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Biphasic kinetics of growth and bacteriocin production with *Lactobacillus amylovorus* DCE 471 occur under stress conditions

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Micro-organisms used during the production of fermented foods are subjected to several abiotic stresses. Microbial survival during these processes strongly depends on the ability of the cells to adapt and become more tolerant to the environmental conditions. Cultivation of *Lactobacillus amylovorus* DCE 471, a potential strain for use during type II sourdough fermentations, at low temperatures, unfavourable pH and high salt concentrations resulted in biphasic growth patterns. In addition, two separate bacteriocin peaks, as well as a dramatic change in cellular morphology, were observed. In general, an increase of the specific bacteriocin production occurred during the second growth phase. Finally, the observed sugar consumption profiles were affected by the applied fermentation temperature. Moreover, the highest bacteriocin activity occurred during maltose consumption at a low constant temperature of 28 °C and a constant pH of 5·4. Plate counts from both growth phases revealed the existence of two colony types. Irregular colonies were found to outnumber smoother colonies during the first growth phase, while the second growth phase was characterized by a greater number of smooth colonies. Electron microscopy was used to investigate the observed morphological switch at the single-cell level. Single, rod-shaped cells changed into elongated cells that grew in chains. Colony and cell morphology changes coincided with the biphasic growth pattern.

INTRODUCTION

Fermentation is one of the oldest preservation techniques used for raw materials such as milk, meat, vegetables and cereals (Caplice & Fitzgerald, 1999). Lactic acid bacteria are the primary group of micro-organisms involved in most food fermentations (Wood, 1997). They are mainly involved in the acidification of the raw material and they also contribute to the development of the texture and unique organoleptic properties of fermented food products. In addition, they are responsible for the production of antimicrobial compounds, such as organic acids (lactate, acetate), ethanol, hydrogen peroxide and bacteriocins (De Vuyst & Vandamme, 1994), which contribute to the inhibition of the background microflora, food spoilers and food-borne pathogens present in the raw materials. However, during fermentation not only the contaminating flora but also the desired microflora, including the starter culture, have to cope with various growth-restricting conditions. For example, during sausage fermentations, bacteria are stressed by the lactic acid produced by the starter cultures and by the addition of nitrate, nitrite and salt, which result in lowered water activity (Leistner, 1995). In contrast, the main stress factor encountered during sourdough fermentation results from the production of lactic and acetic acid. During food processing, microbial survival depends on sensing these environmental changes as well as on the ability of the starter bacteria to adapt quickly and to become more tolerant to environmental stresses. This phenomenon is referred to as stress hardening (Lou & Yousef, 1997).

Lactobacillus amylovorus DCE 471, an isolate from corn steep liquor, has been shown to produce the bacteriocin amylovorin L471 (Callewaert *et al.*, 1999). This bacteriocin has been identified as being small, thermostable and strongly hydrophobic, with antagonistic activity towards closely related strains (De Vuyst *et al.*, 1996b). The homofermentative producer strain has also been reported to be a fast and strong acidifier that confers a competitive advantage (De Vuyst *et al.*, 1996a, b). The growth and bacteriocin production kinetics of *L. amylovorus* DCE 471 in MRS

Abbreviations: CDM, cell dry mass; SSM, sourdough simulation medium.

medium have been the subject of previous investigations (De Vuyst *et al.*, 1996a, b; Lejeune *et al.*, 1998; Callewaert *et al.*, 1999). Recently, it was reported that the temperature and pH conditions that prevail during sourdough fermentations correspond to the range of conditions for good growth, acidification and bacteriocin production by *L. amylovorus* DCE 471 (Messens *et al.*, 2002). Therefore, this strain may be useful as a starter culture in the competitive cereal environment, provided it withstands particular environmental stresses. *L. amylovorus* DCE 471, however, was found to exhibit biphasic growth kinetics when cultivated under suboptimal growth or stress conditions. These growth patterns are characterized by two distinct exponential-growth phases and two separate bacteriocin-production phases.

In this study, the growth limits and the stress hardening capacity of *L. amylovorus* DCE 471 were explored from a kinetic point of view. First, biokinetic parameters characteristic for growth and bacteriocin production were calculated to describe the influence of environmental stress caused by unfavourable temperature, pH and salt conditions that resulted in biphasic fermentation kinetics. Second, the biphasic fermentation kinetics were explained by a stress response and stress resistance that coincided with an altered colony and cell morphology.

METHODS

Micro-organisms and media. *L. amylovorus* DCE 471 was the bacteriocin-producing strain used throughout this study. *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901^T was used as the indicator organism to determine the amylovorin L471 activity levels (De Vuyst *et al.*, 1996b). Both strains were stored at −80 °C in de Man–Rogasa–Sharpe (MRS) medium (Oxoid), containing 25 % (v/v) glycerol as a cryoprotectant. A series of fermentations was performed in a sourdough simulation medium (SSM) (Messens *et al.*, 2002) containing (l^{-1}): 10 g tryptone (Oxoid); 12 g yeast extract (VWR International); 5 g Lab-Lemco (Oxoid); 0·5 g cysteine/HCl; 0·2 g $MgSO_4.7H_2O$; 0·05 g $MnSO_4.H_2O$; 2 g KH_2PO_4; 1 ml Tween 80; 10 g maltose plus 10 g fructose as energy sources. Before experimental use, fresh cultures of *L. amylovorus* and *L. delbrueckii* subsp. *bulgaricus* were prepared by propagating the strains twice in SSM and MRS broth, respectively. The cultures were always incubated at 37 °C for 12 h. Bacterial cells were enumerated after plating onto media containing 15 g granulated agar l^{-1} (Oxoid).

