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DISINTEGRATION OF MICROORGANISMS

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ABSTRACT

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The effects of ionized air in this study were determined for protein content and disintegration of microorganisms in a Baker's yeast-sodium chloride-solution. The Bradford method was used for determining the protein content and the Koch method was used for determining the microorganisms. Non-ionized air and ionized air was used in both methods, so the effects of ionized air could be compared with the non-ionized air. Ionized air came from an ionizator, which made micro bubbles in to the solution.

There is previous research of disintegration of microorganisms with using ozone and it is very effective for that. In this work were found out if ionized air or ozone is better to use taking into account every sides of them; economy, toxicity and effectiveness. The results for this comparison were that disintegration of microorganisms is as successful with both airs. Ozone is more toxic and economically more expensive than ionized air, but it suits better to bigger amount of disintegration of microorganisms.

The results for the impact of ionized air were as same as for ozone, but only with doing disintegration with Bradford method and using bovine serum albumin (BSA) solution. The results of yeast-sodium chloride solution were not successful. The Koch method gave only one expected result, so they are not enough for saying that the method worked.

Key words
Bradford method, ionized air, Koch method, microorganism, protein, yeast cell

KESKI-POHJANMAAN AMMATTIKORKEAKOULU
MELLERSTA ÖSTERBOTTENS YRKESHÖGSKOLA

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Tässä työssä määritettiin ionisoidun ilman tehokkuus proteiinien määrästä sekä tutkittiin mikro-organismien tuhoamista liuoksessa, joka sisälsi hiivaa ja natriumkloridia. Proteiinien määrää määritettäessä käytettiin Bradfordin menetelmää ja mikro-organismien määrittämiseen käytettiin Kochin menetelmää. Molemmissa menetelmissä käytettiin sekä ionisoimatonta että ionisoitua ilmaa, jotta ionisoidun ilman tehokkuutta voitiin vertailla. Ionisoitu ilma syntyi ionisaattorissa, joka teki mikrokuplia liuokseen.

Aikaisemmin on tutkittu mikro-organismien tuhoamista käyttämällä otsonia ja se on todettu olevan tehokas tähän tarkoitukseen. Tässä työssä selvitettiin onko ionisoitu ilma vai otsoni parempi käytettäväksi, ottaen huomioon ionisoidun ilman sekä otsonin hyvät ja huonot puolet, kuten taloudellisuus, myrkyllisyys ja tehokkuus. Tämän vertailun tulokseksi saatiin että molemmat ilmat ovat tehokkaita mikro-organismien tuhoamiseen. Otsoni on myrkyllisempi ja kalliimpi kuin ionisoitu ilma, mutta se sopii paremmin suurempien määrien mikro-organismien tuhoamiseen.

Ionisoidulla ilmalla saadut tulokset ovat samanlaiset kuin otsonilla, mutta vain tehtäessä Bradfordin menetelmällä ja käytettäessä bovine serum albumin (BSA)-liuosta. Hiivan ja natriumkloridin liuoksessa tulokset eivät olleet odotusten mukaiset. Kochin menetelmä antoi vain yhdet odotetut tulokset, joten ei voida päätellä että menetelmä toimi.

Asiasanat
Bradfordin menetelmä, hiivasolu, ionisoitu ilma, Kochin menetelmä, mikro-organismi, proteiini

PREFACE

We would want to give the greatest thanks for Management Center Innsbruck that we got the possibility to do this final thesis there. It was the best time and in the same time the hardest time in Innsbruck when we made this; spending very long days in laboratory, sometimes even twelve hours.

Especially thank you goes to our supervisors Master of Science Marco Rupprich and Diploma Engineer Thomas Obholzer. They helped us always when help was needed and had patience to teach, although language problems came many times against us. And thank you goes also to Marc and Benjamin, they were also very helpful.

The greatest thank you goes to our supervisor Master in Philosophy Jana Holm and Licentiate in Philosophy Esko Johnson. They helped us very well, so we got good help to write this thesis. And most important thing they did not pressurize us too much, so everything went great with own weight.

The warmest thank you goes to our families and friends. Especially our common friends, who we got to know in Austria. They were very interested in our thesis and were asking all the time when the thesis is finished. Now it is finished, because we got so motivated from friends. And of course we motivated each other hard working with many long phone calls.

Concept words

Absorbance: In spectroscopy the absorbance is also called optical density. It describes the solution's ability to absorb the light in some specific wavelength.

Agar: Agar is made by brown algae and it is acid polysaccharide. It is used to solidify the liquid growth media and it is both chemically and microbiologically strong enough.

Autoclave: Autoclave is a pressured vessel and used to sterilize with overpressure water steam.

Baker's yeast: Usually Baker's yeast is called *saccharomuces cerevisiae*. It is used in microbiology and also baking.

Calibration curve: Calibration curve is a general method for determining the concentration of a substance in an unknown sample by comparing the unknown sample to the linear line of standard samples, which concentrations are known.

Cell culture: Microbe cells forming in this culture.

Centrifugation: Centrifugation is a separation method based on gravitation from different weights of solutions or solid substances in liquid.

Colony: Colony is made of cells and it is a group of cells in a basic plate. The colony is shown in the plate and can be seen without a micrograph.

Correlation coefficient: Correlation coefficient describes dependence of two variables and means correlation of observations from experience.

Coomassie: Coomassie dyes are commonly used to stain proteins in sodium dodecyl sulfate and blue native polyacrylamide electrophoresis gels. For determining protein concentration in a solution with the Bradford test coomassie dyes are an integral component.

Culture: Microbes or cells in the solid or liquid culture.

Fungi: Eukaryotic organisms that include microorganisms as yeasts and molds.

Homogeneous: In the homogeneous solution the solid substances are mixed very well to the liquid, so that all particles are dissolved.

Heterogeneous: Heterogeneous is opposite to homogeneous. It is a solution with different kind of particles, which can be seen with bare eyes.

Microorganism: Microorganism, also called microbe, is a micrographic small organism belonging to the group of protozoa, bacteria, algae, fungi and virus.

Pathogens: Pathogens, potentially present in wastewater, can be divided into three separate groups, which are the viruses, bacteria and the pathogenic protozoan/helminthes.

Protozoa: Unicellular eukaryotes classified from microorganisms.

Sludge: Originates from wastewater treatment process or industrial processes. Sludge tends to concentrate heavy metals and poorly biodecrable trace organic compounds and potentially pathogenic organisms, such as viruses or bacteria, present in wastewater.

Van der Waals forces: Van der Waals forces are much weaker than chemical bonds. They can be shared in three different groups (dispersion forces, bond strength and electric attractions) depending on atoms or molecules.

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1 INTRODUCTION

This final thesis' topic was given by Management Center Innsbruck Technical Unit in Austria. This thesis looks into how ionized air destroys microorganisms with Baker's yeast. It has been already researched that ozone is effective for disintegration of microorganisms but if ionized air is usable for disintegration of microorganisms too, it is better to use it. Ozone is more toxic and expensive than ionized air. The main task in this thesis is to study how ionized air destroys microorganisms and protein content. The Bradford method is used in this research for determining the protein content and the Koch method for determining the disintegration of microorganisms.

In both methods are used non-ionized air and ionized air, so the effects of ionized air could be compared. Ionized air comes from a compressor to the solution via an ionizator but the non-ionized air comes directly from a compressor. The non-ionized and ionized air comes to the solution as micro bubbles. The suitable pressure is needed to find out for a successful disintegration of microorganisms. From the solution are taken the samples in different time points for the measurements.

The Bradford method is based on the sample's absorbance measured with a spectrophotometer. In this case is used a spectrophotometer with wavelength 595 nanometer. The samples should be directly comparable to time, which means that after the sample has been taken, the absorbances should decrease after every minute. Before measuring the samples, is needed to do a calibration with the spectrophotometer. From these results is made a calibration curve with excel, which is needed when the experiments results are analyzed. In the Bradford method is used as solution water, Baker's yeast and bovine serum albumin (BSA), which is a Bradford's protein assay. It is needed to find out the amounts of Baker's yeast and BSA, which are suitable for disintegration of microorganisms. Filtration and centrifugation for samples are used in the Bradford method for finding out how they affect to the results.

The Koch method is based on the amount of microorganisms in the sample. In this method the sample is directly comparable to time, which means that after the sample has been taken, the amount of microorganisms on the plate should decrease after every minute. Water, sodium chloride and Baker's yeast are used as solution in the Koch method.

Based on the results, it is compared, if ionized air or ozone is better for protein content and disintegration of microorganisms in a solution with sodium chloride and Baker's yeast, taking into account every sides of them; economy, toxicity and effectiveness. The aim of this study is to find out if the ionized air disintegrates the microorganisms and how effective it is.

