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Modelling the Effect of Different Substrates and Temperature on the Growth and Lactic Acid Production by *Lactobacillus amylovorus* DSM 20531^T in Batch Process

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Summary

Amylolytic lactic acid bacterium *Lactobacillus amylovorus* DSM 20531^T utilised glucose, sucrose and starch as a sole carbon and energy source. The three substrates were completely depleted from MRS medium during batch cultivations carried out in a laboratory scale stirred tank bioreactor at constant temperature (40 °C) and pH value (5.5). Under the tested conditions, the bacterium was capable of conducting simultaneously starch hydrolysis and fermentation. A mixture of two stereoisomers, D-(–)- and L-(+)-lactic acid, was produced in all cases by highly efficient homofermentative bioprocess with 0.93 to 1 g of lactate produced per g of total (consumed) substrate. The effect of temperature on the kinetics of cell growth and lactic acid production by the amylolytic strain in the starch-containing medium was also investigated. Efficient simultaneous saccharification and fermentation (SSF) was obtained at 35, 40 and 45 °C with completely degraded complex carbohydrate in 8 to 12 h and the product yield coefficient in the range from 0.91 to 0.93 g/g. Maximum values for substrate consumption rate (0.89 h^{-1}), maximum specific growth rate (0.87 h^{-1}), product formation rate (2.01 h^{-1}), and productivity of lactic acid (1.45 g/(L·h)) were obtained at 45 °C, while maximum biomass concentration (4.38 g/L) was attained at 40 °C. The ratio of the two stereoisomeric forms of produced lactic acid was strongly affected by the temperature. Unstructured kinetic model was used to describe the consumption of the three substrates, bacterial biomass formation and lactic acid production by *L. amylovorus* DSM 20531^T. The dependence of biokinetic parameters on temperature was described by cardinal temperature model. The applied models successfully predicted all experimental data.

Key words: amylolytic lactic acid bacterium, *Lactobacillus amylovorus*, glucose, sucrose, starch, batch process, cultivation temperature, D/L-lactic acid, unstructured kinetic model, cardinal temperature model

Introduction

Lactic acid (2-hydroxypropionic acid, $C_3H_6O_3$) is one of the most useful chemicals with versatile applications in food, pharmaceutical and chemical industry (*1*). Lactic acid naturally exists in two optical isomers: D-(–)- and L-(+)-lactic acid. On the industrial scale, lactic acid has been produced by chemical synthesis (*2*) or by microbial processes (*2,3*). In fermentation by lactic acid bacteria (LAB), it is possible to reach high growth rate, high product yield and high product specificity. Due to the production of a single product – lactic acid, homofermentative LAB, mainly from genus *Lactobacillus*, have been employed succesfully in industrial production of lactic acid. Therefore, bacterial production holds long-term promise of offering sustainable and environmentally friendly pro-

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duction of lactic acid. Besides conventional use of fermentatively produced lactic acid, new technologies for the production of polylactic acid, polymers of lactic acid, used for manufacturing of plastic, fibres, packing and other special textile materials, have been established (*1*).

Carbon source is the most important contributor to the cost of lactic acid fermentation (*4*). Traditionally, relatively expensive glucose is a preferred substrate in fermentative processes, as well as sucrose from molasses (*5*), and lactose from whey (*6*).

Starch has been used in a two-stage industrial production of lactic acid (*7*). The starch material is, first, chemically and/or enzymatically hydrolyzed to glucose, which is then fermented by LAB (*8,9*) in the second stage. The ability of amylolytic lactic acid bacteria (ALAB) to hydrolyze starch and then to ferment maltose and glucose has earned much attention and has been explored for one-step one-pot lactic acid production. ALAB produce amylases, enzymes capable of cleaving α-1,4-glycosidic linkages between α-D-glucopyranosyl residues in the molecules of a complex carbohydrate and, thus, can directly produce lactic acid from starch and its derivatives (*3,9–12*). According to Taxonomic Outline of the Procaryotes (*13*), the bacterium *Lactobacillus amylovorus* DSM 20531^T belongs to the genus *Lactobacillus*, family Lactobacillaceae, order Lactobacillales, class Bacilli, phylum Firmicutes. The key characteristic of this phylogenetic group of obligate homofermentative bacteria is fermentation of the following carbohydrates: amygdalin, cellobiose, galactose, maltose, mannose, salicin, sucrose, trehalose, fructose, glucose (without formation of CO_2), sorbitol and esculin. ALAB can ferment a wide variety but not all mono- and disaccharides present in renewable and waste materials. *L. amylovorus* strains cannot ferment pentoses, lactose, mannitol, melibiose and raffinose. Only a few publications highlight physiological patterns of *L. amylovorus* strains (*10,12,14-16*). Unlike glucose, fructose and starch, sucrose is not a substrate of choice for efficient production of lactic acid by wild-type amylolytic bacterium *L. amylovorus* JCM 1126 (*17*). Another strain from this genus, *Lactobacillus amylovorus* DCE 471, cannot ferment sucrose either (*14*). Very limited information is available on invertases produced by *Lactobacillus* sp. The invertase from *L. reuteri* (*18*) and the invertase from *L. amylovorus* YF43 (*17*) have been purified and partially characterized. Besides induction of invertase activity, transport of sucrose into the cells can be a rate-limiting step in the substrate utilisation (*17*). It is possible to transport sucrose by permease or phosphotransferase system (PTS) into the cell and (phosphorylate and) hydrolyze to fructose and glucose 6-phosphate, both intermediates of glycolytic pathway (*19*). Based on genomic analyses, it seems that fermentative capabilities of some LAB include PTS transporter for sucrose and activity of invertase (EC 3.2.1.26) (*20*). Invertase from *L. amylovorus* DSM 20531^T has not been purified and characterized yet. Sucrose can also be degraded by phosphorolysis catalyzed by sucrose phosphorylase (EC 2.4.1.7) to fructose and glucose 1-phosphate (*21*), but evidence describing such reaction catalyzed by *L. amylovorus* cells is not available. Maltose and glucose, products of complete degradation of starch, can be translocated into LAB cells by active transport, catabolyzed *via* the glycolysis (homofermentative) to pyruvate and then to lactic acid (*22*).

The aim of this work is to define the capacity of ALAB *Lactobacillus amylovorus* DSM 20531^T to ferment simple sugars, glucose and sucrose, primary carbon sources present in many potential cheap bulk materials. Furthermore, the capability of this amylolytic strain to conduct simultaneously starch saccharification and fermentation (SSF) has been investigated. The effect of temperature on biokinetic parameters and the ratio of the two stereoisomeric forms of produced lactic acid was characterized. Unstructured kinetic model (UKM) was upgraded by cardinal temperature model (CTM), and it was shown that this model is suitable for describing fermentative activity of *L. amylovorus* DSM 20531^T.

