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Flow cytometry assessment of *Lactobacillus rhamnosus GG* (ATCC 53103) response to non-electrolytes stress

E.O. Sunny-Roberts, E. Ananta and D. Knorr
Department of Food Biotechnology and Process Engineering, Berlin University of Technology, Berlin, Germany

Abstract

Purpose – *Lactobacillus rhamnosus GG*, a probiotic of human origin, known to have health beneficial effects can be exposed to osmotic stress when applied in food production as important quantities of sugars are added to the food product. The aim of this study is to assess the mode of action of non-electrolytes stress on its viability.

Design/methodology/approach – Investigations were carried out on stationary phase cells treated with 0-1.5 M sugars, by means of flow cytometric method (FCM) and plate enumeration method. Osmotically induced changes of microbial carboxyfluorescein (cF)-accumulation capacity and propidium iodide-exclusion were monitored. The ability of the cells to extrude intracellularly accumulated cF upon glucose energization was ascertained as an additional vitality marker, in which the kinetics of dye extrusion were taken into consideration as well. Sugar analysis by HPLC was also carried out.

Findings – The results of FCM analysis revealed that with sucrose, only cells treated at 1.5 M experienced membrane perturbation but there was a preservation of membrane integrity and enzymatic activity. There was no loss of viability as shown by plate counts. In contrast, the majority of trehalose-treated cells had low extent of cF-accumulation. For these samples a slight loss of viability was recorded on plating ($\log N/N_o \sim -0.45$). At 0.6 M, cells had similar extrusion ability as the control cells upon glucose energization. However, 20 per cent of sucrose-treated cells and 80 per cent of trehalose-treated cells extruded the dye in the first 10 min.

Originality/value – This finding pointed out the importance of trehalose to enhance the dye extrusion activity, which is regarded as an analogue of the capability of cells to extrude toxic compounds. Sugars exert different effects on the physiological and metabolic status of LGG but none caused a significant viability loss. LGG can be a choice probiotic bacterium in sugar-rich food production e.g. candies, marmalade etc., in which exposure to high osmotic pressure is be expected.

Keywords Food products, Food testing, Bacteria

Paper type

Introduction

Lactic acid bacteria (LAB) constitute a heterogeneous group of bacteria that are traditionally used to produce fermented foods. The industrialization of food bio-transformations increased the economical importance of LAB as they play a crucial role in the development of the organoleptic and hygienic quality of fermented foods (van de Gutche *et al.*, 2002). The use of micro-organisms as probiotic products is being given an adequate importance in the industrial world and moreover, researches on probiotics are gaining more interest in the scientific communities based on the

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numerous advantages associated with these group of organisms. Probiotics are defined as live organisms that are used as dietary supplements with the aim of benefiting the health of the hosts by positively influencing the intestinal microbial balance (Fuller, 1989). The genera *Bifidobacterium* and *Lactobacillus* are the main focus of probiotic interest.

The production, storage, and use of LAB impose environmental stresses on the bacterial cells (Bunthof *et al.*, 1999) and it is well known that during industrial fermentation, LAB encounter a number of stress conditions such as low temperature, low pH, and low water activity (Sandine, 1996; Baati *et al.*, 2006). Though the application of physical stress to micro-organisms, is the most widely used method to induce cell inactivation and promote food stability. To survive, micro-organisms have evolved both physiological and genetic mechanisms to tolerate some extreme conditions. This is clearly of significance to the food industry in relation to survival of pathogens or spoilage organisms (Beales, 2004). Interestingly, of recent, the application of these stresses to improve the viability and stability of probiotics is being given a keen interest.

In their various applications in the food and feed industry, LAB can be exposed to osmotic stress when important quantities of salt or sugar are added to the product (Poolman and Glaasker, 1998). Thus, they need to adapt to such a change in their environment in order to survive so that adequate quantities of cells can be made available to the consumer for health benefits purposes. It has been reported that they do so by accumulating compatible solutes (uptake or synthesis) under hyper osmotic conditions and releasing (or degrading) them under hypo osmotic conditions. Compatible solutes may also stabilize enzymes and thereby provide protection not only against osmotic stress but also against high temperature, freeze thawing, and drying (Kets *et al.*, 1996; Poolman and Glaasker, 1998; Panoff *et al.*, 2000; Gouesbet *et al.*, 2001; Leslie *et al.*, 1995; Conrad *et al.*, 2000).

Determination of the impact of treatment on bacterial strains have been made mainly by the use of classical plate count methods, however, this method bears a major draw back in the sense that it only indicates how many cells replicate under the conditions provided for growth and its long-term determination (Ritz *et al.*, 2001; Ben Amor *et al.*, 2002). Moreover, bacteria may occur in chains and clumps, resulting in underestimation of bacterial numbers. In addition, cell injury and dormancy may result in low viable counts (Barer and Harwood, 1999; Kell *et al.*, 1998).

However, flow cytometric method (FCM) is a rapid and sensitive technique that can determine cell numbers and measure various physiological characteristics of each individual cell using appropriate probes. The differentiation of the viable states of cultures are made possible by the use of specific fluorescent probes, into four classes viz a viz reproductively viable, metabolically active, intact, and permeabilized (Hewitt and Nebe-von-Caron, 2001). The applied probes include nucleic acid probes such as propidium iodide (PI), SYTO9, carboxyflourescein diacetate (cFDA), and bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4^{(3)}$) (Ananta *et al.*, 2004; Ananta and Knorr, 2004; Alakomi *et al.*, 2005; Auty *et al.*, 2001).

Many researches have been conducted on the effect of salt stress (and few on sugar stress) on LAB probably due to the requirements of a high quantity of sugar needed to obtain an equiosmolar with salt (amongst other factors). However, it is expedient to assess the extent to which probiotics remain viable in the presence of the commonly used sugars in our food products.

The subject of this study was the assessment, by FCM, of survival and viability of LGG when exposed to osmotic stress (sugars). Plate counts were performed to ensure that the populations indicated as live by FCM viability assay were indeed culturable.

Materials and methods

Test strains and growth conditions

The bacterial strains used in this study, *Lactobacillus rhamnosus GG* (ATCC 53103) was obtained from Valio R & D, Helsinki, FL. The culture which was sent in freeze-dried form in glass ampoule was later stored as glass beads cultures (Roti$^{(R)}$_Store, Carl-Roth, Karlsruhe, D) in a −80 °C freezer (U101, New Brunswick Scientific, Nürtingen, D) for long-term maintenance.