2 WASTEWATER

Between storm water and sanitary wastes the characteristics of the waste water effluent depend upon the population and industrial sector served, land uses, groundwater levels and degree of separation. Various gases, organic and inorganic compounds comprise the chemical waste water. Proteins, fat and greases, carbohydrates, oils, surfactants, pecticides and fenols are organic components. Heavy metals, phosphorus, nitrogen, chlorides, pH, sulfur alkalinity and toxic compounds are inorganic components. Methane, hydrogen sulfide, ammonia, oxygen, nitrogen and carbon dioxide are the common waste waters dissolved gases. Dissolved gases in waste water are commonly hydrogen sulfide, methane, ammonia, oxygen, carbon dioxide and nitrogen. Biologically, wastewater incorporates diverse microorganisms. Protista, animals and plants are the biggest concern, where protista consist bacteria, protozoa, fungi and algae, and plants from mosses, seed plants, ferns and liverworts. Animals consists from vertebrates and invertebrates and the most important category in wastewater treatment are the protista, bacteria, protozoa and algae. (The Green Lane 2002.)

Microorganisms role in disease transmission and in biological treatment processes are significant in water and wastewater. There should be no pathogenic microorganisms in drinking water. The microbial quality of drinking water is controlled by specified treatment techniques and monitoring for the presence of coli form bacteria. (Spellman 2003, 307.)

2.1 Industrial wastewater

Several industrial production effluents can cause major disturbance of the biological equilibrium by consumption of dissolved oxygen in a water resource because they are polluted by various organic substances. (DAS Environmental Expert GmbH 2009.)

Released effluent water into the sewer or a water resource is set limits to the biological oxygen demand (BOD) and chemical oxygen demand (COD). Effluent has to be therefore treated

partially or completely before releasing it into the sewer or into a water resource and can also be a way to recycle process water. (DAS Environmental Expert GmbH 2009.)

2.2 Biological technologies for wastewater treatment

By the growth of aerobic organisms, most compounds will be completely converted to CO_2 and water can be removed from effluent waste water in this way at relatively low cost. The demand of industrial effluents oxygen is often in the range of 1000 to 10000 mg/l when normally the solubility of oxygen in water is only a few mg/l. Using modern methods, high concentrations of biomass can be reached but the air has to be introduced against the pressure of the water column and causes high operation costs. Anaerobic methods operate in the absence of oxygen, but start-up and stable operation of these methods are difficult. (DAS Environmental Expert GmbH 2009.)

2.3 Trickle-Flow reactor

The Trickle-Flow reactor can, by biological microorganisms, treat the organic load (BOD, COD) of industrial production effluent. Easily transportable materials are used, small grains, which are overgrown with a highly active mixed population and adapts to the specific operating conditions. The carrier material can be aerated easily, meanwhile to the conventional technology, because it is not submersed in bulk water. Ambient air is ventilated from the bottom and the effluent water trickles continuously from top to bottom over the packed bed. The carrier material does not need to be exchanged because it is stable and growing biomass is flushed out in an automatic regeneration cycle. The basis for the high and stable biological degradation of pollutants is the high density of microorganisms and the optimal conditions for chemical transfer between biomass, water pollutants and oxygen in excess. The investment cost is low because of the simple principle and the high efficiency. Also the operating cost is low due to minimal operating and maintenance needs. The design is advantageous since

corrosion protection is not necessary and maintenance times are low. (DAS Environmental Expert GmbH 2009.)

GRAPH 1. Trickle-Flow reactor (adapted from Penz 2008, 7)

3 OZONE AND IONIZED AIR

3.1 Ozone and ozonation

Ozone (O_3) consists of three oxygen atoms. It is an unstable gas with strong oxidizing power and a short reaction time. Ozone has harmful effects against bacteria, viruses and protozoa. It can eliminate not only inorganic, organic and microbiological problems, but also taste, odor and color problems. (Wilkes University 2009.)

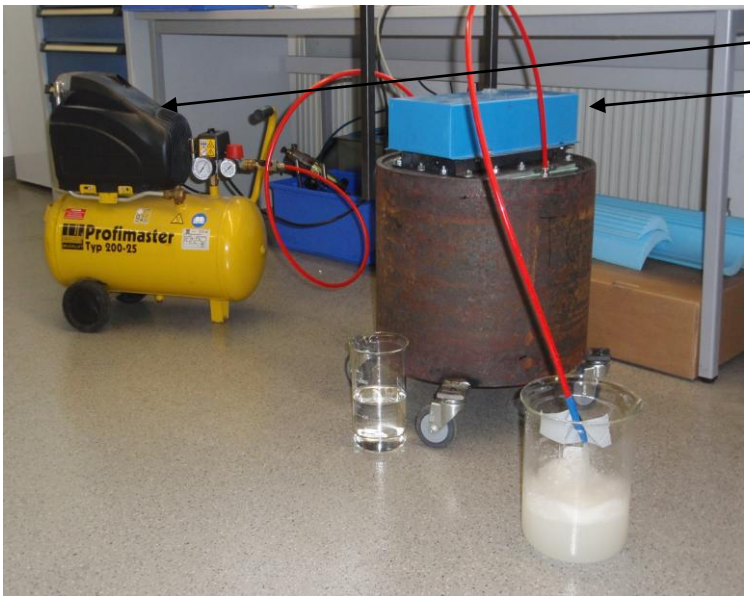
Ozone, a strong oxidizing gas, is an effective disinfectant and an oxidant of taste and odor compounds. It has a rapid reaction in inactivating microorganisms (Hammer, M. & Hammer, M. Jr. 2001, 263). Ozone can extremely affect bacteria. It penetrates the cell wall and damages the cell membrane also destroying the zoogloea structures by oxidizing the extracellular polymeric substances (EPS) and bridging matters. The solid organic components of sludge are then transformed to soluble substances and can further to be biologically degraded when the ozonated sludge is returned to the process of wastewater treatment. (Zhang, Yang, Liu & Zhang 2009.)

Ozone is using in water treatment; purification, odor, taste and color. It has both advantages and disadvantages for disintegrating microorganisms. Comparing for other purification methods ozone's advantage is that it is very effective against aerobic microorganisms. It kills bacteria very quickly and many different kind of styles. (Ozair 2007.) Ozone's disadvantages for exposing to it, are that it can damage the lungs and other different kind of reactions to human. It is dangerous for environment too, especially for plants. (International Chemical Safety 2005.)

Ozonation destroys bacteria and other microorganisms in waste water process through molecules to high electrical voltages. Ozone does not remain in water very long, but it is a very strong disinfectant so chlorine has to be added to protect the water while it is in the distribution system. (Southern Nevada Water Authority 2008.)

3.2 Air ionizator and micro bubbles

An air ionizer is a negative ion generator which uses high voltage to ionize air molecules. These negative ions, which are anions, are particles with one or more extra electrons. These electrons give a net negative charge to the particle. (Air ioniser 2009.)



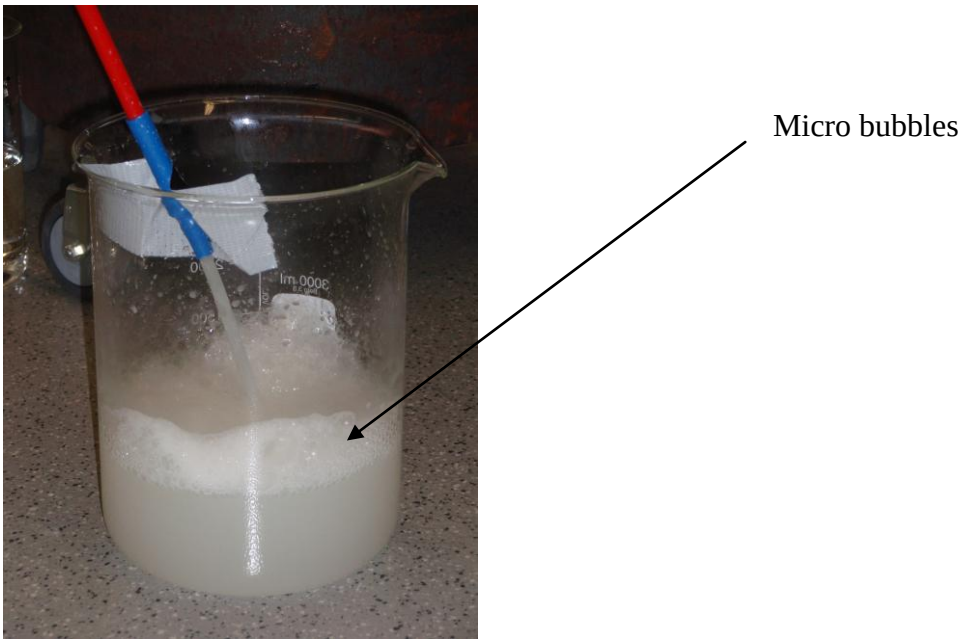
GRAPH 2. The experiment with ionized air to a yeast solution (Photo: authors)

Micro bubbles are tiny gas bubbles in the water, with diameters less than 50 μm. In recent years there has been a lot of research on micro bubbles. The micro bubbles can remain suspended in the water for an extended time. The micro bubbles with the gas, in this case ionized air, dissolves into the water. (Suwa Precision Group 2009.)

