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Cell length alternations as a stress indicator for *Lactobacillus johnsonii* NCC 533

Lena-Katrin Krieger [a,*], Antje Litzmann [a], Alexander Mathys [b,c], Edwin Ananta [b], Dietrich Knorr [a]

[a] Department of Food Biotechnology and Food Process Engineering, Berlin University of Technology, Koenigin-Luise-Str. 22, D-14195 Berlin, Germany
[b] Nestlé Research Center Lausanne, CH-1000 Lausanne 26, Switzerland
[c] German Institute of Food Technologies, Prof.-von-Klitzing-Str. 7, D-49610 Quakenbrück, Germany

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ABSTRACT

The aim of this work was to evaluate whether cell length alternations of *Lactobacillus johnsonii* NCC 533 can be used to monitor the response of this bacterium towards various stress factors during its growth. The selected stressors were heat shock (46 to 55 °C for 15, 25 min) and pulsed electric fields (PEF; at 15, 20 kJ kg^{-1} with 7 to 20 kV cm^{-1}). These stress factors were applied either at the beginning or at the end of the log-phase in MRS broth. The cell length changes were also monitored during normal growth conditions. It was found, that the cell lengths of bacteria changed in the course of growth; with clear differences between lag, log and stationary growth phases. Heat shock led to significant cell elongation, whereas PEF-stress resulted in cell shrinkage. The stress treatments by heat and PEF were more influential on cell length changes at the beginning than at the end of the log-phase. The magnitude of cell length changes correlated with the intensity of the applied stresses. *Industrial relevance:* Microbial fermentations require a reliable online monitoring, which rapidly gives information about occurring stresses, their extent and nature as well as the cell damage, the capability of cell recovery and the possibility of reaching the desired cell count. The present investigations and method developments could improve the understanding of these mechanisms to lead to better controlled industrial fermentations.

1. Introduction

The biomass development of an industrial microbial fermentation can be monitored by the online measurement of the optical density (OD). However, OD value gives no exact information about the cell condition, such as vitality, length or volume. So an online monitoring parameter has to be found, which gives more information about the cell condition and occurring stress. It was assumed that natural variations in microbial growth rates during the fermentation can be influenced by stress resulting in the metabolism of different protective substances, such as H_2O_2 (Pridmore, Pittet, Praplan, & Cavadini, 2008), expression of thioredoxin reductase (Elli, Morelli, & Zink, 2002), increase of *dnaK*-specific mRNA (Zink et al., 2000), specific proteins (Gouesbet, Jan, & Boyaval, 2002; Walker, Girgi, & Klaenhammer, 1999) or verolic acid (Guerzoni, Lanciotti, & Cocconcelli, 2001). As for heat stress, the appliance of 10 °C above the normal growth temperature is followed by the transient induction of HSPs in *Lactobacillus johnsonii*, *L. helveticus*, *L. casei*, and *L. acidophilus* (Broadbent, Oberg, Wang, & Wie, 1997; Di Cagno, De Angelis, Limitone, Fox, & Gobbetti, 2006; Smeds, Varmanen, & Palva, 1998; Zink et al., 2000). Also Walker et al. published in 1999 that a temperature of 55 °C applied for 60 min to *L. johnsonii* VPI 11088 caused a maximal expression of *gro*ESL, a surface associated heat shock protein (Bergonzelli et al., 2006), which is also included in the genome of *L. johnsonii* NCC 533 (Gupta, 1995; Pridmore et al., 2004). Subsequently, if environmental stress, such as oxygen induction, nutrient deprivation or elevated temperature is introduced to *L. johnsonii* NCC 533, an enhanced transcription of the surface associated heat shock protein could probably delay the natural cell division due to significant cell elongation (Räsänen, Elväng, Jansson, & Lindström, 2001). In 1935, Pederson found evidence of varying morphology of *L. plantarum* cells depending on growth conditions.

