Differential enumeration of subpopulations in concentrated frozen and lyophilized cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus*

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ABSTRACT

Differential enumeration of subpopulations in concentrated frozen and lyophilized cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus* ND02 derived from 2 propagation procedures was determined. The subpopulations consisted of 3 categories (physiological states): viable cells capable of forming colonies on agar plates (VC+), viable cells incapable of forming colonies on agar plates (VC−), widely referred to as viable but nonculturable (VBNC) cells, and nonviable or dead cells (NVC). Counts of VC+ were recorded using a conventional plate count procedure. A fluorescent vital staining procedure that discriminates between viable (VC+ and VC−) and NVC cells was used to determine the number of viable and nonviable cells. Both propagation procedures had 2 variables: in procedure (P)1, the propagation medium was rich in yeast extract (4.0%) and the pH was maintained at 5.7; in P2, the medium was devoid of yeast extract and the pH was maintained at 5.1. The results showed that post-propagation operations—concentration of cells by centrifugation and subsequent freezing or lyophilization of cell concentrate—induced different degrees of transience from VC+ to VC− states in cells derived from P1 and P2. Compared with cells derived from P2, cells from P1 were more labile to stress associated with centrifugation, freezing, and lyophilization, as revealed by differential counting.

Key words: nonculturable viable cells, differential count, *Lactobacillus delbrueckii* ssp. *bulgaricus*, starter culture technology

INTRODUCTION

Lactobacillus delbrueckii ssp. *bulgaricus* (*L. bulgaricus*) is widely used in the production of yogurt and cheese varieties as a component of starter cultures (Batista

et al., 2015). In modern, large-scale, high-throughput dairy plants, concentrated frozen or lyophilized starter cultures are preferred to set large volumes of bulk starters or for direct setting of production tanks for yogurt or cheese vats. Ready-made concentrated frozen and lyophilized starters are manufactured in the specialized starter culture industry. To produce such concentrated cultures, proper selection of starter strains for robust acid production and flavor generation in the dairy product is necessary (Cruz et al., 2012, 2013). Additionally, it is important to optimize the technology to produce functionally efficient concentrated cultures. Technology for starter manufacture involves a series of operations consisting of propagation, concentration of cells, freezing or lyophilization, packaging, quality control, and proper transportation. Constant improvement in all of the operations is necessary to meet the demands of the highly competitive dairy industry.

Among various lactic acid bacteria used in starters, *L. bulgaricus* is extremely labile to various operations used to produce concentrated cultures (Wright and Kl-aenhammer, 1983a; Santos et al., 2014; Meneghel et al., 2017). Loss of viability at every stage—concentration, freezing, and lyophilization—occurs with *L. bulgaricus*, even when high populations are attained at the end of propagation. Various growth media and growth parameters have been used to alleviate the loss of viability and acid-producing characteristic of this *Lactobacillus* species. In a previous study (Shao et al., 2014), we described a procedure that yielded highly viable and functionally efficient concentrated lyophilized *L. bulgaricus* cultures. In that paper, we grouped the various operations in the production of concentrated starter cultures into 4 phases: propagating the culture in a fermentor to obtain high cell numbers; harvesting cells by centrifuging; preconditioning the cell concentrate by inducing heat-shock and (or) cold-shock proteins; and preserving either by deep-freezing or lyophilization. The focus of the work was to study the effects of propagation with respect to media composition and fermentation parameters, and to precondition the cell concentrate by inducing heat- or cold-shock proteins on the resili-

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ience of cell concentrates to subsequent preservation. The results indicated that both fermentation medium and preconditioning had profound positive effects on the survival and functional properties of the preserved end-product.

This research is an extension of the earlier work (Shao et al., 2014). In this investigation, we focused on exploring the effect of variable fermentation parameters on the physiological status of the harvested cells. Physiological status here relates to phenotypic expression signified by colony formation on agar media. Phenotypic expression based on colony-forming ability on agar media can be used to categorize stages in the progressive loss of viability: viable cells capable of colony formation on agar (**VC+**), viable cells incapable of colony formation on agar (**VC−**), and nonviable or dead cells (**NVC**). This phenomenon can be depicted as shown below:

where *a* is a nonreversible phenomenon, and *b* is a reversible phenomenon; reversal occurs on repair of injury. The **VC−** cells are often referred to as viable but nonculturable (VBNC) cells, in which the cells are alive and metabolically active but they fail to grow using conventional culture methods (Su et al., 2016). In this paper, the **VC−** designation will be used. Salma et al. (2013) reported on such progressive loss of viability among 60 species of gram-positive and gram-negative bacteria in response to various environmental factors (Cunningham et al., 2009; Oliver, 2010; Chen et al., 2012; Pasquaroli et al., 2013; Prakash et al., 2013; Ayrappetyan et al., 2015; Ayrappetyan and Oliver, 2016). Most of the reported studies on this phenomenon are related to pathogenic bacteria; however, a few studies have used lactic acid bacteria to demonstrate the occurrence of the **VC−** state (Moreno et al., 2006).

