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„Effect of stress on cellular properties of probiotic microorganisms“

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Contents

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Furthermore, I would like to express my gratitude to my family and best friends, without their help I would have not been able to finish my studies.

1. Introduction

Probiotics

Illustration 1 Enterococcus faecium

Probiotic products are formulations containing living microorganisms thought to be healthy for the host organism. According to the currently adopted definition by FAO/WHO, probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" [1]. Most probiotics are bacteria from the genera *Lactobacillus* or *Bifidobacterium*, although other genera, including *Escherichia* and *Enterococcus* have been also marketed as probiotics.

The requirements for a microorganism to be considered as probiotic are as follows: the microorganism must be alive when administered, must be documented to have a health benefit and must be administered at levels shown to the beneficial. At a minimum, probiotic products should be safe, effective, and should maintain their effectiveness. This aim could be reached if the producer and the consumer comply with the rules. The producer must identify strains and conditions of storage that will assure consumers that the probiotic will stay alive at efficacious levels through the end of the product's shelf life. The consumer must also store and use the product according to manufacturers' instructions [2].

The beneficial effects of probiotics include antagonistic effects and immune effects. The use of probiotic bacterial cultures stimulates the growth of preferred microorganisms, crowds out potentially harmful bacteria and reinforces the body's natural defense mechanism [3].

Several investigations have shown that the application of probiotic bacteria can:

- Help the reduction of the risk of certain diarrheal illnesses [4]
- Assist people with lactose intolerance [5]
- Enhance the immune function [6]

Enterococcus faecium

Originally classified in the 1930s as Group D *Streptococci, Enterococci* were officially given genus status in 1984 after hybridization studies that established a more distant relationship to *Streptococci*. *Enterococci* are gram-positive, spherical bacteria that colonize in groups or chains. They are naturally found as part of the digestive tract

flora in many organisms, including humans. They are robust microorganisms able to tolerate relatively high salt and acid concentrations. They also seem to be able to withstand low levels of detergents, explaining why inadequate cleaning procedures can promote Enterococcus infections [7].

Enterococcus faecium is contained in several probiotic products which are meant for the prevention and treatment of intestinal disorders. One of the well-known products is Bioflorin, which contains *Enterococcus faecium* SF-68 and helps to rebuild the intestinal flora after diarrheal illnesses [8].

Several studies reported on the application of *E. faecium* in veterinary medicine. Marcináková et al. recommended “Enteroferm Gel” containing *E. faecium* NCIB 10415 in cases of diarrhea caused by feed intolerance, after antibiotic treatment, stress conditions or a change of diet [9]. The same product was analyzed in another study [10], where dogs were treated with *E. faecium*. The treatment affected a decrease in injurious bacteria in dog’s gastrointestinal tract. Probiotic products containing *Enterococcus faecium* are also used for other animals, for example rabbits [11] or piglets [12].

Stabilization of probiotic microorganisms

In the last decade, the interest in probiotic products has been growing due to the increasing evidence of health benefits associated with their use. The essential step for the development of such products is the stabilization of probiotic microorganisms.

Cell stability is still the major challenge associated with the production of probiotic formulations, particularly because of the unavoidable induction of different kinds of stress like heat or mechanical forces on the cells during the process. Optimized manufacturing procedures deliver good product quality. For decades, the preservation of micro-organisms by desiccation has been the preferred method for long term storage of cultures for decades. In addition to culture collections, food and pharmaceutical industries have utilized drying technologies as preferred methods for preserving a multitude of different food and drug preparations in bulk quantities [13].

The classical methods for cell stabilization are lyophilization and spray drying. Lyophilization is the most efficient, but at the same time due to its batch-wise principle, the most expensive method. It has been used to preserve micro-organisms for decades and is the preferred method for the preparation, transporting and storage of culture collections worldwide, including the American Type Culture Collection (ATCC) and the National Collection of Type Cultures (NCTC). The main drawbacks of this method are the high expense, the length of product time and the low temperature forces.

It is generally recommended to freeze dry concentrated cultures of $>10^7$ cells to ensure that there are sufficient cells remaining after the freeze drying process, long term storage and reconstitution for successful propagation of the strain [14].

Spray drying is a predominant process in the dairy industry. It can be used to produce large amounts of dairy ingredients at a relatively low cost, compared to freeze drying. “Spray drying produces dry granulated powders from a slurry solution, by atomizing the suspension at high velocity within a chamber. The air within the chamber is heated to temperatures of up to 200 °C, which then blasts the slurry from a nozzle into granules “[15]. This process is generally used for drying of products such as polymers, surfactants, inorganic salts, pigments and also some food products such as milk, whey and corn [16]. It is also a common method for drying the probiotic microorganisms.

Cell injury and loss of viability have been reported as common side effects after the freeze drying or spray drying of probiotic microorganisms [17, 18]. Moreover, in most situations the dried cell mass does not show the appropriate physical properties for processing into pharmaceutical or nutraceutical formulations. Therefore, a second processing step, which additionally affects the cell viability, is required. In regards to these disadvantages, the main aim of this study was the investigation of suitability of fluid bed technology as an alternative method for drying the probiotic microorganisms.

Conventional fluid bed technology is an approved method for gentle drying, granulation and coating purposes [18].

With this type of processing, feed material is placed into a process container and held in the fluidized state by a controlled airstream. Following the direction of the fluidization air flow, the main parts of a fluid bed equipment can be defined as: air inlet container, air distributing base plate, process container, spraying system and exhaust container [Illustration 2].

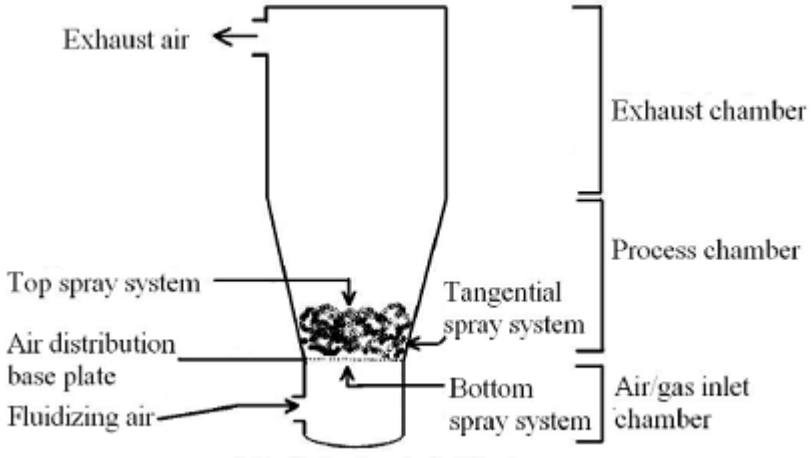
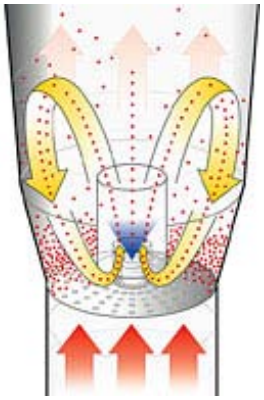


Illustration 2 Main parts of the fluidized bed equipment with different processing methods [18]

In fluid bed technology, processing methods are mainly characterized by the spraying direction and the design principle of fluidized air distribution into the processing container. Different processing methods are: top spray, bottom spray, wurster and rotor processing.

In the case of the top spray method, the spray nozzle is installed in the upper part of the process container, above the fluidized bed. The liquid is sprayed onto the fluidized particles. This method is more suitable for granulation. However, the uncontrolled fluidized bed does not guarantee optimal distribution of coating liquid on the bed. Therefore, the conventional top spray method is not suitable for preparing dosage forms with modified release profiles.

In the case of the bottom spray method, the nozzle is installed at the bottom of the processing container and sprays upwards into the fluidized bed.



The wurster method, which was introduced in 1959 by Wale Wurster, is based on the bottom spray method. The construction contains a small inner column, a “wurster”, which is mounted in the centre of the distributing base plate and surrounds an up-ward facing nozzle. The perforations in the air distribution base plate are larger in the centre, which enables stronger air stream through the inner column, weaker airstream towards the periphery, and larger again along the outermost circle. This construction allows controlled and homogeneous circulation of feed material in the container [illustration 3].

Illustration 3 Wurster tube

In the case of the rotor method, the particles are forced into a rotating movement by either the design of air distribution base plate and the resulting direction of airflow or the rotation of disc plate. This construction is responsible for the circular movement of the feed material. The coating liquid is sprayed tangentially through the bed. Conventional rotor processing is the approved method for pelletization and drug layering.

Generally, the bottom spray processing including the wurster system and the rotor method are the technologies of choice for coating. The wurster process is also utilized for granulation processes due to the defined movement of particles in the process chamber and the more homogeneous distribution of the spraying liquid compared to the top spray method [19].

2. Aims of the survey

The aim of the survey was to investigate the feasibility of fluid bed technology as an alternative method for drying *Enterococcus faecium* M-74. The effect of stress involved with different process parameters was studied on culturability and cellular properties of *E. faecium* M-74.

Furthermore, the cells were pre-treated with following excipients: sucrose, skim milk, glucose and dextrin in different concentrations based on the wet cell mass. The protective effect of these excipients on cellular properties and culturability during fluidized bed drying was investigated.

In order to broaden the knowledge about the impact of different sources of stress on *E. faecium* M-74, the second part of this thesis examined the effect of cryo-stress on cellular properties and culturability.

The culturability was studied using the plate count method.

The cellular properties were investigated by means of fluorimetric and flow cytometric measurements after the staining of the cells with fluorescent dyes.

3. Materials and methods

List of used equipment

Autoclave: Varioklav Type 400 H+P, Labortechnik GmbH (Germany)

Laminar-Air-Flow: Variolab Mobilien W90 Type SWB, Waldner Electronics (Germany)

Drying oven: Model 500, Memmert GmbH + Co KG (Germany)

Centrifuge: High Speed Centrifuge Type RC5C, Sorvall Instruments (Switzerland)

Vortex Heidolph REAX 2000, (Germany)

Thermomixer comfort (Eppendorf AG, Germany)

Microplate reader: Infinite®200, Tecan Österreich GmbH (Austria)

Flow Cytometer EPICS® XL-MCL™, Beckman Coulter (USA)

Incubator shaker innov 4000, New Brunswick Scientific Co. Inc. (USA)

Cultivation of *Enterococcus faecium* M-74

MRS-broth (Merck, Austria) with following compositions was used as growing medium:

Peptone from casein	10.0
Meat extract	8.0
Yeast extract	4.0
D(+)-Glucose	20.0
di-Potassium hydrogen phosphate	2.0
Tween®80	1.0
di-Ammonium hydrogen citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.2
Manganese sulfate	0.04

52.2 g of the dry medium were dissolved in 1000 ml distilled water. Test tubes were filled with 10 ml of prepared medium. The test tubes were closed with cap-o-test caps, autoclaved at 121 °C for 15 minutes and stored at 4 °C until use.

Enterococcus faecium M-74 was grown in the fluid culture medium MRS-broth at 37 °C.

At the beginning of the stationary phase (24 h, according to pre-studies), the cell suspension was inoculated to appropriate volumes of MRS-broth under laminar air flow.

Harvesting and washing of *Enterococcus faecium* M-74

In order to investigate the stress induced by fluid bed drying, *E. faecium* M-74 was cultivated in batches of 2 l MRS-broth.

At the beginning of stationary phase the suspension was centrifuged at 8000 rpm for 10 minutes at 4 °C. Afterwards the cells were washed twice with phosphate buffer (pH 6.8).

Phosphate buffer (pH 6.8):

di-Sodium hydrogen phosphate · $2H_2O$	68.52 g
Sodium dihydrogen phosphate · 2 H_2O	18.25 g

Na_2HPO_4 · $2H_2O$ (Merck, Austria) and NaH_2PO_4 · $2H_2O$ (Merck, Austria) were dissolved in distilled water to 5000 g. pH was adjusted to 6.8 with a solution of citric acid in distilled water.

9.0 ml of buffer were filled into test tubes. The test tubes were autoclaved at 121 °C for 15 minutes and stored at 4 °C until use.

For flow cytometric measurement only particle-free phosphate buffer was used. For this purpose 500 ml of phosphate buffer were filtered through a membrane filter with an pore diameter of 0.22 µm under laminar airflow.

Determination of colony forming units

Colony forming units were counted on Kanamycin esculin azide agar (Merck, Austria) plates.

Nutrient agar:

Kanamycin esculin azide agar (g/l):

Peptone from casein	20.0

Yeast extract	5.0
Sodium chloride	5.0
Sodium citrate	1.0
Sodium azide	0.15
Kanamycin sulfate	0.02
Esculin	1.0
Ammonium iron(III) citrate	0.5
Agar-agar	5.0

47.5 g of the dry medium were suspended in 1000 ml distilled water, autoclaved at 15 minutes at 121 °C and plates were poured. They were stored at 4 °C until use.

3.1. Fluid bed process

For each fluid bed drying experiment 4.4 g of wet mass of harvested cells were resuspended in 100 ml of distilled water. Microcrystalline cellulose pellets (Pharmatrans sanaq, Switzerland) were used as carrier. In each run, the cell suspension was layered on 500 g pellets.

The process parameter adjustment, used as standard-setting, was as bellow:

Spraying rate = 7 g/min

Product bed temperature = 37 - 38 °C

Atomizing air pressure = 1.5 bar

After each experiment 2 g of layered pellets with dried cells were given in 9 ml phosphate buffer and vortexed. The supernatant was prepared for analytical experiments, including culturability and cellular properties measurements.

3.1.1. Protective impact of excipients on the viability of cells during fluid bed drying

The efficiency of following protective agents: glucose, sucrose, dextrin and skim milk for protection of *E. faecium* M-74 against the general stress involved with the fluid bed process have been investigated.

Concentrations of 10%, 50% and 100% of each protectant (based on the wet cell mass) were added to each suspension of cells in 100 ml destilled water. Each batch was fluid bed dried using the standard process parameter settings described above.

2 g of layered pellets with dried cells were given in 9 ml phosphate buffer and resuspended for analytical measurements.

3.1.2. Effect of process parameters on the viability of protected *E. faecium* M-74

After investigating the protective impact of mentioned excipients on *Enterococcus faecium* M-74 during fluid bed drying and selecting the best protectant, the effect of various process parameters on the culturability and cellular properties of protected cells were investigated.

Process parameters were varied as follows:

Product bed temperature: 40 °C, 50 °C, 60 °C

Atomizing air pressure: 1 bar, 1.5 bar, 2.5 bar, 3.5 bar

Process time: 7, 15, 30, 45 minutes

Suspensions of 4.4 g wet cell mass in 100 ml destilled water, protected with a concentration of 100% (based on the wet cell mass) sucrose were sprayed on 500 g microcrystalline cellulose pellets (Pharmatrans sanaq, Switzerland). After each process 2 g of loaded pellets were given in 9ml phosphate buffer and prepared for analytical experiments.

3.1.3. Quantitative DNA-Isolation

After each run a quantitative DNA-isolation was made to normalize the results on 500 µg DNA/ml. For measuring the DNA-content the InstaGene Matrix kit by BIO-RAD was used.

To prepare the DNA from bacteria 10 ml of the pellets' supernatant was centrifuged for 10 minutes at 8.000 rpm. Afterwards the supernatant was removed and the sedimentation was resuspended in 1ml of autoclaved water in a microfuge tube. It was centrifuged for 1 minute at 12.000 rpm and the supernatant was removed.

A volume of 200 µl of InstaGene matrix was added to the pellet and it was incubated at 56 °C for 30 minutes.

The tube was vortexed at high speed for 10 seconds and placed in a 100 °C heat block for 8 minutes. The tube was vortexed again for 10 seconds and centrifuged for 3 minutes at 12.000 rpm.

10 µl of the resulting supernatant was suspended in 1ml water and the absorbance was measured in 260 nm. For a negative control water was used.

The DNA concentration of samples was calculated based on the fact that 50 µg DNA/ml water has an absorbance of 1.0 in 260 nm.

3.1.4. Investigation of culturability

After each fluid bed drying process, a dilution sery was made with the supernatant by the preparation of 1/10 dilutions. A volume of 100 µl of appropriate dilution step was spreaded onto agar plates (n=3).

After incubation at 37 °C for 72 h, the colony forming units were determined.

3.1.5. Investigation of cellular properties

From the first dilution of the supernatant 500 µl containing approximately $3*10^{10}$ colony forming units were taken for fluorescence measurements and incubated with fluorescent dyes in the dark.

Propidium iodide and dihydrorhodamine 123 were used for the investigation of the level of cell membrane damage and alteration in the hydrogen peroxide of cells after fluid bed drying, respectively.

Propidium iodide (PI)

PI is a fluorescent molecule that can be used to stain DNA. It is the most commonly used dye to quantitatively assess DNA content. Propidium iodide binds to DNA by intercalating between the bases without sequence preference.

PI is membrane impermeant and generally excluded from viable cells. PI is commonly used for identifying damaged or dead cells in a population.

The absorption maximum for PI is 535 nm and the fluorescence emission maximum is 617 nm when bound to nucleic acids.

For the stock solution from the solid form, PI was dissolved in deionized water at a concentration of 1 mg/ml (1.5 mM), stored at 4 °C and protected from light. The working solution was made of 1.2 µl stock solution per 1 ml phosphate buffer freshly before use [20].

Dihydrorhodamine 123 (DHR 123)

DHR 123 is the nonfluorescent reduced form of rhodamine 123, a mitochondrion-selective probe with a strong green fluorescence.

DHR 123 enters most of the cells and is oxidized by oxidative species or by cellular redox systems to rhodamine 123.

It is soluble in DMSO and has to be stored at -20 °C and protected from light and air.

The absorption maximum for DHR 123 is 500 nm and the fluorescence emission maximum is 536 nm.

The stock solution had a concentration of 5 mM in DMSO and the working solution was made of 5 µl per 1 ml phosphate buffer freshly before use [21].

After adding the working solutions to the samples, they were mixed and incubated at 37 °C under exclusion of light. Dihydrorhodamine 123 was incubated for one hour, propidium iodide for 15 minutes.

96 well plates were used for fluorimetric measurements. 50 µl of each sample was pipetted into wells in quadruplicate. Intensity of fluorescence was measured with a microplate reader.

The photometric measurement principle is based on detecting the light emitted as a result of fluorescence. The excitation light passes through the sample containing the fluorescent substance.

3.2. Effect of cryo-stress on the viability of *Enterococcus faecium* M-74

In order to investigate the effect of cryo-stress on the culturability and cellular properties of *E. faecium* M-74, samples were prepared as follows. At the begin of the stationary phase cells were centrifuged at 8000 rpm for 10 minutes and washed twice with phosphate buffer. Afterwards they were resuspended in 10 ml phosphate buffer and the first dilution was made. A volume of 1.5 ml each were pipetted into cryo vials and frozen under different conditions: in fluid nitrogen, at -20 °C, -80 °C and controlled freezing at -80 °C with a rate of -1 °C/minute.

Controlled freezing was arranged by giving the samples in a freezing container filled with 200 ml of isopropanol and freezing at -80 °C.

After 24 hours all samples were thawed and 500 µl each containing approximately $1*10^{12}$ colony forming units pipetted into save lock tubes and prepared for analytical experiments.

3.2.1. Investigation of culturability

A dilution sery was made as described in chapter 3.1.5., and spreaded on agar plates, colony forming units were calculated after 72 h incubation at 37 °C.

3.2.2. Investigation of cellular properties

For the investigation of cellular properties 500 µl of the working solution of the fluorescent dyes were added to each of the samples. The lock tubes were covered with aluminium foil to make sure that light could not influence the stability of the fluorescent dyes.

In each case five different fluorescent dyes were used: Propidium iodide, fluorescein diacetate, dihydrorhodamine 123, 3,3'-dihexyloxacarbocyanine iodide and carboxy fluorescein diacetate succinimidyl ester.

Whereas PI and FDA were used for the investigation of alteration in membrane integrity and esterase activity, changes in hydrogen peroxide production, membrane potential and intracellular pH were measured using DHR 123, DioC6(3) and CFDASE, respectively.

The staining procedure of cells with PI and DHR 123 was as same as the procedure of staining the fluid bed dried cells (see 3.1.6.).

Fluorescein diacetate (FDA)

FDA is a non-fluorescent hydrophobic fluorescein derivate that can pass through the cell membrane whereupon intracellular esterases hydrolyze the diacetate group and the highly fluorescent product fluorescein is generated.

The fluorescein molecules accumulate in cells that have intact membranes so the green fluorescence can be used as a marker of cell viability. Cells without intact cell membrane cannot accumulate the fluorescent product and therefore do not exhibit green fluorescence.

The substance can be dissolved in DMSO or acetone. The absorption maximum for FDA is 494 nm and the fluorescence emission maximum is 521 nm.

The stock solution contained 5 mg FDA per ml. A working solution of 4 µl stock solution per 1 ml phosphate buffer was freshly prepared before use [22].

3,3'-Dihexyloxacarbocyanine iodide (DioC6(3))

DioC6(3) is a carbocyanine derivate with short alkyl tails. It accumulates on hyperpolarized membranes, shifts into the lipid bilayer and fluorescence decreases. It is widely used for cell membrane potential measurements.

DioC6(3) is soluble in DMSO and DMF. The absorption maximum is 484 nm and the fluorescence emission maximum is 501 nm.

The concentration for the stock solution was 1 mM in DMSO. A working solution in phosphate buffer with the concentration of 20 µM was freshly prepared before use [23].

Carboxy fluorescein diacetate succinimidyl ester (CFDASE)

The intracellular pH of bacteria is very important for various cellular processes. CFDASE is one of the most commonly used fluorescent probes for measurements of intracellular pH. It is highly pH dependent.

CFDASE is soluble in DMSO. The absorption maximum is 492 nm and the fluorescence emission maximum is 517 nm.

The concentration used for the stock solution was 4 mg/ml DMSO. A working solution in phosphate buffer with the concentration of 10 µM was freshly prepared before use [24].

After having added the working solution of a fluorescent dye, the samples were mixed using a vortex mixer and incubated at 37 °C under exclusion of light. All dyes except propidium iodide were incubated for one hour. For PI an incubation time of 15 minutes was sufficient.

The samples were analyzed using fluorimetric as well as flow cytometric measurement.

For flow cytometric measurements, 1 ml of particle free filtered phosphate buffer was pipetted into a provided tube and 50 µl of the sample were added. After mixing using a vortex mixer the assay was undertaken. The flow cytometer was adjusted to measure 3000 cells per sample (n=4).

Flow cytometry

Flow cytometry is a method for quantitative analysis of components or structural features of cells primarily by optical means. Although it makes measurements on one cell at a time, it can process thousands of cells in a few seconds. Since different cell types can be distinguished by quantitating structural features, flow cytometry can be used to count cells of different types in a mixture.

For flow cytometric measurements a single cell suspension is required. The single cells pass through a laser beam by continuous flowing of the suspension.

Each cell scatters some of the laser light, and also emits fluorescent light excited by the laser. The cytometer typically measures several parameters simultaneously for each cell:

- low angle forward scatter intensity, approximately proportional to cell diameter
- orthogonal (90 degree) scatter intensity, approximately proportional to the quantity of granular structures within the cell
- fluorescence intensities at several wavelengths

Fluorescence intensities are typically measured at several different wavelengths simultaneously for each cell. Fluorescent probes are used to report the quantities of specific components of the cells [25].

3.3. Data analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San-Diego, USA) and the Microsoft Excel integrated analysis tools. Data were analyzed using one-way ANOVA with post-hoc Tukey test cross-comparing all study groups. Values of $p<0.05$ were considered significant.

4. Results

4.1. Effect of stress induced during fluid bed drying on the viability of *Enterococcus faecium* M-74

The effect of fluid bed drying on the culturability and cellular properties of *E. faecium* M-74 was investigated. The cellular properties studied in this part included the cell membrane permeability and the hydrogen peroxide production following incubation of rehydrated cells with PI and DHR 123 and fluorimetric measurement of the fluorescence intensity. The measurement of esterase activity, membrane potential, and intracellular pH was failed because of the insignificant results.

Flow cytometric measurements were not undertaken, due to the self-fluorescence of MCC-Pellets used as carrier.

4.1.1. Effect of rehydration medium and time

In order to investigate the effect of stress during fluid bed drying, the dried cells should rehydrate in suitable medium. The effect of different rehydration media and rehydration time intervals on the cellular properties has been studied.