Materials and Methods

Bacterial strain, maintenance, inoculum preparation and medium

The homofermentative ALAB *Lactobacillus amylovorus* DSM 20531^T (ATCC 33620, NRRL B-4540) (Braunschweig, Germany), a D/L-lactate producer (*23*), was used throughout this study. MRS medium containing three different substrates: glucose (MRS-glc, S_0=20 g/L), sucrose (MRS-suc, S_0=10 g/L), or soluble starch (MRS-starch, S_0=10 g/L) was used for culture maintenance, inoculum preparation and fermentation experiments. MRS-glc (Biolife, Milan, Italy) was used as supplied, and all ingredients of MRS-suc and MRS-starch media were separatelly weighed. Concentrations of all components of the MRS medium, except sucrose and starch, were unchanged (*24*). Unless otherwise stated, all chemicals used in this work were purchased from Merck (Darmstadt, Germany). The medium was sterilized at 121 °C for 20 min. *L. amylovorus* DSM 20531^T was maintaned in the MRS broth and on the MRS agar (both as MRS-glc, MRS-suc, and MRS-starch; 10 mL) at 4 °C for maximum seven days and propagated twice in the MRS broth at 40 °C for 12 h ($A_{600\ \mathrm{nm}}$ ≈0.8) prior to use as inoculum for preliminary cultivations and fermentation experiments. Inoculum preparation for preliminary cultivations (400 mL) in Erlenmeyer flasks (500 mL) was carried out in MRS-glc, MRS-suc and MRS-starch broth (10 mL) in test tubes. The medium was inoculated by overnight culture (0.1 mL) pregrown in the corresponding MRS medium at 40 °C and, after 12 h of incubation at 40 °C, the cell count was in the range from 10^7 to 10^8 CFU/ mL ($A_{600\ \mathrm{nm}}$=0.6–0.8, γ=0.2–0.3 g/L). Described procedure for inoculum preparation with pregrown inocula first in the test tubes and then in Erlenmeyer flasks was used for fermentation experiments. Aliquots of bacterial suspension were freeze-dried (Christ Alpha 1–2 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and stored at –20 °C.

Fermentation experiments

A stirred tank bioreactor with control system (Chemap AG, Volketswil, Switzerland) was used for batch fermentations. The Chemap *in situ* sterilizable bioreactor (V=6 L) was filled with 5 L of MRS medium with initial pH value of 6.2±0.2. After inoculation (2.5 %), due to the activity of the bacterial strain, pH decreased to 5.5 and then, the value was maintaned at 5.5±0.2 through the automatic addition of 10 moL/L NaOH. First, fermentations in MRS-glc, MRS-suc and MRS-starch media were carried out at 40 °C, and, afterwards, lactic acid was produced by the starch-hydrolyzing bacterium only in MRS-starch medium at different temperatures of cultivation (30, 35, 40, 45 and 50 °C). The fermentations were conducted without aeration and with constant agitation speed (400 rpm) to keep the medium homogeneous. Samples were withdrawn aseptically from the fermentation medium at regular time intervals and analyzed as described below.

Analysis of cell growth

Absorbance of the withdrawn sample suspension was determined at 600 nm ($A_{600\ \text{nm}}$) (spectrophotometer Cary 13E Varian, Mulgrave, Australia). After centrifugation of the sample (4000 rpm/20 min/4 °C; Harrier 18/80, Sanyo, UK), the supernatant was removed, the biomass dried at 105 °C for 24 h and biomass dry mass (BDM) was determined. Corresponding pairs of BDM (hereafter instead of BDM symbol X is used) and $A_{600\ \text{nm}}$ values were taken together in order to calculate their dependence, and linear correlations for fermentations carried out in MRS-glc, MRS-suc and MRS-starch medium were obtained (data not shown). Viable cell numbers were enumerated by plating on MRS agar and expressed as CFU per millilitre. Maximum viable cell number of $8.9\cdot10^9$ CFU/mL was determined in MRS-glc medium after 12 h of cultivation at 40 °C.

Substrates and product concentration determination

Sample pretreatment

Equal volumes of supernatant and zinc sulphate heptahydrate solution (γ=500 g/L) were vigorously mixed and left at room temperature for 20 min (*25*). Precipitated proteins were removed by centrifugation (12 000 rpm/15 min; Tehtnica HC-240, Železniki, Slovenia), the resulting supernatant was filtered through a nylon syringe filter (0.2 μm; Carl Roth GmbH, Karlsruhe, Germany) and analyzed by ion-exchange high-pressure liquid chromatography (see below).

Pretreatment of the supernatants, which were obtained by centrifugation of samples withdrawn during fermentations carried out in MRS-starch medium, included also acid hydrolysis of the remaining soluble starch. Briefly, hydrochloric acid (7 mL, γ=210 g/L) and distilled water (10 mL) were added to 5 mL of the supernatant, then the mixture was heated in boiling water for 40 min, afterwards another portion of hydrochloric acid (4 mL) was added and the mixture was allowed to react for 30 min at room temperature. After neutralization (NaOH, γ=200 g/L), the final volume of the mixture of 50 mL in a volumetric flask was adjusted by the addition of distilled water. The obtained solution was used for determination of concentration of reducing sugars (RS) and part of the solution, after filtration, for HPLC analysis, as described below.

Concentration of substrates

Total concentration of carbohydrates in the MRS medium was calculated from the initial concentration of the main carbon source (glucose S_0=20 g/L, sucrose S_0=10 g/L and soluble starch S_0=10 g/L) and from the total concentration of RS (S_0=(0.8±0.04) g/L) from yeast extract, meat extract and peptone (*26*), standard ingredients of the MRS medium. Further, total hydrolysis of 1 g of starch resulted in 1.11 g of glucose. Therefore, theoretical total concentration of hexose equivalents in the MRS medium (S_0) was 20.8 g/L in MRS-glc, 11.4 g/L in MRS-suc and 11.9 g/L in MRS-starch. Results for the concentrations obtained by using two methods, determination of reducing sugars and HPLC analysis, were in the range of standard deviation of ±5 % according to the theoretical value, proving thus that starch hydrolysis used in these methods was complete.

Reducing sugars (RS)

In all the supernatants, concentration of RS was determined by the reaction with a copper salt. Modification of a method described elsewhere (*27*) was used. Briefly, to the supernatant were added, first, distilled water, then 10 mL of Fehling's solution I ($CuSO_4\cdot5H_2O$, γ=69.3 g/L) followed by 10 mL of Fehling's solution II ($KNaC_4H_4O_6$, γ=346 g/L). The mixture was heated for several minutes, brought to a boil and the boiling was continued for exactly 2 min. To the cooled mixture, 10 mL of potassium iodide solution (γ=300 g/L), 10 mL of sulphuric acid solution (γ=260 g/L), and 2 mL of starch indicator solution (γ=10 g/L) were added. The mixture was titrated with standard 0.1 mol/L sodium thiosulphate solution. Two blank determinations were conducted in identical manner substituting the supernatant with distilled water (blank 1) or glucose solution (γ=10 g/L; blank 2). Concentration of RS is expressed as the concentration of glucose in g/L. In the supernatants from the fermentations carried out in MRS-starch medium, quantitative analysis was performed before and also after acid hydrolysis of the remaining starch in order to determine the concentration of undepleated starch and higher oligosaccharides.

HPLC analysis

Concentrations of different substrates and the concentration of produced lactate were determined by high-performance liquid chromatography (HPLC) using Shimadzu Class-VP LC-10A$_{VP}$ system (Shimadzu, Kyoto, Japan) with Supelcogel H precolumn (5 cm×4.6 mm, i.d. 9 μm; Sigma-Aldrich, USA), Supelcogel C-610H column (30 cm×7.8 mm, i.d. 9 μm; Sigma-Aldrich, St. Louis, MO, USA) and a refractive index detector. All standards for HPLC analysis (glucose, fructose, sucrose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, lactic acid, acetic acid and ethanol) were obtained from Sigma-Aldrich (Taufkirchen, Germany). After injecting the standard or sample solution (20 μL) in the equilibrated chromatographic system, analyses were perfomed at a temperature of 30 °C and the elution was done using isocratic mobile phase (0.1 % H_3PO_4) conditions at a flow rate of 0.5 mL/min.