One bead was transferred into 10 ml MRS broth (Oxoid, Basingstoke, UK) and incubated overnight at 37 °C. For growth analysis, an overnight culture was inoculated into MRS broth (50 ml) at OD_{600} of 0.1 and samples were collected at intervals for optical density determination (Graphicord uV-240, Schimadzu, Jpn) for 24 h.

For flow cytometry measurements, stationary phase cells were washed in Ringer's solution and subsequently concentrated to a theoretical value of 10 (cell concentration $\sim 3 \times 10^9$) with Ringer's solution.

Determination of sublethal and lethal levels of osmotic stress

MRS broth (10 ml) containing 0-1.5 M trehalose (Roth) and sucrose (Merck) was prepared and the osmotic strength of the media was measured (51308 Vapor Pressure Osmometer Wescor Inc.). Inoculation was done at an initial OD_{600} of 0.1 and incubated at 37 °C for 24 h. Samples were collected at intervals and optical density determined.

Assessment of response of exponential-phase cultures against osmotic stress

Exponential-phase cultures (OD_{600} of 0.5) were harvested by centrifugation (1,600 g × 10 min) and re-suspended in MRS broth containing 0-1.5 M sugars. Incubation was carried out for a period of 1 h at 37 °C after which OD and CFU were determined. Kinetics of sugar treatment was carried out by withdrawing samples at 0, 15, 30, 45, and 60 min.

Flow cytometry

Stress conditions. Equal volume of the cell concentrate was treated with equal volume of the sugar solutions (0-1.5 M) at 37 °C for 30 min, and 60 min. After incubation, the cells viability was assessed by plate count enumeration and flow cytometric measurements.

Plate enumeration method. Treated samples were serially diluted in Ringer's solution (No. 15525, Merck, Darmstadt, D) and drop plated in duplicate on MRS agar (Oxoid, Basingstoke, UK). The viable cell numbers were determined after 48 h of incubation at 37 °C under anaerobic conditions produced by anaerobic kits (Anaerocult® A, Merck, Darmstadt, D).

The impact of osmotic treatment on cell viability, as assessed by plate count method was expressed as the logarithmic value of relative survivor fraction ($\log N/N_o$). N refers to the bacterial count following osmotic exposure, while N_o refers to the initial count before osmotic exposure.

Fluorescence labelling

Esterase activity and membrane integrity. Osmotically treated cells were incubated with 50 μm cFDA (Molecular Probes Inc., Leiden, NL) at 37 °C for 10 min. cFDA is an esterase substrate that yields the fluorescent carboxyfluorescein (cF) upon hydrolysis. Cells were washed to remove excess cFDA, 30 μm PI (Molecular Probes Inc., Leiden, NL) was added and incubated in ice bath for 10 min to allow labelling of membrane-compromised cells (Ananta *et al.*, 2004).

cF extrusion activity. cF-stained cells were further incubated together with 20 mM glucose for 20 min at 37 °C in order to measure the performance of treated cells in extruding intracellular accumulated cF (Bunthof *et al.*, 1999).

Kinetics of cF extrusion. cF labelled cells were incubated at 37 °C in the presence of 20 mM glucose and samples were withdrawn every 5 min for 20 min (Ananta *et al.*, 2004) to monitor the kinetics of cF release from glucose energized cells.

Flow cytometric measurement. Analysis was performed on a Coulter® EPICS® XL_MCL flow cytometer (Beckman Coulter Inc., Miami, FL, USA) equipped with a 15 mW 488 nm air-cooled argon laser. Cells were delivered at the low flow rate, corresponding to 400-600 events. Forward scatter (FS), side scatter (SS) green (FL1) and red fluorescence (FL3) of each single cell were measured, amplified, and converted into digital signals for further analysis. cF emits green fluorescence at 530 nm following excitation with laser light at 488 nm, whereas red fluorescence at 635 nm is emited by PI-stained cells.

A set of band pass filters of 525 nm (505-545 nm) and 620 nm (605-635 nm) was used to collect green fluorescence (FL1) and red fluorescence (FL3), respectively. All registered signals were logarithmically amplified. A gate created in the density plot of FS vs SS was preset to discriminate bacteria from artefacts. Data were analysed with the software package Expo32 ADC (Beckman-Coulter Inc., Miami, FL, USA). All detectors were calibrated with FlowCheck™ Fluorospheres (Beckman-Coulter Inc., Miami, FL, USA).

Data analysis

Density plot analysis of FL1 vs FL3 was conducted as described by Ananta *et al.* (2004). Density plot was used to resolve the fluorescence properties of the population measured by flow cytometer (Figures 1-5). The population was differentiated and gated as described in Table I.

Residual esterase activity following osmotic treatment was calculated using equation (1):

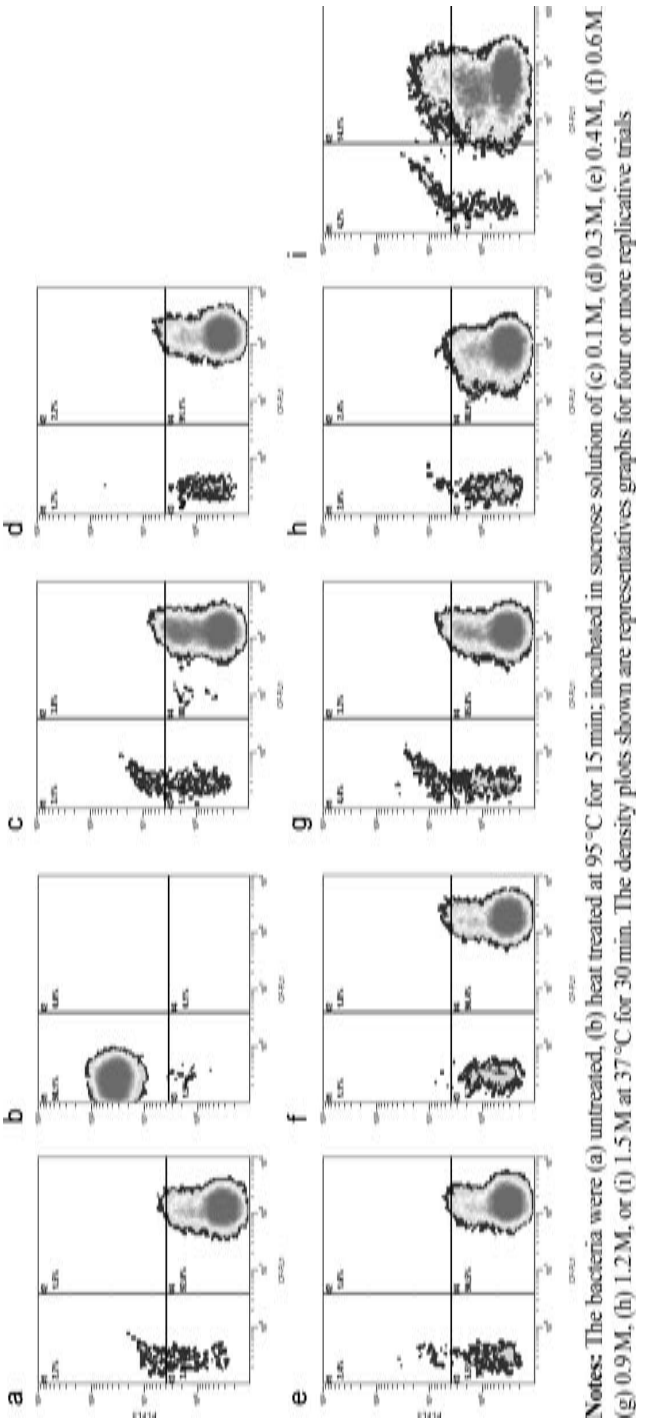
$$\mathrm{EA}(\%) = (\mathrm{A4_p}/\mathrm{A4_{ctrl}}) \times 100 \quad (1)$$