Inoculum preparation. This consisted of two steps. First, 10 ml SSM was inoculated with 0·1 ml of a freshly prepared *L. amylovorus* culture and incubated at 37 °C for 12 h. This pre-culture was used to inoculate 90 ml SSM. After incubation at 37 °C for 12 h, this second pre-culture was used to inoculate the fermenter.

Fermentation experiments. *In vitro* fermentation experiments were carried out in a 15 l laboratory fermenter (Biostat C; B. Braun Biotech International) with a working volume of 10 l. Preparation of the fermenter and on-line control of the fermentation process (temperature, pH, agitation) were performed as described previously (Messens *et al.*, 2002).

The influence of sublethal environmental conditions on cell growth and bacteriocin production by *L. amylovorus* DCE 471 was assessed. Therefore, the strain was cultivated at a constant pH of 5·4 and a temperature of 28 and 31 °C, a constant temperature of 37 °C and a constant pH of 6·4, and in the presence of 3 % (w/v) NaCl at a constant temperature of 37 °C and a constant pH of 5·4. All experiments performed under these conditions resulted in biphasic kinetics for growth, sugar consumption and bacteriocin production.

Assays. At regular time intervals, samples were aseptically withdrawn from the fermentation vessel and immediately cooled on ice. The optical density of the samples was measured at 600 nm (Uvikon 923; Kontron Instruments). Determination of biomass concentration (X), colony-forming units (c.f.u.), total lactic acid concentration (L), residual maltose (M) and fructose (F) concentrations, and bacteriocin activity levels (B) were performed as described elsewhere (De Vuyst *et al.*, 1996a, b; Lejeune *et al.*, 1998; Messens *et al.*, 2002). In brief, biomass (as cell dry mass; CDM) was determined gravimetrically after membrane filtration. Bacteriocin activity was determined by a twofold critical dilution method. Activity was expressed in arbitrary units (AU) per ml or mega arbitrary units (MAU) per litre. The lactic acid concentration and the residual fructose and maltose concentrations were determined by HPLC using a Waters chromatograph. The standard deviations for the maltose, fructose, lactic acid and CDM measurements were 0·040, 0·035, 0·025 and 0·11 g l^{-1}, respectively.

Modelling. The mathematical equations represented in Table 1 were used to describe growth and product formation by *L. amylovorus* DCE 471. These equations were integrated with the Euler integration technique in Microsoft Excel 2000. To avoid unrealistic fitting solutions without physiological relevance, and computational solving problems (e.g. convergence problems), all parameters needed for the modelling were estimated by manual adjustment until a good visual fit of the curves was obtained.

Biphasic fermentation kinetics. To study biphasic fermentation kinetics in detail, the fermentation carried out in SSM containing 3·0 % (w/v) NaCl was taken as an example. The inoculum culture as

Table 1. Equations used for the primary model development

X, Biomass concentration (g CDM l^{-1}); X_{max}, maximum attainable biomass concentration (g CDM l^{-1}); μ_{max}, maximum specific growth rate (h^{-1}); t, time (h); λ, duration of the lag phase (h); n, inhibition exponent; α, specific rate of death (h^{-1}); M, residual maltose concentration (g maltose l^{-1}); F, residual fructose concentration (g fructose l^{-1}); m_M, maintenance coefficient of maltose [g maltose (g CDM)$^{-1}$ h^{-1}]; m_F, maintenance coefficient of fructose [g fructose (g CDM)$^{-1}$ h^{-1}]; $Y_{X/M}$, cell yield coefficient based on maltose [g CDM (g maltose)$^{-1}$]; $Y_{X/F}$, cell yield coefficient based on fructose [g CDM (g fructose)$^{-1}$]; L, amount of lactic acid produced (g lactic acid l^{-1}); $Y_{L/S}$, yield coefficient for lactic acid production based on fructose and maltose [g lactic acid (g fructose + g maltose)$^{-1}$]; B, soluble bacteriocin activity (AU l^{-1}); k_B, specific bacteriocin production [AU (g CDM)$^{-1}$]; k_{inact}, apparent rate of bacteriocin inactivation [l (g CDM)$^{-1}$ h^{-1}]. AU, arbitrary unit.

Model	Equation
Cell growth	$dX/dt=[\mu_{max}\times(1-X/X_{max})^n-\alpha]\times X$ with $t>\lambda$
Sugar consumption	$dM/dt=-1/Y_{X/M}\times dX/dt-m_M\times X$
	$dF/dt=-1/Y_{X/F}\times dX/dt-m_F\times X$
Lactic acid production	$dL/dt=-1/Y_{L/S}\times(dM/dt+dF/dt)$
Bacteriocin production	$dB/dt=k_B\times dX/dt-k_{inact}\times X\times B$

well as samples taken at the beginning, the middle and the end of each growth phase were analysed by plating and scanning electron microscopy to follow the switch in colony morphology at the onset of the second growth phase. The samples were also analysed by PCR amplification of repetitive bacterial DNA elements (rep-PCR fingerprinting) and PFGE to exclude the presence of a possible contaminant. Cell enumeration was performed by plating onto SSM containing maltose plus fructose as energy source.

rep-PCR fingerprinting. Total DNA from cells obtained through microcentrifugation (13 000 r.p.m., 15 min) of 1·5 ml samples was extracted as described previously (Gevers *et al.*, 2001). PCR amplifications were performed with a DNA thermal cycler GeneAmp PCR System 9600 (Perkin Elmer) as described previously (Versalovic *et al.*, 1994), using Goldstar DNA polymerase (Eurogentec) and the primer $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′). Electrophoresis of the PCR products was performed in a 1·5 % agarose gel (15 × 20 cm) for 16 h at a constant voltage of 2 V cm^{-1} in 1 × TAE buffer (40 mM Tris/acetate, 1 mM EDTA, pH 8·0) at 4 °C. The rep-PCR profiles were observed after staining of the gel with ethidium bromide and visualization under UV light, followed by digital image capturing using a CCD camera (Fotodyne). The resulting fingerprints were analysed using the BIONUMERICS version 2.0 software package (Applied Maths). The similarity among digitized profiles was calculated using the Pearson correlation. A mean linkage (UPGMA) dendrogram was derived from the profiles.