Phosphate buffer (pH 6.8), distilled water and 10% sucrose solution were used as rehydration media. Dried cells were rehydrated in each medium for 2, 15 or 30 minutes. Afterwards the cellular properties and culturability of cells were investigated.

Neither the variation of rehydration medium nor the time of rehydration influenced the cellular properties of *Enterococcus faecium* M-74. After this cognition phosphate buffer was chosen to be used through the whole work. 2 minutes were set for rehydration time.

4.1.2. Effect of fluid bed drying on the viability of unprotected cells

The fluid bed drying was processed using following parameter adjustments:

Spraying rate= 7 g/min

Product bed temperature= 37-38 °C

Atomizing air pressure= 1.5 bar

4.1.2.1. Culturability

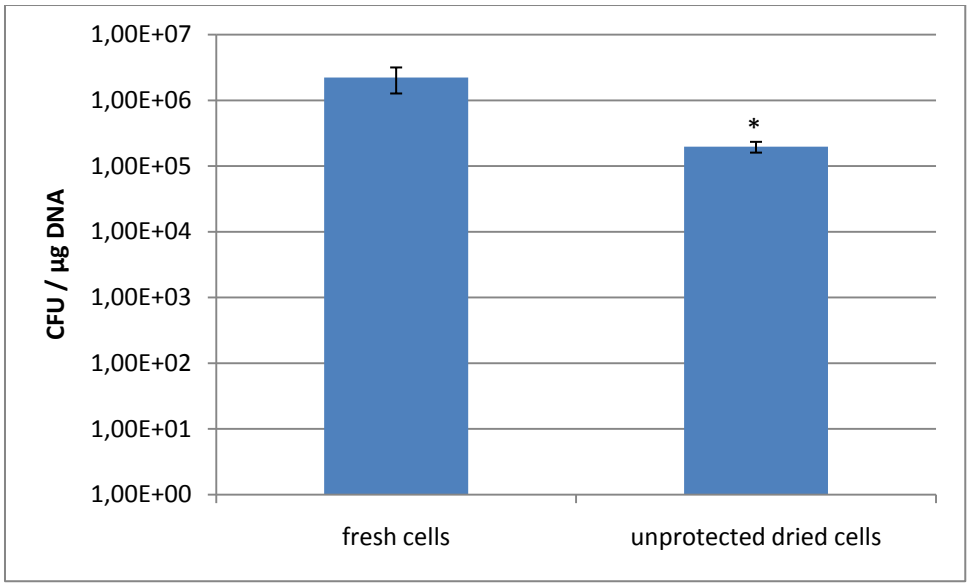


Figure 1 Colony forming units / µg DNA of unprotected *E. faecium* M-74 after fluid bed drying using standard process parameters compared to freshly harvested cells, * p<0.05 significance between the CFU / µg DNA of freshly harvested and unprotected dried cells

Figure 1 shows the impact of fluid bed drying on the culturability of unprotected *E. faecium* M-74. The process affected the cell culturability and a loss of 1.01 log colony forming units (CFU) was observed.

4.1.2.2. Cellular properties

- Cell membrane permeability

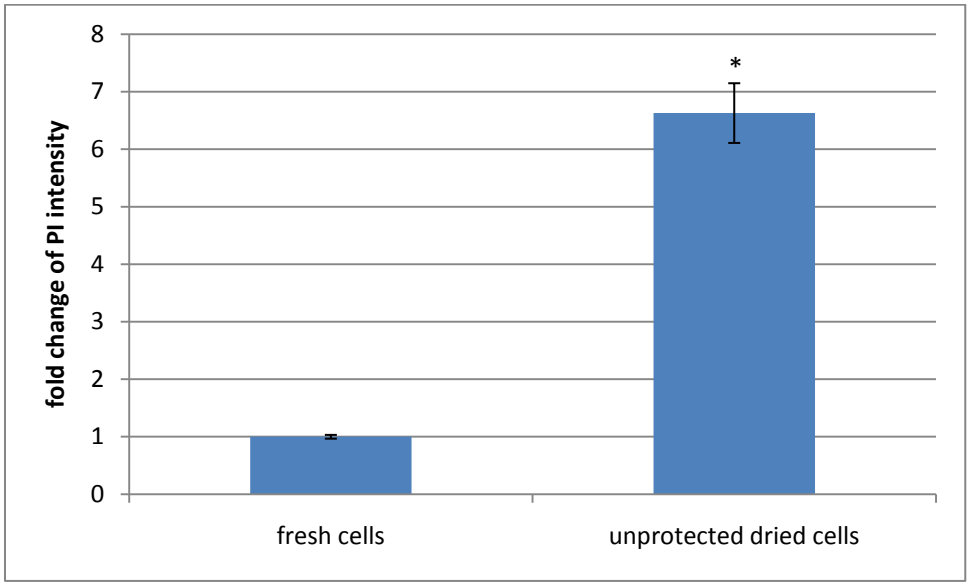


Figure 2 relative PI intensity of unprotected *E. faecium* M-74 after fluid bed drying using standard process parameters compared to freshly harvested cells, * p<0.05 significance between the relative PI intensity of freshly harvested and unprotected dried cells

Fluid bed drying had a remarkable impact on the membrane integrity of *E. faecium* M-74. As illustrated in Figure 2 the relative PI intensity of unprotected cells increased 6.63 times after fluid bed drying as compared to the intensity of freshly harvested cells, which indicates the increased cell membrane integrity.

- Hydrogen peroxide production

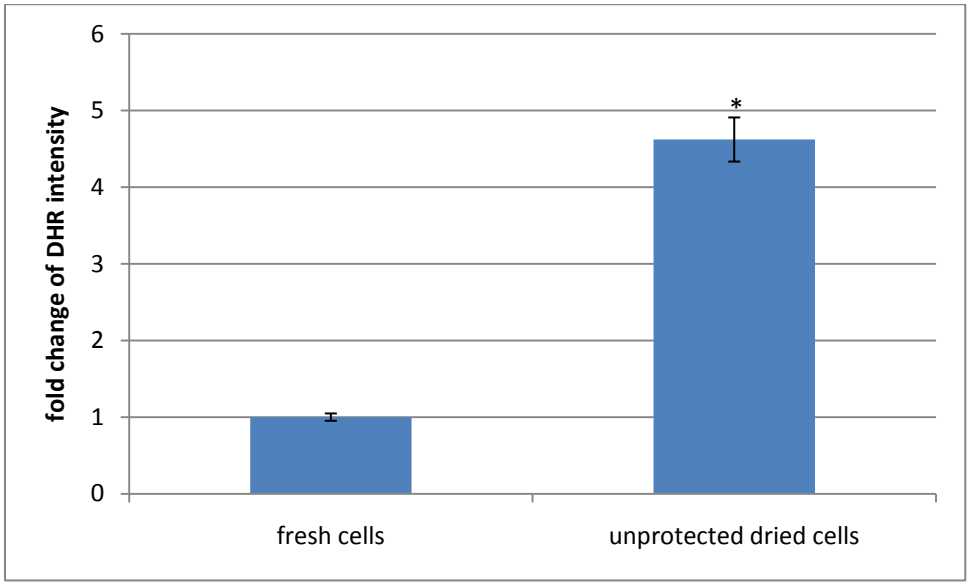


Figure 3 relative DHR 123 intensity of unprotected *E. faecium* M-74 measured after fluid bed drying using standard process parameters compared to freshly harvested cells, * $p<0.05$ significance between the relative PI intensity of freshly harvested and unprotected dried cell

As shown in figure 3, the relative DHR 123 intensity of unprotected dried cells was 4.62-fold higher than the intensity of freshly harvested cells, indicating the higher production of hydrogen peroxide after drying process due to the induced stress to the cells.

4.1.3. Effect of fluid bed drying on the viability of protected *E. faecium* M-74

4.1.3.1. Culturability

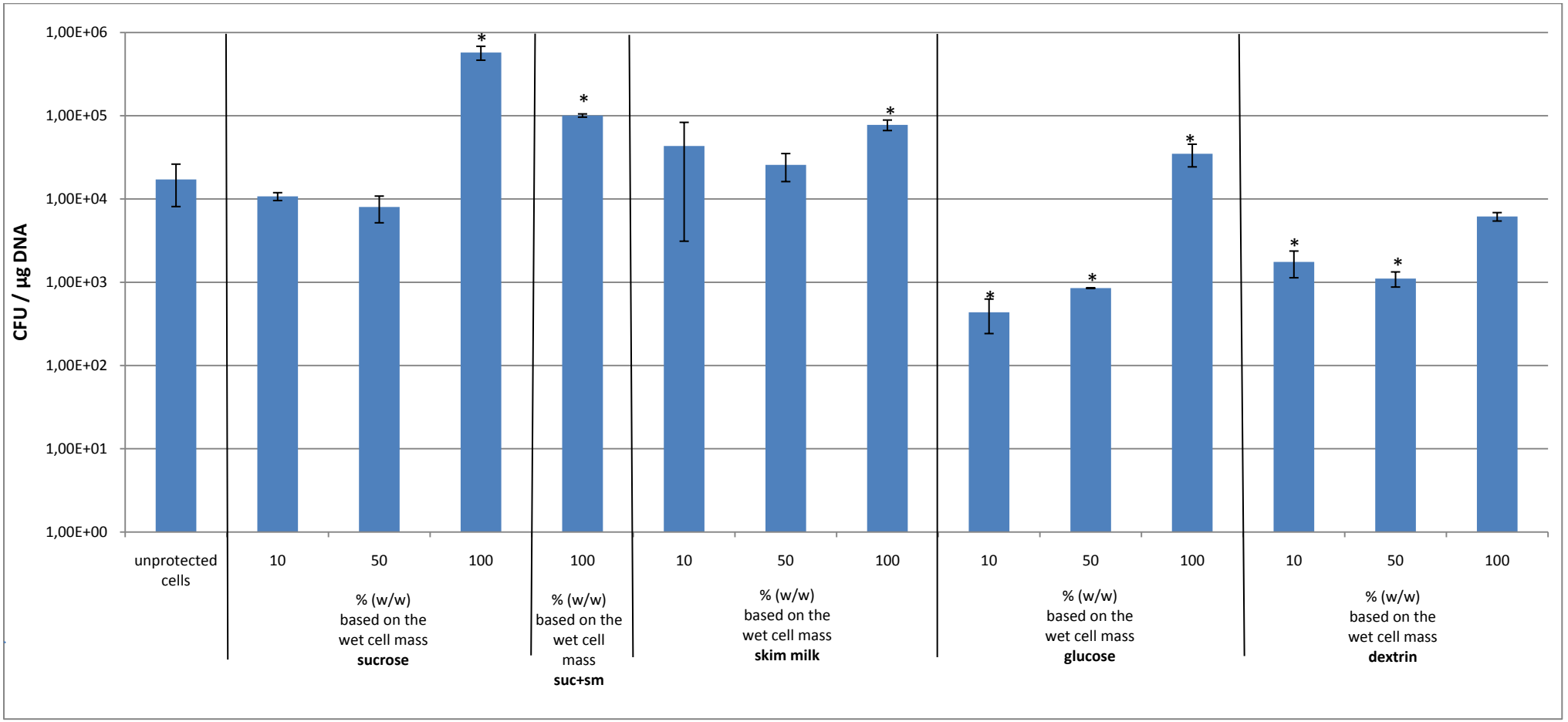


Figure 4 Colony forming units / µg DNA of unprotected and protected *E. faecium* M-74 with different protectants after fluid bed drying using standard process parameters, suc = sucrose, sm = skim milk * $p<0.05$ significance between the CFU / µg DNA of protected and unprotected cells.

Figure 4 shows the impact of different excipients on the cell culturability during fluid bed drying. To get detailed information, the effect of individual excipients on the cell culturability after fluid bed drying is shown in separate figures.

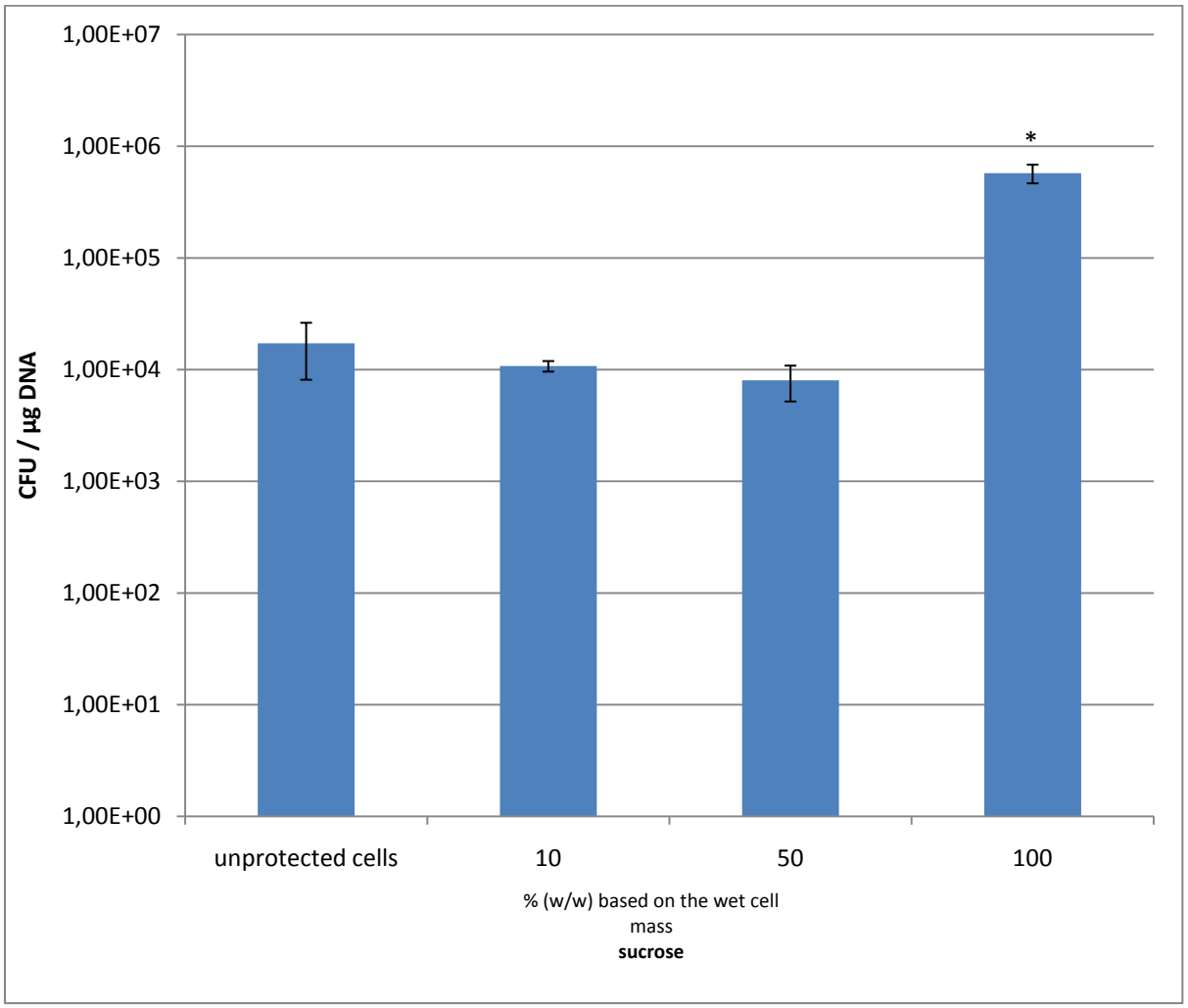


Figure 5 colony forming units / µg DNA of unprotected *E. faecium* M-74 and protected with different concentrations of sucrose after fluid bed drying using standard process parameters, * $p<0.05$ significance between the CFU / µg DNA of protected and unprotected cells

Figure 5 illustrates the effect of different concentrations of sucrose as protectants on the culturability of fluid bed dried *Enterococcus faecium* M-74. As can be seen using 100% sucrose provided the best protective effect ($p<0.05$).

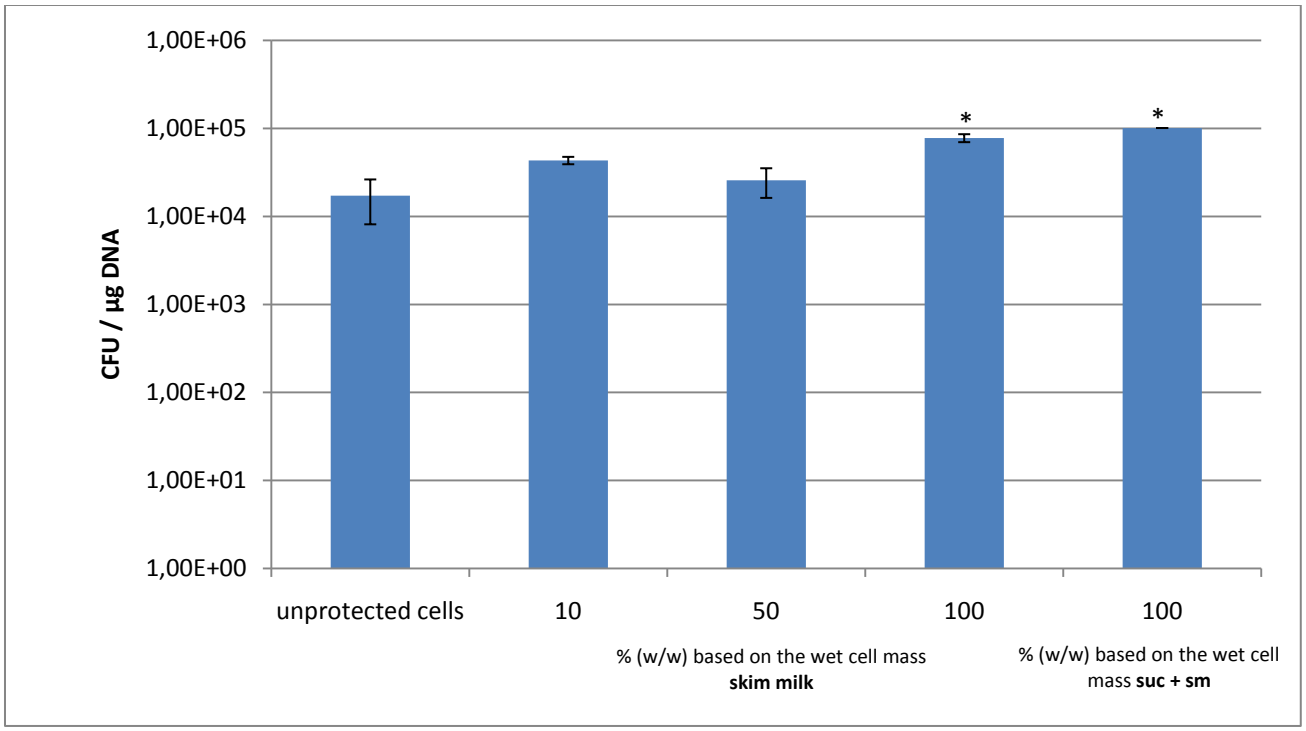


Figure 6 colony forming units / µg DNA of unprotected *E. faecium* M-74 and protected with different concentrations of skim milk or equal amount of sucrose and skim milk to gain 100% wet cell mass (su + sm) based on the wet cell mass after fluid bed drying using standard process parameters, suc = sucrose, sm = skim milk, * $p<0.05$ significance between the CFU / µg DNA of protected and unprotected cells

Figure 6 shows the protective effect of skim milk in different concentrations and a 1:1 mixture of sucrose and skim milk. A concentration of 100% skim milk based on the wet cell mass had the best protective effect, as using this concentration the culturability of *E. faecium* M-74 after drying was significantly higher ($p<0.05$) than the culturability of unprotected dried cells or in the case of pretreated cells with 10 or 50% skim milk. The equal amount of sucrose and skim milk to gain 100% of wet cell mass protected *E. faecium* M-74 as well. These two compositions had significantly the best protective impact on the cell culturability compared to other protectants and to other concentrations of skim milk.

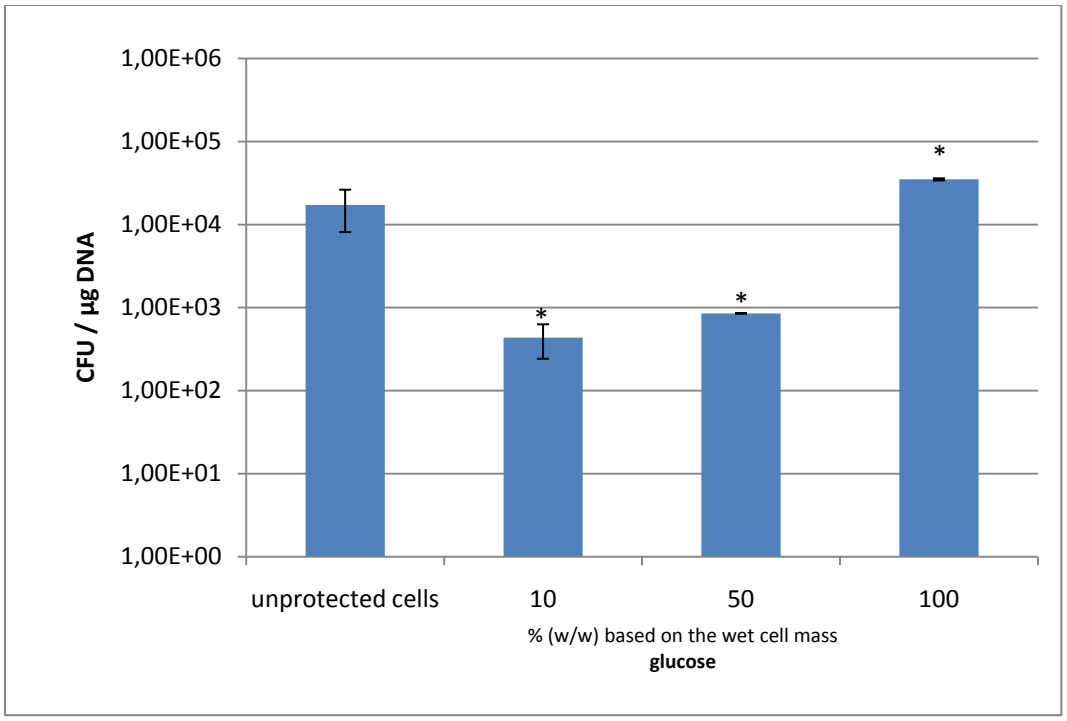


Figure 7 colony forming units / µg DNA of unprotected *E. faecium* M-74 and protected with different concentrations of glucose based on the wet cell mass after fluid bed drying using standard process parameters, * $p<0.05$ significance between the CFU / µg DNA of protected and unprotected cells

Pre-treatment of cells with a concentration of 100% glucose (based on the wet cell mass), the culturability of dried cells was higher than those of unprotected dried cells. However, using 10% or 50% glucose (based on the wet cell mass) did not protect the cells during fluid bed drying. As indicated in figure 7, the culturability of pretreated cells with 10% or 50% glucose was significantly lower than those of unprotected cells ($p<0.05$).

