimetrically. The cell dry weight (g/L) was obtained by filtration of a known sample volume through a cellulose acetate filter (0.2 µm,), washed with distilled water and dried to a constant weight. Cell viability was estimated by counting with an epifluorescent microscope after vital staining (Lange *et al.*, 1995; Plihon *et al.*, 1995). Viable cell concentration (X g/L) was defined as dry weight multiplied by % of viability. Glucose was analysed by means of YSI enzymatic method with glucose-oxidase (Yellow Spring Instruments).

Experimental design. A Plackett and Burman design was used. 7 factors, X1 to X7, were studied with two levels (-1 and +1 coded values) each one. These factors were respectively: temperature, pH, peptone, yeast extract, glucose, Na-acetate and Na-citrate. The experimental matrix is defined by the coded values of these seven variables and four mid-points (level 0) and shown in Table 1. Table 2 shows the true value of each factor for different levels. Statistical examination of results was made using STATITCF program.

Table 1: Coded values of the experimental design

Experiment	X1	X2	X3	X4	X5	X6	X7
1	+	+	+	-	+	-	-
2	-	+	+	+	-	+	-
3		-	+	+	+	-	+
4	+	-	-	+	+	+	
5	-	+	-	-	+	+	+
6	+	-	+	-	-	+	+
7	+	+	-	+	-	-	+
8	-	-	-	-	-	-	-
9	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0

Table 2: Real values of the factors

	Level -1	Level 0	Level +1
X1 = Temperature (°C)	30.0	33.5	37.0
X2 = pH	5.5	6.0	6.5
X3 = Peptone (g/L)	5.0	12.5	20.0
X4 = Yeast extract (g/L)	5.0	12.5	20.0
X5 = Glucose (g/L)	40.0	50.0	60.0
X6 = sodium-acetate (g/L)	1.0	3.0	5.0
X7 = sodium-citrate (g/L)	1.0	2.0	3.0

RESULTS AND DISCUSSION

1. Kinetics of growth in the standard conditions

Standard conditions correspond to the central values of the variables. Figure 1 shows the kinetics of growth and glucose consumption obtained in this case (mean of the 4 experiments). Glucose was not exhausted at the end of the culture. After 8 hours, dead cells started to appear, reaching about 30% of the total biomass at the end of the culture. At the same time, during the last 4 hours, biomass concentration was not increased despite glucose consumption. This could indicate a limitation in nutrients other than glucose and shows that the point for the harvest of cells must be judicially done at the right moment. The growth kinetics (viable biomass) shows that the lag phase lasts about 4 hours. The specific growth rate μ is defined as the ratio of growth rate (dX/dt) to the cell dry weight concentration (X g/l); μmax is the maximun value.It is reached after 4 hours.

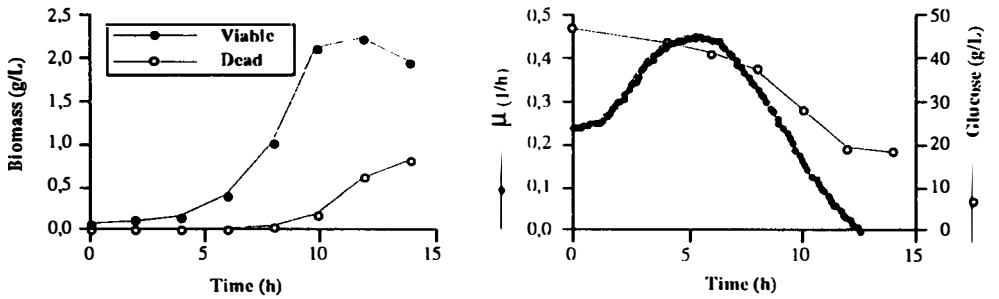

Figure 1: Kinetics in standard conditions.

2. Experimental design

2.1 Results and statistical analysis

Three responses were measured for each experiments: viable Xmax (g/L), μmax (1/h) and viable biomass productivity Px (g/L h). The observed values can be found in Table 3.

Table 3: Values of the responses and viability for each experiment

Experiment	1	2	3	4	5	6	7	8	9	10	11	12
μmax (1/h)	0.45	0.24	0.29	0.38	0.11	0.47	0.47	0.14	0.39	0.41	0.39	0.42
Xmax (g/L)	0.11	3.3	3.2	0.7	2.6	1.3	0.7	0.2	1.2	1.4	0.7	1.0
Pmax (g/Lh)	0.01	0.134	0.133	0.058	0.09	0.133	0.05	0.012	0.10	0.117	0.06	0.088
Viability (%)	4	92	92	95	90	38	23	63	41	46	23	23

To emphasise the importance of the effects a Pareto chart (Haaland, 1989) was built for each response (Figures 2 to 4). The magnitude of the effect of each variable is shown in Pareto chart in decreasing order. The probability of the effect being significant is also plotted on each figure as an histogram.