where EA is the residual enzymatic activity in response to a particular osmotic treatment, $\mathrm{A4_p}$ is the percentage of population in gate A4 following osmotic treatment and $\mathrm{A4_{ctrl}}$ is the percentage of population in gate A4 prior to osmotic treatment.

The performance of cells in extruding intracellularly accumulated dye is calculated using equation (2):

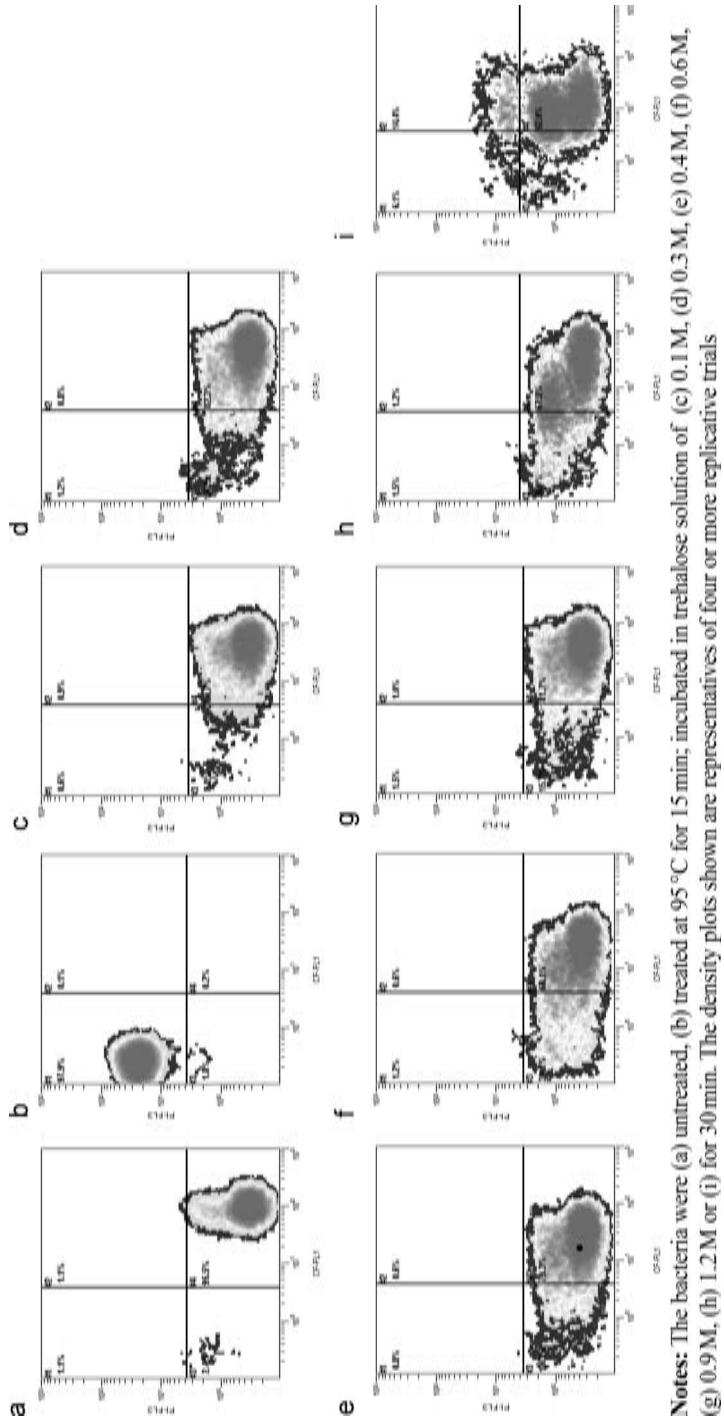
$$\mathrm{cFA}(\%) = (1 - \mathrm{A4_{Glu}}/\mathrm{A4}) \times 100 \quad (2)$$

where cFA is the measure of performance in extruding cF, $\mathrm{A4_{Glu}}$ is the percentage of population in gate A4 following glucose addition and 20 min incubation and A4 is the percentage of population in gate A4 prior to glucose addition.



