Flow-cytometric assessment of damages to *Acetobacter senegalensis* during freeze-drying process and storage

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Abstract

Downstream processes have great influences on bacterial starter production. Different modifications occur to cellular compounds during freeze-drying process and storage of bacterial starters. Consequently, viability and culturability (multiplication capacity) undergo some changes. In this study, the effects of freeze-drying process and storage conditions were examined on cell envelope integrity, respiration and culturability of *Acetobacter senegalensis*. Freezing of cells protected with mannitol (20% w/w) did not affect cell multiplication and respiration considerably; however, 19% of cells showed compromised cell envelope after freezing. After drying, 1.96×10^{11} CFU/g were enumerated, indicating that about 34% of the cells could survive and keep their culturability. Drying of the cells induced further leakage in cell envelope and finally 81% of cells appeared as injured ones; however, 87% of the dried cells maintained their respiration capacity. Storage temperature had significant effect on cell multiplication ability; higher storage temperature (35°C) caused 8.59-log reduction in cell culturability after nine-month period of storage. Collapse of cell envelop integrity and respiration was observed at 35°C. At lower storage temperature (4°C), the culturability decreased about one-log reduction after nine months. Cell envelope integrity was subjected to minor changes during a period of nine month-storage at 4°C whereas a heterogeneous population of cells with different respiration capacity emerged at 4°C. These results indicate that a major part of cells undergone drying process and storage entered into viable but non-culturable state. In addition, usage of different culture media didn't improve resuscitation. Besides, it seems that sub-lethal damages to cell envelope caused uptake of propidium iodide, however these kinds of injuries could not impress cell multiplications and respiration.

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Introduction

Preservation of microorganisms by desiccation has been the preferred method for long term storage of cultures for decades.[1] Freeze-drying is the favorite method of drying in industrial biotechnology, pharmaceutical and bio-products industry.[2,3] However, freezing and drying are known to injure microbes and bio-products.[4] One of the most important drawbacks of freeze-drying is the loss of viability.[2]

Freeze-drying tolerance of microorganisms depends on freeze-drying medium, the physiological state of the cells, freezing rate, freeze-drying parameters, rehydration conditions, and initial cell concentration.[5] Compared to gram positive bacteria, gram negative ones generally demonstrate lower survival rate after freeze-drying.[6]

Viable but non-culturable (VBNC) state is induced in cells by natural stress such as elevated temperature,[7] or by different industrial processes used for production of dried biomass.[8] Bacteria in the VBNC state fail to grow on the routine bacteriological media on which they would normally grow and develop into colonies, but are alive and capable of renewed metabolic activity.[7]

Freezing process and especially the rate of cooling have great influences on physical appearance and viability of cells after drying,[1,2] however, the effect of freezing rate may differ for different microbes.[9] Moreover, Dumont and co-workers have shown that viability can be preserved equally at ultra-rapid and low cooling rates.[10,11]

Although the inhibition of metabolic activities and other reactions at cryogenic temperature is beneficial for long term storage of biomaterials, the process of freezing and thawing are often deleterious.[12] Since water molecules contributes to stabilization of proteins, DNA, and lipids as well as conferring structural order upon cells, removal of water (especially bound water) affects on viability.[13] All the cellular sites such as cell wall, chromosome, ribosomes, membranes and enzymes are subjected to physical treatments such as freezing and drying.[4] It is therefore

extremely difficult to detect a single key factor that causes cell injury or death; hence most likely a multiplicity of defects is involved.[14]

Cellular membranes are often damaged during the process of freezing and thawing, or during desiccation and rehydration.[15] Changes in permeability have led to the suggestion that certain treatments, such as heating, freezing, drying, and radiation create small pores in the outer membrane.[16] A theory was proposed by Crowe *et al.* about the damages of phospholipids during dehydration. According to this theory, the removal of hydrogen-bonded water from the phospholipid bilayer results in the increase in the head-group packing of membrane lipids, leading to increased opportunities for Van der Waals interaction among hydrocarbon chains. Therefore, the lipids change from liquid crystalline to gel phase in dry membranes because of the increase in melting temperature of membrane lipid. Consequently, it is likely that cell envelope damage during drying occurs only when cells lose free water, and the damage is most pronounced at a critical range of water content.[17]

Changes in the water content of the cells can produce dysfunction in specific enzymes or promote the development of chemical reactions that normally would not occur in a fully hydrated cell. Thus, under normal metabolic conditions, free radicals would be trapped by the antioxidant defense systems; however, under water stress, these mechanisms could be inactive.[2] Dehydrogenases are especially heat sensitive. Glycosylases and endonucleases, enzymes involved in DNA repair are heat labile at mild temperatures of 40 to 50°C.[16] This unfavorable effect of temperature on proteins should be considered during storage at high temperature. Indeed, ATPase inactivation has been reported during storage under controlled humidity condition.[18]

Although the decrease in water content during freeze-drying leads to some cell injuries; however remaining of water in dried cells also promotes unfavorable reactions, therefore many authors have reported the maximum and minimum amount of water concentration below which cells have to be stored.[14] There is no doubt that storage stability increases at lower temperature.[14] Lipid compositions of freeze-dried cells usually change due to oxidation reactions and a decrease in ratio of the unsaturated to saturated fatty acid content of the cells are observed.[18,19] Under oxidative storage conditions, molecular oxygen is introduced into unsaturated fatty acid such as linoleic (18:2) and linolenic (18:3) acids, leading to the formation of the corresponding hydroperoxy polyunsaturated fatty acids.[20] Furthermore, by incorporation of florescent probes and using confocal laser scanning microscopy, Carlsen and coworkers showed that lipid oxidation was not the main responsible for the initial bacteria cell deterioration; however radical formation during initial days of storage can be an evidence for oxidation reactions instability of freeze-dried bacteria.[21]

