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Growth and exopolysaccharide yield of Lactobacillus delbrueckii ssp. bulgaricus DSM 20081 in batch and continuous bioreactor experiments at constant pH

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Some *Lactobacillus delbrueckii ssp. bulgaricus* strains are able to synthesize exopolysaccharides (EPS) and are therefore highly important for the dairy industry as starter cultures. The aim of this study was to investigate nutritional requirements for growth and EPS production of *Lactobacillus delbrueckii ssp. bulgaricus* DSM 20081. The developed medium emanates from a semi-defined medium (SDM) in which glucose was replaced by lactose, and combinations of different mixtures of supplements (nucleobases, vitamins, salts, sodium formate and orotic acid) were added. Constant pH batch fermentation with the modified medium resulted in an EPS yield of approx. 210 mg glucose equivalents per liter medium. This is a 10-fold increase compared with flask cultivation of this strain in SDM. Although not affecting cell growth, the mixture of salts enhanced EPS synthesis. Whereas specific EPS production was approx. 12 mg EPS per g dry biomass without salt supplementation, a significantly higher yield (approx. 20 mg EPS per g dry biomass) was observed after adding the salt mixture. In continuous fermentation a maximal EPS production was obtained at a dilution rate of 0.3/h (57 mg/g dry biomass).
Many lactic acid bacteria are able to produce extracellular polysaccharides (EPS). Although function and relevance for the bacteria are not completely understood, it has been suggested that EPS may play a significant role in the protection of cells against dehydration, phagocytosis, phage attacks or toxic compounds. EPS may also contribute to the adhesion of microorganisms onto solid surfaces and to intercellular communication. There is, however, a general agreement that they are not important as energy source for the bacteria (1, 2). Exopolysaccharides may alter technofunctional properties (e.g., viscosity, water binding capacity) of fermented foods such as yogurt, cheese or sourdough (3-6) and, consequently, sensory properties of these products (7). Therefore, lactic acid bacteria (LAB) which show the capacity to excrete EPS are frequently used to replace thickeners and stabilizers such as polysaccharides of animal (gelatine), plant (starch, pectin) or other microbial origin (xanthan). A further advantage is their GRAS (Generally Recognized As Safe) status, meaning that LAB and their metabolites are considered as safe and there is no need for declaration when added to food.

The metabolism of EPS synthesis has been in the focus of research for years (e.g., 8, 9). Different strains of *Lactobacillus delbrueckii* ssp. *bulgaricus*, including strain DSM 20081, and *Streptococcus thermophilus* used in yogurt production are able to produce EPS (10), the amounts of which strongly depend on the growth medium (carbohydrate and nitrogen source, C/N ratio, vitamins, salts and other supplements). Moreover, fermentation conditions such as temperature, broth pH and the presence of oxygen do also show a significant impact on EPS synthesis (11, 12). Data on EPS amounts which were produced during batch fermentation in defined media by *L. delbrueckii* ssp. *bulgaricus* strains vary from 50 - 350 mg/L (13-18; Table 1). Larger quantities of EPS are synthesized in natural media such as skim milk or whey but, for studying the influence of growth parameters on EPS production, a well-defined medium
which satisfies the high nutritional demands of LAB is necessary. This is of special
importance when EPS production is growth associated (2).

The aim of this study was to determine microbial growth and EPS production of *L. delbrueckii* ssp. *bulgaricus* DSM 20081, which was originally isolated from Bulgarian yogurt, in a defined medium by adding various supplements of nutritional relevance.

After selecting the most promising media on the basis of flask cultivations, bioreactor experiments in batch mode were carried out to achieve higher EPS yield, and continuous fermentations were performed to obtain information on the kinetics of growth and EPS production.

**MATERIALS AND METHODS**

**Strain management and cell count determination** *L. delbrueckii* ssp. *bulgaricus* DSM 20081 (ATCC 11842, NCDO 1489) was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The freeze-dried strain was cultured in MRS broth (Merck KGaA, Darmstadt, Germany) at 40 °C in an incubator (Binder GmbH, Tuttingen, Germany) under anaerobic conditions (flushing gas composition: 10 % CO₂, 10 % H₂, 80 % N₂). After incubation, portions of the medium were transferred to cryotubes and stored at -80 °C. For short-term storage up to four weeks, cells were streaked on Reconstituted Skim Milk (RSM) agar plates (100 g/L skim milk powder, 10 g/L casein peptone, tryptic digest (CP), 5 g/L yeast extract, 15 g/L agar) and anaerobically incubated at 40 °C. Prior to use, bacteria were anaerobically propagated in a semi-defined medium (SDM; 19) with lactose instead of glucose at 40 °C for 48 h. Lactose-SDM (L-SDM) contained 36 g/L (100 mmol/L) lactose monohydrate, 10 g/L CP, 5 g/L yeast nitrogen base (YNB), 1 g/L Tween®80, 5 g/L sodium acetate, 2 g/L dipotassium hydrogen phosphate, 2 g/L diammonium hydrogen citrate, 0.2 g/L magnesium sulfate heptahydrate and 0.05 g/L manganese sulfate.
Cell count was monitored by measuring the optical density at 577 nm on a Helios UV/VIS spectrophotometer (Thermo Electron Corporation, Cambridge, UK). Via Neubauer improved counting chamber (Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany) a calibration curve was established to relate absorbance to cell count. When necessary, the fermentation media were diluted with 0.95 % (w/v) sodium chloride in water. Biomass was determined by subjecting 40 mL fermentation broth to centrifugation (1900 x g, 15 min, 20 °C). The pellet was washed twice with demineralized water, dried to mass constancy in an oven (103 ± 1 °C) and weighed.

