Kinetic growth parameters of different amylolytic and non-amylolytic *Lactobacillus* strains under various salt and pH conditions

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Abstract

Four *Lactobacillus* species were studied for their ability to grow at high NaCl concentrations and different initial pH values. Among these strains, *Lactobacillus plantarum* strains 541 and A6 indicated to be the most salt tolerant. Both strains were able to ferment glucose up to 8% salt and produce lactic acid even at 10% salt. For strain 541, the specific rate of lactate production (q_{lac}) and the yield of lactic acid relative to substrate ($Y_{p/s}$) remained constant up to 6% salt whereas the yield of biomass relative to substrate ($Y_{x/s}$) decreased at higher salt concentrations. In contrast, for strain A6, $Y_{p/s}$ decreased up to 6% salt whereas $Y_{x/s}$ did not vary markedly. At 8% salt, decreasing performance in final biomass and lactic acid production was observed. Both strains were able to reduce pH to values lower than 4.0 within 24h. A factorial design was applied to study combined effects of salt and initial pH on the fermentation parameters. It is shown that salt and pH do not interact significantly within the established experimental domain, and that pH is the more dominant factor. Considering overall performance, it is concluded that 4% salt and pH between 6.0-6.6 can be taken as appropriate conditions, for the use of both strains as starter in processes where higher salt concentrations are preferred.

Abbreviated title: *Lactobacillus* salt tolerance

Keywords: *Lactobacillus, lactic acid, salt tolerance*

INTRODUCTION

Fermentation using lactic acid bacteria is widely used in food fermentation. The lactic acid bacteria need to withstand varying environmental conditions including differences in temperature, pH and salinity, depending on the specific application. An enhanced salt and pH resistance of lactic acid bacteria is attractive in shrimp waste, fish, vegetables and seafood fermentation.

Glucose is frequently used as a carbon source in lactic acid fermentation. For instance, it had been used to promote lactic acid fermentation of shrimp wastes (Rao et al. 2000). Alternatively, the use of amylolytic lactic acid bacteria as starter cultures, that have ability to hydrolyse starch (Giraud et al. 1991) or glycogen (Pintado et al. 1999), might allow the use of a cheaper and locally available carbon source (e.g. cassava flour), reducing the costs of the process. For application of these strains in processes where high salt concentrations are required or are naturally present, their salt tolerance should be studied. Combined effect of low initial pH and salt addition may present additional advantages.

This work investigates the salt tolerance of the three amylolytic lactic bacteria (ALAB) compared to one non amylolytic lactic acid bacterium. The three (ALAB) strains have been selected on the basis of their reported efficiency in hydrolysing starch: *Lactobacillus manihotivorans* OND 32^T (Morlon-Guyot et al. 1998, Guyot et al. 2001), *Lactobacillus plantarum* A6 (Giraud et al. 1991) and *Lactobacillus fermentum* Ogi E1 (Agati et al. 1998). The non-amylolytic strain is *Lactobacillus plantarum* 541 previously used for shrimp waste

fermentation. To obtain comparable growth kinetic parameters from salt tolerance experiments all strains were grown on glucose medium. The effect of salt concentration and the interactions between salt and pH on kinetic parameters were investigated on the most salt tolerant strains, by using a factorial design.

Materials and methods

Microorganisms and cultivation methods

The non-amylolitic *strain Lactobacillus plantarum* 541 was obtained from Thailand Institute of Scientific and Technological Research Centre, Bangkok, Thailand (Rao et al., 2000). The amylolytic strains *Lactobacillus plantarum* A 6 (LMG 18053, BCCM, Gent, Belgium) was isolated from the process of cassava retting (Giraud et al., 1991), *Lactobacillus fermentum* OGI E1 (I-2028, CNCM, Paris, France) was isolated from maize sourdough (Agati et al., 1998) and *Lactobacillus manihotivorans* OND 32 (LMG 18010^{T}, BCCM, Gent, Belgium) was isolated from cassava sour starch (Morlon Guyot et al., 1998). Strains were stored in 40% glycerol at -80°C. All strains were routinely cultivated in Lactobacilli MRS medium (DeMan et al., 1960), using glucose as substrate (20 g/l). Overnight cultures in Erlenmeyer flasks were used as inoculum (5% v/v). All media were sterilized for 20 min at 115 °C.