On the contrary, electroporation would induce cell shrinkage due to cytoplasm loss (Raso & Heinz, 2006; Tsong, 1991; Zimmermann, 1986; Zimmermann, Pilwat, & Riemann, 1974). Hence, as another possible online monitoring parameter for fermentations, cell length alternations after stress induction were investigated with heat and PEF as model stressors to see the post-stress impact on growth behavior. Stressor related stress responses could help to find and eliminate an unknown stress during the fermentation as well as to evaluate the cell condition and regeneration capability. Consistently the cell length measurement could be used to monitor industrial processes, although this method is not yet mature enough.

2. Material and methods

2.1. Bacterial strain and pre-culture growth conditions

The culture of *L. johnsonii* NCC 533 was obtained from the Nestlé Culture Collection NCC (Lausanne, Switzerland) and grown for 24 h at 37 °C in De Man Agarosa Sharpe (MRS) broth under anaerobic

* Corresponding author at: DIN e. V., Am DIN-Platz, Burggrafenstr. 6, 10787 Berlin, Germany. Tel.: +49 30 2601 2810, fax: +49 30 2601 42810.
E-mail address: lena.krieger@din.de (L.-K. Krieger).

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conditions using an Aerocult A (Merck KGaA, Darmstadt, Germany) anaerobiosis generating kit.

2.2. Fermentation

A BIOSTAT® Q 500 laboratory-fermenter (B. Braun Biotech International GmbH, Melsungen, Germany) was used for the experiments. The fermenter was filled with 370 ml MRS broth. The temperature was kept at 37 °C. The stirring rate was 150 rpm. Each batch was inoculated with the inoculum, targeting an initial OD of 0.1. OD measurement was performed using LAMBDA 25 UV/Vis spectrophotometer (PerkinElmer®, Rodgau, Germany) at 620 nm. Prior to inoculation the vessel headspace was purged with CO_2 to obtain anaerobic conditions.

2.3. Treatment time points

The stress induction for heat and PEF trials was performed 180 (t_1) or 390 min (t_2) after inoculation; at the beginning or the end of the log-phase, respectively. Both time points were determined by monitoring unstressed fermentations of *L. johnsonii* NCC 533 with FPIA and OD.

2.4. Heat stress treatment and equipment

Four sterile 100 ml *Schott* flasks were tempered in a water bath at 37 °C. Ten minutes before the treatment those flasks were purged with CO_2 for 20 s and then filled with 50 ml of *L. johnsonii* NCC 533 suspension from the fermenter. Turbulently flowing water baths, one for each temperature, were concurrently tempered at 46, 48, 50 or 55 °C. The suspension for the reference sample remained in the fermenter at 37 °C. The temperature up to 55 °C was selected since it was reported that log-phase cells of *L. johnsonii* VPI 11088 experienced maximum *groESL* transcription after temperature-shift from 37 to 55 °C (Walker et al., 1999). Heat shocks were applied for 15 or 25 min (Walker et al., 1999) by permanently shaking the flasks within the tempered water. Heat-up and cooling times were about 2 min. Afterwards, these flasks were put into a 37 °C warm water bath to continue the fermentation at optimal conditions. Every 2 h the flasks were purged with CO_2 for 10 s.

2.5. PEF instruments and treatment

For the continuous treatment of the *L. johnsonii* NCC 533 sample suspensions a co-linear PEF-treatment chamber with two treatment zones and an electrode distance (d) of 4 mm was used (Töpfl, 2006). After disinfection with 70% ethanol and rinsing with MRS broth, the implemented field strength (E; Eq. (1)) and pulse frequency (f) were adapted to the specific energy input (W_{spec}; Eq. (4)) (Table 1) and a mass flow of 5 l h^{-1} with MRS broth. Thereafter the fermenter was connected to the treatment system and the sample was pumped through the treatment chamber using a flexible-tube pump. The sample was conveyed from the fermenter through the treatment chamber into three tempered (37 °C), CO_2 purged 100 ml *Schott* flasks; one for each treatment. The untreated suspension remaining in the fermenter was the reference. Following to the stress treatment, the fermenter was connected to the BIOSTAT® Q control tower again. The three 100 ml *Schott* flasks containing around 60 ml treated *L. johnsonii* NCC 533 suspension were purged with CO_2 for 20 s and put into a water bath at 37 °C for further fermentation. Every 2 h the CO_2 purging was renewed.

