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Production of D- and L-Lactic Acid by Mono- and Mixed Cultures of *Lactobacillus* sp.

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Summary

Batch cultivation of monoculture of *Lactobacillus* sp. and two–strain mixed culture of *Lactobacillus* sp. and *Lactobacillus amylovorus* DSM 20531^T was carried out with the aim of producing L-(+)- and D-(–)/L-(+)-lactic acid to be implemented in poly(lactic acid) polymer production. Metabolic capacity of two *Lactobacillus* strains to ferment different carbon sources (glucose, sucrose or soluble starch) during cultivation in MRS medium at 40 °C, in a laboratory-scale stirred tank bioreactor was defined. *Lactobacillus* sp. showed similar affinity towards mono- and disaccharide substrates, which were homofermentatively converted mostly to L-(+)-lactic acid. *L. amylovorus* DSM 20531^T has been characterized as a D/L-lactate producer and it is capable of conducting simultaneous saccharification and fermentation. Due to the interaction of *Lactobacillus* sp. with *L. amylovorus* DSM 20531^T, starch was hydrolysed and fermented to the mixture of L-(+)- and D-(–)-lactic acid. Modified Luedeking-Piret kinetics used for the description of substrate utilization, growth of mono- and mixed cultures and production of lactic acid stereoisomers showed good agreement with experimental data.

Key words: monoculture, mixed culture, *Lactobacillus* sp., batch fermentation, L-(+)- and/or D-(–)-lactic acid, bacteriocin, amensalism, mathematical model

Introduction

On the industrial scale, lactic acid is mainly produced by microbial fermentations of simpler carbohydrates (*1*). Diverse applications of this organic acid are generally determined by the ratio of its stereoisomeric forms, L-(+)- and D-(–)-lactic acid, isolated from fermentation broth (*2*). Besides other applications, purified stereoisomers of lactic acid are used in production of biodegradable polylactide polymers – poly(lactic acid) (PLA), already well established in pharmaceutical, medical, biomedical and packaging industries (*3,4*). High molecular mass PLA can be produced by: (*i*) direct condensation polymerization of L-(+)- and/or D-(–)-lactic acid, (*ii*) azeotropic dehydrative condensation, or (*iii*) commercially applied polymerization through lactide formation. Properties of PLA, such as mechanical, physical, optical and degradation properties depend on its molecular mass and stereochemistry of the backbone. It is known that L-(+) isomer can be produced by lactic acid bacteria (LAB) of genera *Streptococcus*, *Pediococcus*, *Lactococcus* and *Lactobacillus*, while D-(–) isomer can be produced only by particular strains of *Lactobacillus* genus (*5*). Mixture of two stereoisomers is usually produced by homofermentative LAB that belong to genera *Pediococcus* and *Lactobacillus*.

The lactic acid production by monocultures of *Lactobacillus*, wild-types or mutants, has already been studied. High yields of lactic acid (≥1.8 mol of lactate per mol of hexose) were obtained during cultivation in media containing different mono- or disaccharides (*6–9*). Cocultivations of two different microbial strains, one with hydro-

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lytic and another with fermentative activity, have been employed in direct conversion of polymeric carbohydrates (*10*). In such bioprocesses, *i.e.* simultaneous saccharification and (homolactic) fermentation (SSF), complex substrates from rather inexpensive renewable materials are converted to lactic acid. In the cocultivations of the two-strain mixed culture, the interaction between the strains also has to be taken into account. Several types of associations among microbial strains – members of mixed populations, are known: neutralism, competition, amensalism, parasitism, predation, commensalism, protocooperation, and mutualism (*11,12*).

Amylolytic LAB (ALAB) possess both activities required for the completion of simultaneous saccharification and fermentation (SSF), hydrolytic and fermentative, and therefore have been widely studied for implementation in sustainable production of one or a mixture of both stereoisomeric lactate forms (*6,13–15*). ALAB *Lactobacillus amylovorus* DSM 20531^T can utilize a wide variety of different substrates including starch, and it catalyzes highly efficient SSF to D/L-lactic acid (*16*). Percentage of produced lactate isomers depends on the carbon source and temperature of cultivation. For L-(+) form it varied between 37.1 and 56.3 %, and for D-(–) form it was between 43.7 and 62.9 % (*6*). The ratio of L-(+)- and D-(–)-lactate can be further tuned by using raw materials (data to be published) and less common process parameters, *e.g.* relatively low temperature (*6,17–19*).