Fermentation conditions

Fermentation was carried out at 30° C in capped 250 ml Erlenmeyer flasks with 150 ml MRS broth media (Difco, USA) containing different concentrations of added salt (NaCl)

and inoculated with 10% (v/v) cultures incubated at 30°C for 12 h. To allow the comparison between non-amylolytic and amylolytic lactic acid bacteria and the calculation of kinetic parameters, glucose was used as a substrate to all cultures. In the experiments with pH and salt combinations, the pre-specified initial pH values was achieved by adding phosphate-citrate buffer (0.2 M sodium hydrogenophosphate and 0.1 M citric acid mixed in different ratios). The final pH adjustment was made by adding 5 N NaOH.

Analytical methods

Samples were obtained at regular intervals under sterile conditions. Optical density (OD) at 600 nm was measured after appropriate dilution using a Jenway spectrophotometer (6405UV/Vis., Bioblock, France). The values of OD_{600} were corrected by subtracting the OD_{600} of inoculum added to the cultures. The biomass (g/l) was determined based on individual biomass-OD_{600} calibration curves obtained for each strain. Biomass was collected after two centrifugation steps at 5500 rpm for 20 minutes each, with intermediate washing. The biomass was subsequently dried at 105°C for 24 h. For chemical analysis, 1.3 ml of broth culture was transferred to microtubes containing either 0.2 ml 5N NaOH or 0.2 ml 2N H_2SO_4 (for assay of reducing sugars and lactic acid, respectively) and then centrifuged at 10,000 rpm for 10 min. The supernatant was frozen at -20°C until further analysis. The consumption of reducing sugars (RS) was analysed using the DNS method (Miller 1959). Lactic acid (LA) was analysed by HPLC (Waters, Milford, USA) after membrane filtration (Whatman 0.22 μm, cellulose acetate membrane) using an Aminex HPX 87 H column (Biorad Laboratories, Richmond, CA, USA) with a Cation - H refill ref:

125-0129 and a refractive index detector (Phillips PU 4026). Flow rate of 0.8 ml/min of 6mM H_2SO_4 as carrier was maintained at 65°C (Waters 515 HPLC pump).

Kinetics

Kinetic parameters and growth yields were determined for cultures at different pH and salt concentrations. Maximum specific growth rate (μ_{max}, h^{-1}) was calculated as the slope of ln(biomass) vs. time during the exponential growth phase. Growth and lactate yields on substrate ($Y_{x/s}$ and $Y_{p/s}$, respectively) were calculated as the slope of the linear regression ($r_{0.01}$) of either produced biomass or lactate vs consumed substrate. Specific rate of lactate production (q_{lac}) was estimated as indicated by Pirt (1985). All experiments were conducted in duplicate.

Factorial Design

The combined effect and interaction between salt concentrations and initial pH on the growth parameters (μ_{max}, X_{max} and P_{max}) for the two selected strains were studied. X_{max} and P_{max} represent the maximal biomass and product concentration obtained during the fermentation period. A second order rotatable plan, with five replicates at the centre of the domain was used to obtain empirical models (Box et al. 1978). The values of salt concentrations and pH levels used in the rotatable matrix are presented in Table 1. The criterion for model acceptance was based on the Student's t test ($\alpha<0.05$) for the coefficients, and on the Fisher's F test ($\alpha<0.05$), applied to the ratios : experimental error/total error and lack of fit/experimental error, for global validity.

Results and discussion

Salt tolerance of different Lactobacillus strains

L. plantarum 541 and A6, *L. fermentum* Ogi E1 and *L. manihotivorans* OND 32^T were tested on MRS media at 30°C with concentrations of salt of 0, 2, 5, 10, 20 and 30% (w/w). The comparison of growth, based on OD_{600} values of the samples obtained at 24h (Fig. 1), showed that strains 541 and A6 of *L. plantarum* had higher salt tolerance. Both strains could grow, although at different rates, in MRS with added NaCl concentrations as high as 10 %. On the other hand the two strains OND 32 and OGI E1 did not show any remarkable resistance to salt. At 24 h fermentation in the presence of 10% salt, *L. plantarum* strains 541 and A6 produced only low amounts of biomass (0.1 and 0.5 g/l, respectively) and lactic acid (1.3 and 4.8 g/l, respectively). But upto 8% salt, reasonable growth and lactic acid production were observed (Fig. 2). Due to their salt resistance, one non-amylolytic and one amylolytic *L. plantarum* strains (541 and A6, respectively) were selected for further investigation.