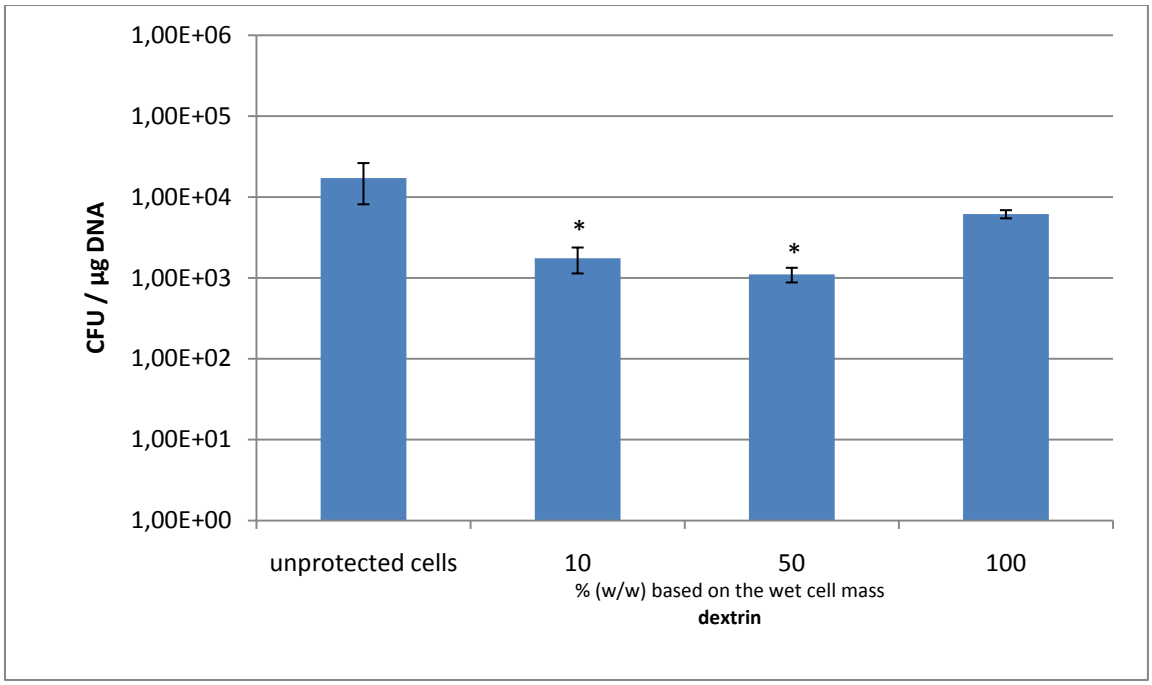


Figure 8 colony forming units / µg DNA of unprotected *E. faecium* M-74 and protected with different concentrations of dextrin based on the wet cell mass after fluid bed drying using standard process parameters, * $p<0.05$ significance between the CFU/ µg DNA of protected and unprotected cells

Dextrin was not convenient as a protectant regardless to its concentration as shown in figure 8. Using any concentration of dextrin the number of colony forming units of dried cells was even significantly lower than the number of unprotected dried cells ($p<0.05$).

Summing up this first part of experiments it became obvious that 100% sucrose protected cells best. Cells treated with 100% sucrose showed the highest amount of colony forming units [figure 5] followed by 100% skim milk based on the wet cell mass. Dextrin did not seem to be convenient protective agents.

4.1.3.2. Cellular properties

- Cell membrane permeability

The cell membrane damage and the change in hydrogen peroxide production of cells have been investigated after exposure of rehydrated cells to the fluorescent dyes propidium iodide and dihydrorhodamine 123, respectively, using fluorimetry.

Because of self-fluorescence of micro crystalline cellulose, flow cytometric measurements were not undertaken for fluid bed drying experiments.

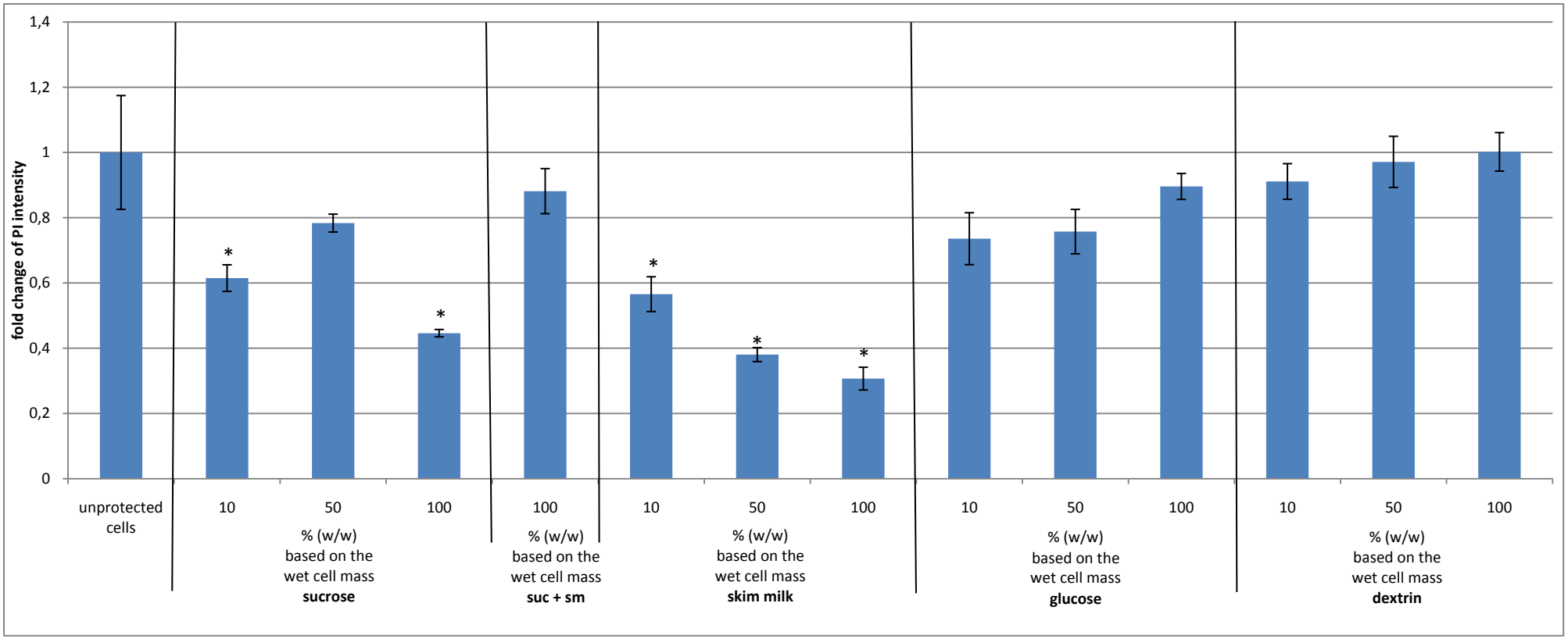


Figure 9 relative PI intensity of unprotected and protected *E. faecium* M-74 using different protectants after fluid bed drying using standard process parameters, suc = sucrose, sm = skim milk, * $p<0.05$ significance between the relative PI intensity of protected and unprotected cells

Figure 9 indicates the relative PI intensity of unprotected dried cells as well as those of dried cells pretreated with different protective agents in different concentration.

The impact of different excipients is shown in separate figures.

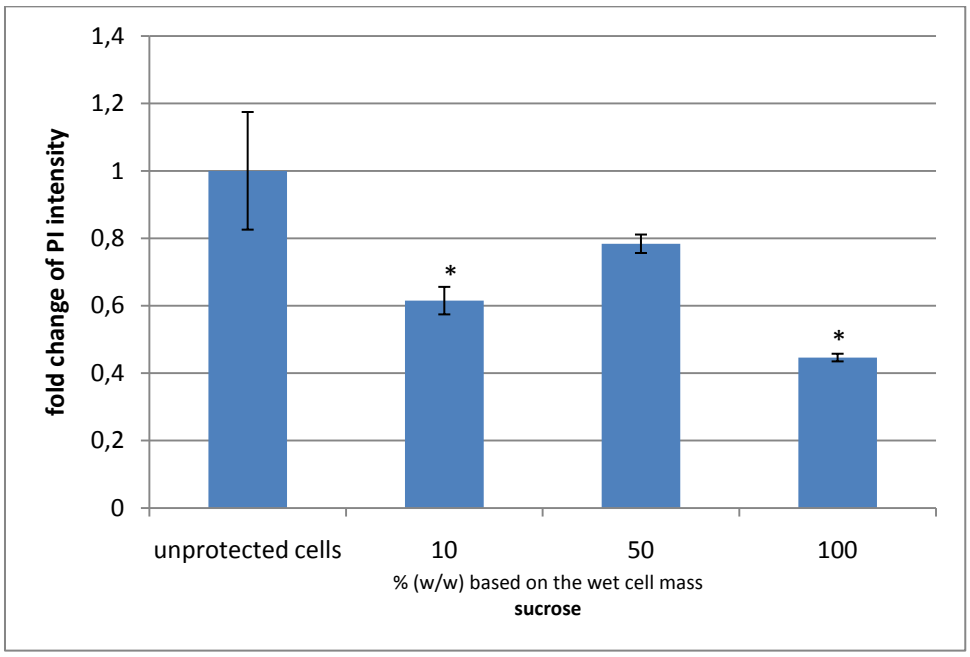


Figure 10 relative PI intensity of unprotected *E. faecium* M-74 and protected with different concentrations of sucrose based on the wet cell mass after fluid bed drying using standard process parameters, * $p<0.05$ significance between relative PI intensity of protected and unprotected cells

Figure 10 shows the relative PI intensity of dried cells protected with different amounts of sucrose after fluid bed drying.

A concentration of 100% sucrose based on the wet cell mass showed the best protective effect. The relative PI intensity of these cells were 0.4 fold lower than those of unprotected and dried cells ($p<0.05$). This relative PI intensity was also significantly lower than the PI intensity of dried cells, pretreated with 10% or 50% sucrose based on the wet cell mass.

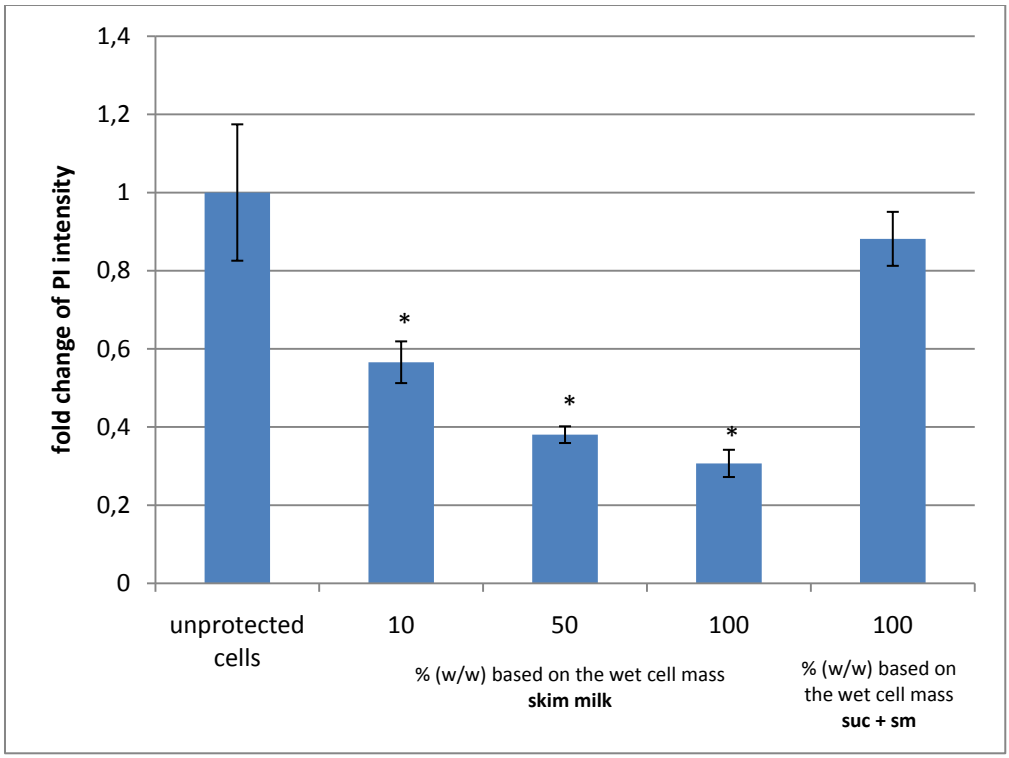


Figure 11 relative PI intensity of unprotected *E. faecium* M-74 and protected with equal amount of sucrose and skim milk to gain 100% wet cell mass or different concentrations of sucrose based on the wet cell mass after fluid bed drying using standard process parameters, suc = sucrose, sm = skim milk, * $p<0.05$ significance between relative PI intensity of protected and unprotected cells

From figure 11 it becomes obvious that skim milk was an adequate protectant. Equal amounts of skim milk and sucrose to gain 100% of the wet cell mass had no significant protective impact on the cell membrane. The relative PI intensity of cells pretreated with 50% or 100% skim milk based on the wet cell mass were comparable with each other ($p>0.05$) and they were significantly lower than those of unprotected dried cells or dried cells pretreated with equal amounts of skim milk and sucrose.

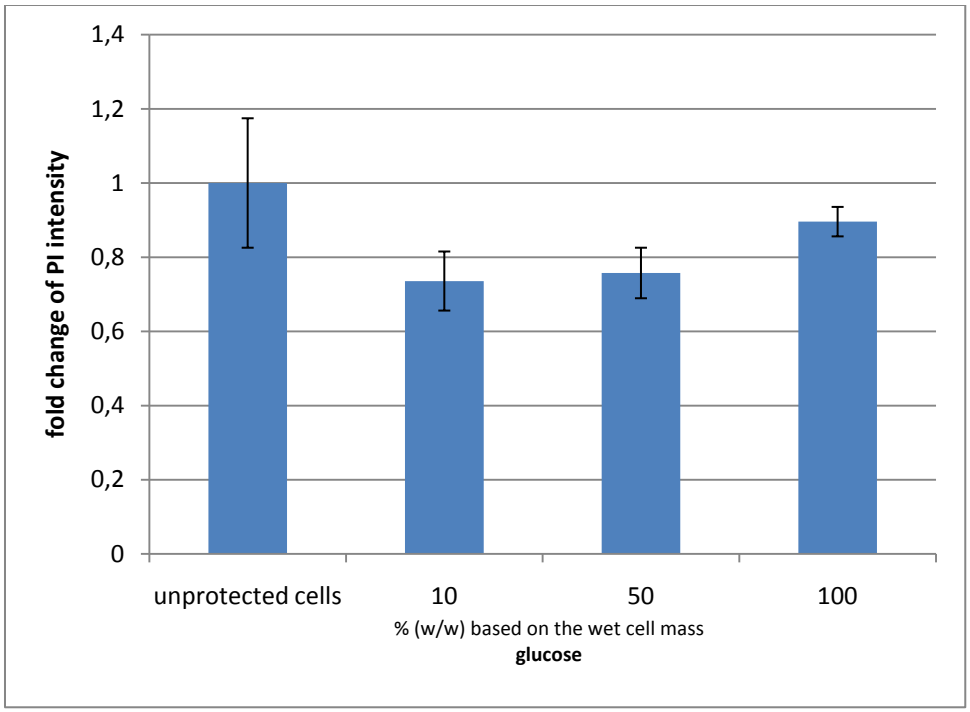


Figure 12 relative PI intensity of unprotected *E. faecium* M-74 and protected with different concentrations of glucose based on the wet cell mass after fluid bed drying using standard process parameters, * p<0.05 significance between relative PI intensity of protected and unprotected cells

Relative PI intensity of dried samples pretreated with glucose was not significantly lower than the PI intensity of unprotected dried cells, indicating the less membrane damage of pretreated cells [figure 12]. However, this protective impact was less effective than the protective impact of sucrose or skim milk.

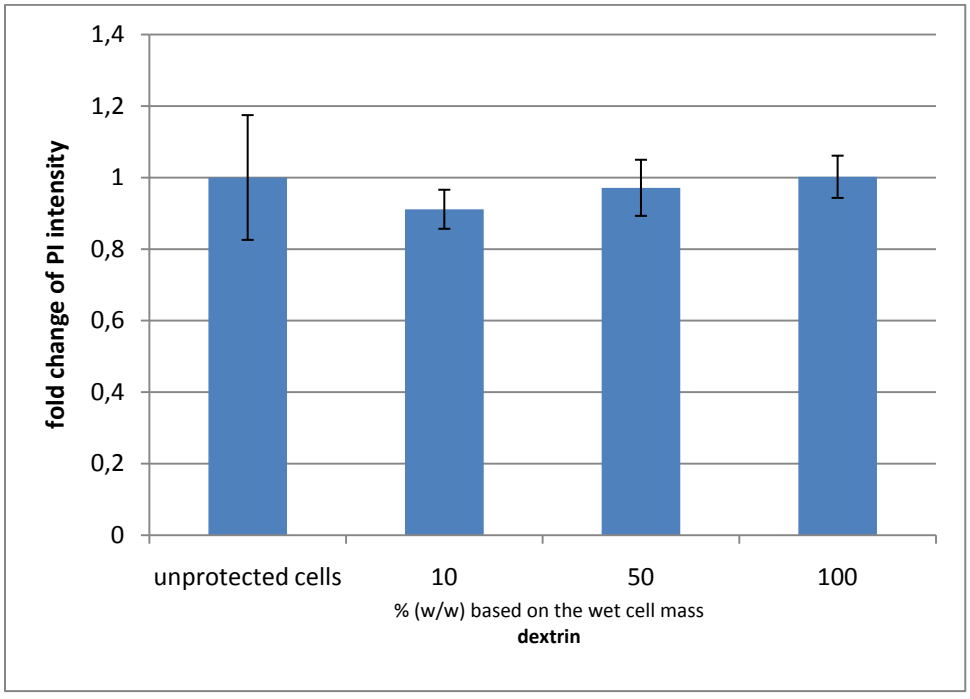


Figure 13 relative PI intensity of unprotected *E. faecium* M-74 and protected with different concentrations of dextrin based on the wet cell mass after fluid bed drying using standard process parameters, * p<0.05 significance between relative PI intensity of protected and unprotected cells

Dextrin had no protective impact on the cell membrane of *E. faecium* M-74 as shown in figure 13. The relative PI intensity of dried cells pretreated with dextrin was comparable with the PI intensity of unprotected dried cells ($p<0.05$).

Summarizing the results, we concluded that 100% sucrose or 100% skim milk based on the wet mass of cells protected the cell membrane against the stress during fluid bed drying better than other used protective agents and concentrations

- Hydrogen peroxide production

The alteration in hydrogen peroxide production of *Enterococcus faecium* M-74 after fluid bed processing was investigated after staining of cells with dihydrorhodamine 123.

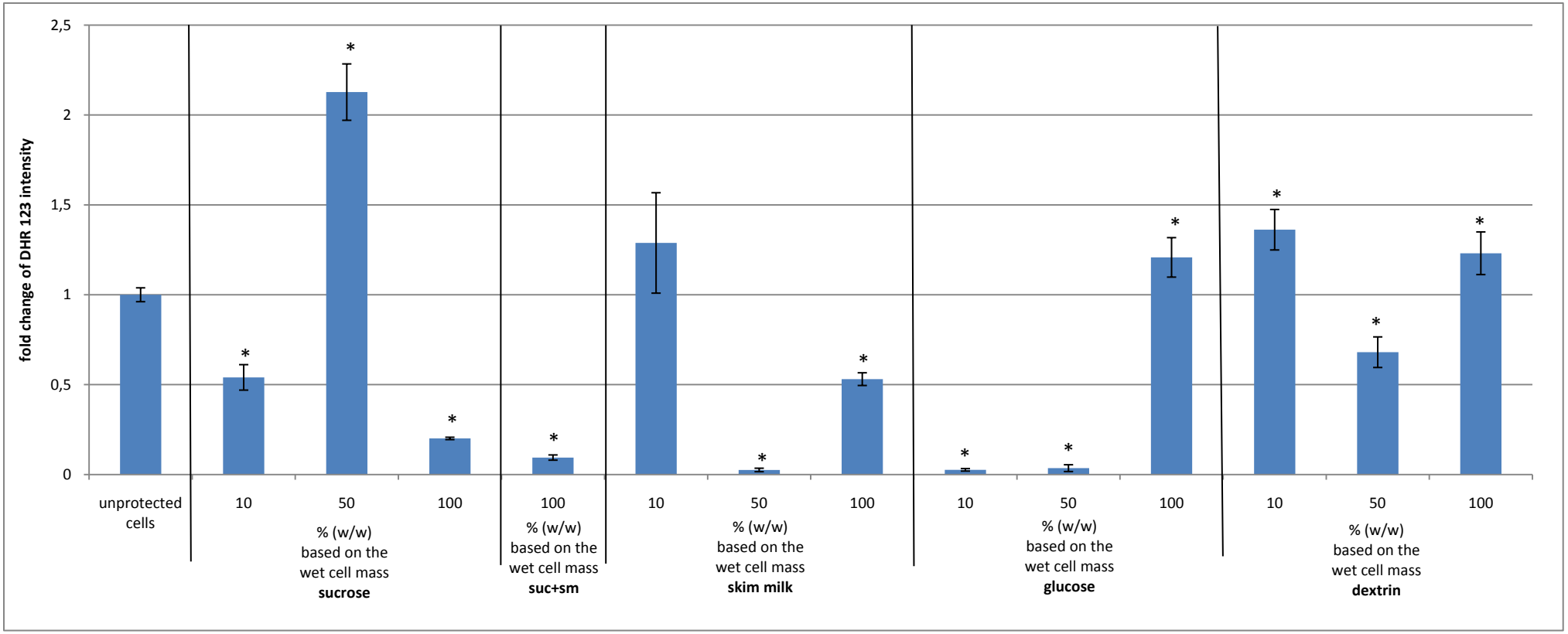


Figure 14 relative DHR 123 intensity of unprotected *E. faecium* M-74 and protected with different protectants after fluid bed drying using standard process parameters, suc = sucrose, sm = skim milk, * $p<0.05$ significance between relative DHR 123 intensity of protected and unprotected cells

Figure 14 indicates the alteration in the DHR 123 intensity of dried cells pretreated with different excipients. To get detailed information, the impact of different protective agents on the hydrogen peroxide production of cells after fluid bed drying is shown in separate figures.

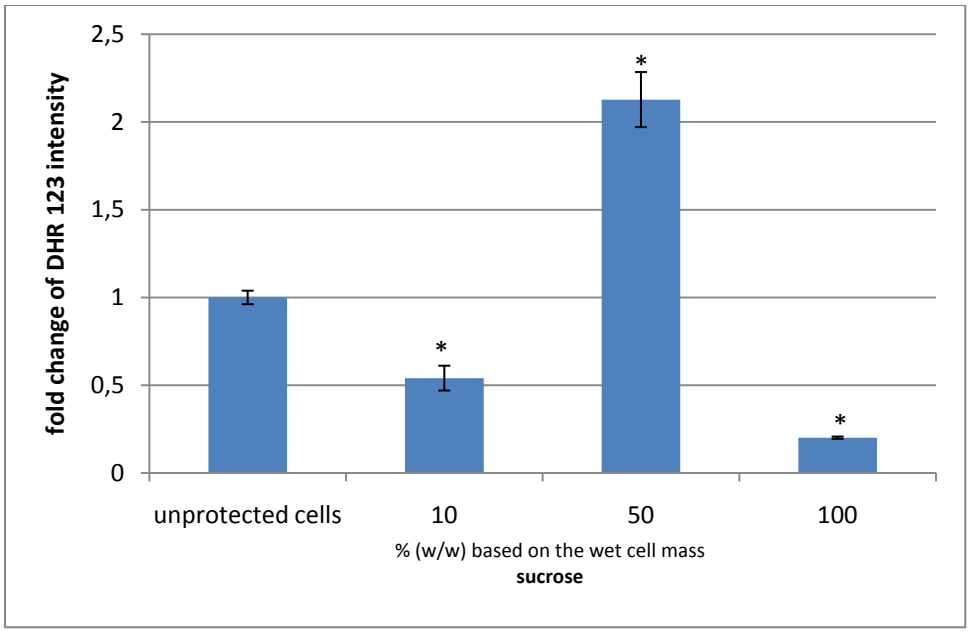


Figure 15 relative DHR 123 intensity of unprotected *E. faecium* M-74 and protected with different concentrations of sucrose based on the wet cell mass after fluid bed drying using standard process parameters, * $p<0.05$ significance between relative DHR 123 intensity of protected and unprotected cells

Figure 15 shows the relative DHR 123 intensity of dried cells pretreated with different concentrations of sucrose. Pretreatment of cells with 50% sucrose based on the wet cell mass resulted in 2.12-fold increased DHR 123 intensity of cells compared to the intensity of unprotected dried cells. The lowest DHR 123 intensity belonged to the cells pretreated with a concentration of 100% sucrose based on the wet cell mass.

