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FERMENTATION, CELL CULTURE AND BIOENGINEERING

Scalable temperature induced stress for the large-scale production of functionalized *Bifidobacteria*

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Abstract The application of sub-lethal stresses is known to be an efficient strategy to enhance survival of probiotic bacteria during drying processes. In this context, we previously showed that the application of heat stress upon the entry into stationary phase increased significantly the viability of *Bifidobacterium bifidum*. However, this heat shock has been considered only in small-scale bioreactor and no information is available for a possible scaling-up strategy. Five different operating scales (0.2, 2, 20, 200 and 2000 L) have thus been tested and the results showed that the viability of *B. bifidum* increases from 3.15 to 6.57 folds, depending on the scale considered. Our observations pointed out the fact that the heat stress procedure is scalable according to the main outcome, i.e., increases in cell viability, but other factors have to be taken into account. Among these factors, dissolved carbon dioxide seems to play a significant role, since it explains the differences observed between the test performed at laboratory scale and in industrial conditions.

Keywords Exopolysaccharide · Scale-up · Bioreactor · Heat stress · Probiotics · Carbon dioxide · *Bifidobacteria*

Abbreviations

Cfu	Colony-forming unit
EPS	Exopolysaccharide
GC	Gas chromatography
LAB	Lactic acid bacteria
MRS	Man–Rogosa–Sharpe medium
RCM	Reinforced clostridial medium
L	Litre
HPLC	High-performance liquid chromatography
PBS	Phosphate buffer saline

Introduction

Cultivation of lactic acid bacteria and *Bifidobacteria* has been the subject of numerous studies considering their importance as probiotics [17, 21, 24, 26]. Besides this application, some specific strains of *Bifidobacteria* are also becoming popular as functional foods [19]. According to this diversification at the level of the fields of application, reliable industrial processes for the production of *Bifidobacteria* are needed [11]. However, these bacteria are particularly sensitive to stress conditions arising during the production process. The most widely applied method for their production starts with a cell propagation step carried out in a batch anaerobic bioreactor. Cell recovery is then performed either by microfiltration or by centrifugation, followed by a freeze-drying step. Drying and stabilization

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of these microorganisms are actually the main bottleneck for the development of reliable and cost-effective probiotic production processes [12]. It has been shown that the robustness of *Bifidobacteria* and LAB can be increased by applying sub-lethal stresses of different nature (i.e., temperature, osmotic shock,…) during the stationary phase of the culture [11]. We have shown previously that the application of a sub-lethal heat stress at 42 °C during the culture of *Bifidobacterium bifidum* increased significantly cell recovery at the end of the process and after freeze drying [15]. This increase in cell recovery was attributed to the excretion of EPS after exposure to heat shock. These EPSs, and more precisely those bounds to microbial cells, offer an effective protection against potential damaging agents or stresses, such as those encountered during downstream processing operations [8, 15]. To highlight the industrial feasibility of such heat stress procedure, this work is based on a scaling-up study with bioreactors of different operating scales, ranging from 0.2- to 2000-L. Indeed, hydrodynamics and heat transfer performances vary widely across these operating scales due to an alteration of the mixing quality when the operating volume is increased [1, 14]. More precisely, when the bioreactor volume is increased, the time required to raise the temperature of the broth is also increased, leading to a different temperature profile. Since microbial physiology is affected by temperature gradients field, it is thus of importance to determine whether the induction of protective EPS synthesis can be scaled up. The same heat stress procedure will thus be applied for all the bioreactor operating scales considered in this work and its effect at the level EPS excretion and final cell viability will be estimated.