In this research, the possibility of the application of partially characterized *Lactobacillus* strain, isolated from silage, in the production of one or both stereoisomeric forms of lactic acid from mono- and disaccharide is examined. Since the bacterial monoculture produced mostly L-(+)-lactic acid, it was cocultivated with ALAB *L. amylovorus* DSM 20531^T in order to obtain the defined ratio of L-(+)- and D-(–)-lactate by utilizing starch as a carbon source. Unstructured kinetic models which were established in order to describe these systems showed good agreement with experimental data.

Materials and Methods

Preparation of microorganisms, media and inocula

Lactobacillus sp. belongs to the culture collection of Laboratory of Biochemical Engineering, Industrial Microbiology, Malting and Brewing Technology at the Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, Croatia. It was originally isolated from silage and maintained on a medium containing (in g/L): molasses 120, malt sprouts 10, and $CaCO_3$ 60. After Gram staining, pairs or chains of rod-shaped blue cells of *Lactobacillus* sp. of max. 10 μm in length were visible under microscope (image not shown). Amylolytic lactic acid bacterium *Lactobacillus amylovorus* DSM 20531^T (ATCC 33620, NRRL B-4540), a bacteriocin producer, was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The monocultures were maintained at 4 °C for max. 7 days in MRS broth medium (glucose, γ_0=20 g/L) (Biolife, Milan, Italy) or in MRS-based media in which glucose was replaced with sucrose (γ_0=10 g/L) or soluble starch (γ_0=10 g/L) (for *L. amylovorus* DSM 20531^T). After sterilization at 121 °C for 20 min, the three media (initial pH value of 6.2±0.2) were used for inoculum preparation and fermentation experiments. Each bacterial strain or mixed culture were propagated twice in corresponding MRS-based medium at 40 °C for 12 h ($A_{600\ nm}$=0.6–0.8, N=10^7–10^8 CFU/mL, γ_X= 0.2–0.3 g/L) prior to inoculation of fermentation broth.

Lactic acid production

Production of stereoisomers of lactic acid was investigated in a laboratory-scale stirred tank bioreactor (Chemap AG, Volketswil, Switzerland) at 40 °C. The *in situ* sterilizable bioreactor (V=6 L) was loaded with 5 L of MRS medium which was inoculated with culture suspension (125 mL) pregrown overnight in the corresponding MRS medium at 40 °C. The pH value of 5.5±0.2 was maintained by the automatic addition of 10 mol/L NaOH. After cultivation of *Lactobacillus* sp. and production of mainly L-(+)-lactic acid in the MRS media containing glucose or sucrose, the production of D/L-lactic acid by the mixed culture of the two *Lactobacillus* strains was carried out in the MRS medium with starch as a carbon source. Batch processes were conducted at constant agitation speed (400 min^{-1}).

Analytical methods

Absorbance of the fermentation broth samples was determined at 600 nm ($A_{600\ nm}$) (spectrophotometer Cary 13E Varian, Mulgrave, Australia). After centrifugation of the suspensions (3500×g/20 min/4 °C; Harrier 18/80, Sanyo, UK), the supernatant was used for determination of substrate and product concentrations, while bacterial biomass was dried at 105 °C for 24 h to determine the dry mass. Viable cell concentrations were enumerated by plating on MRS agar (Biolife, Italy).

Before determination of the concentration of substrates and products, supernatants were pretreated. In short, the concentration of proteins was reduced by precipitation with zinc sulphate. In the samples withdrawn during SSF from the medium with polymeric carbohydrate, the remaining starch was completely hydrolysed by hydrochloric acid (*6*). After the pretreatment of samples, concentration of carbohydrates in the MRS media was determined by using two methods: concentration of glucose, sucrose and starch (glucose after complete hydrolysis of starch) by HPLC analysis (*6*), and concentration of reducing sugars by the method described by Somogyi (*20*). Concentration of produced lactic acid was determined by HPLC analysis, as described previously (*6*). Concentrations of D-(–)- and L-(+)-lactic acid in the supernatant were measured with the D-lactic acid (D-lactate) and L-lactic acid (L-lactate) kit supplied by Megazyme (*21*).