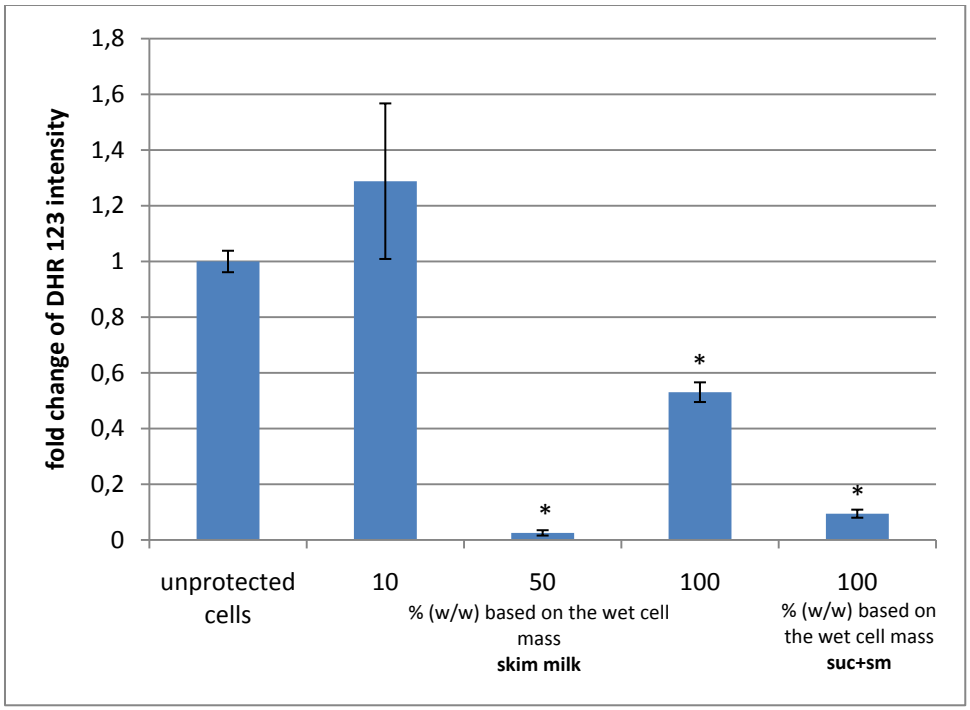


Figure 16 relative DHR 123 intensity of unprotected *E. faecium* M-74 and protected with equal amount of sucrose and skim milk to gain 100% wet cell mass or different concentrations of skim milk based on the wet cell mass after fluid bed drying using standard process parameters, suc = sucrose, sm = skim milk, * $p<0.05$ significance between relative DHR 123 intensity of protected and unprotected cells

Using skim milk or a mixture of 50% skim milk and 50% sucrose had a significant effect on hydrogen peroxide production [figure 16]. Pre-treatment of cells with 50% skim milk and the equal amount of sucrose and skim milk to gain 100% of wet cell mass resulted in the 0.05-fold and 0.1-fold decrease in relative dihydrorhodamine 123 intensity, respectively, as compared to the relative dihydrorhodamine 123 intensity of unprotected cells ($p<0.05$).

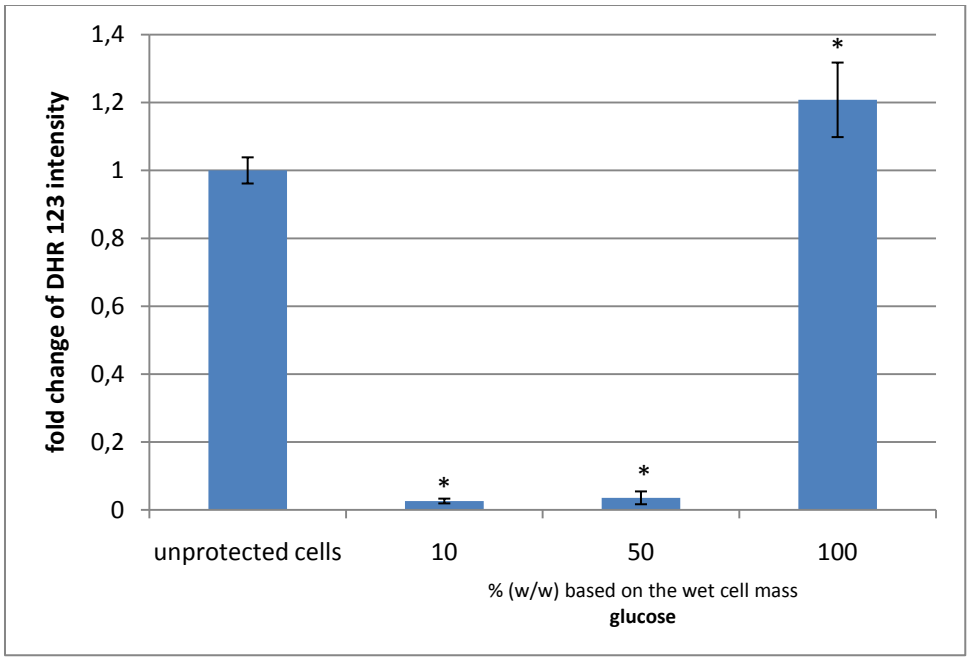


Figure 17 relative DHR 123 intensity of unprotected *E. faecium* M-74 and protected with different concentrations of glucose based on the wet cell mass after fluid bed drying using standard process parameters, * $p<0.05$ significance between relative DHR 123 intensity of protected and unprotected cells

The impact of glucose on the hydrogen peroxide production of *E. faecium* M-74 after drying is showed in figure 17. Fluid bed drying of cells pretreated with 10% or 50% glucose resulted in the reduction of fluorescence intensity as compared to the intensity of unprotected dried cells. In contrary using 100% glucose we have observed a significant increase of relative dihydrorhodamine 123 intensity of pretreated dried cells compared with the relative intensity of unprotected dried cells.

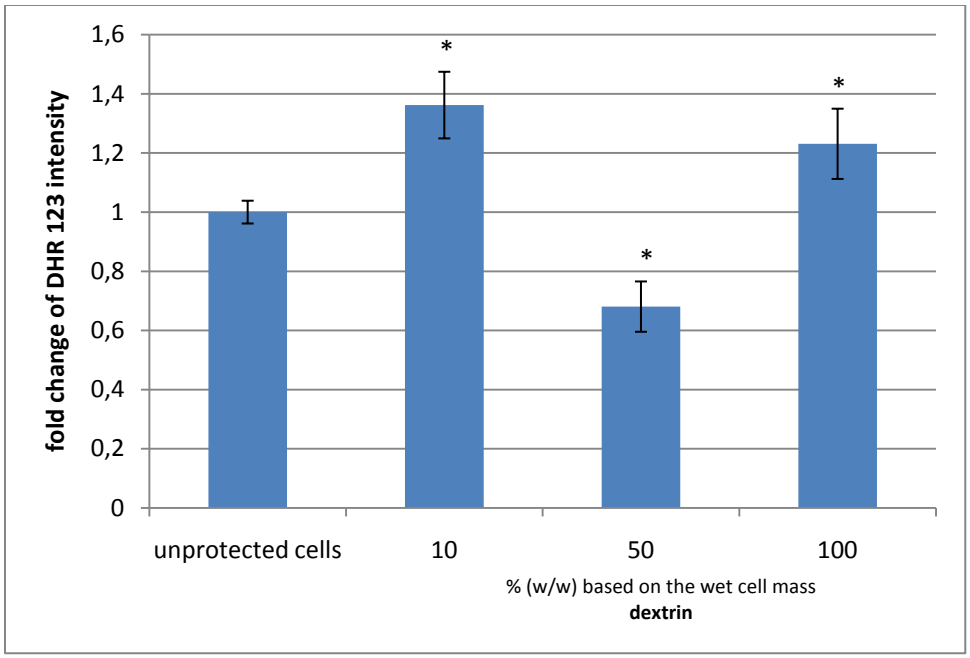


Figure 18 relative DHR 123 intensity of unprotected *E. faecium* M-74 and protected with different concentrations of dextrin based on the wet cell mass after fluid bed drying using standard process parameters, * $p<0.05$ significance between relative DHR 123 intensity of protected and unprotected cells

Using dextrin as protectant affected the hydrogen peroxide production of dried cells. As illustrated in figure 18, pretreatment of cells with 10% dextrin based on the wet cell mass resulted in 1.38-fold increase of the DHR 123 intensity of dried cells, compared to the intensity of unprotected dried cells, indicating the inadequate protective impact of 10% dextrin on cells. However, the concentration of 50% (based on the wet cell mass) protected the cells against the induced stress involved with the drying process, as the DHR 123 intensity of these cells was 0.7 fold of the intensity of unprotected cells. This indicates the reduced production of hydrogen peroxide in the protected and dried cells.

Comparing the DHR 123 intensity of protected cells with different protectants showed that the intensity of protected cells with 50% sucrose was higher than the intensity of unprotected cells. Pretreatment of cells with 100% sucrose, 50% skim milk, 10% or 50% glucose or the mixture of sucrose and skim milk resulted in lower DHR 123 intensity of dried cells as compared to the intensity of unprotected dried cells.

4.2. Effect of the drying process parameters on the viability of *Enterococcus faecium* M-74

Analysis of the effect of different protective agents with different concentrations during fluid bed drying on the culturability and cellular properties of Enterococcus *faecium* M-74 have shown that 100% sucrose provides the best protective effect. Using 100% sucrose as protectant the effect of following process parameters: process time, product bed temperature and atomizing air pressure on the cell viability were investigated.

The following parameter settings were used as standard setting:

Product bed temperature: 40 °C

Process time: 15 min

Atomizing air pressure: 1.5 bar

The impact of process parameters on the viability of cells was investigated by the variation of each parameter, keeping other parameters constant on the standard setting. After each process the fluorescence intensity of dried cells was related to the intensity of cells, dried at standard parameter settings.

4.2.1. Effect of the variation of product bed temperature on the viability of *Enterococcus faecium* M-74

Firstly, the product bed temperature was varied, keeping other parameters constant.

4.2.1.1. Culturability

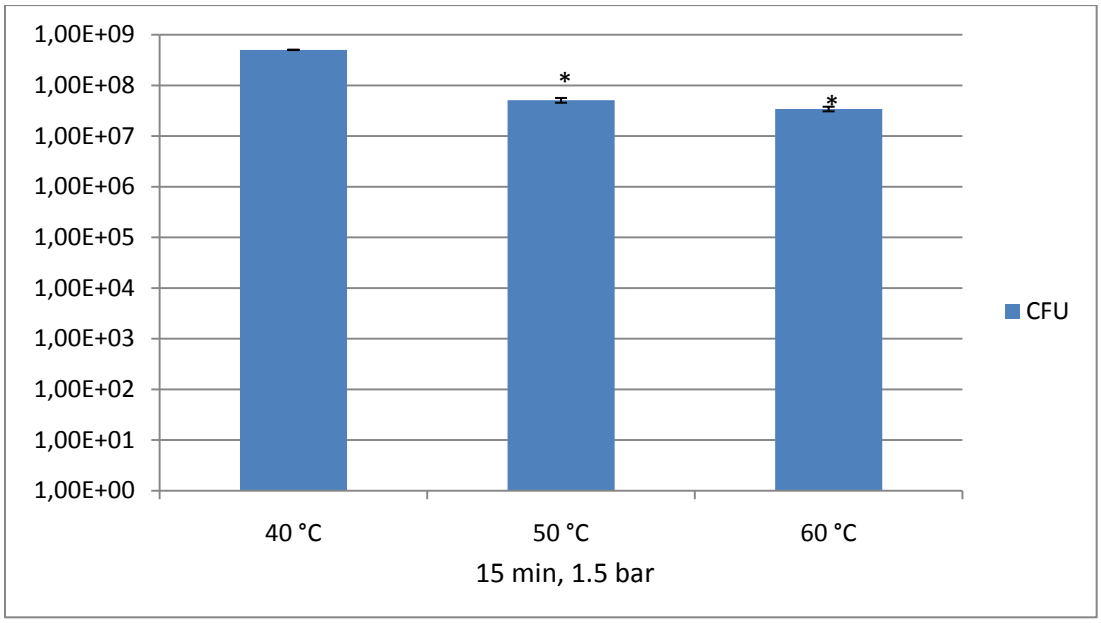


Figure 19 colony forming units / µg DNA of *E. faecium* M-74 after fluid bed drying using different product bed temperatures, * p<0.05 significance between CFU / µg DNA of cells dried with 40 °C and different product bed temperatures

Figure 19 indicates the impact of product bed temperature on the culturability of *E. faecium* M-74. Increasing the temperature from 40 °C to 60 °C resulted in significantly lower numbers of colony forming units ($p<0.05$).

4.2.1.2. Cellular properties

- Cell membrane permeability

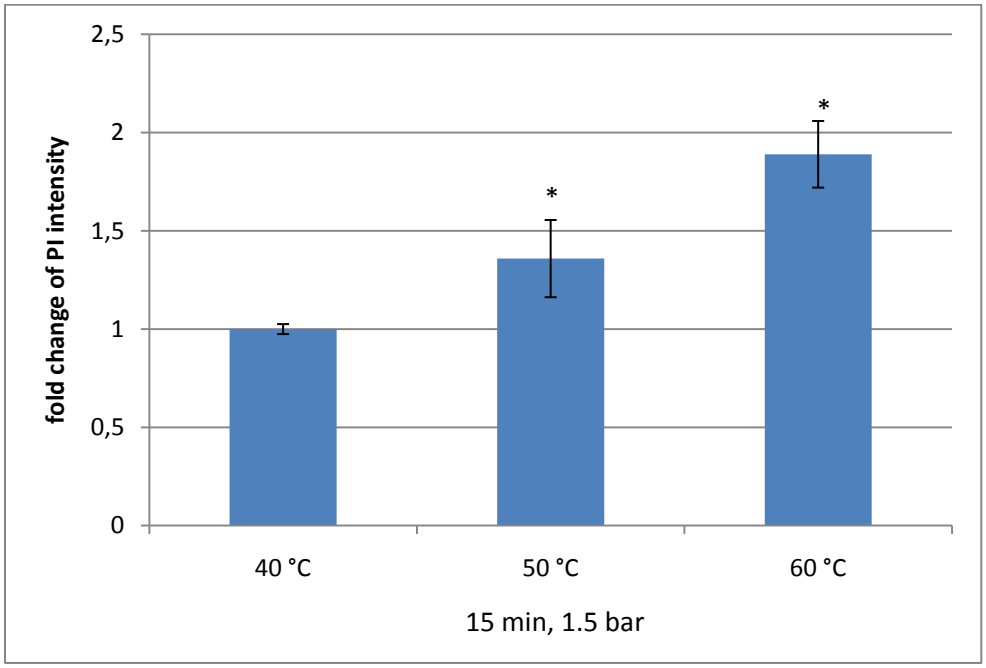


Figure 20 relative PI intensity of *E. faecium* M-74 after fluid bed drying using different product bed temperatures, * $p<0.05$ significance between relative PI intensity of cells dried with 40 °C and different product bed temperatures

As illustrated in figure 20, increasing the product bed temperature resulted in increased relative PI intensity of dried cells. Using product bed temperature of 60 °C, the PI intensity of dried cells increased to 1.89- fold of the intensity of cells dried at 40 °C, indicating the higher level of membrane damage at 60 °C.

- Hydrogen peroxide production

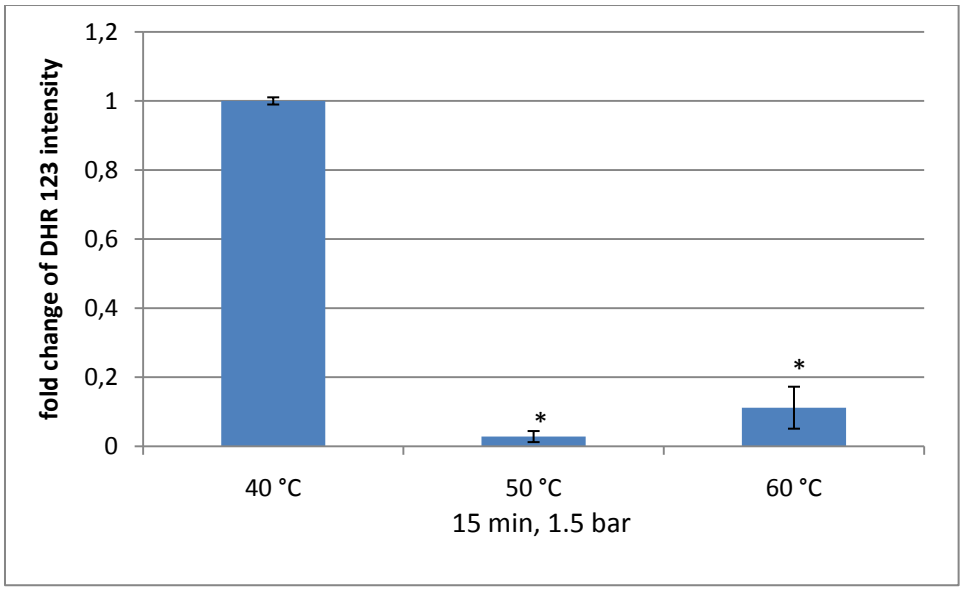


Figure 21 relative DHR 123 intensity of *E. faecium* M-74 after fluid bed drying using different product bed temperatures, * p<0.05 significance between relative DHR 123 intensity of cells dried with 40°C and different product bed temperatures

The impact of product bed temperature on the DHR 123 intensity of fluid bed dried cells is shown in figure 21. As can be observed, increasing the temperature resulted in the decrease of fluorescence intensity. This indicated the lower production of hydrogen peroxide which may be due to the collapse of the cellular redox system.

4.2.2. Effect of the variation of process time on the viability of *Enterococcus faecium* M-74

4.2.2.1. Culturability

During the next investigation process time was varied, while the other parameters were kept constant. Process time was varied between 7 and 45 minutes.

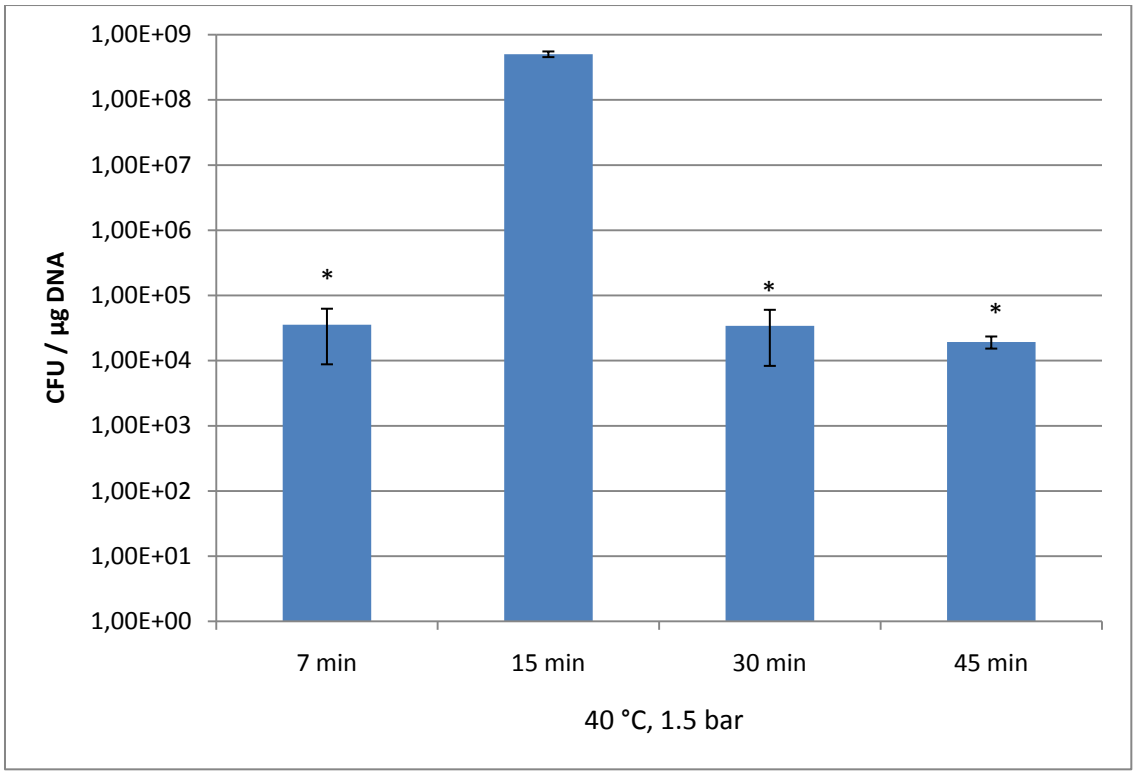


Figure 22 colony forming units / µg DNA of *E. faecium* M-74 after fluid bed drying using different process time settings, * p<0.05 significance between colony forming units / µg DNA of cells dried for 15 min and with different process time settings

Figure 22 illustrates the impact of different processing periods of times on the number of colony forming units of *E. faecium* M-74. It seems that a process time of 15 minutes results the highest culturability of cells. Between the other results there was no significant difference (p>0.05).

4.2.2.2. Cellular properties

- Cell membrane permeability

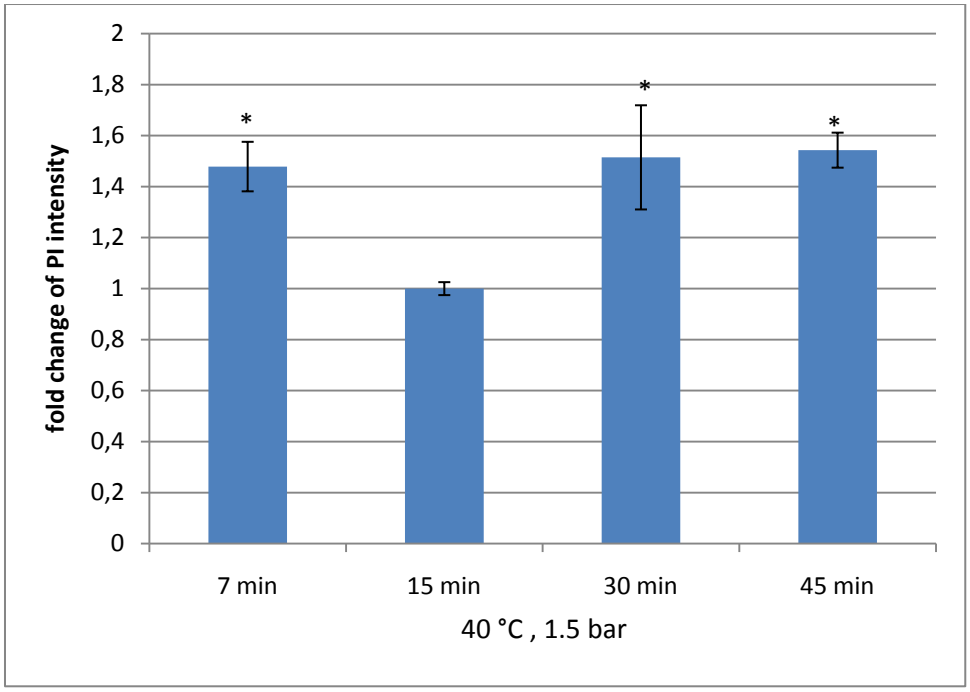


Figure 23 relative PI intensity of *E. faecium* M-74 after fluid bed drying using different process time settings, * $p<0.05$ significance between relative PI intensity of cells dried with 15 min and different process time settings

Using different processing times by keeping the other parameters constant caused different levels of membrane damage. As illustrated in figure 23, the PI intensity of cells dried during 15 min was significantly lower than the intensity of cells dried using other processing times ($p<0.05$), indicating the less membrane damage of the rather cells.