Enzymatic assay

D- and L-lactic acid in the supernatants were measured with the 'D-lactic acid (D-lactate) and L-lactic acid (L-lactate) assay procedures' determination kit by Megazyme (*28*).

Values for biokinetic parameters r_S, μ and r_P were calculated by using experimental data, as described by Doran (*29*):

$$\ln S=\ln S_0+r_S t \qquad /1/$$

$$\ln X=\ln X_0+\mu t \qquad /2/$$

$$\ln P=\ln P_0+r_P t \qquad /3/$$

Unstructured kinetic model (UKM)

For the purpose of unstructured modelling of batch fermentation of three different substrates to lactic acid by the amylolytic lactic acid bacterium, some simplifications were introduced: in the medium with starch as a sole substrate, hydrolysis is not separated from fermentation, and glucose is considered to be the only substrate in the fermentation process; starch hydrolysis is not a limiting step for fermentation.

Consumption of three different substrates, growth of the amylolytic strain and lactic acid production by the bacterium were modelled using the following set of equations:

$$\frac{dS}{dt}=-\frac{1}{Y_{X/S}}\cdot\frac{dX}{dt}-m_s\cdot X \qquad /4/$$

$$\frac{dX}{dt}=\mu\cdot X-k_d\cdot X \qquad /5/$$

$$\mu=\mu_{max}\cdot\frac{S}{S+K_S}\left(\frac{K_1}{K_1+P}\right) \qquad /6/$$

$$k_d=k_{d_0}\cdot(1+C\cdot P) \qquad /7/$$

$$\frac{dP}{dt}=\alpha\cdot\frac{dX}{dt}+\beta\cdot X \qquad /8/$$

Eq. 4 implies that substrate was used for growth and maintenance of bacterial cells (*30*). Biomass growth (Eq. 5) depends on the correlation between biomass formation (Eq. 6) and biomass death rate (Eq. 7). Noncompetitive product inhibition term was included in Monod equation for specific growth rate (Eq. 6) (*31*). Specific death rate was described by Eq. 7 (*32*). Production of lactic acid depends on instantaneous concentration of biomass and its growth rate, which is described in Eq. 8 (*33*). Ordinary differential equations were simultaneously solved by the Runge-Kutta 4 integration technique of Berkeley Madonna software (*34*). All parameters needed for the modelling were limited to realistic values to avoid unrealistic fitting solutions without physiological relevance and optimised with the functions 'multiple curve fit' and 'parameter slide'.

Cardinal temperature model (CTM)

In this model proposed by Rosso *et al.* (*35*) biokinetic parameters μ_{max}, r_S and r_P, derived from the experimental data, were expressed as a function of the temperature:

$$\mu_{max}=\begin{cases}\theta<\theta_{min},\ 0.0\\ \theta_{min}<\theta<\theta_{max},\ \mu_{opt}\cdot\tau(\theta)\\ \theta<\theta_{max},\ 0.0\end{cases} \qquad /9/$$

$$\tau(\theta)=\frac{(\theta-\theta_{max})\cdot(\theta-\theta_{min})^2}{(\theta_{opt}-\theta_{min})\cdot[(\theta_{opt}-\theta_{min})\cdot(\theta-\theta_{opt})-(\theta_{opt}-\theta_{max})\cdot(\theta_{opt}+\theta_{min}-2\cdot\theta)]} \qquad /10/$$

The above equation can be summarized as:

$$\mu_{max}(\theta)=\mu_{opt}\cdot\tau(\theta) \qquad /11/$$

where θ_{min} is the temperature below which no growth occurs, θ_{opt} is the temperature at which the μ_{max} is optimal, and θ_{max} is the temperature above which no growth occurs. Temperature dependence of biokinetic parameters X_{max}, $Y_{X/S}$, and $Y_{P/S}$ was described by empirical equations. When appropriate, coefficient of determination (R^2) is given. Modified Arrhenius equation was used to calculate activation energies (E_a), as described by Yuwono and Kokugan (*36*):

$$\ln k=\ln A-\frac{E_a}{R}\cdot\frac{1}{T} \qquad /12/$$

where k can be μ_{max}, r_S, r_P, Pr_X or Pr_P.

Results and Discussion

Preliminary experiments

In batch cultivations of *Lactobacillus amylovorus* DSM 20531^T in MRS-glc medium in Erlenmeyer flasks, the strain was adapted for approx. 7 h of lag phase, and then grown exponentially for 5 h, until it reached the stationary phase (data not shown). Maximum biomass concentration of 5 g/L was attained at the end of exponential growth phase after 12 h of cultivation. According to the pattern of pH decrease from 6 to 3.8, lactic acid production followed the growth curve and most of lactate was produced during the exponential growth phase. The bacterial strain was also adapted to MRS-suc medium and MRS-starch medium in the same way, and similar patterns of growth and fermentative activity of the bacterium were observed (data not shown). Bacterial suspensions were cultivated in MRS-glc, MRS-suc, and MRS-starch medium in Erlenmeyer flasks and used as inocula for fermentation experiments in MRS medium with corresponding substrate in the stirred tank laboratory bioreactor.

Effect of different carbon and energy sources on the growth and lactic acid production

The ALAB strain used in this work, *Lactobacillus amylovorus* DSM 20531^T, utilized glucose, sucrose and soluble starch from MRS medium as a sole carbon and energy source. During batch cultivations in stirred tank bioreactor at a constant temperature (40 °C) and maintaned pH value (5.5), the ALAB grew, consumed all available substrates and fermented them almost stoichiometrically producing D/L-lactate.

Under the described conditions, *L. amylovorus* DSM 20531^T utilized glucose from MRS medium at the rate of 0.38 h^{-1}. Glucose was consumed faster than starch (r_S=0.29 h^{-1}) or sucrose (r_S=0.24 h^{-1}) (Table 1) and it (S_0=20 g/L) was depleted from the MRS-glc medium during approx. 12 h of batch cultivation (Fig. 1). Metabolic energy (ATP) was generated through the substrate level phosphorylation and reducing equivalents were used for biomass formation and its maintenance. Exponential growth of bacterial cells in the MRS-glc medium was observed after 5 h of lag phase and biomass production

Table 1. Effect of substrate on the biokinetic parameters of *L. amylovorus* DSM 20531^T grown in MRS medium at a constant temperature (40 °C) and pH=5.5. Values obtained by the unstructured kinetic model are given in parentheses

Substrate	t_{gp}/h		μ	X_{max}	r_S	$Y_{X/S}$	Pr_x	r_P	P_{max}	$Y_{P/S}$	$Y_{P/X}$ (α)	Pr_P	K_S	K_I	β	m_S	k_{D_0}
	lag	exp	h^{-1}	g/L	h^{-1}	g/g	g / (L · h)	h^{-1}	g/L	g/g	g/g	g/(L·h)	g/L	g/L	g/(g·h)	h^{-1}	h^{-1}
glucose	5	5	0.41 (0.58)	4.90 (5.62)	0.38	0.45 (0.32)	0.25	0.40	18.56 (19.27)	0.93	3.79 (3.40)	1.69	(1.68)	(40)	(0.01)	(0.01)	(0.011)
sucrose	7	7	0.24 (0.32)	3.73 (4.61)	0.24	0.31 (0.50)	0.37	0.23	10.08 (10.66)	1.01	2.70 (1.80)	0.84	(1.40)	(20)	(0.03)	(0.00)	(0.000)
starch^a	2	6	0.67 (0.61)	4.38 (4.26)	0.29	0.44 (0.44)	0.44	0.61	10.52 (10.34)	0.95	2.40 (2.30)	1.05	(0.80)	(38)	(0.01)	(0.00)	(0.004)

t_{gp} – duration of growth phases, lag (lag) and exponential (exp); ^{a}experimental data presented also in Table 4 for comparison