**Culture media in flask fermentation for maximizing EPS production** In flask experiments we studied the influence of broth composition and environmental parameters on the growth of *L. delbrueckii* ssp. *bulgaricus* DSM 20081. Fermentation was carried out in SDM and L-SDM, either without or with supplementation. Supplements were added separately or in combinations and comprised a mixture of nucleobases (adenine, guanine, xanthine, uracil at 50 mg/L each), a mixture of vitamins (0.03 mg/L folic acid, 0.2 mg/L riboflavin, 0.01 mg/L cobalamin), and a mixture of inorganic and organic salts (0.8 g/L sodium dihydrogen phosphate, 0.36 g/L calcium chloride dihydrate, 0.5 g/L sodium thioglycolate). Further supplements were 0.3 g/L sodium formate and 0.5 g/L orotic acid. Unless mentioned otherwise, all media were adjusted to pH 6.0 and autoclaved for 15 min at 121°C. Carbohydrates were autoclaved separately, and vitamins were added after sterile filtration. Additionally, the carbon/nitrogen ratio was varied in unsupplemented or supplemented L-SDM by increasing CP and YNB concentration.

Inocula for flask experiments were prepared in the broth which was used for subsequent fermentation. 100 mL broth inoculated with a single colony from RSM agar
was incubated for 48 h at 40 °C under anaerobic conditions and subsequently diluted to obtain a cell count of $10^7$/mL. Inoculation strength was 1 % (v/v) to ensure a starting cell count of $10^5$/mL in all flask experiments.

To study the influence of carbon source (glucose, lactose) and concentration, and additional broth supplementation on cell growth and EPS yield, fermentation was performed at 40 °C. Out of these media the one showing best microorganism growth was selected for the determination of the impact of fermentation temperature. The same medium was used to evaluate the effect of carbon dioxide on cell growth; for that purpose we compared flushing with gas mixture or with pure N$_2$ for maintaining anaerobiosis. Samples were taken at pre-selected intervals to determine cell count, pH and EPS content. All fermentation experiments were carried out in duplicate.

**Batch fermentation** For fermentation in a 5 L laboratory bioreactor (working volume 2.5 L; Applikon Biotechnology B.V., Schiedam, NL), undiluted precultures were used for inoculation in different quantities. Prior to use, the bioreactor was sterilized at 121°C for 15 min. The system was monitored using the BioXpert R software. pH in the fermentation broth was maintained constant at 6.0 by automatic addition of 10 mol/L NaOH, and temperature was kept constant at 40 °C. The medium, which was aerated with 0.1 L/min N$_2$ to obtain anaerobic conditions and agitated continuously at 200 rpm, was selected on the basis of the results of the flask experiments and contained either 100 or 200 mmol/L lactose. Samples were taken during fermentation at defined time intervals for the determination of cell count, EPS content, and lactic acid, galactose, glucose and residual lactose concentration. pH and amount of NaOH added to the system were monitored on-line. The concentration of lactose, glucose, galactose and lactic acid during fermentation was determined in the culture supernatant using enzymatic test assays (R-Biopharm AG, Darmstadt, Germany).
Continuous fermentation The impact of dilution rate on EPS production was investigated at 40 °C and pH 6.0 in an 1 L bioreactor (Applikon Biotechnology B.V., Schiedam, NL) filled with 400 mL of fully supplemented L-SDM containing 100 mmol/L lactose. After inoculation with 6 % (v/v) preculture, batch fermentation was performed for 24 h before continuous fermentation was started. The feed medium was transferred to the fermentation vessel by a peristaltic pump (Masterflex, L/S digital drive, Cole Palmer, USA) and the volume was kept constant by pumping out excess volume. Samples for biomass, EPS, lactic acid, galactose, glucose and lactose determination at each dilution rate were taken when 4 fermentor volumes had been replaced and steady state conditions were achieved. Dilution rate range was adjusted to 0.05 - 1.2/h. For the balancing it was assumed that one mol lactose is cleaved into 1 mol glucose and 1 mol galactose. Specific conversion rates were calculated as mol carbohydrate per g dry biomass, hour and liter.

EPS quantification Isolation and quantification of exopolysaccharides was performed according to Petry et al. (13) and Aslim et al. (20). 5 mL of fermented medium was treated with 0.6 mL 80 % (w/v) trichloroacetic acid (TCA) and heated to 90 °C for 10 min. After cooling in ice water, the samples were centrifuged (1900 x g, 20 min, 4 °C) to remove cells and proteins. The resulting pellets were resuspended in 10 % (w/v) TCA and centrifuged again. The supernatants of both centrifugation steps were pooled and treated with 5 volumes of chilled ethanol (4 °C) overnight. Subsequently, precipitated exopolysaccharides were collected through centrifugation (1900 x g, 20 min, 4 °C). The pellets were dissolved in demineralized water, transferred into dialysis tubes (molecular weight cut-off 8-10 kDa; Karl Roth GmbH & Co KG, Karlsruhe, Germany) and dialyzed for 48 h against demineralized water; water was changed twice.
a day. The dialysates were then freeze-dried (Alpha 1-2, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany).

For EPS quantification the phenol-sulphuric acid method (21) was used, and the assay was calibrated using glucose as standard. Freeze-dried EPS were dissolved in water, diluted, and 0.2 mL aliquots were mixed with 0.2 mL 5 % (w/v) phenol in water and 1 mL 98 % (v/v) sulfuric acid. Absorbance was measured at 490 nm, and EPS content is expressed as mg glucose equivalent (GE) per L medium. All assays were performed in duplicate. The uninoculated media served as blank and its absorbance was subtracted from that of the fermented sample.