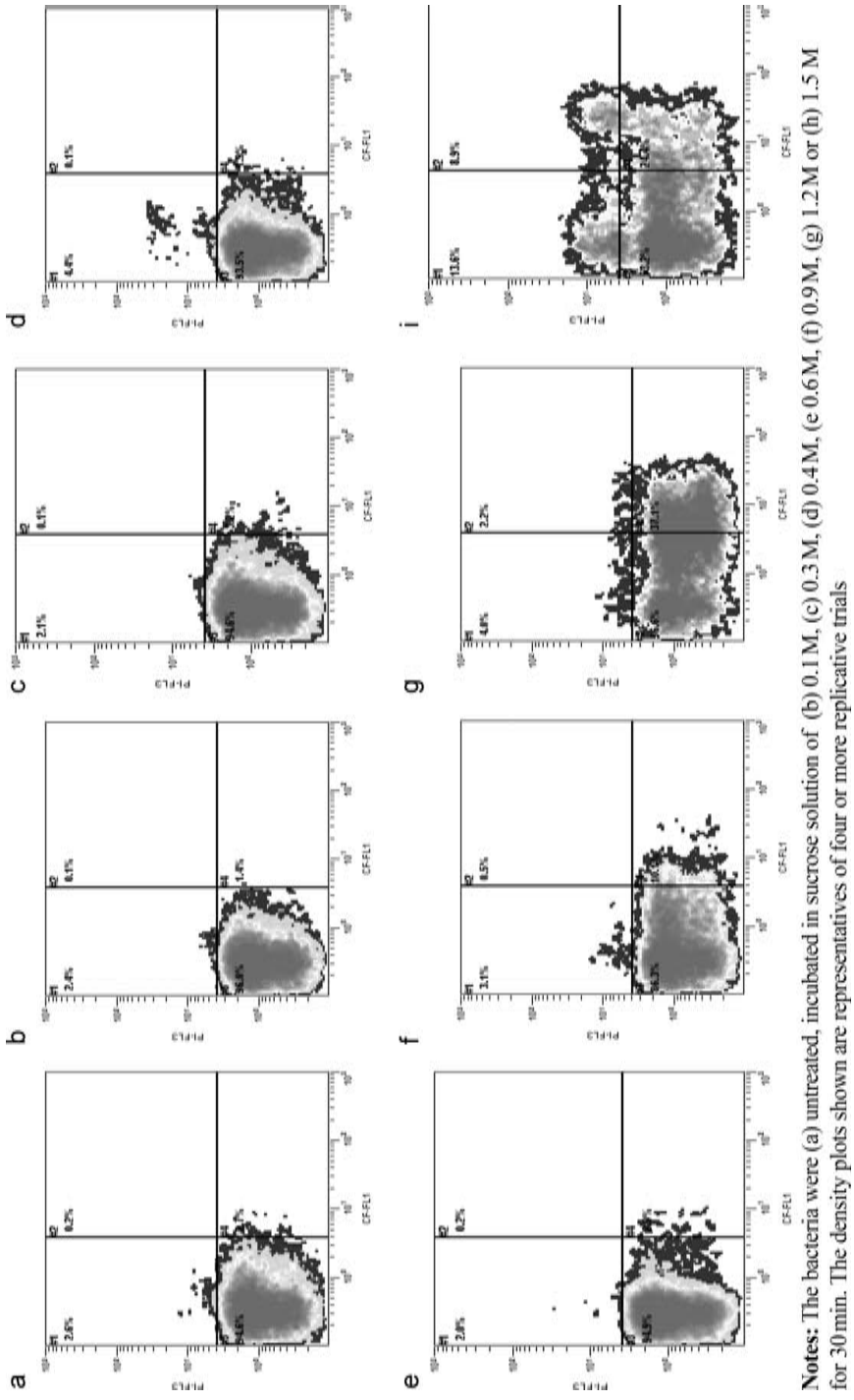
Notes: The bacteria were (a) untreated, (b) heat treated at 95 °C for 15 min; incubated in sucrose solution of (c) 0.1 M, (d) 0.3 M, (e) 0.4 M, (f) 0.6 M (g) 0.9 M, (h) 1.2 M, or (i) 1.5 M at 37 °C for 30 min. The density plots shown are representatives graphs for four or more replicative trials

Figure 1.
Flow cytometry density plots of FL1 vs FL3 of *Lactobacillus rhamnosus GG* for evaluating the impact of incubation in sucrose solution at different molarities on their membrane integrity and cF-accumulation capacity



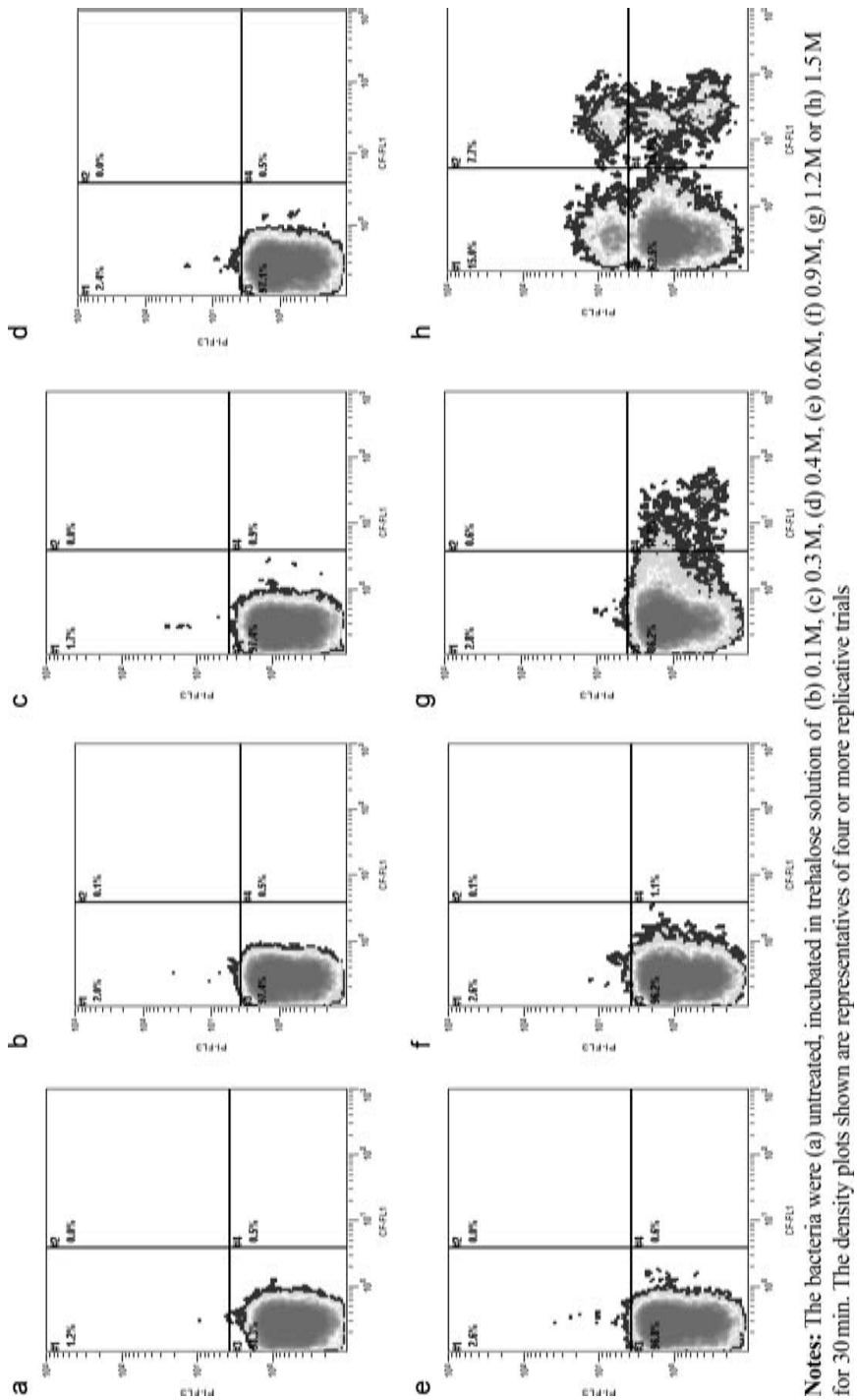
Notes: The bacteria were (a) untreated, (b) treated at 95 °C for 15 min; incubated in trehalose solution of (c) 0.1 M, (d) 0.3 M, (e) 0.4 M, (f) 0.6 M, (g) 0.9 M, (h) 1.2 M or (i) for 30 min. The density plots shown are representatives of four or more replicative trials