PFGE of chromosomal DNA. The preparation of genomic DNA was performed *in situ* in agarose blocks by the method of Hung & Bandziulis (1990) with slight modifications. At regular intervals, cells were harvested from 10 ml fermentation liquor by microcentrifugation (13 000 r.p.m., 15 min), washed with 50 mM EDTA (pH 8·5) and suspended in 300 µl per OD_{600} unit of the same buffer. After pre-heating 1·1 % low-melting-point (LMP) agarose prepared in 50 mM EDTA (pH 8·5) and cooling to 45 °C, 125 µl of the cell suspension was mixed with 750 µl LMP agarose. The mixture was solidified in a plug mould for at least 15 min at 4 °C. The plugs were incubated overnight at 37 °C in lysis buffer (50 mM EDTA, pH 8·5; 0·05 % *N*-laurylsarcosine; 2 mg lysozyme ml^{-1}; 12·5 U mutanolysin ml^{-1}). Proteinase K treatment was performed overnight at 50 °C in NDS buffer (10 mM Tris, pH 8·0; 1 % SDS; 0·5 M EDTA, pH 8·5; 2 mg proteinase K ml^{-1}). Plugs containing lysed cells were washed six times in 50 mM EDTA (pH 8·5) at room temperature. Before restriction enzyme digestion, the plugs were soaked in TE buffer (10 mM Tris-base, 1 mM EDTA, pH 8·0) for 1 h and then slowly shaken for 1 h in an appropriate restriction enzyme buffer. Restriction enzyme digestion with *Sma*I was performed overnight at 30 °C. Electrophoresis was carried out with a CHEF mapper (Bio-Rad) in 1·1 % PFGE-certified agarose (Bio-Rad) and using 0·5 × TBE electrophoresis buffer (0·045 M Tris/borate, 0·001 M EDTA). The switch time was 2–30 s, the current was 5·3 V cm^{-1}, the temperature was 14 °C and the running time was 24 h. The agarose gel was further treated and analysed as described above, except that the similarity among digitized profiles was calculated using the dice correlation.

Scanning electron microscopy. To prevent crystallization of the salt in the fermentation liquor, samples (1·5 ml) were microcentrifuged (13 000 r.p.m. for 15 min) first. The recovered pellet was resuspended in 1·5 ml sterile water, and appropriate dilutions were prepared. Then, 100 µl of cell suspension were smeared onto the surface of a microscope slide, which was carefully passed through a heating source without boiling the liquor. The samples were coated with carbon. Cells were examined and photographed at a magnification of 2000 with a JEOL-JSM 6400 electron microscope (JEOL, Tokyo, Japan) operating at a voltage of 20 kV.

RESULTS

Fermentation profiles

The relationship between cell growth and bacteriocin production by *L. amylovorus* DCE 471 as influenced by suboptimal temperature and pH values was assessed. Fig. 1 represents an example of a fermentation trial at a controlled temperature of 37 °C and a constant pH of 5·4. Both the experimental as well as the modelled values of the biomass concentrations and bacteriocin activity levels (Fig. 1a), and of the residual maltose and fructose concentrations, and lactic acid production (Fig. 1b) are shown. As previously observed in MRS medium (Callewaert *et al.*, 1999; De Vuyst *et al.*, 1996a; Lejeune *et al.*, 1998), amylovorin L471 was detected early in the exponential-growth phase and was produced continuously during this phase. The highest amylovorin L471 activity was reached at the end of the exponential phase, and corresponded with the maximal cell density. Amylovorin L471 activity decreased rapidly when cells entered the stationary phase. Hence, the primary metabolite kinetics of amylovorin L471 production, followed by an apparent inactivation of the bacteriocin, were confirmed in SSM. Maltose and fructose were consumed simultaneously and converted into lactic acid.

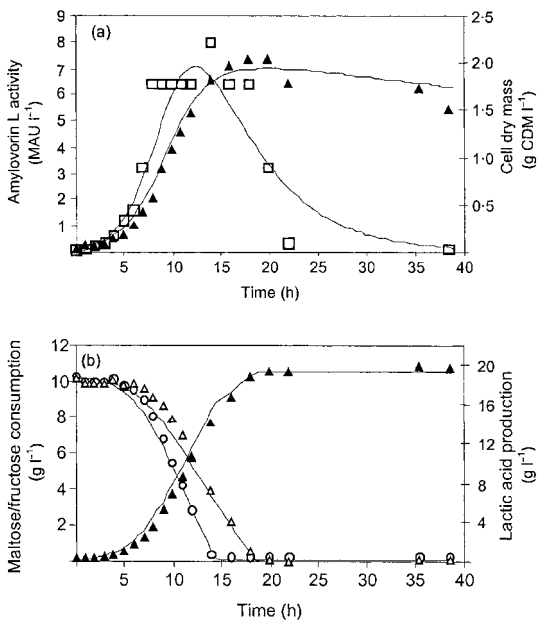

Fig. 1. Modelling of (a) the biomass (▲) and bacteriocin production (□) and (b) the maltose consumption (△), fructose consumption (○) and lactic acid formation (▲) of *L. amylovorus* DCE 471 in SSM at a controlled temperature of 37 °C and at a constant pH of 5·4. Symbols represent the experimental values; full lines are drawn according to the model. Amylovorin L, amylovorin L471; MAU, mega arbitrary units.