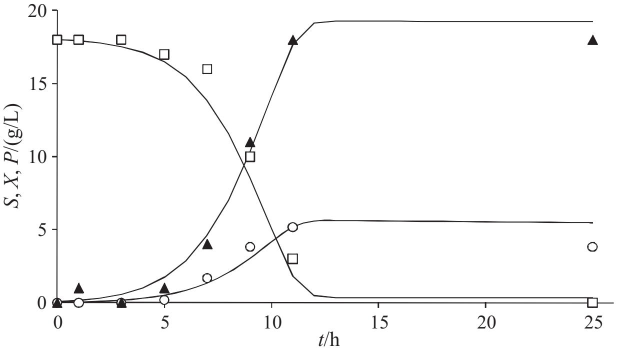

Fig. 1. Modelling of substrate consumption (*S*, □), biomass formation (*X*, ○) and lactic acid production (*P*, ▲) by *L. amylovorus* DSM 20531^T in MRS-glc medium at a constant temperature (40 °C) and pH=5.5. In all figures the symbols represent experimental values and full lines were drawn according to the unstructured kinetic model

occured at maximum specific growth rate (μ_{max}) of 0.41 h^{-1}. Considerably higher μ_{max} (0.64 to 0.71 h^{-1}) was obtained for *L. amylovorus* DCE 471 grown in glucose-based modified MRS medium with similar process parameters (*14*).

Glucose as a single carbon and energy substrate was converted to a racemic mixture of D- and L-lactic acid (Table 2). Any other product, *e.g.* acetate or ethanol, was not determined by HPLC analysis. Due to depletion of the substrate, lactic acid production is solely associated with the growth of the amylolytic bacterium. Lactate formation rate (r_P=0.40 h^{-1}) is proportional to glucose consumption rate (r_S), as it is well known for fermentation processes (*37*). In MRS-glu medium the r_P:r_S ratio was approx. 1 (Table 1). In highly efficient batch process, product yield coefficient ($Y_{P/S}$) was 0.93 g/g.

Table 2. Fraction (*w*) of L- and D-lactic acid produced by *L. amylovorus* DSM 20531^T grown in MRS medium with three different substrates at different temperatures and at a constant pH value (5.5)

Substrate	θ °C	*w*(L-lactic acid) %	*w*(D-lactic acid) %
glucose	**40**	**49.9**	**50.1**
sucrose	**40**	**51.6**	**48.4**
starch	30	56.3	43.7
	35	50.9	49.1
	40	**48.0**	**52.0**
	45	50.3	49.7
	50	37.1	62.9

In the samples withdrawn during batch cultivation in MRS-suc medium, sucrose was the only substrate whose peak was detected by HPLC analysis, while fructose or glucose were not detected. Sucrose (S_0=10 g/L) was depleted from MRS medium in approx. 16 h (Fig. 2). No clearly distinct lag (≈7 h) or exponential growth phase (≈7 h) were observed in MRS-suc medium. Similar to glucose from MRS-glu medium, sucrose from MRS-suc medium was also converted to a mixture of D- and L-lactic acid (Table 2) with lactate formation rate (r_P=0.23 h^{-1}) equal to sucrose consumption rate (r_S=0.24 h^{-1}), and $Y_{P/S}$ of 1.01 g/g (Table 1).

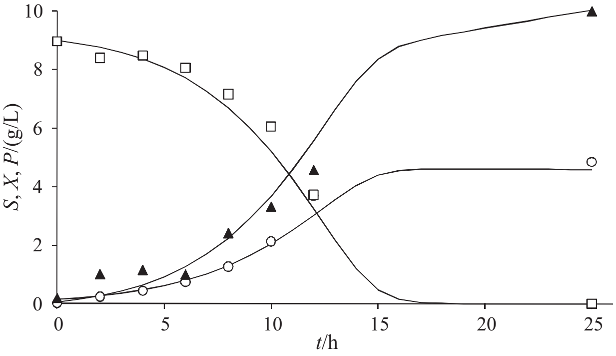

Fig. 2. Modelling of substrate consumption (*S*, □), biomass formation (*X*, ○) and lactic acid production (*P*, ▲) by *L. amylovorus* DSM 20531^T in MRS-suc medium at a constant temperature (40 °C) and pH value (5.5)

During lactic acid production in MRS-starch medium by *L. amylovorus* DSM 20531^T, the concentration of starch (glucose determined by HPLC after acid hydrolysis of starch) was decreasing until it was depleted after 10 h (40 °C; Fig. 3). As a result of starch hydrolysis, RS increased to 0.72 g/L in 2 h, were kept approx. constant (0.72–0.92 g/L) for 8 h, and after starch depletion, they were utilized completely. In these experiments starch was depleted completely, contrary to some results in literature (*10*). Xiaodong *et al.* (*10*) reported hydrolysis of

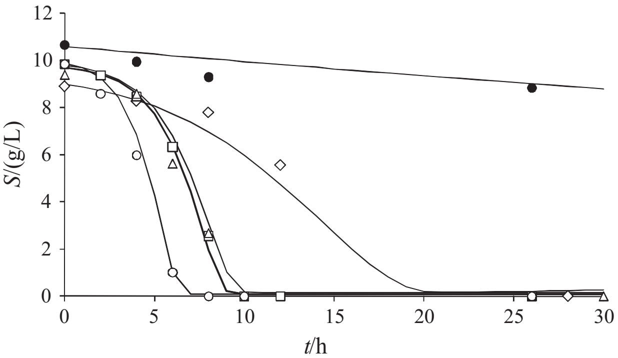

Fig. 3. Modelling of starch consumption (S) from MRS-starch medium by *L. amylovorus* DSM 20531^T at different temperatures (◊ 30 °C, □ 35 °C, **Δ 40 °C**, ○ 45 °C and ● 50 °C) and at a constant pH value (5.5)