- Hydrogen peroxide production

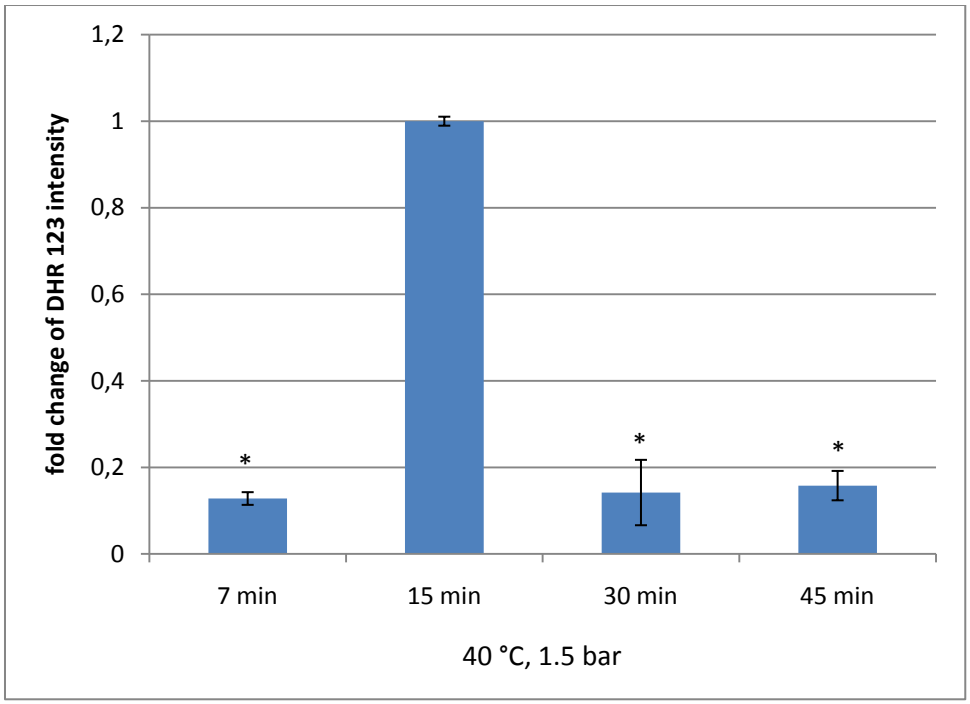


Figure 24 relative DHR 123 intensity of *E. faecium* M-74 after fluid bed drying using different process time settings * $p<0.05$ significance between relative DHR 123 intensity of cells dried with 15 min and different process time settings

Figure 24 shows the effect of different process times on the relative intensity of dihydrorhodamine 123 of dried cells. During 15 minutes the relative fluorescence intensity of dried cells was significantly higher than the relative intensity of cells dried during 7, 30 or 45 minutes ($p<0.05$).

4.2.3. Effect of the variation of atomizing air pressure on viability of *Enterococcus faecium* M-74

Atomizing air pressure was varied as the last parameter, keeping the other parameters constant. Atomizing air pressure was varied from 1.0 to 3.5 bar.

4.2.3.1. Culturability

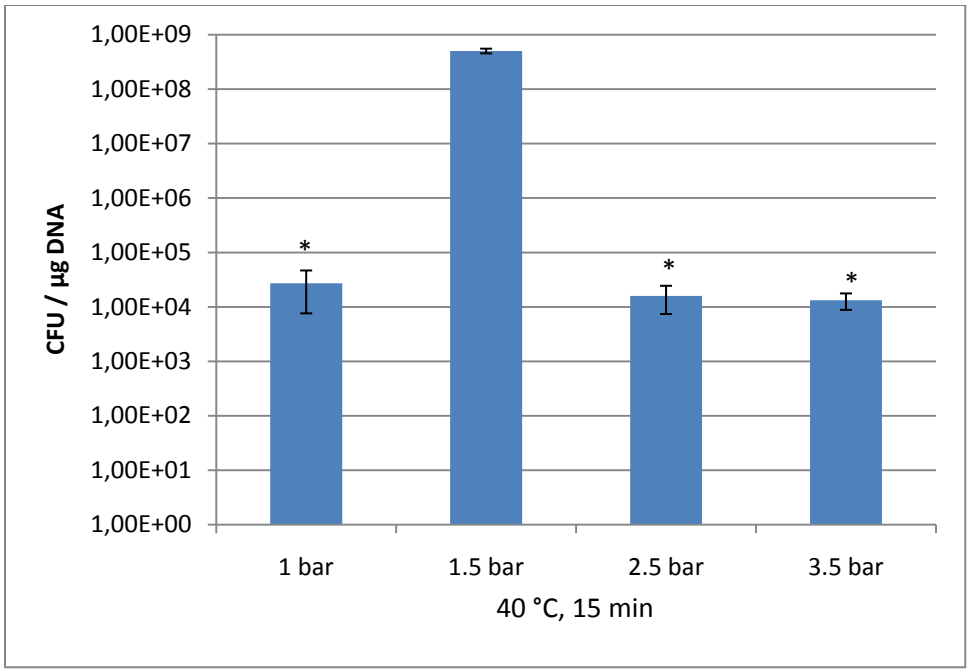


Figure 25 colony forming units / µg DNA of *E. faecium* M-74 after fluid bed drying using different atomizing air pressure settings, * $p<0.05$ significance between colony forming units / µg DNA of cells dried with 1.5 bar and different atomizing air pressure settings

Using atomizing air pressure of 1.5 bar, the culturability of dried cells was 4.03 log units higher than the culturability of cells, dried using other atomizing air pressures [figure 25].

4.2.3.2. Cellular properties

- Cell membrane permeability

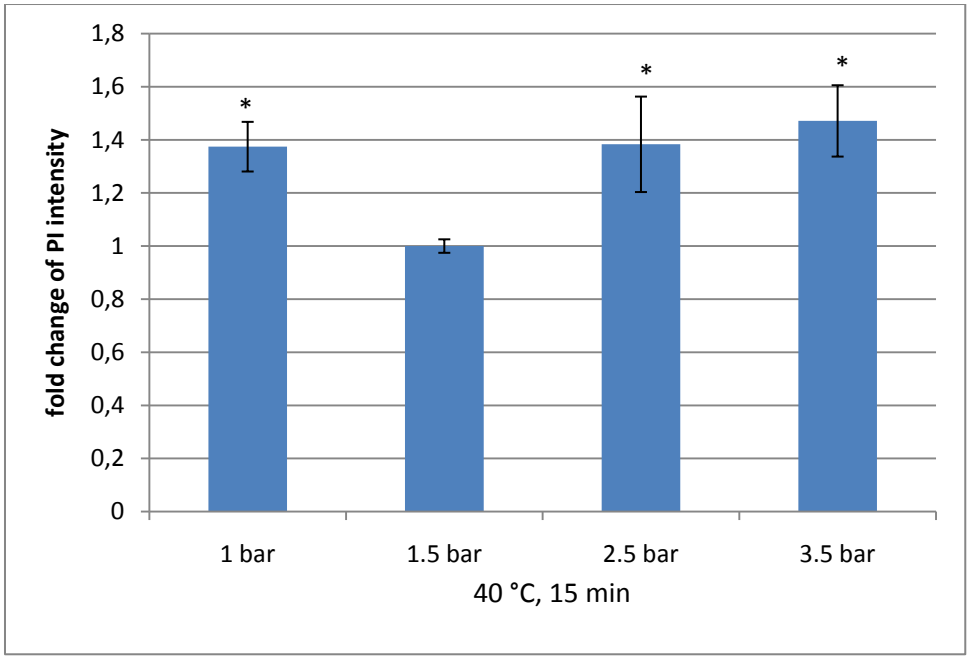


Figure 26 relative PI intensity of *E. faecium* M-74 after fluid bed drying using different atomizing air pressure settings, * p<0.05 significance between relative PI intensity of cells dried with 1.5 bar and different atomizing air pressure settings

The relative propidium iodide intensity of dried cells using atomizing air pressure of 1.5 bar was significantly less than those of dried cells using other atomizing air pressures, indicating the less membrane damage of these cells [figure 26].

- Hydrogen peroxide production

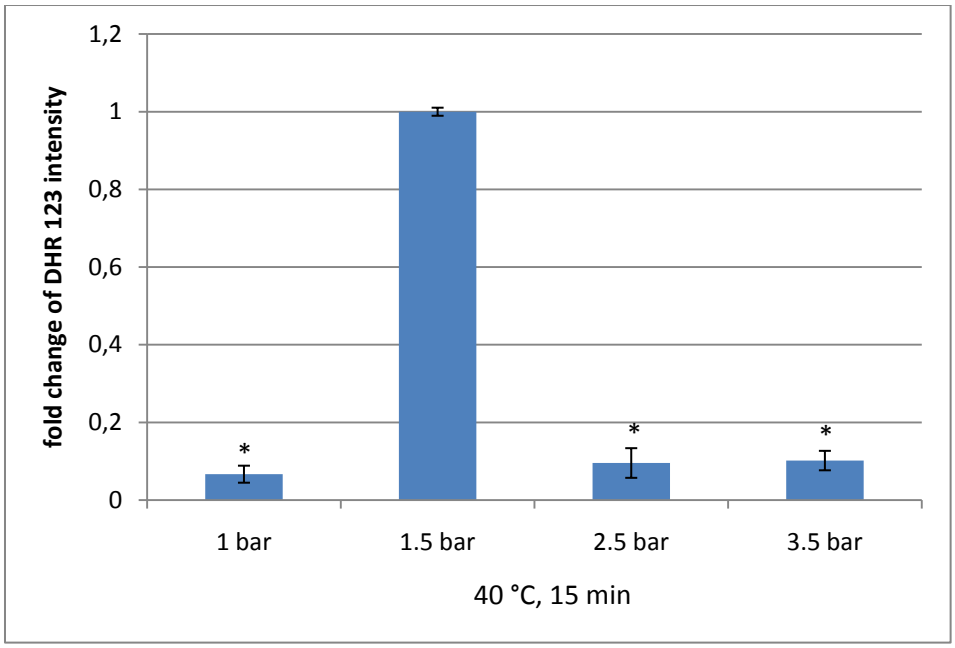


Figure 27 relative DHR 123 intensity of *E. faecium* M-74 after fluid bed drying using different atomizing air pressure settings, * p<0.05 significance between relative DHR 123 intensity of cells dried with 1.5 bar and different atomizing air pressure settings

The impact of atomizing air pressure on the intensity of DHR 123 of fluid bed dried cells is illustrated in Figure 27.The intensities of dried cells processed with 1.0, 2.5 or 3.5 bar were comparable with each other and were significantly lower than the intensity of dried cells processed using 1.5 bar.

We have concluded that the parameter setting of

Product bed temperature: 40 °C

Processing time: 15 min

Atomizing air pressure: 1.5 bar

was the suitable setting for fluid bed drying of *E. faecium* M-74.

4.3. Effect of cryo-stress on viability of *Enterococcus faecium* M-74

The impact of cryo-stress on the culturability and cellular properties of *E. faecium M-74* was studied. In this part of study the alterations in cellular properties were investigated using fluorimetry and flow cytometry. The membrane damage, changes in esterase activity and hydrogen peroxide production was measured after staining the cells with PI, FDA and DHR 123, respectively using both flow cytometry and fluorimetry methods. In addition the alterations in membrane potential and intracellular pH were studied after staining the cells with Dioc6(3) and CFDASE.

4.3.1. Culturability

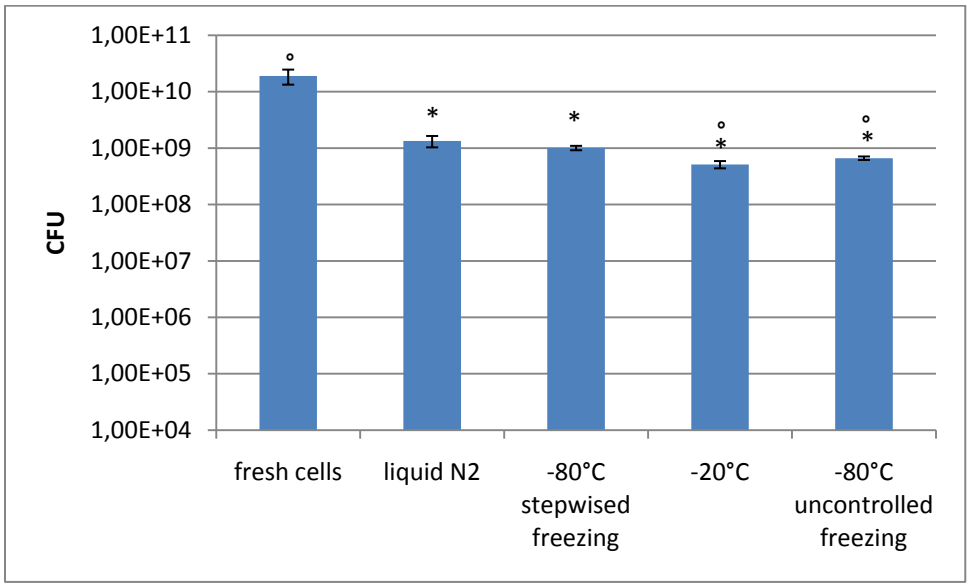


Figure 28 colony forming units of *E. faecium* M-74 frozen under different conditions compared to fresh cells, * $p<0.05$ significance between colony forming units of fresh harvested and frozen cells, ° $p<0.05$ significance between colony forming units of cells frozen with liquid nitrogen and other frozen cells

Figure 28 shows the effect of different freezing conditions on the culturability of *E. faecium M-74*. Regardless the freezing conditions the culturability of frozen cells decreased about more than 1 log unit as compared to freshly harvested cells.

Comparing the impact of different freezing conditions on the cell culturability showed that using liquid nitrogen harmed the culturability of unprotected cells less than using -20°C or -80°C ($p<0.05$) (figure 28). The effect of stepwise freezing of cells at -80°C was comparable with the effect of liquid nitrogen ($p>0.05$).

4.3.2. Cellular properties

4.3.2.1. Effect of cryo-stress on cellular properties of *Enterococcus faecium* M-74, fluorimetric measurements

The effect of different freezing conditions, i.e. -20°C, -80°C and liquid nitrogen, on cellular properties of *Enterococcus faecium* M-74 was studied. The frozen cells were thawed, incubated with different fluorescent dyes as described in chapter “Materials and Methods”.

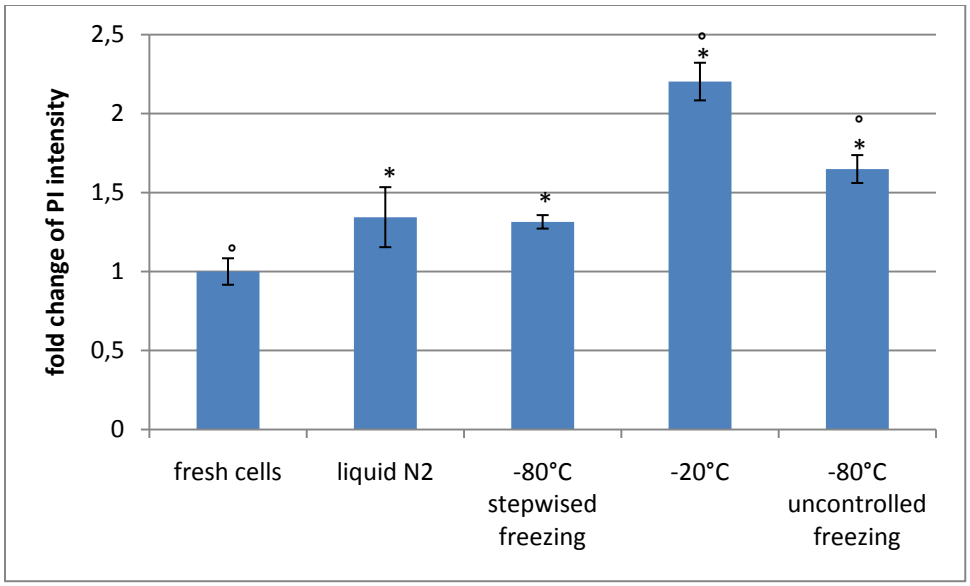


Figure 29 relative PI intensity of frozen *E. faecium* M-74 under different freezing conditions compared to fresh cells, * p<0.05 significance between relative PI intensity of fresh harvested and frozen cells, ° p<0.05 significance between relative PI intensity of cells frozen with liquid N2 and other frozen cells

Generally the cryo-stress affected the cell membrane of *E. faecium M-74*. Frozen cells showed higher fluorescence intensity after staining with propidium iodide as compared to freshly harvested cells.

Freezing the cells at -20°C damaged the cell membrane more than other conditions ($p<0.05$). As demonstrated in figure 29 the PI intensity of cells frozen at -20 °C was 2.2-fold higher than the intensity of freshly harvested cells. Freezing the cells at liquid nitrogen or stepwise freezing at -80 °C had comparable effect on the cell membrane. Moreover using these conditions the fluorescence intensities of frozen cells were less than in the case of freezing in -20 °C or -80 °C indicating the less membrane damage ($p<0.05$).

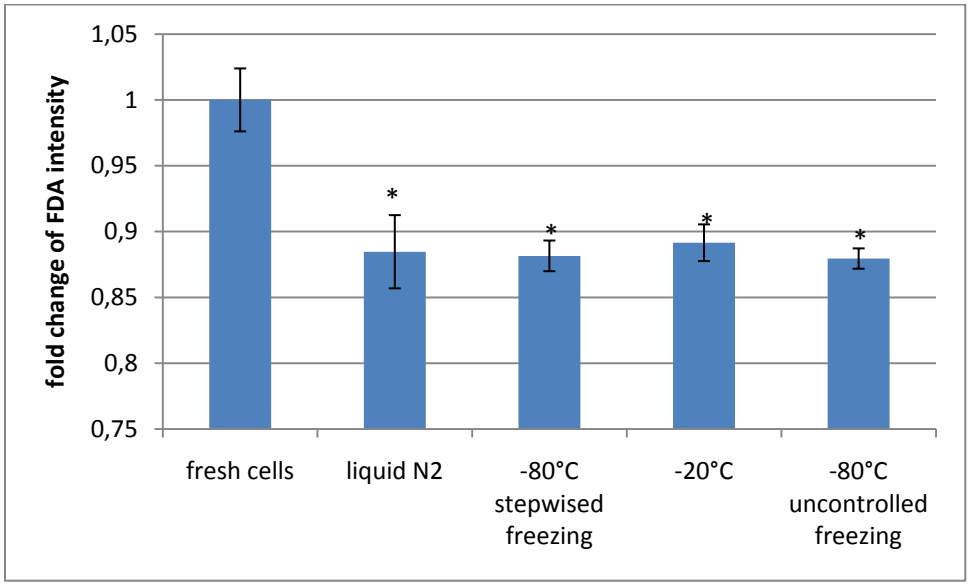


Figure 30 relative FDA intensity of frozen *E. faecium* M-74 under different freezing conditions compared to fresh cells, * p<0.05 significance between relative FDA intensity of fresh harvested and frozen cells

Using fluorescein diacetate it became obvious that generally cryo-stress caused significant decreasing of esterase properties in *Enterococcus faecium* M-74 ($p<0.05$). As indicated in figure 30, the relative FDA intensity of frozen cells was significantly lower than those of freshly harvested cells.

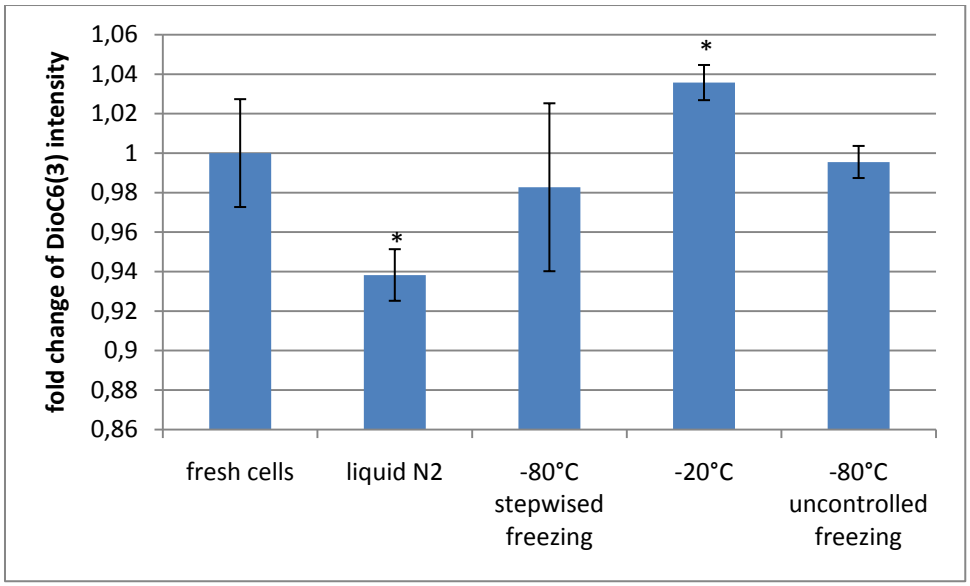


Figure 31 relative Dioc6(3) intensity of frozen *E. faecium* M-74 under different freezing conditions compared to fresh cells, * $p<0.05$ significance between relative Dioc6(3) intensity of fresh harvested and frozen cells

Freezing the cells affected the membrane potential. Exposing *Enterococcus faecium* M-74 to -20°C harmed the most again ($p<0.05$). Figure 31 shows changes in membrane potential, cells frozen in liquid nitrogen showed the lowest fluorescence intensity ($p<0.05$).

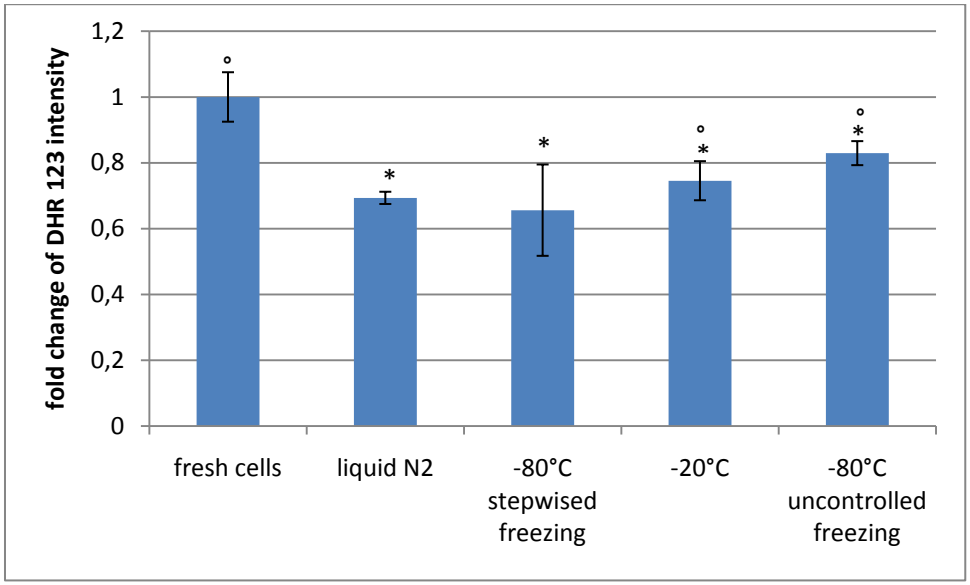


Figure 32 relative DHR 123 intensity of frozen *E. faecium* M-74 under different freezing conditions compared to fresh cells, * $p<0.05$ significance between relative DHR 123 intensity of fresh harvested and frozen cells, ° $p<0.05$ significance between relative DHR 123 intensity of cells frozen in liquid N2 and other frozen cells

Generally, the fluorescence intensity of frozen cells was significantly lower than the intensity of freshly harvested cells, regardless the freezing condition. However, the cells frozen at -20 °C or -80 °C under uncontrolled freezing showed significantly lower intensities, as compared to the intensity of cells frozen at liquid nitrogen. The DHR 123 intensities of cells frozen stepwised at -80 °C and at liquid nitrogen were comparable.

4.3.2.2. Effect of cryo-stress on cellular properties of *Enterococcus faecium* M-74, flow cytometric measurements

The impact of freezing on the cells was also investigated using flow cytometry. In the context of this survey, cell size has not been investigated. The focus lay on the fluorescence of the cells and its intensity.

Investigation of the impact of freezing on the esterase activity, membrane potential and hydrogen peroxide of *E. faecium* M-74 failed due to insignificant alterations of FDA, DioC6(3), and DHR 123 intensity, respecively.