The LIVE/DEAD *BacLight* Bacterial Viability Kit (Molecular Probes Inc., Eugene, OR) has been widely used for quantitative determination of **VC−** cells in various bacterial species (Nicolò et al., 2011; Malmo et al., 2013; Tawakoli et al., 2013; Fernandes et al., 2014). The differentiation of dead cells from viable cells by this technique is based on cell-membrane integrity. The kit has 2 fluorescent stains, the first of which is SYTO 9, a green fluorescent nucleic acid stain that stains bacteria with both intact and damaged cell membranes. The second reagent, propidium iodide (**PI**), can penetrate only bacteria with damaged cell membrane, staining them red (Stiefel et al., 2015). Propidium iodide, however, is capable of reducing the green fluorescence of SYTO 9.

Consequently, the green fluorescent-stained live bacteria (with intact membranes) can be distinguished from red-stained dead bacteria (having damaged cell membrane). Using a fluorescent microscope and a counting chamber, quantitative determination of live (**VC+** and **VC−**) and **NVC** subpopulations is possible.

To our knowledge, this is the first report to establish the occurrence of **VC−** state in *L. bulgaricus* in response to stress factors. Quantitative determination of the **VC+**, **VC−**, and **NVC** states of cells in culture could be determined by the combined use of conventional agar plating and a direct microscopic count (**DMC**) using a fluorescent vital staining procedure that distinguishes viable cells from nonviable (dead) cells. This procedure was used herein to compare the relative proportions of **VC+**, **VC−**, and **NVC** cells in concentrated lyophilized preparations derived from 2 propagation processes to discriminate between viable and nonviable cells, which would aid in developing efficient technology for starter culture production.

MATERIALS AND METHODS

Bacteria and Culture Conditions

Lactobacillus delbrueckii ssp. *bulgaricus* ND02 was obtained from the culture collection at the Key Laboratory of Dairy Biotechnology and Engineering, Inner Mongolia Agricultural University (Hohhot, Inner Mongolia, China). This strain was chosen because it is extremely sensitive to concentration by centrifugation, freezing, and lyophilization. A freeze-dried stock culture of this strain was activated by passage through sterile reconstituted 10% nonfat dry milk (incubated at 37°C for 24 h), followed by another transfer in sterile de Man, Rogosa, and Sharpe (**MRS**) broth under similar incubation conditions.

Propagation in Fermentors

Two 5-L fermentors (Eastbio, GUJS-5, Zhenjiang East Biotech Equipment and Technology Co. Ltd., Zhenjiang, China), designated F1 and F2, were installed in parallel. Fermentor F1 was designated for process (**P**)1, in which **MRS** broth containing 4% yeast extract was the propagation medium, and the pH was maintained at 5.7. In F2, designated for **P**2, the medium was **MRS** broth free of yeast extract, and the pH was controlled at 5.1. The 2 fermentors were separately filled with 3 L of sterile propagation medium (**MRS** broth with or without yeast extract). The initial pH of the medium in both fermentors was adjusted to 6.5 using 25% NH_4OH as the neutralizer. The incubation temperature for fermentors was set at 37°C. After setting the respective

pH controls for F1 and F2, the vessels were inoculated with the activated culture at a rate of 1%. Cessation of neutralizer demand, as indicated by the pH tracing on the chart, marked the end-point for both P1 and P2.

Sampling

Samples (5-mL aliquots) were taken from F1 and F2 and analyzed separately. A portion of each sample was used for VC+ counts by plating on MRS agar (incubated at 37°C for 48 h); the remaining samples were used for differential vital fluorescent staining technique for determining the counts of living cells (VC+ and VC− cells) and NVC. After being harvested by centrifugation ($5,116 \times g$ for 10 min at 4°C), the cells were washed twice in 0.8% saline. The washed cells were suspended in a cryoprotective mixture (349.2 g) consisting of 10% nonfat milk and 1% sodium glutamate (1:6 wt/wt). The cell concentrates from P1 and P2 were divided into 20-g portions and frozen at −40°C. After 2.5 h, each of the concentrated cell suspensions (from P1 and P2) was lyophilized over a 36-h period. The remaining frozen samples were retained for differential enumeration in freeze–thaw experiments. The lyophilized preparations were stored at −18°C.