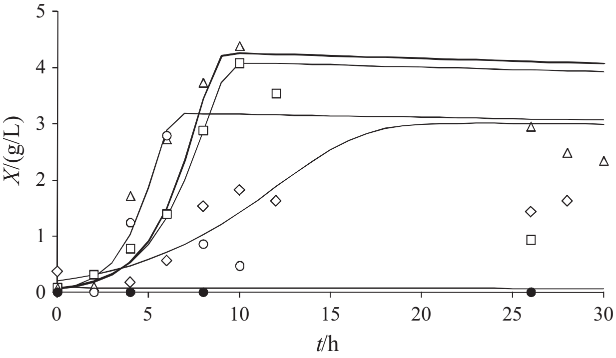

Fig. 4. Modelling of biomass production (X) of *L. amylovorus* DSM 20531^T in MRS-starch medium at different temperatures (◊ 30 °C, □ 35 °C, **Δ 40 °C**, ○ 45 °C and ● 50 °C) and at a constant pH value (5.5)

different kinds of raw starches (S_0=10 g/L) and accumulation up to 4.5 g/L of glucose during direct production of lactic acid by *L. amylovorus* ATCC 33620 at 40 °C in Erlenmeyer flasks. Taken together, these data clearly show that this amylolytic strain simultaneously catalyzed the hydrolysis of starch to RS and the fermentation of a portion of resulting RS to lactic acid, while the remaining portion of RS was accumulated. It can be assumed that the detected accumulation of RS occurs due to: (*i*) uncoordinated rate of starch hydrolysis to RS and the rate of fermentation of RS to lactic acid, (*ii*) problems in transport into *L. amylovorus* DSM 20531^T cells, and/or (*iii*) fragmented consumption of maltose and glucose. Additional experiments have been carried out in order to clarify the correlation between the two processes, starch hydrolysis and fermentation of simple sugars to lactic acid, which were simultaneously conducted by the amylolytic strain, and the related regulation phenomena (results to be published). Under selected conditions, complex carbohydrate (S_0=10 g/L) was depleted from MRS-starch medium by *L. amylovorus* DSM 20531^T in shorter period of batch cultivation (10 h, 40 °C; Fig. 3) than glucose (12 h; S_0=20 g/L) and sucrose (16 h; S_0=10 g/L). When comparing the three media, 2-hour lag phase determined in MRS-starch medium was the shortest and it was followed by 6 h of exponential growth. The most efficient biomass production (μ_{max}=0.67 h^{-1}) and the fastest production of the two stereoisomers of lactic acid (r_P=0.61 h^{-1}) (Table 1), with slightly higher portion of D-lactic acid (52 %) than L-lactic acid (48 %) (Table 2), were determined in the experiments carried out in MRS-starch medium.

As seen in Figs. 1-5, unstructured kinetic model fitted experimental data for glucose, sucrose and soluble starch consumption, biomass formation and lactic acid production in MRS medium at constant temperature (40 °C) and pH value (5.5). Correlation of the model with the experimental values was satisfactory with the correlation coefficient (R^2) in the range from 0.91 to 0.99 (Table 3).

The model was also used to predicit the values of biokinetic parameters: K_S, K_I, β, m_S, and k_{D_0} (Table 1). According to the predicted data, *L. amylovorus* DSM 20531^T showed the highest affinity towards starch as a substrate (K_S=0.80 g/L), while the affinity to glucose and sucrose was lower and similar for mono- and disaccharide (K_S=1.68 and 1.40 g/L, respectively). The highest inihibition by the product was predicted to be in MRS-suc medium (K_I=20 g/L) and significantly lower inhibition in MRS-glc and MRS-starch media (K_I=40 and 38 g/L, respectively).

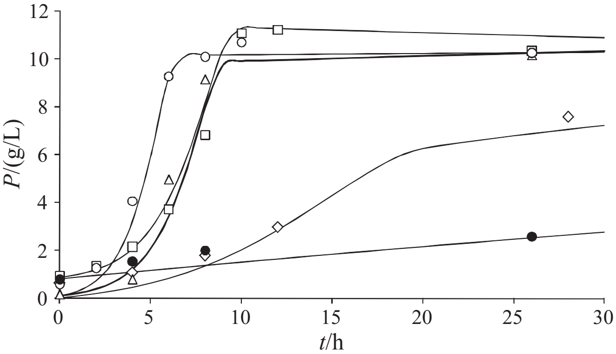

Fig. 5. Modelling of lactic acid production (P) in MRS-starch medium by *L. amylovorus* DSM 20531^T at different temperatures (◊ 30 °C, □ 35 °C, **Δ 40 °C**, ○ 45 °C and ● 50 °C) and at a constant pH value (5.5)

Table 3. Correlation coefficients (R^2) for the substrate (S), biomass (X) and product (P) calculated by comparing experimental data and the model defined by Eqs. 4-8

MRS medium	θ / °C	R^2		
		S	X	P
MRS-glc	**40**	**0.9872**	**0.9068**	**0.9915**
MRS-suc	**40**	**0.9858**	**0.9903**	**0.9775**
MRS-starch	30	0.9518	0.8587	0.9264
	35	0.9982	0.9786	0.9906
	40	**0.9324**	**0.9742**	**0.9609**
	45	0.9955	0.9775	0.9967
	50	0.8010	n.e.	0.8372

n.e. – not estimated

Effect of different temperatures on the growth and lactic acid production in MRS-starch medium

Moderately thermophilic amylolytic lactic acid bacterium *L. amylovorus* DSM 20531^T showed activity in

MRS-starch medium at a constant pH value of 5.5 and over entire range of tested temperatures starting with 30 °C and, with shifts of 5 °C, ending at 50 °C. Experimental data are presented in Figs. 3-5 and Table 4. The biokinetic parameters of *L. amylovorus* DSM 20531^T increased strongly with temperature in the range of 30–45 °C and all values decreased rapidly at 50 °C. The highest values for r_S, μ_{max}, r_P and Pr_P (0.89 h^{-1}, 0.87 h^{-1}, 2.01 h^{-1} and 1.45 g/(L·h), respectively) were obtained at 45 °C. At lower temperatures, 30, 35 and 40 °C, substrate consumption rate was approx. 60 % lower (r_S=0.26–0.30 h^{-1}) than the corresponding maximum, and at 50 °C the rate was estimated to be only 0.02 h^{-1} (Table 4). Soluble starch from MRS-starch medium was depleted by *L. amylovorus* DSM 20531^T at temperatures of 35, 40 and 45 °C for 8 to 12 h (Fig. 3). At 30 °C the depletion took place after approx. 20 h. At 50 °C the growth and RS consumption were negligible although significant amylolytic activity took place. Only ≈24 % of soluble starch were used from MRS-starch medium at 50 °C. During batch cultivations carried out in MRS-starch medium at 30–45 °C, the concentration of maltose and glucose (RS) was in the range of 0.3 to 1 g/L. These results support the earlier proposed hypotheses for the accumulation of RS: uncoordinated rate of starch hydrolysis and rate of fermentation, bottleneck in transport, and possible glucose repression.

Shift in temperature from 35 to 40 °C and from 40 to 45 °C resulted in linear increase of the maximum rate of biomass synthesis (μ_{max}=0.26 h^{-1}, 0.67 h^{-1}, and 0.87 h^{-1}, respectively). From experimental data obtained during batch cultivation at 50 °C, it was not possible to estimate the slope of biomass curve (Fig. 4, not estimated in Table 4). After relatively short lag phase (2–4 h), *L. amylovorus* DSM 20531^T grew exponentially for 4 to 7 h in MRS-starch medium. Maximum concentrations of bacterial biomass were reached at the end of exponential phase and they were in the range of 2.23–4.38 g/L. Here it can be assumed that temperature around 40 °C is optimal for the growth of *L. amylovorus* DSM 20531^T in MRS medium. The same temperature is experimentally confirmed to be optimal for the growth of *L. amylovorus* DCE 471 (*38*). After exponential growth in MRS-starch medium at 35, 40, and 45 °C, the concentration of biomass decreased rapidly and stationary phase did not occur (experimental data; Fig. 4). The stationary phase in MRS-starch medium can be seen only at 30 °C. Besides inhibitory effect of the produced lactic acid (Fig. 5), it seems that the consumption rate of the substrate as well as the absence of soluble starch strongly influence the viability of active bacterial cells. In contrast to that, in spite of or as a consequence of the depletion of glucose (Fig. 1) and sucrose (Fig. 2), *L. amylovorus* DSM 20531^T showed stationary phase. This interesting occurrence involved in the physiology of ALAB has to be further investigated.