- Cell membrane permeability

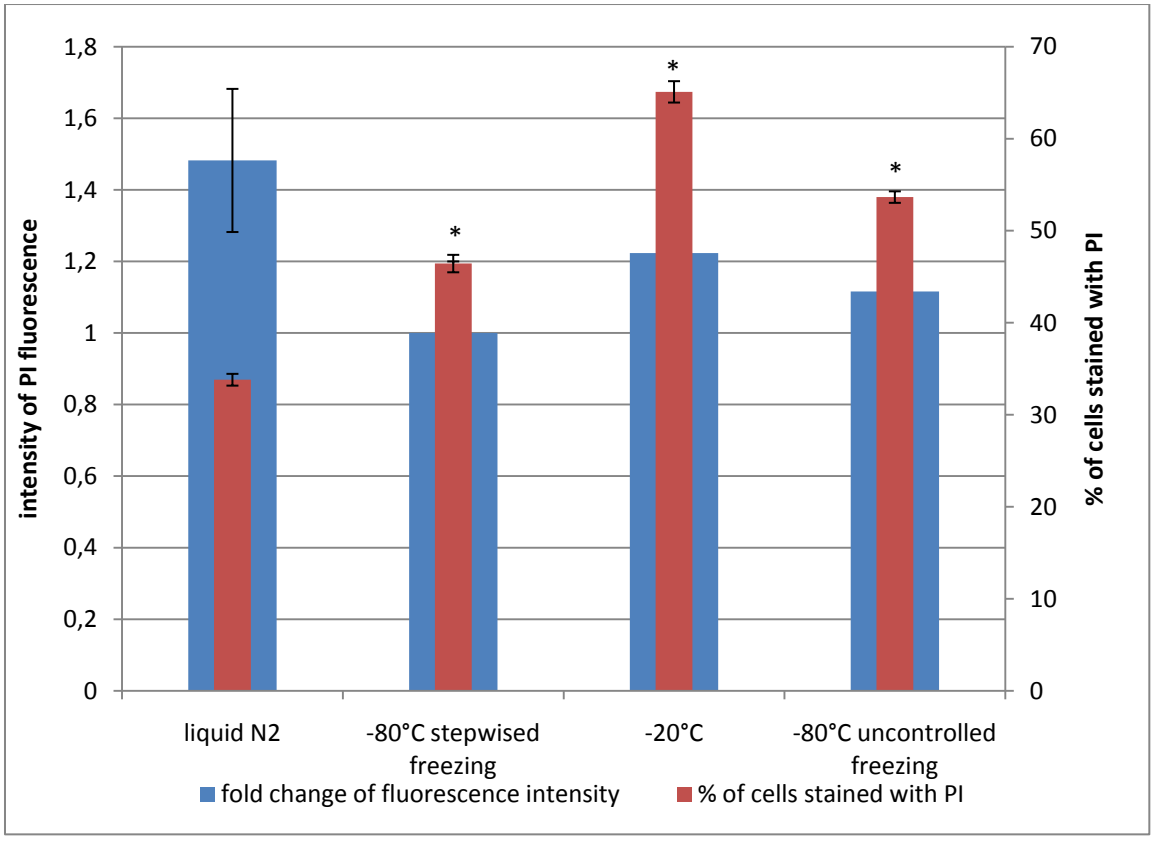


Figure 33 relative PI intensity of frozen *E. faecium* M-74 vs. percentage of stained frozen cells under different freezing conditions, * p<0.05 significance between amount of cells stained with PI of cells frozen in liquid nitrogen and other frozen cells

Figure 33 shows the impact of freezing on the percentage of stained cells and their fluorescence intensity. The percentage of stained cells after freezing in liquid nitrogen was significantly lower than the percentage of stained cells frozen under the other conditions.

- Intracellular pH

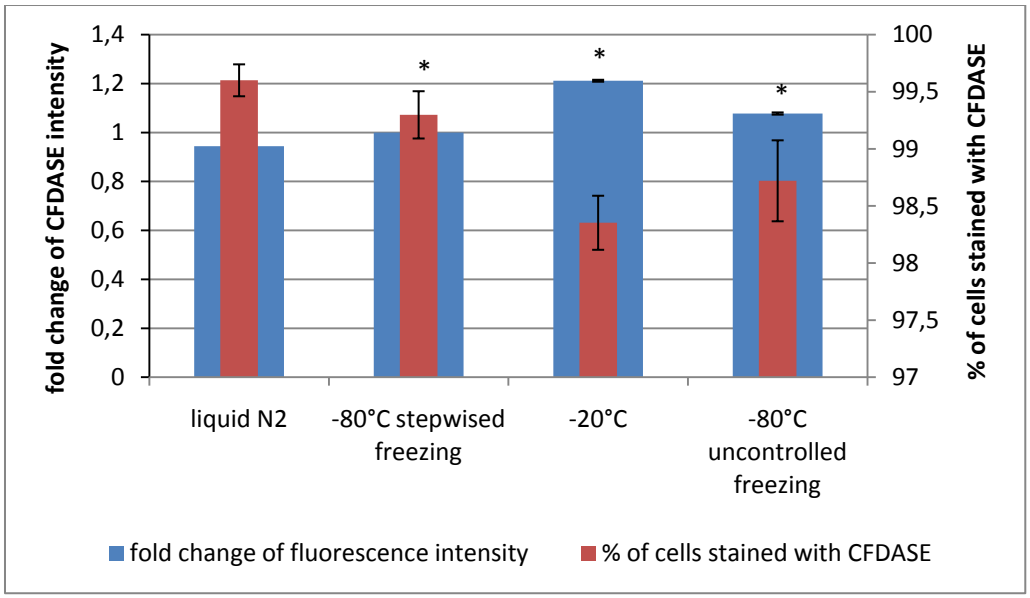


Figure 34 relative CFDASE intensity of frozen *E. faecium* M-74 vs. percentage of stained frozen cells under different freezing conditions, * p<0.05 significance between relative CFDASE intensity cells frozen in liquid nitrogen and other frozen cells

Carboxy fluorescin diacetate succinimidyl ester (CFDASE) tagged nearly all cells, regardless of their previous exposure to stress. Intracellular pH changed when *Enterococcus faecium* M-74 was stressed at different freezing conditions (figure 34).

Flow cytometric investigations were based on the measurement of the percentage of the stained cells as well as the fluorescence intensity of cells. In the case of staining the cells with PI, the percentage of stained cells gave us more meaningfull results than the fluorescence intensity. In contrary, staining the cells with CFDASE, the fluorescence intensity was more noticeable.

We have concluded that freezing of cells stepwise at -80 °C or liquid nitrogen affected the cell viability less than freezing conditions of -20 °C or uncontrolled freezing at -80 °C.

Next we have studied the effect of cryoprotectants on the viability of *E. faecium* M-74 after freezing. Liquid nitrogen or uncontrolled freezing at -80 °C were chosen as the best or conventional freezing conditions, respectively. As used cryoprotectants we decided on sucrose and glucose in 50% and 100% concentrations based on the wet mass of cells.

4.3.3. Effect of cryo-protectants on viability of *Enterococcus faecium* M-74 after freezing

The cryo-protective impact of sucrose and glucose on the cells was investigated, using the concentrations of 50% or 100% based on the wet cell mass. After treatment of cells with these ingredients, cells were frozen at -80 °C or in liquid nitrogen for 24 h.

4.3.3.1. Culturability

There were no significant differences in the culturability of pre-treated frozen cells, regardless to the protective agent and the freezing condition. Therefore, the data are not shown.

4.3.3.2. Impact of protectants on the cellular properties of *E. faecium M-74* after freezing, fluorimetric measurements

- Cell membrane permeability

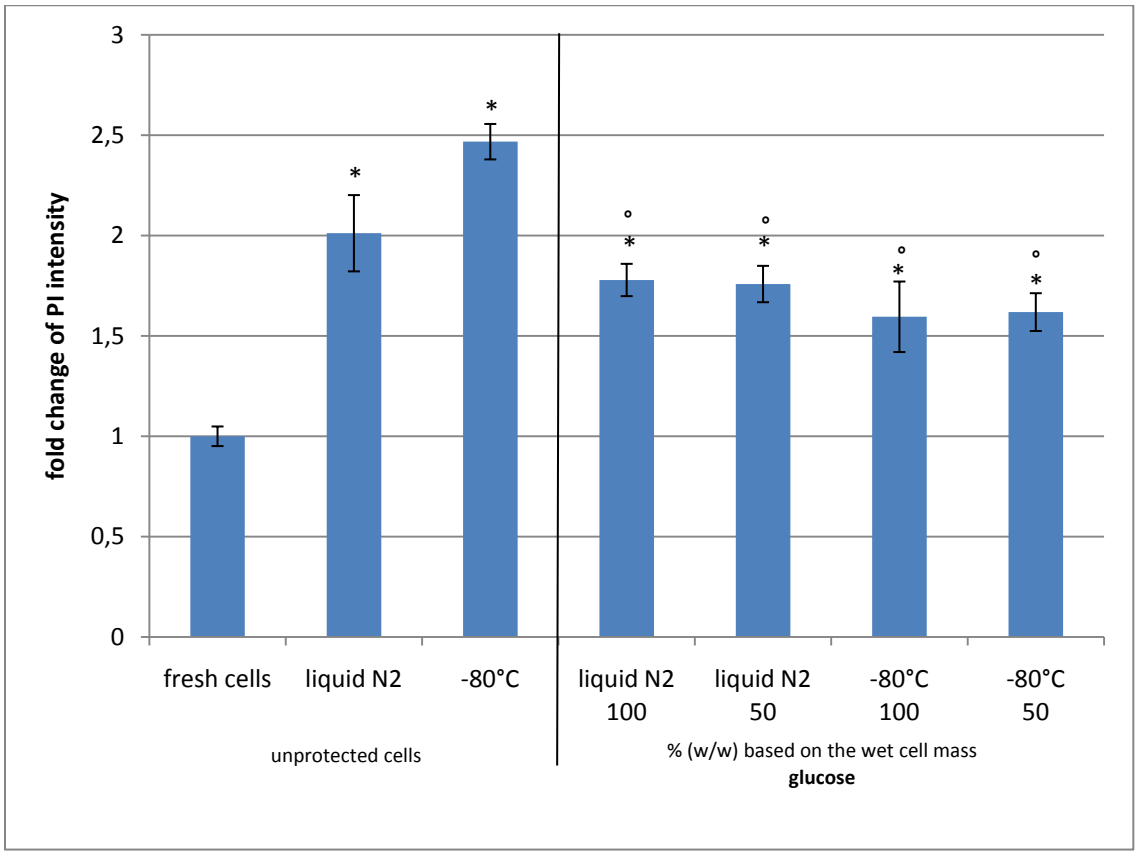


Figure 35 relative PI intensity of *E. faecium* M-74 after cryo-stress using different concentrations of glucose based on the wet cell mass, * p<0.05 significance between relative PI intensity of fresh and frozen cells, ° p<0.05 significance between relative PI intensity of cells protected and frozen cells with unprotected frozen cells

Figure 35 indicates the relative PI intensity of pre-treated cells with glucose and frozen at -80 °C or in liquid nitrogen. Using glucose in both concentrations protected the cell membrane against the cryo-stress involved with the freezing, regardless to

the freezing condition. As can be observed in figure 35, the PI intensity of protected and frozen cells was significantly lower than the intensity of unprotected frozen cells.

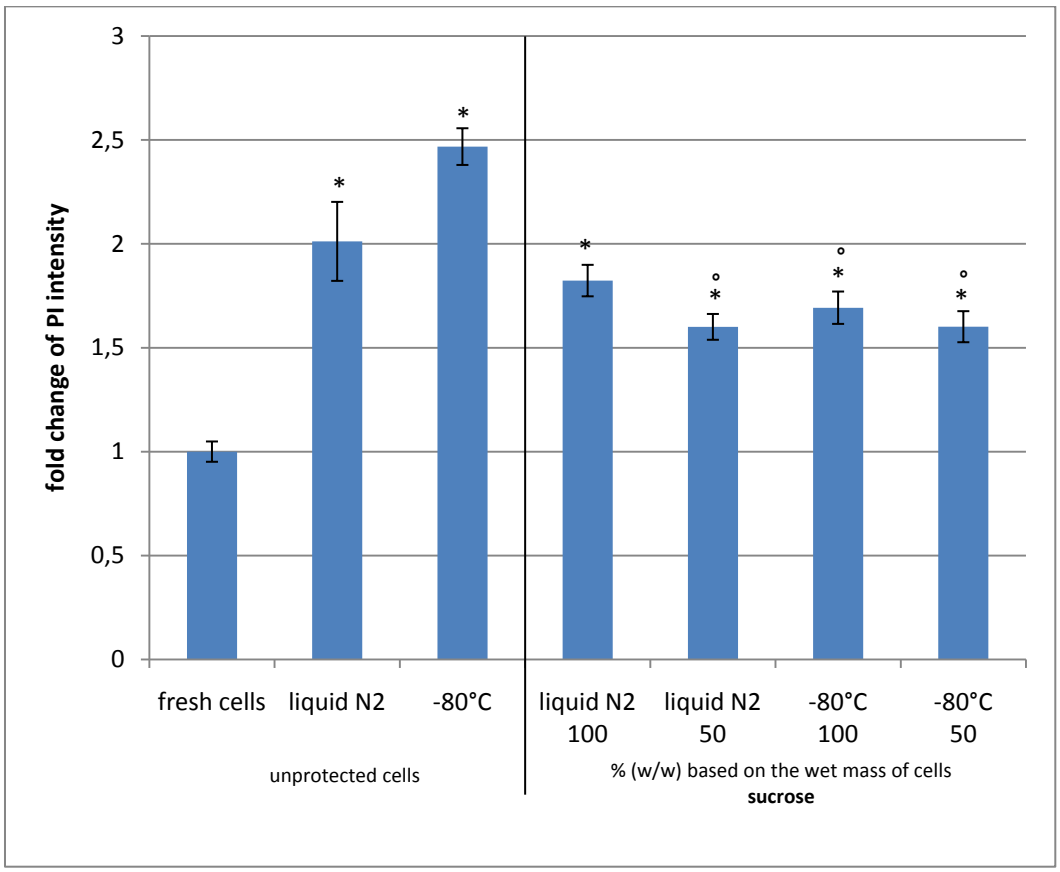


Figure 36 relative PI intensity of *E. faecium* M-74 after cryo-stress using different concentrations of sucrose based on the wet cell mass, * p<0.05 significance between relative PI intensity of fresh and frozen cells, ° p<0.05 significance between relative PI intensity of cells protected and frozen cells with unprotected frozen cells

The relative PI intensity of unprotected frozen cells was compared with the intensity of pre-treated cells with sucrose after freezing (figure 36). The concentration of 50% sucrose protected cells significantly better than 100% sucrose (p<0.05), when cells were frozen in liquid nitrogen.

Generally, the relative PI intensity of frozen cells, pre-treated with sucrose was lower than those of cells, pre-treated with glucose, indicating the superior cryo-protective effect of sucrose on the cell membrane (figure 35 and 36).

Pre-treatment of cells with sucrose at the concentration of 50% based on the wet cell mass protected the cell membrane more than the concentration of 100 %.

- Esterase activity

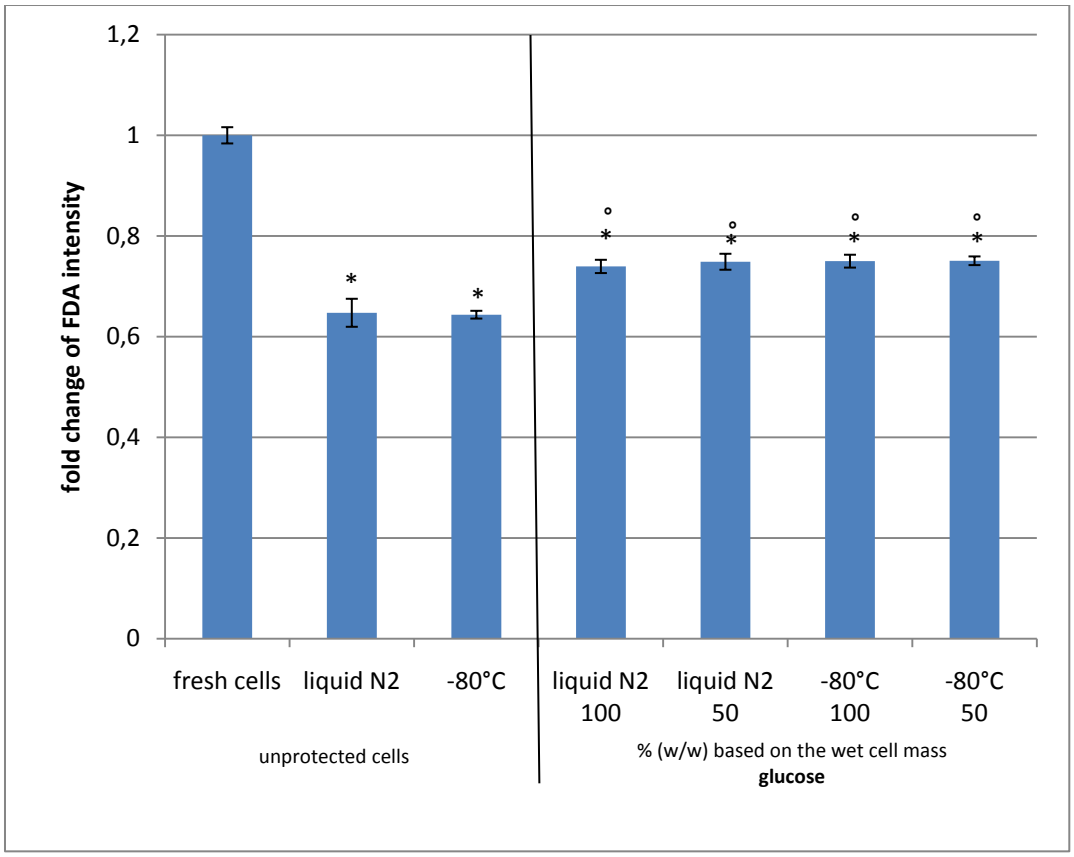


Figure 37 relative FDA intensity of *E. faecium* M-74 after cryo-stress using different concentrations of glucose based on the wet cell mass, * p<0.05 significance between relative FDA intensity of fresh and frozen cells, ° p<0.05 significance between relative FDA intensity of cells protected and frozen cells with unprotected frozen cells

Figures 37 and 38 indicate the relative FDA intensity of frozen cells, pre-treated with glucose and sucrose, respectively.

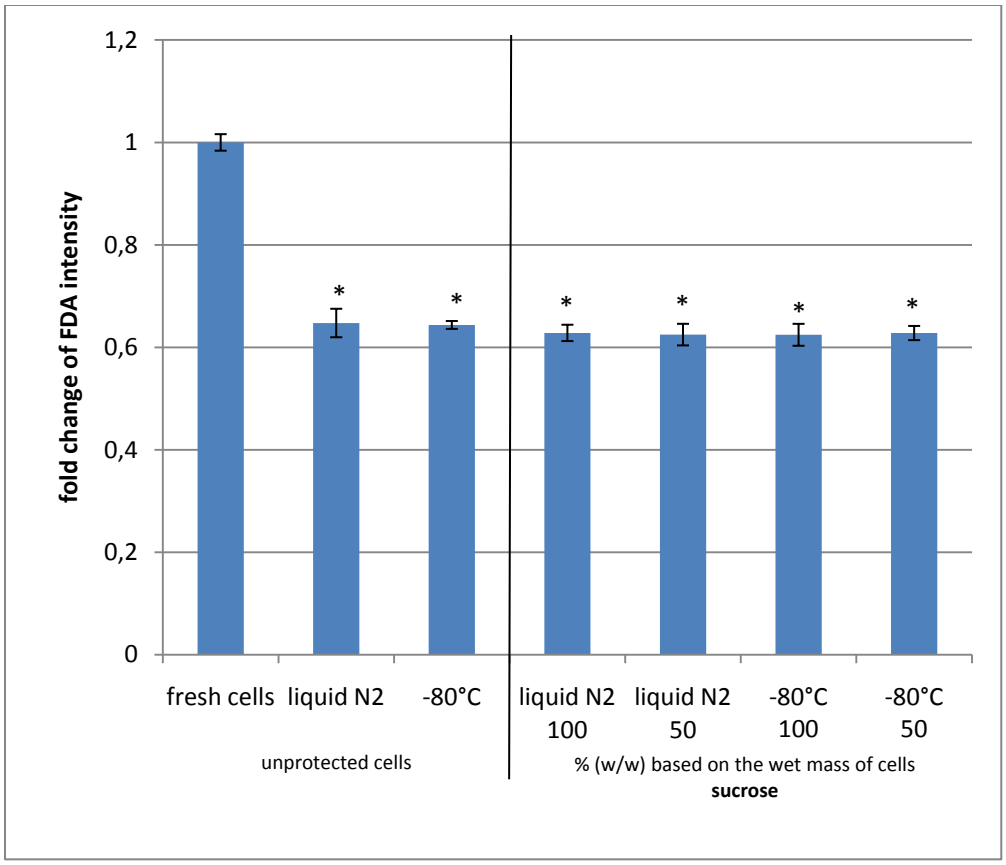


Figure 38 relative FDA intensity of *E. faecium* M-74 after cryo-stress using different concentrations of sucrose based on the wet cell mass, * p<0.05 significance between relative FDA intensity of fresh and frozen cells, ° p<0.05 significance between relative FDA intensity of cells protected and frozen cells with unprotected frozen cells

In all cases the FDA intensities of pre-treated and frozen cells were significantly lower than those of freshly harvested cells, indicating the decrease of esterase activity after freezing. However, the fluorescence intensity of pre-treated cells with glucose after freezing was significantly higher than the intensity of unprotected frozen cells.

- Membrane potential

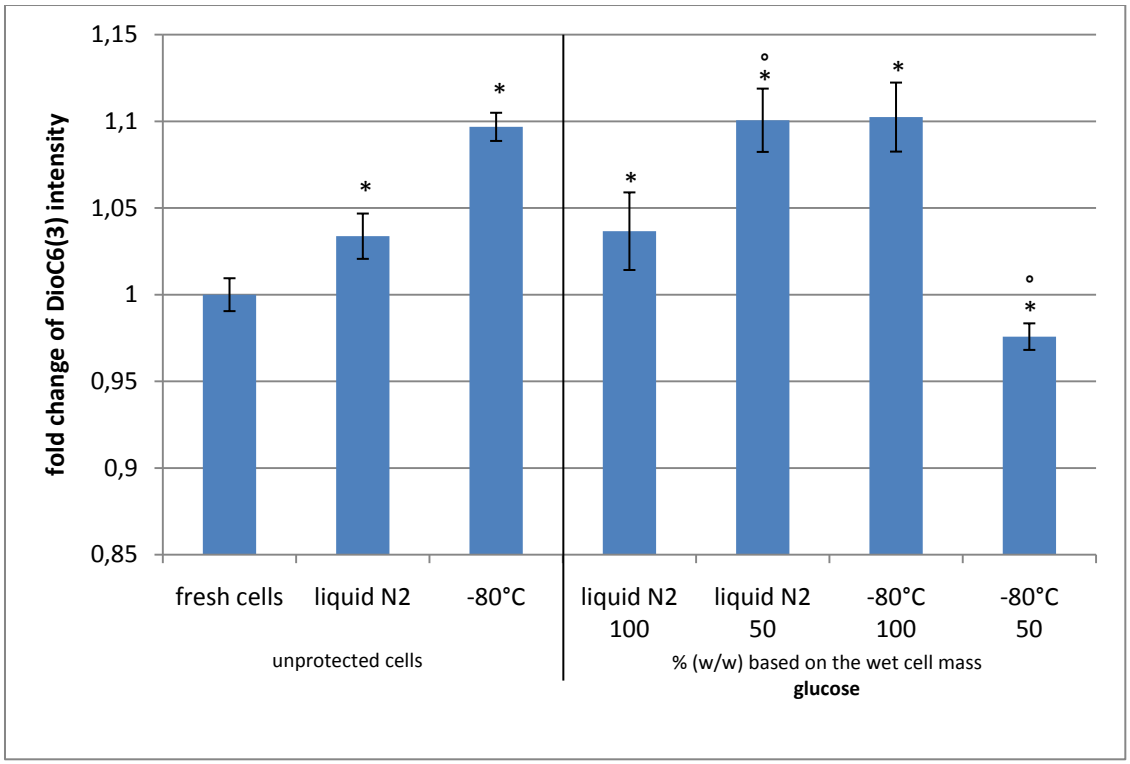


Figure 39 relative DioC6(3) intensity of *E. faecium* M-74 after cryo-stress using different concentrations of glucose based on the wet cell mass, * p<0.05 significance between relative Dioc6(3) intensity of fresh and frozen cells, ° p<0.05 significance between relative DioC6(3) intensity of cells protected and frozen cells with unprotected frozen cells

Alterations in the relative DioC6(3) intensity of frozen cells, pre-treated with glucose is shown in figure 39. The fluorescence intensity of pre-treated cells with a concentration of 50% glucose (based on the wet cell mass) and frozen at -80 °C was lower than the intensity of freshly harvested cells. In the contrast, using other conditions resulted in the significantly higher fluorescence intensity of frozen cells ($p<0.05$), indicating alterations in the membrane potential due to the membrane damage.