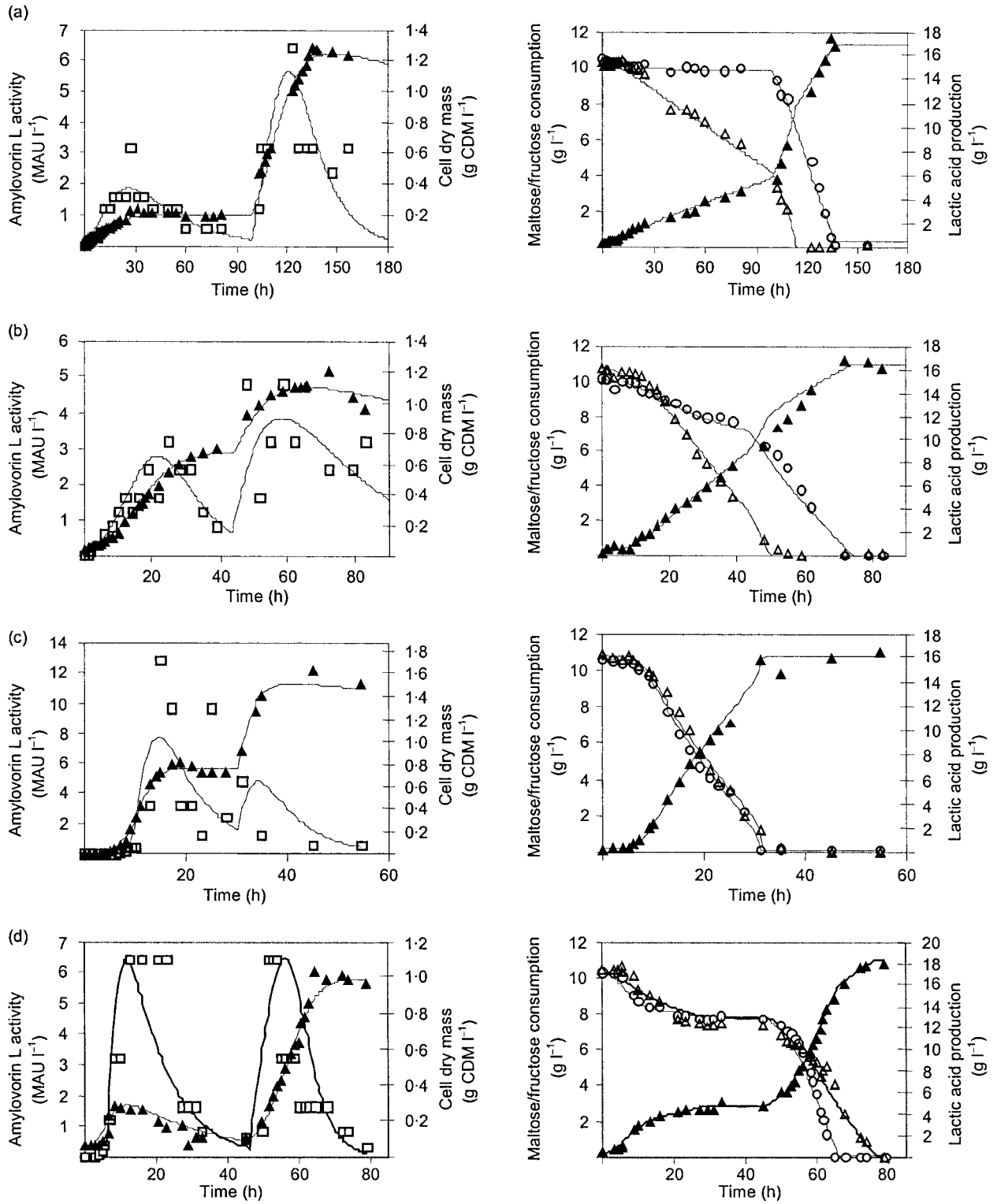

Fig. 2. Modelling of the biomass (▲) and bacteriocin production (□) (left) and the maltose consumption (△), fructose consumption (○) and lactic acid formation (▲) (right) of *L. amylovorus* DCE 471 in SSM at (a) a controlled temperature of 28 °C and a constant pH of 5·4, (b) a controlled temperature of 31 °C and a constant pH of 5·4, (c) a controlled temperature of 37 °C and a constant pH of 6·4 and (d) a controlled temperature of 37 °C, a constant pH of 5·4 and 3·0 % (w/v) NaCl. Symbols represent the experimental values; full lines are drawn according to the model. Amylovorin L, amylovorin L471; MAU, mega arbitrary units.

Biphasic growth kinetics and modelling

At suboptimal conditions for growth of *L. amylovorus* DCE 471, biphasic growth patterns were observed (Fig. 2). Each growth phase was characterized by an exponential and a stationary growth phase as well as by a bacteriocin activity peak. This was the case at low temperatures (at both 28 and 31 °C at constant pH 5·4) (Fig. 2a, b), high pH values (constant pH 6·4 at 37 °C) (Fig. 2c) and high salt concentrations [3 % (w/v) NaCl and constant temperature and pH of 37 °C and pH 5·4, respectively] (Fig. 2d).