Materials and methods

Microbial strain and cultivation medium

Bifidobacterium bifidum THT 0101 was obtained from a probiotic producer (THT, Gembloux, Belgium). Glycerol stock solutions were composed of 30 % of glycerol and kept at −80 °C. Before operating bioreactors, *B. bifidum* THT 0101 was precultured anaerobically (Gaspak, BD, USA) in reinforced clostridial agar (RCM), provided from Merck (Darmstadt, Germany). A single colony was transferred into a tube containing 10 mL of Man-Rogosa-Sharpe medium supplemented with 0.5 g/l of L-Cysteine (MRSC). MRS was composed of 10 g/L of casein peptone, 5 g/L of meat extract, 5 g/L of yeast extract, 20 g/L of glucose, 1 g/L of tween 80, 2 g/L of potassium dihydrogen phosphate, 5 g/L of sodium acetate, 2 g/L of diammonium citrate, 0.5 g/L of L-cysteine, 0.1 g/L of $MgSO_4$, and 0.1 g/L of $MnSO_4$, and incubated at 37 °C for 24 h in an anaerobic jar. Cells were then transferred into Erlenmeyer flask 500 mL containing 300 mL sterile MRSC and incubated for 24 h at 37 °C. For large-scale cultures (i.e., in 200- and 2000-L bioreactors), the same precultivation procedure was used, but was extended to flask containing with a total capacity of 5 L. For each bioreactor operating procedure, inoculation was made on the basis of 1 mL of inoculum per L of volume. Prior to inoculation, flasks and bioreactor were degassed with carbon dioxide for 5 min. The culture in bioreactors was developed on MRSC medium.

To assess the anaerobic conditions, the redox potential was adjusted to −50 mV by carbon dioxide stripping (Hamilton redox probe, ChemoTrode ORP 150) before each experiment. Since *B. bifidum* is strictly anaerobic, the whole cultivation medium was stripped with CO_2 after sterilization and before inoculation. During the cultures carried out on MRSC medium, pH was maintained to 6.8 (regulation by addition of NaOH 12.5 M), temperature was kept at 37 °C, and agitation rate was set up at 80 rpm.

Bioreactor operating scales

The scale-up procedure was tested on five different operating scales (Table 1). Different bioreactor systems were used for this purpose: 0.2 L (DASGIP Control System for 4 Vessels bioreactor, Eppendorf, Germany); 2 L (Biostat B-Twin,

Table 1 Geometry, operating conditions and effectiveness parameters for the different bioreactor set up used in this work

Bioreactor operating parameters and design	Bioreactor 0.2-L	Bioreactor 2-L	Bioreactor 20-L	Bioreactor 200-L	Bioreactor 2000-L
Stirrer diameter d (mm)	30	52.5	100	250	450
Type of impeller	RDT6	RDT6	RDT4	RDT6	RDT6
Number of impellers	2	2	2	3	3
Effective volume (L)	0.3	1.6	16	160	1800
Tank diameter (mm)	64	130	220	495	1000
d/D ratio	2.1	2.4	2.2	2.2	2.2
Volumetric power (W/m^3)	503	504	502	502	509
Mixing time (s)	ND	<3	13	25	49

The scale-up factor considered was the volumetric power (*RDT* Rushton disk turbine)

Sartorius, Germany); 20 L: (stirred bioreactors, Biolafitte, France); 200 L (F3 AG chem, AG Switzerland); 2000 L (stirred bioreactor AMOS, Angleur-Belgium). All the bioreactors used in this work exhibited a similar geometry, i.e., with a ratio between the vessel diameter and the impeller diameter of 2.2–2.4, and a liquid height equal to the vessel diameter multiplied by the number of agitation stages. Technical details about the different bioreactor configurations used in this work can be found in Table 1. Basically, the scale-up procedure was performed by keeping the volumetric power constant at a value of 500 W/m^3. Mixing time values reporter in Table 1 were extracted from previous studies [5, 6].

Freeze drying

Cells were collected from culture by centrifugation (Sorvall, USA) at 10000×*g* for 30 min at 4 °C, then washed with sterile demineralized water and resuspended in water prior to freeze drying. Cell concentrates were then put into stainless steel freeze-drying plates and congealed at −20 °C. The plates were then placed in the freeze dryer where the following protocol was applied. For the first phase, temperature was decreased to −44 °C in 2 h at 149 Pa. The second phase involved a temperature increase to −20 °C in 4 h and this temperature was then maintained for 12 h. The third phase involved a progressive increase of the temperature to 15 °C at a rate of 5 °C/h.