For μmax the only important factor is the temperature which has a positive effect; for Xmax the more important but negative factor is also temperature; peptone, yeast extract, acetate and citrate are also important but positive; for Px the variables having a great effect are acetate, citrate and peptone.

For all the responses glucose and pH are not very important confirming that the glucose must be in excessive concentration in the medium. This concentration could be decreased in an optimised medium. In many experiments (1 and 6 to 12), at the end of the culture viability dropped to very low values. This was accompanied by the precipitation of solid particles at the bottom of the fermentor. This was already been reported for *L. acidophilus* (Fernadez-Murga *et al.*, 1994) and was attributed to the autolytic activity of the bacteria depending on the pH of the medium.

2.2 Statistical model

The STATITCF program allow us to fit a statistical model giving the variation of the response as a function of the factors and to calculate R^2. It gives the following results:

$$\mu max = 0.34 + 0.12\ X1 \quad 0.0012\ X2 + 0.044\ X3 + 0.026\ X4 \quad 0.0113\ X5 - 0.019\ X6 + 0.016\ X7 \quad (R^2 = 0.94)$$

$$X_{max} = 1.36 - 0.79\ X1 + 0.15\ X2 + 0.47\ X3 + 0.46\ X4 + \quad 0.12\ X5 + 0.45\ X6 + 0.43\ X7 \quad (R^2 = 0.97)$$

$$P_x = 0.082 - 0.015\ X1 - 0.006\ X2 + 0.025\ X3 + 0.0163\ X4 \quad - 0.005\ X5 - 0.026\ X6 + 0.024\ X7 \quad (R^2 = 0.95)$$

The larger the R^2, the more accurately the value of the reponse can be predicted by the model. R^2 is considered good if it is above 0.9 and close to 1 (Haaland, 1989).

In our case, the effect of the 7 factors studied can be modeled by a first degree polynomial for each factor (linear variation).

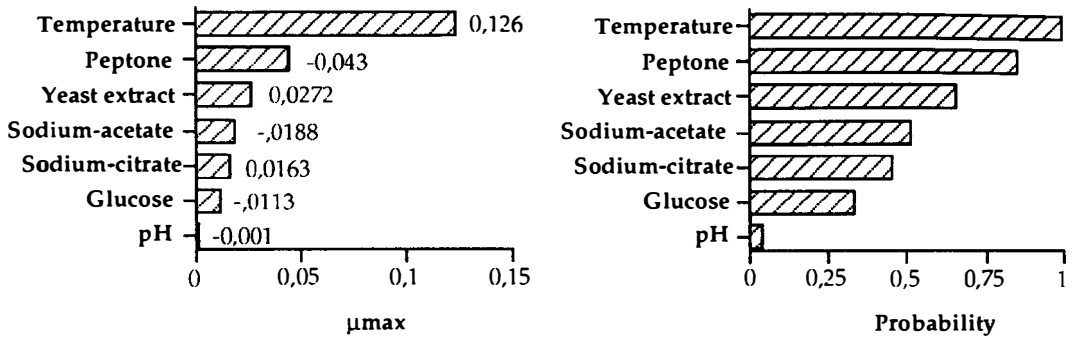

Figure 3: Estimation of the effect of each factor on μmax (1/h).

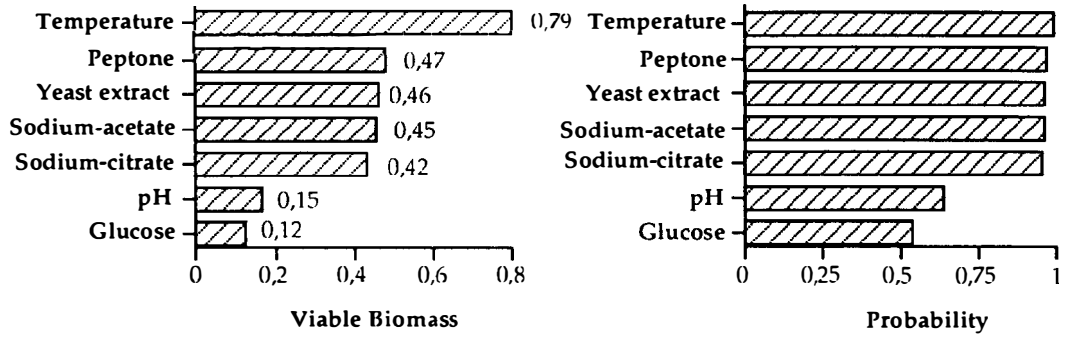

Figure 4: Estimation of the effect of each factor on Xmax (g/L).