Bacteriocin sensitivity measurement

Suspension of *L. amylovorus* DSM 20531^T pregrown in MRS medium (overnight, 40 °C) was centrifuged (3500×g/20 min/4 °C; Harrier 18/80, Sanyo, UK), bacterial biomass was removed and the pH value of the supernatant was brought to 6.5 by adding 5 mol/L NaOH. The supernatant is then filtered (nylon syringe filter, 0.2 μm; Carl Roth GmbH, Karlsruhe, Germany). Sterile supernatant (5 mL) was aded to 50 mL of MRS broth in

an Erlenmeyer flask and the medium prepared in the described way was inoculated with *Lactobacillus* sp. (2.5 % by volume). The suspension was incubated at 40 °C, samples were withdrawn at regular time intervals under aseptic conditions and absorbance ($A_{600\ nm}$) was determined. As control, *Lactobacillus* sp. was incubated under the same conditions but without the addition of sterile supernatant of *L. amylovorus* DSM 20531^T. Distilled sterile water (5 mL) was added to the control instead of the supernatant of *L. amylovorus* DSM 20531^T.

Kinetic model

Primary modelling describing substrate utilization, the growth of mono- and mixed culture, product formation and synthesis of bacteriocins was performed by using the following set of equations:

$$\frac{d\gamma_S}{dt} = -\frac{1}{Y_{X/S}} \cdot \frac{d\gamma_X}{dt} - m_S \cdot \gamma_X \qquad /1/$$

$$\gamma_X = \gamma_{X_1} + \gamma_{X_2} \qquad /2/$$

$$\frac{d\gamma_X}{dt} = \frac{d\gamma_{X_1}}{dt} + \frac{d\gamma_{X_2}}{dt} \qquad /3/$$

$$\frac{d\gamma_{X_1}}{dt} = \mu_{max_1}\left(1 - \frac{\gamma_{X_1}}{\gamma_{X_{max_1}}}\right)^{n_1} - k_{d1} \cdot \gamma_{X_1} \qquad /4/$$

$$\frac{d\gamma_{X_2}}{dt} = \mu_{max_2}\left(1 - \frac{\gamma_{X_2}}{\gamma_{X_{max_2}}}\right)^{n_2} - k_{d2} \cdot \gamma_{X_2} - k_{DB} \cdot \gamma_B \cdot \gamma_{X_2} \qquad /5/$$

$$k_d = k_{d_0} \cdot (1 + C \cdot \gamma_P) \qquad /6/$$

$$\frac{d\gamma_P}{dt} = \alpha \cdot \frac{d\gamma_X}{dt} + \beta \cdot \gamma_X \qquad /7/$$

$$\frac{d\gamma_B}{dt} = \alpha_B \cdot \frac{d\gamma_{X_1}}{dt} + \beta_B \cdot \gamma_{X_1} - k_B \cdot \gamma_B \cdot \gamma_{X_2} \qquad /8/$$

Consumption of substrates by mono- or mixed culture was expresed by the Pirt equation (Eq. 1) (*22*). For the conversion by mixed culture, the biomass (γ_X) consisted of two variables – *L. amylovorus* DSM 20531^T biomass (γ_{X_1}), and the biomass of presumably sensitive *Lactobacillus* sp. (γ_{X_2}) (Eqs. 1–5). Eq. 4 was used when *Lactobacillus* sp. fermented glucose and sucrose. Lactic acid production (Eq. 7) and bacteriocin synthesis by bacteriocinogenic DSM 20531^T strain were described by Luedeking-Piret kinetics (Eq. 8) (*23*). All calculations were done by using Berkeley Madonna (*24*), as already described (*6*). Values obtained for the parameters of the kinetic model are listed in Table 1. Parameters μ_{max}, $\gamma_{X_{max}}$, $Y_{X/S}$ and $\alpha(Y_{P/X})$ were fixed to their experimental values and not fitted by the software (bolded in Table 1). Kinetic parameters that were not determined from the experimental data were optimized by using multiple curve fit function (not bolded in Table 1) and limited to realistic values applicable for fermentations by *Lactobacillus* species. For parameter estimation, Berkeley Madonna employs a simplex method to obtain parameter values that minimise the root mean square (RMS) deviation (Table 1) of the experimental and simulated data.

Table 1. Optimal parameters of the kinetic model for fermentation of mono- and disaccharide by bacterial monoculture and SSF of polysaccharyde by two-member mixed culture to L-(+)- and D-(–)-lactic acid (θ=40 °C; pH=5.5) in the stirred tank bioreactor. Experimental values used as fixed values in the model are bolded