Determination of cell viability

For the determination of the survival cells after freeze drying, *Bifidobacterium bifidum* THT 0101 cells suspension was prepared from 1 g of powder dissolved in 9 ml of sterile physiological water (casein peptone 1 g, NaCl 8.5 g, Tween 80 1 mL, demineralized water 1 L and adjusted to pH 7). This suspension was held at 37 °C for 30 min under agitation 50 rpm. Survival cells were enumerated after spreading on Reinforced Clostridial Medium (RCM) (Merk, Germany). Petri dishes were maintained for 48 h at 37 °C in anaerobic jar with anaerobic gaspak (BD, USA). Survival rates after freeze drying were calculated as the ratio N/N_o, with N being the total number of bacteria with sub-lethal temperature stress in 1 g of powder after freeze drying, and N_o the total number of bacteria without sub-lethal temperature stress in 1 g of powder after freeze drying in the same volume of bioreactor setup.

Determination of EPS quantity and composition

EPSs were extracted from cell suspensions collected at the end of the culturing carried out in the different bioreactor operating conditions according to the method described by Nguyen et al. [15]

Crude EPS total produced by *Bifidobacterium bifidum* was harvested after 24 h by mixing 25 mL of culture with an equal amount of NaOH 2 M and was gently stirred overnight at room temperature. Supernatants were then recovered by centrifugation at 8400 g for 20 min and crude EPSs were precipitated from the supernatants by adding twice the volume of 96 % (v/v) cold ethanol. The precipitation was carried out at 4 °C for 48 h. After a second centrifugation step at 8400 g for 30 min, EPS was dried at 55 °C until constant weight. Total EPS quantification was then determined gravimetrically. In a second time, the free (non-attached to cells) EPS fraction was determined. After centrifugation at 8400 g for 20 min of fresh culture, supernatants were collected from the different bioreactors' operating conditions after 24 h of culture. Proteins were removed by precipitation with trichloroacetic acid to a final concentration of 5 % (w/v). The precipitate was then removed by centrifugation (8400×*g* for 30 min). EPSs were precipitated by gradually mixing twice the volume of cold 96 % cold ethanol. The precipitation was carried out at 4 °C for 48 h. The precipitate was collected by centrifugation (8400×*g* for 30 min). EPS preparations were dried at 55 °C until constant weight and free EPS fraction was determined gravimetrically. The EPS bound to microbial cells was determined as the differences between the total EPS and the free EPS fraction. Quantitative analysis of EPS regarding to their monosaccharide composition was carried out by Gas Chromatography [9]. For this purpose, dried EPS sample was resuspended in a solution of H_2SO_4 1 M and hydrolyzed for 3 h at 100 °C. After hydrolysis, 2-Deoxy-D-glucose 3 mg/ml (Merk, Germany) was added as the internal standard. GC analysis was then performed on an HP 6890 GC (HP-1 dimethylpolysiloxane capillary column) equipped with a flame ionization detector (FID) (Agilent, USA). The temperature of column was maintained at 325 °C throughout the analysis (injector temperature 290 °C, detector temperature 320 °C). The results were processed using the Agilent ChemStation A.08.03 software.

Results and discussion

Evolution of the heat transfer performances according to the bioreactor operating scales

It has been reported previously that the application of a sub-lethal heat stress promotes cell viability and recovery after freeze drying [7, 15, 18, 22]. The experiments were conducted at a relatively small scale, and it should be interesting to test whether the bioreactor operating scale affects the results. Indeed, heat transfer performances are affected by the bioreactor volume and configuration (i.e., number of agitation stages). In this context, five operating scales have