Freezing and freeze-drying processes have been used by researchers for production of vinegar starter for long time.[22-24] According to the strains and the protective components used through the studies, different survival rates have been reported after freezing or freeze-drying.[22-24] Most of the studies used spread plate techniques to enumerate the number of culturable cells,[22,24] or most probable number methods for estimation of viable cells.[23]

Commercial production of vinegar in tropical regions needs large quantities of water for cooling of bioreactors which in turn increases the cost of production. Thus, application of an efficient thermo-tolerant strain may reduce. Ndoye and co-workers isolated a thermo-tolerant acetic acid bacterium, *Acetobacter senegalensis*[25,26] and this strain was used for production of freeze-dried starter.[24] This starter could produce 8% (w/v) acetic acid in a pilot plant scale acetifier at elevated temperature (35°C).[27] However, further studies in the context of sub-lethal and lethal injuries to bacteria during downstream process are required to improve the production of vinegar starter.

In this study, the influences of freeze-drying process and storage on culturability (multiplication ability), respiration and cell envelope integrity of *A. Senegalensis* were investigated. In addition, the relation between culturability and viability was studied.

Materials and Methods

Bacterial strain

Acetobacter senegalensis CWBI-B418 (=LMG 23690^T=DSM 18889^T), a thermo-tolerant acetic acid bacterium isolated from mango fruit, was used through this study.[26]

Culture media and growth conditions

GY medium was used as a pre-culture medium to cultivate *A. senegalensis*: GY contained: glucose 20 g/L, yeast extract 7.5 g/L, Mg SO_4.7 H_2O 1.0 g/L, and $(NH_4)_2HPO_4$ 1.0 g/L (pH 6.0). pH was set by potassium hydroxide (KOH).

Pre-culture was prepared in 1000 mL three-baffled flask containing 100 mL of the GY medium and incubated on shaker (120 rpm) at 30°C.

GY agar contained all the above components plus 15 g/L agar. GYE agar had all the components of GY agar plus 5% (w/v) of ethanol. pH of the media (5.0) was adjusted by using phosphoric acid and KOH. The mentioned solid media were used for enumeration of culturable cells during freeze-drying process and also storage period.

Biomass production

For production of biomass, a fermentation process was performed in a Lab-scale bioreactor (Bio Lafitte, Poissy, France), with total volume of 15 L and working volume of 10 L. The bioreactor was equipped with three probes that were used for monitoring dissolved oxygen, temperature and pH. The aeration system consisted of two marine-blade impellers (left-handed orientation) and a cross-shaped sparger. The air inlet equipped with a flow meter and set at 1 vvm. The amount of dissolved oxygen was regulated automatically by changing the marine-blade impeller rotation speed according to the demanded oxygen during bacterial growth. The critical threshold for dissolved oxygen was set at 30%. The considered pH (4.8) was regulated by dosing pumps automatically using KOH (1.5 N) and H_3PO_4 (1.5 N) as regulator. Temperature was set at 30±0.5°C.

GY medium containing 20 g/L glucose was used for production of biomass under above conditions. Cells harvested as the residual glucose concentration reached about 0.5 g/L.

Freeze-drying process

Harvested cells were centrifuged (4500 g, +4°C, and 10 min) to get a concentrated cream (about 6% dry matter). The cream was divided into 100 mL portions and mixed with 20% (w/w) of D-Manitol (VWR international company, Leuven, Belgium), then it was frozen at an average rate of -1°C/min to -20°C. Frozen cells were dried in an industrial freeze-drier machine (Louw Koeltechniek BvBa, Rostelaar, Belgium). The drying procedure applied during the present study was: sublimation during 20 h at an average temperature ramp about +1.5°C/h and then 4 h at 10°C and finally the secondary drying was carried out at 20°C (at an average ramp about +5°C/h) for 8 h.

Packaging and storage of freeze-dried cells

Freeze-dried cells were crushed and then dispensed into glass vials. The vials were sealed with septum and screw caps. Vials were kept at different temperatures (-21, +4 and +35°C) in darkness. At least three vials were preserved for each storage conditions.

Determination of culturability and total number of cells

Spread plate technique was used to study the effect of freeze-drying and storage conditions on culturability of cells. For this purpose, GY agar and GYE agar were used as growth media. Freeze dried cells and stored cells were rehydrated in GYP broth (15 min, 30°C) and spread on GY agar and GYE agar with incubation at 30°C for 72-96 h. Log_{10} of CFU per gram of dried powder was reported in result section.

Total number of cells was determined by Bürker slide (Lo-laboroptic Ltd., Lancing, UK) using a phase contrast microscope (Olympus, Tokyo, Japan). The difference between total number of cells per mL and plate count (CFU/mL) used for calculation of non-culturability.

Non-culturability is defined as the percentage of cells which cannot grow on solid media under defined conditions. Non-culturability during freeze-drying process and storage period were calculated by the following formulas:

Calculation of non-culturability during freeze-drying process:

$$\%\text{Non-culturability} = \frac{\text{Number of cells counted by Bürker - CFU on solid media}}{\text{Number of cells counted by Bürker}} \times 100$$

Calculation of non-culturability during storage at different temperature:

$$\%\text{Non-culturability} = \frac{\text{CFU on solid media after drying} - \text{CFU after storage at specific temperature}}{\text{CFU on solid media after drying}} \times 100$$

Preparation of cell suspension for flow cytometry

In order to determine the effect of ethanol and glucose on respiration system and cell envelope integrity, rehydrated cells were introduced to broth GY and broth GYE media for 90 min, then the cells harvested and used for flow cytometry analyses.