Both growth phases were modelled separately (Fig. 2). All biokinetic parameters derived from the primary modelling in the first and second growth phase are listed in Table 2. When cultivated at 28 °C (Fig. 2a) the biomass was lower in the first growth phase compared to the fermentation at 31 °C (Fig. 2b). An increase in the duration of the intermediate lag phases, indicating an enhanced stress, was observed at lower temperatures (98·5 h at 28 °C compared with 44 h at 31 °C). During the first growth phase the maximum specific growth rate (μ_{max}) was lower at lower temperatures (Table 2). Remarkably, the observed μ_{max} values during the second growth phase were identical to the ones observed during the first growth phase. The first growth phase of the fermentation at 37 °C and constant pH 6·4 was characterized by a maximum obtainable biomass concentration that was comparable to the one found at 31 °C and constant pH 5·4 (Table 2). Furthermore, during the fermentation at 37 °C and constant pH 6·4, it only took 30 h before the second growth phase began. At 31 °C and constant pH 5·4, and at 37 °C and constant pH 6·4, X_{max} obtained in the first growth phase was about half the X_{max} value reached at the end of the fermentation. At 28 °C and constant pH 5·4, only 14 % of the X_{max} value reached at the end of the fermentation was achieved after the first growth phase (Table 2). In general, bacteriocin activity was highest in the second growth phase, except for the fermentation at 37 °C and constant pH 6·4 (Fig. 2). On the other hand, the maximum specific bacteriocin production (k_B) was lower during the second growth phase due to the higher X_{max} values encountered during this part of the fermentation (Table 2). The cell yield coefficients for fructose ($Y_{X/F}$) and maltose ($Y_{X/M}$) and the maintenance coefficient for maltose (m_M) were found to remain constant from one growth phase to the next (Table 2). As the maintenance coefficient for fructose (m_F) and μ_{max} decreased at lower temperatures, maintenance by slow-growing cells was less dependent on fructose. In the first growth phase, a lower m_F value was observed than in the second growth phase. Hence, maintenance on fructose became more important in the second growth phase. Sugar consumption profiles changed with the temperature: at 37 °C and pH 6·4, maltose and fructose were consumed simultaneously, at 31 °C maltose was consumed faster than fructose, and at 28 °C fructose was not consumed during the first growth phase (Fig. 2). It appears that maltose consumption corresponded with highest bacteriocin activity. It has been observed before that maltose was consumed faster than fructose when present as the sole carbohydrate source (P. Neysens, F. Leroy, N. De Cock & L. De Vuyst, unpublished results).

Molecular typing of *L. amylovorus* DCE 471 during biphasic growth

To exclude the possibility of contamination, preliminary experiments (microscopic observations, phenotypic analyses, 16S rDNA sequence analysis) indicated the occurrence of only one bacterium in the fermentation vessel, namely *L. amylovorus* DCE 471 (data not shown). To exclude the existence of subpopulations due to the genetic variability in the population, which would result in different DNA patterns, the DNA extracted from the original stock culture, as well as from the pre-culture of *L. amylovorus* DCE 471 and from 10 samples withdrawn from the fermentation vessel at regular time intervals, was subjected to rep-PCR and PFGE. As shown in Fig. 3(a), identical rep-PCR fingerprints were found for the pre-culture and the 10 samples. For the pre-culture and a representative selection of seven samples, identical DNA restriction patterns were found by PFGE of *Sma*I-digested chromosomal DNA as well (Fig. 3b).

Morphological characteristics of *L. amylovorus* DCE 471 during biphasic growth

To explain the unusual behaviour of biphasic growth of *L. amylovorus* DCE 471 under suboptimal growth conditions, a representative fermentation in the presence of 3 % NaCl was performed (Fig. 1d). Based on this experiment, it was concluded that the number and shape of the different colonies was affected by the stress conditions to which the bacteria were subjected. Plating of the samples, taken during the first growth phase, onto agar containing maltose and fructose resulted in the development of relatively big, flat, irregular colonies, while the colonies during the second growth phase were visually smaller and smoother. This morphological switch matched very well with changes at the single-cell level (Fig. 4). Fig. 5 shows the correlation between the OD_{600} value and plate count enumeration (in c.f.u. ml^{-1}). During the first growth phase, the numbers of c.f.u. of the two bacterial colony types were comparable (Fig. 5a). As an example, at an OD_{600} value of 0·6, $3{\cdot}8 \times 10^7$ and $4{\cdot}4 \times 10^7$ c.f.u. ml^{-1} were found in SSM containing 3·0 % NaCl and in unsupplemented medium, respectively. The bacterial cells present in the inoculum, prepared in SSM without salt, occurred as single, rod-shaped cells (Fig. 4a). Each of these single cells could result in a c.f.u. after plating. However, after inoculation, cells had to adapt to the NaCl-enriched growth medium. This led initially to lysis of the cells that were unable to adapt (Fig. 4b), while some viable cells, obtained during the first active growth phase, had a more curled, twisted shape (Fig. 4c). Growth of this irregular shaped biotype ceased after 9 h fermentation, which was followed by a stationary phase lasting for 37 h. At the onset of this phase, cell lysis could clearly be observed (Fig. 4d),

Table 2. Experimental values for μ_{max}, $Y_{X/M}$, $Y_{X/F}$, m_M, m_F, X_{max}, B_{max}, k_B and k_{inact} of *L. amylovorus* DCE 471 in the first and second growth phase and at a given combination of temperature and pH