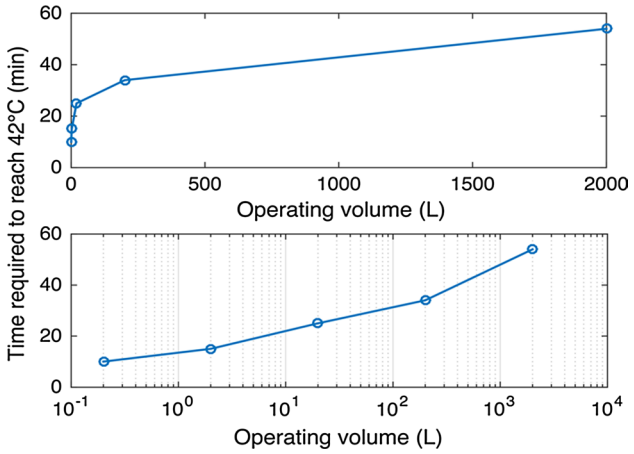

Fig. 1 Evolution of the time required to shift from 37 to 42 °C in stirred tank bioreactor according to the operating scale (*top* representation in linear axis, *bottom* representation in semi-logarithmic scale)

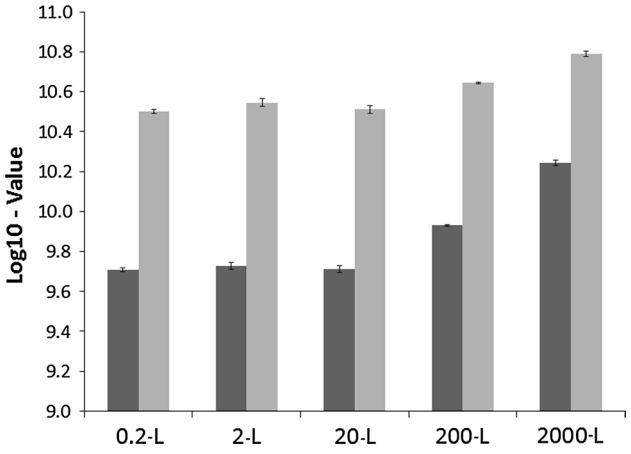

Fig. 2 Evolution of cell viability (cfu counting expressed in log10 scale) according to the different bioreactor operating scales. Bioreactors are operated with (Stressed S) or without (Non-stressed NS) sub-lethal heat stress. Standard deviation is displayed and accounts for three independent experiments

been tested and the results point out significant differences at the level of the time required to reach a temperature of 42 °C (Fig. 1). The tests were performed with the same volumetric power input. However, considering the level of power dissipated in our operating conditions (see Table 1), i.e., 500 W/m^3, this parameter has a limited influence on the global heating rate since conductive heat transfer through the double jacket is the limiting factor [25]. Heat transfer can thus be hardly adjusted from scale to scale, leading to significant differences at the level of the time required to reach the target temperature, with around 10 min for the smallest scale and more than 55 min for the largest one.

Temperature sub-lethal stress induces an increase of EPS production and cell viability for all the bioreactor operating scales

In the previous section, it has been shown that the heat transfer performances between the different bioreactor operating scales are quite different. The temperature profiles are thus different from a scale to another and possibly impact the microbial physiology. This section will be dedicated to the effect of the bioreactor operating scale on the viability of *Bifidobacteria*. The heat stress procedure has thus been applied for all the operating scales considered in this study. It can be observed, for all the operating scales, an increase of cell viability at the end of the process (Fig. 2a). Cell recovery increases also with scale, this effect being particularly marked for the 200 L and 2000 L bioreactors. However, it is important to point out that cell viability before downstream processing operations tends to be higher in cultures carried out in large-scale reactors, as shown by the higher cell recovery values recorded for the 200 and 2000 L bioreactors without applying the heat stress procedure (Fig. 2a). The reason why cell viability tends to be higher in large-scale reactors has to be found at the level of the gas exchange between the headspace of the bioreactor and the liquid volume. This statement will be discussed in the next section. As stated before, *Bifidobacteria* respond to heat stress by synthesizing and excreting EPS. This physiological response is confirmed in our case for all the operating scales investigated (Fig. 3). More precisely, the fraction of EPS bounds to microbial cells increases significantly upon exposure to heat stress, providing the cells with a protective layer [20]. It is also interesting to notice that, for the largest bioreactor, the quantity of EPS is higher than in small-scale ones, even if no heat stress is applied, suggesting that heat stress is not the only mechanism involved in cell survival and EPS excretion.