Statistics Procedures of the SAS® Learning Edition 4.1 (SAS® Institute, Cary, NC, USA) were used for statistical data evaluation.

RESULTS

Influence of carbon source As L. delbrueckii ssp. bulgaricus is evolutionary adapted to grow in milk, media with similar composition are most promising for efficient growth and EPS production. Kimmel & Roberts (19) developed a growth medium especially for Lactobacillus delbrueckii ssp. bulgaricus RR. As L. delbrueckii ssp. bulgaricus DSM 20081 used in our study showed very poor growth in this SDM (100 mmol/L glucose) in preliminary experiments, we consequently performed a series of fermentations with different carbon sources.

The impact of carbon source and concentration on cell count, pH and EPS production in flask fermentation is summarized in Table 2. The data show that, independent of its concentration, growth of L. delbrueckii ssp. bulgaricus DSM 20081 was poor when glucose was provided as sole carbon source. The cell count range for
those experiments was $2.2 \times 10^7 - 4.1 \times 10^7$/mL, and mean pH after 72 h as a measure
for lactic acid formation was $5.37 \pm 0.13$. Cell count was significantly (P < 0.05) higher
in case of lactose as carbon source, increased with increasing lactose concentration, and
reached its maximum at 200 mmol/L lactose ($4.6 \times 10^8$/mL). EPS content was generally
low, but highest in media with 150 or 200 mmol/L lactose. Combining lactose with
glucose, or lactose with galactose did not improve cell growth or EPS production.

**Modification of Lactose-SDM to improve EPS production in static cultivation**

By using different LAB, including *L. delbrueckii ssp. bulgaricus*, Chervaux et al. (22)
and Petry et al. (13) pointed on the importance of supplementation for cell growth
stimulation and EPS synthesis. Therefore, we added several supplements to introduce
new compounds (e.g., nucleobases or cobalamin) to SDM or L-SDM, or to increase the
concentration of others (e.g., folic acid or riboflavin).

Among these supplements, the nucleobases had the most stimulating effect on cell
growth; cell count increased from $3.0 \times 10^8$/mL in medium number 1 (m#1) to $5.6 \times
10^8$/mL (m#2). The additional supplementation with the vitamin mixture resulted in a
further increase of cell count, independent of the addition of salts ($8.5 \times 10^8$/mL, m#5
vs. $8.1 \times 10^8$/mL, m#8); pH after 72 h fermentation was approx. 4.0 (Table 3).

Supplementation of L-SDM with orotic acid led to higher cell counts of *L.
*delbrueckii* ssp. *bulgaricus* DSM 20081 than supplementation with sodium formate
(m#10 and m#9, respectively). When both were combined, a further increase in growth
was observed ($4.0 \times 10^8$/mL; m#11). As can be seen from the comparison of m#1 - m#8
with m#11 - m#18, there is a general tendency that additional supplementation of L-SDM with either nucleobases, vitamins or salts or combinations thereof with sodium
formate and orotic acid lead to an increase in the respective cell counts.

*L. delbrueckii ssp. bulgaricus* DSM 20081 did not grow when YNB concentration
was increased to 10 or 20 g/L (m#19, m#20). Neither a higher YNB level combined
with full supplementation (m#21), nor the increase of casein peptone to 20 or 30 g/L
(m#22, m#23, m#24) resulted in a cell count higher than 8 x 10^{8}/mL. Upon doubling
nitrogen compound concentration (10 g/L YNB, 20g/L CP), supplementation was
necessary for cell growth. A 50 % increase of lactose in fully supplemented L-SDM
(m#27 vs. m#18) did not further stimulate cell growth. For glucose, supplementation
was responsible for a ten-fold increase in cell count (m#28 and m#29 vs. m#0).
However, cell counts were in the range of m#1 (100 mmol/L lactose, no supplements).

For the six media with cell counts higher than 7.5 x 10^{8}/mL, EPS content and dry
biomass were determined. EPS concentrations were 66 ± 8 mg GE/L and 75 ± 6 mg
GE/L in m#5 and m#15, respectively, which was significantly (P < 0.05) lower than EPS
contents in the other four media (i.e., m#8, m#18, m#26, m#27; Fig. 1). As can be
expected from cell counts, dry biomass was on a comparable level (5.47 - 6.05 g/L; P >
0.05). Consequently, EPS productivity was also lower for m#5 and m#15 (~12 mg
EPS/g biomass) compared with the other four media (17.6 - 19.8 mg EPS/g biomass).
Relating EPS productivity with broth composition (see Table 3) implies that compounds
of the mixture of inorganic and organic salts were essential for enhanced EPS yield. The
higher pH after fermentation in m#26 can be attributed to the higher buffering capacity
of the medium because of an increased casein peptone content.

**Influence of temperature and carbon dioxide** Static flasks experiments

performed with medium #18 reveal temperature dependency of cell count and EPS
content (fermentation was started at pH = 6.0 and terminated at pH = 4.1 ± 0.1; Table 4).
Because of poor growth of *L. delbrueckii* ssp. *bulgaricus* DSM 20081 at 45°C, this
fermentation was terminated at pH 4.58; cell count (2.6 x 10^{8}/mL) and EPS content (45
mg GE/L) were significantly (P < 0.05) lower than for other fermentations. Highest cell
counts and EPS contents were observed at 37 °C, and time to reach the end pH was similar in experiments at 37 °C and 40 °C. After fermentation at 30 °C, cell count and EPS content were comparable to 40 °C, but 120 h were needed to achieve pH 4.16. Flushing the incubator either with pure N₂ or a mixture of 10 % CO₂, 10 % H₂ and 80 % N₂ did not affect growth of L. delbrueckii ssp. bulgaricus DSM 20081, and EPS yield was similar (63 ± 7.9 and 66 ± 8.7 mg GE/L, respectively; P > 0.05).

