DIPLOMARBEIT

Titel der Diplomarbeit

Survey of heat stress response of Enterococcus faecium using fluorimetry and flow cytometry

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1. Introduction

Probiotics

The World Health Organisation defines probiotics as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'[1]. Most probiotic products contain bacteria from the genera Lactobacillus or Bifidobacterium, although other genera, including Escherichia, Enterococcus, Bacillus and Saccharomyces have also been marketed as probiotics [2]. For informing consumers about the product, it is recommended that the following information be described on the label of probiotics: [1]

- Genus, species and strain designation. Strain designation should not mislead consumers about the functionality of the strain.
- Minimum viable numbers of each probiotic strain at the end of the shelf-life
- The suggested serving size must deliver the effective dose of probiotics related to the health claim.
- Health claim(s)
- Proper storage conditions
- Corporate contact details for consumer information

It is known that microorganisms in the large intestine complete the digestion process on any food components that were not digested in the small intestine, such as lactose in lactose intolerant people or fibers resistant to the enzymes present in the small intestine. There is evidence of non-digestive microbial activities as well. Certain intestinal microorganisms are known to produce vitamins. Also, in studies done with special microorganism-free laboratory animals, evidence is strong that without normal microbial populations, the immune system functions poorly, and resistance to pathogenic bacteria is greatly reduced [2].

Effects of probiotics

Probiotics exert their benefits through several mechanisms; they prevent colonization, cellular adhesion and invasion by pathogenic organisms, they have direct antimicrobial activity and they modulate the host immune response. The strongest evidence for the clinical effectiveness of probiotics has been in their use for the prevention of symptoms of lactose intolerance, treatment of acute diarrhea, attenuation of antibiotic-associated gastrointestinal side effects and the prevention and treatment of allergy manifestations [3]. Although several products are

existing for prevention of travelers' diarrhea, dental caries and for pediatric application, more research needs to be carried out to clarify conflicting findings on the use of probiotics for these applications.

Microorganisms in probiotics

Practically all organisms used in probiotic foods or food supplements are representatives of the genera Lactobacillus, Enterococcus or Bifidobacterium. Food products or supplements as well as pharmaceutical preparations containing viable probiotic strains are supplied on the market either as fermented food commodities or in lyophilized form.

In Germany, species such as Lactobacillus rhamnosus, Enterococcus faecium and Enterococcus faecalis have been grouped into risk group 2. The investigators responsible for this classification, however, concede that strains of these species with a documented safe history may belong to a risk group 1 (species constituting no risk). Such strains that have found application in food fermentations or certain probiotic products for a long time are considered as safe [4].

Risk group classification of prokaryotes

The European Community classification of the risk groups of prokaryotes is based on the lists published by the French Official Gazettes and the European Council Directives.

Group 1	A biological agent that is most unlikely to cause human disease
Group 2	A biological agent that may cause human disease and which might be a hazard to laboratory workers but is unlikely to spread in the community. Laboratory exposure rarely produces infection and effective prophylaxis or treatment is available.
Group 3	A biological agent that may cause severe human disease and present a serious hazard to laboratory workers. It may present a risk of spread in the community but there is usually effective prophylaxis or treatment.
Group 4	A biological agent that causes severe human disease and is a serious hazard to laboratory workers. It may present a high risk of spread in the community and there is usually no effective prophylaxis or treatment.

Figure 1: Risk group classification of prokaryotes

[5]

The genus Enterococcus

Enterococci have first been described by Thiercelin (1899) as well as by Thiercelin and Jouhaud (1903) as gram positive diplococci which are immotile.

Besides the resistance of Enterococci against ampicillin and aminoglycosides, resistance against glycopeptides has been noticed. Many strains of Enterococcus faecalis and Enterococcus faecium show resistance against various antibiotics and are discussed as a cause of nosocomial infections like endocarditis, sepsis and urinary tract infections.

Enterococci can be found in milk and milk products except in ultra high temperature milk or unless the products have been sterilized. They further appear in cheeses which have been manually produced. In some types of cheese, like the Greek Kefalotyri or Spanish Cebreiro, Enterococci can be ranked among the dominating microorganisms [6].

About 80 selective media for the detection of enterococci are known. Currently, Slanetz-Bartley agar and Kanamycin esculin azide agar are being considered most suitable. The formation of black colonies on Kanamycin esculin azide agar is an indicator for the presence of enterococci [6].

Enterococcus faecium

Enterococcus faecium and Enterococcus faecalis are the most frequently detected enterococcal species in the human gastrointestinal tract.

Since the late 1970s, Enterococcus faecium and Enterococcus faecalis have been identified as causes of nosocomial infections, inducing increased incidence of endocarditis, bacteremia, urinary tract, and neonatal infections. Other enterococci have rarely been reported to be pathogenic to humans.

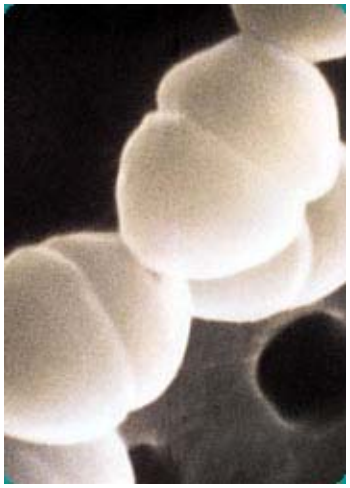

Figure 2: Enterococcus faecium

On the other hand, these two enterococcal species occur in raw and processed meats, as well as in fermented meat and dairy products, in particular, traditional cheeses in which they may contribute to ripening and product flavor [7].

Enterococcus faecium is contained in several probiotic products which are meant for prevention and treatment of intestinal disorders, especially in animal health products.

Formulation of probiotic products

Beside the capsule as the most popular dosage form for probiotics, probiotic tablets, powders and drops are available. The microorganisms contained need to be alive for showing their beneficial effects. However, during the steps of processing, certain sources of stress such as mechanical forces, temperature or osmotic stress can occur.

One of the methods used is microencapsulation, described by Champagne and Fustier [8]. According to their review, at least five microencapsulation methods have been applied to probiotics: spray-coating, spray-drying, extrusion, emulsion and gel-particle technologies. Most commercial products are spray-coated products. Spray-coating technology is promising, considering large-scale production possibilities when selecting a system for study. However, spray-coating is a difficult technique to master and most information on the technology is proprietary. In addition to spray-coating and gel-particle technologies, methodologies that employ extrusion, emulsion and spray-drying have also been explored for the microencapsulation of probiotics [8].

Spray-drying is an economical process for preparing industrial scale quantities of viable microorganisms. It offers high production rates and low operating costs and is a method commonly used to prepare food adjuncts, which are stable, dry and occupy small volume. However, the spray-drying of probiotic bacteria presents a number of challenges, in particular, the requirement to maintain culture viability, given the high processing temperatures encountered [9].

In order to adapt cells to high temperatures, it is necessary to find out more about their tolerance against heat. For this reason, they can be kept in cell cultures, so as to investigate their features and their cellular activity.

Bacterial cell culture

In laboratories, bacteria can be kept at -20°C in their lyophilised form or in suspension with culture media and protectant. For working with them, bacteria from these frozen cultures have to be transferred into fresh media. Depending on the bacteria, an appropriate temperature for growth has to be chosen.

Culture media

Microbiological culture media are used to detect and enumerate bacteria and other micro-organisms in clinical, food, and environmental samples. Production laboratories in the food and pharmaceutical industries use culture media to monitor hygiene standards within the factory and to measure the microbial content of both raw materials and finished product [10].
Different species of bacteria have different needs and this is why a variety of culture media has been developed. They consist of nutrients like anorganic salts, but can also contain organic components.
Culture media can be fluid as well as solid. In fluid media, bacteria are in suspension; in solid media, bacteria can form colonies on the surface.
In these cultures, bacteria can grow and when the stationary growth phase is reached, they can be used for investigation.

Growth phases of bacteria

Bacteria added to fresh media typically go through four more or less distinct phases of growth: [11]

- Lag phase
- Log (logarithmic or exponential) phase
- Stationary phase
- Decline (death) phase

Lag phase

Transferring bacteria from one medium to another - chemically different - medium, typically results in a lag in cell division. This lag in division is associated with a physiological adaptation to the new environment by the cells, prior to their resumption of division.

Log phase (logarithmic phase, exponential phase)

Lag phase is followed by log phase during which binary fission occurs. This phase of growth is called logarithmic or exponential because the rate of increase in cell number is a multiplicative function of cell number.

The rate of increase is a function of absolute cell number - the more cells are present, the faster the population of cells increases in size during log phase.

Stationary phase

Stationary phase is a steady-state equilibrium where the rate of cell growth is exactly balanced by the rate of cell death. Cell death (or, at least, lack of cell growth) occurs because of a loss of limiting nutrients or an accumulation of toxins.

Decline phase (death phase)

Stationary phase, in a standard bacterial growth curve, is followed by a die-off of cells. Cell death in bacteria cultures basically means that the cells are unable to resume division following their transfer to new environments. Typically this die-off occurs exponentially.

Cell death occurs because vegetative cells can survive exposure to harsh conditions (few nutrients or too many toxins) only for a limited period of time.

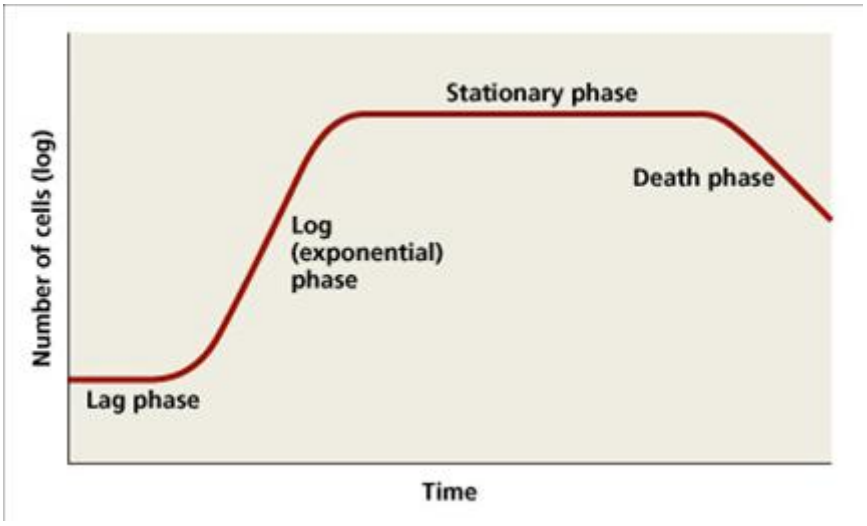

Figure 3: growth phases of bacteria

Aims of the thesis

The aim of this thesis was to investigate the effect of heat stress on cell viability and activity of the probiotic microorganism Enterococcus faecium M-74. Specific fluorescent dyes were selected to monitor changes in cell membrane permeability, esterase activity, ROS generation, membrane potential and intracellular pH of Enterococcus faecium M-74 exposed to heat. The cellular characteristics were studied using both fluorimetry and flow cytometry techniques.

Enterococcus faecium was chosen as a model for robust probiotic microorganisms. The results of the current study should be compared to the stress responses going on more sensitive probiotic strains.

Fluorescent dyes

Five different fluorescent dyes were used during the survey: propidium iodide (PI), fluorescein diacetate (FDA), dihydrorhodamine 123 (DHR 123), 3,3'-dihexyloxacarbocyanine iodide (DioC6(3)) and carboxy fluorescein diacetate succinimidyl ester (CFDASE). Each of them was meant to show changes in bacterial cells due to heat stress.

Propidium iodide

PI (MW 668.4) binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA. PI also binds to RNA. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20- to 30-fold. When bound to nucleic acids, the absorption maximum for PI is 535 nm and the fluorescence emission maximum is 617 nm [12].

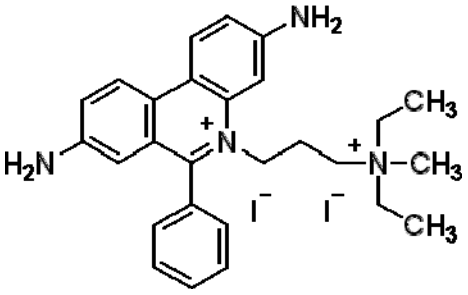

Figure 4: Propidium iodide

Propidium iodide is suitable for fluorescence microscopy, confocal laser scanning microscopy, flow cytometry and fluorimetry.

In general, PI is membrane impermeant and excluded from viable cells but can penetrate impaired cell membranes of dying or dead cells. Therefore, PI is commonly used for identifying dead cells in a population and as a counterstain in multicolor fluorescent techniques.

PI is a known mutagen. Solutions containing PI should be poured through activated charcoal before disposal. The charcoal must then be incinerated to destroy the dye.

Fluorescein diacetate

FDA (MW 416.38) is a non-fluorescent hydrophobic fluorescein derivative that can pass through the cell membrane whereupon intracellular esterases hydrolyze the diacetate group producing the highly fluorescent product fluorescein. The fluorescein molecules accumulate in cells that possess intact membranes so the green fluorescence can be used as a marker of cell viability. Cells that do not possess an intact cell membrane or an active metabolism may not accumulate the fluorescent product and therefore do not exhibit green fluorescence.

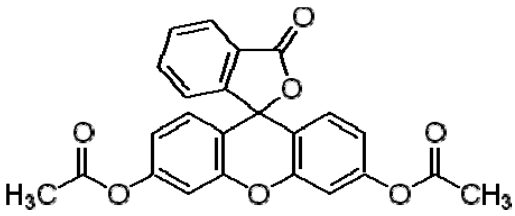

Figure 5: Fluorescein diacetate

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 [13].

Dihydrorhodamine 123

DHR 123 (MW 346) is the reduced form of rhodamine 123, which is a commonly used fluorescent mitochondrial dye. DHR 123 itself is nonfluorescent, but it readily enters most of the cells and is oxidized by oxidative species or by cellular redox systems to the fluorescent rhodamine 123 that accumulates in mitochondrial membranes.

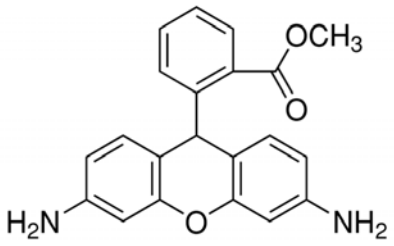

Figure 6: Dihydrorhodamine 123

The white solid DHR 123 is useful for detecting reactive oxygen species including superoxide (in the presence of peroxidase or cytochrome c) and peroxynitrite. It is soluble in DMSO and has to be stored at -20°C and protected from air and light, especially when in solution [14].

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

3,3'-Dihexyloxacarbocyanine iodide

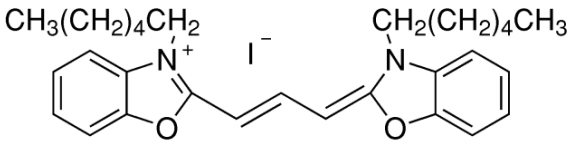

Figure 7: 3,3'Dihexyloxacarbocyanine iodide

DioC6(3) (MW 572.53) is a carbocyanine derivative with short alkyl tails (<7 carbon atoms). It accumulates on hyperpolarized membranes by translocation into the lipid bilayer and aggregation that decreases their fluorescence. It is widely used for cell membrane potential measurements in flow cytometry.

DioC6(3) is soluble in DMSO and DMF. The absorption maximum is 484 nm and the fluorescence emission maximum is 501 nm [15].

Carboxy fluorescein diacetate succinimidyl ester

The intracellular pH of bacteria is crucial for various cellular processes. CFDASE (MW 557.46) is one of the most commonly used fluorescent probes for measurements of intracellular pH, since its fluorescence intensity is highly pH dependent [16].

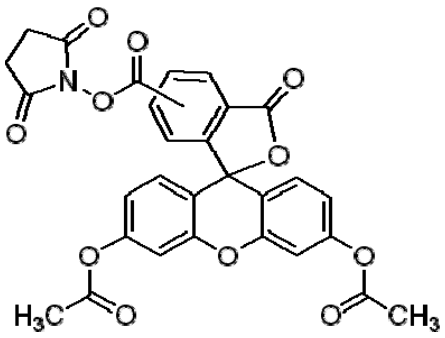

Figure 8:
Carboxyfluorescein diacetate succinimidyl ester

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

2. Materials and Methods

Fluorescent dyes were purchased from Sigma, other chemicals and culture media were purchased from Merck, Germany, unless otherwise stated.

Cultivation of Enterococcus faecium M-74

Enterococcus faecium M-74 (Chr. Hansen, Sweden) was grown at 37°C in the fluid culture medium MRS broth.

MRS broth (g/l):

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

100 µl of bacterial suspension were transferred to 10 ml of MRS broth under laminar airflow after 24 hours of growth.

Harvesting and washing of Enterococcus faecium M-74

After centrifugation at 8000 rpm for 10 minutes, the cells were washed twice with phosphate buffer (pH 6.8) and subsequently resuspended in 10 ml of the same phosphate buffer.
Hereafter, whenever “phosphate buffer” is mentioned, phosphate buffer with citric acid (pH 6.8) of the below described composition is meant, unless otherwise stated.

Phosphate buffer with citric acid (pH 6.8)

di-Sodium hydrogen phosphate · $2H_2O$	13.70 g/l
Sodium diyhdrogen phosphate · $2H_2O$	3.65 g/l

Determination of colony forming units

In order to determine the amount of bacteria in samples, colony forming units were counted on Kanamycin esculin azide agar plates.

Nutrient medium:

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

A dilution series was made, adding 1 ml of the suspension of bacteria to 9 ml of phosphate buffer and mixing them. Then, 1 ml was taken from this dilution and added to another 9 ml of phosphate buffer. With this method, seven dilutions were prepared. For transferring bacteria to agar plates, 0.1 ml was taken from one of the dilutions and spreaded onto an agar plate using a glass spreader. Three agar plates were prepared following this procedure from dilutions five, six and seven.

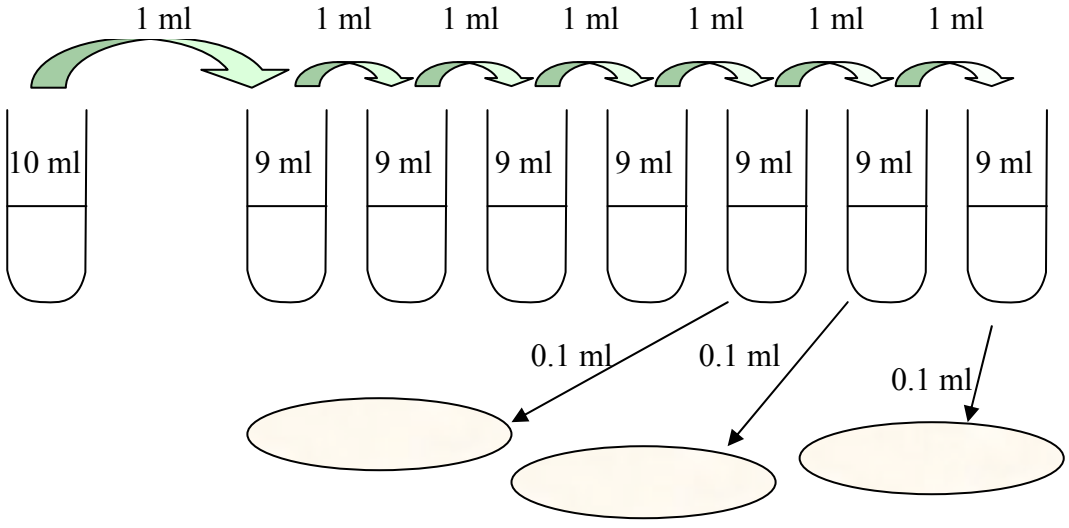

Figure 9: Dilution series for determination of colony forming units of E. faecium M-74

Investigation of the period of stationary phase culturing Enterococcus faecium M-74

To find out when the stationary phase of Enterococcus faecium M-74 was reached, samples were taken every two hours, beginning 20 hours after transferring Enterococcus faecium M-74 into MRS broth. The longest interval investigated was 52 hours.