Microorganisms attract suspended floating particles very effectively by having electrical charges. This appropriate feature has been used in sludge treatment by using the micro bubbles to capture and float organic matters and it does not need so much time for sludge treatment. (Suwa Precision Group 2009.)

In this thesis the used air was inserted to the solution true an ionizator, which converted the air to ionized air. The ionized air was inserted as micro bubbles to our solution. A very low

pressure was applied, so that in the solution appeared only few bubbles. This is because in the first experiments the used pressure was too high and the amount of ionized air were too much, so the results were not successful. (Suwa Precision Group 2009.)



GRAPH 3. Ionized micro bubbles to a yeast solution (Photo: authors)

3.3 Difference between ionized air and ozone

Both ionizers and ozone generators devices operate in a similar way but they should not be mixed. Ionizers produce positively or negatively charged gas ions. Ozone generators use either a corona discharge tube or UV light to attract an extra oxygen ion to the O_2 molecule. All ionizers produce a small amount of ozone, and ozone generators gaseous ions of molecules other than ozone. (Air ioniser 2009.)

Pure ozone is a highly toxic and extremely reactive gas and can damage odor bulb cells directly. At high concentrations it can also be toxic to air-borne bacteria and may destroy or kill these sometimes infectious organisms. Ionized air is not as toxic as ozone. (Air ioniser 2009.)

4 PROTEINS

Proteins are polymers of amino acids covalently bonded by peptide bonds. Dipeptide constitutes two together bonded amino acids, tripeptide three amino acids and so on. Many of these amino acids are covalently linked together via peptide bonds and to form a polypeptide. Proteins are consisted of one or more polypeptides. (Wiesmann, Choi & Dombrowski 2007, 45.)

Protein plays key roles in cell function and the major classes of proteins are catalytic proteins (enzymes) and structural proteins. The catalysts for the wide variety of chemical reactions that occurs in cells are enzymes. Structural proteins become integral parts of the structures of cells in membranes, walls and cytoplasmic components. From bacterial cell mass nearly 50% is proteins. (Wiesmann et al. 2007, 45.)

In living organisms nearly all reaction steps are catalyzed by a special enzyme, which are biocatalytic proteins. Without the effect of enzyme, no organism would survive. Each reaction step must be catalyzed by a special enzyme. (Wiesmann et al. 2007, 45.)

4.1 The Bradford method for determining the protein content

The Bradford method is used for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis. (Caprette 2005.) It is a spectroscopic analytical procedure used to measure the concentration of protein in a solution, but also dependent on the amino acid composition of the measured protein. The Bradford method is based on a solution's ability to absorb the light on the wavelength from λ_{max} = 465 to 595 nanometer. (Waldmann & Janning 2006, 120.)

Absorbances can be calculated from formula 1,

$$A_\lambda = -\log_{10}\left(\frac{I}{I_o}\right) \quad (1)$$

where A_λ is the calculating absorbance, I is the intensity of light at the specified wavelength λ that has passed through the sample. I_o is the intensity of light before it enters the sample or incident the light intensity. (Absorbance 2009.)

The Bradford assay is a colorimetric protein assay. It is based on an absorbance transition in the dye coomassie when it changes and stabilizes by the binding of protein from the red form coomassie reagent into coomassie blue. Two types of bond interaction take place during the formation of this complex. The red form of coomassie dye first donates its free proton to the ionizable groups on the protein. It causes a disruption of the protein's native state, consequently exposing its hydrophobic pockets. On the protein tertiary structure, these pockets bind non-covalently to the non-polar region of the dye via van der Waals forces positioning the positive amine groups in proximity with the negative charge of the dye. Further, the bond is strengthened by the ionic interaction between the two. The binding of protein stabilizes the blue form of coomassie dye. Thus the amount of complex present in solution is a measure for the protein concentration by use of an absorbance reading. (Bradford protein assay 2009.)

The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 (see the manual by Roti®-Nanoquant in Appendix 1) transfers from 465 nm to 595 nm, and appears binding to the protein. Hydrophobic and ionic interaction stabilizes the anionic form of the dye and causes a visible color change. Since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range, the assay is necessary. (Caprette 2005.) Sodium dodecyl sulfate (SDS) is a common detergent and could be found in protein extract because it is used in lysing cells by disrupting the membrane lipid bilayer. Sodium dodecyl sulfate tends to bind well with protein, inhibiting the protein binding sites for dye reagent. (Bradford protein assay 2009.)

4.2 Spectrophotometer for measuring the samples absorbances

A spectrophotometer consists of two instruments. A spectrometer produces light of any selected wavelength. A photometer is for measuring the intensity of light. The liquid in a cuvette can be placed between the spectrometer beam and the photometer and the amount of light passing through the tube is measured by the photometer. A voltage signal is delivered to a display device, normally a galvanometer, by the photometer and the signal changes as the amount of light absorbed by the liquid changes. Concentration can be measured by determining the extent of absorption of light at the appropriate wavelength if development of color is linked to the concentration of a substance in solution. (Caprette 2005.)

GRAPH 4. A spectrophotometer SHIMADZU UV Mini 1240 used in the Bradford method for measuring the protein content (Photo: authors)

4.3 Protein assay standard

To choose a protein standard can be the largest source of fault in any protein assay. A purified, known concentration of the predominant protein found in the sample is the best choice for a standard. But the highly purified version of the protein is not always available or it is too

expensive to use as the standard. In this study bovine serum albumin (BSA) was used for general protein assay work. BSA works well for a protein standard because it is widely available in high purity and it is not very expensive. BSA standard is the most widely-used protein assay standard. It is sterile-filtered, stable and consistent.

Comparing sample assay response to determine protein concentration to that of a standard whose concentration is known, is a typical protein assay. Protein samples and protein standards are processed by mixing them with the assay reagent and using a spectrophotometer to measure the absorbances. Table 1 shows how BSA solution standards are made. (Thermo Fischer Scientific 2009.)

TABLE 1. BSA solution standards (Praktikum umwelt technology 2009)

Standard	Protein concentration [μg/ml]	BSA Standard solution [μl]	0,9% NaCl [μl]
1	0	0	1000
2	1	10	990
3	2.5	25	975
4	5	50	950
5	10	100	900
6	15	150	850
7	20	200	800
8	25	250	750
9	30	300	700

4.3.1 Determining the protein content with the Bradford method

The first experiment of this thesis was determining the protein content with Bradford method. About 2 grams of yeast and 0.1 bar pressure with the ionized air were used for the first experiment. Several experiments were made with different amounts of yeast and different amounts of ionized air. The experiments were made with and without filter (0.45 μm) and also with using centrifuge (1 min and 5000 rpm).

4.3.2 Preparing the solution for the experiment

One litre of 0.9% sodium chloride (NaCl) solution was first made, where the yeast were added and mixed with magnetic mixer to a homogenous solution. When the solution was homogenous, a zero sample was taken and adding ionized air was started. The ionized air was taken with help of a compressor through an ionizator in a very low pressure. The ionized air was added in to the solution for 1.5 hours and taken about 8 ml samples in different time points: 0, 5, 10, 15, 20, 30, 40, 50, 60 and 90 minutes. The same experiment was made also with non-ionized air. In some experiments half of the samples were filtered and in some experiments 1 ml from the samples was centrifuged. Absorbance from all samples was measured by using a spectrophotometer.

4.3.3 Calibration for the spectrophotometer

The protein content was determined with a spectrophotometer. First a calibration had to be made with the spectrophotometer. A volume of 1 ml samples were made, from nine different protein concentrations, with different amount of NaCl and protein assay reagent (BSA). A volume of 200 µl of Roti®-Nanoquant was pipetted to cuvettes, and by using this substance reproducible protein amounts could be analyzed. Then 800 µl of the sample 1–9 was added and in five minutes after inserting the first protein concentration sample, the absorbance was measured with the spectrophotometer at 595 nm. From these results a calibration curve could be made with Excel, which was needed when the experiments' results were analyzed.