Temp (°C)	pH	NaCl (w/v)	Growth phase	Modelled value for							
				μ_{max} (h^{-1})	$Y_{X/M}$ [g CDM (g maltose)$^{-1}$]	$Y_{X/F}$ [g CDM (g fructose)$^{-1}$]	m_M [g maltose (g CDM)$^{-1}$ h^{-1}]	m_F [g fructose (g CDM)$^{-1}$ h^{-1}]	X_{max} (g CDM l^{-1})	k_B[kAU (g CDM)$^{-1}$]*	k_{inact} [l (g CDM)$^{-1}$ h^{-1}]
28	5·4	0	First	0·19	0·35	0·32	0·32	0·00	0·22	18 000	0·170
28	5·4	0	Second	0·19	0·35	0·32	0·32	0·18	1·60	9 000	0·050
31	5·4	0	First	0·27	0·45	0·42	0·35	0·06	0·85	8 900	0·170
31	5·4	0	Second	0·27	0·45	0·42	0·35	0·17	1·70	7 000	0·025
37	6·4	0	First	0·55	0·35	0·35	0·45	0·48	0·80	19 000	0·150
37	6·4	0	Second	0·55	0·35	0·35	0·45	0·48	1·60	7 000	0·080
37	5·4	3	First	0·49	0·35	0·20	0·30	0·10	0·40	50 000	0·240
37	5·4	3	Second	0·30	0·35	0·15	0·25	0·20	1·38	51 000	0·600

*kAU, Kilo arbitrary units.

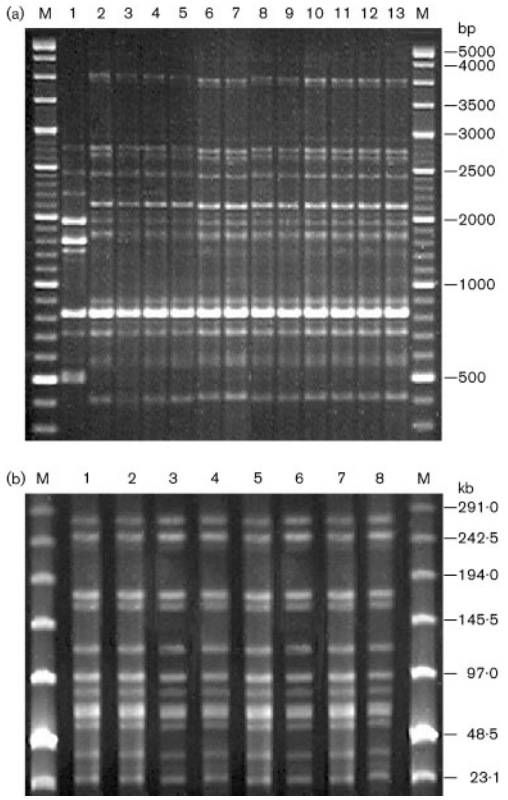

Fig. 3. (a) rep-PCR fingerprints using the $(GTG)_5$ primer with *L. amylovorus* LMG 9496^T (lane 1), *L. amylovorus* DCE 471 (lane 2), the pre-culture of *L. amylovorus* DCE 471 used to inoculate the fermenter (lane 3) and 10 samples from the fermentation vessel (lanes 4–13). (b) PFGE patterns (*Sma*I digest of chromosomal DNA) of the pre-culture of *L. amylovorus* DCE 471 used to inoculate the fermenter (lane 1) and seven representative samples from the fermentation broth in the presence of 3·0 % (w/v) NaCl (lanes 2–8). M, molecular mass marker.

followed by the gradual recovery of the remaining bacterial population (Fig. 4e, f). During the stationary phase, a switch to a better-adapted, less NaCl-sensitive, smooth biotype occurred. During this phase and at an equivalent OD_{600} value, the number of c.f.u. of the stressed bacterial population was 2·0–2·5 times lower than that observed for the bacteria that grew optimally (Fig. 5b). For example, at an OD_{600} value of 2·8, no more than $9{\cdot}3 \times 10^7$ c.f.u. ml^{-1} were found during the second growth phase, whereas $2{\cdot}4 \times 10^8$ c.f.u. ml^{-1} were observed under optimal growth conditions. Scanning electron microscopy results of this smooth biotype showed an elongated, less-curved, filamentous and cluster-forming morphology, with the bacterial cells tending to form chains (Fig. 4g–j). This morphology resulted in decreased cell counts. These experiments and observations were reproducible and similar for stresses implied by suboptimal temperature and pH values (data not shown).