**Batch fermentation** Batch fermentations were conducted with fully supplemented L-SDM (m#18) at 40 °C under anaerobic conditions. Upon the first inoculation with 1 % preculture we could not observe any growth as indicated by acid formation within 32 h. In total, 3 inocula (1 %, 2 % and another 2 %) were needed until lactic acid production started (data not shown). In a second experiment, the inoculum was raised to 3 %. Monitoring cell count revealed a decrease in the first fermentation stage; after inoculating again with 3 %, cell growth started immediately (Fig. 2, indicated by arrows). In the third fermentation batch, an inoculation with 6 % preculture induced cell propagation after approx. 12 h. Cell counts at the end of fermentation were in a comparable range for batch 2 and 3 (6.3 x 10⁸/mL and 7.0 x 10⁸/mL, respectively), although batch 2 was started with 100 mmol/L lactose, and batch 3 with 200 mmol/L. Growth rate in the exponential phase was 0.29/h and 0.33/h for fermentation 2 and 3, respectively. EPS production mainly took place during exponential phase, and maximum levels were 215 mg and 210 mg GE/L. Initial lactose concentration in the broth did not affect the EPS yield significantly (P < 0.05). It appears unlikely that the delayed growth in batch 1 and 2 was caused by an extended lag phase because the time span between subsequent inoculations was at least 30 h. It is also evident that, although no cell growth took place, lactose was cleaved after the first inoculation in batch 2. Consequently, glucose and galactose were accumulated in the medium (Fig. 3A),
presumably by the action of β-galactosidase which was released during autolysis (23). When cell growth started after the second inoculation (32 h), glucose was already available for metabolism. Whereas lactose and glucose were entirely consumed during fermentation, galactose appears to be not utilized.

Fig. 3B shows the evolution of carbohydrate content for batch 3 which was carried out with 200 mmol/L lactose. After inoculation with 6 % preculture and a 12 h lag phase where lactose was partially hydrolyzed, exponential growth resulted in a steep increase of lactic acid content. Lactose was fully hydrolyzed, whereas approx. 60 mmol/L glucose remained in the medium after fermentation.

Continuous fermentation The influence of growth rate on EPS production was investigated in a continuous culture between 0.05 and 1.2/h. The concentration of residual lactose, glucose and galactose, and lactic acid are shown in Fig. 4A as a function of dilution rate. At the lowest dilution rate all lactose was cleaved into glucose and galactose. Despite complete conversion of lactose, concentration of biomass remained low at this point (0.77 g/L). It is likely that the high lactic acid concentration (184 mmol/L) caused growth inhibition, as we could also observe in batch fermentation, and therefore glucose partly remained in the medium. With increasing dilution rate D, lactose started to accumulate in the medium, whereas glucose, galactose and lactic acid concentrations decreased; glucose was taken up as preferred carbon source as can be seen from its lower concentration. Lower lactic acid levels resulted in reduced growth inhibition and, consequently, biomass concentration rose until an optimum (1.63 g/L) was reached at D = 0.3/h (Fig. 4B). Higher dilution rates caused biomass to decrease again until, at D = 1.21/h biomass was washed out completely and, hence no conversions could take place. At this point lactose concentration at the outlet of the reactor equals inlet concentration, and neither glucose, galactose, lactic acid, nor EPS
were measurable. Generally, the concentration of EPS roughly correlated with biomass concentration, exhibiting an optimum at $D = 0.3$/h.

To obtain deeper insight into growth and product formation kinetics, specific turnover rates for carbohydrates (cleavage and consumption rate), and EPS and lactic acid (productivities) were calculated. After normalizing EPS concentration to biomass, productivity data reveal that EPS formation is growth associated (Fig. 5). However, higher dilution rates do not necessarily lead to higher EPS concentration. Fluctuation in biomass concentration affects EPS production in a more pronounced way, so that highest EPS concentration in continuous fermentation was obtained at a rather low dilution rate of $0.3$/h where biomass concentration peaks. Lactose conversion rate correlates almost linearly with dilution rate. The slope is, however, too small to make up the increase of $D$, as the specific lactose conversion rate rose by a factor of 3.25 from the lowest to the highest dilution rate, while the dilution rate increased by a factor of 18. This means that the amplified lactose conversion rate cannot compensate for the intensified dilution of the cleaved monosaccharides by the flux through the reactor. Consequently the volumetric conversion rate has a flat optimum between $D = 0.3$/h and $0.56$/h (data not shown). This strongly suggests that, in this continuous culture, a limited cleavage of lactose was responsible for the decrease of biomass above $D = 0.3$/h.

Specific glucose consumption was fairly constant up to $D = 0.3$/h, probably because of growth inhibition by lactic acid. At higher dilution rate the uptake correlated with $D$ to cope with the need for faster growth. For galactose we measured a low but constant specific uptake. When glucose became limiting at $D = 0.56$/h galactose uptake increased which means that the bacteria co-metabolize glucose and galactose to a certain extent; cells appear to regulate up the galactose transporters to compensate for glucose limitation.

Specific lactic acid production rose constantly with dilution rate, indicating that
the energy metabolism of the cells was up-regulated with increasing growth rate.
However, productivity increase is again smaller than dilution rate, which is responsible for the decreasing lactic acid content with increasing D. On the other hand it shows that lower biomass concentrations at higher dilution rates are caused by the limited supply of carbon source. At $D = 0.56/h$, the specific lactic acid formation flattens, probably because of a change in cell metabolism with increased galactose uptake. A wash out of the bacteria at this dilution rate appears unlikely, since the turn over rates of the carbon hydrates are still high.