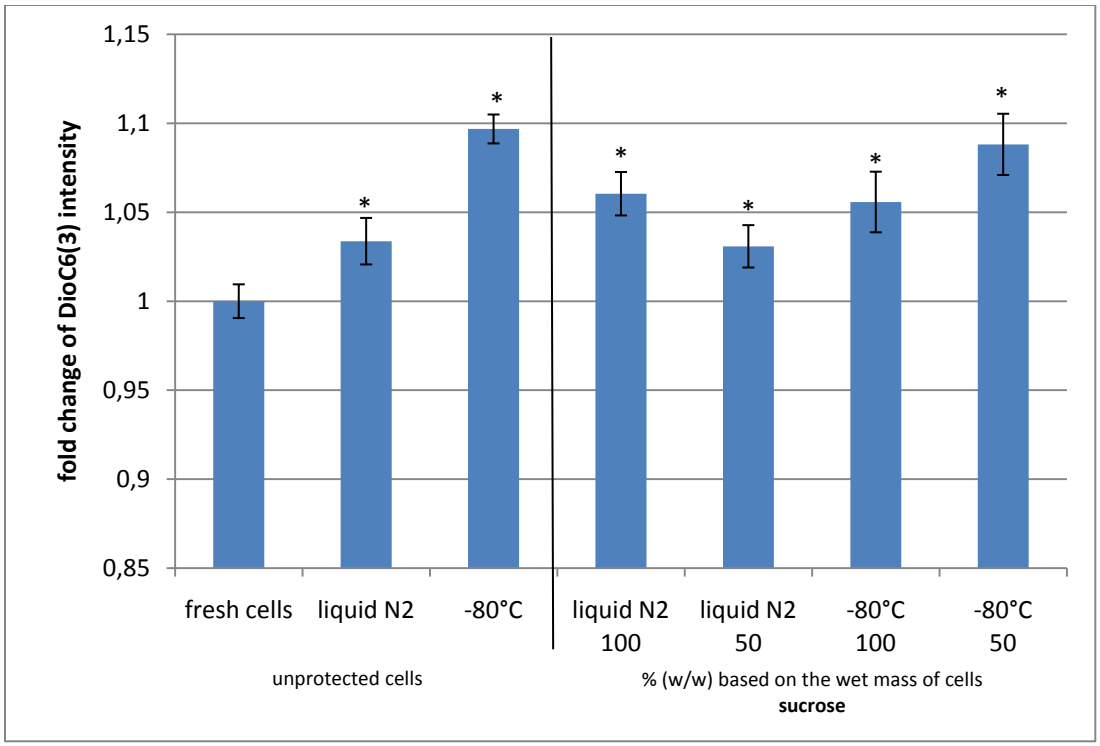


Figure 40 relative DioC6(3) intensity of *E. faecium* M-74 after cryo-stress using different concentrations of sucrose based on the wet cell mass, * $p<0.05$ significance between relative Dioc6(3) intensity of fresh and frozen cells, ° $p<0.05$ significance between relative DioC6(3) intensity of cells protected and frozen cells with unprotected frozen cells

Pre-treatment of cells with sucrose resulted in higher fluorescence intensities than freshly harvested cells ($p<0.05$). The DioC6(3) intensity of pre-treated cells with 50% sucrose and frozen in liquid nitrogen was significantly lower than using 100% sucrose.

- Hydrogen peroxide production

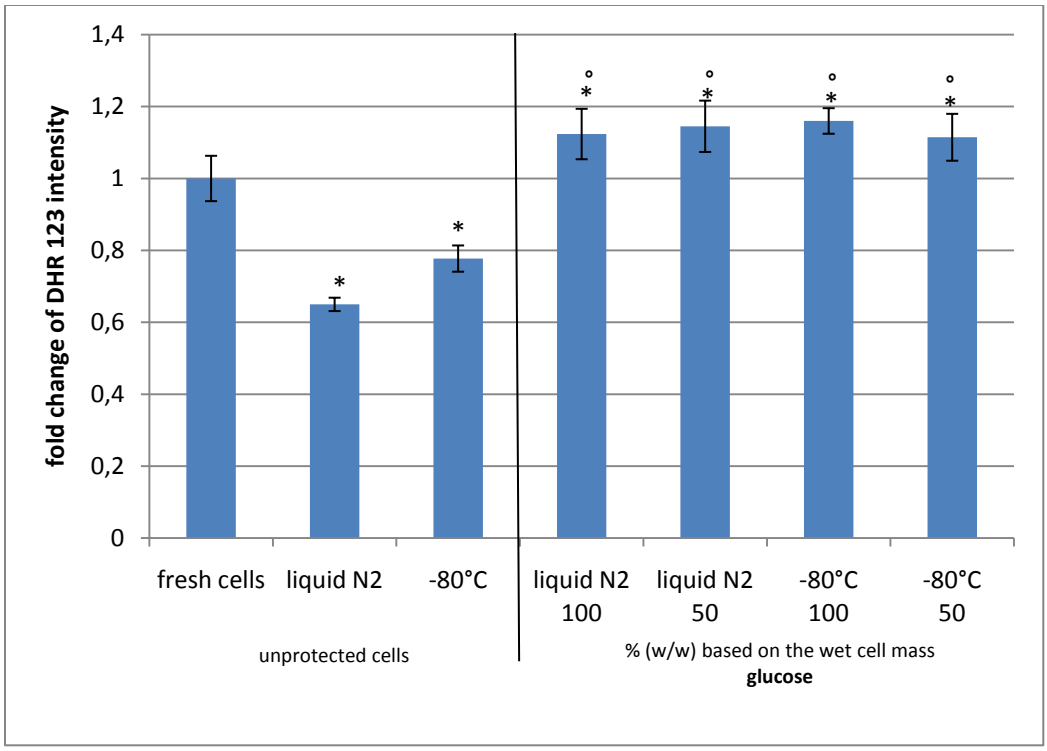


Figure 41 relative DHR 123 intensity of *E. faecium* M-74 after cryo-stress using different concentrations of glucose based on the wet cell mass, * p<0.05 significance between relative DHR 123 intensity of fresh and frozen cells, ° p<0.05 significance between relative DHR intensity of cells protected and frozen cells with unprotected frozen cells

Whereas the DHR 123 intensity of unprotected frozen cells was significantly lower than the intensity of freshly harvested cells, pre-treatment with glucose resulted in higher fluorescence intensities.

In preliminary experiments we have observed an increase of relative DHR 123 intensity of *E. faecium* M74 in response to moderate stress. Increasing the stress intensity caused the decrease of fluorescence intensity, which might be due to the collapse of the cellular redox system.

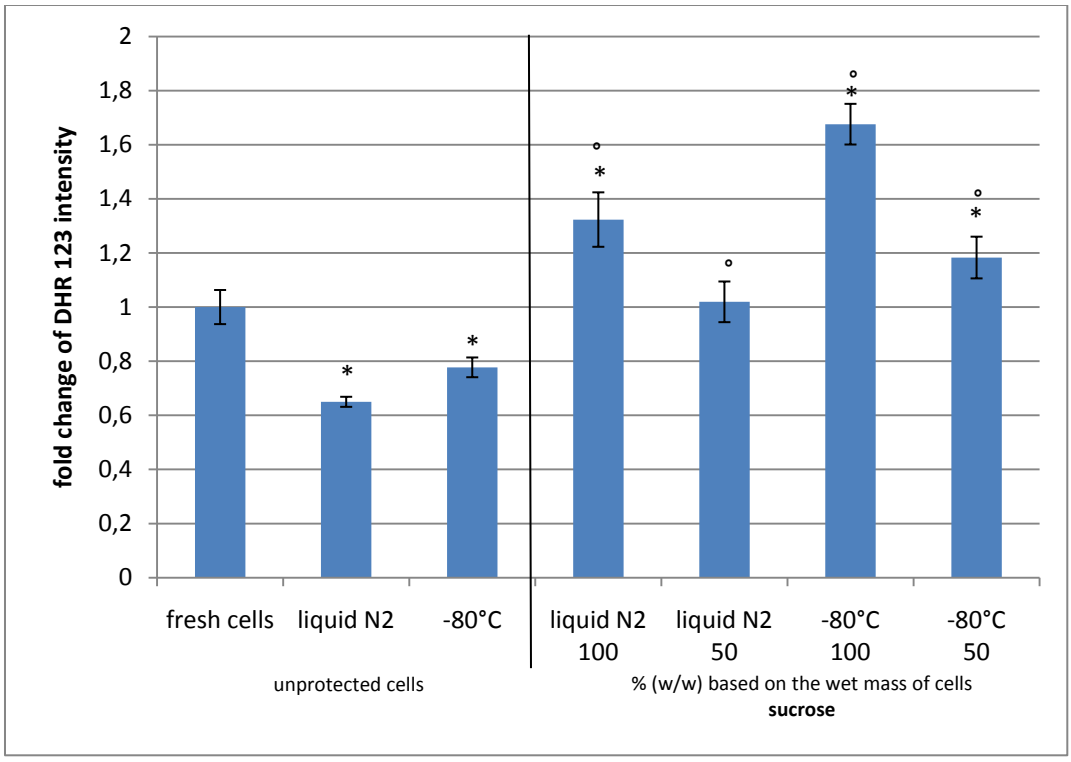


Figure 42 relative DHR 123 intensity of *E. faecium* M-74 after cryo-stress using different concentrations of sucrose based on the wet cell mass, * p<0.05 significance between relative DHR 123 intensity of fresh and frozen cells, ° p<0.05 significance between relative DHR 123 intensity of cells protected and frozen cells with unprotected frozen cells

Generally, pre-treatment of cells with a concentration of 50% sucrose (based on the wet cell mass) protected the cells against the induced stress more than a concentration of 100 % sucrose. As indicated in figure 42, the DHR 123 intensities of frozen cells pre-treated with 50% sucrose (based on the wet cell mass) were significantly lower than the intensities of frozen pre-treated cells with 100% sucrose. Of note, the intensity of pre-treated cells with 50% sucrose and frozen in liquid nitrogen was comparable with the intensity of freshly harvested cells.

4.3.3.2.2. Impact of protectants on the cellular properties of *E. faecium M-74* after freezing, flow cytometric measurements

Flow cytometric investigations were based on the measurement of the percentage of the stained cells as well as the fluorescence intensity of cells. In the case of staining the cells with PI, the percentage of stained cells gave us more meaningfull results than the fluorescence intensity. In contrary, staining the cells with CFDASE, the fluorescence intensity was more noticeable.

- Cell membrane permeability

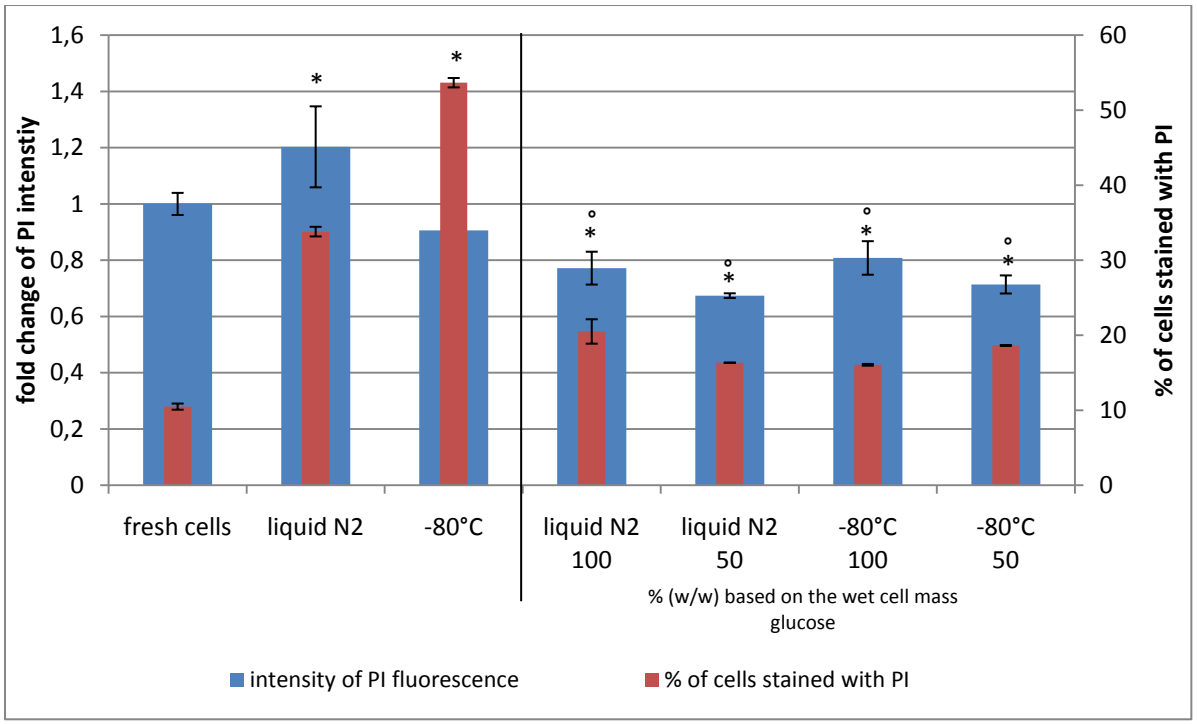


Figure 43 relative PI intensity of frozen *E. faecium* M-74 vs. percentage of stained frozen cells under different freezing conditions protected with different concentrations of glucose based on wet cell mass, * $p<0.05$ significance between amount of cells stained with PI of frozen to freshly harvested cells, ° $p<0.05$ significance between amount of cells stained with PI of cells protected and frozen cells with unprotected frozen cells

Using 50% glucose as protectant and freezing in liquid nitrogen affected the cell membrane significantly ($p<0.05$) less than protecting with 100% glucose (figure 43). Samples pretreated with 100% glucose and frozen in -80 °C were stained less than pretreated with 50% glucose.

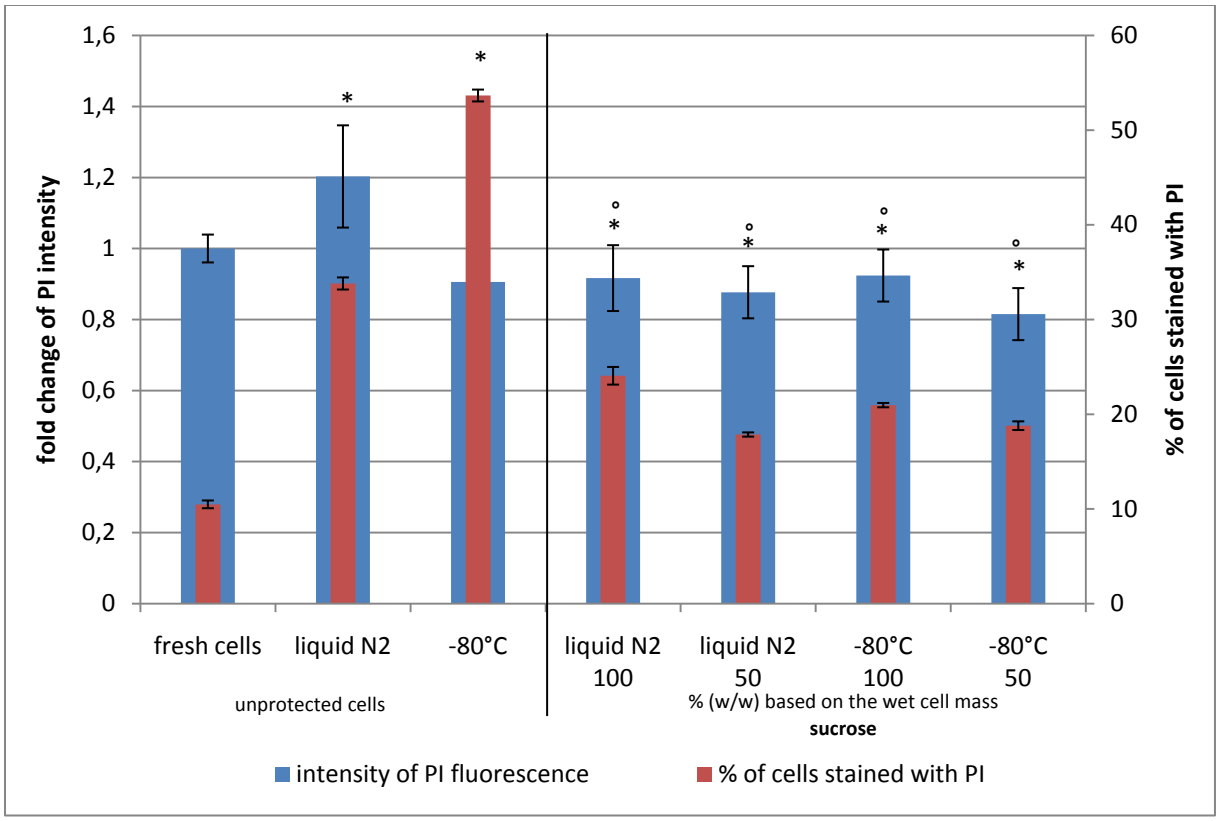


Figure 44 relative PI intensity of frozen *E. faecium* M-74 vs. percentage of stained frozen cells under different freezing conditions protected with different concentrations of sucrose based on wet cell mass, * $p<0.05$ significance between amount of cells stained with PI of frozen to freshly harvested cells, ° $p<0.05$ significance between amount of cells stained with PI of cells protected and frozen cells with unprotected frozen cells

As can be observed in figures 43 and 44, pre-treatment of cells with both glucose and sucrose protected the cells against the cryo-stress. The percentage of the stained cells pre-treated with sucrose or glucose at any concentration was comparable with each other and they were significantly lower than the percentage of unprotected frozen cells.

The fluorimetric measurements showed the protective impact of sucrose and glucose as well. However, those results showed the lower PI intensity of cells protected with sucrose, as compared to those protected with glucose.

- Intracellular pH

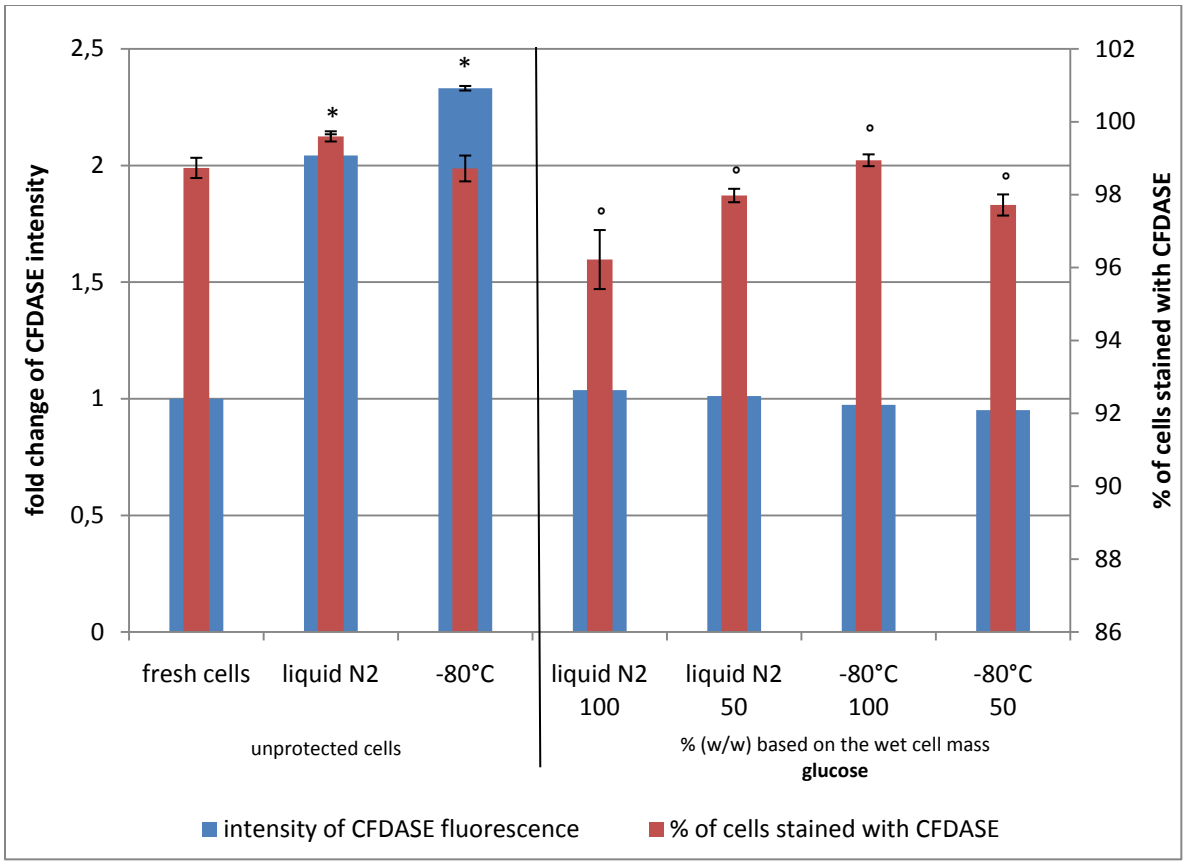


Figure 45 relative CFDASE intensity of frozen *E. faecium* M-74 vs. percentage of stained frozen cells under different freezing conditions protected with different concentrations of glucose based on wet cell mass, * $p<0.05$ significance between relative CFDASE intensity of frozen to freshly harvested cells, ° $p<0.05$ significance between relative CFDASE intensity of cells protected and frozen cells with unprotected frozen cells

As indicated in figure 45, after freezing of unprotected cells the intensity of CFDASE significantly increased more than 2-fold compared to the intensity of stained fresh cells. Using glucose resulted in a significant drop of CFDASE intensity and the intensity was comparable with the intensity of freshly harvested cells. Preliminary studies of intracellular pH showed a light drop of CFDASE intensity in the case of mild stress. Increasing the intensity of stress resulted in increased of intracellular pH, indicated by increasing CFDASE intensity.

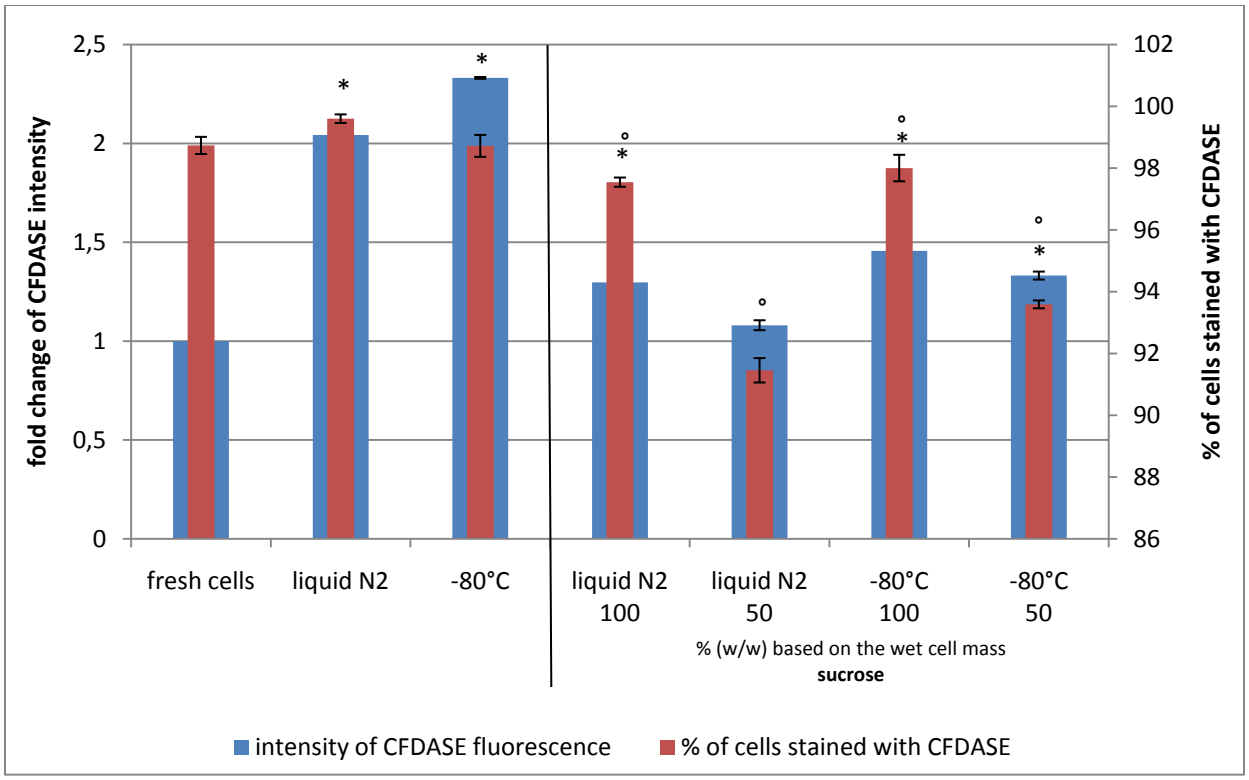


Figure 46 relative CFDASE intensity of frozen *E. faecium* M-74 vs. percentage of stained frozen cells under different freezing conditions protected with different concentrations of sucrose based on wet cell mass, * $p<0.05$ significance between relative CFDASE intensity of frozen to freshly harvested cells, ° $p<0.05$ significance between relative CFDASE intensity of cells protected and frozen cells with unprotected frozen cells

Observing figure 46 samples pretreated with 100% sucrose showed higher fluorescence intensities ($p<0.05$). Among each other the results showed significant differences comparing 100% and 50% sucrose. The samples pretreated with 100% sucrose showed the highest amount of cells stained with CFDASE, whereas cells pretreated with 50% sucrose and frozen in liquid nitrogen showed the lowest amount of stained cells.