For this purpose, 1 ml of bacterial suspension in MRS broth was transferred into 9 ml of phosphate buffer and thus diluted. Six more dilutions were made, always adding 1 ml of the previous dilution to 9 ml of phosphate buffer. Between these steps, the dilutions were always mixed with a vortex mixer. Three agar plates - using Kanamycin esculin azide agar - were prepared of dilutions five, six and seven. These plates were incubated at 37°C for at least 48 hours.

Heat stress

In order to prepare samples for heat stress, the first dilution (D1) of the original 10-ml-suspension of bacteria was used. 500 µl each were pipetted into safe lock tubes.

Heat stress was generated using a thermomixer suitable for safe lock tubes with a volume of 1.5 ml. The tubes filled with 500 µl of D1, were placed in the thermomixer and, under rotation at 650 rpm, were heated for certain durations at predefined temperatures.

For fluorescence measurement using a microplate reader:

50°C: 1’, 2’, 4’, 10’, 30’, 1h

60°C: 1’, 2’, 4’, 6’, 10’

70°C: 1’, 2’, 4’, 6’, 10’

80°C: 1’, 90’’, 2’, 3’, 4’

90°C: 30’’, 1’, 90’’, 2’, 4’

For fluorescence measurement using a flow cytometer:

50°C: 1’, 2’, 4’, 10’

60°C: 1’, 2’, 4’, 6’, 10’

70°C: 1’, 2’, 4’, 6’, 10’

80°C: 30’’, 1’, 2’, 4’

90°C: 30’’, 50’’, 1’, 90’’, 2’

The durations of heat stress used for flow cytometry were based on the results attained with fluorescence measurement by means of microplate reading. For each experiment, one sample of unstressed bacteria was prepared as control.

Fluorescent dyes

Working solutions of fluorescent dyes were prepared by dilution of stock solutions with phosphate buffer. 500 µl of the working solution were added to each of the samples.

The safe lock tubes were covered with aluminium foil to make sure that light could not influence the stability of the fluorescent dyes.

One sample containing a fluorescent dye was prepared as control.

Propidium iodide

To make a stock solution from the solid form, PI was dissolved in deionized water at 1 mg/ml (1.5 mM), stored at 4°C and protected from light. A working solution of 1.2 µl stock solution per 1 ml phosphate buffer was freshly prepared before use.

For flow cytometric measurements, PI was measured in slot 3.

Fluorescein diacetate

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

For flow cytometric measurements, FDA was measured in slot 1.

Dihydrorhodamine 123

The concentration used for the stock solution was 5 mM in DMSO. A working solution in phosphate buffer with the concentration of 25 µM was freshly prepared before use.

For flow cytometric measurements, DHR 123 was measured in slot 1.

3,3'-Dihexyloxacarbocyanine iodide

The concentration used 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.

For flow cytometric measurements, DioC6(3) was measured in slot 1.

Carboxy fluorescein diacetate succinimidyl ester

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.

For flow cytometric measurements, CFDASE was measured in slot 1.

After having added 500 µl of the working solution of a fluorescent dye, the samples were mixed with a vortex mixer and incubated at 37°C under exclusion of light. For all fluorescent dyes, with the exception of PI, an incubation time of one hour was used. For PI, an incubation time of 15 minutes was sufficient. Before measuring, the samples were mixed again with a vortex mixer.

Influence of pH value on fluorescence of CFDASE

Buffers of several pH values between 0.91 and 10.00 were prepared. The solutions used were 0.1 M HCl (pH 0.9), 3% acetic acid (pH 2.9), phosphate buffer (pH 6.4), HEPES buffer (pH 7.0) and borate buffer (pH 9.0). For receiving a wider range of pH values, further solutions were prepared adding NaOH to the solutions of 3% acetic acid, HEPES buffer and borate buffer, whereas HCl was added to phosphate buffer. As in the assays with Enterococcus faecium M-74, the samples were incubated at 37°C for one hour in order to simulate the conditions of the previous assays with the bacteria.

Fluorimetry

96 well plates were used for measuring fluorescence in a microplate reader. 50 µl of each sample was pipetted into wells in quadruplicate.

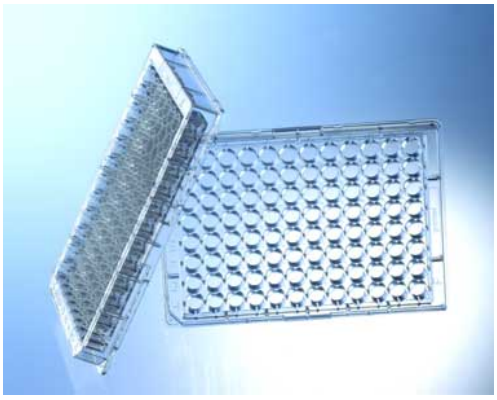

Figure 10: 96 well microplate

The photometric measuring principle is based on detecting the light emitted as a result of fluorescence. The excitation light passes through the sample containing the fluorescent substance. The latter converts part of the incident light of wavelength λ into fluorescent light of a greater wavelength λ_1. The light created in this manner radiates in all directions and can be measured with a detector set perpendicular to the axis of the incident light.

For low concentrations, the radiant flux of fluorescent light is proportional to the concentration of the fluorescing substance. The main reasons for using fluorescence measurement are its relatively high selectivity and sensitivity compared with absorption measurement [17].
To receive more reliable data with the microplate reader than in the initial trials, triplicates of samples were used. This means, that in every assay, three identical samples were prepared, stressed and mixed with a working solution of a fluorescent dye. These samples were prepared and measured simultaneously.

Flow cytometry

Flow cytometry is a method for quantitating 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 also be used to count cells of different types in a mixture.
Flow cytometers have been commercially available since the early 1970's, and their use has been increasing since then. The most numerous flow cytometers are those used for complete blood cell counts in clinical laboratories - these do not employ fluorescence. More versatile research instruments employ fluorescence, hence may be distinguished as flow cytofluorometers. The cells may be alive or fixed at the time of measurement, but must be in monodisperse (single cell) suspension. They are passed through a laser beam by continuous flow of a fine stream of the suspension. Each cell scatters some of the laser light, and also emits fluorescent light excited by the laser [18].

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 certain wavelengths

Flow cytometers involve sophisticated fluidics, laser optics, electronic detectors, analog to digital converters, and computers. The optics deliver laser light focused to a beam a few cell diameters across. The fluidics hydrodynamically focus the cell stream to a small fraction of a

cell diameter, and, in sorters, break the stream into uniform-sized droplets to separate individual cells. The electronics quantitate the faint flashes of scattered and fluorescent light, and, under computer control, electrically charge droplets containing cells of interest so that they can be deflected into a separate test tube or culture wells. The computer records data for thousands of cells per sample, and displays the data graphically. [18]

For measuring fluorescence with the flow cytometer, 1 ml of sterile-filtered phosphate buffer was pipetted into a provided tube and 50 µl of the sample were added. After mixing with a vortex mixer, the assay could be started. The flow cytometer measured 3000 cells per sample (n = 3).

Effect of heat stress on reproduction

In order to find out how heat stress affects the ability of reproduction of Enterococcus faecium M-74, samples of Enterococcus faecium M-74, which had been centrifuged, and washed with phosphate buffer, were resuspended in 10 ml of phosphate buffer. Then, they were exposed to heat using the thermomixer.

50°C: 1', 2', 4', 6', 30', 1h

60°C: 1', 2', 4', 6'

70°C: 1', 2', 4', 6'

80°C: 30'', 1', 2', 4', 6'

90°C: 30'', 1', 2', 4'

Dilutions of these stressed samples were prepared (1 ml + 9 ml). 100 µl of a sample were spreaded onto a Kanamycin agar plate. Three dilutions were chosen for this purpose and three plates per dilution were prepared.

Figure 11: petri dishes

After an incubation of at least 48 hours, the plates chosen to count showed 0 to 300 colony forming units. The stressed samples were compared to an unstressed sample, which was utilized as initial value for "0 minutes stressed".

The stress trials for the temperatures 50°C, 60°C, 70°C and 80°C were performed on the same day and thus, are comparable. The stress trials for 90°C were done another day and at this occasion, the number of colony forming units in the unstressed sample was lower than in the

previous one. In order to ensure better comparability, all figures of the 90°C stress trials were adapted to the figures of the previous trials, using the initial number of unstressed bacteria of the previous trials and calculating the corresponding relative amount of bacteria of the stressed samples.

Statistics

Calculations were performed with the Microsoft Excel XP integrated statistics tools. The significance of differences in the resulting data was evaluated with the t-test. p-values < 0.05 were considered significant.

Preparation of reagents

Preparation of MRS broth

52.2 g of the dry medium were dissolved in 1000 ml distilled water and 10.0 ml each were filled into test tubes. The test tubes were closed with cap-o-test caps and autoclaved at 118°C for 15 minutes. They were stored at 4°C until use.

Preparation of phosphate buffer

$Na_2HPO_4 \cdot 2H_2O$ and $NaH_2PO_4 \cdot 2H_2O$ were dissolved in distilled water. 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 closed with cap-o-test caps and autoclaved for 15 minutes at 121°C and stored at 4°C until use.

For flow cytometry, only sterile-filtered phosphate buffer was used. For this, 500 ml of phosphate buffer were filtered through a sterile filter with a mesh opening of 0.22 µm under laminar airflow.

Preparation of Kanamycin esculin azide agar

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

Equipment

Incubator (Memmert GmbH + Co KG, Germany)

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

Safety bench (DIN 12950 Variolab Mobilien W90 Type SWB Waldner Electronics, Germany)

Vortex (Heidolph REAX 2000, Germany)

Petri dishes (Sterilin, Bibby Sterilin Ltd., UK)

Safe lock tubes 1.5ml (Eppendorf AG, Germany)

Thermomixer comfort (Eppendorf AG, Germany)

pH-meter (HANNA instruments PH211, Microprocessor pHMeter, Mauritius)

Centrifuge instrument (Sorvall®instruments, RC5C, Switzerland)

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

96 well polystyrene microplate, transparent (Greiner Bio-One, Austria)

Steritop® (0.22 µm, Millipore, Durapore™, USA)

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

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

3. Results

3.1. Investigation of the period of stationary phase of Enterococcus faecium M-74

According to literature, Enterococcus faecium enters the stationary growth phase after about 24 hours of growth in MRS broth. Early stationary growth phase is already reached after 18 hours [19], but this duration can vary due to the use of different nutrient media [20].

The beginning of stationary growth phase is the ideal time for harvesting bacteria, as they are more sensitive to stress in the phase of growth [21] and less numerous in the decline phase. In these other phases, the results of measurements could vary greatly and might not be reproducible. So harvesting the bacteria in the stationary phase is a way to ensure the reproducibility of the assays.

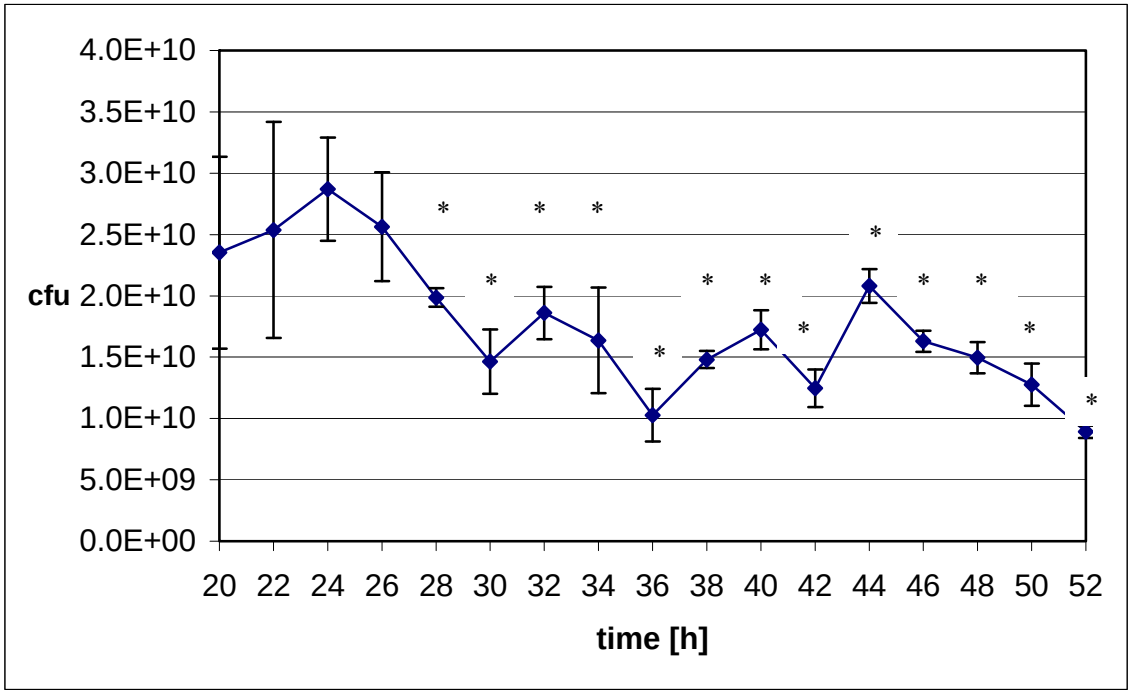

Figure 12: Determination of the stationary phase of E. faecium M-74

Significant differences (as compared to growth for 24 hours) are marked with asterisks ($p < 0.05$).

Samples were taken at certain times after transferring Enterococcus faecium M-74 to new MRS broth.

Figure 12 shows that after 20 hours of growth, averagely $23.5*10^9$ (± 33.24%) bacteria are alive. After 24 hours, the mean quantity lies at $28.7*10^9$ (± 14.63%). Only four hours later, the average number falls to $19.87*10^9$ (± 3.81%), which is a total drop of 30.78% in comparison to the number of bacteria counted after 24 hours of growth. These results show that the stationary phase is reached between 20 and 26 hours of growth.

3.2. Effect of heat stress on reproduction

The samples were stressed with temperatures from 50°C to 90°C for certain durations, as described in chapter 2. In order to determine the effect of heat stress on the reproduction of Enterococcus faecium M-74, samples of stressed bacteria were spreaded onto agar plates and colony forming units were counted.

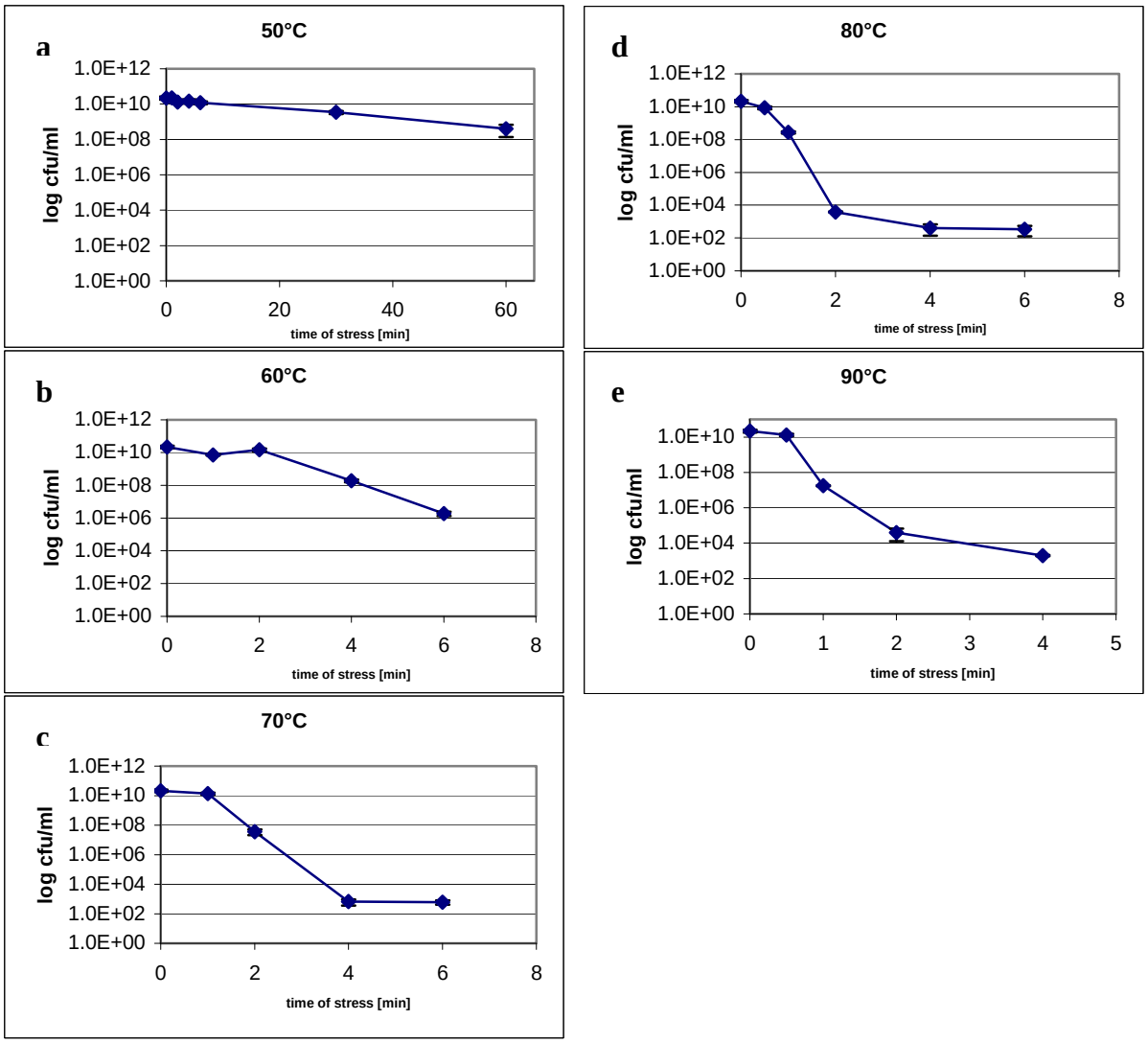

Figure 13: Effect of 50°C (a), 60°C (b), 70°C (c), 80°C (d) and 90°C (e) on reproduction of E. faecium M-74

When Enterococcus faecium M-74 was stressed with 50°C (figure 13a), only little effect on reproduction could be seen in comparison to the other stress temperatures. However, also heat stress of 50°C showed a significantly reduced number of living bacteria. Figure 13b, which depicts the effect of heat stress of 60°C, clearly shows that after two minutes of stress, the number of living bacteria was reduced significantly.

At a temperature of 70°C (figure 13c), averagely 63.33% of the bacteria were still alive after one minute of heat stress, which is a significant decrease in living bacteria. Figure 13d shows that due to a temperature of 80°C, after 30 seconds of heat stress, only 39.09% of the bacteria

were still alive on the average. At a heat stress of 90°C (figure 13e), the most rapid fall of colony forming units during the first minute of stress could be detected, as only 0.08% of the bacteria survived the heat they were exposed to.

From these experiments it can be concluded that heat stress has an effect on the ability of reproduction of Enterococcus faecium M-74. In general, elevated temperatures caused a reduction of colony forming units.

The curves in the figures drop earlier, the higher the temperature applied. Although the reduction of colony forming units due to exposure to 50°C was significant, using higher temperatures in shorter stress periods had more dramatic effects on the reduction of colony forming units.

3.3. Investigation of heat stress using fluorimetry

To determine the effect of different temperatures on Enterococcus faecium M-74, the bacteria were exposed to 50°C, 60°C, 70°C, 80°C and 90°C for certain durations as described in chapter 2.

Figure 14 shows that a temperature of 50°C does not impair certain cellular features of Enterococcus faecium M-74. It is visible that membrane permeability (PI), activity of esterases (FDA) and production of ROS (DHR 123) were not changed significantly, even when the heat stress was carried out for one hour.