Values calculated for r_P increased exponentially with temperature in the range of 30–45 °C and the increase can be described by the following equation:

$$r_P=0.0485e^{0.8994\theta} \quad R^2=0.9870 \qquad /13/$$

Analogous values at 30 and 50 °C were significantly lower and similar at both temperatures (r_P=0.13 h^{-1} and 0.12 h^{-1}, respectively). At these two temperatures, the lowest and the highest tested in this work, maximum concentrations of produced lactic acid were 7.61 and only 0.80 g/L, which gives values for $Y_{P/S}$ of 0.69 and 0.40 g of lactic acid per g of consumed starch, respectively. Slight differences in highly efficient lactic acid production from a complex carbohydrate was determined at 35, 40 and 45 °C ($Y_{P/S}$=0.93, 0.95 and 0.91 g/g, respectively), although the highest productivity of 1.45 g/(L·h) was estimated at 45 °C. Values for lactic acid productivity in media with hydrolyzed complex substrates were reported (*1*) and due to their experimental set up, they cannot be compared to the data obtained in our experiments.

The impact of three different substrates (glucose, sucrose and starch) on the ratio of D-(–)- and L-(+)-lactic acid produced by *L. amylovorus* DSM 20531^T in MRS medium at a constant temperature of 40 °C and constant pH value of 5.5 was not detected. However, temperature had strong effect on the ratio of the two stereoisomers produced by simultaneous starch hydrolysis and glucose fermentation in MRS-starch medium at a constant pH value of 5.5 (Table 2). Increase in temperature from 30 to 50 °C resulted in the decrease of a portion of L-(+)-lactic acid from 56.3 to 37.1 %, therefore, portion of D-lactic

Table 4. Effect of temperature on the biokinetic parameters of *L. amylovorus* DSM 20531^T grown in MRS-starch medium at different temperatures and at a constant pH value (5.5). Values obtained by the unstructured kinetic model are given in parentheses

θ	t_{gp}/h		μ	X_{max}	r_S	$Y_{X/S}$	Pr_x	r_P	P_{max}	$Y_{P/S}$	$Y_{P/X}(\alpha)$	Pr_P	K_S	K_I	β	m_S	k_{D_0}
°C	lag	exp	h^{-1}	g/L	h^{-1}	g/g	g/(L·h)	h^{-1}	g/L	g/g	g/g	g/(L·h)	g/L	g/L	g/(g·h)	h^{-1}	h^{-1}
30	4	6	0.24 (0.28)	2.23 (3.01)	0.26	0.22 (0.32)	0.22	0.13	7.61 (7.07)	0.69	3.41 (2.10)	0.25	(2.0)	(10)	(0.017)	(0.002)	(0.001)
35	3	7	0.26 (0.46)	4.07 (4.08)	0.30	0.41 (0.42)	0.41	0.28	10.27 (11.31)	0.93	2.52 (2.67)	1.03	(0.7)	(32)	(0.003)	(0.005)	(0.001)
40^a	**2**	**6**	**0.67 (0.61)**	**4.38 (4.26)**	**0.29**	**0.44 (0.44)**	**0.44**	**0.61**	**10.52 (10.34)**	**0.95**	**2.40 (2.30)**	**1.05**	**(0.8)**	**(38)**	**(0.010)**	**(0.005)**	**(0.004)**
45	2	4	0.87 (0.82)	3.20 (3.18)	0.89	0.32 (0.32)	0.53	2.01	10.12 (10.30)	0.91	3.17 (3.20)	1.45	(1.0)	(38)	(0.007)	(0.005)	(0.004)
50	n.e.	n.e.	n.e. (0.01)	0 (0.08)	0.02	n.e. (0.12)	n.e.	0.12	0.80 (0.93)	0.40	n.e. (0.01)	0.07	(2.5)	(36)	(0.880)	(0.870)	(0.007)

t_{gp} – duration of growth phases, lag (lag) and exponential (exp); ^{a}experimental data already presented in Table 1 are presented again here for comparison; n.e. – not estimated acid increased for 19.2 %, from 43.7 to 62.9 %. Based on these data, it might be suggested that at different temperatures the two stereospecific lactate dehydrogenases (LDHs; EC 1.1.99.–) in *L. amylovorus* DSM 20531^T cells show different activities. Cytoplasmic NAD-independent LDHs as well as cytoplasmic NAD-dependent L-(+)-LDH (EC 1.1.1.27) and D-(–)-LDH (EC 1.1.1.28) enzymes, which have been found in LAB, are known to have different characteristics in different strains of the same species (*39*). None of them has been isolated from *L. amylovorus* species. In addition, lactate racemase (EC 5.1.2.1) has been isolated from some DL-lactate-forming species from genus *Lactobacillus* (*40*). This enzyme catalyzes interconversion of D- and L-lactate and it has not been purified from *L. amylovorus* species yet.

The unstructured kinetic model successfully predicts all aspects of the growth and lactic acid production in MRS-starch medium at temperatures in the range of 30–45 °C (R^2=0.85–1) (Table 3). At 50 °C the fitting for kinetics of poor starch consumption (Fig. 3), negligible growth of *L. amylovorus* DSM 20531^T (Fig. 4) and lactic acid production (Fig. 5) was unsatisfactory (R^2=0.80–0.84). Values of biokinetic parameters K_S, K_I, β, m_S and k_{D_0} were estimated by the model and the data are presented in Table 4. To the best of our knowledge, there are not comparable published data for these values. It may be possible to compare these values with the data obtained during batch and fed-batch cultivation of *L. amylovorus* DCE 471 in rich glucose-based medium with significantly higher initial concentration of substrate (S_0=40 g/L) (*41*). From experimental data for *L. amylovorus* DCE 471, K_S for glucose at 37 °C was estimated to be 0.7 g/L, and for *L. amylovorus* DSM 20531^T in MRS-starch medium at 35 and 40 °C, K_S of 0.7 and 0.8 g/L were obtained. Values for K_I of 32 and 38 g/L were predicted for the temperature range of 35–50 °C and are similar to the inhibition constant of lactic acid of 30 g/L for *L. amylovorus* DCE 471.

Cardinal temperature model (CTM)

The CTM was used to calculate the dependence of μ_{max}, r_S and r_P in MRS-starch medium on the temperature (Fig. 6). Estimated values obtained by CTM were θ_{min}=19 °C, θ_{opt}=46 °C and θ_{max}=49.5 °C for μ_{max}; θ_{min}=19 °C, θ_{opt}=45 °C and θ_{max}=49.5 °C for r_S; and θ_{min}=26.5 °C, θ_{opt}=46 °C and θ_{max}=49.5 °C for r_P. These data resemble those determined by Nakamura (*23*). Corresponding temperatures for the growth of *L. amylovorus* DCE 471 calculated by using the model were θ_{min}=20 °C, θ_{opt}=44 °C and θ_{max}=49.8 °C (*41*). The CTM fitted well the temperature dependence of μ_{max}, r_S and r_P with R^2 of 0.95, 0.71 and 0.85, respectively. Mathematical relationships describing the response of X_{max}, $Y_{X/S}$ and $Y_{P/S}$ in MRS-starch medium at a constant pH value of 5.5 (Fig. 7) are given in Table 5. In the tested range of temperatures, the estimated values for θ_{min}, θ_{opt} and θ_{max} were 30, 38.4 and 50 °C, respectively, for X_{max}; for $Y_{X/S}$ were estimated to be θ_{min}=30 °C, θ_{opt}=38.9 °C and θ_{max}=50 °C, and for $Y_{P/S}$ θ_{min}=30 °C, θ_{opt}=41.3 °C and θ_{max}=50 °C.