Flow cytometry analysis

Cell envelope integrity and also respiration ability (total dehydrogenase activity) of cells were analyzed by using two different methods. All experiments were performed using a BD FACSCalibur™flow cytometer (Becton & Dickinson Co., Franklin Lakes, NJ, USA) equipped with an air-cooled argon laser and a red diode laser. Data were analyzed using BD CellQuest™ Pro software (Becton & Dickinson). Instrument settings were described after explanation of each method (see below).

Cell envelope integrity

Thiazole orange (TO) (λ_{ex} 509 nm; λ_{em} 530 nm) and propidium iodide (PI) (λ_{ex} 482 nm; λ_{em} 608 nm) were used for the determination of non-injured cells, injured and dead bacterial cells according to the guidelines provided by BD Biosciences® Company (Chicago, IL, USA) for the BD cell viability kit. Briefly, PI penetrates through compromised cellular membranes whereas intact cell membrane inhibits PI penetration. TO is a permeant dye which enters freely in dead and alive cells. Since injured cells show different level of damages which cause leakage in cell membranes, therefore different amount of PI uptake by cells exhibit various levels of viability in a bacterial population.

A stock solution of 10 mg/mL of propidium iodide in filtrated deionized water and also a stock solution of 42 μmol/L TO in dimethyl sulfoxide (DMSO) were prepared. A 10 times-diluted TO solution was prepared in DMSO just before staining of cells.

Prepared cells for flow cytometry (see above) were harvested by centrifugation at 4500 g for 10 min, and washed twice with phosphate buffer solution (PBS, pH 7.4). Washed cells were resuspended in PBS to get 5×10^7 cells/mL. 500 μL of the bacterial suspension was mixed with 5 μL of diluted TO solution and incubated at 30°C for 5 min, afterwards 5 μL of PI solution was added and incubated at 30°C for 5 min. Finally cells were washed with PBS and resuspended in PBS.

Cell suspensions were delivered at the low flow rate, corresponding to 900-1000 events/s [in the forward scatter (FSC) *vs* side scatter (SSC) plot]. TO was detected in the FL1 channel whereas PI was detected in the FL3 channel. A combination of FSC *vs* SSC was used to discriminate bacteria from background. Gating using SSC *vs* FL2 was used for a better identification of bacteria. All signals were collected by using logarithmic amplifications: FSC- E01, SSC-551 V, FL1-690 V, FL2-620 V, and FL3-829 V. Threshold was set on the SSC signal. The total number of events counted for each sample was 20,000 from SSC *vs* FL2.

Cell respiration capacity

5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) (λ_{ex} 450 nm; λ_{em} 630 nm) and TO (λ_{ex} 509 nm; λ_{em} 530 nm) were used to determine the respiration capacity of cells. CTC is a redox dye which may be converted to a solid formazan dye by cellular dehydrogenases. Solid formazan emits red fluorescence. A stock solution of 50 mmol/L CTC was prepared in filtrate deionized water.[28] TO stock solution was prepared as mentioned in previous section. Prepared cell suspension for flow cytometry was resuspended in saline phosphate buffer solution containing 18 mM glucose, to get 5×10^7 cells/mL. 45 μL of CTC solution was mixed with 450 μL of cell suspension and incubated at 30°C for 120 min on a shaker (130 rpm) in darkness, then 5 μL of diluted TO (10-fold in DMSO) was added to each sample and incubation was continued for 5 min at 30°C in darkness. Cell suspensions were delivered at the low flow rate, corresponding to 900-100 events/s (FSC *vs* FL1). CTC was detected in the FL3 channel whereas TO was detected in the FL1 channel. A combination of FSC and SSC was used to discriminate bacteria from background. All signals were collected by using logarithmic amplifications: FSC-E01, SSC-443 V, FL1-551 V and FL3-937 V. Threshold was set on the FSC signal. The total number of events counted for each sample was 20,000 from FSC *vs* SSC.

Results

The effect of freezing process on cellular features

In order to investigate the influence of freezing and drying on *A. senegalensis*, the non-culturability of cells (see materials and methods section) was determined. Non-culturable cells are dead or they are viable but cannot grow, in other words they have entered into VBNC state. In order to decide about death or non-culturability, the viable count methods (respiration system and cell envelope integrity) were also examined by using flow-cytometric methods.

Table 1 shows total number of cells and the number of culturable cells after each process (before and after freeze-drying and also during storage period). As it can be seen, there is no considerable difference between the number of cells counted directly and CFU on GY agar before and after freezing indicating that freezing at rate of 1°C/min did not cause any detrimental effect on cells and also it did not disorder culturability of bacterial cells. In addition, by using ethanol as carbon source in GYE agar, no significant difference in culturability (colony formation) was observed meaning that produced cells were also able to survive and grow in the presence of ethanol.

Cell envelope integrity was also analyzed after freezing process. Normally, in intact cells, cell membranes limit propidium iodide uptake. As Figure 1 shows, this property underwent some changes during freezing step. Freezing process caused cells to become more permeable to propidium iodide and consequently more propidium iodide penetrated into the cells, so 63.25% of cells appeared as injured cells (Figure 1A).

This trend was also observed in frozen cells which encountered ethanol after thawing (Figure 1B). However as shown in Tables 1 and 2, this kind of damage to cell envelope couldn't cause cells to lose their culturability and almost all the cells maintained their multiplication capacity.