Figure 2.
Flow cytometry density plots of FL1 vs FL3 of LGG for evaluating the impact of inclubation in trehalose solution at differnet molarities on their membrane integrity and cF-accumulation capacity



Notes: The bacteria were (a) untreated, incubated in sucrose solution of (b) 0.1 M, (c) 0.3 M, (d) 0.4 M, (e 0.6 M, (f) 0.9 M, (g) 1.2 M or (h) 1.5 M for 30 min. The density plots shown are representatives of four or more replicative trials

Figure 3.
Flow cytometry density plots of FL1 vs FL3 of *Lactobacillus rhamnosus GG* to assess the impact of incubation in sucrose at different molarities on their cF-extrusion activity



Notes: The bacteria were (a) untreated, incubated in trehalose solution of (b) 0.1 M, (c) 0.3 M, (d) 0.4 M, (e) 0.6 M, (f) 0.9 M, (g) 1.2 M or (h) 1.5 M for 30 min. The density plots shown are representatives of four or more replicative trials

Figure 4.
Flow cytometry density plots of FL1 vs F13 of *Lactobacillus rhamnosus GG* to assess the impact of incubation in trehalose at different molarities on their cF-extrusion activity

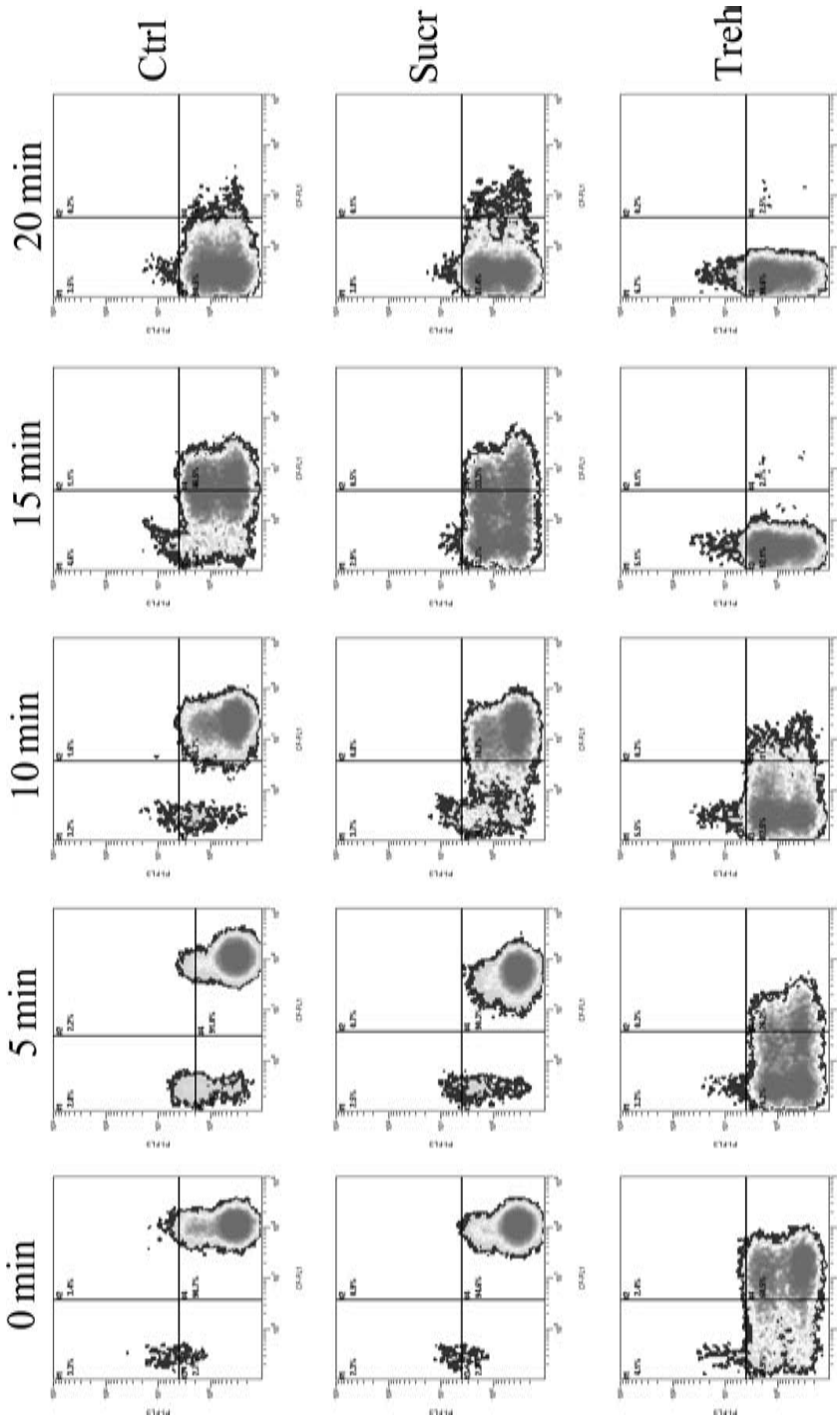


Figure 5.
Flow cytometric assessment of the kinetics of cF-efflux upon energization by glucose addition as shown by the density plot fluorescence pattern (FL1 vs FL3) of the untreated, 0.6 M sucrose-treated, and 0.6 M trehalose-treated cells

Gate	Fluorescence properties of cells collected in each gate	Possible explanation of the status of involved cellular mechanism
A1	CF^- and PI^+	Esterase activity not detectable; membrane compromised
A2	CF^+ and PI^+	Active esterase; membrane minimally damaged
A3	CF^- and PI^-	Esterase inactivated or cF extrusion out of the cells; intact membrane
A4	CF^+ and PI^-	Active esterase; intact membrane

Source: Extracted from Ananta *et al.* (2004)

Table I.
Gate designation of cells stained with cF and PI

The kinetics of relative number of population extruding the intracellularly accumulated dye is calculated in equation (3) thus:

$$\mathrm{RcF}(\%) = (\mathrm{A4}_{t-\mathrm{Glu}}/\mathrm{A4}_{t=0}) \times 100 \quad (3)$$

where RcF is the relative number of cells still stained with cF in gate A4 following glucose addition, $\mathrm{A4}_{t-\mathrm{Glu}}$ is the percentage of cells still stained with cF in gate A4 following glucose addition and incubation t min and $\mathrm{A4}_{t=0}$ is the percentage of cells still stained with cF in gate A4 prior to glucose addition.