Parameter	Monoculture		Mixed culture
	glucose	sucrose	starch
μ_{max}/h^{-1}	**0.25±0.02**	**0.29±0.02**	–
$\gamma_{X_{max}}/(g/L)$	**6.83±0.28**	**4.50±0.26**	–
n	1.23	0.40	–
m_s/h^{-1}	0.0172	0.0002	0.0329
$Y_{X/S}/(g/g)$	**0.27±0.01**	**0.45±0.04**	**0.26±0.01**
k_{d_0}/h^{-1}	0.0035	0.0042	–
C_0	0.8284	0.28	–
$\alpha(Y_{P/X})/(g/g)$	**2.84±0.15**	**2.10±0.12**	**1.20±0.04**
$\beta/(g/(g\cdot h))$	0.003	0.049	0.046
μ_{max_1}/h^{-1}	–	–	0.29
μ_{max_2}/h^{-1}	–	–	0.18
$\gamma_{X_{max1}}/(g/L)$	–	–	2.7
$\gamma_{X_{max2}}/(g/L)$	–	–	0.7
n_1	–	–	1.3
n_2	–	–	1.5
k_{d_1}/h^{-1}	–	–	0.0050
k_{d_2}/h^{-1}	–	–	0.0081
$k_{DB}/(L/(AU\cdot h))$	–	–	0.07
C_1	–	–	1.4
C_2	–	–	3.9
$\alpha_B/(AU/g)$	–	–	1.1
$\beta_B/(AU/(g\cdot h))$	–	–	0.00003
$k_B/(L/(g\cdot h))$	–	–	0.9
R^2_S	0.9813	0.9818	0.9703
R^2_X	0.9918	0.9806	0.8854
R^2_P	0.9880	0.9859	0.9586
RMS	1.4573	2.0144	0.8968

Determination of bioprocess parameters

Substrate consumption rate (r_S), specific growth rate (μ), product formation rate (r_P), biomass yield coefficient ($Y_{X/S}$), product/biomass yield coefficient ($Y_{P/X}$), and product yield coefficient ($Y_{P/S}$) were estimated according to the following equations (*25*):

$$\ln\gamma_S = \ln\gamma_{S_0} + r_S t \qquad /9/$$

$$\ln\gamma_X = \ln\gamma_{X_0} + \mu t \qquad /10/$$

$$\ln\gamma_P = \ln\gamma_{P_0} + r_P t \qquad /11/$$

$$Y_{X/S} = \frac{(\gamma_X - \gamma_{X_0})}{(\gamma_{S_0} - \gamma_S)} \qquad /12/$$

$$Y_{P/S} = \frac{(\gamma_P - \gamma_{P_0})}{(\gamma_{S_0} - \gamma_S)} \qquad /13/$$

$$Y_{P/X} = \frac{(\gamma_P - \gamma_{P_0})}{(\gamma_X - \gamma_{X_0})} \qquad /14/$$

$$Pr_X = \frac{\gamma_X - \gamma_{X_0}}{t} \qquad /15/$$

$$Pr_P = \frac{\gamma_P - \gamma_{P_0}}{t} \qquad /16/$$

Results and Discussion

In this investigation, cultivation of mono- and mixed cultures of two *Lactobacillus* strains was carried out in a laboratory-scale stirred tank bioreactor at constant temperature (40 °C) and stirrer speed of 400 min^{-1}. pH value was maintained at 5.5±0.2. In order to determine the suitability of the selected substrates for the production of lactic acid, the monoculture of *Lactobacillus* sp. was cultivated in two MRS media, the first one containing glucose and the second one sucrose. It turned out that under the selected conditions, the test strain produced mainly L-(+)--lactic acid. Additional experiment proved that this strain isolated from silage cannot hydrolize starch (data not shown). Therefore, fermentation of soluble starch by the mixed culture of *Lactobacillus* sp. and *Lactobacillus amylovorus* DSM 20531^T, with the equal portions of both bacterial suspensions (1:1) in the inoculum added to the same culture medium, was carried out. Namely, production of different ratios of L-(+)- and D-(–)-lactic acid by ALAB *L. amylovorus* DSM 20531^T from soluble starch had already been described (*6*). In the produced D/L-mixture, the portion of L-(+)-lactate varied between 0.371 and 0.563, while the portion of D-(–)-lactate was in the range from 0.437 to 0.629. Therefore, the two-strain bacterial culture was used in order to move the ratio of two stereoisomeric forms in favour of L-(+)-lactate.

Production of lactic acid by the monoculture of Lactobacillus sp.

In the first experiments glucose and sucrose were fermented by *Lactobacillus* sp., a Gram-positive rod-shaped strain isolated from silage and maintained in our laboratory in the molasses/malt sprouts/$CaCO_3$ medium. The strain has not been further characterised so far. This homofermentative strain was able to use mono- and disaccharide, grow and produce mainly (≈90 %) L-(+)-lactic acid (Figs. 1 and 2, Table 2).