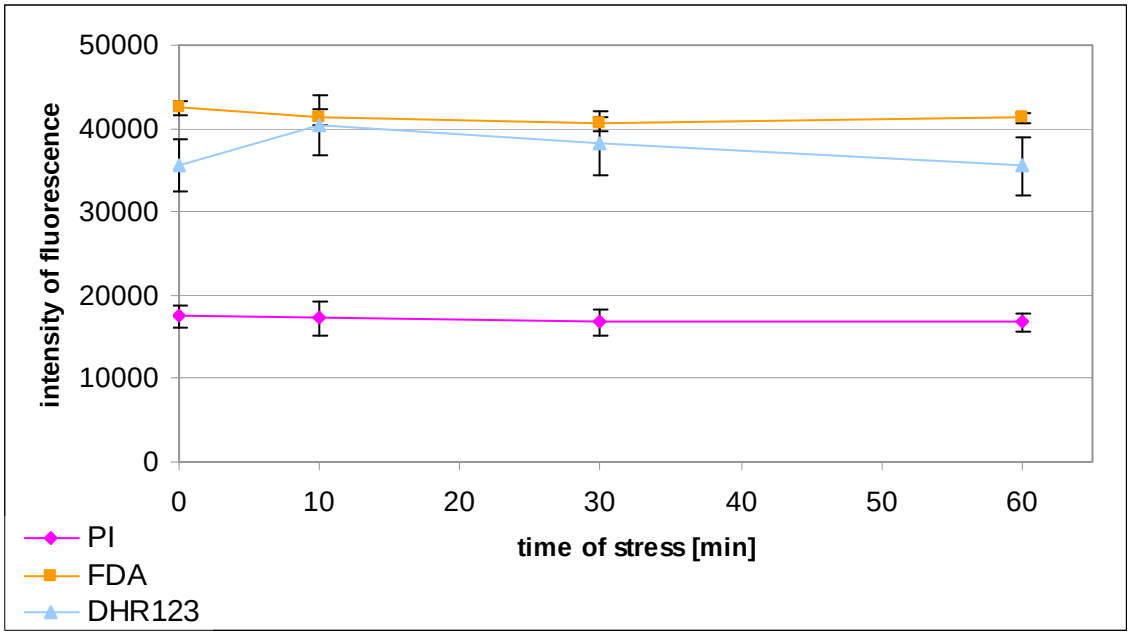

Figure 14: Comparison of fluorescence intensity of E. faecium M-74 stressed with 50°C and incubated with PI, FDA and DHR 123 using the microplate reader

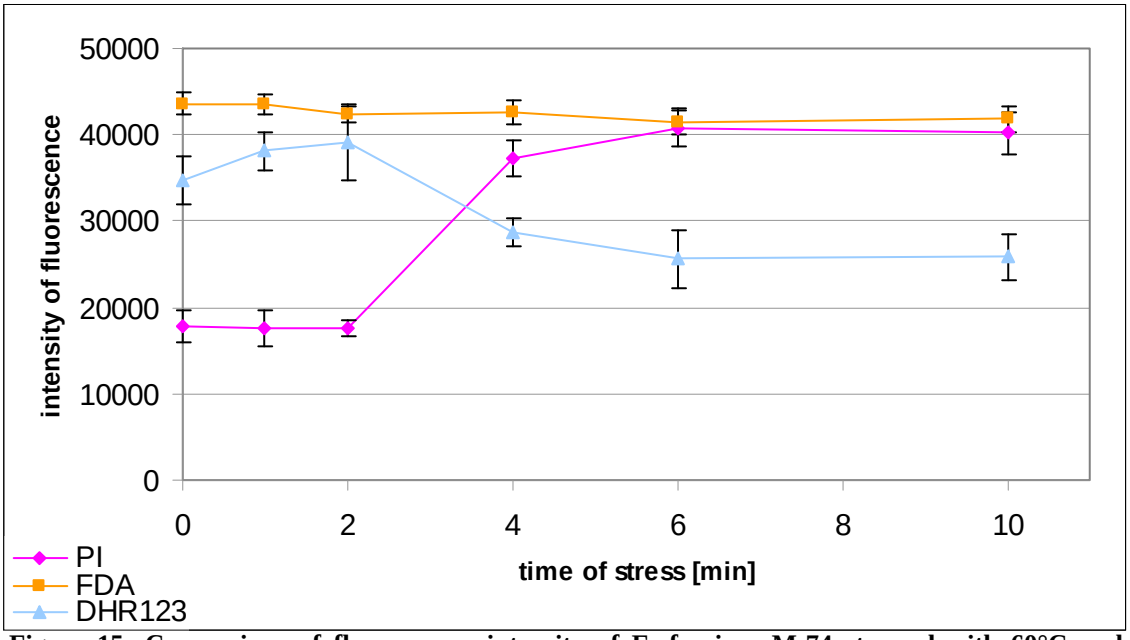

Figure 15: Comparison of fluorescence intensity of E. faecium M-74 stressed with 60°C and incubated with PI, FDA and DHR 123 using the microplate reader

Figure 15 shows that Enterococcus faecium M-74 can apparently be stressed with 60°C for two minutes without experiencing changes in membrane integrity. The fluorescence of PI increased from 17535.17 (± 945.77) at two minutes of stress to 37342.67 (± 2074.33) when Enterococcus faecium M-74 was stressed for four minutes. In the same time interval, the fluorescence of DHR 123 decreased significantly from 39084.67 (± 4409.61) to 28785.58 (± 1627.34). The changes in fluorescence of FDA were small, but significant.

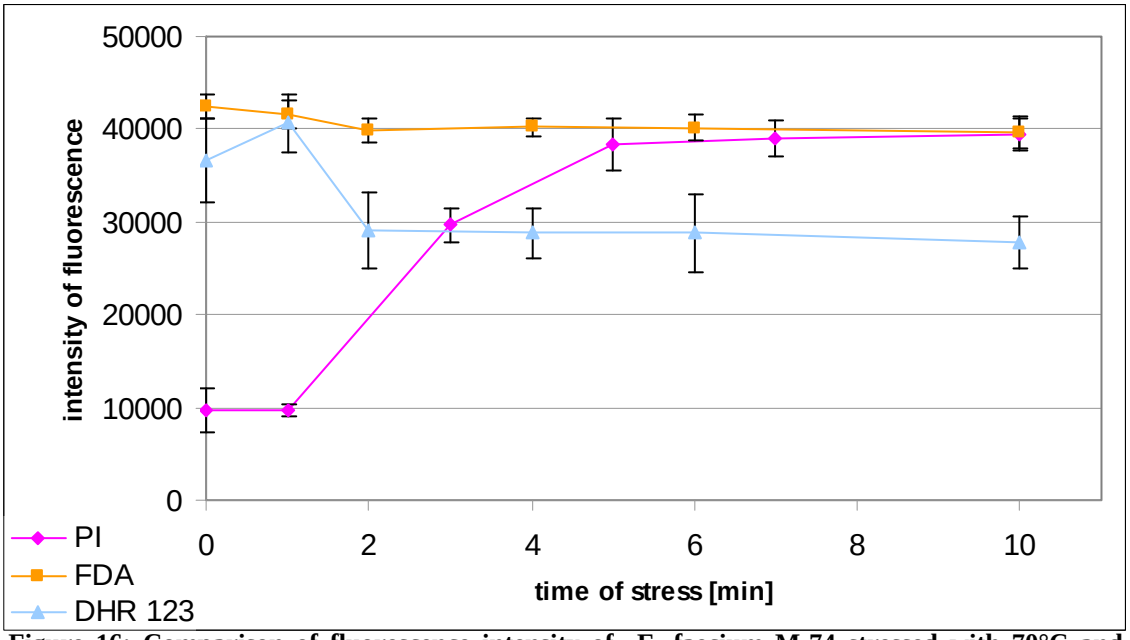

Figure 16: Comparison of fluorescence intensity of E. faecium M-74 stressed with 70°C and incubated with PI, FDA and DHR 123 using the microplate reader

In figure 16, the impact of a temperature stress of 70°C is shown. The fast rise in the intensity of fluorescence of PI is noticeable. Five minutes at 70°C caused a 3.93 fold increase of fluorescence in comparison to the unstressed sample.

The fluorescence of DHR 123 dropped from the value of the unstressed sample of 36677.58 (± 4524.12) to 29098.17 (± 4150.53) at a stress of two minutes at 70°C. From this point on, the fluorescence of DHR 123 remained at the same level. The fall in the fluorescence of FDA, comparing the unstressed sample and the one which had been stressed for ten minutes was small, but significant.

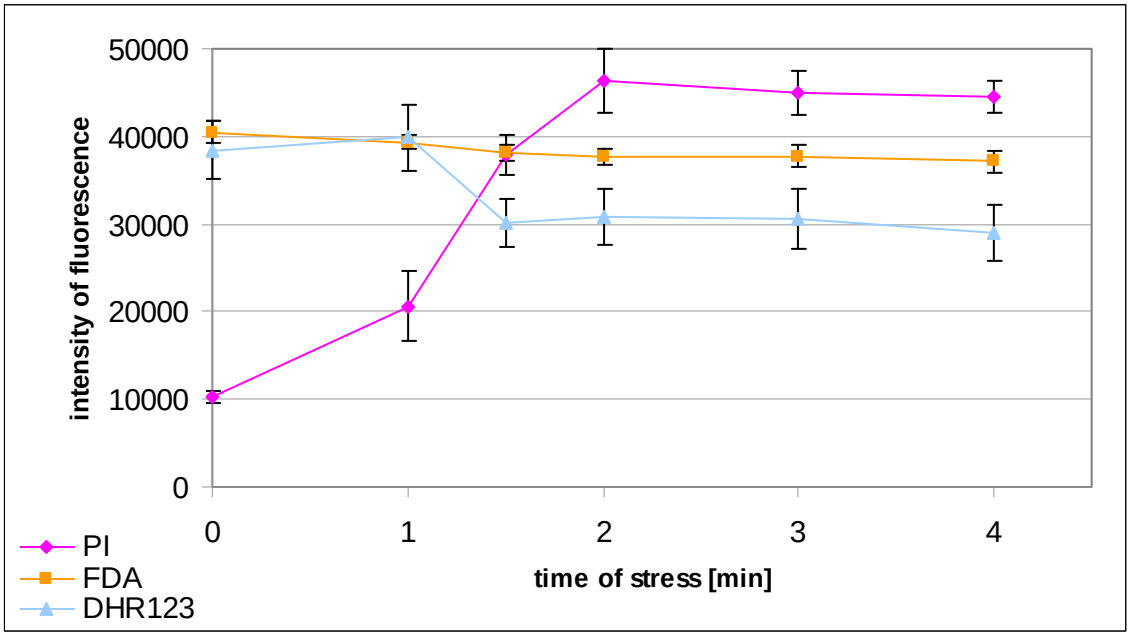

Figure 17: Comparison of fluorescence intensity of E. faecium M-74 stressed with 80°C and incubated with PI, FDA and DHR 123 using the microplate reader

In figure 17, the rise of the fluorescence of PI is even more dramatic, as a stress level of only two minutes at 80°C caused a 4.51 fold increase of fluorescence compared to the unstressed sample. The fluorescence of DHR 123 decreased 1.25 fold in the same stress period.

The fluorescence of FDA decreased constantly, when Enterococcus faecium M-74 was stressed. Although the drop in the curve is very small, it is significant.

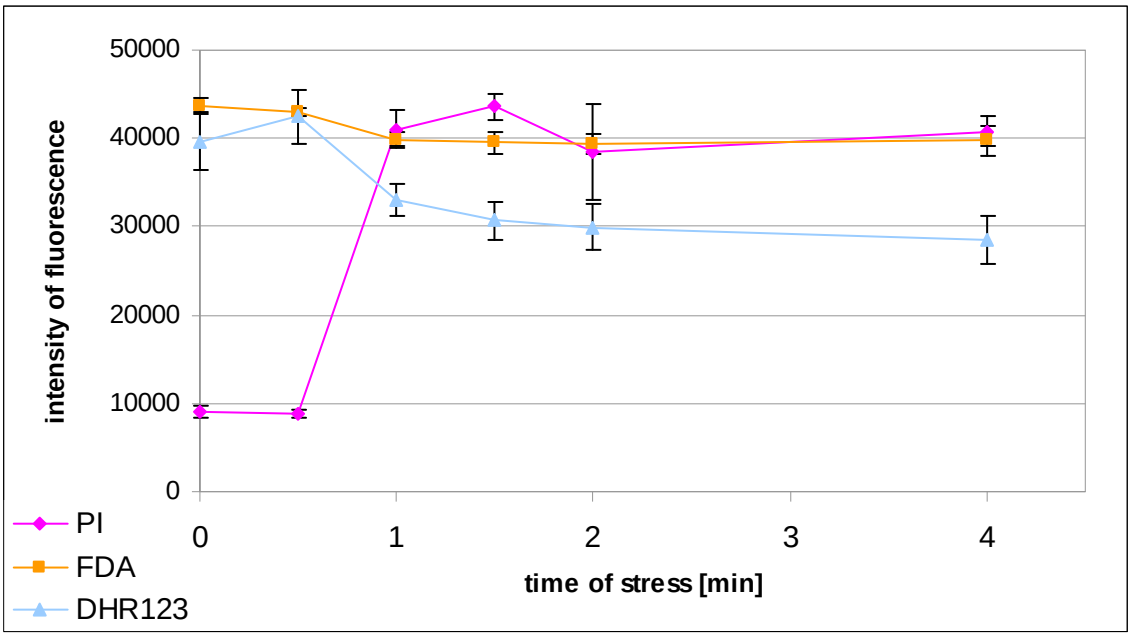

Figure 18: Comparison of fluorescence intensity of E. faecium M-74 stressed with 90°C and incubated with PI, FDA and DHR 123 using a microplate reader

Figure 18 shows the changes in fluorescence, when Enterococcus faecium M-74 was stressed with 90°C. The fall in the curve of FDA was small, but it already dropped significantly when the bacteria were stressed for 30 seconds.

The fluorescence of DHR 123 showed a significant decrease in fluorescence, whereas the fluorescence of PI increased rapidly. Exposure of bacteria to 90°C for half a minute did not seem to have an effect, but already one minute after the stress had started, the bacteria reacted strongly with PI, which resulted in a 4.55 fold rise in fluorescence. The drop in the fluorescence of DHR 123 was 1.39 fold, comparing the unstressed sample with the bacteria which had been stressed with 90°C for four minutes. The most dramatic fall happened between 30 seconds and one minute of stress with 90°C.

3.4. Investigation of heat stress using flow cytometry

The information generated by flow cytometry differs from that revealed by the microplate reader. Since the acquired data include cell size, fluorescence of the cells and intensity of the fluorescence, only the fluorescence associated with cells is detected.

In the context of this thesis, the cell size has not been investigated. The focus lay on the fluorescence of the cells and its intensity.

In the experiments with the flow cytometer, no significant data for FDA were obtained. For this reason, only the measurements for PI, DHR 123, DioC6(3) and CFDASE are presented.

In order to illustrate the results received with the flow cytometer, diagrams with two y-axes were designed. The y-axis on the left describes the percentage of the cells stained by the fluorescent dye, whereas the y-axis on the right shows the mean values of the fluorescence intensity.

Propidium iodide

When Enterococcus faecium M-74 was stressed with a temperature of 50°C (figure 19), less than 10% of the cells were stained with PI and even when the cells were confronted with 50°C for ten minutes, only 5.36% (± 0.36) were stained with the fluorescent dye. the intensity of the fluorescence did not vary significantly.

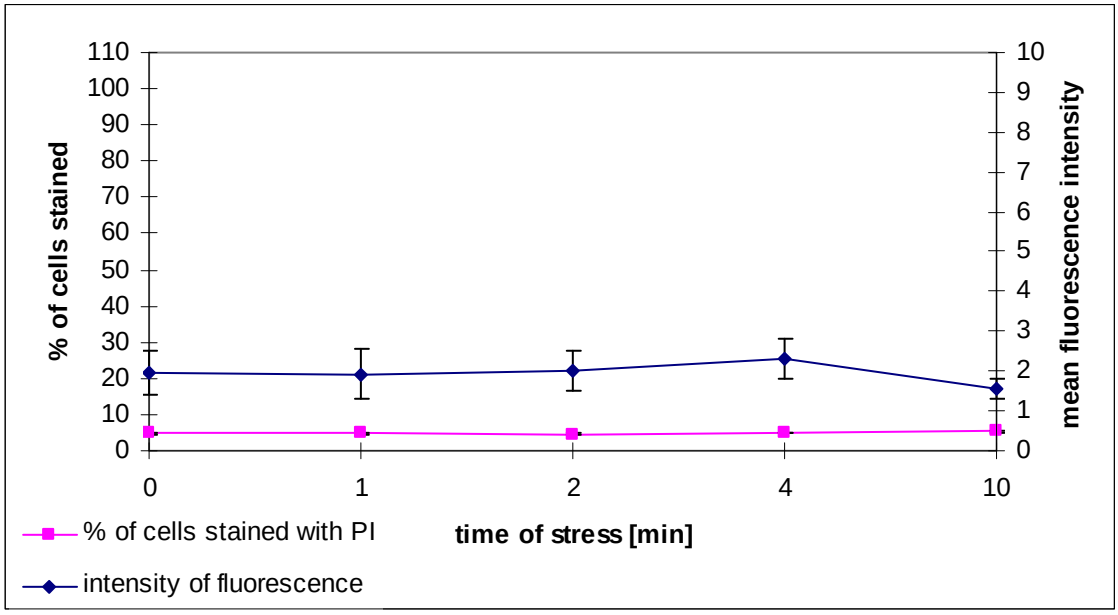

Figure 19: Flow cytometric measurement of PI after heat stress of E. faecium M-74 at 50°C

The amount of cells stained with PI increased after they had been stressed longer than two minutes at 60°C (figure 20). At a stress duration of two minutes, 7.98% (± 0.25) of the cells were stained. However, when they were exposed to 60°C for ten minutes, 66.41% (± 3.59) of the bacteria were marked with the fluorescent dye. Also, the mean fluorescence intensity increased significantly after two minutes of stress. The increase between a stress duration of two minutes and of four minutes was 3.05 fold.

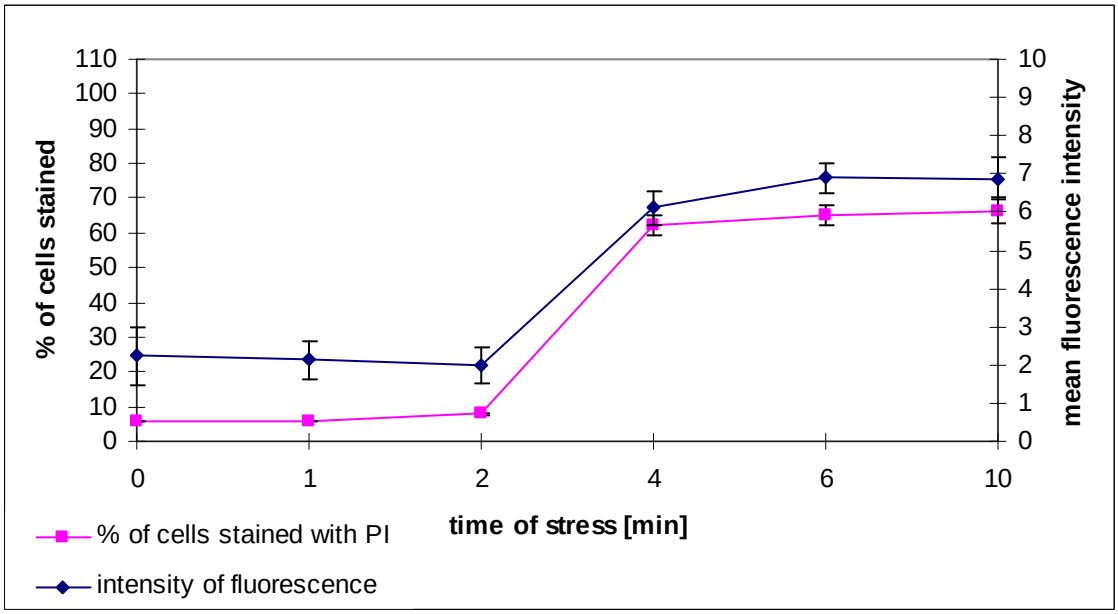

Figure 20: Flow cytometric measurement of PI after heat stress of E. faecium M-74 at 60°C

The effect of heat stress at 70°C is shown in figure 21. Concomitantly, changes in the fluorescence intensity were measurable. The rise in the curves depicts the significant changes

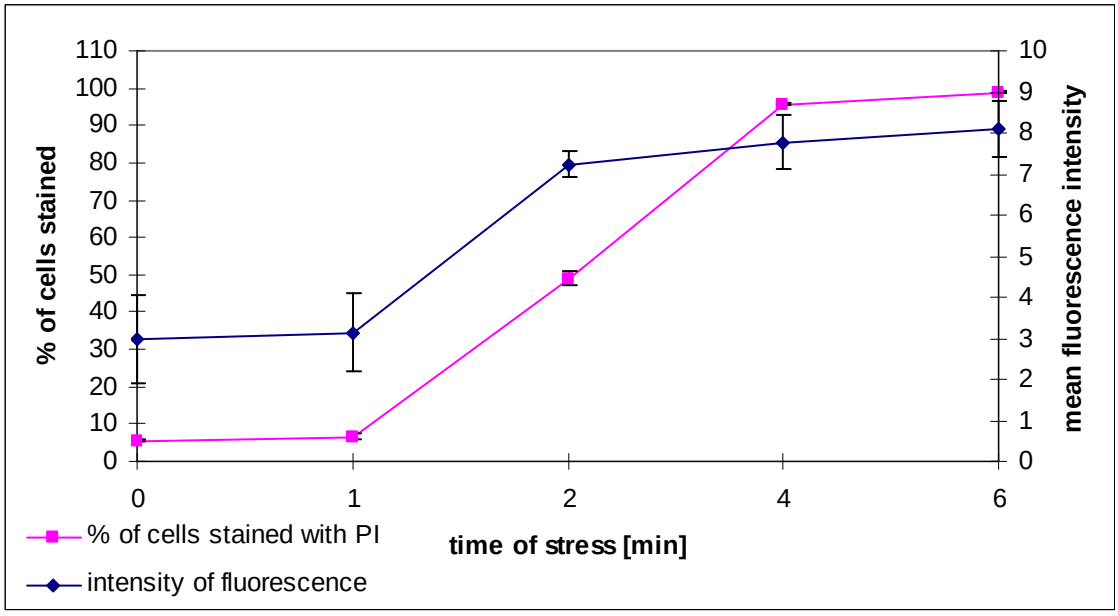

Figure 21: Flow cytometric measurement of PI after heat stress of E. faecium M-74 at 70°C

which happened in an interval of only three minutes. When Enterococcus faecium M-74 was stressed with 70°C for one minute, 6.61% (± 0.65) of the cells were stained. After two minutes, 49.07% (± 1.96) of the cells were tagged whereas six minutes of stress caused staining of 98.96% (± 0.10) of the bacteria.