Statistical analysis. The correlation between the cell viability and osmotic-induced changes on the physiology of LGG was tested by one-way ANOVA test. Differences were considered significant at $p < 0.05$ level of probability. This was performed with Origin7 software package (Origin Lab, Northampton, MA, USA).

Results and discussion

Growth kinetics of L. rhamnosus GG

LGG was cultivated in batch culture to determine the times taken to reach the exponential and stationary phases of growth. These were the required stages needed to determine the stress responses of this organism. LGG reached the exponential phase at approximately 4 h and the stationary phase at about 12 h (data not shown).

Determination of the sublethal and lethal osmotic conditions

The measurement of growth of cells signified a decrease with increase in molarity of the medium. At the end of the incubation period, an appreciable level (though less than the control) of growth was observed up to 1.2 M. As shown by the sugar fermentation pattern (API 50 CH system) our study strain utilizes trehalose and not sucrose but both had same influence on survival rates. The environmental factors that signal to the bacteria the transition from the logarithmic phase to the stationary phase may have a considerable effect on the survival rates during the stationary phase (Wetzel *et al.*, 1999), thus a starvation signal, triggered by depletion of carbon sources, appears to be much more favourable for survival than a low pH in the presence of sufficient carbon source (Heller, 2001; Corcoran *et al.*, 2005). However, the cells adjusted to the environmental stress by accumulation of sugars to balance their internal turgor pressure. Sugars prepared at 0.6 M were thus taken as the sublethal condition while 1.5 M was taken as the lethal because at this level, no growth was observed (data not shown).

Though the organism was cultivated in MRS broth containing glycine betaine (a compatible solute) derived from yeast extract, the uptake of sugar together with accumulation of betaine eventually might have resulted in the hyperosmolarity of the cytoplasm, which would then be compensated by net exit of glycine betaine. Glycine betaine does not protect against sugar stress (Glaasker *et al.*, 1998).

Response of exponential-phase cells to osmotic stress

Subjection of these cells to osmotic shock resulted in a slight loss of viability, even at the highest molarity, log $N/N_o \sim -0.6$ was recorded (Figure 6). Kinetically, cells were able to balance their internal pressure with that of their environment after 30 min incubation. Several investigations showed that the logarithmic phase are more susceptible to environmental stresses than are bacteria from stationary phase (Saarela *et al.*, 2004; Kim *et al.*, 2001; Gouesbet *et al.*, 2001), however in this study, there was no significant difference on the reaction of the two phases of growth to the stress (Figures 6 and 7). Similar observation was made on stress response of *Lb. plantarum* to NaCl stress (Kim *et al.*, 2001). HPLC analysis of sugars revealed that the cells responded by sugar uptake. It was assumed that the period of treatment might give little or no opportunity to sugar metabolism.

Esterase activity and membrane integrity

The percentage of cF-stained cells was used to estimate the viability of osmotic stressed cells (Figures 7-11). Sucrose (0.1-1.2 M) treated cells possessed residual esterase activity as the control cells. This is shown in Figure 9 by the presence of a high

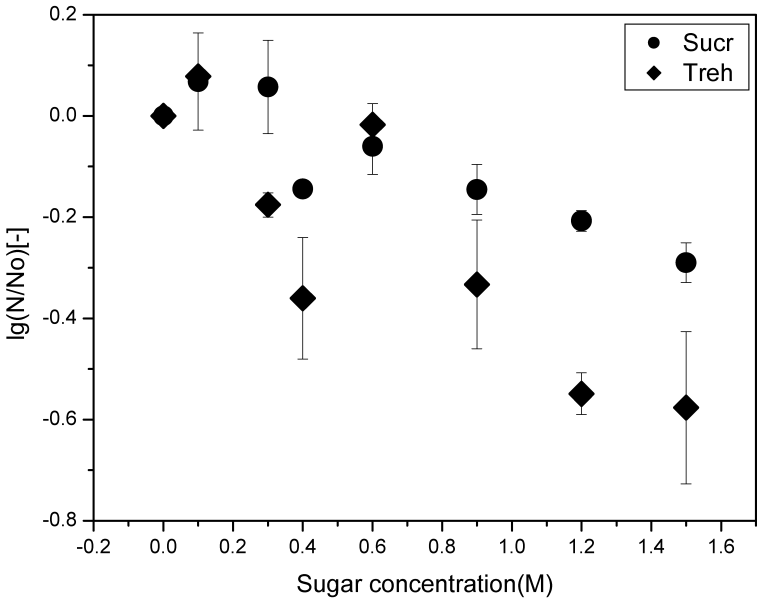
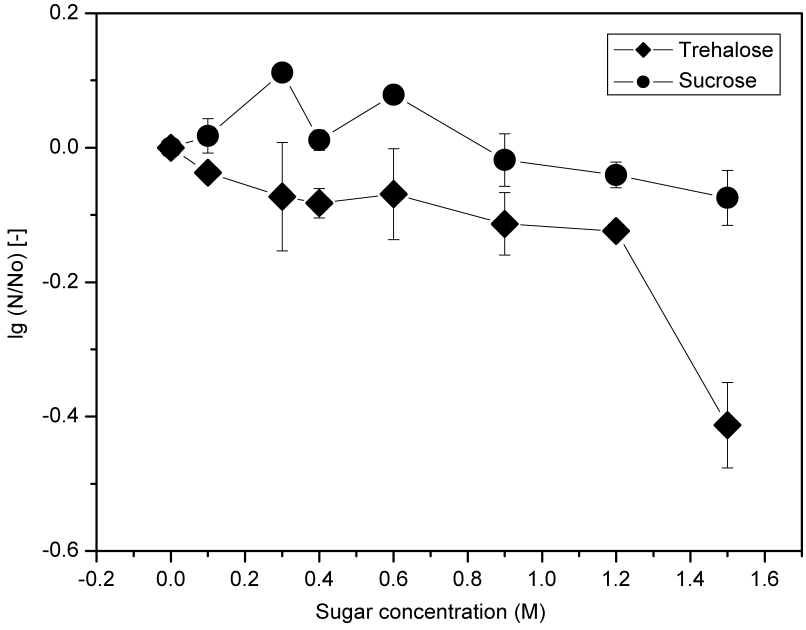