**DISCUSSION**

Semi-defined media such as that developed for *L. delbrueckii ssp. bulgaricus* RR (19) are frequently used for growth and metabolism studies. However, *L. delbrueckii ssp. bulgaricus* DSM 20081 showed a very limited growth in this medium so that adaption was necessary. In flask fermentation experiments with falling pH, EPS yield was increased approx. 6-fold from 19 mg GE/L in SDM (Table 2, 100 mmol/L glucose) to 108 mg GE/L in fully supplemented L-SDM (Table 3, m#18). Schiraldi et al. (16) mentioned 100 mg/L EPS for *L. delbrueckii ssp. bulgaricus* DSM 20081 during batch fermentation at 37 °C, pH = 6.5, using glucose as sole carbon source whereas our experiments with lactose resulted in an EPS yield of approx. 210 mg GE/L.

To stimulate cell growth it was necessary to spike L-SDM with a number of supplements, including nucleobases and vitamins, to comply for the high nutritional requirements of *L. delbrueckii ssp. bulgaricus* DSM 20081. Orotic acid is naturally present in milk in a concentration up to 100 mg/L (13) and seems to be important for the growth of *L. delbrueckii ssp. bulgaricus* as it is needed for RNA synthesis and formation of new cells (24, 25). Sodium formate, produced by *S. thermophilus* in classical yoghurt starters in amounts of 40 - 600 mg/L (24, 26, 27), is also an essential factor for RNA
generation (28) and growth stimulation of *L. delbrueckii* ssp. *bulgaricus* (29).

The mixture of salts we have added to our growth medium emerged as important supplement especially for EPS production and not only for cell growth. Sodium thioglycolate is a reducing agent which has been reported as being protective against toxic oxygen derivatives produced during growth (22). It is also likely that sodium thioglycolate influences enzymes that are specific for EPS production, thus leading to higher amounts of excreted EPS at constant cell count. α-phosphoglucomutase and UDP-glucose pyrophosphorylase are two key enzymes in the EPS pathway (30), which can be activated by reducing agents such as cystein or thioglycolate (31, 32).

In our experiments lactose was better utilized than glucose, presumably because of LAB adaption to milk (28); galactose accumulated almost completely during fermentation. This carbohydrate preference is in line with Chervaux et al. (22) and Welman & Maddox (17) but in contrast with others (13, 19, 33). Various experiments showed a faster enzymatic lactose transport in *S. thermophilus*, and it was suggested that carbohydrate metabolism of *L. delbrueckii* ssp. *bulgaricus* is similar (34, 35). Chervaux et al. (22) also presumed different transport systems for sugars other than lactose and concluded that glucose import is inefficient in some strains. The presence of galactose or glucose besides lactose seemed to inhibit cell growth and EPS production. Growth inhibition of *L. delbrueckii* ssp. *bulgaricus* in the presence of galactose was also observed (22). Some *L. delbrueckii* ssp. *bulgaricus* strains import lactose by a lactose/galactose antiport transport system (17, 36), similar to *S. thermophilus* LY03. Hutkins and Poone (37) observed a restricted lactose uptake in the presence of extracellular galactose due to the competitive inhibition of lactose binding or inhibition of the galactose efflux. Free glucose in a medium may inhibit β-galactosidase activity and repress lactose transport (28), as has been described for *L. plantarum* ATCC 8014 (38).

Mesophilic LAB seem to produce EPS under non-optimum growth whereas EPS
production in thermophilic LAB largely appears to be growth-associated (11, 17, 39, 40). Optimum growth temperature for *L. delbrueckii* ssp. *bulgaricus* strains has been expressed to range from 35 - 45 °C (16, 18). For *L. delbrueckii* ssp. *bulgaricus* DSM 20081, we observed optimum growth at anaerobic incubation at 37 - 40 °C. Kimmel et al. (18) mentioned an optimum at 38 °C for EPS production by *L. delbrueckii* ssp. *bulgaricus* RR, whereas others found higher EPS yields at temperatures up to 45 °C (20, 41). Whether N₂ or CO₂ was present in the atmosphere had no impact on cell count and EPS production. This is in line with Grobben et al. (15) and Koch et al. (42) who could not detect any influence of CO₂ on the growth of *L. delbrueckii* ssp. *bulgaricus*.

EPS yields were higher in bioreactor fermentation than in flasks, presumably because of controlled pH and stirring which leads to a better medium homogenization and improved mass transfer. Several other authors mentioned that the EPS amount is higher when microorganisms are cultivated in bioreactors (2, 39, 43, 44). Inocula usually vary between 1 % and 5 % (13, 23, 45). It turned out that, for bioreactor fermentation with *L. delbrueckii* ssp. *bulgaricus* DSM 20081, sufficiently high inoculum strength was essential for rapid initial cell growth. Kang et al. (46) explained poor growth associated with a low inoculation volume by a massive autolysis of *L. delbrueckii* ssp. *bulgaricus* UL12 cells, the reason of which could be preculture state, stressed cells, or a strain-dependent behavior (47). In our experiments, cell count decreased after inoculation with 3 % (Fig. 2) which suggests that autolysis started immediately; when starting with a higher cell density, this effect did not occur. A further explanation arises from reports which discuss communication of cells as a function of cell density (48, 49).