Comparison of glucose fermentation by Lactobacillus plantarum strains 541 and A6 at different salt concentrations

For both strains 541 and A6, biomass production decreased similarly as salt concentration increased (Fig. 2). A decrease in glucose conversion efficiency was also observed at 8% salt but there was still a significant lactic acid production (Fig. 2). At 6 and 8% salt, strain 541 was able to convert more efficiently glucose and to produce more lactic acid than strain

A6. Growth started without any lag phase at salt concentrations lower than 6%. At 6% salt, a 3 h lag phase was observed for both strains. At 8% salt, 5h and 4h lag phases were observed for strain 541 and A6, respectively. For both the strains the final pH at different salt concentrations was lower than 4.0 (Fig. 2). Kinetics parameters were further analysed to enable better comparisons between both strains.

Kinetic parameters of strain 541 at different salt concentrations

For strain 541, the yield of biomass on substrate ($Y_{x/s}$) decreased from 0.30 to 0.18 (g biomass per g consumed glucose) at increasing salt concentrations (Fig. 3) suggesting that carbon assimilation was negatively affected. Product yields related to substrate consumption ($Y_{p/s}$, g lactic acid/g consumed glucose) remained constant for strain 541 at all salt concentrations. It was also observed that specific lactic acid production rate (q_{lac}) remained constant up to 6% salt, whereas specific growth rate (μ) progressively decreased between 2 and 8% salt (Fig. 3). These data suggests uncoupling between growth and energy generation. Energy expenditure in other functions than growth, could be explained by an increased ATP requirement to expel out of the cell Na^+ ions and for other maintenance functions. Furthermore, it has been reported for *Lactococcus lactis* that heat shock proteins (DnaK, GroEL, GroES) were also induced by salt stress (Kilstrup et al. 1997). In the hypothesis that other lactic acid bacteria could similarly produce such salt stress induced proteins at high salt concentrations, and since protein synthesis requires the highest ATP expenditure compared to other cell macromolecules (Verduyn et al. 1990), it can thus be

expected that change in biomass composition, i.e. synthesis of additional proteins, would decrease ATP availability for growth.

Kinetic parameters of strain A6 at different salt concentrations

Effect of high salt concentration on specific growth rate was similar for strain A6 (Fig. 3). However, from the other parameters it was concluded that strain A6 had different physiological characteristics at high salt concentrations (Fig. 3). For strain A6, $Y_{x/s}$ did not vary markedly for salt concentrations up to 6%. In the same range of salt concentrations, it was observed that both $Y_{p/s}$ and q_{lac} decreased (from 0.79 to 0.60 for $Y_{p/s}$, from 1.1 to 0.7g g^{-1} biomass h^{-1} for q_{lac}). These results suggest that unlike strain 541, the energy yielding pathway in strain A6 is being progressively affected at increasing salt concentrations (up to 6% salt) but that enough energy is still available for cell component synthesis.

Combined effect of salt and initial pH on L. plantarum 541 and A6

Since interactions between salt concentration and initial pH can be expected, their combined effect was studied by means of a complete second-order factorial plan, using an orthogonal rotatable design whose experimental domain and coding criteria are shown in Table 1. This approach allowed us to obtain, with a limited number of experiments, empirical equations that represented the interaction and weightage of the two variables, in the following form:

$$Y = a_1 + a_2.pH + a_3.\ NaCl + a_4.\ pH.NaCl + a_5.\ (pH)^2 + a_6.\ (NaCl)^2 \qquad (1)$$

The values of the coefficients a_1, a_2, a_3, a_4, a_5 and a_6 obtained for the parameters X_{max} (maximal biomass, g/l), P_{max} (maximum lactic acid concentration, g/l) determined at 12h and μ_{max} (maximum specific growth rate, h^{-1}) are presented in Table 2. Results of the experimental plan for *L. plantarum* strains 541 and A6 are presented in Table 3.

Combined effect of salt and pH on Lactobacillus plantarum 541

The values of the coefficients for X_{max} (maximum biomass), μ_{max} and P_{max} (maximum lactic acid concentration), indicate that among the two variables, pH was the dominant factor. The coefficients for pH were high in comparison to those for NaCl. The effect of pH is stronger for X_{max} and P_{max} than for μ_{max}. Unlike expected, the interaction between salt and pH was not significant for the three parameters.