4.3.4 Calibration curve with Excel

A graph was made in the excel sheet from standard protein concentrations and absorbances from the measured samples. A linear line was drawn through the points. The absorbances must rise constantly to get a linear line. From the linear line a correlation coefficient (R^2) was created and equation of linear line could be calculated from formula,

$$y = kx + b \quad (2)$$

where k is angular coefficient and b is constant value. The points from the measured samples needed to be as near to the linear line as possible. The correlation coefficient of calibration curve must be close to 1. The calibration curve was needed to calculate samples concentrations. The correlation coefficient showed how well the calibration went. It is not needed later in calculations. The angular coefficient and constant value were needed in the calculations.

4.3.5 Measuring the samples with the spectrophotometer

After making the calibration, the measurement of the samples could be made. First 200 µl of Roti®-Nanoquant was pipette into the cuvettes and then 800 µl of the sample and mixed them. After five minutes, since inserting into the cuvette the first sample, the absorbance was measured. With the results and with help of the calibration curve it could be measured what the protein contents were in the samples. The protein concentration was determined from the unknown sample with comparing the results with the calibration curve. From the calibration curve constant value (b) and angular coefficient (k) were determined. The dilution was needed to take into account. Protein concentrations in the solution were calculated with previous information with formula 3.

$$\text{Concentration [mg/l]} = \frac{\text{Absorbance [A] - Constant value (b)}}{\text{Angular coefficient (k)}} \quad (3)$$

Tables 21–32 present concentrations from pure and diluted samples. The concentrations are calculated by using the formula in theory part (formula 3). In the 1:10 dilutions concentrations, the dilution has taken into consideration too with calculating them also with the formula 4. In the dilutions 1:10 was usually 1000 µl of ion changed water and 100 µl of pure sample. In

some dilutions was 900 µl of ion changed water and 90 µl of pure sample. Formula for these diluted samples concentrations are:

$$\text{Concentration [mg/l]} \times \frac{\text{Ion changed water (µl)}}{\text{Sample amount (µl)}} \quad (4)$$

5 MICROBIOLOGY AND MICROORGANISMS

Microbiology is the research of microorganisms, also called microbes, a massive and versatile group of microscopic small organisms that exist as single cells or cell groups. Microorganisms belong to group of protozoa, bacteria, algae, fungi and virus. Microbial cells are thus different from the cells of animals and plants, which are unable to live alone in nature and can exist only as parts of multicellular organisms. Microbiology is about microbial versatility and evolution, about how different kinds of microorganisms arose and why. After a fashion, microorganisms affect all other life forms in Earth. (Madigan et al. 2003, 1)

5.1 Disintegration of microorganisms

Disintegration of microorganisms can be made by three different methods. The first is a biological method, which includes disintegration with enzyme, bacteriophage and autolyse. The second method is a chemical method, which handles disintegration with acids or base, solvent, surfactant, chelating agenda, antibiotic and ozone. The third is a physical method, and it has two different ways: non-mechanical and mechanical. To non-mechanical methods include osmotic shock, congeal, drying and decompression. To mechanical methods include high-pressure-homogenizer and ultrasound-homogenizer. (Kampen & Michel 2009, 3–4.) This thesis focuses on how to disintegrate microorganisms with ionized air. Disintegration of microorganisms with ionized air is inexpensive and easy way to destroy the yeast cell's wall.

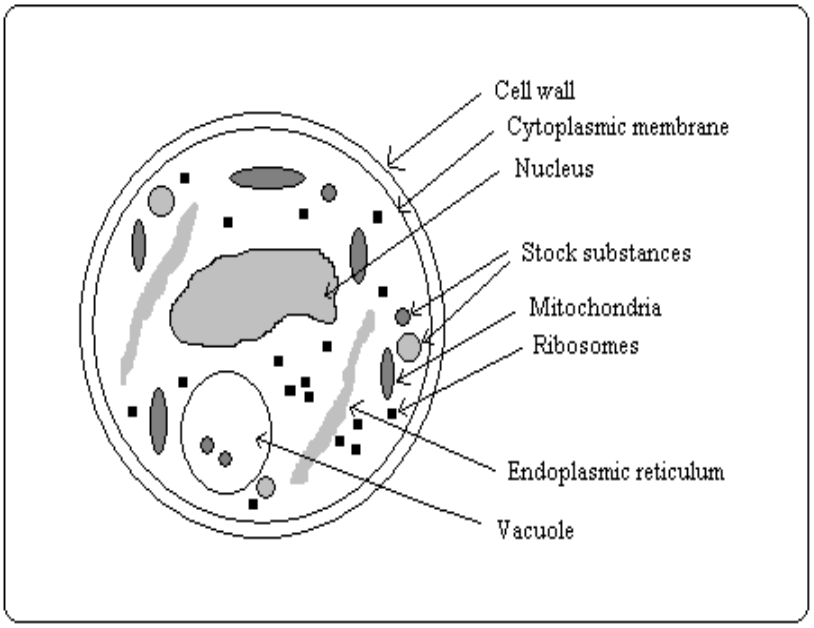
5.2 Microorganisms as cells

A fundamental unit of life is cell. A single cell is a cloistered and isolated from other cells by a cell membrane and contains a variety of chemicals and subcellular structures, which make it possible for the cell to function. The cell membrane separates the inside of the cell from outside. (Madigan et al. 2003, 2–3.) All cells are made up of proteins, nucleic acids, lipids and polysaccharides, which are called macromolecules. Nucleous or nucleoid, where the genetic

information (deoxyribonucleic acid, DNA) is, are the key structures. Every cell has a distinct structure and size. Thus a cell is a dynamic unit, constantly undergoing change and replacing its parts. (Madigan et al. 2003, 2–3.) A cell may be acquiring material from its environment and incorporating them into its own fabric even when it is not growing. It can at the same time discard waste products into its environment. (Madigan et al. 2003, 2–3.)

5.3 Yeast and a yeast cell

Yeast cell is typically 5–10 μm big. A yeast cell consists of 70–85% water and its drying weight is mostly polymer: protein 50%, cell wall 10–20%, RNA 10–20%, DNA 3–4% and lipid 10%. The cell wall from yeast cell is mostly made up of bacteria. (Kampen & Michel 2009, 4)



GRAPH 5. Structure of a yeast cell (adapted from Kampen & Michel 2009, 4)

5.4 Agar for a substrate to the microorganisms

Agar is a polysaccharide derived from red algae and it was widely used in 19th century as a gelling agent. An agar diffusion method is a commonly used procedure for studying antimicrobial action. For solid media is added usually about 1,5% of agar as a gelling agent, before sterilizing the media. In the sterilization process the agar melts and the molten medium is poured into sterile Petri dishes and allowed to solidify before use. (Madigan et al. 2003, 15.)

The sterilization has to be done before use because microorganisms are to be found everywhere. Usually this is done by heating, typically by moist heat in a large pressure cooker called an autoclave. In every step aseptic techniques have to be practiced for successful cultivation of pure cultures of microorganisms. (Madigan et al. 2003, 109.)

5.5 An autoclave for the sterilization

An autoclave is normally used for sterilizing solutions and equipment in laboratory. It is a large pressure cooker. Normal temperature in an autoclave is 120˚C or more, and it has to be kept in this temperature for about 20 minutes. For more than 1–2 liter sterilization, more sterilization time is needed. (Aittomäki, Leisola, Ojamo, Suominen & Weymarn 2002, 138.)

GRAPH 6. An autoclave (Photo: authors)

5.6 Culturing the microorganisms in a laboratory

Culturing microorganisms can be done in number of ways. In this thesis Petri dishes were used, where individual cells that were spread out on the plate, grow and divide to form colonies. In order to sustain life processes, microorganisms must carry out one or more series of reactions that conserve energy. (Madigan et al. 2003, 109.)

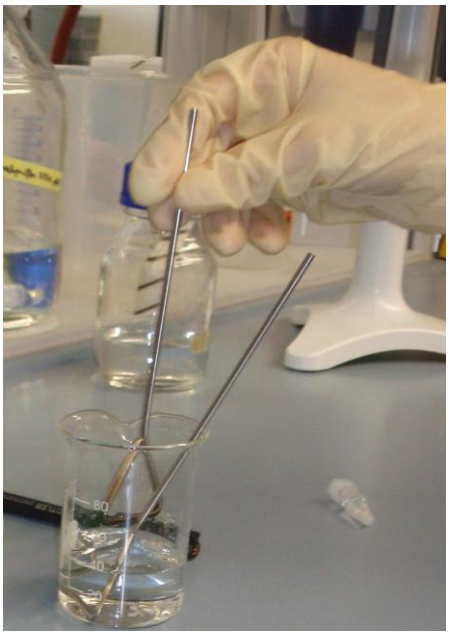
The nutrient solutions, which are used to grow microorganisms in the laboratory, are culture media. Microbiology uses broad classes of culture media that are chemically defined and undefined. In chemically defined media, precise amounts of highly purified inorganic or organic chemicals are added to distilled water. (Madigan et al. 2003, 107.)