We observed highest EPS production during exponential growth, which implies a correlation between cell growth and EPS formation. In accordance with other reports (20, 33) EPS were not degraded in the stationary phase. Nevertheless, we determined a
more pronounced cell lysis in batch fermentation with 200 mmol/L lactose immediately after exponential growth. This could be a question of osmotic pressure because of high residual sugars, metabolites and buffering base concentration in the medium or cell stress because of micronutrient limitation. Usually, an increase of sugar concentration results in increased EPS production (11, 16, 30). The fact that an increase in lactose concentration in fully supplemented L-SDM did neither enhance cell count nor EPS might be an indicator for a limitation caused by the change in the C/N ratio (2, 12), a limitation of secondary substrates (vitamins, nucleobases, salts), or excessively high lactic acid concentration (23, 50). A carbon limitation can be excluded as residual lactose remained in the medium after exponential growth, and glucose remnants were present in the broth after fermentation was stopped.

Because data from batch cultivation did not allow drawing a clear picture of the kinetics we performed continuous fermentation. Here we could show that EPS formation correlates to both, biomass concentration and growth rate. The latter becomes only obvious when EPS formation is normalized to biomass concentration, since large fluctuations in biomass concentration occurred. This effect is discussed controversially: Keller & Gerhardt (51) explained that product inhibition may partly cause low biomass concentration, while others make the complex nutritional requirements of Lactobacillus strains responsible (17, 52).

Our turnover rates strongly suggest that the kinetics of growth and biomass concentration is governed by two effects: 1.) at low dilution rate, a high concentration of lactic acid inhibits L. delbrueckii ssp. bulgaricus DSM 20081 growth; 2.) with increasing dilution rate, lactose cleavage increases slower than dilution rate. Hence, lactose accumulates and the availability of the monosaccharides becomes limiting. An additional decrease in the concentration of monosaccharides is given by the flux through the reactor, which also causes a decrease of lactic acid below the inhibiting
concentration, but biomass formation is limited by lower amounts of glucose and galactose. For the monosaccharides we were able to show that both are constantly co-metabolized, with glucose being the main carbon source. When the latter is limited, the bacteria were able to increase galactose uptake to partly compensate for the lack of glucose. A growth limitation by secondary substrates is not likely because we obtained much higher biomass concentrations in batch cultivations with the same medium.

In summary, *L. delbrueckii* ssp. *bulgaricus* DSM 20081 showed high nutritional demands. To enhance EPS yield, it was necessary to modify a semi-defined medium reported in literature; glucose was replaced by lactose, and several supplements were added. It turned out that the mixture of salts enhanced EPS synthesis although not affecting cell growth. Higher amounts of EPS were achieved in batch fermentation with constant pH than in flask cultivation with falling pH. EPS production was comparable with that of other *L. delbrueckii* ssp. *bulgaricus* strains, despite clear differences in growth behavior. Results of continuous fermentation indicate that optimal EPS production takes place at growth rates of 0.1 - 0.3/h. To achieve high EPS production it is desirable to reach high growth rates and biomass concentrations, and maintain lactic acid concentration low.

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Figure captions

Figure 1: Relationship between specific EPS formation, dry biomass and EPS content for media resulting in cell counts higher than $7.5 \times 10^8$/mL (media explanation, see Table 3). Open symbols, specific EPS formation; closed symbols, dry biomass.

Figure 2: Anaerobic batch fermentation of *L. delbrueckii* ssp. *bulgaricus* DSM 20081 in fully supplemented L-SDM #18 ($T = 40 ^\circ C$, $pH = 6.0$). Circles, EPS concentration; squares, cell count. Initial lactose concentration: open symbols, 100 mmol/L; closed symbols, 200 mmol/L. Initial inoculation was 3 % and 6 % for fermentation with 100 and 200 mmol/L lactose, respectively. Arrows indicate a second inoculation (3 %).

Figure 3: Development of sugar and lactic acid concentration in batch fermentations with (A) 100 mmol/L lactose, 3 + 3 % inoculation and (B) 200 mmol/L lactose, 6 % inoculation. Open circles, lactose; open square, glucose; closed circles, galactose; closed squares, lactic acid.

Fig. 4: Influence of dilution rate during continuous fermentation of *L. delbrueckii* ssp. *bulgaricus* DSM 20081 in fully supplemented L-SDM #18 ($T = 40 ^\circ C$, $pH = 6.0$). (A) open circles, lactose; closed circles, glucose; closed squares, galactose; open squares, lactic acid; (B) open triangle, EPS; closed triangle, dry biomass.

Fig. 5: Influence of dilution rate during continuous fermentation of *L. delbrueckii* ssp. *bulgaricus* DSM 20081 in fully supplemented L-SDM #18 ($T = 40 ^\circ C$, $pH = 6.0$) on the productivity of: Open circles, lactose; closed circles, glucose; closed squares, galactose; open squares, lactic acid; open diamond, EPS.
Table 1: EPS concentrations produced by different *L. delbrueckii* ssp. *bulgaricus* strains during batch fermentation at constant pH

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium¹</th>
<th>EPS (mg/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRNZ 1187</td>
<td>CDM</td>
<td>107</td>
<td>Petry et al. (13)</td>
</tr>
<tr>
<td>CRNZ 416</td>
<td>CDM</td>
<td>174</td>
<td>Petry et al. (13)</td>
</tr>
<tr>
<td>CNRZ 397</td>
<td>CDM</td>
<td>75</td>
<td>Petry et al. (14)</td>
</tr>
<tr>
<td>LB 18</td>
<td>CDM</td>
<td>90</td>
<td>Petry et al. (14)</td>
</tr>
<tr>
<td>NCFB 2772</td>
<td>CDM</td>
<td>95</td>
<td>Grobben et al. (15)</td>
</tr>
<tr>
<td>DSM 20081</td>
<td>SDM</td>
<td>100</td>
<td>Schiraldi et al. (16)</td>
</tr>
<tr>
<td>NCFB 2483</td>
<td>SDM</td>
<td>160</td>
<td>Welman and Maddox (17)</td>
</tr>
<tr>
<td>RR</td>
<td>SDM</td>
<td>354</td>
<td>Kimmel et al. (18)</td>
</tr>
</tbody>
</table>