For μ_{max} and P_{max} there is no first-order effect of NaCl. Only a negative second order effect is significant, which reflects that salt concentration, within the experimental domain, may have negative effects on growth, biomass and product formation at high values. Furthermore, for X_{max}, NaCl has a negative first order effect, which means a higher sensibility of this parameter against salt.

The response surfaces reflect the weightage of pH and salt effects (Fig. 4). The growth is more affected by the pH and relatively less by the higher salt concentration up to 5 %. The optimum values for pH and salt, calculated from the equations, are respectively, for μ_{max}: 6.60 and 2.50%, for X_{max}: 6.32 and 1.27% and for P_{max}: 6.45 and 2.50%. This also indicates

that the effect of salt on growth and lactic acid production are more significant than the effect of salt on production of biomass. .

Combined effect of salt and pH on *Lactobacillus plantarum A6*

The values of the coefficients for the parameters, X_{max}, μ_{max} and P_{max} (12h) indicate similar trends as those for strain 541. The pH proved to be the determining factor with high values of coefficients for pH in comparison to those with NaCl. The effect of pH is stronger for X_{max} and P_{max} than for μ_{max} and there was no significant interaction between these three parameters. For μ_{max} and X_{max}, there was a negative first order effect of NaCl. Negative second order effect was significant for pH too.

As indicated by the response surfaces (Fig. 5), the growth is dominated by pH and only limited by salt concentration. The optimum values for pH and salt, calculated from the equations, are for μ_{max}: 6.14 and 1.31% (respectively), for X_{max}: 6.29 and 2.5% and for P_{max}: 6.34 and 2.50%. Unlike strain 541, strain A6 required lower salt concentration for growth whereas higher salt concentrations were required for maximum lactic acid and biomass production, furthermore optimum pH were slightly lower for strain A6 than for strain 541.

Discussion

L. plantarum is known to grow at low pH. For instance, *L. plantarum* WSO and *L. plantarum* A6 can grow at pH values as low as 4.5 (McDonald et al. 1990, Guyot et al.

2000). This feature allows *L. plantarum* strains to participate in the last stage of natural lactic acid fermentations of plant material (Daeschel and Nes, 1995). But as shown in Fig. 5 and 6, specific growth rates may significantly vary according to the strain and pH value at the onset of fermentation. Strain 541 is more tolerant than strain A6 at extreme pH values (Fig. 5 and 6). Kinetics parameters emphasised different physiological behaviours between both strains, which deserve further investigation. Variability in salt tolerance has also been shown for other *L. plantarum* strains. At initial pH 6.4, Montaño et al. (1993) reported that non-amylolytic *L. plantarum* strains H4 and 221, isolated from olive fermentation, show respectively growth or no growth at 6% salt. This indicates a great intraspecies variability in response to stress induced by technological conditions, and that a particular care must be taken in selecting strains to be used as starter culture in high salt containing food systems.

Conclusions

Both *Lactobacillus plantarum* strains can produce lactic acid and reduce the pH to values lower than 4.0 at salt concentrations up to 8%. At high salt concentrations uncoupling between growth and energy production indicates that lactic acid production is still possible even in detrimental conditions for growth. Factorial designs indicate for both *L. plantarum* strains, that salt and pH did not interact significantly within the experimental domain and that pH was the most determining factor for glucose fermentation, in the range of salt concentration tested.

Depending on the strains and parameters, maximum specific growth rate, cell biomass and lactic acid production were at salt concentrations of either 1.3% or 2.5%. However since the ability of both strains to grow without lag phase up to 4% salt, it is concluded that a 4% salt concentration and initial pH between 6.0 and 6.6 can be a good compromise for their use. These more drastic conditions would still allow an efficient lactic acid production to prevent growth of spoilage microorganisms. Considering that either *L. plantarum* 541 or A6 present potential to be used in processes containing high salt concentration, the choice of one of the two strains for raw material processing will depend mainly on the type of carbohydrate to be added in. If glucose is planned to be used as a growth substrate for acidification, strain 541 would be preferred to strain A6. Notwithstanding, for economical reasons the use of other alternative carbon sources, containing complex carbohydrates such as starch or glycogen, may be preferred. For instance, the ability of strain A6 to ferment glycogen contained in mussel processing wastes has been reported (Pintado et al. 1999). The use of this type of wastes would present economical advantages. Other types of sea-food processing wastes might also be considered for lactic acid production, which opens a broad range of applications to be investigated.