Examination of cellular dehydrogenases after freezing showed that this process did not interfere with respiration system and 87.3% of the frozen cells were able to do respiration after thawing (Figure 2A). It is also important to point out that no adverse effect on respiration system was observed where ethanol was used as carbon source after thawing of cells (Figure 2B).

In brief, by comparing the cells before and after freezing, it can be deduced that freezing process did not change culturability and viability. Moreover, the percentage of nonviable cells (Figures 1 and 2) and non-culturable cells (Tables 1 and 2) are comparable after freezing.

The effect of drying on the main cellular features

In contrast to freezing, drying of frozen cells had greater impacts on culturability, dehydrogenase activity and cell envelope integrity. As it can be seen in Table 1, a dry matter about 91.60 % was achieved after drying which represented a normal final point for freeze-drying process and insured a safe range of water content for dried bacteria. As Table 2 shows, drying of cells increased the non-culturability up to 66%, this represents that dehydration of cells during drying damaged the cells in a way that multiplication ability was got away from most of the cells and even under favorite conditions cells were not able to repair and multiply. Using of ethanol or glucose as a carbon sources to resuscitate the VBNC did not show a significant differences (Tables 1 and 2). Therefore, it can be deduced that ethanol (5% w/v) or glucose can be considered as a carbon source for this kind of starter. However, on the basis of colony size, it seems that ethanol could increase the rate of growth. As Figure 1 shows, 81.9% of the cells appeared as injured cells after drying of cells meaning that cell envelope integrity decreased considerably, however more than 86% of dried cells could demonstrate cellular respiration in the presence of glucose and ethanol (Figure 2). In addition, as Table 1 shows, multiplication ability of cells was affected considerably by drying and about 1.96×10^{11} CFU was detected on GY agar indicating that just about 34.4% of cells were able to grow and form colonies on a solid media.

The effect of storage conditions on cellular features

As Table 1 shows, the amount of moisture content didn't change due to proper packaging during nine-month period of storage, so it is possible to assume that the whole changes in culturability and viability were due to storage temperature.

The percentage of non-culturability increased as the storage temperature elevated. At 35°C, the culturability decreased about 8.5 log units on GY agar; in comparison, we observed a lower non-culturabilty change at -21°C after nine months (Table 2). Interestingly, just 1.02 log-reduction was observed for cells stored at +4°C.

As it can be seen in Table 1, the number of observed CFU on a medium containing ethanol as carbon source is rather equal to the number of CFU counted on a medium containing glucose, therefore it can be concluded that ethanol can be used as one of the alternative carbon sources but it cannot improve the resuscitation of VBNC formation during storage periods.

Figure 3 shows the analysis of cell envelope integrity of freeze-dried *A. senegalensis* at various storage temperatures. According to this figure, cell envelope integrity was subjected to extensive changes during storage period. Comparison of Figures 3A and B indicates that cells maintained their cell envelope integrity at lower temperature (-21 and 4°C). However, as Tables 2 shows, the percentage of non-culturability is considerably lower at -21°C. In other words, more cells were able to grow despite of damages to cell envelope (Table 1). In contrast, Figure 3C shows that storage of freeze-dried bacteria at higher temperature, 35°C, compromised cell envelope extensively and consequently all the cells (100%) were permeable to propidium iodide extremely, and as a result, they emerged as dead cells. Thus, it is possible to assume that injures to cell envelope at lower storage temperature were sub-lethal ones because it did not cause great loss of culturability.

Figure 4A represents the effect of ethanol on cell envelope integrity. Based on obtained results, cellular property was not affected by ethanol and also using glucose or ethanol as carbon sources had the same influence on cell envelope integrity. No correlation was observed between function of respiration system and cell culturability at-21°C. As already represented in Table 1, 1.42E+11 cells kept at -21°C for nine months were considered as culturable cells. However, as Figure 3D shows, more than 82% of cells kept at -21°C were viable according to

Table 1. The number of culturable cells, total number of cells and residual dry matter during freeze-drying process and nine-month storage period.

	Freeze-drying process			Storage temperature		
	Before freezing	After freezing	After drying	-21°C	+4°C	+35°C
Direct count (% CV)	6.52E+10 (8.7)	6.16E+10 (7.0)	5.69E+11 (4.4)	5.94E+11 (6.1)	6.53E+11 (6.1)	5.57E+11 (6.1)
CFU on GY agar* (% CV)	6.21E+10 (18.6)	5.80E+10 (17.9)	1.96E+11 (14.9)	1.42E+11 (14.4)	1.83E+10 (15)	5.00E+02 (34)
CFU on GYE agar* (% CV)	6.71E+10 (11.2)	5.51E+10 (10.2)	1.86E+11 (12.3)	1.30E+11 (15.3)	1.23E+10 (11.9)	6.53E+02 (17.1)
Dry matter (%)°	5.67±0.55	5.8±0.45	91.60±1.64	92.07±1.53	90.42±0.98	91.62±1.50

*CFU is the number of counted colonies in 1 mL of liquid cream (before and after freezing) or 1 g of dried cells (after drying or stored samples). °Data are mean±standard deviation of at least three independent experiments. CV, coefficient of variance; GY, medium contains glucose and yeast extract; GYE, medium consists of glucose, yeast extract and ethanol.

Table 2. Non-culturability of *A. senegalensis* during freeze-drying process and after nine-month period of storage.