¹ CDM, chemically defined medium; SDM, semi-defined medium
Table 2: Cell count, pH and EPS production of *L. delbrueckii* ssp. *bulgaricus* DSM 20081 after 72 h anaerobic cultivation in flasks in semi-defined media with different carbon sources

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Type</th>
<th>Concentration (mmol/L)</th>
<th>Cell count (x 10^6/mL)</th>
<th>pH</th>
<th>EPS (mg GE/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLU</td>
<td>50</td>
<td>22^a ± 20</td>
<td>5.36</td>
<td>7^a ± 2</td>
<td></td>
</tr>
<tr>
<td>GLU</td>
<td>100</td>
<td>26^a ± 4</td>
<td>5.59</td>
<td>19^b ± 7</td>
<td></td>
</tr>
<tr>
<td>GLU</td>
<td>150</td>
<td>41^a ± 3</td>
<td>5.35</td>
<td>20^b ± 2</td>
<td></td>
</tr>
<tr>
<td>GLU</td>
<td>200</td>
<td>40^a ± 18</td>
<td>5.56</td>
<td>20^b ± 1</td>
<td></td>
</tr>
<tr>
<td>LAC</td>
<td>50</td>
<td>212^b ± 41</td>
<td>4.86</td>
<td>12^a ± 4</td>
<td></td>
</tr>
<tr>
<td>LAC</td>
<td>100</td>
<td>303^b ± 16</td>
<td>4.56</td>
<td>24^b,c ± 2</td>
<td></td>
</tr>
<tr>
<td>LAC</td>
<td>150</td>
<td>404^c ± 32</td>
<td>4.51</td>
<td>30^b ± 5</td>
<td></td>
</tr>
<tr>
<td>LAC</td>
<td>200</td>
<td>459^c ± 54</td>
<td>4.38</td>
<td>40^b ± 1</td>
<td></td>
</tr>
<tr>
<td>GLU+LAC</td>
<td>100+100</td>
<td>157^b ± 9</td>
<td>4.72</td>
<td>12^a ± 2</td>
<td></td>
</tr>
<tr>
<td>GAL+LAC</td>
<td>100+100</td>
<td>190^b,c ± 19</td>
<td>4.69</td>
<td>12^a ± 2</td>
<td></td>
</tr>
</tbody>
</table>

1 GLU, glucose; LAC, lactose; GAL, galactose
2 pH is the arithmetic mean, and cell count and EPS are arithmetic mean ± half range from two fermentation experiments. Mean values marked by different superscripts differ significantly (P < 0.05)
3 EPS, extracellular polysaccharides expressed as glucose equivalents
Table 3: pH and cell count of *L. delbrueckii* ssp. *bulgaricus* DSM 20081 after 72 h anaerobic cultivation in media with different composition and supplementation