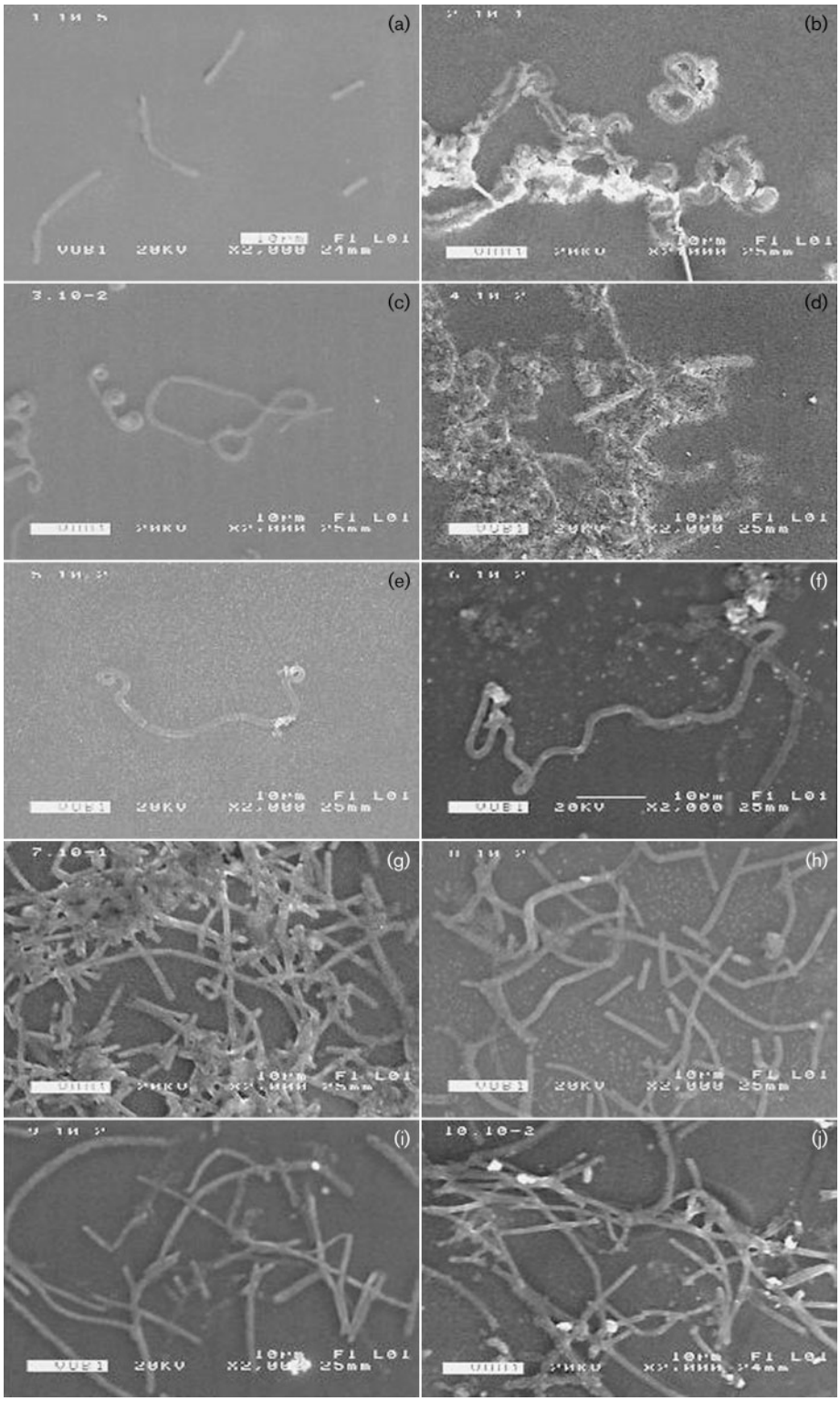

Fig. 4. Influence, at single-cell level, of 3·0 % (w/v) NaCl on the morphological characteristics of *L. amylovorus* DCE 471 in SSM at a constant temperature of 37 °C and pH 5·4 as determined by scanning electron microscopy. All observations were done at a magnification of 2000. Samples were taken at specific time intervals after the start of fermentation: (a) pre-culture used for inoculation; (b) after 6 h; (c) after 10 h; (d) after 21 h; (e) after 33 h; (f) after 45 h; (g) after 56 h; (h) after 63 h; (i) after 74 h; (j) after 80 h.

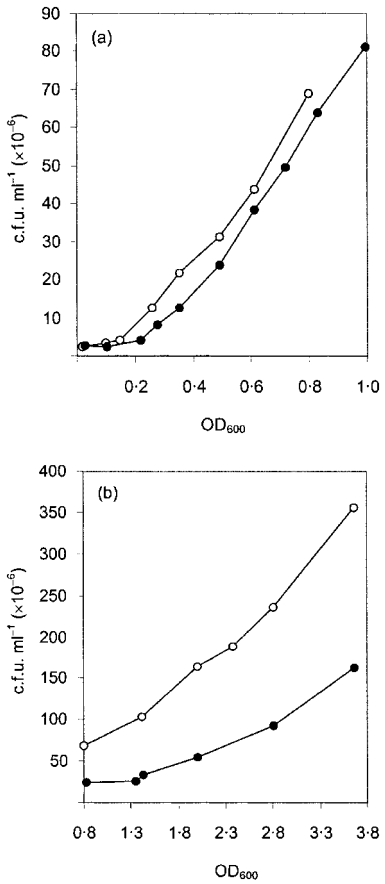

Fig. 5. Correlation between OD_{600} value and c.f.u. per ml of *L. amylovorus* DCE 471 grown at a controlled temperature of 37 °C and a constant pH of 5·4 in the absence (○) and in the presence (●) of 3·0 % (w/v) NaCl during (a) the first growth phase and (b) the second growth phase.

DISCUSSION

The homofermentative species *L. amylovorus* has been isolated frequently from type II sourdough fermentations based on rye as the main cereal (Vogel *et al.*, 1999; Müller *et al.*, 2001). Some strains of this species produce bacteriocins, which may improve their competitiveness in the complex cereal ecosystem, and hence make them interesting starter cultures for cereal fermentations (De Vuyst *et al.*, 1996b; Messens *et al.*, 2002). However, starter cultures undergo several stress situations during fermentation such as nutrient limitation, low pH and decreased water activity (Juillard *et al.*, 1995; Helinck *et al.*, 1997; Leroy & De Vuyst, 1999, 2002). Adaptation to unusual or unfavourable growth conditions may explain the occurrence of biphasic growth patterns for *L. amylovorus* DCE 471 in SSM under stress conditions. This two-stage fermentation profile was never observed in MRS (De Vuyst *et al.*, 1996a, b), underlying the appropriateness of MRS as a cultivation medium for lactic acid bacteria (De Man *et al.*, 1960). However, omitting meat extract from MRS also resulted in biphasic growth kinetics of *L. amylovorus* DCE 471 (Messens *et al.*, 2002). On the other hand, Calderon *et al.* (2001) reported biphasic growth profiles upon cultivation of *Lactobacillus fermentum* Ogi E1 in MRS broth. Exhaustion of essential growth factors or their limited availability in this complex broth were proposed as possible reasons. A limited availability of oxygen has been indicated as a possible reason for the observed double growth response of *Lactobacillus sanfranciscensis*, namely the production of less ethanol and more acetate, based on the initial amount of dissolved oxygen acting as an electron acceptor during a first growth phase, followed by growth on the medium supplemented with fructose as electron acceptor, resulting in high amounts of acetate during a second growth phase (Stolz *et al.*, 1993; Gobbetti *et al.*, 1996).