Non-culturability (%)*	Freeze-drying process			Storage temperature		
	Before freezing	After freezing	After drying	-21°C	+4°C	+35°C
On GY medium	4.8±0.4	5.8±0.4	65.6±3.2	27.6±4.0	90.2±6.0	100
On GYE medium	0	10.6±0.7	67.3±3.0	30.1±4.1	93.4±5.6	100

*For the calculation of nonculturabiliy, see *Materials and Methods* section. Data are presented as means±standard deviation. GY, medium contains glucose and yeast extract; GYE, medium consists of glucose, yeast extract and ethanol.

respiration test. These results indicate that almost all the stored cells at -21°C were viable (active respiration) but just 1.42E+11 were culturable. In other words, even low temperature could not inhibit VBNC formation but the rate of VBNC formation was undoubtedly dependent upon storage temperature. Similarly, as Figure 3E shows, a heterogeneous cell population was formed when the cells kept at +4°C indicating that some of them were able to do respiration while others lost this property or did it in slower rate, however more than 90% of these cells were unable to form colonies on solid media. On the contrary, cells kept at +35°C for nine months, were neither capable to do respiration nor grow and form colonies on media (Table 2 and Figure 3C).

Discussion

In the present study, we examined the effect of freeze-drying process and different storage temperatures on cell viability (cell membrane integrity, respiration) and also culturability (cell multiplication) as the main observable fact. Additionally, the relationship between culturability and viability, in particular, was investigated.

Before freezing

The impacts and mechanisms of natural stresses and industrial downstream processes (which are similar in great extent to some

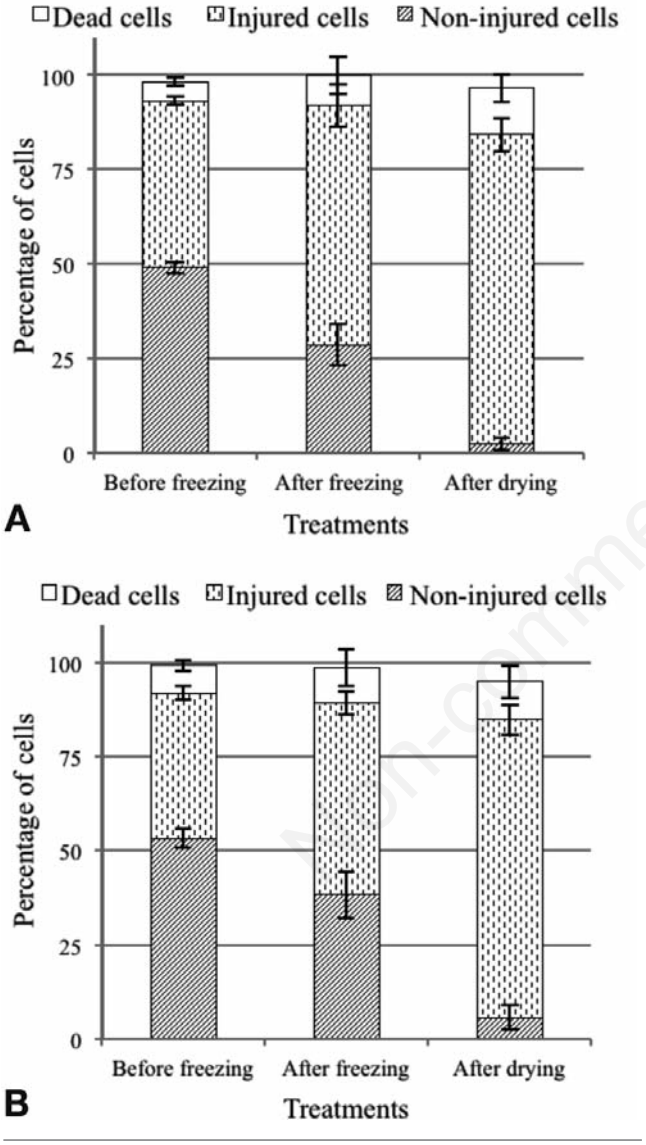

Figure 1. Cell envelope integrity of *A. senegalensis* before and after freeze-drying process. A) Cells incubated in glucose medium after rehydration. B) Cells incubated in ethanol containing medium after rehydration. Cells exhibiting propidum iodide uptake were considered as dead ones (no-fill) whereas cells absorbing just thiazole orange were considered as non-injured cells (dark upward diagonal). Injured cells also exhibited propidium uptake (dashed vertical), however the intensity of uptake was different from dead cells. Values are means±standard deviation of at least three independent experiments.