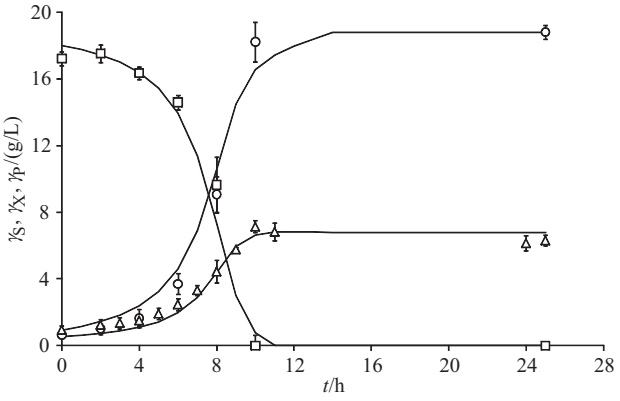

Fig. 1. Fermentation of glucose to lactic acid by *Lactobacillus* sp.: substrate concentration (γ_S, □), dry biomass (γ_X, Δ) and lactic acid concentration (γ_P, ○). In all figures symbols represent experimental data and lines are drawn according to the model

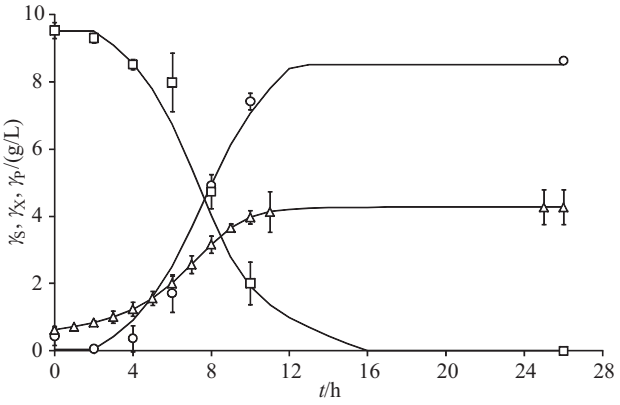

Fig. 2. Fermentation of sucrose to lactic acid by *Lactobacillus* sp.: substrate concentration (γ_S, □), dry biomass (γ_X, Δ) and lactic acid concentration (γ_P, ○)

Both conversions were rather fast and complete. Glucose (γ_0=20 g/L) was totally fermented in 10 h, while for complete conversion of sucrose (γ_0=10 g/L) approx. 12 h were needed. *Lactobacillus* sp. was adapted in 4 h to the

Table 2. Fine-tuning of the L-(+)- and D-(–)-lactic acid ratio by using mono- and mixed cultures during batch fermentation of different substrates from MRS medium at constant pH value (5.5±0.2) and different temperatures of cultivation (30–50 °C)

	Substrate	Temperature/°C	*w*(L-(+)-lactic acid)	*w*(D-(–)-lactic acid)
monoculture	glucose	40	0.926±0.020	0.074±0.021
(*Lactobacillus* sp.)	sucrose	40	0.887±0.002	0.113±0.026
mixed culture (*Lactobacillus* sp.+*L. amylovorus* DSM 20531^T)	starch	40	0.728±0.004	0.272±0.019
monoculture (*L. amylovorus* DSM 20531^T)*	starch	30	0.563±0.008	0.437±0.017
	sucrose	40	0.516±0.018	0.484±0.022
	starch	35	0.509±0.006	0.491±0.005
	starch	45	0.503±0.004	0.497±0.009
	glucose	40	0.499±0.002	0.501±0.002
	starch	40	0.480±0.008	0.520±0.013
	starch	50	0.371±0.008	0.629±0.011

*data from Trontel *et al.* (*6*)

conditions and grew exponentially the following 6 h by using substrates from the MRS medium. Similar specific growth rates (μ=0.25 and 0.29 h^{-1}, respectively) and substrate consumption rates (both r_S=0.24 h^{-1}) were observed. Twice higher initial concentration of glucose did not result in considerably higher biomass concentration ($\gamma_{X_{max}}$= 5.34 g/L for glucose and 4.50 g/L for sucrose) and, consequently, biomass yield coefficient was almost doubled in the medium with sucrose ($Y_{X/S}$=0.45 g/g) compared to the medium with glucose ($Y_{X/S}$=0.27 g/g). The described parameters brought fermentative activity of *Lactobacillus* sp. very close to the performance of *L. amylovorus* DSM 20531^T in the medium with sucrose (*6*).