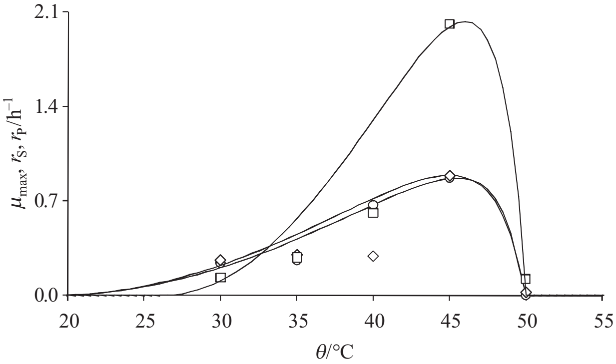

Fig. 6. Effect of temperature on maximum specific growth rate (μ_{max}, ○), substrate consumption rate (r_S, ◇), and the product formation rate (r_P, □) by *L. amylovorus* DSM 20531^T grown in MRS-starch medium at a constant pH value (5.5)

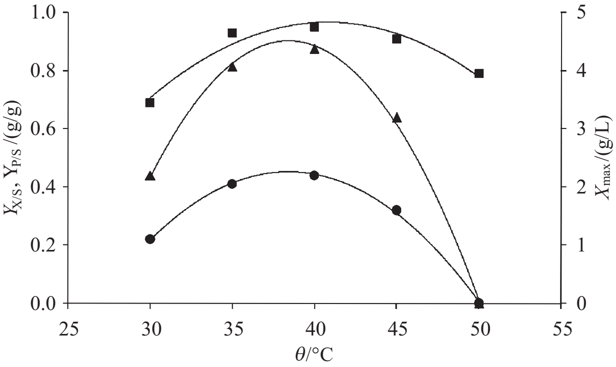

Fig. 7. Effect of temperature on maximum biomass concentration (X_{max}, ▲), biomass yield coefficient ($Y_{X/S}$, ●), and product yield coefficient ($Y_{P/S}$, ■) in MRS-starch medium at a constant pH value (5.5)

Table 5. Mathematical relationships describing the response of the three biokinetic parameters, X_{max}, $Y_{X/S}$, and $Y_{P/S}$, of *L. amylovorus* DSM 20531^T grown in MRS-starch medium at different temperatures and at a constant pH value (5.5)

Parameter	Mathematical relationship	R^2
X_{max}/(g/L)	$-0.0332\theta^2+2.5529\theta-44.518$	0.9979
$Y_{X/S}$/(g/g)	$-0.0033\theta^2+0.2568\theta-4.4794$	0.9983
$Y_{P/S}$/(g/g)	$-0.0022\theta^2+0.1819\theta-2.7443$	0.9507

According to the Arrhenius equation, the maximum temperature at which ln μ_{max}, r_S, r_P, Pr_X and Pr_P remained linear (ascending part) was 45 °C and E_a for all these parameters was estimated (77.0, 68.8, 143.9, 48.2, 96.9 kJ/mol, respectively). Values of E_a for μ_{max} and Pr_P strongly depend on medium composition (*36*), and E_a for μ_{max} is comparable to the value obtained for *L. amylovorus* DCE 471 of 84.7 kJ/mol (*38*).

Conclusions

Lactobacillus amylovorus DSM 20531^T has shown advantages compared to strains from the same genus and species when employed to convert different carbohydrates to lactic acid. These advantages are especially related to its amylolytic activity and ability to catalyze simultaneous saccharification and fermentation (SSF) of starch.

Three substrates, glucose, sucrose and starch, added to MRS medium at initial concentrations of 10 or 20 g/L were completely utilized and stoichiometrically fermented to the mixture of D-(–)- and L-(+)-lactic acid by the strain. The shortest adaptation of the ALAB strain of only 2 h, and the highest predicted affinity to the substrate as well as the fastest depletion of the substrate were determined in starch medium. Also, the highest rates of biomass synthesis and production of lactic acid were obtained in MRS-starch medium. Consequently, starch materials can be used for one-step one-pot production of lactic acid by *L. amylovorus* DSM 20531^T. Optimization of bioprocess parameters, such as temperature, and mathematical modelling of the bioprocess can result in improved and economically atractive lactic acid production.

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List of symbols

A	pre-exponential factor in Eq. 11
C	constant in Eq. 7
E_a	activation energy/(kJ/mol)
k	constant in Eq. 11
k_{D_0}	constant in Eq. 7/h^{-1}
k_D	specific death rate/h^{-1}
K_I	product inhibition constant/(g/L)
K_S	Monod constant/(g/L)
m_S	maintenance energy coefficient/h^{-1}
P	product concentration/(g/L)
Pr_X	productivity of biomass/(g/(L·h))
Pr_P	productivity of lactic acid/(g/(L·h))
R	gas constant/(J/(mol·K))
r_S	substrate consumption rate/h^{-1}
r_P	product formation rate/h^{-1}
S	substrate concentration/(g/L)
t	time/h
T	temperature/K
X	biomass concentration/(g/L)
$Y_{X/S}$	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	the coefficient for growth-associated term/(g/g)
β	coefficient for non-growth-associated term/(g/(g·h))
μ	specific growth rate/h^{-1}
τ	function of temperature
θ	temperature/°C