Stressing Enterococcus faecium M-74 with a temperature of 80°C resulted in a fast increase of stained cells, as depicted in figure 22. When the cells were stressed at 80°C for only half a minute, no significant changes could be detected - neither in the percentage of cells tagged with PI, neither in the intensity of fluorescence. However, when the cells were stressed for one minute, the amount of stained cells increased to 48.43% (± 0.92) in comparison to the stress of half a minute (6.86% (± 0.71)). Treating the cells with 80°C for two minutes lead to the staining of 99.52% (± 0.23) of cells.
The intensity of the fluorescence experienced an increase between the stress durations of half a minute and one minute, but did not change significantly any more at longer stress durations.

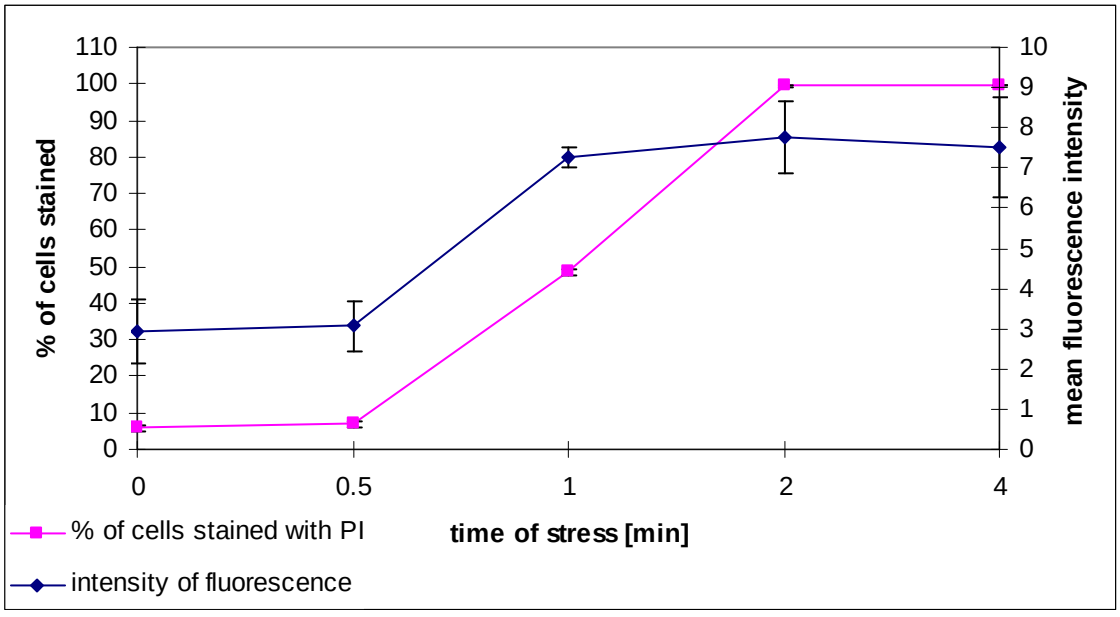

Figure 22: Flow cytometric measurement of PI after heat stress of E. faecium M-74 at 80°C

Heat stress at 90°C shows the fastest changes in the curves (figure 23). The most remarkable changes in this figure happened during a very short interval. Half a minute of stress at 90°C did not seem to cause changes in membrane integrity, as only 5.74% (± 0.61) of the cells were tagged. When Enterococcus faecium M-74 was kept just 20 seconds longer at this temperature, already 66.31% (± 3.70) of the bacteria appeared to be damaged. As soon as the bacteria had been stressed for one and a half minutes, all cells were tagged with PI.

At all stress durations longer than 30 seconds, the changes in the intensity of fluorescence differed significantly from the fluorescence of unstressed cells.

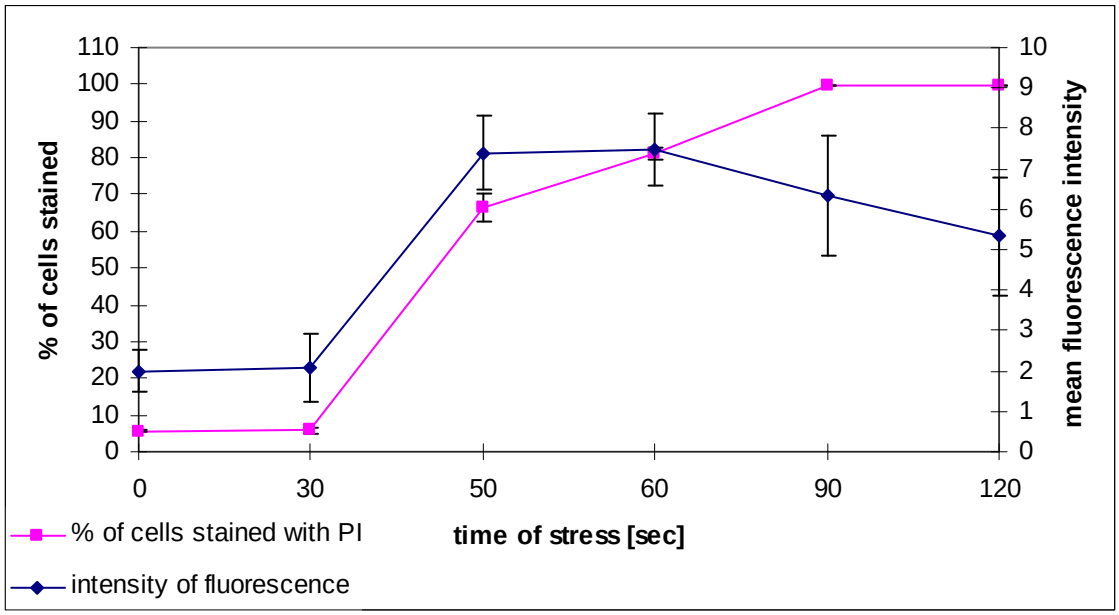

Figure 23: Flow cytometric measurement of PI after heat stress of E. faecium M-74 at 90°C

Dihydrorhodamine 123

Figure 24 shows that 41.83% (± 2.25) of unstressed bacteria were stained with DHR 123, indicating that these cells produced ROS. The amount of bacteria stained increased to 52.40% (± 5.80) when the bacteria were stressed with 50°C for four minutes and further to 56.10% (± 5.09) when they were stressed for 10 minutes, which is a significant increase.

The change in the intensity of fluorescence seems very small, but the difference between unstressed bacteria and bacteria which have been stressed for four or ten minutes is significant.

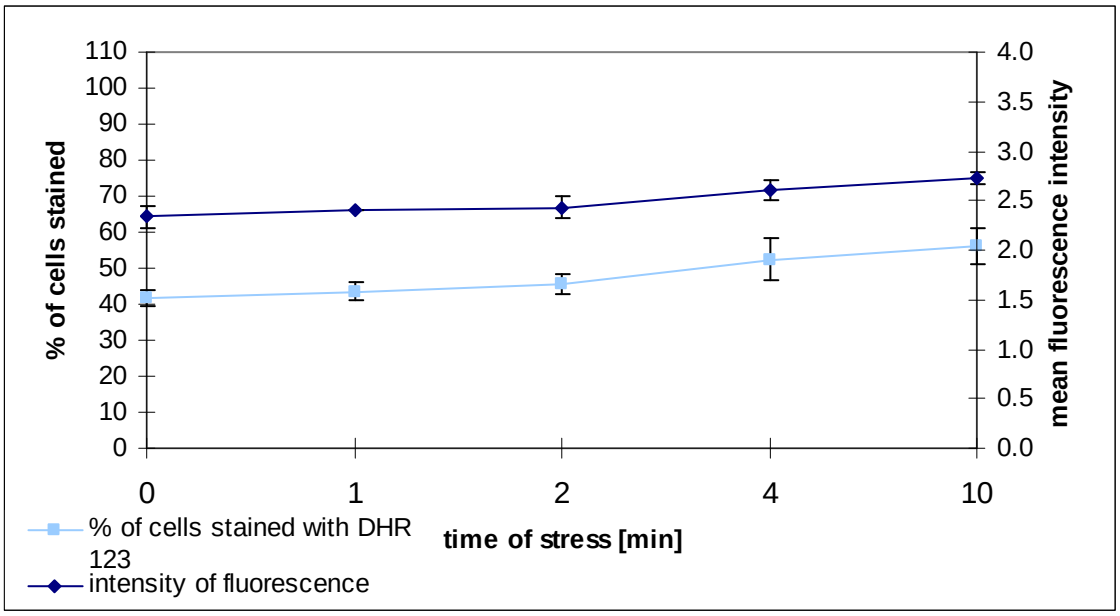

Figure 24: Flow cytometric measurement of DHR 123 after heat stress of E. faecium M-74 at 50°C

As found out already during the trials with the microplate reader, Enterococcus faecium M-74 seems to produce ROS in its healthy state. The results obtained with the flow cytometer support this finding.

The curves in figure 25 show a slight, but significant rise when Enterococcus faecium M-74 is stressed with 60°C for two minutes. At a stress of 60°C for two minutes, significantly more (57.38% (± 4.98)) cells were stained with DHR 123 compared to unstressed cells (42.72% (± 3.05). Longer heat stress durations lead to constant decrease in stained cells.

The interesting features of figure 25 are the peaks of both curves when Enterococcus faecium M-74 was stressed for two minutes.

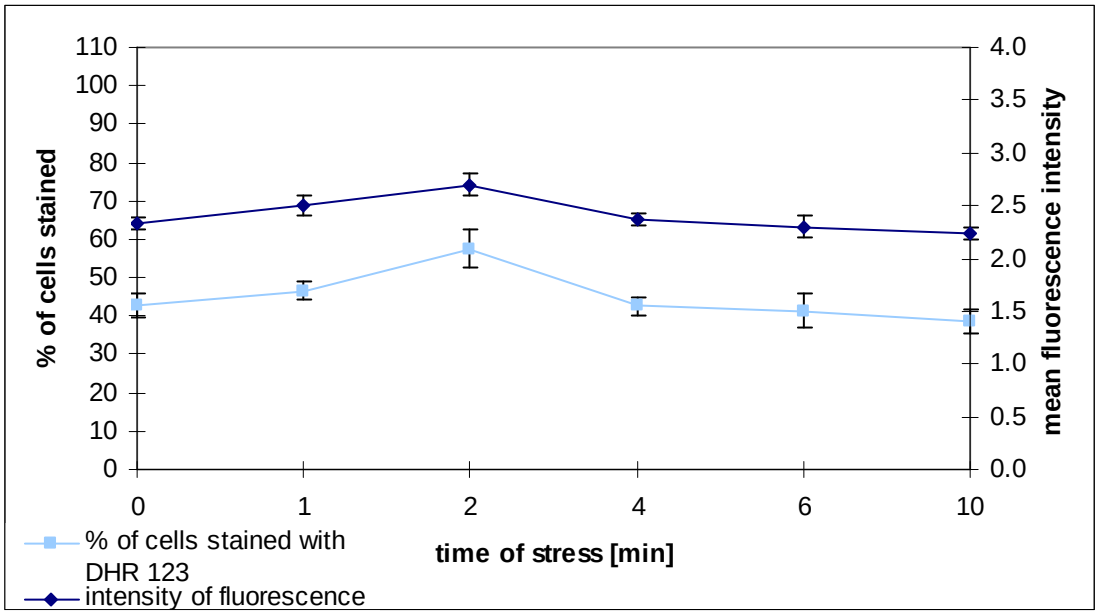

Figure 25: Flow cytometric measurement of DHR 123 after heat stress of E. faceium M-74 at 60°C

Figure 26 depicts that when Enterococcus faecium M-74 was kept at 70°C for one minute, the initial rise in the number of stained cells showed an increased production of ROS. Due to heat stress for one minute 68.73% (± 7.50) of the bacteria were stained with DHR 123. However, as the cells were stressed for longer durations, a continuous drop in the number of stained cells could be detected. When the cells had been stressed for six minutes, 7.42% (± 1.94) of them were marked with the fluorescent dye.

The peak of the curve depicting the intensity of the fluorescence also shows a peak at a stress duration of one minute.

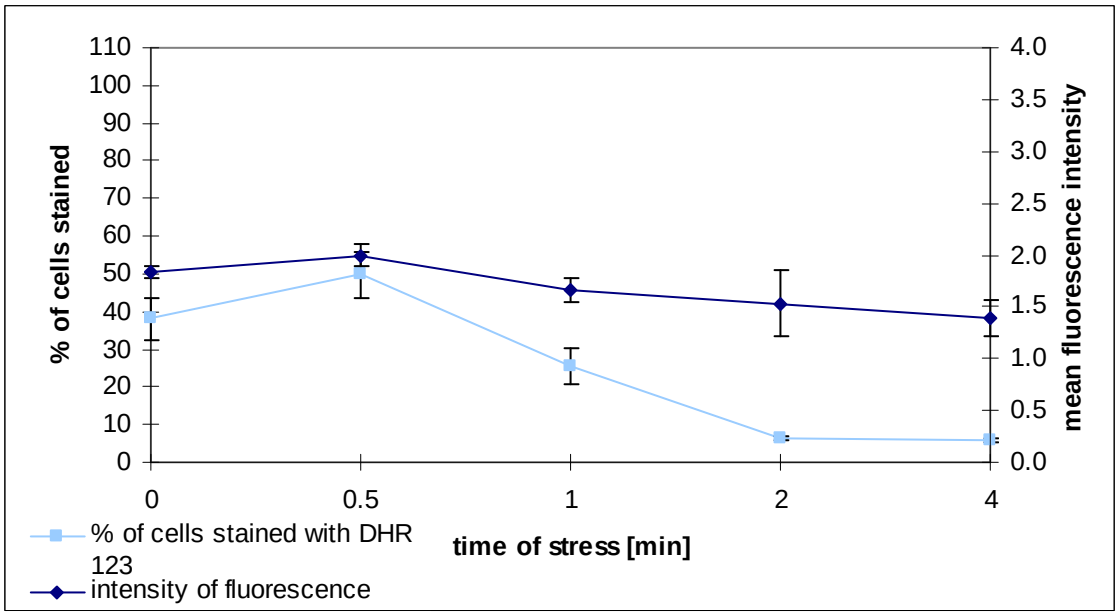

Figure 26: Flow cytometric measurement of DHR 123 after heat stress of E. faecium M-74 at 70°C

The effects of heat stress at a temperature of 80°C are depicted in figure 27. The peak in the curve which describes the percentage of cells stained with DHR 123 (49.74% (±6.05)) appears at a stress duration of half a minute, but it is not significant.

The number of cells stained with DHR 123 decreased when Enterococcus faecium M-74 was exposed to heat for one minute. The lowest level of stained cells (6.23% (± 0.65)) was reached as soon as the heat stress was carried out for two minutes. Compared to the fluorescence of the unstressed sample, the intensity of fluorescence decreased significantly when Enterococcus faecium M-74 was stressed for four minutes at 80°C.

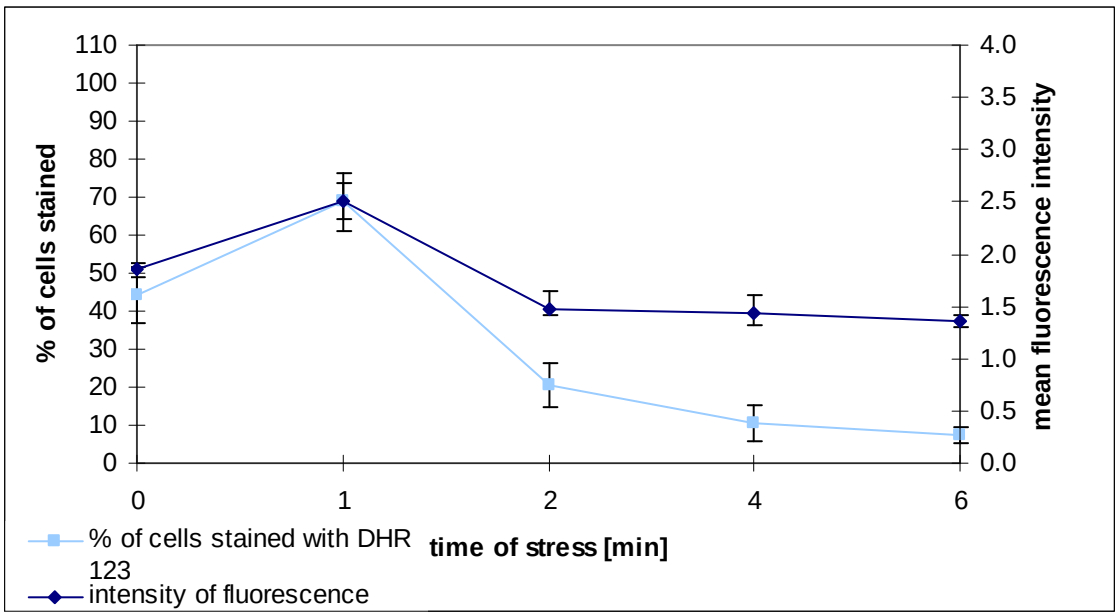

Figure 27: Flow cytometric measurement of DHR 123 after heat stress of E. faecium M-74 at 80°C

Challenging Enterococcus faecium M-74 with 90°C (figure 28) had a similar effect on ROS production as stressing it with 80°C. After 30 seconds of heat stress at 90°C, 57.31% (± 8.43) of the cells were tagged and the lowest percentage of stained cells was reached when Enterococcus faecium M-74 was heated with 90°C for one and a half minutes - then, 5.86% (± 0.66) of the cells were marked with DHR 123.

When Enterococcus faecium M-74 was stressed at a temperature of 90°C for half a minute, the intensity of fluorescence increased significantly.