Luedeking-Piret kinetics (*23*) is clearly followed during the production of L-(+)-lactic acid by *Lactobacillus* sp. (Figs. 1 and 2). Fermentation of glucose and sucrose resulted in similar product formation rates (r_P=0.41 and 0.50 h^{-1}, respectively) and this clearly indicates that the strain was inhibited neither by the initial concentration of the substrates nor by the produced lactate. Further, equal product yield coefficients (both $Y_{P/S}$=0.91 g/g) were obtained, although approx. 17 % less biomass was estimated in the medium with sucrose. Significantly different productivity of L-(+)-lactic acid was observed during the fermentation of glucose (Pr_P=1.82 g/(L·h)) and sucrose (Pr_P=0.82 g/(L·h)). These values are in agreement with the analogue values obtained in lactic acid production by *Lactobacillus* sp. (*10*).

Production of lactic acid by the mixed culture of Lactobacillus sp.

Starch was completely hydrolysed but not completely fermented by *L. amylovorus* DSM 20531^T (Fig. 3). At the end of fermentation, the concentration of the remaining glucose and/or maltose from starch hydrolysis, as determined by HPLC analysis and reducing sugars (RS) method, was 0.5 g/L. This is quite different from the results obtained in starch fermentation by the monoculture of *L. amylovorus* DSM 20531^T under the same conditions when the substrate was completely depleted from the medium in 10 h (*6*). Accorrding to Carr *et al.* (*5*), some members of group A obligate homofermentative lactobacilli are able to use maltose, with or without delay. All together, it implies that the remaining maltose/glucose was not fermentatively converted by *Lactobacillus* sp. or by *L. amylovorus* DSM 20531^T.

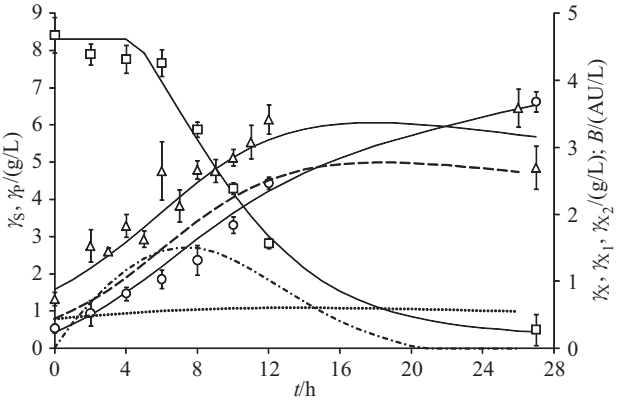

Fig. 3. Simultaneous saccharification and fermentation of starch to D/L-lactic acid by the mixed culture: substrate concentration (γ_S, □), dry biomass (γ_X, Δ) and lactic acid concentration (γ_P, ○). Changes in the concentration of *L. amylovorus* DSM 20531^T (γ_{X_1}, dashed line) and *Lactobacillus* sp. (γ_{X_2}, dotted line) biomass in mixed culture and bacteriocin (*B*, dashed/dotted line) were predicted by the model

Morphological differences between the two bacterial strains of the same genus, *Lactobacillus* sp. and *L. amylovorus* DSM 20531^T, have not been observed (image of the two-member mixed culture in the MRS broth under microscope after Gram staining not shown). Maximum viable cell number of $8.3 \cdot 10^8$ and $1.1 \cdot 10^9$ CFU/mL for *Lactobacillus* sp. monoculture and for two-strain mixed culture, respectively, were determined in the MRS medium with glucose after 12 h of cultivation at 40 °C. The biomass of two strains growing together in the medium was quantified. The interrelation of dry biomass and $A_{600\ nm}$ value was linear (y=0.5804x+1.6794; R^2=0.9517) and similar to that yielded for *Lactobacillus* sp. monoculture (y= 0.5634x+0.9026; R^2=0.9719 for glucose and y=0.8017x+ 0.6531; R^2=0.9498 for sucrose) (Fig. 4). Bacterial biomass grew without lag phase and reached its maximal value of 3.5 g/L after 12 h of batch cultivation.