<table>
<thead>
<tr>
<th>Medium code</th>
<th>Medium macronutrients&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Supplements&lt;sup&gt;2&lt;/sup&gt;</th>
<th>After fermentation&lt;sup&gt;3,4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carbohydrate C&lt;sub&gt;YNB&lt;/sub&gt; (g/L)</td>
<td>C&lt;sub&gt;CP&lt;/sub&gt; (g/L)</td>
<td>B V S F O pH Cell count&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Source C&lt;sub&gt;CH&lt;/sub&gt; (mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Glucose 100 5.0 10.0</td>
<td>- - - - - -</td>
<td>5.59 26 ± 4</td>
</tr>
<tr>
<td>1</td>
<td>Lactose 100 5.0 10.0</td>
<td>+ - - - -</td>
<td>4.56 303 ± 16</td>
</tr>
<tr>
<td>2</td>
<td>Lactose 100 5.0 10.0</td>
<td>+ - - - -</td>
<td>4.06 560 ± 6</td>
</tr>
<tr>
<td>3</td>
<td>Lactose 100 5.0 10.0</td>
<td>- + - - -</td>
<td>4.52 261 ± 0</td>
</tr>
<tr>
<td>4</td>
<td>Lactose 100 5.0 10.0</td>
<td>- - + - -</td>
<td>4.65 192 ± 6</td>
</tr>
<tr>
<td>5</td>
<td>Lactose 100 5.0 10.0</td>
<td>+ - - - -</td>
<td>3.92 851 ± 19</td>
</tr>
<tr>
<td>6</td>
<td>Lactose 100 5.0 10.0</td>
<td>+ - - - -</td>
<td>4.13 620 ± 22</td>
</tr>
<tr>
<td>7</td>
<td>Lactose 100 5.0 10.0</td>
<td>+ - - - -</td>
<td>4.47 300 ± 6</td>
</tr>
<tr>
<td>8</td>
<td>Lactose 100 5.0 10.0</td>
<td>+ + - - -</td>
<td>4.06 807 ± 6</td>
</tr>
<tr>
<td>9</td>
<td>Lactose 100 5.0 10.0</td>
<td>- - - - -</td>
<td>4.59 220 ± 3</td>
</tr>
<tr>
<td>10</td>
<td>Lactose 100 5.0 10.0</td>
<td>- - - - +</td>
<td>4.34 347 ± 3</td>
</tr>
<tr>
<td>11</td>
<td>Lactose 100 5.0 10.0</td>
<td>- - - + +</td>
<td>4.33 398 ± 16</td>
</tr>
<tr>
<td>12</td>
<td>Lactose 100 5.0 10.0</td>
<td>+ - - + +</td>
<td>4.31 376 ± 13</td>
</tr>
<tr>
<td>13</td>
<td>Lactose 100 5.0 10.0</td>
<td>- + - + +</td>
<td>4.18 632 ± 10</td>
</tr>
<tr>
<td>14</td>
<td>Lactose 100 5.0 10.0</td>
<td>- - + + +</td>
<td>4.25 461 ± 10</td>
</tr>
<tr>
<td>15</td>
<td>Lactose 100 5.0 10.0</td>
<td>+ + - + +</td>
<td>4.06 886 ± 3</td>
</tr>
<tr>
<td>16</td>
<td>Lactose 100 5.0 10.0</td>
<td>- + + + +</td>
<td>4.13 575 ± 22</td>
</tr>
<tr>
<td>17</td>
<td>Lactose 100 5.0 10.0</td>
<td>- + + + +</td>
<td>4.13 705 ± 13</td>
</tr>
<tr>
<td>18</td>
<td>Lactose 100 5.0 10.0</td>
<td>+ + + + +</td>
<td>4.08 842 ± 35</td>
</tr>
<tr>
<td>19</td>
<td>Lactose 100 5.0 10.0</td>
<td>+ + + + +</td>
<td>6.02 n.g.&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>20</td>
<td>Lactose 100 5.0 10.0</td>
<td>- - - - -</td>
<td>6.02 n.g.</td>
</tr>
<tr>
<td>21</td>
<td>Lactose 100 20.0 10.0</td>
<td>+ + + + +</td>
<td>5.81 717 ± 25</td>
</tr>
<tr>
<td>22</td>
<td>Lactose 100 5.0 10.0</td>
<td>- - - - -</td>
<td>4.30 442 ± 29</td>
</tr>
<tr>
<td>23</td>
<td>Lactose 100 5.0 10.0</td>
<td>- - - - -</td>
<td>5.02 210 ± 36</td>
</tr>
<tr>
<td>24</td>
<td>Lactose 100 5.0 10.0</td>
<td>- + + + +</td>
<td>4.42 531 ± 51</td>
</tr>
<tr>
<td>25</td>
<td>Lactose 100 5.0 10.0</td>
<td>- - - - -</td>
<td>5.96 n.g.</td>
</tr>
<tr>
<td>26</td>
<td>Lactose 100 10.0 20.0</td>
<td>+ + + + +</td>
<td>4.38 781 ± 51</td>
</tr>
<tr>
<td>27</td>
<td>Lactose 150 5.0 10.0</td>
<td>+ + + + +</td>
<td>4.06 781 ± 13</td>
</tr>
<tr>
<td>28</td>
<td>Glucose 150 5.0 10.0</td>
<td>+ + + + +</td>
<td>4.85 300 ± 6</td>
</tr>
<tr>
<td>29</td>
<td>Glucose 150 5.0 10.0</td>
<td>+ + + + +</td>
<td>4.77 315 ± 3</td>
</tr>
</tbody>
</table>

---

<sup>1</sup> C<sub>CH</sub>, carbohydrate concentration; C<sub>YNB</sub>, yeast nitrogen base concentration; C<sub>CP</sub>, casein peptone concentration

<sup>2</sup> B, nucleobases; V, vitamins; S, inorganic salts and sodium thioglycolate; F, sodium formate; O, orotic acid

<sup>3</sup> Temperature was 40 °C, and starting pH was 6.0

<sup>4</sup> pH is the arithmetic mean, and cell count is arithmetic mean ± half range from two fermentation experiments

<sup>5</sup> n.g., not grown
<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time&lt;sup&gt;1&lt;/sup&gt;</th>
<th>pH</th>
<th>Cell count</th>
<th>EPS&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>(°C)</td>
<td>(h)</td>
<td></td>
<td>(x 10&lt;sup&gt;6&lt;/sup&gt;/mL)</td>
<td>(mg GE/L)</td>
</tr>
<tr>
<td>30</td>
<td>120</td>
<td>4.16</td>
<td>831&lt;sup&gt;a&lt;/sup&gt; ± 30</td>
<td>101&lt;sup&gt;b&lt;/sup&gt; ± 20</td>
</tr>
<tr>
<td>37</td>
<td>72</td>
<td>4.08</td>
<td>952&lt;sup&gt;a&lt;/sup&gt; ± 50</td>
<td>136&lt;sup&gt;b&lt;/sup&gt; ± 2</td>
</tr>
<tr>
<td>40</td>
<td>72</td>
<td>4.08</td>
<td>830&lt;sup&gt;a&lt;/sup&gt; ± 23</td>
<td>108&lt;sup&gt;b&lt;/sup&gt; ± 14</td>
</tr>
<tr>
<td>45</td>
<td>144</td>
<td>4.58</td>
<td>264&lt;sup&gt;b&lt;/sup&gt; ± 64</td>
<td>45&lt;sup&gt;a&lt;/sup&gt; ± 14</td>
</tr>
</tbody>
</table>

<sup>1</sup> Time until pH = 4.1 ± 0.1 was reached. Fermentation at 45 °C was terminated after 6 d.

<sup>2</sup> pH is the arithmetic mean, and cell count and EPS are arithmetic mean ± half range from two fermentation experiments. Mean values marked by different superscripts differ significantly (P < 0.05).

<sup>3</sup> EPS, extracellular polysaccharides expressed as glucose equivalents.
Figure 1

Dry biomass (g/L)

<table>
<thead>
<tr>
<th>Specific EPS formation (mg GE/g biomass)</th>
<th>Exopolysaccharide content (mg GE/L)</th>
</tr>
</thead>
</table>

- m#5
- m#15
- m#8
- m#18
- m#26
- m#27