Enumeration by Plate Count

Suitable decimal dilutions of the respective fermentation broths at the end of propagation were plated by pour plate technique using MRS agar; then, dilutions were incubated at 37°C for 48 h and colonies were counted. For frozen samples, suitable decimal dilutions were plated on MRS agar after thawing, incubated at 37°C for 48 h and colonies were counted. For lyophilized powders, a 0.5-g sample was used to make serial decimal dilutions and plated, and colonies were counted as described. Plate counts for both the fermentation broths and lyophilized samples were made in triplicate.

Differential Enumeration

Samples from F1 and F2 (25 mL each) at the end of fermentation were centrifuged at $12,000 \times g$ for 10 min at 4°C. The cell pellet was washed 3 times in Ringer's solution, and the washed cells were suspended in 5 mL of 0.85% NaCl. For differential counting, the LIVE/DEAD BacLight Bacterial Viability Kit (L7012) was used according to the kit instructions. Reagents SYTO 9 (3.34 mM) and PI (20 mM) were mixed in a ratio of 1.2:1 (vol/vol) in an Eppendorf vial and stored in the dark. One microliter of the dye mixture was added to 500 μ L of the prepared cell suspension, mixed well,

and then held in the dark at room temperature for 15 min. After incubation, the stained cells were loaded into a microscopic cell counting chamber, and observed under a fluorescent microscope (Leica DM 4000B; Leica Microsystems Ltd., Heerbrugg, Switzerland) at a total magnification of 400 $\times$. The fluorescent image was captured using the software LAS V3.7 (Leica Microsystems Ltd.). The length of the bacterial cells was measured using the subsidiary software (Leica Qwin V3). Differential counting was done in triplicate for both the fermentation broths and the lyophilized powders. For lyophilized samples, 0.5 g of each powder was first suspended uniformly in 25 mL of Ringer's solution and then the cells were centrifuged and treated in the same way as the fermentor liquor from F1 and F2.

Counting Cells

The area of the counting chamber is 1.0 mm². Within that area, there are 25 medium-sized grids, which are subdivided into 16 smaller grids, such that there are a 400 small grids in the counting chamber. The depth of the chamber is 0.1 mm. The sample volume loaded into the counting chamber was 0.1 mm³. Bacterial cells in 5 small grids in each of 20 microscopic fields were counted (a total of 100 small grids). Suitable dilutions of the samples were made before loading the chamber so that an accurate count could be made. The following formula was used to determine the cell count in the freeze–thaw study:

$$\text{Cell density} = \frac{\text{No. of cells in 100 small grids}}{100} \times \text{sum of small grids} \times \frac{\text{dilution factor}}{\text{volume of chamber}}.$$

Freeze–Thaw Study

Temperature fluctuations, and even freeze–thaw cycles, often occur as frozen cultures move through marketing channels. Similar fluctuations could occur because of mishandling of frozen cultures in dairy plants. Such cyclic phase transitions from frozen state to partial or complete liquid state and the reverse are detrimental to bacterial cell viability. To simulate the freeze–thaw cycles that could occur in the marketing chain, and to determine the effect of repeated freeze–thaw cycles on cell viability, samples of concentrated frozen cultures (held at −40°C) were subjected alternately to thawing at 20°C for 4 h and refreezing, holding at −40°C for 20 h, and repeating the process through several cycles. At every thaw stage, plate counts and differential counts were made. The process was repeated until no colonies

Figure 1. Fluorescent images of living (green; brighter) and dead (red; duller) bacteria in P1 fermented broth (A) and after freeze-thaw storage (B). Color version available online.

were detected on plate counts even when samples were plated directly without any dilution.

Statistical Analysis

Statistical analysis of data was performed using R program (v 3.3.0; <https://www.r-project.org/>), and significant differences between the 2 samples were evaluated by *t*-test. The experiments were done in triplicate and the results are expressed as means $\pm$ standard deviations. Figure 2 was plotted using GraphPad Prism 5.0 (GraphPad Inc., San Diego, CA).