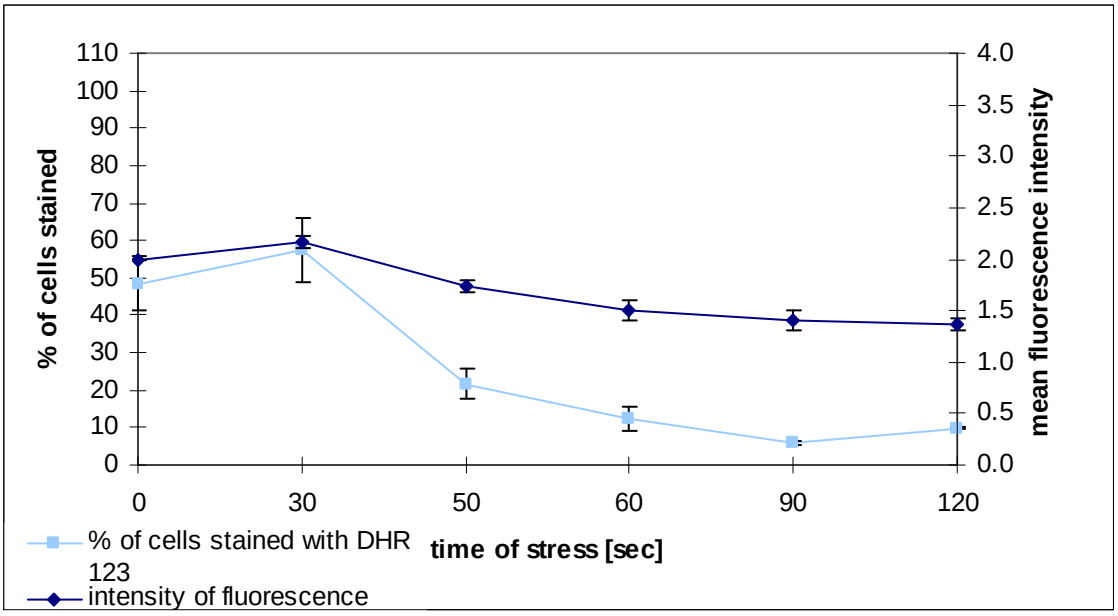

Figure 28: Flow cytometric measurement of DHR 123 after heat stress of E. faecium M-74 at 90°C

3,3'-Dihexyloxacarbocyanine iodide

The application of DioC6(3) should show changes in membrane potential due to heat stress. The fluorescence of DioC6(3) drops when cell membranes are hyperpolarized.

In figure 29, it is shown that a temperature of 50°C did not have an influence on the membrane potential, when Enterococcus faecium M-74 was stressed for ten minutes.

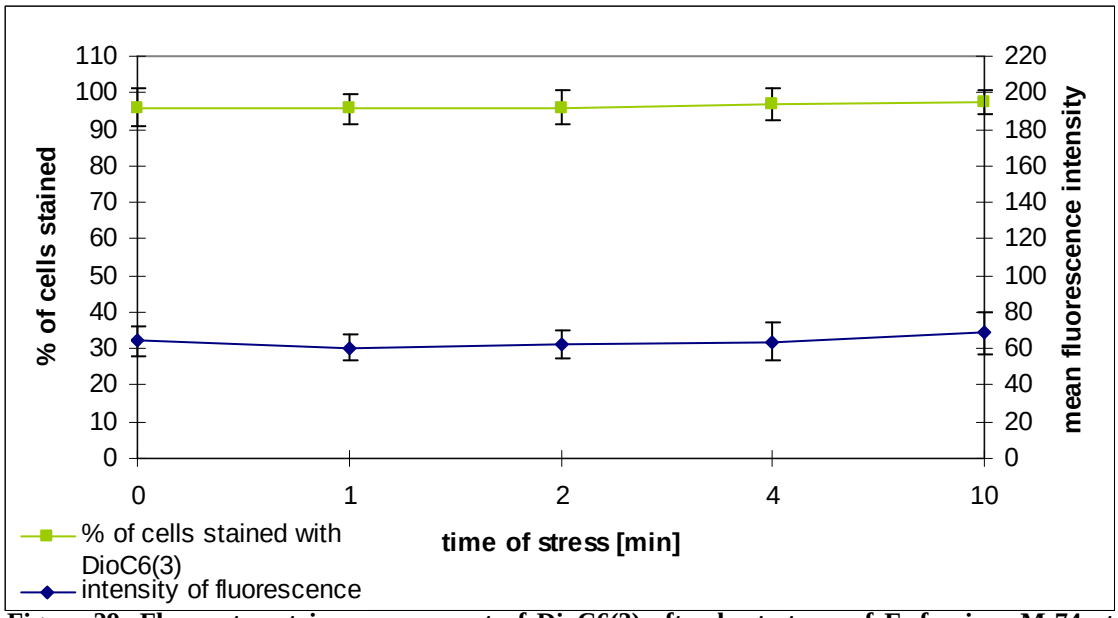

Figure 29: Flow cytometric measurement of DioC6(3) after heat stress of E. faecium M-74 at 50°C

Figure 30 shows that the percentage of cells stained with DioC6(3) was not affected by temperature stress of 60°C. The intensity of fluorescence, however, changed. As Enterococcus faecium M-74 was heated, the intensity of the fluorescence was enhanced significantly after 6 minutes.

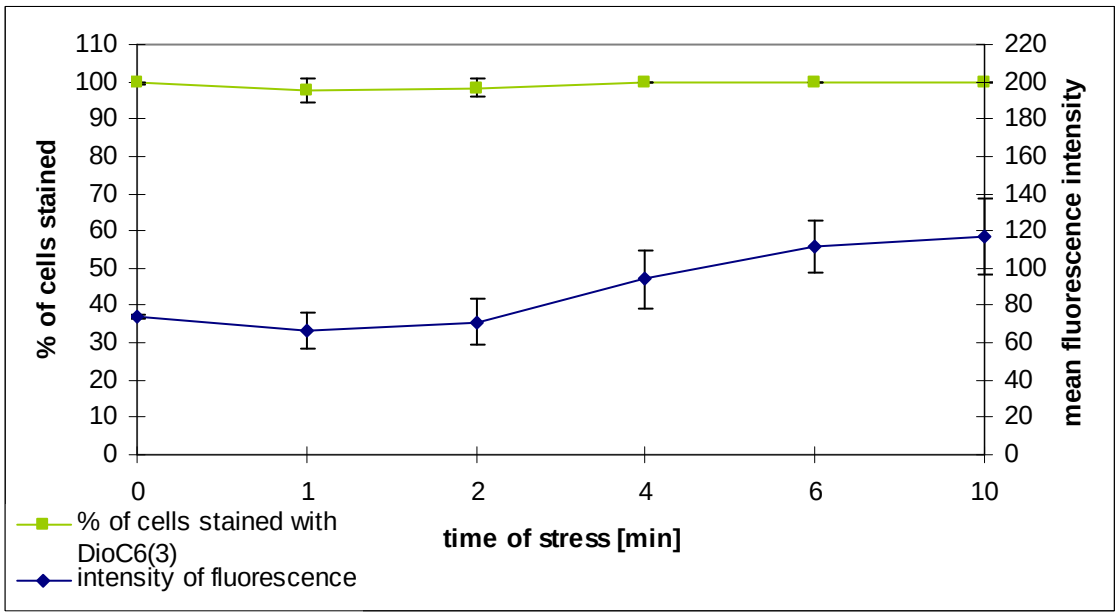

Figure 30: Flow cytometric measurement of DioC6(3) after heat stress of E. faecium M-74 at 60°C

Figure 31 depicts that the fluorescence of DioC6(3) increased as the cells were stressed with 70°C. From the initial intensity of 63.67 (± 9.79), the curve experiences a fast increase until a stress of four minutes, when a significantly higher intensity of 160.17 (± 15.42) is reached.

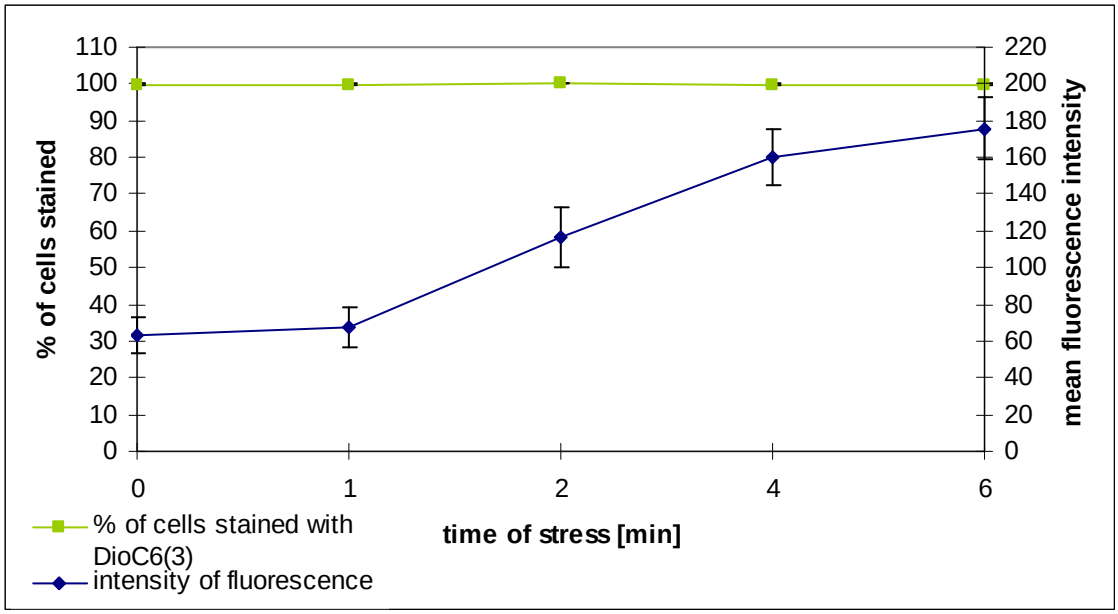

Figure 31: Flow cytometric measurement of DioC6(3) after heat stress of E. faecium M-74 at 70°C

Figure 32 shows that unstressed cells had a mean fluorescence intensity of 70.43 (± 8.32). One minute of stress at 80°C did not show a significant rise in the curve, whereas two minutes at this temperature already made the intensity increase to 162.90 (± 12.83). At a stress duration of four minutes, the fluorescence intensity of 189.57 (± 15.71) was reached, which shows a 2.69 fold rise in comparison to the intensity of fluorescence of unstressed cells.

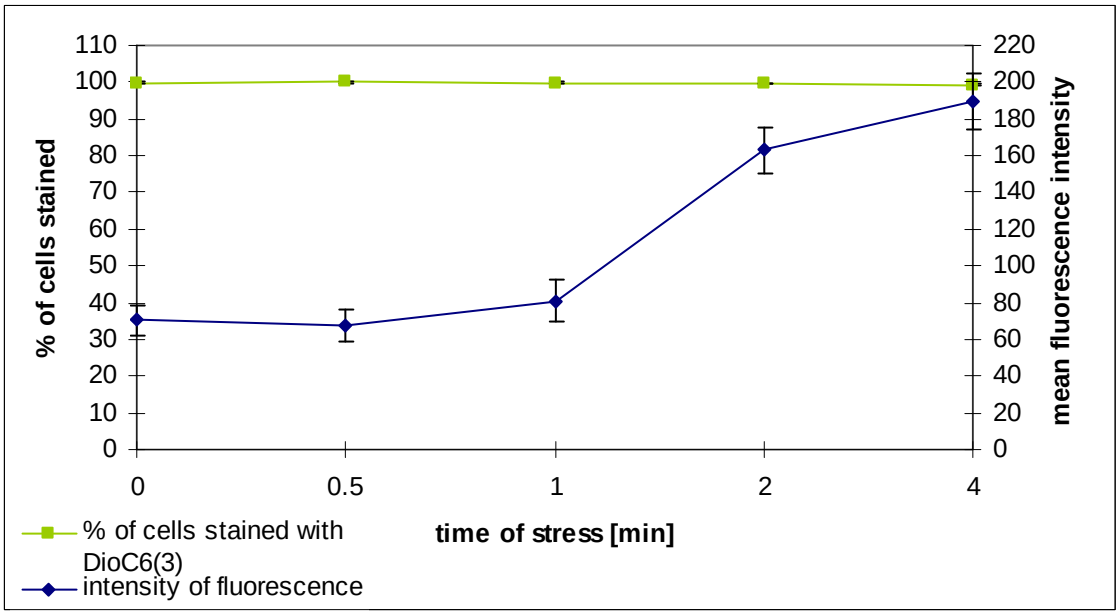

Figure 32: Flow cytometric measurement of DioC6(3) after heat stress of E. faecium M-74 at 80°C

The increase of fluorescence intensity was even more explicit when Enterococcus faecium M-74 was stressed with 90°C, as illustrated in figure 33.

The intensity of fluorescence started to change significantly when the bacteria were exposed to heat for 50 seconds and longer. While unstressed cells showed a mean fluorescence intensity of 71.37 (± 6.47), cells stressed for 50 seconds had a fluorescence intensity of 94.77 (± 8.11). Challenging the cells with 90°C for two minutes caused a rise to 192.10 (± 11.18).

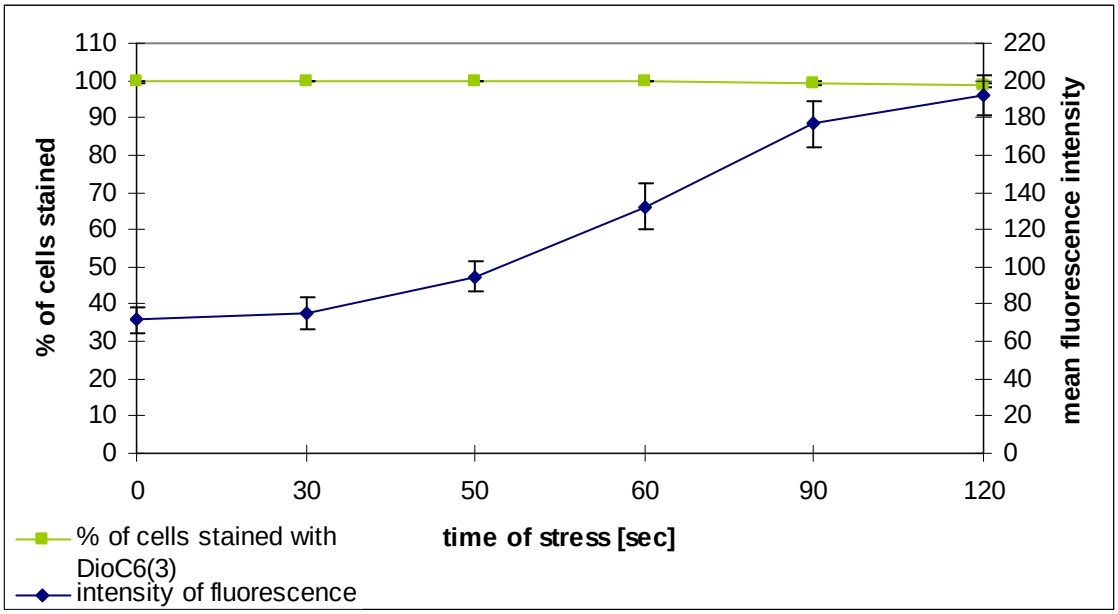

Figure 33: Flow cytometric measurement of DioC6(3) after heat stress of E. faecium M-74 at 90°C

Carboxy fluorescein diacetate succinimidyl ester

Similar to DioC6(3), CFDASE tagged all cells, regardless of their previous exposure to heat stress.

Temperature stress of 50°C did not cause changes in the intensity of fluorescence either, as depicted in figure 34. Thus, at a stress level of 50°C, the intracellular pH did not seem to experience any alterations.

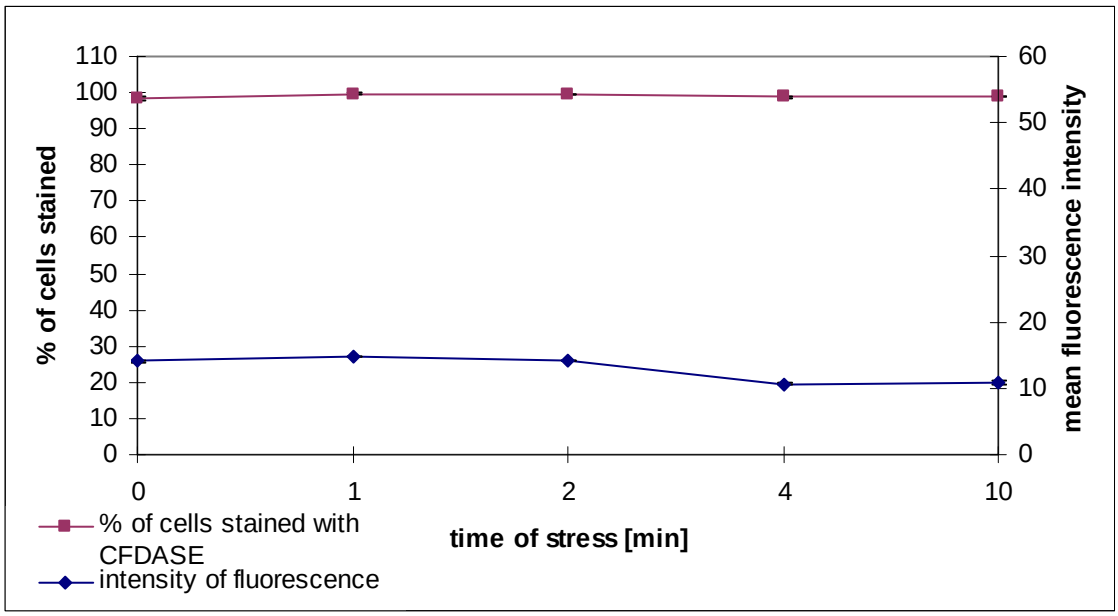

Figure 34: Flow cytometric measurement of CFDASE after heat stress of E. faecium M-74 at 50°C

Figure 35 presents the effects of a temperature of 60°C on intracellular pH of Enterococcus faecium M-74. The intensity of the fluorescence of unstressed cells was 12.17 (± 0.12). When the cells were stressed for four minutes, the value increased to 20.43 (± 0.15), which is a significant rise, and it increased further to 27.93 (± 0.46) when the bacteria were heated for ten minutes.

Regarding the percentage of cells tagged with CFDASE, figure 35 indicates that 96.81% (± 0.20) of unstressed cells were tagged. 88.26% (± 0.58) of the cells confronted with 60°C for two minutes were tagged, which results in a significant drop in the curve. At a stress duration of four minutes, however, again almost all the cells (99.16% (± 0.16)) were tagged with the fluorescent dye.

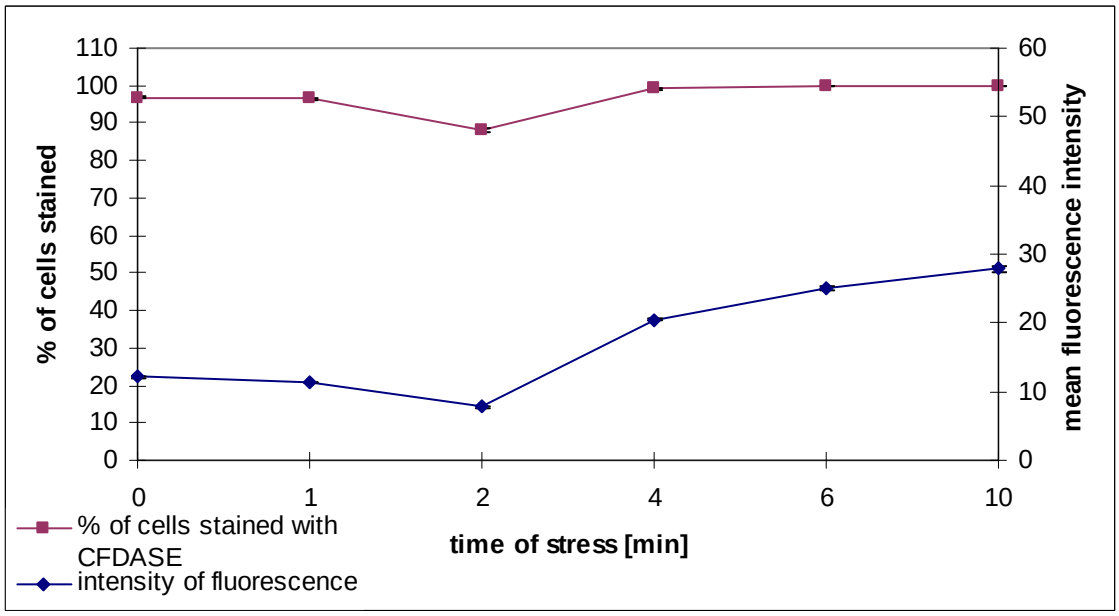

Figure 35: Flow cytometric measurement of CFDASE after heat stress of E. faecium M-74 at 60°C

Figure 36 presents the effects of a heat stress of 70°C on the intracellular pH of Enterococcus faecium M-74. The curve describing the amount of cells stained with CFDASE shows a significant drop when Enterococcus faecium M-74 was stressed for one minute. At longer durations of stress, almost all cells were stained.