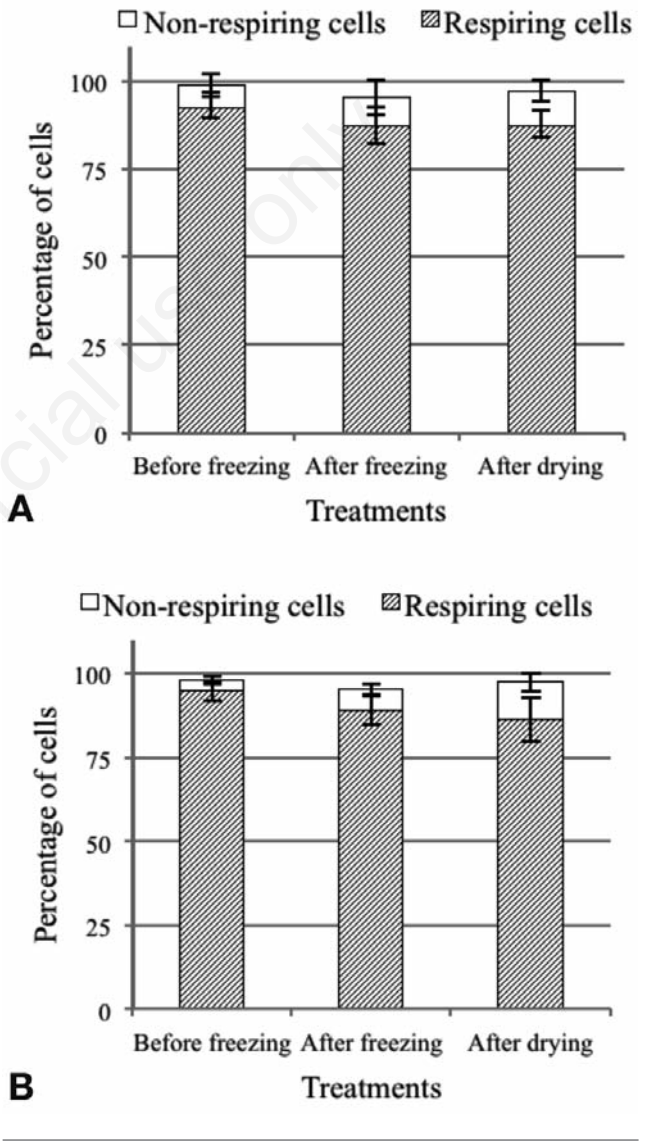

Figure 2. Respiration (dehydrogenase activity) of *A. senegalensis* before and after freeze-drying process. A) Cells incubated in a medium containing glucose after rehydration. B) Cells incubated in a medium containing ethanol after rehydration. Cells reducing 5-cyano-2, 3-ditolyl tetrazolium chloride were considered as cells contain active dehydrogenases (dark upward diagonal) whereas cells which absorbed just thiazole orange were considered as cells with inactive dehydrogenases (unfilled). Values are mean±standard deviation of at least three independent experiments.

stress conditions) on biological membranes have been studied and reviewed by many researchers.[13,29-32]

In the present study, 44% of fresh cells (before freezing)took in propidium iodide and therefore were emerged as injured ones (Figure 1) whereas the culturability and respiration were intact. These results are in agreement with the previous report by Ulmer and coworkers. They observed that some dyes such as ethidium bromide which were used for discrimination of dead and alive cells, penetrated into the bacterial cells before complete loss of viability.[33] Baena-Ruano and *et al.* also reported that the result of membrane integrity assessment by flow cytometry on acetic acid bacteria was not correlated with cell dividing as assessed by plate count method.[34]

Since fresh cell, before freezing, underwent harvesting process (centrifugation and washing) so it seems these processes damaged cell envelope. These results are also in agreement with previous observations by Santivarangkna and co-workers. They observed small marks on the cell surface of *Lactobacillus helveticus* before drying and they attributed these marks to the harvesting step, which includes washing and centrifugation.[17]

Freezing

Since freezing and drying cause low water stress for cells, they are regarded as similar processes.[13] Damage to and modification of the cell membrane are associated with almost all forms of physical stress including injury by chilling, freezing, or heating.[16]

As explained in result section, freezing with a rate of 1°C/min did not have any detrimental effect on culturability, however it could compromised the cell envelope to some extent (in 19% of cells) and therefore the number of injured cells increased up to 63% after freezing.

One of the factors which minimize the detrimental effect of freezing is the rate of cooling during freezing.[1] An optimal cooling rate should be low enough to avoid intracellular ice formation but high enough to minimize the solution effect.[13] Cell viability during freezing depends on the cell size,[10,35] the water permeability properties, and the presence of a cell wall[10] as well as the composition of cell membrane.[13,36] For gram negative bacteria such as *E. coli,* the favorite cooling rate has been reported between 2.1-5°C/min[10,13] and this range was considered as slow cooling rates whereas for *Pseudomonas fluorescens*, the preferred rate is 223°C/min.[13] Therefore, since in this study, the culturability did not change after freezing, it seems that 1°C/min (under the conditions used in this study) can protect cells from ice formation damages or the solution effect.

We also observed that even injured cells (discriminated by compromised cell envelope) were able to grow on solid media (Table 1 and Figure 1). Membrane integrity demonstrates the protection of cell constituents and its potential to generate gradients that endow ability to live. Multiplication of cells as the most stringent proof of viability requires metabolic activity and in turn membrane integrity.[37] In addi-