RESULTS AND DISCUSSION

The results of the plate and differential counts on the propagation broths from F1 and F2 and the respective bacterial cell lengths are summarized in Table 1. More living bacteria were found at the end of propagation in samples from F1 than samples from F2. In sample F1, plate counts showed a count of 1.06×10^{10} cfu/mL (VC+). The count of living bacteria (VC+ and VC−) by differential DMC was 1.45×10^{10} cells/mL (Figure 1A), which was significantly ($P < 0.05$) greater than the plate count results. This indicates that cells in F1 had transitioned from the VC+ to the VC− state. In

contrast, the sample from F2 at the end of fermentation had only 4.30×10^9 cfu/mL, and by DMC, the viable cell count was 4.32×10^9 cells/mL. The difference between the plate count and the DMC was not significant ($P > 0.05$), indicating that very few or none of the cells had transitioned from the VC+ to the VC− state.

The disparity in cell counts between samples F1 and F2 was large. The lower count in sample F2 could be attributed to the lack of sufficient protein and nutrients (i.e., lack of yeast extract) in the F2 propagation formula and the more acidic environment (pH 5.1) during propagation. The growth medium in F1 had 4% yeast extract and pH control during propagation was 5.7. Whereas the culture in F1 reached the end-point (cessation of demand for neutralizer) by 6.5 h, the culture in F2 reached the end-point after more than 11 h.

The most important finding in the study was that the propagation conditions (medium composition and pH control during fermentation) had a profound effect on the colony-forming ability of the cells. Thus, the propagation conditions in F1 (medium with 4% yeast extract content and pH control at 5.7 during fermentation) predisposed the cells to a declining path in viability. The conditions in F2 yielded cells that retained the VC+ phenotype. This shows that establishing optimum conditions for propagation is of primary importance in

Table 1. Differential count of subpopulations in the fermentation broth at the end of fermentation

Fermentation stream ¹	Fermentation duration (h)	Cell length (μm)	DMC ² (log cells/mL)	Plate count (log cfu/mL)	<i>P</i> -value ³
MRS (4% yeast extract); pH = 5.7	6.50	5.00 $\pm$ 0.18	10.16 $\pm$ 0.013 (1.45×10^{10} cells/mL)	10.03 $\pm$ 0.008 (1.06×10^{10} cfu/mL)	0.0004
MRS (yeast extract-free); pH = 5.1	11.25	2.40 $\pm$ 0.23	9.64 $\pm$ 0.016 (4.32×10^9 cells/mL)	9.63 $\pm$ 0.022 (4.30×10^9 cfu/mL)	0.91

¹MRS = de Man, Rogosa, and Sharpe.

²Direct microscopic count.

³*P*-values show the statistically significant differences between the cell counts detected by the DMC and plate count.

Table 2. Enumeration of subpopulations in the lyophilized cultures

Fermentation stream ¹	Cell length (μm)	DMC ² (log cells/g)	Plate count (log cfu/g)	<i>P</i> -value ³
MRS (4% yeast extract); pH = 5.7	4.81 $\pm$ 0.12	10.65 $\pm$ 0.028 (4.42×10^{10} cells/g)	9.58 $\pm$ 0.012 (3.81×10^9 cfu/g)	0.000027
MRS (yeast extract-free); pH = 5.1	2.07 $\pm$ 0.09	11.03 $\pm$ 0.025 (1.07×10^{11} cells/g)	11.00 $\pm$ 0.006 (1.01×10^{11} cfu/g)	0.22

¹MRS = de Man, Rogosa, and Sharpe.

²Direct microscopic count.

³*P*-values show the statistically significant differences between the cell counts detected by the DMC and plate count.

developing an efficient technology for production of concentrated *L. bulgaricus* cultures.

The deleterious effect of high concentrations of yeast extract on viability needs further study. Cogan et al. (1968) found that although tomato solids in low concentrations stimulated growth of *L. bulgaricus*, in high concentrations, it inhibited acid production in milk by the bacteria. Cogan et al. (1968) identified xylose and an adenine-containing nucleotide in tomato solids to be the incriminating components for inhibition. A similar nutrient toxicity might be involved in causing progressive loss of viability with high yeast extract levels in propagation medium for high-density cultures of lactobacilli. Yeast extracts are known to contain high concentrations of nucleotides (Peppler, 1982).