When the culture medium has been prepared, the microorganisms can be added and incubated under conditions that will support microbial growth. In a pure culture is appeared growth in most cases, which is containing only a single kind of microorganisms. To obtain or maintain a pure culture it is essential that other organisms, called contaminants, are prevented from entering it. Microbiological techniques are designed to avoid contaminants. For obtaining pure cultures and for assessing the purity of a culture, a major method is the use of solid media, specifically, solid media in the Petri plate. (Madigan et al. 2003, 108–109.)

5.7 The viable count for cells

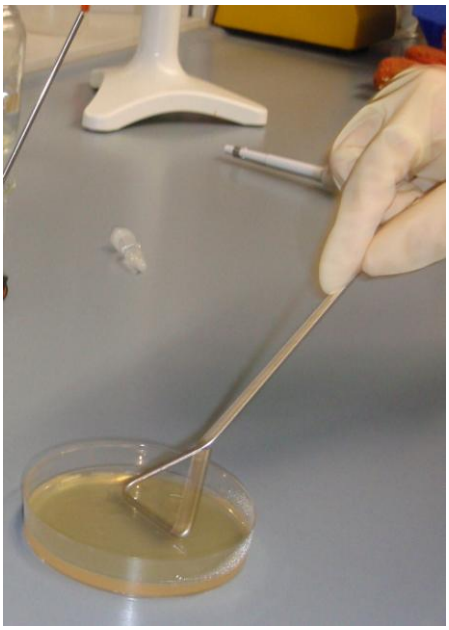
The viable cell counting method can be easily used to count only the living cells. Viable count is often called the plate count or colony count because the number of cells in the sample capable of forming colonies on a suitable agar medium can be determined. The two ways of performing a plate count are called the spread plate method and the pore plate method. In this thesis the first of these methods was used. (see Madigan et al. 2003, 146.)

GRAPH 7. Pipetting the sample on the agar (Photo: authors)



GRAPH 8. Steeping the spreader in to ethanol (Photo: authors)

GRAPH 9. Burning the ethanol in the flame (Photo: authors)



GRAPH 10. Spreading the sample over the agar (Photo: authors)

In Graph 7, a volume of 200 µl of an appropriately diluted culture is spread over the surface of an agar plate using a sterile pipette. The spreader was steeped into ethanol for sterilizing the spreader in Graph 8 and burned the ethanol away in a flame in Graph 9. In Graph 10 the sample was spread evenly over the surface of agar with the sterile spreader so long that it was dried up. After spreading the sample over the agar, the plate was incubated until the colonies appeared and the number of colonies was counted. It took about 24 to 30 hours that all the colonies were appeared.

5.8 Diluting the samples

The colony number must not be very high because counting the colonies is impossible from plates with over 500 colonies. But the number of colonies cannot be very low either so there should be over 30 colonies on the plate. That is why the sample should almost always be diluted, and with more than one dilution, before plating. It is common to use several 10-fold dilutions of the sample. The dilutions were done with in an autoclave sterile made 0,9% NaCl solution.

5.9 Determining the microorganisms with the Koch method

With the Koch method it is possible to determine the number (not exact number) of living cells. For this, defined amounts of cell suspension containing agar are needed to pour to Petri dishes, then to incubate them and to count the grown microorganism colonies. It is necessary to use a homogeneous cell suspension with a total microorganism number between 30 and 500 to get reproducible results. The cells have to be diluted with physiological 0,9% brine in 1:10 steps after sampling to avoid the growth of the microorganism.

Even though nutrient gelatin is a marvelous culture medium for the isolation and study of various bacteria, it does not remain solid at body temperature (37˚C) which is the optimum temperature for growth of most human pathogens. (Madigan et al. 2003, 15.)

5.9.1 Preparing the yeast solution

One liter of 0,9% NaCl solution with few grams of yeast was used for the experiment. Nine grams of NaCl and one gram of Baker's yeast were mixed to one liter of ion changed water with a magnetic mixer that the solution was homogeneous. The yeast did not dissolve completely.

Ionized air was added to the solution through an ionizator with a low pressure for 60 minutes, so that only few bubbles appeared. The mixing was continued for the whole procedure. The first sample was taken at zero point, when the ionized air was added to the solution. After this the samples were taken after 5, 10, 15, 20, 30, 40, 50 and 60 minutes. The same procedure was repeated also with non-ionized air. The samples were kept in a fridge in the mean while when preparing the plates for the next procedure.

5.9.2 Making the agar solution

An agar solution was used in this thesis as culture media. Microorganisms were cultured from the yeast solution to these agar plates. The solution was made in three laboratory media bottles, which were autoclavable. Autoclaving was used to make the solutions sterilized. A volume of 333 ml of ion changed water and the right amount of substances were weighed in every bottle as follows: 1 g yeast extract, 1 g malt extract broth (liquid medium for cultivation of yeasts and moulds), 1,67 g peptone (soya beans), 3,33 g glucose and 5 g agar bacteriological (gelling agent selected for solidifying the microbiological culture media). The agar had to be added into the solution last because it did not dissolve into the solution without heat but it dissolved in the autoclaving.

The autoclave was a steam autoclave. The caps on the bottles had to be little bit opened so that it would not explore from the effect of pressure and that the steam could get in to the bottle. Making autoclaving in this way, the solution became sterilized. A tape destined for autoclaving was put to the bottles. The tape was permanent imprinted graduations and marking

spots and its color changes in the autoclaving so that it was seen what had already been autoclaved. The autoclaving took approximately two hours.

5.9.3 Preparing the agar plates

After autoclaving the plates were prepared. Making the plates was done in sterilized conditions so the plates were prepared next to a gas flame. The mouth of the bottle was heated for a few seconds in the flame before decanting the solution in to the Petri dish, so that it was sterile (Graph 11). The solution was poured to the plate so that the bottom was covered and placed a cap on it. The plates must solidify before they could be used in the experiments (Graph 12). Heating the mouth of the bottle was done before decanting the solution to every Petri dish.

GRAPH 11. Heating the bottle mouth in a flame to make it sterile (Photo: authors)

GRAPH 12. Pouring the agar solution to the Petri dish (Photo: authors)

More solutions than needed were sometimes made. Then the bottles were kept in a locker, because the whole agar solution was not needed to use at the same time. The solution solidified in room temperature, but it became usable liquid again by warming it up in a microwave. In this way, more plates could be done later.

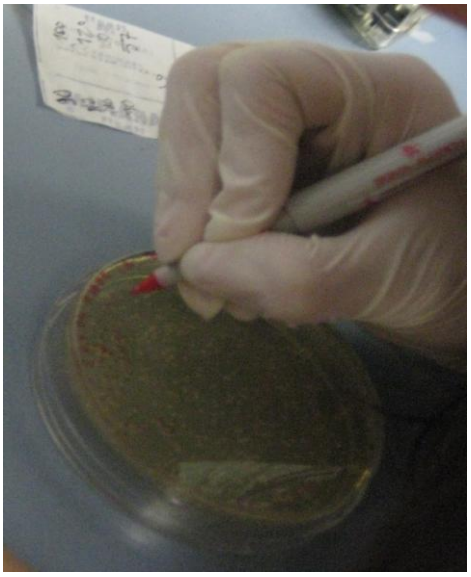
5.9.4 Making the plates from the samples

When preparing the plates with the samples, the agar plates were first dried in a sterile fume hood for about 10 minutes. Then the samples were diluted with 0,9% sodium chloride, which had been autoclaved to make them sterile. In the first experiment, the solution was diluted for 1:10, 1:100 and 1:1000; in other words, one part of the sample and 10 parts of 0,9% sodium chloride, for example. The amounts were 90 µl of solution and 900 µl of NaCl. The further dilutions were made the same way from the previous dilution.

Preparing the plates was done in very sterile conditions. The table was cleaned with ethanol and the whole work was done near a gas fire. Also sterile, autoclaved pipettes were used. A volume of 200 µl of the diluted sample was pipetted to the plate, on the agar. Then a cultivation spreader was taken and dunked to ethanol and the ethanol was burned from it in the gas fire, so that it was as sterile as possible. The sample was distributed with the cultivation rod until it was dried and it was then distributed on the whole plate. The plate was placed into an oven of 30˚C and kept there 24 hours. Finally the microorganisms were counted.

5.9.5 Counting the microorganisms

The microorganisms grown on the plate were counted after 24–48 hours. Counting the microorganisms was done with a counter and pen. The microorganisms were colored with the pen so that the counting was exact. Only 500 or fewer microorganisms could be counted. It was impossible to count microorganisms more than 500.