Initially, the intensity of the fluorescence was 7.57 (± 0.12). One minute of heat stress caused a significant drop to 5.77 (± 0.06), but already after two minutes of heating the cells at 70°C, the intensity significantly increased to 12.83 (± 0.15). The intensity after six minutes of heating was 19.57 (± 0.45).

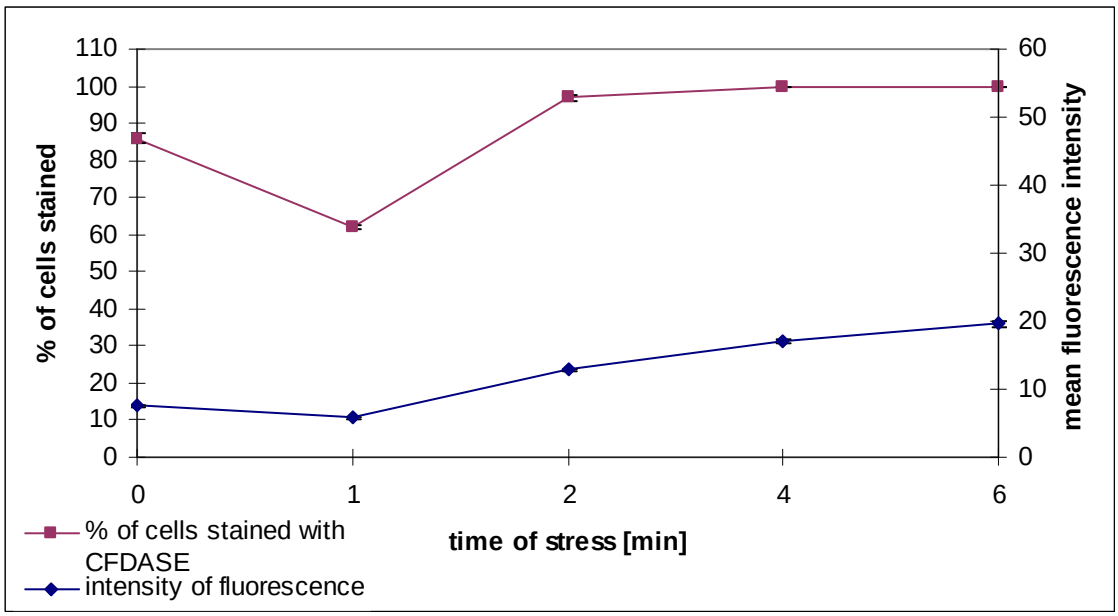

Figure 36: Flow cytometric measurement of CFDASE after heat stress of E. faecium M-74 at 70°C

In figure 37, which illustrates the flow cytometric measurement after heat stress at 80°C, the fluorescence intensity decreases significantly from the initial fluorescence of 9.17 (± 0.06) to 8.03 (± 0.06) because of heat stress for 30 seconds and then increases to 12.83 (± 0.29) due to heat stress for one minute and further to 24.93 (± 0.40) when the bacteria were heated for four minutes.

The curve showing the percentage of cells shows a significant drop to 90.21% (± 0.36) at one minute of heat stress. When Enterococcus faecium M-74 was stressed with 80°C for two minutes, 99.69% (± 0.19) of the cells were stained.

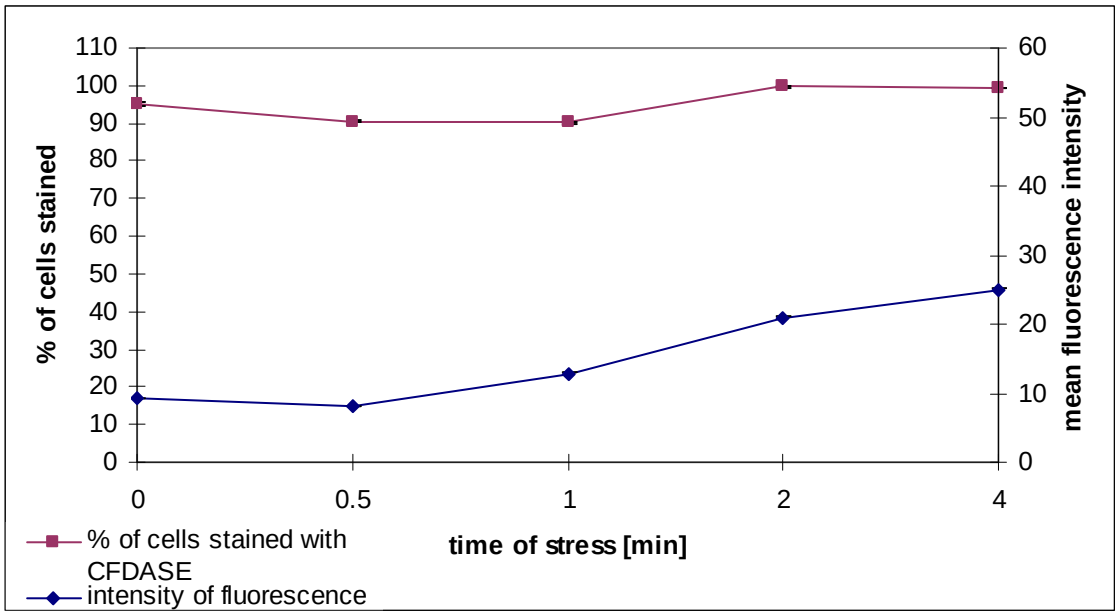

Figure 37: Flow cytometric measurement of CFDASE after heat stress of E. faecium M-74 at 80°C

Figure 38 shows, that in this assay, the mean fluorescence intensity of unstressed cells was higher than previously. From the initial value of 19.93 (± 1.19), it significantly decreased to 10.33 (± 0.23) when the cells were stressed for half a minute. Further temperature stress led to a significant increase in fluorescence intensity. After two minutes at 90°C, it was at 45.37 (± 0.87).

The amount of cells stained with CFDASE during this assay was 99.53% (± 0.52%) on the average.

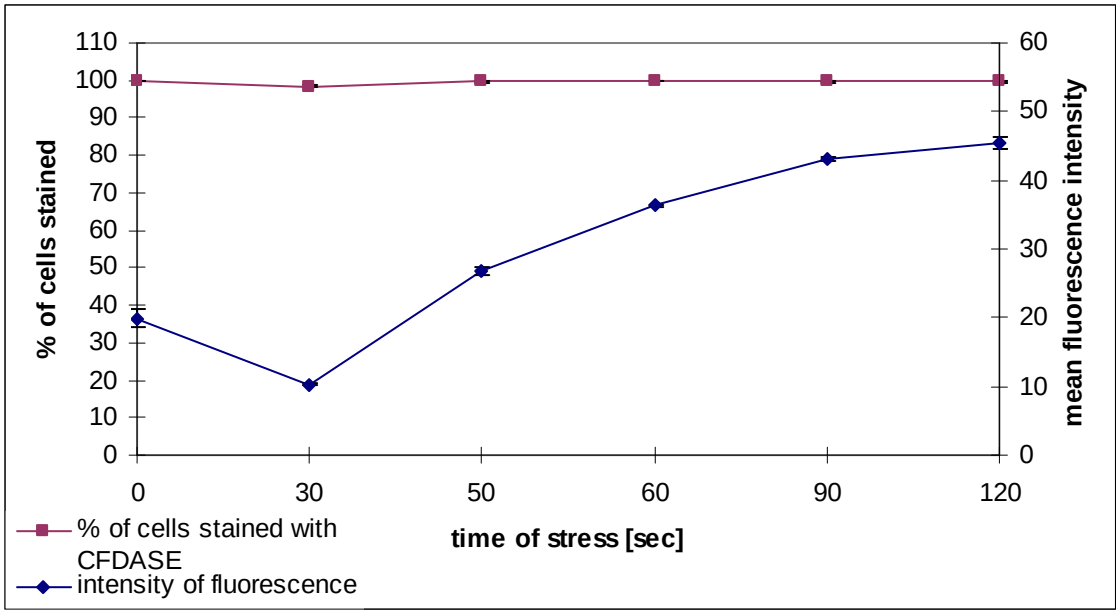

Figure 38: Flow cytometric measurement of CFDASE after heat stress of E. faecium M-74 at 90°C

Influence of pH value on fluorescence of CFDASE

The figures of the assays with CFDASE (figures 34-38) imply that the intracellular pH of Enterococcus faecium M-74 was altered by heat stress.

In order to interpret the altering fluorescence intensities of CFDASE in more detail, it was necessary to monitor the influences of different pH values on the fluorescent dye. Since the flow cytometer only measured fluorescence in and on cells, it was not suitable for this experiment. For measuring the direct effect of pH on the fluorescent dye, the microplate reader was needed.

Figure 39 clearly shows that the fluorescence intensity of CFDASE increased with rising pH. Up to a pH of 7.4 the fluorescence of CFDASE remained stable. At a pH of 0.91, for instance, the fluorescence was 8493.5 (± 246.83) and at a pH of 7.4 it was 10023.5 (± 147.12).

A pH of 9 made the fluorescence rise to 23931.00 (± 380.82), which is more than twice as intense. A pH of 10 had an even greater effect, as a fluorescence of 42890.25 (± 560.30) could be measured.

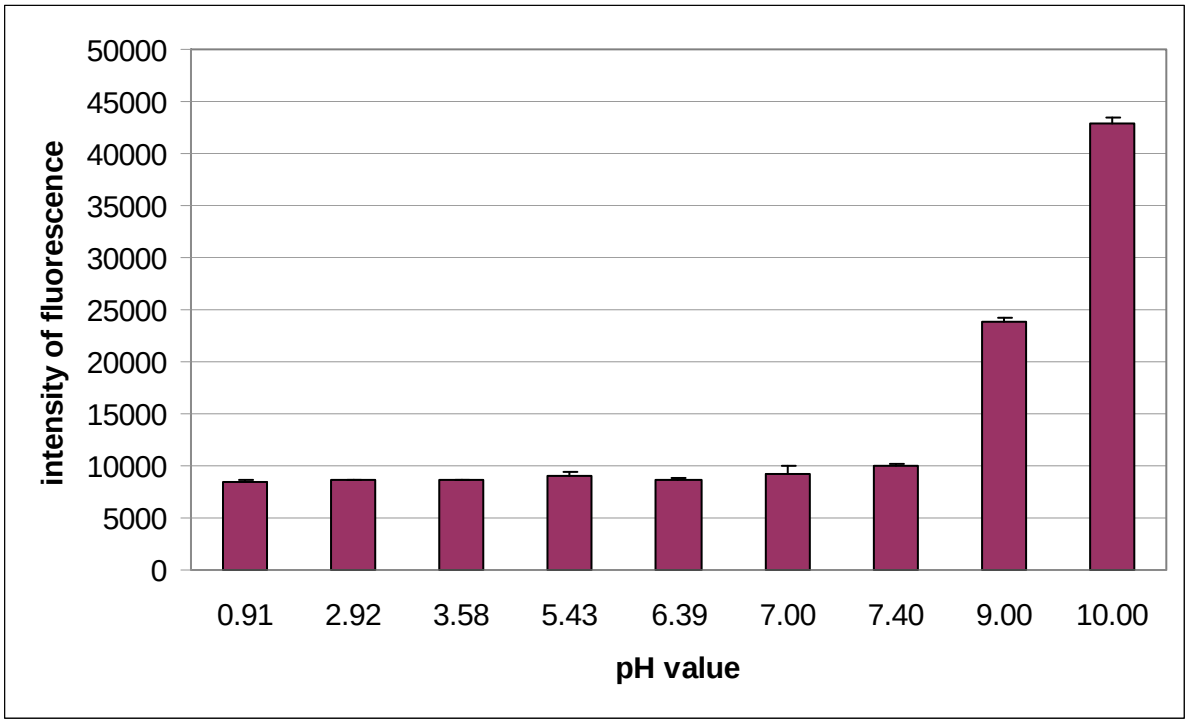

Figure 39: pH dependent fluorescence of CFDASE

4. Discussion

4.1. Cell viability

In this survey, the assay about the ability of reproduction in Enterococcus faecium M-74 showed that the bacteria were affected when increasing temperature. The temperatures applied were 50°C, 60°C, 70°C, 80°C and 90°C.

The viability of enterococci has been a topic of interest for a long time as enterococci are known to cause nosocomial infections. For example, Kearns et al. have done research on the resistance of enterococci to heat and sodium hypochlorite [22]. Comparing six isolates of Enterococcus faecium, they found out that all six isolates of Enterococcus faecium used survived 65°C for 20 minutes, and 71°C and 75°C for 3 minutes. Four isolates survived 80°C for 3 minutes. All of their isolates were killed by exposure to 75 and 80°C for 10 minutes, but one isolate survived 71°C for 10 minutes. It is interesting to see that six isolates of Enterococcus faecium could survive 65°C for as long as 20 minutes in these previous assays. The time frame used in the current survey for investigating the ability of reproduction of Enterococcus faecium M-74 at 60°C was smaller, as the maximum exposure time did not exceed 6 minutes. It was shown that the number of living bacteria started to decline after two minutes of exposure to 60°C. A temperature of 70°C induced an earlier reduction in the number of living bacteria, as the initial number only remained the same until one minute of exposure to heat. Heating Enterococcus faecium M-74 at 80°C already showed a difference at half a minute of heat stress. 90°C also started to show a decrease after half a minute. Heat stress of 90°C was not carried out in the survey of Kearns et al., as the aim was to investigate temperatures and holding times specified by the Department of Health for the disinfection of ‘foul and used’ or ‘infected’ linen. Differently, the focus of the current assay lay on the duration in which Enterococcus faecium was not affected by heat stress and its biomolecular activity due to exposure to different heat levels.

Another survey by Bradley and Fraise revealed that the thermal tolerance of enterococci is strain dependent [23]. In their experiments they proved that some strains they used showed the reduction in viable count which was required for the definition of disinfection. However, other strains could even survive the heat stress of 80°C for over three minutes.

Another example which proves the interest of scientists in enterococci is the survey by Orr et al. [24]. They showed that under laboratory conditions, many strains of enterococci were able to survive the time/temperature combinations recommended for the decontamination of hospital laundry. The temperatures tested were 65°C, 71°C, 75°C, 80°C and 85°C. The

studies of enterococcal survival during processing in 10 working hospital laundries, suggested that in practice hospital laundries were able to decontaminate linen experimentally contaminated with enterococci.

As enterococci have thus been proved to be thermotolerant to a certain extent, it was interesting to find out more about their ability to survive and the changes they go through when exposed to heat. Previous studies have investigated Enterococcus faecium due to its capacity of being a nosocomial pathogen or its ability to produce bacteriocins. Others have investigated the effects of pH, temperature and salt concentration on the bacterium, but the methods applied analysed effects other than cellular activity.

4.2. Cellular activity

In this survey, the aim was to investigate the effect of heat stress on the cellular activity of Enterococcus feacium M-74. This knowledge may help to find out more about how to make this species resistant against elevated temperatures as encountered during technological formulation processing. An approach of making Enterococcus faecium restistant to heat was suggested by Maninez et al., who provided data on the effects on culture temperature and physiological state of cells on heat resistance of Enterococcus faecium ATCC 49624, which may be useful in establishing pasteurization procedures. They, however, did not only apply heat when the cells were in the stationary phase, but grew them at different temperatures, monitoring them at various stages of growth [25].

4.2.1. Fluorimetry

In the fluorometric assays, the three fluorescent dyes PI, FDA and DHR 123 were successfully employed. In the assays with PI it could be observed that as temperature stress was carried out, the fluorescence was altered. As PI only fluoresces strongly when it can enter cells, the high fluorescence proved that due to heat, the cell membrane of Enterococcus faecium M-74 had become permeable for the fluorescent dye. This means, that the cell membrane was damaged.

FDA has to be cleaved by esterases in order to increase its fluorescence. The fluorescence measured in heat-stressed cells was lower than in unstressed cells. However, the fluorescence decreased only to a small extent. Thus, it was not clear if FDA was a suitable fluorescent dye for this assay and if the esterases were influenced by heat stress.

DHR 123 should prove if the ROS generation in Enterococcus faecium M-74 was changed due to heat stress. Interestingly, the ROS generation was higher in unstressed cells than in cells which had been extensively exposed to heat. This seems to be in line with previous studies by Huycke et al., showing that superoxide production occurs in certain enterococci, such as Enterococcus faecium [26]. To assess the frequency of extracellular superoxide production by enterococci, multiple species were surveyed by them in a whole organism assay for their ability to reduce ferricytochrome C in a superoxide dismutase-inhibitable fashion. For stool and clinical enterococcal isolates and 12 type strains, only Enterococcus faecalis (87/91 isolates and ATCC 19433), Enterococcus faecium (5/13 isolates), Enterococcus casseliflavus (ATCC 25778), and Enterococcus gallinarum (ATCC 35038) produced extracellular superoxide. However, Henderson and Chappell have investigated the ability of DHR 123 as a fluorescent probe for superoxide generation and have demonstrated that this fluorescent dye reacts with hydrogen peroxide and not superoxide anions [27]. They have shown that it is hydrogen peroxide which oxidises the non-fluorescent DHR 123 to the fluorescent rhodamine. This reaction is slow but can be catalysed by an enzyme with a peroxidase activity, resulting in a greatly enhanced rate of increase in fluorescence. Due to these results it can be concluded that in the current survey DHR 123 detected the presence of hydrogen peroxide which had been generated by Enterococcus faecium M-74.

DioC6(3) and CFDASE were also measured with the microplate reader. In these assays, however, no evident differences could be detected between the fluorescence of samples of unstressed and stressed bacteria.

4.2.2. Flow cytometry

Propidium iodide and dihydrorhodamine 123

For investigating Enterococcus faecium M-74 using flow cytometry, four different fluorescent dyes were used. PI and DHR 123, which were applied both in fluorometric measurements and in flow cytometry, showed that due to stress, the amount of stained cells changed. In order to accentuate how the various parameters investigated in this survey are correlated, figures depicting the ability of reproduction of Enterococcus faecium M-74 when exposed to heat stress, are shown connected with the figures of PI and DHR 123.

Figure 40 shows the comparison of the assays with PI and DHR 123, when Enterococcus faecium M-74 was heated with 50°C. The fluorescence of PI was low, which means that membrane permeability did not change when Enterococcus faecium M-74 was confronted with a temperature of 50°C. The exposure of cells to 50°C for ten minutes caused a significant rise in the number of cells stained with DHR 123. The generation of ROS increased after two minutes of heat stress.