The determination of the number of pulses n, which could strike a cell during the retention time inside the treatment chamber during the treatment was calculated with Eq. (2). The energy per pulse W_p is regulated by the capacity (C) and the voltage (U) according to Eq. (3).

$$E = \frac{U}{d} \tag{1}$$

$$n = 2 \cdot t_R \cdot f \tag{2}$$

Table 1
Operated PEF-treatment parameters for all three operated trials; W_{spec} — specific energy input, t — point of stress induction, E — field strength, f — frequency, W_p — energy per pulse, t_R — retention time, n — pulse number.

W_{spec} kJ/kg	t	E kV/cm	f Hz	W_p 10E + 4 kJ	t_R s	n
15	t_1	7	144	1.45	72.4	10426
15	t_1	10	71	2.95	72.4	5140
15	t_1	15	31	6.66	72.4	2244
20	t_1, t_2	10	95	2.95	72.4	6878
20	t_1, t_2	15	42	6.66	72.4	3041
20	t_1, t_2	20	24	11.8	72.4	1738

$$W_p = U^2 \cdot C \cdot \frac{1}{2} \tag{3}$$

$$W_{spec} = \frac{W_p \cdot f}{\dot{m}} \tag{4}$$

2.6. Sampling and preparations

3.2 ml of sample were taken through the sampling port. To dissolve chains of *L. johnsonii* NCC 533, 2 ml cell suspension were inserted into a polypropylene CELLSTAR® tube (15 ml, Greiner Bio-One GmbH, Hungary) and treated with ultrasound using a Sonopuls HD 70 generator (BANDELIN electronic, Type: GM 70, Ger) with a UW 70 sonotrode frequency of 20 kHz for 8 s.

2.7. Cell length measurement, OD and cell count detection

After the ultrasound treatment the sample was immediately inserted into the measurement unit of the Sysmex FPIA-3000 (Malvern Instruments, Worcestershire, UK) to investigate size and shape of the bacteria. The instrument took pictures of all particles in the inserted sample with a CCD camera as shown in Fig. 1. By analyzing the different greyscale levels of pixels in the pictures, the shape and size parameters of the bacteria were calculated. As the diameter of a circle with the same area as the particle weighted by the number (N) of particles, the mean CE diameter (N) was calculated as an output. An experiment with standardized latex spheres (1 μm, 2 μm, 3 μm) showed best results for the CE diameter in comparison with the measured "maximum distance parameter" (data not shown). That CE value was used to determine the area of circle (A_{circle}) as stated in Eq. (5).

$$A_{circle} = \pi \cdot \frac{CE^2}{4} = \frac{L_{ellipsoid} \cdot b_{ellipsoid} \cdot \pi}{4} = A_{ellipsoid} \tag{5}$$

$$L_{ellipsoid} = \frac{CE^2}{b_{ellipsoid}} \tag{6}$$

Adjacent the cell length (CL) was determined by the calculation of the length of an ellipsoid ($L_{ellipsoid}$) with an equal area ($A_{ellipsoid}$) shown in Eqs. (5) and (6); where $b_{ellipsoid}$ is the shorter axis of the ellipsoid with a length of 0.8 μm (at average; Axelsson, 1998). Those calculated cell lengths are graphed in Fig. 2. To measure single cells and not chains or cell fragments, the size parameter CE (N) was adjusted between 1.133 and 3.359 μm; as for determining the shape, bacteria with circularity from 0.53 to 0.95 were investigated. These length and circularity ranges were determined due to the analysis of captured pictures of ultrasound treated *L. johnsonii* NCC 533by the FPIA-3000.

With the FPIA-3000 the cells within the sample suspension were counted while measuring the CE (N). The amount of detected cells is referred to as the cell count (CC). The optical density (OD) of 1 ml of microbial suspension was measured hourly at 620 nm in semi-micro