Another variation noted between cells from F1 and F2 was the difference in cell dimensions. Cells from F1 (5.00 μm) were twice as long as those from F2 (2.40 μm). This confirmed our previous observations (Shao et al., 2014). Wright and Klaenhammer (1983a), working with *L. bulgaricus* 1243, examined the effect of Ca^{2+} ions and environmental pH on the cell morphology of this bacterium. In the presence of Ca^{2+} and an environmental pH of ≤ 6.0 , cells of *L. bulgaricus* 1243 existed as short rods. At pH 7.0, even in the presence of Ca^{2+} , the cells assumed a long filamentous morphology. The filaments consisted of cells in chains, which failed to separate into individual units. Further, the cells in filaments displayed spherical protrusions of the cell membrane at discrete intervals on the cell surface. Wright and Klaenhammer (1983a) proposed that the elongated cell structure and distorted cell membrane protrusions could render the cells sensitive to freezing. In general among lactobacilli, it has been observed that the shorter the cell, the better their cryo-stability (Wright and Klaenhammer, 1983b).

The data for lyophilized concentrated cultures are shown in Table 2. Following freeze-drying, only 8.7% of the population from the F1 stream were recovered on agar plating (VC+). In the F1 freeze-dried powder, DMC revealed that viable cells totaled 23.7% of the population. This means that the VC− phenotype ac-

counted for more than 60% of the total viable count (VC+ and VC−) as recorded by DMC. At the end of propagation in F1, a large proportion of the cells had transitioned to the VC− state. A further attrition of VC+ cells during the freeze-drying process to the VC− phenotype and concomitant loss of viability resulted in a very low plate count for sample F1 (only 8.7% of the population). The viable count of F2 powder as determined by plate count (VC+) did not significantly ($P > 0.05$) differ from counting by the vital fluorescent stain method, indicating that very little or no loss in viability occurred in that sample. This again points to the importance of optimizing initial propagation of cells to ensure maximum viability of *L. bulgaricus* cultures through preservative processes such as concentration and freezing (and sale as frozen cells) or as lyophilized powder.

Although the F2 fermentation yielded cells that were stable through centrifugation and lyophilization, the initial cell population was relatively low at the end of propagation. The absence of yeast extract, a known stimulant of bacterial growth, probably explains the low cell numbers. This could be remedied by using other stimulatory ingredients such as corn steep solids, malt extract, or tomato solids in limited amounts (Cogan et al., 1968). Further research in this area is necessary.

As mentioned earlier, freeze-thaw experiments were conducted to simulate extreme conditions that frozen cultures could undergo through marketing channels and at the site of application. In these trials, the viable counts as determined by plate counts declined steadily for both F1 and F2 frozen concentrates (Figure 2). It took 8 d for F1 concentrate to reach the targeted end-point, where no colonies were seen, even in direct plating of the concentrate (i.e., without dilution). However, at that stage, a small number of “live” cells (VC−; 3.10×10^3 cells) were detected by the vital staining technique (Figure 1B). The concentrate from the F2 stream took 12 d to reach the end-point. There were <100 “live” cells in the F2 sample at that stage by the vital staining technique. In this stress test, cells derived from the F2 stream showed greater resilience.

Figure 2. Changes in subpopulations of the concentrated frozen cultures sampled from (A) fermentor F1 and (B) fermentor F2 during the freeze-thaw process. DMC = direct microscopic count. Color version available online.

In industrial-scale starter culture production, starter bacteria undergo greater stress when fermentation stream is directed through “desludging” centrifuges. Within such centrifuges, the temperature of the fermentation stream can reach as high as 30°C. The cell concentrate discharged from the centrifuge needs to be cooled rapidly to prevent cell damage. In membrane separation or ceramic ultrafiltration, bacterial cells are subjected to shear forces. In large-scale freeze-drying, cell concentrates frozen in large pans as sheets (>1–2 cm in thickness) are loaded onto shelves in industrial lyophilizers. To aid efficient sublimation, controlled heat is applied to the shelves in contact with the pans bearing the frozen sheet of cell concentrate. All of these factors affect cell viability, which should be taken into account in developing a suitable technology for production of starter cultures on an industrial scale. In this paper, we present evidence to show that differential counting, using plate counts and direct microscopic differential counting using vital fluorescent staining, would aid in developing efficient technology for starter culture production. This procedure could be adapted for other industrially important food and dairy bacteria, which are difficult to concentrate and preserve by freezing or freeze-drying. Examples include probiotic *Lactobacillus* spp. and other lactobacilli.

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