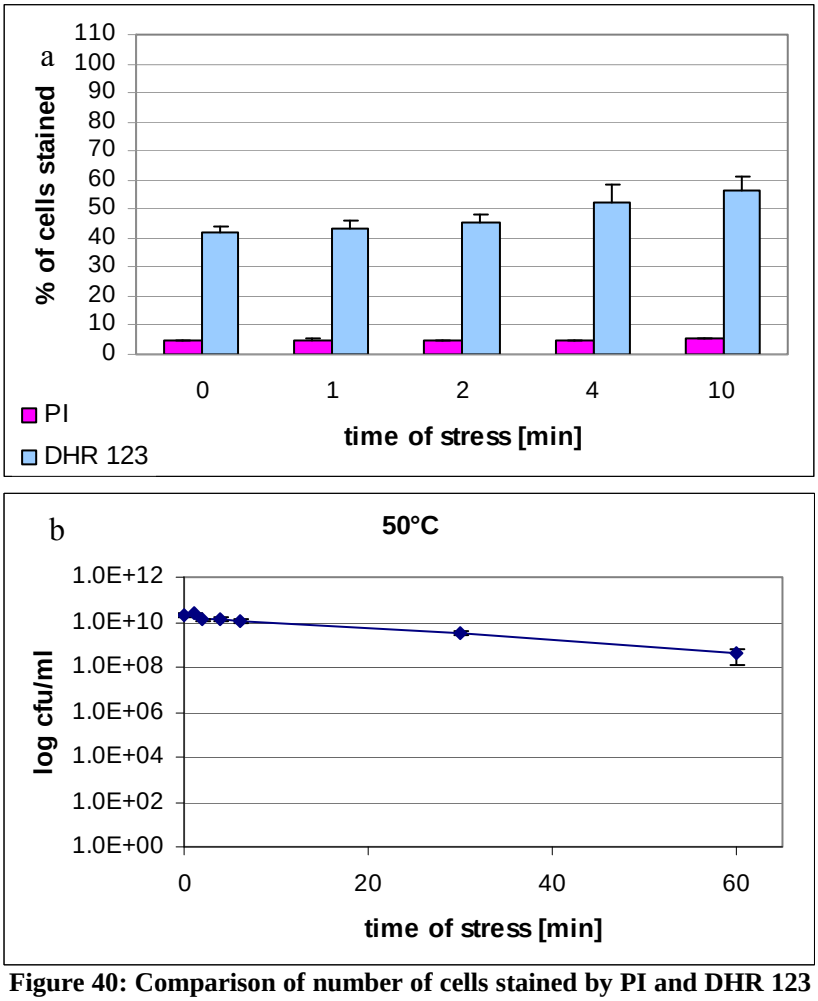

Figure 40: Comparison of number of cells stained by PI and DHR 123 after heat stress of E. faecium M-74 at 50°C (40a), effect of 50°C on reproduction of E. faecium M-74 (40b)

When Enterococcus faecium M-74 was exposed to 60°C for two minutes, less than 10% of the cells showed altered membrane permeability, whereas due to stress for four minutes, over 60% of the cells had damaged cell membranes, as presented in figure 41.

The fluorescence of DHR 123 changed within the same stress durations. Among bacteria which had been stressed for two minutes, 57.38% (± 2.7%) produced ROS, but after a stress of four minutes, this percentage declined.

These findings correlate with the effects of 60°C on reproduction of Enterococcus faecium M-74, as the bacteria start losing their ability of reproduction after two minutes of heat stress.

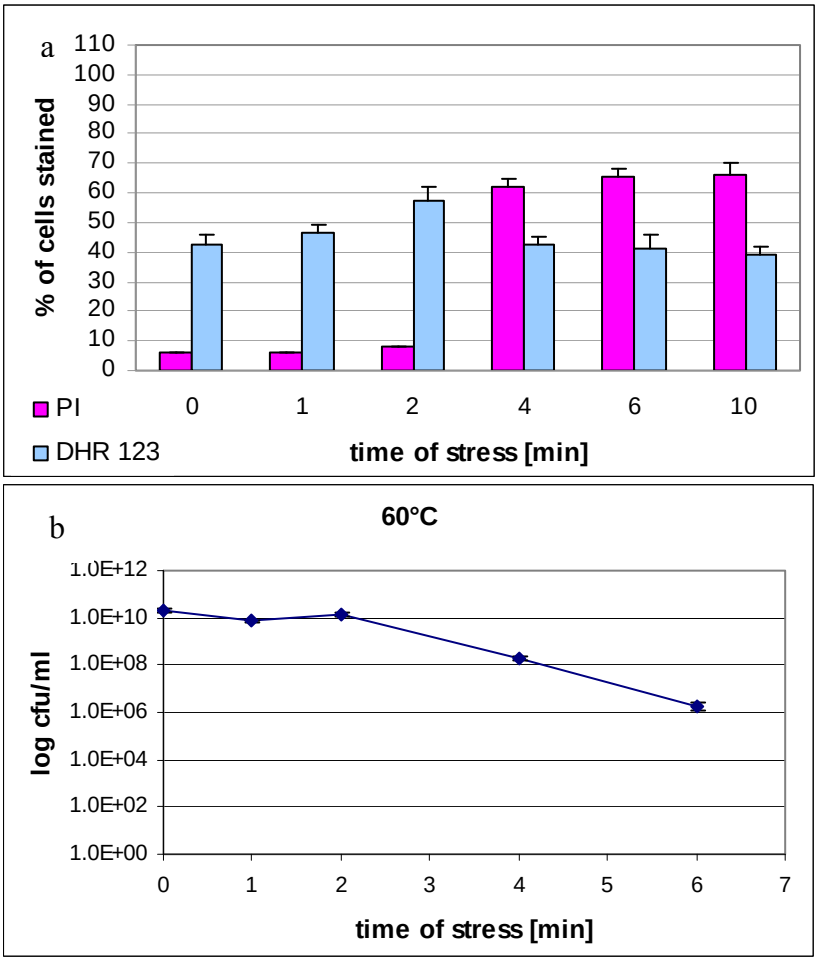

Figure 41: Comparison of number of cells stained by PI and DHR 123 after heat stress of E. faecium M-74 at 60°C (41a), effect of 60°C on reproduction of E. faecium M-74 (41b)

Figure 42 shows that a stress of two minutes at 70°C destroys the cell membranes of 49.07% (± 1.96%) of the bacteria and six minutes caused an increase to 98.96% (± 0.10%).

Regarding the amount of cells producing ROS, there was a rise until one minute of heat stress. When the cells were stressed for two minutes, ROS generation was decreased. Also cell viability decreased after one minute of heat stress at 70°C.

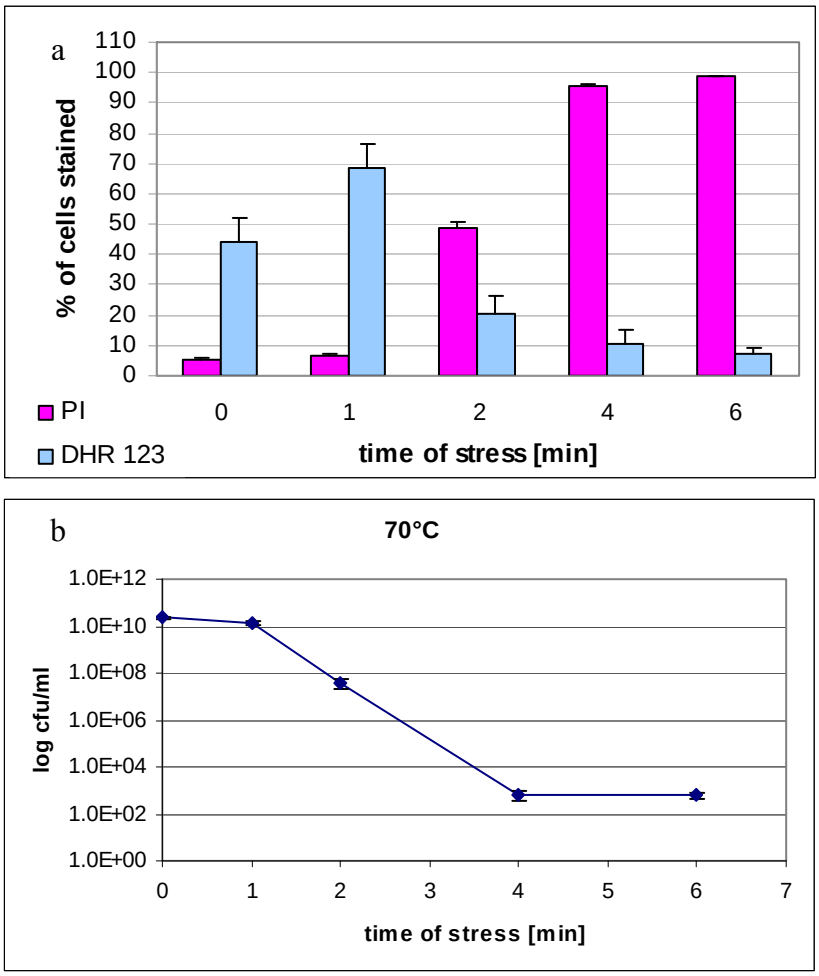

Figure 42: Comparison of number of cells stained by PI and DHR 123 after heat stress of E. faecium M-74 at 70°C (42a), effect of 70°C on reproduction of E. faecium M-74 (42b)

The changes are even more definite in figure 43, when Enterococcus faecium M-74 was stressed with 80°C. At half a minute of stress, less than 10% of the cell membranes were permeable and after more than one minute of heat stress the membranes of all bacteria were damaged.

Stressing Enterococcus faecium M-74 with 80°C also had a significant effect on ROS production, as the fluorescence of DHR 123 clearly varied when the cells had been exposed to heat for different durations. At 30 seconds of stress about half of the cells generated ROS, but one minute at 80°C made the number decrease. After two minutes of stress - when all cell membranes were damaged - less than 10% of the bacteria produced ROS.

The data received about the viability of Enterococcus faecium when heated with 80°C also show that as soon as membrane permeability increased, the ability of reproduction was lowered.

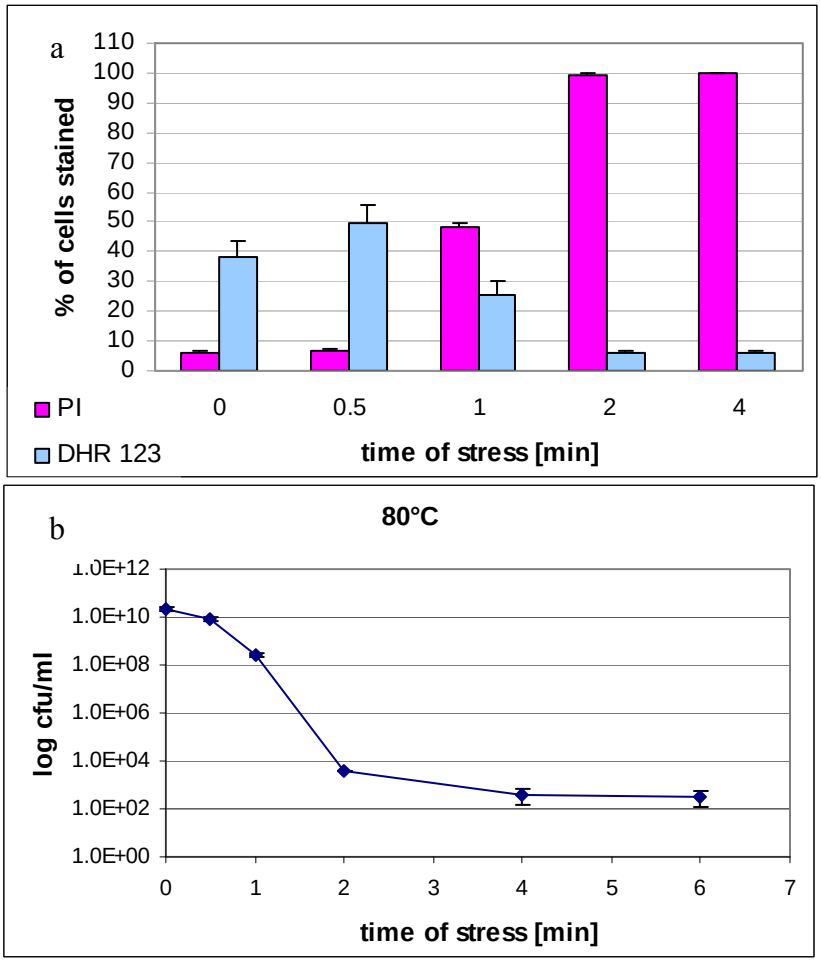

Figure 43: Comparison of number of cells stained by PI and DHR 123 after heat stress of E. faecium M-74 at 80°C (43a), effect of 80°C on reproduction of E. faecium M-74 (43b)

Figure 44 illustrates the comparison of membrane permeability and ROS generation when Enterococcus faecium M-74 was stressed with 90°C.

The amount of cells with damaged cell membranes increased very quickly, until all cells were damaged after 90 seconds of exposure to 90°C. The production of ROS increased until Enterococcus faecium M-74 was stressed for 30 seconds. At longer stress durations, the generation of ROS was reduced.

Again, the ability of reproduction in Enterococcus faecium M-74 correlated with the amount of cells with damaged cell membranes.

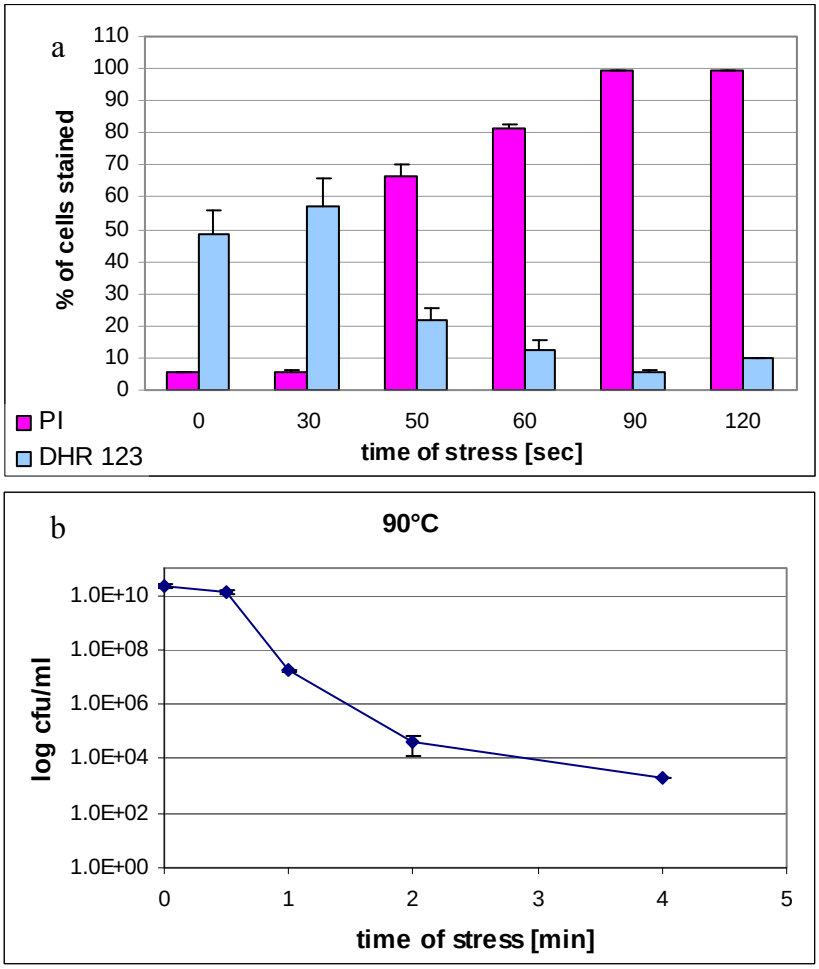

Figure 44: Comparison of number of cells stained by PI and DHR 123 after heat stress of E. faecium M-74 at 90°C (44a), effect of 90°C on reproduction of E. faecium M-74 (44b)

The comparison of the figures above (figures 40-44) indicates that before membrane permeability rises due to heat stress, more ROS are produced by Enterococcus faecium M-74. As the membrane becomes permeable, the ROS concentration decreases significantly. When the amount of cells stained with PI - showing the damage of the cell membrane - rises up to 100%, less than 10% of the cells are stained with DHR 123, which implies that the concentration of ROS is significantly reduced.

These results suggest that before bacteria are damaged by heat, the cells react to protect themselves against the changing in their environment with the physiological mechanism of ROS production.

Comparison of fluorescence of stressed cells using different fluorescent dyes

To compare flow cytometric results of all fluorescent dyes PI, DHR 123, DioC6(3) and CFDASE in figures, relative values were calculated. The fluorescence intensity of unstressed cells was set to 1.

The fluorescence intensity of the fluorescent dyes DioC6(3) and PI did not change significantly when the cells were treated with 50°C, as can be observed in figure 45. This shows that membrane integrity and membrane potential were not influenced. The intensity of CFDASE and DHR 123 changed, which means that intracellular pH and ROS production were affected by heat stress of 50°C.

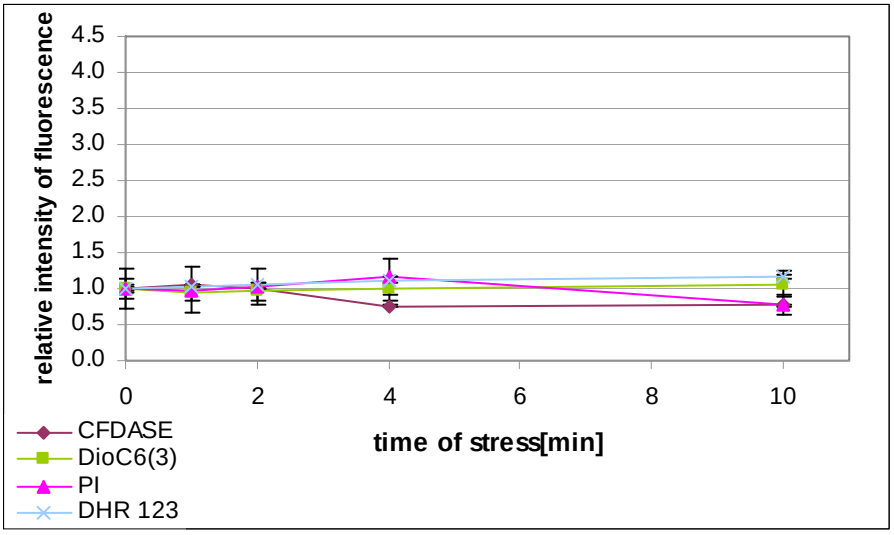

Figure 45: Comparison of CFDASE, DioC6(3), PI and DHR 123 after heat stress of E. faecium M-74 at 50°C

In figure 46, it is interesting to see that the curves of all four fluorescent dyes seem to undergo changes and reach a steady state at the same time. This finding implies that a heat stress of 60°C induces changes in cellular activity affecting membrane permeability, ROS generation, membrane potential and intracellular pH.

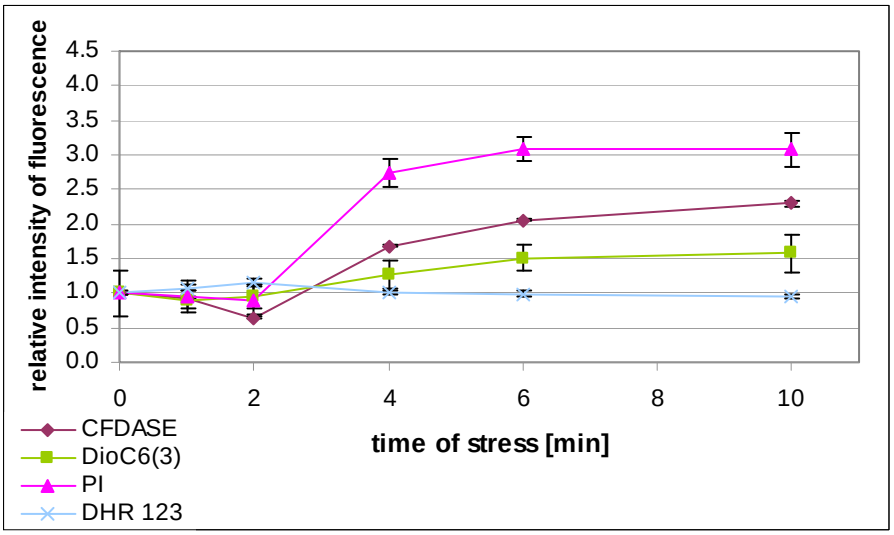

Figure 46: Comparison of CFDASE, DioC6(3), PI and DHR 123 after heat stress of E. faecium M-74 at 60°C

Figure 47 shows that when Enterococcus faecium M-74 was exposed to 70°C for one minute, membrane permeability and membrane potential did not change significantly. However, the production of ROS was enhanced and the intracellular pH was lowered. After one minute of treatment with 70°C, all four parameters changed.