GRAPH 13. Counting the microorganisms (Photo: authors)

6 RESULTS

The results are presented in three sections. The first part discusses the calibrations, which have been made with the spectrophotometer with the BSA standard solution. The calibrations curves are needed in the second part's calculations, using the Bradford method for determining the protein contents. Different kind of proving was made in the test, like how centrifuging, filtering, different amount of yeast and BSA affects on the protein content. Some tests were just determined ionized air impacts comparing to non-ionized air. The third part study looks into how microorganisms disintegrate with the Koch method.

6.1 Calibration curves for calculating the protein concentrations

Calibration of the Bradford method needed to be done before almost every experiment. Calibrations were sometimes done before and after the tests to see if the calibrations were similar. In some calibrations, average values were calculated for the calculations. A calibration curve could be made from the calibration's absorbances with Excel and this calibration curve was used in the calculations for determining the protein content. Absorbances in the calibration must increase constantly so that the line is linear and useful for the second part, which is the Bradford method for determining the protein content. The closer to linear the line is, the better the calibration, so the correlation coefficient of calibration curve must be as near as possible to 1. Many calibrations had to be done sometimes, to get enough successful calibration curves. The calibrations were made several times in the beginning, almost every time when the tests were done. But then were used the same calibration curve many times, because it was not necessary to do the calibration every time again. The previous calibration curve what have been done needed to use in the samples' concentrations calculations.

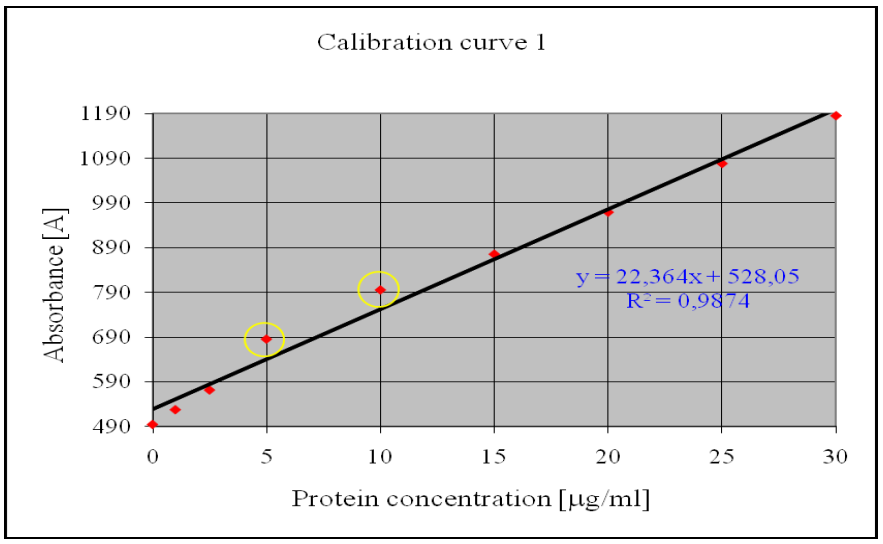
Doing the calibration, even one small mistake ruined the whole calibration curve. Mistakes could be easily done by weighting the BSA, because it just stuck to the spoon and float with the air current. Weighting 0.01 g of the BSA was so punctual without its floating away. Pipetting the BSA-, NaCl- or Roti Nanoquant solution needs to be done very carefully to get

the exact amount of these. The calibration was very sensitive with even very small wrong drop with every substance. Also time was very important thing, for that the reaction time after mixing the substances is same with every sample. After pipetting protein standard to the first assay reagent sample, an exact time needed to be taken from this point to the point when the first sample was measured in the spectrophotometer. This same time were needed in every spectrophotometer measurement, both in calibration and test samples (5 minutes). Mixing the substances in cuvettes need to be done very carefully, so that even every small drop is staying in the cuvette and it needs to be done the same way every time.

The first calibration (see Table 2) shows that the values increased almost constantly. In the calibration curve had some mistake in samples 4 and 5, which made the correlation coefficient lower. Graph 14 indicates that samples 4 and 5 diverged from the linear line more than the other points. This calibration curve was still useable and was used in some experiments calculations.

TABLE 2: Absorbances for calibration 1

Sample no:	Protein conc. [μg/ml]	Absorbance [A]
1	0	494
2	1	527
3	2.5	571
4	5	685
5	10	795
6	15	875
7	20	969
8	25	1078
9	30	1185

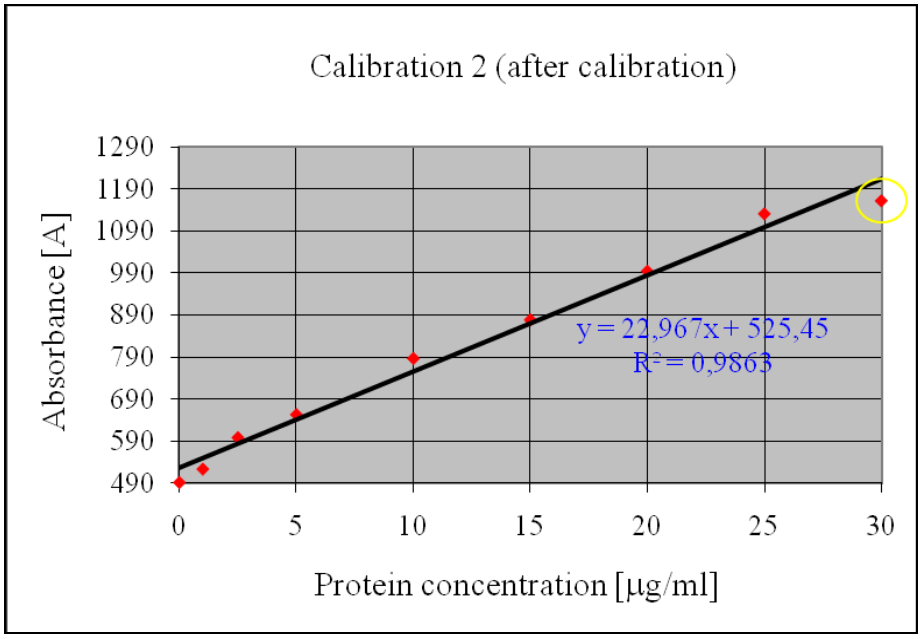


GRAPH 14. Calibration curve 1

Calibration was sometimes necessary to ensure, that could be sure that the calibration before the test is right. Table 3 presents absorbances the after calibration. They increased constantly but sample number 9 was not on the line. The correlation coefficient is 0,9863 which divorces 0,0011 decimal compared to the previous calibration. And the equation of linear line in Graphs 14 and 15 was almost the same. This means that the post-calibration is not changing considerably from the pre-calibration, so the pre-calibration could be used in the calculations in the future experiments.

TABLE 3. Absorbances for calibration 2

Sample no:	Proteinconc. [µg/ml]	Absorbance [A]
1	0	491
2	1	523
3	2.5	598
4	5	653
5	10	787
6	15	879
7	20	995
8	25	1132
9	30	1163

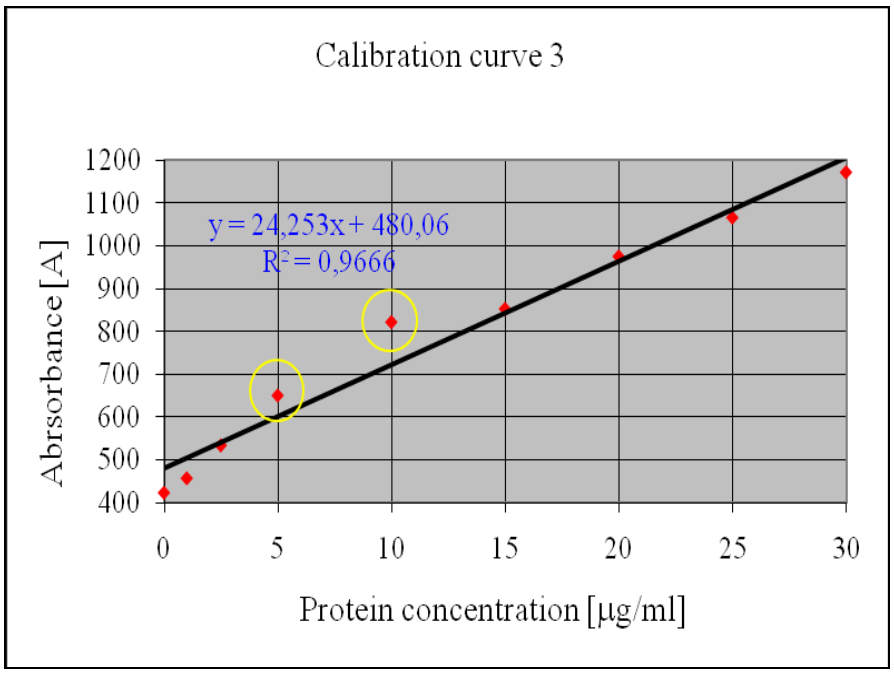


GRAPH 15. Calibration curve 2

The absorbances in the third calibration were increased constantly, but again sample 4 and 5 diverged from the linear line. The correlation coefficient was only 0,9666, so the post-calibration was made to get better average from these calibrations to the calculations in future experiments.