Large-scale bioreactor promotes EPS production: effect of gas–liquid mass transfer

Previous results have been discussed by assuming that temperature profiles and mixing times are the only differences between the bioreactor set-ups. However, physiology of *Bifidobacteria* is also strongly affected by the redox potential of the medium, as well as by the availability of carbon dioxide [10, 13, 16]. Indeed, our observations point out a strong re-incorporation of dissolved oxygen in small-scale bioreactor, by comparison with largest ones. This effect can be attributed to the ratio between the free surface S (cross-sectional area of the bioreactor, representing the free liquid surface in contact with the headspace) and the working volume V of the bioreactor. The S/V ratio tends to decrease during scale-up according to the relative dependency to the characteristic diameter of S (progressing according

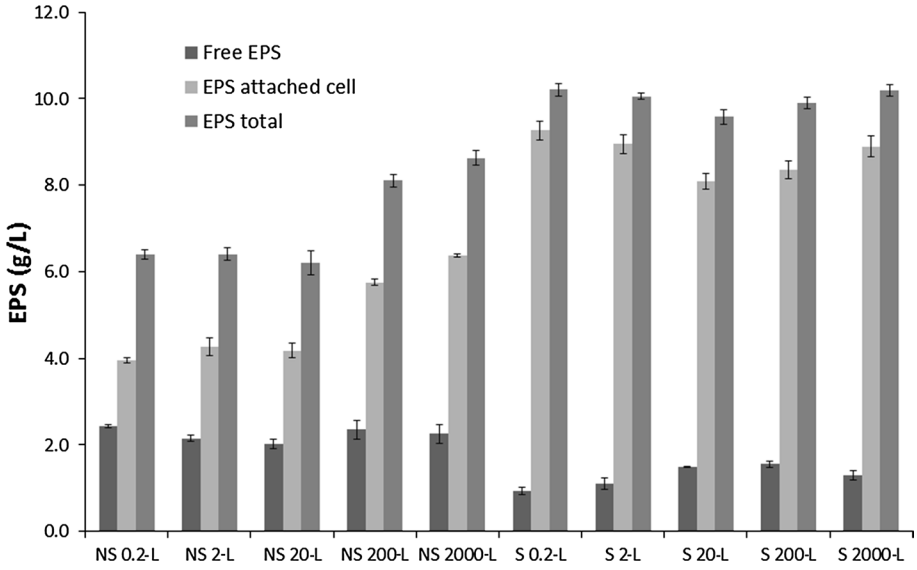

Fig. 3 Excretion of EPS according to the different bioreactor operating scales investigated and operated with (Stressed S) or without (Non-stressed NS) sub-lethal heat stress

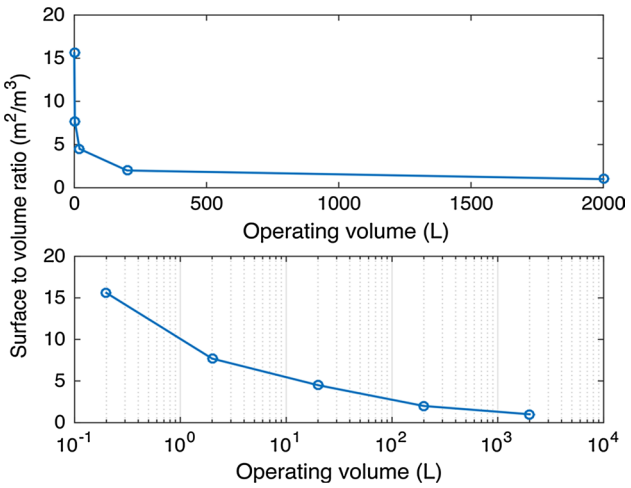

Fig. 4 Evolution of the S/V ratio during scale-ups, the surface S representing the section of the bioreactor and the volume V being the working volume (*top* relationship in linear axis, *bottom* relationship in semi logarithmic scale)