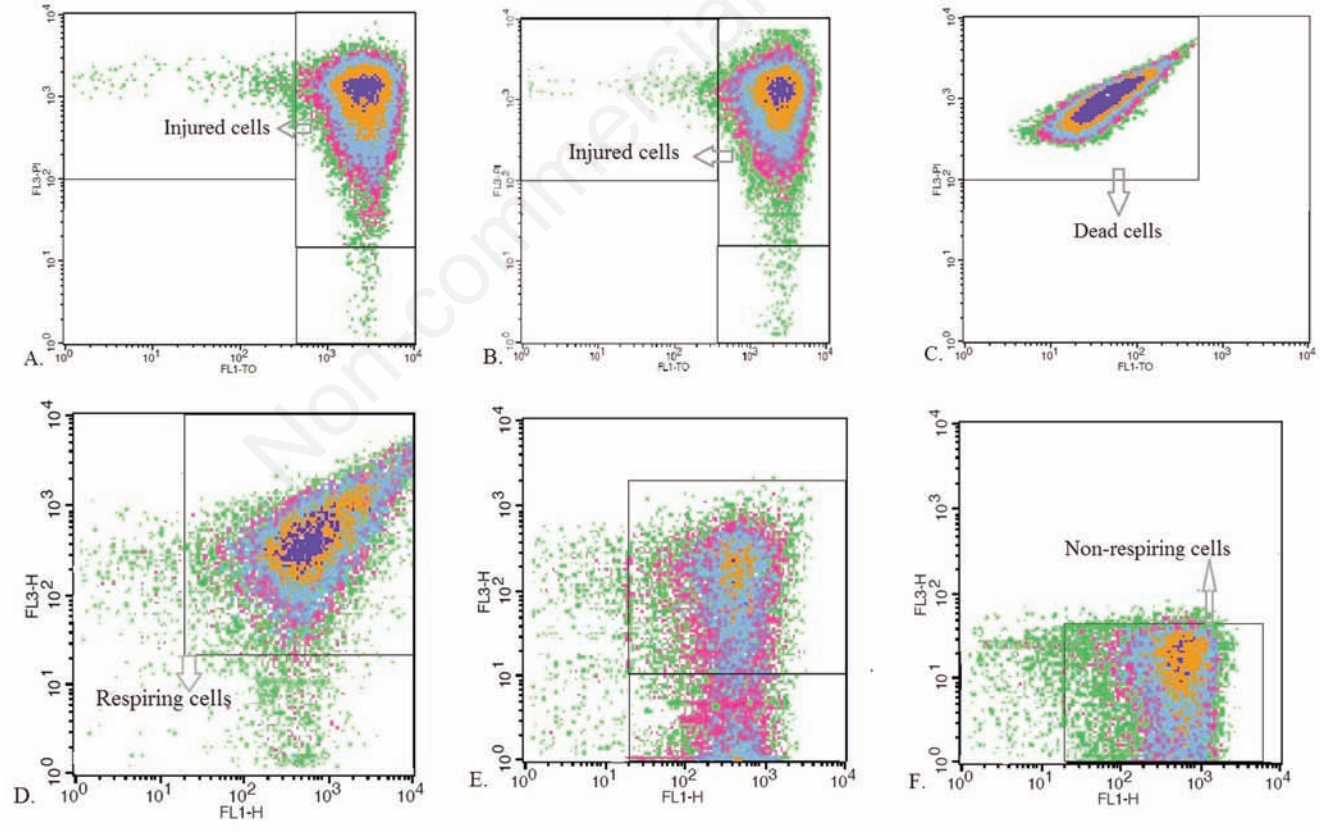

Figure 3. Flow cytometric assessment of freeze-dried *A. senegalensis* kept at various temperatures for nine months. A, B and C represent the cell envelope integrity for cells preserved at -21, +4 and +35°C, respectively. Cells which took in both thiazole orange and propidium iodide were considered as injured or dead cells on the basis of propidum iodide uptake intensity. D, E and F demonstrate the respiration system of cells kept at -21, +4 and +35°C, respectively. Cells reducing 5-cyano-2, 3-ditolyl tetrazolium chloride in the presence of glucose were considered as respiring cells.

tion, cells that reduce tetrazolium dyes (such as CTC which was used in this study) are generally considered viable (able to perform all essential cell function necessary for survival under certain condition), but cells which fail to do so are not necessarily non-viable,[38] since the injured cells (discriminated by compromised cell envelope) were also able to respire (Figures 1 and 2), it can be deduced that the damages should be considered as sub-lethal ones. Sub-lethal injury is a consequence of exposure to a chemical or physical process that damages but does not kill a microorganism; sub-lethal injuries cause loss of cell function that may be transient or permanent.[16]

Drying

According to the obtained results, after drying, cell envelope integrity and the number of cells which were able to multiply decreased significantly (Figure 1 and Table 1), however respiration was not affected (Figure 2). These differences in assessment of cell functions seem normal since each method addresses specific cellular function.[37] Furthermore, these dissimilarities suggest different inactivation mechanisms.[17]

Since number of cells with compromised cell envelope increased to 81.9% after drying (Figure 1), so probably drying condition affected cell envelope. Cytoplasmic membrane damages are generally considered as the main mechanisms of dehydration damages.[12-14] Dehydration of cells makes cell membranes more susceptible to the reactive oxygen species attack.[2] A study on *Lactobacillus helveticus* byatomic force microscopy, showed cracks on the surface of cells after drying, in addition main changes in FT-IR spectra were attributed to the damage in cell envelope.[17]

In one hand, since 66.6% of dried cells lost their culturability (Table 2), so it seems that the damages to some cellular sites were so severe that did not allow cells to repair the damages. In other hand, it is possible to assume that 34.4% of cells which kept their culturability and respiration capacity but had compromised cell envelope were sublethally injured. Ulmer and coworkers also reported that sublethally injured cells (after treatment by high pressure) were metabolically active, and loss of metabolic activity corresponded to the decrease of cell viability.[33] Metabolically active cells or intact cells might not be capable of resuming growth on the agar medium, if they are damaged at the genetic level or in the cytoplasm.[17] DNA and protein damages have been shown to occur during drying and heating processes, respectively.[16]

It should be pointed out that drying by itself may cause some protein denaturation which causes loss of the biological activity after rehydration.[2] Since in this study, after rehydration of dried cells, the respiratory dehydrogenases reduced CTC, therefore it seems that rehydration medium had no or very little adverse influence on these enzymes.

Resuscitation of starter cultures is a key features for most kind of this products.[8,39] According to our results, drying process caused VBNC formation in majority of cells. Bacteria in the VBNC state fail to grow on the routine bacteriological media on which they would normally grow and develop into colonies.[7] Since a valuable feature for vinegar starter is its ability to grow, we tried to improve resuscitation by using different carbon sources (ethanol and glucose), however it seems that other conditions are necessary for induction of resuscitation.