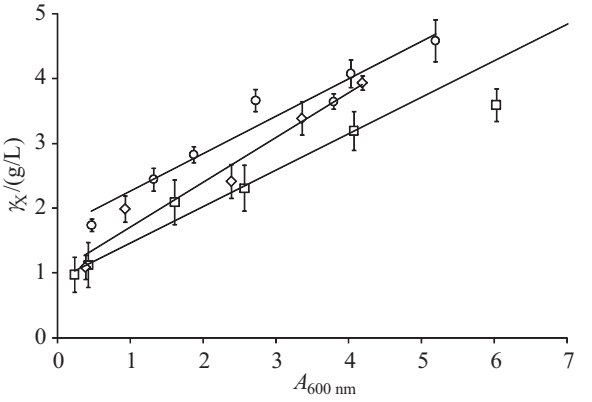

Fig. 4. Ratio between the corresponding pairs of $A_{600\ nm}$ and dry biomass (γ_X) during exponential growth phase of *Lactobacillus* sp. monoculture in MRS medium with glucose (□) and sucrose (◊), and two-member mixed culture in the medium with starch (○)

It seems that hydrolytic activity of *L. amylovorus* DSM 20531^T and fermentative activity of the mixed culture ocurred simultaneously since the D/L-mixture (0.272/ 0.728) was produced at a defined constant ratio from the begining of the cultivation (Fig. 3). Overall starch consumption rate (r_S) and D/L-lactate formation rate (r_P) were equal, with estimated value of 0.17 h^{-1}. This bioprocess was less efficient than the two fermentations conducted by the monoculture and SSF of soluble starch catalyzed by *L. amylovorus* DSM 20531^T (*6*). Maximal concentration of the produced lactate of 6.60 g/L, product yield coefficient ($Y_{P/S}$) of 0.63 g/g and productivity (Pr_P) of 0.61 g/(L·h) were achieved. Fermentative activity of *Lactobacillus* sp., as a member of the mixed culture, was confirmed by the ratio of produced isomeric forms of lactic acid (Table 2). Three quarters of maltose/glucose yielded during starch hydrolysis were fermented to L-(+)-lactic acid (w=0.728).

Initial concentration of acetate in the MRS media was 3.6 g/L and it remained at the same level during batch conversions of the three substrates by the mono- and mixed culture, as proved by HPLC analysis (Fig. 5). Other fermentative products were not identified by HPLC analysis. Based on this, it can be concluded that the mono- and mixed culture of *Lactobacillus* strains conducted homolactic fermentation.

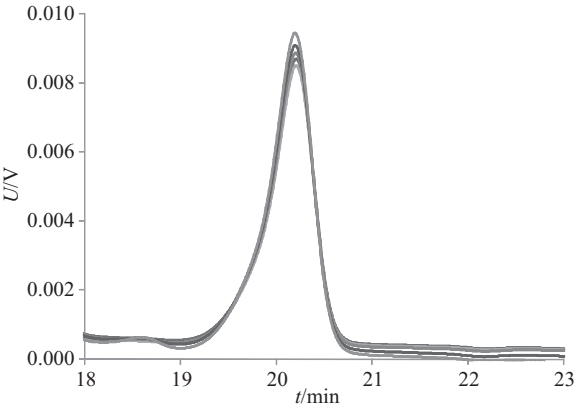

Fig. 5. Part of chromatograms (retention time, t_R=18–23 min) obtained by HPLC analysis of samples withdrawn during batch cultivation of two-strain mixed culture; t_R of acetic acid is 20.225 min (*6*)

Compatibility of experimental data and modelled values

Both *Lactobacillus* strains grown together in the mixed culture could not be morphologically distinguished on the MRS agar and therefore could not be selectively counted. Several members of *L. amylovorus* species are bacteriocin producers (*19,26–28*). Bacteriocin sensitivity measurement was performed to prove the sensitivity of the tested *Lactobacillus* sp. strain to bacteriocins of *L. amylovorus* DSM 20531^T. Growth of the sensitive strain was inhibited by 50 % with the addition of supernatant (9 % by volume) of the bacteriocin producer (data not shown). In one of the previous studies, a model describing killer/sensitive mixed cultures comprising bacterial amensalism (*11*) was used. In the model, the biomass was divided into two components – bacteriocin-producing *L. amylovorus* cells (γ_{X_1}) and sensitive *Lactobacillus* sp. cells (γ_{X_2}). The model predicted growth dynamics of both cultures and bacteriocin synthesis (Fig. 3). Based on the modelled data, it can be assumed that *L. amylovorus* DSM 20531^T grew faster and represented the main part of the biomass. The concentration of sensitive *Lactobacillus* sp. cells did not change significantly, probably due to the accumulation of bacteriocins, but was high enough to move the D/L-lactate ratio from 0.520/0.480 (*6*) to 0.272/0.728 (Table 2).

Compatibility of experimental and modelled data (correlation coefficient, R^2, and root mean square, RMS) are given in Table 1.

Based on the two parameters, R^2 and RMS (Table 1), it can be concluded that the simple kinetic model successfully describes the production of defined ratios of D-(–)- and L-(+)-lactic acid by mono- and mixed bacterial cultures.