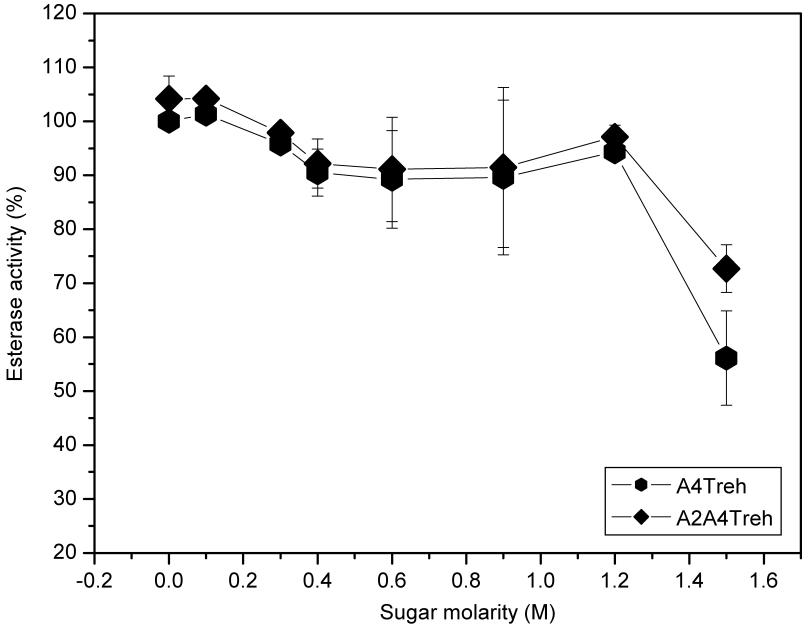
Figure 6.
Effect of increasing sucrose and trehalose concentration in MRS growth medium on LGG viability

Notes: Each point is an average of four independent experiments. Standard deviation is shown by bars



Notes: Results are means of at least three independent experiments

Figure 7.
The impact of osmotic treatment on the viability of LGG assessed by plate count method and exhibited as the logarithmic value of the surviving cells (N/N_o)



Notes: Results are means of at least 3 independent experiments

Figure 8.
Relative changes of esterase activity as affected by trehalose treatments at different molarities

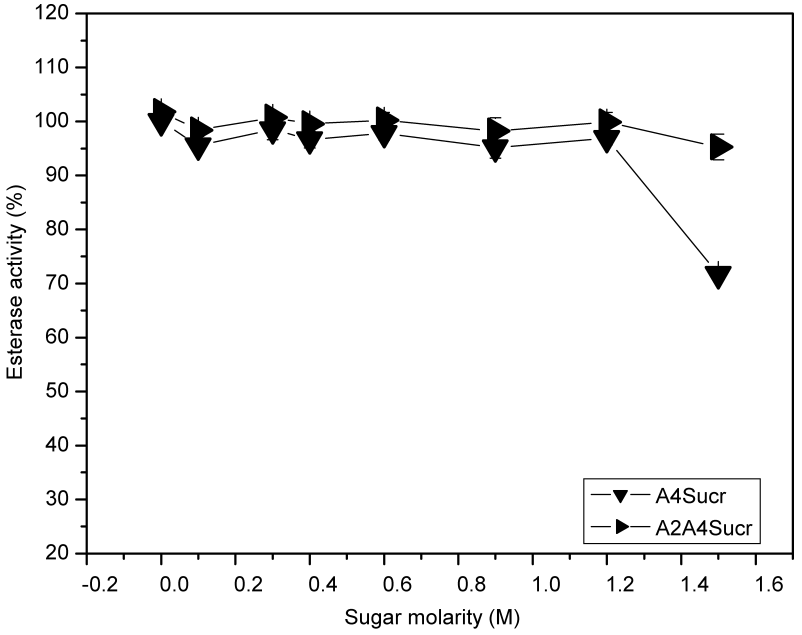


Figure 9.
Relative changes of esterase activity as affected by sucrose treatment at different molarities

Notes: Results are means of at least three independent experiments

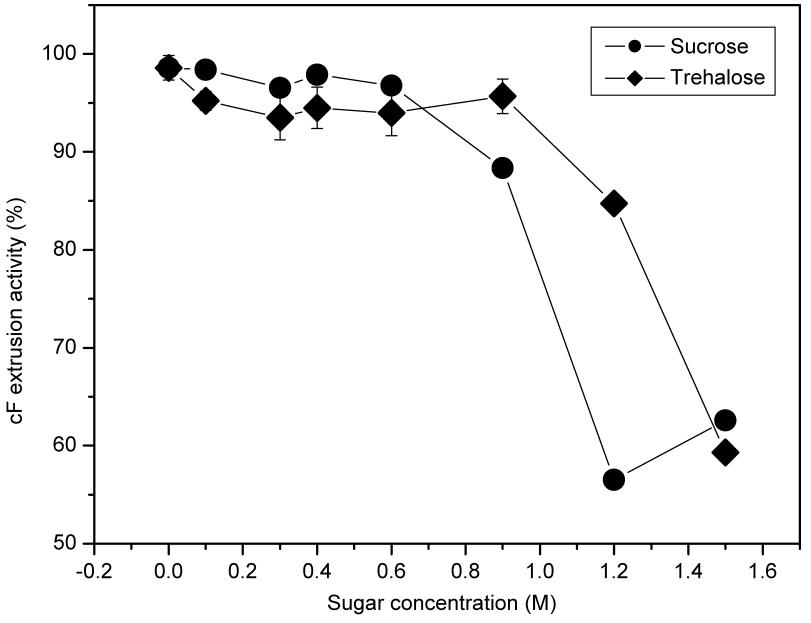


Figure 10.
Graphical representation of cF-extrusion activity as affected by osmotic treatments

Notes: These results were determined by the fluorescence properties of population in gate A4. Results were means of at least three independent experiments