Subscripts

0	initial
min	minimum
max	maximum
opt	optimum

References

1. R.P. John, G.S. Anisha, K.M. Nampoothiri, A. Pandey, Direct lactic acid fermentation: Focus on simultaneous saccharification and lactic acid production, *Biotechnol. Adv. 27* (2009) 145–152.
2. N. Narayanan, P.K. Roychoudhury, A. Srivastava, L (+) lactic acid fermentation and its product polymerization, *Electron. J. Biotechnol. 7* (2004) 167–179.
3. B.J. Naveena, M. Altaf, K. Bhadrayya, S.S. Madhavendra, G. Reddy, Direct fermentation of starch to L(+) lactic acid in SSF by *Lactobacillus amylophilus* GV6 using wheat bran as support and substrate – Medium optimization using RSM, *Process Biochem. 40* (2005) 681–690.
4. M.I. González, S. Álvarez, F. Riera, R. Álvarez, Economic evaluation of an integrated process for lactic acid production from ultrafiltered whey, *J. Food Eng. 80* (2007) 553–561.
5. Z. Aksu, T. Kutsal, Lactic acid production from molasses utilizing *Lactobacillus delbrueckii* and invertase together, *Biotechnol. Lett. 8* (1986) 157–160.
6. A.W. Schepers, J. Thibault, C. Lacroix, *Lactobacillus helveticus* growth and lactic acid production during pH-controlled batch cultures in whey permeate/yeast extract medium. Part II: Kinetic modeling and model validation, *Enzyme Microb. Technol. 30* (2002) 187–194.
7. R. Otto, Method for the production of lactic acid or a salt thereof by simultaneous saccharification and fermentation of starch. *US patent 0261285* (2008).
8. Y.Y. Linko, P. Javanainen, Simultaneous liquefaction, saccharification, and lactic acid fermentation on barley starch, *Enzyme Microb. Technol. 19* (1996) 118–123.
9. D.X. Zhang, M. Cheryan, Direct fermentation of starch to lactic acid by *Lactobacillus amylovorus*, *Biotechnol. Lett. 13* (1991) 733–738.
10. W. Xiaodong, G. Xuan, S.K. Rakshit, Direct fermentative production of lactic acid on cassava and other starch substrates, *Biotechnol. Lett. 19* (1997) 841–843.
11. C. Vishnu, B.J. Naveena, M. Altaf, M. Venkateshwar, G. Reddy, Amylopullulanase: A novel enzyme of *L. amylophilus* GV6 in direct fermentation of starch to L(+) lactic acid, *Enzyme Microb. Technol. 38* (2006) 545–550.
12. P. Cheng, R.E. Mueller, S. Jaeger, R. Bajpai, E.L. Iannotti, Lactic acid production from enzyme-thinned corn starch using *Lactobacillus amylovorus*, *J. Ind. Microbiol. 7* (1991) 27–34.
13. G.M. Garrity, J.A. Bell, T.G. Lilburn: Taxonomic Outline of the Procaryotes. In: *Bergey's Manual of Systematic Bacteriology*, G.M. Garrity, J.A. Bell, T.G. Lilburn (Eds.), Springer-Verlag, New York, NY, USA (2004).
14. F. Leroy, T. De Vinter, T. Adriany, P. Neysens, L. de Vuyst, Sugars relevant for sourdough fermentation stimulate growth of and bacteriocin production by *Lactobacillus amylovorus* DCE 471, *Int. J. Food Microbiol. 112* (2006) 102–111.
15. P. Neysens, W. Messens, L. de Vuyst, Effect of sodium chloride on growth and bacteriocin production by *Lactobacillus amylovorus* DCE 471, *Int. J. Food Microbiol. 88* (2003) 29–39.
16. C. Castillo Pompeyo, M.S. Gómez, S. Gasparian, J. Morlon-Guyot, Comparison of amylolytic properties of *Lactobacillus amylovorus* and of *Lactobacillus amylophilus*, *Appl. Microbiol. Biotechnol. 40* (1993) 266–269.

17. Y. Oda, M. Ito, Characterization of a mutant from *Lactobacillus amylovorus* JCM 1126^T with improved utilization of sucrose, *Curr. Microbiol. 41* (2000) 392–395.

18. C.S. de Ginés, M.C. Maldonado, F.G. de Valdez, Purification and characterization of invertase from *Lactobacillus reuteri* CRL 1100, *Curr. Microbiol. 40* (2000) 181–184.

19. A.R. Neves, W.A. Pool, J. Kok, O.P. Kuipers, H. Santos, Overview on sugar metabolism and its control in *Lactococcus lactis* – The input from *in vivo* NMR, *FEMS Microbiol. Rev. 29* (2005) 531–554.

20. T.R. Klaenhammer, R. Barrangou, B.L. Buck, M.A. Azcarte-Peril, E. Altermann, Genomic features of lactic acid bacteria effecting bioprocessing and health, *FEMS Microbiol. Rev. 29* (2005) 393–409.

21. J.H. Lee, Y.H. Moon, N. Kim, Y.M. Kim, H.K. Kang, J.Y. Jung, E. Abada, S.S. Kang, D. Kim, Cloning and expression of the sucrose phosphorylase gene from *Leuconostoc mesenteroides* in *Escherichia coli*, *Biotechnol. Lett. 30* (2008) 749–754.

22. M. Cocaign-Bosquet, C. Garrigues, P. Loubiere, N.D. Lindley, Physiology of pyruvate metabolism in *Lactococcus lactis*, *Antonie van Leeuwenhoek, 70* (1996) 253–267.

23. L.K. Nakamura, *Lactobacillus amylovorus*, a new starch-hydrolyzing species from cattle waste-corn fermentations, *Int. J. Syst. Bacteriol. 31* (1981) 56–63.

24. J.C. de Man, M. Rogosa, M.E. Sharpe, A medium for the cultivation of Lactobacilli, *J. Appl. Bacteriol. 23* (1960) 130–135.

25. C. Polson, P. Sarkar, B. Incledon, V. Raguvaran, R. Grant, Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography-tandem mass spectrometry, *J. Chromatogr. B*, *785* (2003) 263–275.

26. BD Bionutrients^{TM} Technical Manual, BD Biosciences, Sparks, MD, USA (*http://www.bd.com/ds/technicalCenter/misc/br_3_2547.pdf*).

27. M. Somogyi, Notes on sugar determination, *J. Biol. Chem. 195* (1951) 19–23.

28. D-Lactic Acid (D-Lactate) and L-Lactic Acid (L-Lactate) Assay Procedures Determination Kit, Megazyme International Ltd, Wicklow, Ireland (*http://www.megazyme.com*).

29. P. Doran: Presentation and Analysis of Data. In: *Bioprocess Engineering Principles*, P. Doran (Ed.), Academic Press Limited, London, UK (1998) pp. 27–48.

30. S.J. Pirth, The maintenance energy of bacteria in growing cultures, *Proc. R. Soc. Lond. B*, *163* (1965) 224–231.

31. H. Ohara, K. Hiyama, T. Yoshida, Kinetics of growth and lactic acid production in continuous and batch culture, *Appl. Microbiol. Biotechnol. 37* (1992) 544–548.

32. A. Nishiwaki, I.J. Dunn, Performance of a two-stage fermentor with cell recycle for continuous production of lactic acid, *Bioprocess Eng. 21* (1999) 299–305.

33. R. Luedeking, E.L. Piret, A kinetic study of the lactic acid fermentation. Batch process at controlled pH, *J. Biochem. Microbiol. Technol. Eng*. *1* (1959) 393–412.

34. Berkeley Madonna Modeling and Analysis of Dynamic Systems v. 8.3.14, University of California, Berkeley, CA, USA (2007) (*www.berkeleymadonna.com*).

35. L. Rosso, J.R. Lobry, S. Bajard, J.P. Flandrois, Convenient model to describe the combined effects of temperature and pH on microbial growth, *Appl. Environ. Microbiol*. *2* (1995) 610–616.

36. S.D. Yuwono, T. Kokugan, Study of the effects of temperature and pH on lactic acid production from fresh cassava roots in tofu liquid waste by *Streptococcus bovis*, *Biochem. Eng. J. 40* (2008) 175–183.

37. E.L. Gaden, Fermentation process kinetics, *J. Biochem. Microbiol. Technol. Eng*. *1* (1959) 413–429.

38. W. Messens, P. Neysens, W. Vansieleghem, J. Vanderhoeven, L. de Vuyst, Modeling growth and bacteriocin production by *Lactobacillus amylovorus* DCE 471 in response to temperature and pH values used for sourdough fermentations, *Appl. Environ. Microbiol. 68* (2002) 1431–1435.

39. E.I. Garvie, Bacterial lactate dehydrogenases, *Microbiol. Rev. 44* (1980) 106–139.

40. P. Goffin, M. Deghorain, J.L. Mainardi, I. Tytgat, M.C. Champomier-Vergès, M. Kleerebezem, P. Hols, Lactate racemization as a rescue pathway for supplying D-lactate to the cell wall biosynthesis machinery in *Lactobacillus plantarum*, *J. Bacteriol. 187* (2005) 6750–6761.

41. R. Callewaert, L. de Vuyst, Bacteriocin production with *Lactobacillus amylovorus* DCE 471 is improved and stabilized by fed-batch fermentation, *Appl. Environ. Microbiol*. *66* (2000) 606–613.