5. Discussion

Effect of induced stress on *E. faecium* M-74 during fluid bed process

One of the major problems associated with the development of probiotic formulations is the necessity of using several manufacturing steps that exert different kinds of stress on the microorganisms. Commonly used methods for stabilization of probiotics are spray-drying and freeze-drying. Using spray-drying, the exerted high temperature on the microorganisms affects a negative impact on cell viability. It is known that the removal of water during the spray-drying process “causes irreversible changes in the structural of membrane proteins and influences the integrity of bacterial membranes” [26]. Freeze-drying is considered the most convenient dehydration method, allowing easy and inexpensive shipping and handling of probiotic microorganisms. However, cell injury and loss of viability have been reported as common side effects after freeze-drying [16, 27, 28, 29, 30]. Referring to these drawbacks, the main aim of this thesis was the investigation of the feasibility of fluid bed technology as an alternative cell stabilization method.

Y. Mille et al. [31] dried *L. plantarum* and *L. bulgaricus* using a fluid bed dryer. However, as reviewed by Morgan et al. [13] there is insufficient knowledge regarding the suitability of this technology for the drying of probiotic bacteria.

In the present work we have investigated the influence of fluid bed process parameters on the culturability and cellular properties of *E. faecium* M-74. The following process parameters were investigated: Product bed temperature, atomizing air pressure, and process time as a result of spray rate. Before each run, the cells were pre-treated with 100% sucrose based on the wet cell mass. Culturability was studied counting colony forming units. Alterations in cellular properties were analyzed using two different fluorescent dyes. Propidium iodide shows the effect of process on membrane permeability and dihydrorhodamine 123 displays changes in hydrogen peroxide production. *E. faecium* M-74 produces hydrogen peroxide as a mechanism for limiting or eliminating of competitive flora or as a response to stress [32]. Our pre-studies showed an increase of hydrogen peroxide production of *E. faecium* M-74 after inducing a moderate stress, for instance temperature of 60 °C. Increasing the stress intensity resulted in the significant decrease of hydrogen peroxide production, indicating the collapse of the cellular redox system.

First of all the effect of product bed temperature on *E. faecium* M-74 was investigated. 40 °C, 50 °C and 60 °C were chosen for temperature settings. The higher the temperature, the less colony forming units were built by dried cells. Higher degrees of membrane damage and the collapse of cellular redox system were also observed by increasing the product bed temperature up to 60° C, indicating the crucial impact of heat on cell viability.

The mechanism of membrane damage as a result of heat is studied by several authors. According to J.H. Crowe [33] dehydration caused by heat can lead to

several sorts of membrane damage, including fusion, leakage, lateral phase separation, and non-bilayer phases.

X.C. Meng [16] reported on the cell membrane damage after dehydration as the result of removing the hydrogen-bonded water from the hydrophilic part of phospholipid bilayers and consequently increasing the head group packing and forcing the alkyl chains together. The lipid part goes through a transition from lamellar to gel phase, the chains are becoming stiff and fully extended. Thereby the membrane becomes more prone to damage.

Teixeira et al. [34] studied the impact of spray-drying on *Lactobacillus bulgaricus* and proposed that injury was “caused by melting of membrane lipids” around 50 °C. They reported that increased cell permeability during spray-drying was caused by the leakage of intracellular components.

Further, we have studied the impact of atomizing air pressure on cell viability, using 1 bar, 1.5 bar, 2.5 bar and 3.5 bar. Settings lower than 1 bar caused larger droplet size and consequently resulted in higher degree of humidity in process container and agglomeration. Choosing atomizing air pressure higher than 3.5 bar resulted in the drying of atomized cell suspension before reaching the pellets.

Dried cells obtained by using 1.5 bar atomizing air pressure showed higher culturability and lower level of membrane damage than the dried cells using other atomizing air pressures whereas higher pressures lead to mechanical stress and implicated friction forces. Lower atomizing air pressure below 1.5 bar resulted in higher humidity in the process container, which inhibited the complete drying of cells. The consequence of both cases was the loss of culturability and higher degrees of membrane damage of dried cells.

Process time was varied from 7 min (spray rate of 20 g/min) to 45 min (spray rate of 3 g/min). Process times shorter than 7 min required higher spray rates than the other process time settings, which caused larger droplet sizes and resulted in increased humidity in the process container and agglomeration. Process times longer than 45 min were combined with excessive thermal load.

The viability of cells dried during 15 min (spray rate = 7 g/min) was higher than other dried cells, as these cells showed higher culturability and less membrane damage. Comparisons of hydrogen peroxide production in dried cells with freshly harvested cells showed a significant increase of hydrogen peroxide production by dried cells using process time of 15 min. This indicates the induced moderate stress on the cells during the drying process. In contrary the level of hydrogen peroxide production of dried cells obtained by using other processing times was significantly less than freshly harvested cells, indicating the collapse of the cellular redox system.

The spray rate of 20 g/min that resulted in the drying of *E. faecium* M-74 in 7 minutes was too fast and caused higher humidity in the fluid bed process. Using the spray

rate of 3 g/min, i.e. the processing time of 45 min, *E. faecium* M-74 was exposed to heat and mechanical stress for a longer period of time.

As described above, the use of atomizing air pressures lower than 1.5 bar or spray rates higher than 7 g/min caused an increase of residual humidity in the process chamber. Results showed that residual humidity had a huge impact on *E. faecium* M-74, concerning its viability and cellular properties.

The effect of residual humidity on cell viability was investigated by S. Wong et al. [35] and G.F. de Valdez [36]. S. Wong et al. reported on the effect of spray-drying and freeze-drying on *Bifidobacterium Pseudocatenulatum* G4 and declared that lower residual moisture resulted in a higher survival rate but cells needed a minimum amount of water for better viability. G.F. de Valdez et al. researched on the effect of freeze-drying on Lactic Acid Bacteria and stated that the optimum residual moisture content depends on the species and physiological state of cells. They observed that a higher level of residual moisture resulted in a loss of native protein's structure because water interacted with cellular proteins. According to Wong et al. cellular water plays a crucial role in the stability of cells.

Investigation of the impact of fluid bed drying on the viability of unprotected cells showed a loss of 1.01 log units after drying. Moreover, the fluid bed process affected the cell membrane and hydrogen peroxide production of unprotected cells. As the next step, we have investigated the protective effect of different agents on the viability of cells during drying process. Following agents: glucose, sucrose, skim milk, and dextrin with a concentration of 10, 50 or 100% based on the wet mass of cells were used. A mixture of equal amounts of sucrose and skim milk was used as well.

Using a 100% sucrose base on the wet mass of cells provided the best protective effect on the cell membrane and culturability of dried *E. faecium* M-74. The second best protective impact could be observed using 100% skim milk based on the wet mass of cells. Neither glucose nor dextrin showed optimal protecting effect on the culturability and cell membrane during the fluid bed drying.

The protective impact of sucrose and skim milk on the cells during drying was also reported by C.A. Morgan, Crowe et al. and Champagne et al.:

In a review C.A. Morgan [13] discussed the protective impact of different agents, above all sugars such as sucrose on different organisms such as *Escherichia coli* DH5α and *Bacillus thuringiensis* HD-1 during the desiccation. The protective mechanism should be "due to the stabilization of membranes and proteins, by replacing the water around polar residues within these macromolecular structures".

Crowe et al. [33] suggested glucose, mannose, sucrose or ribose to "protect the functional integrity of respiratory proteins during dehydration". Even though they studied the influence of the freeze-drying process, their findings about dehydration can be discussed relating to heat stress. In their study they used sugars inhibited fusion and managed to prevent leakage. They suggested that sugars maintained the

lipids in a fluid phase during dehydration. During rehydration lipids did not pass from gel to liquid crystalline phase resulting no leakage.

The promising protective effect of skim milk is reported by Champagne et al. [37]. They described the ability of proteins containing in skim milk to provide a protective layer on the cell wall and stabilizing the cell membrane. In our experiments the stabilized cell membrane might also be more resistant to the mechanical stress during the process.

Viet et al. [38] reported on the protective effect of dextrin on Bacillus bacteria during spray-drying.

To conclude, we have successfully used fluid bed technology for the drying of *E. faecium* M-74, using the following parameter setting

Product bed temperature: 40 °C

Processing time: 15 min

Atomizing air pressure: 1.5 bar

We provided dried cells with enhanced viability during the drying process. The best protective effect could be achieved by treatment of cells with 100 % sucrose or skim milk (based on the wet cell mass) before fluid bed drying.

Still, the suitability of fluidized-bed technology as well as the kind and the concentration of protective agents for the drying of different probiotic strains should be optimized individually for each strain.

Effect of cryo-stress on *E. faecium* M-74

During the other part of the current thesis we studied the impact of freezing on the viability of *E. faecium* M-74. Freezing is a wide-applied method for the storage of cultures and is the first essential step of lyophilization, the common method for drying of microorganisms.

We have chosen different freezing conditions; *E. faecium* M-74 was frozen in liquid nitrogen at -196 °C, -20 °C and -80 °C. In addition cells were frozen at -80 °C under controlled freezing rate of -1 °C/min. Culturability was investigated by measuring of colony forming units. Alterations in membrane permeability, hydrogen peroxide production, membrane potential and intracellular pH were investigated using PI, DHR 123, DioC3(6), and CFDASE, respectively.

First experiments showed that freezing of unprotected *E. faecium* M-74 at liquid nitrogen affected the cell culturability and membrane permeability less than other freezing conditions. Not only do those cells show lower levels of membrane potential, but also decreased hydrogen peroxide production and lower intracellular pH.

Freezing of cells at -20 °C, by contrast, affected the cell culturability and membrane more than other conditions. These frozen cells showed the highest membrane potential, higher intracellular pH and more hydrogen peroxide production than cells frozen under other conditions, indicating the higher level of cryo-stress on these cells.

Comparing the viability of cells frozen at -80°C under controlled and uncontrolled condition showed the higher culturability and less membrane damage of cells when frozen under controlled condition. While, alterations in membrane potential and hydrogen peroxide production were comparable, the frozen cells under controlled condition showed lower levels of intracellular pH, indicating the lower level of stress involved with the controlled freezing as compared to the uncontrolled condition.

The mechanisms of intracellular freezing are not really clear. K. Muldrew and L.E. McGann [39] reported on the increase of osmotic pressure as the result of removing water from the solution during freezing. They reported that intracellular freezing was a consequence of this damage and plasma membrane was not able to be a barrier against extracellular ice. The osmotic pressure caused an efflux of water from cells.

M. Volkert et al. [40] reported on the effect of freezing rate, location of ice nucleation in the cells, the ice crystal growth, and crystal size. They found out that not only membrane damage increased at high freezing rates due to faster osmotic flux, but also mechanical forces increased. In addition, they reported that cellular damage was related to the concentration of the used solution.

P. Mazur [41] proposed a general explanation of the effect of freezing rate; and discussed a two-factor theory: If freezing is too fast, intracellular water cannot remove, which causes the building of intracellular ice. Slow freezing is injurious as well as ice forms outside of the cells and intracellular freezing leads to destabilized cell membranes. Yet, freezing rate should be optimized for the different cell types.

Following our results *E. faecium* M-74 frozen at -80 °C under controlled condition showed better culturability and less membrane damage than the cells frozen uncontrolled at -80 °C. However, the best results were obtained by freezing the cells in liquid nitrogen. These cells demonstrated higher culturability and a lower level of membrane damage than frozen cells used under any other condition.

Apart from this we have also studied the effect of two cryoprotectants on the viability of *E. faecium* M-74 after freezing. Liquid nitrogen or uncontrolled freezing at -80 °C were chosen as the best or conventional freezing conditions, respectively. Sucrose and glucose in the concentrations of 50% and 100% based on the wet cell mass were used as protectant.

Both glucose and sucrose showed cryo-protective effect on the *E. faecium* M-74, although the impact of sucrose was superior. Results showed that treatment of cells with 50% sucrose protected the cell membrane against the cryo-stress more than other used concentrations. The alterations in membrane potential and membrane

permeability of frozen cells protected with 50% sucrose were less than other frozen cells.

According to Hubalek [42], glucose and sucrose are nonpermeable compounds with extracellular protective mechanism. Such nonpermeable additives form a viscous layer on the cell surface and increase the viscosity of solution. That inhibits the growth of ice crystals. Ray et al. [43] reported on the preventive effect of nonpermeable additives on the lipopolysaccharides on the surface of cells against cryo-stress.

S.B. Leslie et al. [29] proposed in their work possible requirements for the mechanism of cryo-protection. During freeze-drying the headgroups of lipid bilayers were brought closer when water removed. “The increase in attraction between the acyl chains forces the dry bilayer into the gel phase at room temperature, which could lead to phase separation of membrane components. When the dry lipid is rehydrated it undergoes a phase transition from the gel back to the liquid crystalline phase, during which leakage may occur.” Drying with sucrose can maintain the lipid in the liquid phase at room temperature, which could prevent damaging effects.

We should bear in mind that the impact of cryo-stress and the protective effect of different agents on the viability of probiotic microorganisms are different for each strain.

6. Summary

The impacts of fluid bed drying and freezing on the culturability and cellular properties of *E. faecium* M-74 were studied.

E. faecium M-74 was dried in a fluid bed dryer and the efficiency of sucrose, skim milk, glucose and dextrin for protection against the general stress involved with the drying process have been investigated. After each drying process the cell culturability has been determined by spreading the rehydrated cells on kanamycin esculin azide agar plates and calculating the numbers of colony forming units. The cell membrane damage and the change of hydrogen peroxide production of cells have been investigated after exposure of rehydrated cells to the fluorescent dyes propidium iodide and dihydrorhodamine 123. The fluorescent measurements have been undertaken using a microplate reader.

The concentration of 100% sucrose based on the wet mass of cells provided the best protection for the cells against the stress. It could prevent the effect of stress on the culturability and cellular properties of *E. faecium* M-74.

Furthermore, product bed temperature, process time as a result of spray rate and atomizing air pressure have been chosen as individual stress factors involved with the drying process. The effect of these parameters on the cell reproduction and cellular properties of protected *E. faecium* M-74 has been investigated. 100% sucrose based on the wet mass of cells was used to protect the cells from mechanical stress involved with the drying process. The same methods as above were used to investigate cell's culturability and the level of membrane permeability and hydrogen peroxide production.

Product bed temperature had the most noticeable effect on the cell viability, cell membrane permeability and the production of hydrogen peroxide. We have successfully used fluid bed technology for the drying of *E. faecium* M-74, using the following parameter setting

Product bed temperature: 40 °C

Processing time: 15 min

Atomizing air pressure: 1.5 bar

For the second part of the survey *E. faecium* M-74 was frozen at different temperatures. Approximately -200 °C in liquid nitrogen, -80 °C uncontrolled in a freezer, stepwise freezing to -80 °C and -20 °C in a freezer were chosen. Cell culturability and different cellular properties were investigated.

Different fluorescent dyes were used and the measurements were carried out with the microplate reader and the flow cytometer.

Frozen in liquid nitrogen *E. faecium* M-74 suffered the least from cryo-stress. The worst method was freezing at -20 °C. There were differences between freezing the cells controlled or uncontrolled in -80 °C. The controlled loss of temperature affected a lower level of cell damage.

Next sucrose and glucose in 100% and 50% concentration based on the wet mass of cells were chosen to protect *E. faecium* M-74 from cryo-stress. Cryo-stress at -80 °C and liquid nitrogen were investigated and it became obvious that the 50% concentration was the best, both sucrose and glucose.

7. List of abbreviations

E.faecium	**E**nterococcus **f**aecium
MRS-broth	de**M**an, **R**ogosa, **S**harpe
L	**l**iter
g/l	**g**ram per **l**iter
H	**h**our
rpm	**r**otations **p**er **m**inute
Min	**m**inutes
PI	**p**ropidium **i**odide
FDA	**f**luorescein **d**i**a**cetate
DHR 123	**d**i**h**ydro**r**hodamine 123
DioC6(3)	3,3` d**i**hexyl**o**xacarbo**c**yanine iodide
CFDASE	**c**arboxy **f**luorescein **d**i**a**cetate **s**uccinimidyl **e**ster
MW	**m**olecular **w**eight
DMSO	**d**i**m**ethyl **s**ulf**o**xide
CFU	**c**olony **f**orming **u**nits

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Illustrations

Illustration 1: http://efaecium.mlst.net/; accessed March 22nd, 2009

Illustration 2: S. Salar-Behzadi, S. Toegel, H. Viernstein (2008). Innovations in Coating Technology. Recent Patents in Drug Delivery and Formulation, 2, 209-230

Illustration 3: http://www.glatt.com/e/01_technologien/01_04_08.htm; accessed March 22nd, 2009

9. Zusammenfassung

Verschiedenste Präparate enthalten heutzutage probiotische Mikroorganismen. Eine Herausforderung bleibt dabei noch immer die möglichst schonende Verarbeitung. Die klassischen Methoden, auf die oft zurückgegriffen wird, sind Lyophilisation und Sprühtrocknung. Beide Varianten verursachen Stress für die Bakterien, sichtbar anhand koloniebildender Einheiten und zellulärer Eigenschaften. Das Wirbelschichtverfahren bietet eine moderate Methode für Granulierung und Trocknung.

Anhand *Enterococcus faecium* M-74 sollte in dieser Arbeit festgestellt werden, ob das Wirbelschichtverfahren für die Trocknung von Bakterien geeignet ist. Für diesen Zweck wurde *E. faecium* M-74 in einer Wirbelschichtanlage auf kristalline Mikrozellulose-Pellets aufgesprüht.

Der Zustand der Bakterien wurde einerseits durch die Anzahl der koloniebildenden Einheiten auf Agarplatten überprüft, andererseits wurden verschiedene fluoreszierende Farbstoffe verwendet. Der Grad der Zerstörung der Zellmembran wurde mit Propidiumiodid detektiert, mit Dihydrorhodamin 123 wurde die Wasserstoffperoxidproduktion nachgewiesen. Die Fluoreszenzmessungen wurden mit einem Microplate Reader durchgeführt.

Verschiedene Substanzen wie Saccharose, „Skim milk“ (Magermilchpulver), Glukose und Dextrin wurden in unterschiedlichen Konzentrationen (10%, 50% und 100% bezogen auf die Feuchtmasse an Bakterien) hinzugefügt, um die Mikroorganismen vor allzu großer Zerstörung und Stress zu schützen.

Bei dieser Versuchsreihe stellte sich heraus, dass 100% Saccharose bezogen auf die Bakterien-Feuchtmasse die besten Ergebnisse bezüglich der Lebensfähigkeit, des Zustands der Zellmembranen und der Wasserstoffperoxidproduktion zeigten.

100% Saccharose wurde auch ausgewählt, um *Enterococcus faecium* M-74 in der folgenden Versuchsanordnung zu schützen. Der Einfluss der einzustellenden Parameter auf die Mikroorganismen - Prozesstemperatur, Prozesszeit resultierend aus der Sprührate und der verwendete Sprühdruck während des Wirbelschichtprozesses - wurde ebenfalls überprüft. Die Temperatur schwankte zwischen 40 und 60°C, die Prozesszeiten variierten zwischen 7 und 45 Minuten, der verwendete Sprühdruck zwischen 1 und 3 bar. Die gleichen Methoden wie oben erwähnt wurden verwendet, um die Lebensfähigkeit, Zustand der Zellmembran und die Wasserstoffperoxidproduktion zu eruieren.

Die Prozesstemperatur zeigte den größten Effekt auf die Viabilität der Bakterien. Zusammenfassend lässt sich zu dieser Versuchsreihe sagen, dass mit 40 °C, 15 Minuten und 1.5 bar die besten Ergebnisse bezogen auf die Lebensfähigkeit, den Zustand der Zellmembran und die Wasserstoffperoxidproduktion während des Wirbelschichtprozesses für *Enterococcus faecium* M-74 erreicht wurden.

Das Wirbelschichtverfahren bietet eine gute Möglichkeit, *E. faecium* M-74 unter Zuhilfenahme geeigneter Protektantien und Prozesseinstellungen zu stabilisieren.

Neben den Auswirkungen des Wirbelschichtverfahrens auf die Mikroorganismen wurde auch der Effekt von Kältestress untersucht. In einer neuen Versuchsanordnung wurden die Effekte von -20 °C, -80 °C und -200 °C in flüssigem Stickstoff auf *E. faecium* M-74 ermittelt. Bei -80 °C wurden auch verschiedene Arten des Einfrierens untersucht. Einerseits wurden die Bakterien schonungslos im Tiefkühler bei -80 °C gekühlt, andererseits wurden sie langsam auf diese Temperatur mit einer Rate von -1 °C pro Minute gebracht. Bei diesem Teil der Arbeit sind weitere fluoreszierende Farbstoffe zum Einsatz gekommen. Neben den schon erwähnten wurden der Hyperpolarisationsgrad der Membran mit Dihexyl-oxacarbocyaniniodid und der intrazelluläre pH-Wert mit Carboxyfluorescindiacetatsuccinimidylester mit dem Microplate Reader und dem Durchflusszytometer gemessen.

Bei -200 °C zeigten die Bakterien das geringste Maß an Schaden, sie bildeten die meisten kolonieformenden Einheiten aus. Das stufenweise Einfrieren auf -80 °C brachte ebenfalls gute Ergebnisse, als schlechteste Methode entpuppte sich das Einfrieren auf -20 °C.

In einem weiteren Versuch wurde die Eignung von Saccharose und Glucose mit 50% und 100% bezogen auf die Feuchtmasse an Bakterien als Protektantien während des Einfrierens getestet. Die Bakterien wurden in flüssigem Stickstoff und bei -80 °C eingefroren. In diesem Fall zeigten interessanterweise sowohl 50% Saccharose als auch 50% Glucose die besseren Ergebnisse.

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EFFECT OF STRESS ON CELLULAR PROPERTIES OF ENTEROCOCCUS FAECIUM

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During the recent decade, the interest on probiotic products has been growing due to the increasing evidence of health benefits associated with their use. The essential step for the development of such products is the stabilization (drying) of probiotic microorganisms. The classical methods for cell stabilization are lyophilization and spray drying[1].

Fluid bed technology is an approved method for gentle drying as well as for mixing, granulation and coating processes[2].

The aim of this study was the investigation of the feasibility of this technology as an alternative cell stabilization method.

Enterococcus faecium M-74 has been used as probiotic microorganism and dried in a fluid bed dryer. The efficiency of following excipients: glucose, sucrose, dextrin and skim milk for protection of *E. faecium* M-74 against the general stress involved with the drying process have been investigated[3]. Furthermore, the product bed temperature, the process time and the atomizing air pressure have been chosen as individual stress factors involved with the drying process. The cell membrane damage and the change in hydrogen peroxide production of cells have been investigated after exposure of rehydrated cells to the fluorescent dyes propidium iodide and dihydrorhodamine 123, respectively.

The concentration of 100% sucrose, based on the wet mass of cells, provided the best protection for the cells against the stress. The product bed temperature had a significant effect on the cell viability, cell membrane permeability and the production of hydrogen peroxides.

The investigations showed that fluid bed drying is a feasible cell stabilization method. However, the selection of suitable protectants and parameter settings are important for the reliable application of this method for drying probiotic microorganisms.

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