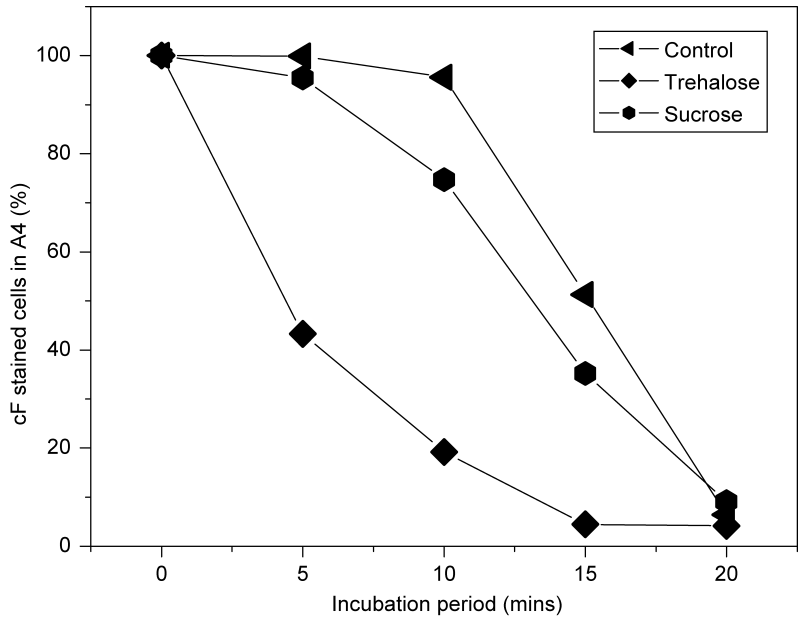


Figure 11.
Graphical representation of the relative number of cF-stained cells at increasing incubation period as derived from the density plots in Figure 5

percentage of the population in gate A2 and A4. Cells solely labelled by cF were found in gate A4. Insignificant percentages of cells were found in gate A2 of 0.1-1.2 M treated cells whereas about 25 per cent was encountered in A2 of 1.5 M.

The presence of such double-stained population at 1.5 M indicated that the cell membranes were irreversibly damaged to a low extent, under which penetration of PI into the cells was allowed but intracellular accumulated cF could still be retained. There was no esterase inactivation despite the perturbation of the cell membranes.

The calculation of the residual esterase activity based on the total percentage of population in gate A2 and A4 showed that despite the membrane damage, cells were still able to accumulate cF thus signifying the preservation of membrane integrity and enzymatic activity. Major part of the population did not accumulate PI but were mostly encountered in gate A4 irrespective of the presence of a double-stained population at this molarity (Figure 1). There was no significant loss in the viability recorded on plates; the organism must have made use of a repair mechanism on the membranes (Figure 7). This physiological status was considered as a transient phase in the progressive change towards cell death. However, death is not irreversible and double-stained cells may recover (Bunthof, 2002).

Treatment of cells with trehalose resulted in the extrusion of cF out of the cells as shown by the movement of cells from gate 4 to gate 3 (Figure 2). Though high fluorescence at FL3 pointed out that in these cells, membranes were not damaged, it was revealed by further investigations that there was a release of cF out of the cells (data not shown), which could have been made possible by membrane permeabilization. The percentage of cF-stained cells (Figure 8) showed same trend as the results obtained by plate counts (Figure 7).

The subjection of LGG to heat treatment at 60 °C for 300 s also revealed the absence of PI labelling accompanied by increasing loss of cF accumulating activity. In this case, the occurrence of esterase inactivation was reported (Ananta, 2005).

Extrusion of intracellular accumulated dye

This probe efflux could be put to use as an additional measure of cell vitality. cF labelling and, subsequently, the efflux could be measured to assess multiple aspects of cell viability, upon energizing. These combined methods could give more information about the physiological condition than cF labelling alone does (Bunthof *et al.*, 1999).

In FL1-FL3 density plot analysis, the extrusion ability of sugar-stressed cells upon glucose addition was determined as a result of the shift of initially stained population from gate 4 to gate 3 by intracellular fluorescence loss (Figures 3 and 4). Sucrose-treated cells up to 0.6 M had a similar extrusion activity as the control samples (Figure 10). Lower percentages were recorded at 1.2 M and 1.5 M but cells treated with trehalose up to 0.9 M had a comparable ability as the control. Culture counts correlated well with the extrusion activity only by trehalose treatment.

Determination of the rate of cF extrusion

A value of 0.6 M sucrose-treated cells exhibited no significant difference ($p > 0.05$) with the control sample in respect to the reproductive, capacity esterase activity, and cF extrusion performance. The kinetics of the movement of cF-stained cells from gate A4 to A3 showed that both the control cells and treated cells had similar extrusion rate (Figures 9 and 5). They completed extrusion in 20 min. This contradicts the report of Ananta *et al.* (2004) on extrusion rate in control cells. This could be variability within the strain due to loss or gain of plasmid leading to inconsistency in the metabolic traits or activity. However, 0.6 M trehalose-treated cells had a higher extrusion rate than both the control cells and sucrose-treated cells. Extrusion was completed in 15 min.

Conclusion

Our results clearly showed the additional value of using fluorescent stains to assess the effect of osmotic treatment on micro-organisms, for example, changes in metabolic activities and cellular events resulting from osmotic treatment which could not be assessed by culture techniques were made explicit.

Survival and viability of our study strain, as shown by plate counts method, revealed a similar protective effect of the stress agents used. The observation made on trehalose-treated cells, as shown by flow cytometry method, could either be explained as its inability to protect esterase activity or an extrusion of cF out of the cells. We confirmed that the movement of cells from gate 4 to gate 3 was as a result of a low degree of membrane permeabilization resulting in the leakage of cF out of the cells into the surrounding medium but PI could not have an access into the cells. This suggests the high level of membrane integrity thus, PI penetration was not permitted to intercalate with nucleic acids. Trehalose was able to give more stabilization to the membrane than sucrose as shown clearly by cF extrusion activity. The activities of the survivors in extruding intracellular accumulated dyes were perturbed beyond 0.6 M sucrose, however, no perturbation was observed in trehalose-treated cells. The higher extrusion rate exhibited by trehalse-treated cells pointed out its importance in enhancing the dye extrusion activity, which is regarded as an analogue of the capability of cells to extrude toxic compounds.

These findings underlined the differences in the protection provided to cells subjected to osmotic stress by compatible solutes of same osmotic strength. The determination of the viability of cells from different metabolic and physiological parameters as shown above is useful in enhancing the stability of starter cultures, probiotics, etc. in food processing and storage.

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Corresponding author

E.O. Sunny-Roberts can be contacted at: igwejis@yahoo.co.uk