TABLE 4. Absorbances for calibration 3

Sample no:	Protein conc. [µg/ml]	Absorbance [A]
1	0	422
2	1	456
3	2.5	533
4	5	650
5	10	822
6	15	853
7	20	976
8	25	1067
9	30	1173

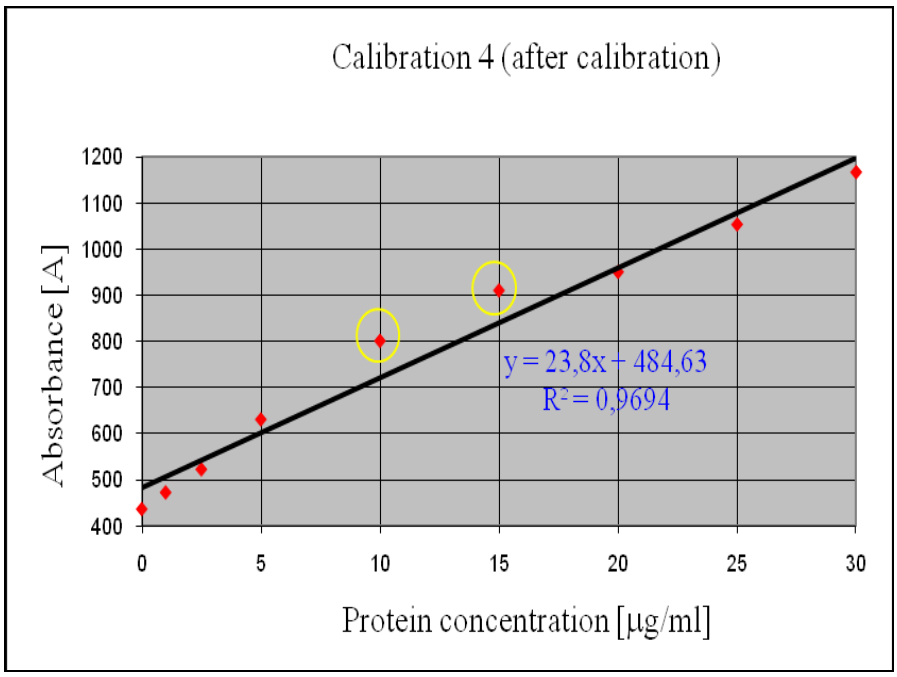


GRAPH 16. Calibration curve 3

The correlation coefficient in Graph 16 (pre-calibration) and in Graph 17 (post-calibration) was not exactly the same. The correlation coefficient was still slightly rising so the average from these values could be calculated and used in the experiments calculations.

TABLE 5. Absorbances for calibration 4

Sample no:	Protein conc. [µg/ml]	Absorbance [A]
1	0	437
2	1	473
3	2.5	523
4	5	631
5	10	801
6	15	910
7	20	950
8	25	1053
9	30	1166

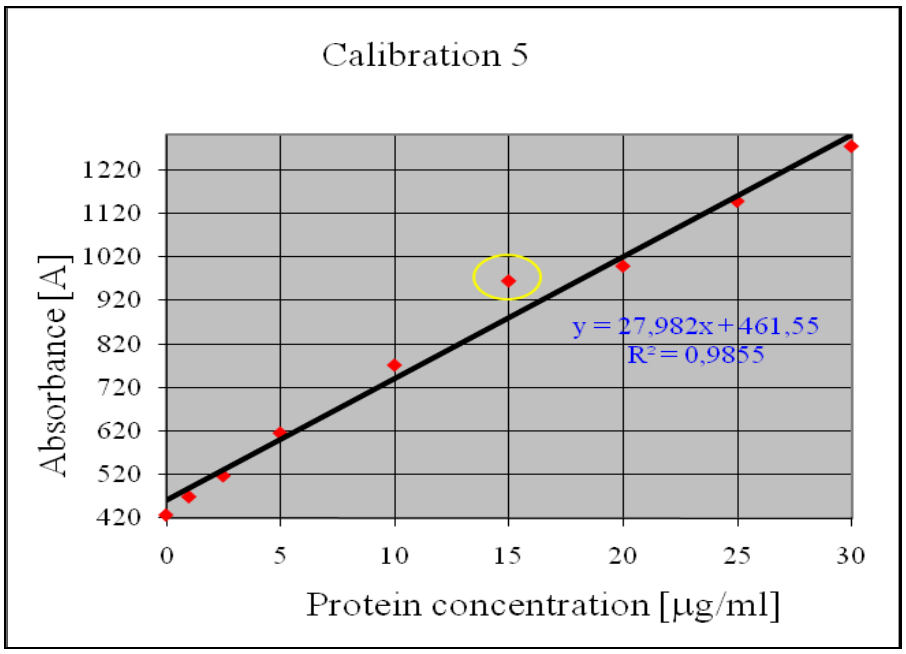


GRAPH 17. Calibration curve 4

Table 6 indicates that absorbances in calibration 5 increased but sample number 6 diverged from the line. Although the points scattered on the line, especially the point for sample number 6, the correlation coefficient was required and could be used it in the tests calculations.

TABLE 6. Absorbances for calibration 5

Sample no:	Protein conc. [µg/ml]	Absorbance [A]
1	0	428
2	1	470
3	2.5	518
4	5	616
5	10	772
6	15	965
7	20	999
8	25	1148
9	30	1274

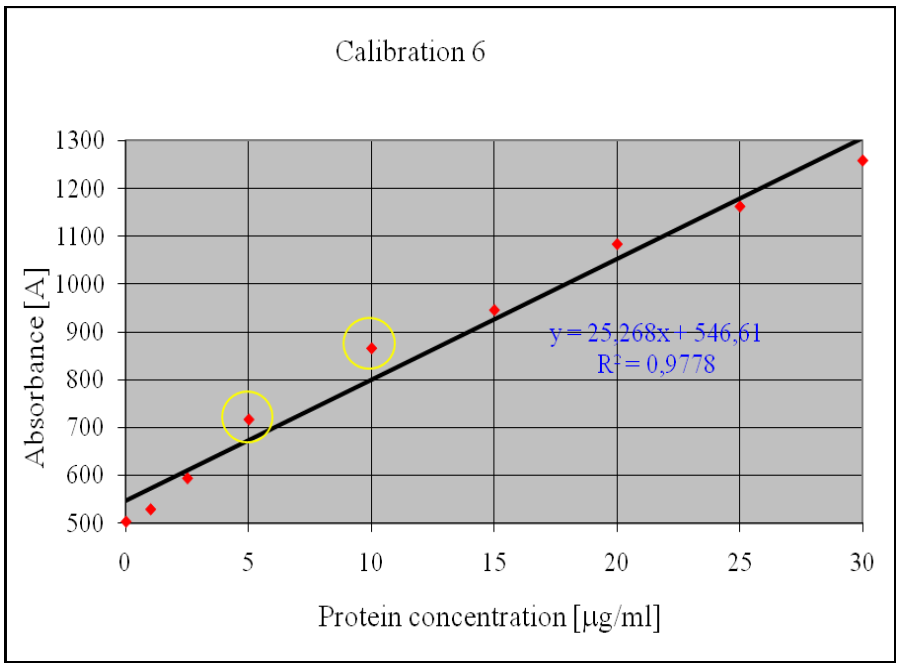


GRAPH 18. Calibration curve 5

Graph 19, for calibration 6 shows that absorbances in samples 4 and 5 diverged from the line again. The correlation coefficient is lower than in calibration 5, but it was still useable and therefore used in the calculations in future experiments.