to D^2 during scale-up) and of V (progressing according to D^3 during scale-up) (Fig. 4). The S/V ratio is strongly different between the two extreme scales considered in this work, with a value of approximately 15 m^2/m^3 for the smallest bioreactor and a value of 1 m^2/m^3 for the largest one. Despite the fact that all the bioreactors have been sparged with the same relative amount of carbon dioxide at the beginning of the experiments, the re-incorporation of oxygen into the liquid phase during cultures carried out at the smallest scale explains the differences between the EPS produced and the resulting viability [16]. To validate

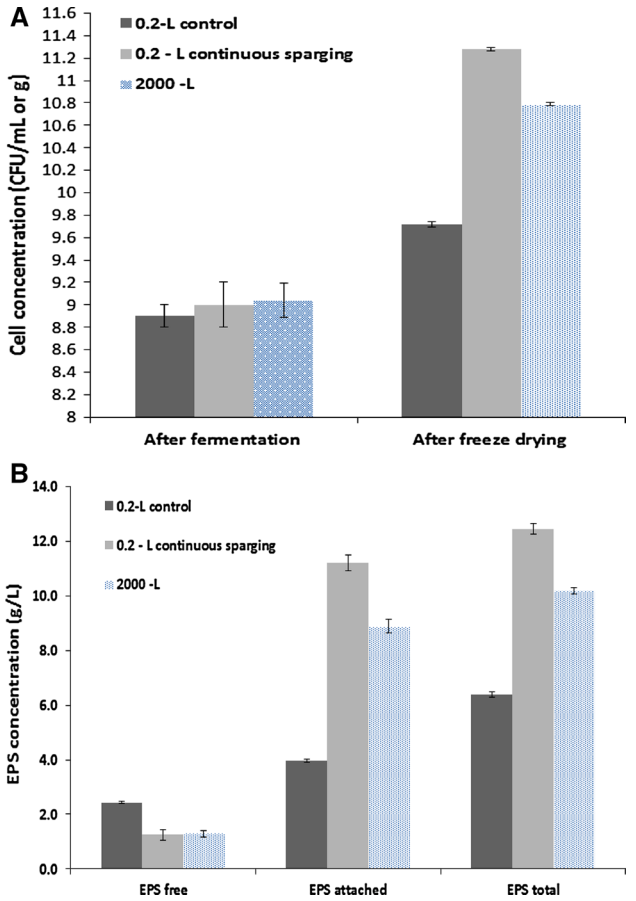

Fig. 5 Effect of carbon dioxide on **a** cell viability and **b** EPS production. Analyses have been performed for a comparison between a 0.2 L bioreactor with continuous sparging with carbon dioxide and a 2000 L bioreactor. Results coming from the 0.2-L bioreactor without continuous carbon dioxide sparging are shown as control

this hypothesis, an additional test has been carried out in a 0.2-L bioreactor with continuous carbon dioxide sparging. In this case, it is observed that the performances of the 0.2-L bioreactor increase and become higher to that found in the largest bioreactor (Fig. 5). Indeed, a largest quantity of EPS is synthesized in this case, resulting in a higher cell recovery at the end of the fermentation, as well as after freeze drying. This result further confirms the relationship between EPS excretion and cell survival after drying operations [15]. This important result points out the fact that intensification of carbon dioxide mass transfer could be used as an efficient strategy for the optimization of *Bifidobacteria* production.