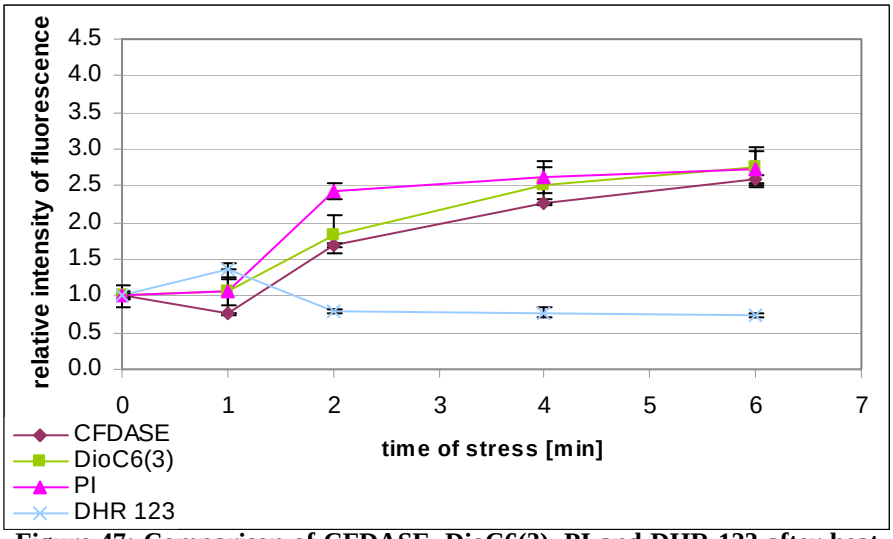

Figure 47: Comparison of CFDASE, DioC6(3), PI and DHR 123 after heat stress of E. faecium M-74 at 70°C

Figure 48 shows that membrane permeability increased significantly after one minute of heat stress at 80°C and ROS production was lowered after two minutes. The intracellular pH decreased when the bacteria were stressed for 30 seconds, but increased significantly at longer stress durations. It took two minutes of heat stress until the membrane potential changed.

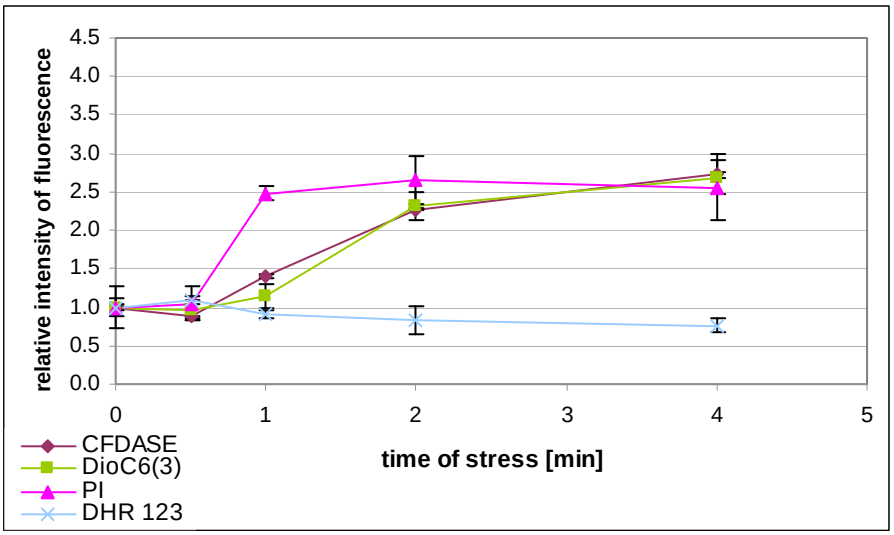

Figure 48: Comparison of CFDASE, DioC6(3), PI and DHR 123 after heat stress of E. faecium M-74 at 80°C

The effects of exposure of Enterococcus faecium M-74 to 90°C are depicted in figure 49. The change in membrane potential started when the cells were stressed for 50 seconds. At the same time the permeability of the membranes increased. At a stress duration of 30 seconds, significantly more ROS were produced than by unstressed cells. Due to longer exposure to 90°C, the production of ROS decreased. Intracellular pH was lowered due to 30 seconds of heat stress, but was significantly elevated when Enterococcus faecium M-74 was stressed for longer durations.

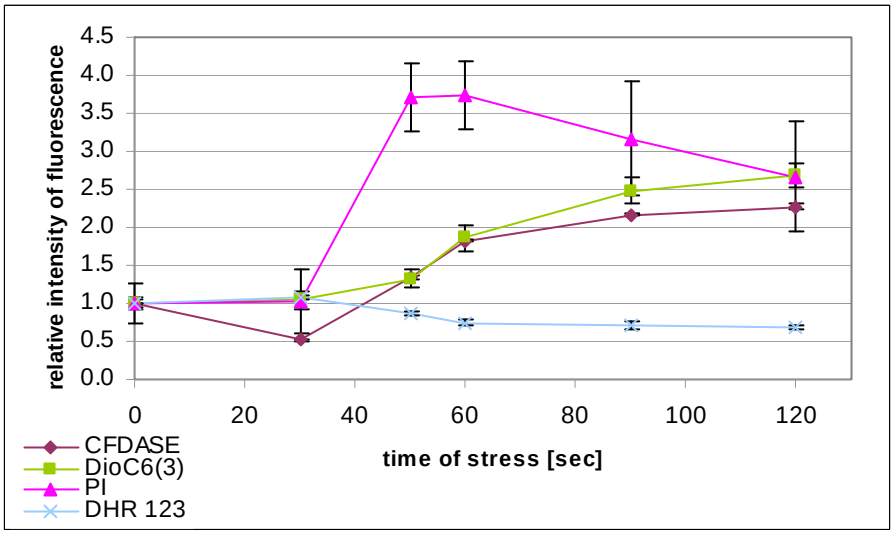

Figure 49: Comparison of CFDASE, DioC6(3), PI and DHR 123 after heat stress of E. faecium M-74 at 90°C

Taken together, the results of the flow cytometer showed the following changes for Enterococcus faecium M-74:
Exposing Enterococcus faecium M-74 to a temperature of 50°C did not cause significant changes in the parameters membrane permeability and membrane potential. ROS generation was slightly, but significantly enhanced when Enterococcus faecium M-74 was stressed for four minutes and intracellular pH was lowered at the same stress duration (figure 45).
Due to stress of 60°C, the parameters were subject to variation until a stress time of six minutes, as shown in figure 46. The only parameter which was still altered after six minutes was intracellular pH.
The temperature of 70°C caused dramatic changes and then generally let the curves remain stable after four minutes of heat stress (figure 47). The only parameter which still changed after four minutes was intracellular pH.
In figure 48, after stress of 80°C, the final values were almost reached after two minutes. Again, the parameter which still increased after this duration was intracellular pH.
Usually, the changes in the parameters were completed after 90 seconds, when Enterococcus faecium M-74 was stressed with 90°C (figure 49). As during the other stress temperatures, intracellular pH was still altered significantly after the other parameters had stopped changing.

A fascinating feature about the figures above is that even before membrane permeability was affected by heat stress, other parameters started to change. Normally, intracellular pH was lowered by short durations of heat stress, but as soon as membrane permeability increased, there was also a significant increase in intracellular pH. Similarly, a rise in ROS before heat stress affected membrane permeability could also be noticed. Membrane potential, however, did not change unless membrane permeability increased.

Basically, the changes in the cellular activitiy of Enterococcus faecium M-74 occurred between 0 and 10 minutes of heat stress at the temperatures applied in this survey. In a survey by Baatout et al., who also investigated temperature induced changes in bacterial physiology, they exposed microorganisms to various temperatures between -170°C and 70°C for one hour and after adding a fluorescent dye, measured fluorescence by means of flow cytometry [16]. Regarding the time in which Enterococcus faecium M-74 was affected by a temperature of 70°C in the current survey, the duration of one hour of stress is very long. However, the

microorganisms employed were not enterococci, which means that the time frame for changing in the cellular activity is different from Enterococcus faecium M-74.

The fluorescent dyes employed in this survey are commonly used. PI, for example, was used by Kim et al. who investigated membrane damage of bacteria by silanols treatment [28]. As it can stain different types of cells, they could stain gramnegative as well as grampositive bacteria, including Enterococcus faecalis. They found that due to the applied stress, the cell membranes of the microorganisms were damaged, which points out that bacterial cell membranes can also be damaged by stresses other than heat.

In the present study, FDA did not show convincing results using flow cytometry. It had been employed by Green et al. [29] in their assay for fluorescein diacetate hydrolytic activity. Their aim was to optimize the assay for FDA hydrolytic activity in soil samples. They chose to work with FDA because ubiquitous lipase, protease, and esterase enzymes were involved in the hydrolysis of this fluorescent dye, which was desirable for a method of measuring overall microbial activity. The optimization of their method revealed that an incubation time of three hours allowed sufficient time for hydrolysis to take place. The fluorescence was measured using a spectrophotometer set. In the current survey, an incubation time of one hour had been chosen during the assays using fluorimetry, which seemed sufficient as significant differences could be detected between unstressed and stressed bacteria. The incubation times used for the assays using fluorimetry were the same as for flow cytometry, but investigating the hydrolysis of FDA with flow cytometry did not confirm the previous results as no significant differences in fluorescence could be detected.

Another fluorescent dye used for studying changes in biomolecular activity was DHR 123. In a survey by Zurgil et al., it was employed for monitoring intracellular levels of reactive oxygen species [30]. They investigated generation of reactive oxygen species in promonocytic cells upon stimulation with hydrogen peroxide and oxidized lipid as this is not only an essential issue for bacterial cells. Important findings about ROS production by Enterococcus faecium were made by Moy et al., who pointed out that Enterococcus faecium produces high levels of hydrogen peroxide as aerobic cultures enter the stationary phase [31]. The release of hydrogen peroxide by bacteria has been shown to inhibit or kill other competing bacteria or host cells. Hydrogen peroxide generation is a common trait among enterococci and according to Moy et al., Enterococcus faecium produces hydrogen peroxide at levels that are high enough to kill the nematode Caenorhabditis elegans. DHR 123 was successfully applied in the current survey, which indicates that it is an appropriate method for measuring hydrogen

peroxide in Enterococcus faecium M-74. An interesting point is that among the unstressed bacteria, only about the half generated hydrogen peroxide. This fact was not detectable with the microplate reader, but as the flow cytometer measured the fluorescence of single cells, it could be found out that not all the cells were stained with the dye.

DioC6(3), the fluorescent dye which can point out changes in membrane potential, has previously been used by Baatout et al. in their survey about the influence of pH stress on Escherichia coli and Cupriavidus metalldurans CH34 [32]. They could show that as the bacteria were exposed to very low or very high pH, DioC6(3) showed increased fluorescence, indicating the loss of membrane potential. Similarly, the assays with DioC6(3) in the current thesis proved the loss of membrane potential caused by heat stress at different temperatures.

Monitoring changes in intracellular pH of microorganisms can be carried out using CFDASE, as did Riondet et al. during their research on Escherichia coli [33]. They suggested CFDASE as a useful means of detecting altered intracellular pH values. In their survey, they measured the fluorescence of CFDASE by means of a spectrofluorometer and they also pointed out that EDTA was needed to ensure sufficient membrane permeability for CFDASE. In the current thesis, no changes in the fluorescence of CFDASE could be detected by fluorimetry, although addition of low concentrations of EDTA had been carried out (data not shown). The flow cytometric measurements using CFDASE were more successful as they could clearly show that the fluorescence intensity of CFDASE was altered in Enterococcus faecium M-74 exposed to stress. In contrast to the flow cytometric method, fluorimetry measurements with CFDASE might not be suitable for the investigation of changes in intracellular pH of Enterococcus faecium M-74 after heat stress.

This shows, that the two methods employed in this survey for showing changes in cellular activity - fluorimetry and flow cytometry -, can complement one another. As two of the fluorescent dyes - PI and DHR 123 - were employed in both methods, it could be proved that the results matched.

5. Conclusion

The effect of heat stress on reproduction of Enterococcus faecium M-74 was studied. It could be confirmed that, the higher the temperatures applied were, the faster the number of viable bacteria was reduced.

These results correlate with those received by fluorescence measurement and flow cytometry. Hence, it is possible to state that Enterococcus faecium M-74 undergoes severe modifications when exposed to heat stress. There, it could also be seen that the changes in the vital functions occurred the sooner, the higher the heat applied was.

By means of fluorescent dyes several vital functions of the bacterium have been investigated. The increasing fluorescence of PI the longer Enterococcus faecium M-74 was confronted with heat indicated that the cell membrane was damaged by the stress, reflecting the results received by testing the effects of heat stress on the ability of reproduction.

The slightly decreased fluorescence of FDA due to high temperatures suggested a lower activity of esterases. Nevertheless, these results obtained with the microplate reader could not be confirmed by flow cytometry as the incubation time with the fluorescent dye might have been too short.

The utilization of DHR 123 helped reveal that Enterococcus faecium M-74 physiologically generated ROS and reduced this production due to heat stress. However, before the cell membrane of Enterococcus faecium M-74 was damaged by heat stress, higher fluorescence of DHR 123 indicated that as a quickly happening reaction to heat stress, the generation of ROS was increased.

The membrane potential also changed when Enterococcus faecium M-74 was heated. According to the fact that the fluorescence of DioC6(3) drops when the cell membrane is hyperpolarized, the conclusion can be made that as Enterococcus faecium M-74 was heated, the cell membrane was depolarized.

CFDASE, which proved to fluoresce more intensely at alkaline pH values, was meant to indicate changes in the intracellular pH of Enterococcus faecium M-74. As the bacterium was

stressed, the intensity of the fluorescence increased, which supported the conclusion that because of heat stress, the intracellular pH became more alkaline.

6. Abbreviations

MRS (broth)	de**M**an, **R**ogosa, **S**harpe
EF, E. faecium	**E**nterococcus **f**aecium
rpm	**r**otations **p**er **m**inute
(30)**''**	second
(1)**'**	minute
(1)**h**	**h**our
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'**di**hexyl**o**xa**c**arbocyanine 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
DMF	**d**i**m**ethyl**f**ormamide
HCl	**h**ydro**c**h**l**oric acid
NaOH	sodium hydroxide
HEPES	4-(2-**h**ydroxy**e**thyl)-1-**p**iperazine**e**thane**s**ulfonic acid
cfu	**c**olony **f**orming **u**nits
EDTA	**e**thylene**d**iamine**t**etraacetic **a**cid
ROS	**r**eactive **o**xygen **s**pecies

7. References

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Figures

Figure 2: www.cerbios.ch/pages/bio.html; accessed July 28th, 2008

Figure 3: http://faculty.ircc.edu/faculty/tfischer/images/bacterial%20growth%20curve.jpg; accessed January 18th, 2009

Figure 4-8: www.sigmaaldrich.com; accessed July 28th, 2008

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Figure 11: http://www.chemtronindia.com/general.html; accessed July 29th, 2008

8. Curriculum vitae

Iris Altenburger

date of birth	December 22, 1983
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Education

October 2003- July 2009	studies of pharmacy, University of Vienna
October 2002- July 2003	Berlitz 4-languages-diploma at Berlitz Austria GmbH (English, French, Spanish, Russian)
September 1994- June 2002	Bundesgymnasium/Bundesrealgymnasium Stockerau

Courses

September 2008	Summer School of TCM, Beijing University of Chinese Medicine

9. Zusammenfassung

Mikroorganismen des Stammes Enterococcus faecium M-74 ist in verschiedenen probiotischen Produkten enthalten. Für die Wirksamkeit dieser Produkte ist es ausschlaggebend, dass vermehrungsfähige Bakterien enthalten sind. Es ist bei den Herstellungsverfahren solcher Produkte wichtig, Kenntnis darüber zu besitzen, wie belastbar die verwendeten Bakterien in Bezug auf verschiedene Stressfaktoren sind. In der vorliegenden Diplomarbeit wurden die Auswirkungen von Hitzebelastung auf Enterococcus faecium M-74 untersucht.

Die Effekte auf die Vermehrungsfähigkeit von Enterococcus faecium M-74 wurden bei fünf verschiedenen Temperaturen (50°C, 60°C, 70°C, 80°C und 90°C) beobachtet. Die Ergebnisse zeigten, dass die Anzahl der lebensfähigen Bakterien umso stärker sank, je höher die Temperatur war, der sie ausgesetzt worden waren.
Unter der Annahme, dass unterschiedliche Veränderungen bzw. Reaktionen in Bakterien statt finden, sobald sie mit hohen Temperaturen konfrontiert werden, wurde Enterococcus faecium M-74 unter Verwendung verschiedener Fluoreszenzfarbstoffe mittels Fluorimetrie und Flowzytometrie untersucht. Aufgrund der unterschiedlichen Eigenschaften der Fluoreszenzfarbstoffe konnten mehrere lebenswichtige Funktionen der Bakterien unter Hitzestress beobachtet werden.
Der Fluoreszenzfarbstoff Propidiumiodid zeigt sehr starke Fluoreszenz, sobald er in Zellen eindringen und an Nukleinsäuren bindet. Da er für gewöhnlich Zellmembranen nicht durchdringen kann, deutet ein Anstieg der Fluoreszenz darauf hin, dass die Zellmembran beschädigt ist. Je länger Enterococcus faecium M-74 einem Hitzestress ausgesetzt wurde, umso stärker war die gemessene Fluoreszenz von Propidiumiodid. Außerdem war die gemessene Fluoreszenz umso stärker, je höher die Stresstemperatur war.
Fluoresceindiacetat ist ein Fluoreszenzfarbstoff, der anzeigt, ob die Esterasen der Zellen noch funktionstüchtig sind, da er fluoresziert, sobald er durch Esterasen gespaltet wird. Da die Fluoreszenz durch Hitzestress sank, wurden die bakteriellen Esterasen offensichtlich in ihrer Funktion eingeschränkt.
Dihydrorhodamin 123, ein Fluoreszenzfarbstoff, der fluoresziert, sobald er oxidiert wird, beweist, ob von einer Zelle reaktive Sauerstoffspezies - vor allem Wasserstoffperoxid - produziert werden. So konnte festgestellt werden, dass Enterococcus faecium M-74 unter physiologischen Bedingungen reaktive Sauerstoffspezies produziert. Durch die Messungen

mittels Flowzytometer wurde jedoch gezeigt, dass nur etwa die Hälfte der Bakterien reaktive Sauerstoffspezies generierte. Durch Hitzestress wurde die Produktion vorerst verstärkt, doch nachdem die Zellmembran geschädigt wurde, sank sie deutlich ab.
Das Membranpotential von Enterococcus faecium M-74 wurde mit dem Fluoreszenzfarbstoff 3,3'Dihexyloxacarbocyaniniodid untersucht. Da die Fluoreszenz dieses Markers stieg, wenn Enterococcus faecium M-74 Hitze ausgesetzt wurde, kann darauf geschlossen werden, dass die Zellmembran durch Stress depolarisiert wurde.
Carboxyfluoresceindiacetat Succinimidylester ist der Fluoreszenzfarbstoff, der zeigte, dass der intrazelluläre pH-Wert von Enterococcus faecium M-74 durch Hitzestress anstieg.

Es konnte im Rahmen der Diplomarbeit festgestellt werden, dass die Veränderungen der Bakterien durch Hitzestress umso schneller geschahen, je höher die angewendete Temperatur war. Die Methoden, die eingesetzt wurden, um die Veränderungen in Enterococcus faecium M-74 zu detektieren, erwiesen sich als geeignet.
Der Zusammenhang zwischen dem Verlust der Vermehrungsfähigkeit und der Zerstörung der Zellmembran sowie der Aktivität der bakteriellen Esterasen, der Produktion von reaktiven Sauerstoffspezies, der Veränderung des Membranpotentials und des intrazellulären pH-Wertes der Zellen wurde bestätigt.
Die Daten über die Reaktion von Enterococcus faecium M-74 auf Hitzestress sollten Aufschluss über die Stressreaktionen in diesem probiotischen Mikroorganismus geben und können der Optimierung von Herstellungsverfahren und Stabilisierungsprozessen probiotischer Produkte dienen. Enterococcus faecium wurde im Rahmen dieser Diplomarbeit ausgewählt, da es ein robustes Bakterium ist und als probiotischer Modellorganismus mit sensibleren Stämmen verglichen werden kann.