Storage

In the present study, the number of viable and culturable cells was dependent upon the storage temperature. At high storage temperature (35°C), viability and other cellular features changed extremely after nine-month storage (Table 1, Figure 3). Basically, dehydrogenases are especially heat sensitive,[16] so loss of respiration during storage at high temperature can be explained. In addition, as Figure 3C shows after nine months of storage at 35°C, cells lost their cell envelope integrity completely and therefore cells took in propidium iodide extensively. It is now well accepted that lipid oxidation occurs during storage of freeze-dried cells.[18-21] It has been reported that free radicals are produced during early days of storage period which promote peroxidation of lipids.[21] Ndoye and coworkers have shown that by using heat stress during production of biomass of *Acetobacter senegalensis,* the heat tolerance improved during storage and this was attributed to the increase of saturated fatty acid.[24] Peroxidation of cellular unsaturated fatty acids leads to formation and accumulation of secondary products collectively named oxylipins which enhances cell death.[20]

Slater reported that in eukaryotic cells, extensive lipid peroxidation can result in membrane disorganization by peroxidizing mainly the highly unsaturated fatty acids leading to changes in the ratio of unsaturated to other fatty acids.[40] The uncontrolled peroxidation of bio-membranes can thus lead to profound effects on membrane structure and function, and may be sufficient to cause cell death.[40] Recently, it has been reported that in eukaryotic cells, lipid peroxiadtion causes that the picture of cell membrane changes from *Fluid Mosaic Model* to the

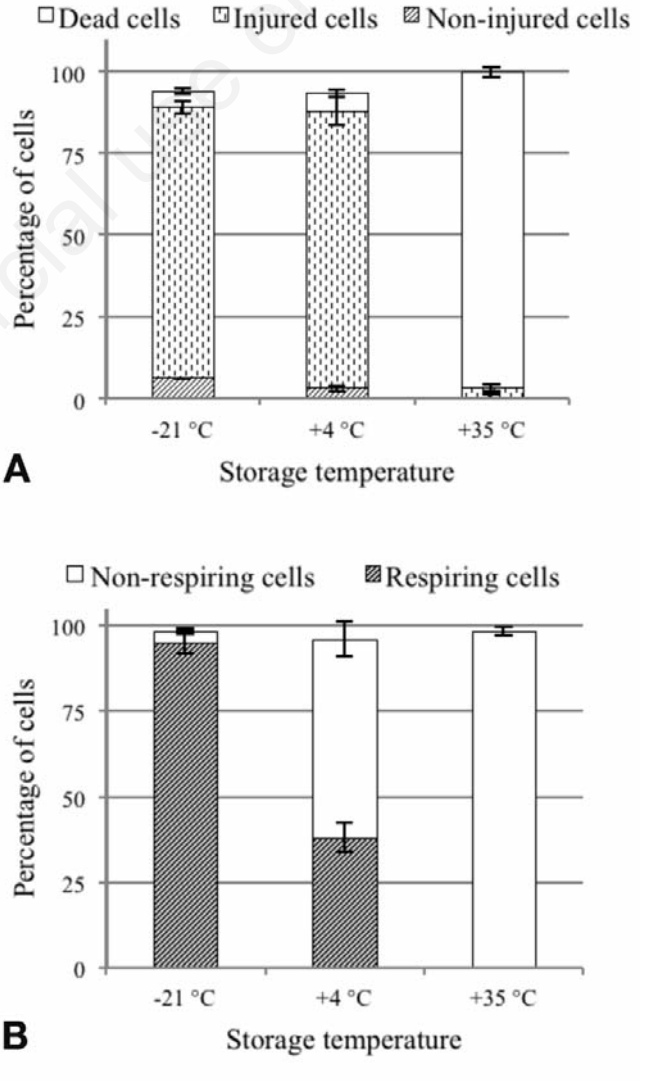

Figure 4. Cell envelope integrity (A) and respiration activity (B) of stored *A. senegalensis*. Cells were inoculated into a medium containing ethanol after rehydration. Values are mean±standard deviation of at least three independent experiments.

Lipid Whisker Model.[41] Lipid peroxidation has been shown to perturb the bilayer structure and modify membrane properties such as membrane fluidity, permeability to different substances, and bilayer thickness.[42] According to these observations, it seems that increase in PI uptake during storage at 35°C can be due to increase in permeability of cells. On the other hand, since increased membrane permeability caused by oxidation of lipids and membrane proteins can disrupt ion gradients,[42] the disruption of respiration system which was observed in the present study (Figures 3 and 4) seems reasonable.

Conclusions

The present study investigated the lethal and sub-lethal injuries that may occur during freeze-drying process and storage period. Sub-lethal damages target different cellular components and functions. We observed that cell envelope, respiration system and multiplication ability are particularly vulnerable to drying process and storage conditions. Our results showed that uptake of PI which may be the consequence of partial damage to cell envelope cannot be considered as a definite indication of cell death and non-culturability. Instead, partial PI uptake can be assumed as a result of sub-lethal damages to cell envelope which may not impair the cell multiplication (culturability) and respiration. Therefore, it can be concluded that: i) assessment of cell viability of freeze-dried starter on the basis of respiration system or cell envelope integrity does not warrant cell culturability, ii) low storage temperature can decelerate viability loss by decreasing the rate of damages to cell envelope integrity and respiration system, but even at low temperature VBNC are formed iii) using of ethanol as the most favorite carbon source for *Acetobacter senegalensis* could not improve resuscitation of VBNC meaning that some other growth conditions should be investigated.

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