It is remarkable that a higher specific bacteriocin production was observed in the first growth phase compared to the second one. This indicates a stress response of the *L. amylovorus* culture, stress to which the bacteria are subjected at the start of the fermentation. It has been observed before that bacteriocin production is stimulated by stress conditions, resulting in lower growth rates, lower cell yields and relatively high bacteriocin activity levels (De Vuyst *et al.*, 1996a; Uguen *et al.*, 1999). Stress caused by a shift of fermentation temperature from 37 to 30 °C has been shown to enhance the specific bacteriocin production by *L. amylovorus* DCE 471 after adaptation to this suboptimal value (Lejeune *et al.*, 1998). Yet, De Vuyst *et al.* (1996a) observed two types of colony morphologies that existed as heterogeneous populations in MRS medium under stable environmental conditions. Therefore, stress is certainly responsible for the fermentation kinetics observed in the SSM used, given the shift in colony morphology. Whitley & Marshall (1999) described that *L. amylovorus* NCFB 2745 exhibits rough (R) and smooth (S) colony forms. The different morphology is not due to genetic variability in the population but to a relatively stable switch in phenotypic expression, depending on different environmental conditions such as incubation temperature, pH, anaerobiosis and media composition. For instance, smooth *L. amylovorus* NCFB 2745 colonies are found on agar plates after five transfers in MRS medium containing glucose and citrate. Smooth cultures revert to rough ones by culturing under aerobic conditions. The rough type ferments glucose homofermentatively, while the smooth type shows patterns of fermentation that are typical of a heterofermentative *Lactobacillus*, producing carbon dioxide and ethanol in addition to lactate. However, De Vuyst *et al.* (1996a) observed a 100 % homofermentative behaviour when growing *L. amylovorus* DCE 471 in MRS medium. Also, in SSM no switch from homo- to heterofermentation could be observed.

Stress-induced morphological changes have been described previously for several non-sporulating Gram-negative and Gram-positive bacteria. Furthermore, it is known that bacterial cells can rely on mechanisms for survival and

resistance against multiple stresses. For instance, upon growth under acidic conditions or exposure to heat shock, a set of inducible responses resulting in a complex regulated expression of genes, in which sigma factors are involved, leads to tolerance or an increased resistance (Foster, 1995; Haldenwang, 1995; Segal & Ron, 1998). This process, along with other stress responses, is known to induce major changes in physiology and morphology (Morita, 1993). A variety of stresses, such as a lowered pH, are known to lead to shrinkage of *Escherichia coli* cells due to the induction of the *bolA* morphogene (Santos *et al.*, 1999). Dramatic morphological changes and a severe decrease in the viability of *Propionibacterium freudenreichii* and *Enterococcus faecalis* are seen upon their exposure to extreme acidic growth conditions (Hartke *et al.*, 1998; Giard *et al.*, 2000; Jan *et al.*, 2001). In the case of *L. amylovorus* DCE 471, the filamentous growth of the more resistant biotype during the second growth phase might be ascribed to an enhanced stress resistance, resulting in a normal multiplication of the surviving cells but hampered cell division. Stress caused by the presence of salt, often present in fermenting foods, has previously been shown to induce an altered morphology of food-borne pathogens as well. For instance, it has been shown that *Listeria monocytogenes* became filamentous upon cultivation in the presence of 10·0 % salt because of a hampered cell multiplication, but nevertheless was able to survive for long periods at high salt concentrations (Brzin, 1975). In the case of *L. amylovorus* DCE 471, salt sensitivity may be the reason why part of the original cell population starved during the intermediate stationary phase. Similarly, other suboptimal and stress-inducing environmental conditions may lead to biphasic growth coinciding with an altered cell and colony morphology.

From an industrial point of view, biphasic profiles observed for growth and bacteriocin production cannot be considered as advantageous since type II sourdoughs are characterized by short fermentation times and need a strong acidification at the beginning of the process. Furthermore, this feature does not contribute to an enhanced competitiveness of the strain since only low biomass concentrations, producing moderate amylovorin L471 levels, are observed during the first growth phase. On the other hand, the temperature dependence of sugar consumption by *L. amylovorus* DCE 471 observed during this study may improve the competitiveness of this strain. A temperature of 28 °C promoted the use of maltose by *L. amylovorus* DCE 471. Fast maltose consumption coincided with high bacteriocin activity levels. Both phenomena may hamper the development of other maltose-dependent micro-organisms in the same ecosystem. This is interesting from a physiological–ecological point of view, because maltose is the most important energy source for *L. amylovorus* in a cereal environment.

In this study, the biphasic growth patterns of *L. amylovorus* DCE 471 were modelled and elucidated in an SSM. It was shown that unfavourable growth conditions caused by low temperatures, high pH and high salt concentrations resulted in biphasic growth patterns. A morphological change coincided with an increased resistance of the bacterial population. However, the underlying molecular mechanism responsible for these phenomena still has to be elucidated in the case of *L. amylovorus* DCE 471.

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