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Captions of Figures.

Figure 1. Effect of salt concentration on growth (OD_{600nm} at 24h) of different *Lactobacillus species*.

Figure 2. Fermentation patterns of glucose by *L. plantarum* strains 541 and A6 at different salt concentrations. □, 0%; ○, 2%; A, 4%; ■, 6%;●, 8%. (X: biomass; RS: glucose as reducing sugar; P: lactic acid).

Figure 3. Growth yield ($Y_{x/s}$, g biomass g^{-1} consumed substrate), product yields ($Y_{p/s}$, g lactic a. g^{-1} consumed substrate), specific growth rate (μ, h^{-1}) and specific lactic acid production rate (q_{lac}, g lactic acid. g $biomass^{-1}$ h^{-1}) at different salt concentrations for *L. plantarum* strains 541 and A6. Δ, $Y_{x/s}$(541); σ, $Y_{x/s}$(A6); ○, $Y_{p/s}$(541); λ, $Y_{p/s}$(A6), A, μ(541); F, μ(A6); Ü, q_{lac} (541); P, q_{lac}(A6).

Figure 4. Effect of NaCl and initial pH on μmax, maximum cell biomass (Xmax) and maximum lactic acid (Pmax) production for *L. plantarum* 541.

Figure 5. Effect of NaCl and initial pH on μmax, maximum cell biomass (Xmax) and maximum lactic acid (Pmax) production for *L. plantarum* A6.

Table 1. Experimental domain and codification criteria of the independent variables used to study the combined effect of salt and pH on *Lactobacillus plantarum* strains 541 and A6.

Coded Values	Natural values	
	Salt (%)	pH
+1.41	5.00	8.0
+1	4.27	7.4
0	2.50	6.0
-1	0.73	4.6
- 1.41	0	4.0
Increments	1.77	1.4

Table 2. Coefficients of empirical equations obtained for Y (μ_{max}, X_{max} and P_{max}) :
$Y = a_1 + a_2.pH + a_3.NaCl + a_4.pH.NaCl + a_5.(pH)^2 + a_6.(NaCl)^2$

Parameter(Y)	a_1	a_2	a_3	a_4	a_5	a_6	R^2
L. plantarum 541							
μ_{max}	0.37	0.06	-	-	-0.07	-0.01	0.99
X_{max} (g/l)	5.38	0.63	-0.25	-	-1.40	-0.18	0.99
P_{max} (g/l)	16.66	2.37	-	-	-3.94	-0.88	0.95
L. plantarum A6							
μ_{max}							
X_{max} (g/l)	0.35	0.03	-0.04	-	-0.15	-0.03	0.92
P_{max} (g/l)	4.46	0.70	-0.45	-	-1.64	-	0.91
	15.87	3.03	-	-	-6.14	-	0.97

T**able 3.** Results of the experimental plan for *L.plantarum* strains 541 and A6

		L. plantarum 541			*L. plantarum* A 6		
PH	NaCl	μ_{max}	P_{max} g/l	X_{max} g/l	μ_{max}	P_{max} g/l	X_{max} g/l
1	1	0.34	13.15	4.06	0.20	13.54	3.81
1	-1	0.35	13.52	4.54	0.23	10.08	3.33
-1	1	0.22	8.12	2.91	0.07	3.80	1.17
-1	-1	0.23	9.25	3.19	0.11	5.28	1.73
1.41	0	0.33	13.59	3.60	0.05	7.74	1.74
-1.41	0	0.17	5.65	1.79	0.05	0.91	0.76
0	1.41	0.36	15.03	4.68	0.20	14.07	2.77
0	-1.41	0.38	15.44	5.58	0.40	15.86	5.28
0	0	0.37	16.17	5.31	0.32	15.62	4.70
0	0	0.37	16.89	5.37	0.37	14.72	4.64
0	0	0.37	16.17	5.47	0.34	15.75	4.00
0	0	0.38	16.97	5.39	0.36	17.34	4.51
0	0	0.37	17.09	5.35	0.35	15.90	4.47