The composition of EPS is affected by the bioreactor operating scale

A comparative analysis of the EPS produced after heat shock has been performed and revealed that EPS composition was affected by the operating scale (Fig. 6). The EPS produced by *B. bifidum* THT 0101 is a heteropolysaccharide which is also found in another species of *Bifidobacterium,* as well as among different species of *Lactobacillus, Streptococcus, B. bifidum* [2–4, 23]. A total 6 types of monosaccharides were detected. Notably, mannose (28.59–72.33 %), glucose (19.96–46.89 %) and galactose (0–21 %) were found in exopolysaccharide. Minor fraction of other monosaccharides was also detected, i.e., rhamnose (0–6.68 %), arabinose (0–4.12 %), and xylose (0.5–1.3 %). Our results point out that the sub-lethal heat shock treatment does not affect significantly the EPS composition. However, significant differences were observed between the two operating scales considered, suggesting that the quality of the EPS can be different. Since EPSs are important for cell adhesion in the gut, such differences can affect the quality of the final product and the functionalization of the probiotics.

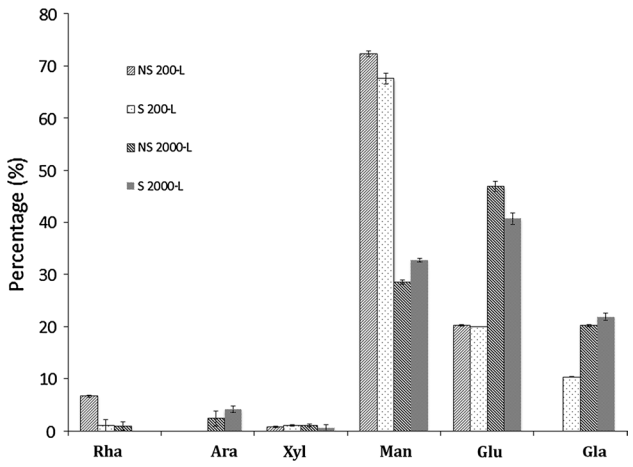

Fig. 6 Composition of EPS collected in 200 L and 2000 L bioreactors operating with (S) and without heat stress (NS), Rhamnose (Rha), Arabinose (Ara), Xylose (Xyl), Manose (Man), Glucose (Glu), Galactose (Gla)

Conclusions

It is well known that the application of heat-lethal stresses (e.g., temperature, pH, osmotic shock…) increases the viability of probiotic bacteria. This is the first study focusing on the impact of the scaling-up procedure on this important phenomenon.

Our results point out that sub-lethal stresses can be used as a scalable technological solution to improve cell viability for the production of *Bifidobacterium bifidum* (see Table 2 for a comparative view of the cell viability results gained in this work). This species is among the most sensitive to industrial conditions and it would be interesting at this level to apply the heat stress procedure to other industrially relevant strains, such as *B. longum*. Indeed, since the heat stress procedure described in this work is very simple and does not require any modifications of the bioreactor design, it should be interesting to estimate its applicability to other types of probiotics and lactic acid bacteria. Our results point out that temperature stress induces the production of EPS. Since EPSs are important for the formation

Table 2 Comparative analysis of cell viability obtained for all the operating scales investigated during this work

Bioreactors	Control		Heat stressed	
	After fermentation CFU/mL	After freeze drying CFU/g	After fermentation CFU/mL	After freeze drying CFU/g
0.2-L	8E+08	5.2E+09	8.2E+08	3.22E+10
2-L	9.5E+08	5.5E+09	9.4E+08	3.61E+10
20-L	1.1E+09	5E+09	1.05E+09	3.15E+10
200-L	1E+09	8.5E+09	1.05E+09	4.39E+10
2000-L	1.1E+09	1.7E+10	1.2E+09	6.01E+10

Results are expressed as a mean value obtained either after fermentation or after freeze drying. Bioreactor experiments have been performed either by considering the sub-lethal heat stress procedure or not

of biofilm and the colonization of the human gut, it can be hypothesized that the bioreactor operating procedure can be designed to improve the functionalization of the microbial cells according to their probiotic effect. EPS can also acts as a prebiotic. However, it has been shown that EPS composition differs from a bioreactor operating scale to another. These qualitative differences should thus be taken into account in future assessment of probiotic efficiency of *Bifidobacteria*.

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