Specific growth rate of *L. amylovorus* DSM 20531^T in the mixed culture estimated by the model (μ_{max}=0.29 h^{-1}; Table 1) was significantly lower than the corresponding value obtained during cultivation of the monoculture under the same conditions (experimental and modelled value of μ_{max} were 0.67 and 0.61 h^{-1}, respectively) (*6*). It is well known that bacteriocin production is usually growth associated (*11,26,28*). Lag phase of growth of the mixed culture was not observed (Fig. 3), assuming that *L. amylovorus* DSM 20531^T was producing bacteriocin (α_B=1.1 AU/g) from the beginning of SSF. Consequently, specific death rate of the sensitive strain due to bactericion activity (k_{DB}) of 0.07 L/(AU·h) and relatively poor growth of *Lactobacillus* sp. (μ_{max}=0.18 h^{-1}) in the mixed culture were obtained by the model. Furthermore, experimentally determined maximal concentrations of the biomass of the two *Lactobacillus* sp. ($\gamma_{X_{max}}$) of 3.5 g/L were divided by the model to $\gamma_{X_{max1}}$=2.7 g/L of bacteriocin-producing strain and to $\gamma_{X_{max2}}$=0.7 g/L of bacteriocin-sensitive strain. Similar values ($\gamma_{X_{max}}$=2.21–2.80 g/L) for *L. amylovorus* DCE 471 had been described previously (*26*). The monoculture biomass concentrations were considerably higher in the media with glucose ($\gamma_{X_{max}}$=5.34 g/L for *Lactobacillus* sp. and $\gamma_{X_{max}}$=4.90 g/L for *L. amylovorus* DSM 20531^T) and sucrose ($\gamma_{X_{max}}$=4.50 g/L for *Lactobacillus* sp. and $\gamma_{X_{max}}$= 3.73 g/L for *L. amylovorus* DSM 20531^T) than those modelled for the growth of mixed culture. In the medium with starch, $\gamma_{X_{max}}$=4.38 g/L was determined for *L. amylovorus* DSM 20531^T (*6*).

Conclusion

Lactobacillus sp. conducts highly efficient homofermentative conversion of glucose and sucrose to mainly L-(+)-lactic acid and therefore represents a good candidate for the industrial production of lactic acid to be used in the advanced PLA manufacturing. During the growth of the strain in the two-strain mixed culture, it strongly interacted with the other member, *L. amylovorus* DSM 20531^T. *L. amylovorus* DSM 20531^T is known as bacteriocin producer, while our test strain (*Lactobacillus* sp.) was sensitive to its bacteriocins. The co-existence of both *Lactobacillus* strains could be described as amensalism. Due to technical limitations, growth dynamics of two interacting bacterial cultures was not experimentally defined, but unstructured kinetic model was able to describe the lactic acid fermentation. Amylolytic activity of *L. amylovorus* DSM 20531^T and its capacity to conduct simultaneous saccharification and ferementation was employed in starch conversion and, in the two-strain mixed culture, for fine-tuning of the ratio of produced D/L-lactic acid. This is another advantage that can be easily implemented in the cost-effective synthesis of biodegradable polymers and production of copolymers, blends and nanocomposites.

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List of symbols

B bacteriocin activity (AU/L)
C constant in Eq. 6
k_B specific consumption rate of bacteriocin (L/(g·h))
k_{DB} specific death rate due to bacteriocin activity (L/(AU·h))
k_{d_0} constant in Eq. 6 (h^{-1})
k_d specific death rate (h^{-1})
m_S maintenance energy coefficient (h^{-1})
γ concentration (g/L)
Pr productivity (g/(L·h))
r_S substrate consumption rate (h^{-1})
r_P product formation rate (h^{-1})
t time (h)
$Y_{X/S}$ biomass yield coefficient (g/g)
$Y_{P/X}$ product/biomass yield coefficient (g/g)
$Y_{P/S}$ product yield coefficient (g/g)

Greek letters

α coefficient for growth-associated term (g/g)
β coefficient for non-growth associated term (g/(g·h))
α_B coefficient for growth associated term for bacteriocin production (g/g)
β_B coefficient for non-growth associated term for bacteriocin production (g/(g·h))
μ specific growth rate (h^{-1})

Subscripts

0 initial
1 related to *Lactobacillus* sp. in the monoculture and to *L. amylovorus* DSM 20531^T in the mixed culture
2 related to *Lactobacillus* sp. in the mixed culture
S substrate
X biomass
P product
min minimum
max maximum

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