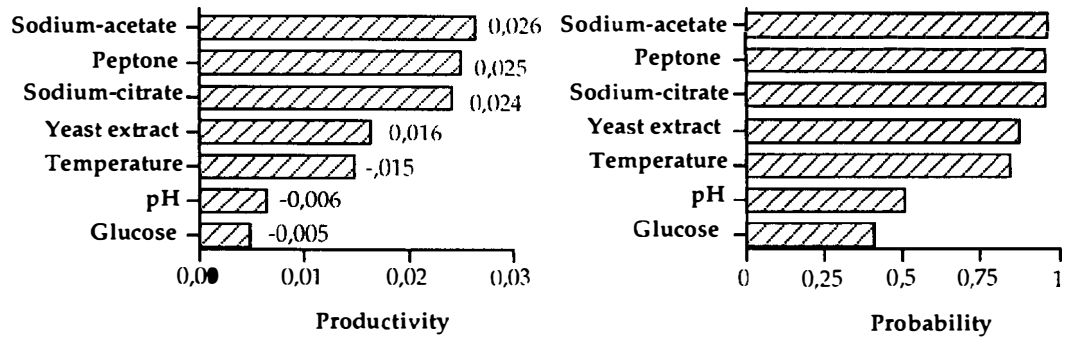

Figure 5: Estimation of the effect of each factor on Px (g/L h).

CONCLUSION

Culture conditions have a great effect on the final viability which is important when the bacteria is produced as a starter culture. Organic acids are well known to stimulate the growth of lactic acid bacteria (Divies *et al.*, 1994; Marshall and Law, 1994). Compared to the classical MRS medium their concentration can be increased a little.

Peptone and yeast extract are necessary for a high production of biomass. When this study was started they were thought being in excess in the culture medium.The experimental design showed that their concentration must be kept at high levels .

The Plackett and Burman design was so an excellent and rapid mean of identifying the main factors from many. However this kind of design does not allow to estimate interactions betweeen factors.

In conclusion, the best conditions for cultivation of this *Lactobacillus* strain are the following: pH, 6.0; temperature, 30°C; 40 g glucose/L; 20 g peptone/L; 20 g yeast extract/L; 5 g sodium-acetate/L; 3 g sodium-citrate/L.

REFERENCES

Champagne, C. and Gardner, N. (1990). *Sc. Alim.* 10, 899-905.

De Man, J. C., Rogosa, M. and Sharpe, M. E. (1960). *J. Appl. Bacteriol.* 23, 130-135.

Divies, C., Frey, L., Hubert, J.C. and de Roissart, H. (1994). Métabolisme d'autres substrats carbonés par les bactéries lactiques. In: Bactéries lactiques, tome I, pp. 291-307, Uriage: Lorica.

Fernadez-Murga, L., De Ruiz Holgado, A. P. and Valdez, G. F. (1994). *Microbiologie Alimentation Nutrition*. 12, 426-428.

Haaland, P. D. (1989). *Experimental Design in Biotechnology*. New York: Marcel Dekker, Inc.

Kandler, O. and Weiss, N. (1986). Regular non-forming spore gram positive rods. In:*Bergey's Manual of determinative Bacteriology*, P.H.A. Sneath, ed. vol. 2 pp.1208-1234, New York: Academic Press.

Lange, H., Plihon, F., Taillandier, P. and Riba, J. P. (1995). *J. Microbiol. Methods*. 24,93-97.

Loones, A. (1994). Laits fermentés par les bactéries lactiques. In: *Bactéries lactiques*, tome II, pp. 135-154, Uriage: Lorica.

Marshall, V.M.E. and Law, B.A. (1984). *Advances in the microbiology and biochemistry of cheese and milk*, F.L. Davies and B.A. Law, eds. Elsevier.

Plihon, F., Taillandier, P. and Strehaiano, P. (1995). *Biotechnol. Technics*. 9, 6, 451-456.