TABLE 7. Absorbances for calibration 6

Sample no	Protein conc. [µg/ml]	Absorbance [A]
1	0	503
2	1	529
3	2.5	594
4	5	717
5	10	866
6	15	946
7	20	1084
8	25	1163
9	30	1259

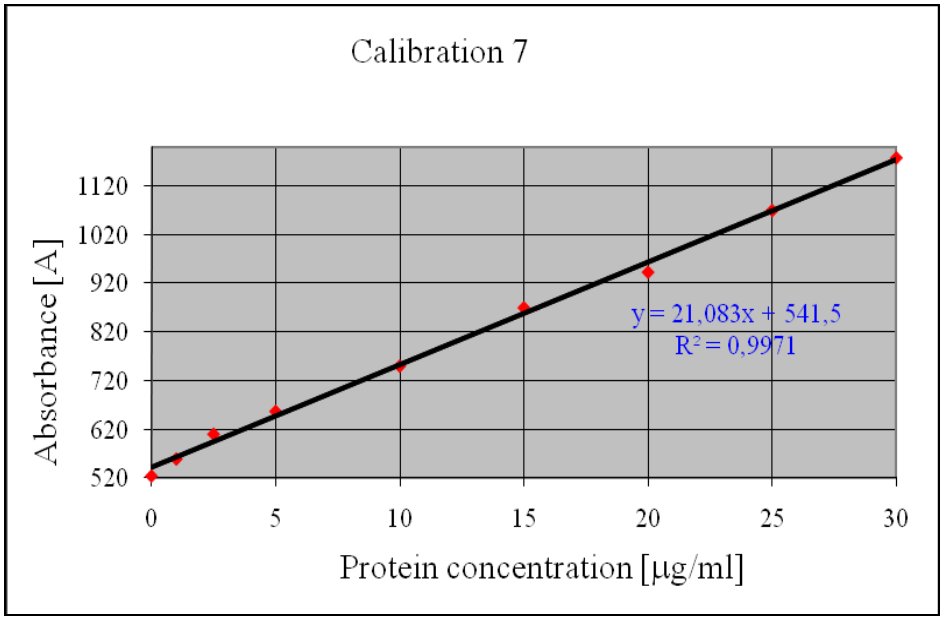


GRAPH 19. Calibration curve 6

Calibration 7 finally succeeded. The calibration curve is linear and the correlation coefficient is 0.9971, which can be seen in Graph 20. The correlation coefficient is better than in any previous calibrations and this calibration was used in the rest of the experiment calculations.

TABLE 8. Absorbances for calibration 7

Sample no:	Protein conc. [µg/ml]	Absorbance [A]
1	0	524
2	1	559
3	2.5	610
4	5	657
5	10	750
6	15	870
7	20	943
8	25	1070
9	30	1178



GRAPH 20. Calibration curve 7

The calibrations in the beginning and in the end were quite successful but the few calibrations between them were not so successful. Still these calibrations were useable and needed in some calculations.

TABLE 9. All calibration curve values, used in tests calculations

Date	Correlation coefficient	Angular coefficient	Constant value
Calibration 1	0.9874	22.364	528.05
Calibration 2 (after calibration)	0.9863	22.967	525.45
Calibration 3	0.9666	24.253	480.06
Calibration 4 (after calibration)	0.9694	23.800	484.63
Calibration 3+4 (average)	0.9680	24.077	482.35
Calibration 5	0.9855	27.982	461.55
Calibration 6	0.9778	25.268	546.61
Calibration 7	0.9971	21.083	541.50

6.2 Determining the protein content with the Bradford method

The Bradford method was used for determining the protein content with in the NaCl-yeast solution. The samples were analyzed in a spectrophotometer. For calculating the protein concentration the calibrations curves from the earlier calibrations were used.

Calibration curve from Graph 14 was used the first test. From its angular coefficient and constant value concentrations were calculated concentrations introduced in the chapter 4.4.5 Measuring the samples (formula 3). The concentrations are negative because the absorbances are lower than the constant value. The constant value needs to be higher than the absorbances, because the value is subtracted from the absorbance and then divided with the angular coefficient. Test 1 looked into how centrifuging and filtering affected the absorbances and concentrations. As can be seen in Tables 10 and 11, the absorbances are not increasing, which means that disintegrations were not happening. The amounts fluctuate, especially the absorbances from the centrifuged solution with non-ionized air, as can be seen in Table 11. Because all the concentrations are negative with ionized air, the experiment was not successful.

TABLE 10. Absorbances and concentrations with ionized air

Time [min]	Absorbance [A]	Concentration [µg/l]	Absorbance [A] Filtered+Centrifuged	Concentration [µg/l]
0	466	-2.77	476	-2.33
5	468	-2.69	504	-1.08
10	475	-2.37	516	-0.54
15	496	-1.43	494	-1.52
20	489	-1.75	527	-0.05
30	462	-2.95	524	-0.18
40	485	-1.92	498	-1.34
50	493	-1.57	484	-1.97
60	485	-1.92	517	-0.49
90	482	-2.06	515	-0.58

TABLE 11. Absorbances and concentrations with non-ionized air

Time [min]	Absorbance [A]	Concentration [µg/l]	Absorbance [A] Filtered+Centrifuged	Concentration [µg/l]
0	487	-1.84	512	-0.72
5	481	-2.10	515	-0.58
10	511	-0.76	1245	32.06
15	497	-1.39	562	1.52
20	484	-1.97	531	0.13
30	477	-2.28	517	-0.49
40	492	-1.61	534	0.27
50	500	-1.25	781	11.31
60	491	-1.66	500	-1.25
90	487	-1.84	483	-2.01

In test 2, all samples were filtered with 0,45 µm filters (see Table 12). The calibration curve from Graph 14 was used there. The protein concentrations were calculated with these values. Table 12 shows that the concentration values increased with ionized air until 60 minutes and after that the effect was opposite. First the protein concentration was rising from the use of ionized air but after a while the ionized air started to destroy the proteins. With non-ionized air the effect is not beneficial, because the values are negative and almost the same during the whole experiment.

TABLE 12. Absorbances and concentrations with ionized and non-ionized air

Time [min]	Absorbance [A] *ionized air*	Concentration [µg/l]	Absorbance [A] *non-ionized air*	Concentration [µg/l]
0	482	-2.06	495	-1.48
5	501	-1.21	490	-1.70
10	563	1.56	506	-0.99
15	698	7.60	505	-1.03
20	787	11.58	501	-1.21
30	884	15.92	530	0.09
40	950	18.87	502	-1.16
50	1050	23.34	498	-1.34
60	1169	28.66	504	-1.08
90	495	-1.48	498	-1.34

In test 3, the 0,45 µm filters were used again (see Table 13). The average from the pre- and post-calibration from Graphs 15 and 16 were used calculations. The correlation coefficient was unexpected in both calibrations and that is why the average was calculated from them. In either of ionized air or non-ionized air there was no effect, because the results were similar and did not change at any time. The experiment was again unsuccessful.

TABLE 13. Absorbances and concentration with ionized and non-ionized air

Time [min]	Absorbance [A] *ionized air*	Concentration [µg/l]	Absorbance [A] *non-ionized air*	Concentration [µg/l]
0	421	-2.55	425	-2.39
5	436	-1.93	431	-2.14
10	424	-2.43	471	-0.47
15	427	-2.30	435	-1.97
20	438	-1.85	427	-2.30
30	418	-2.68	425	-2.39
40	406	-3.18	420	-2.59
50	432	-2.10	427	-2.30
60	426	-2.35	444	-1.60
90	428	-2.26	462	-0.85

The samples in test 4, were filtered again with 0,45 μm filters (see Table 14). The same calibration as in test 3 was used in calculations. Table 14 shows that the results are similar to those in test 3. There was again no effect from ionized air or non-ionized air, because the results were not changing.

TABLE 14. Absorbances and concentrations to ionized and non-ionized air

Time [min]	Absorbance [A] *ionized air*	Concentration [μg/ml]	Absorbance [A] *non-ionized air*	Concentration [μg/ml]
0	496	0.57	501	0.78
5	508	1.07	495	0.53
10	512	1.23	505	0.94
15	504	0.90	531	2.03
20	501	0.78	501	0.78
30	512	1.23	511	1.19
40	505	0.94	503	0.86
50	496	0.57	492	0.40
60	503	0.86	512	1.23
90	517	1.44	500	0.73

Test 5 was looked into how ionized air affected in comparison to non-ionized air. All the samples from both ionized air and non-ionized air were filtered with 0,45 μm filters. The results were not successful, because any difference between them cannot be seen (see Table 15). The calibration curve from the Graph 18 was used for calculating the protein concentrations. The concentrations were again negative, which means that the absorbances were lower that the constant value.

TABLE 15. Absorbances and concentrations with ionized and non-ionized air

Time [min]	Absorbance [A] *Ionized air*	Concentration [µg/l]	Absorbance [A] *non-ionized air*	Concentration [µg/l]
0	455	-0.23	454	-0.27
5	422	-1.41	432	-1.06
10	410	-1.84	432	-1.06
15	420	-1.48	432	-1.06
20	435	-0.95	441	-0.73
30	419	-1.52	443	-0.66
40	449	-0.45	435	-0.95
50	437	-0.88	430	-1.13
60	427	-1.23	431	-1.09
90	441	-0.73	425	-1.31

In test 6, the non-filtered and filtered samples were compared (see Table 16). The experiment times were shortened from 90 minutes to 60 minutes, because after 60 minutes absorbances always went down. 10 ml samples were took in this test and 5 ml of them were filtered with 0,45 µm filters. The rest 5 ml from the 10 ml samples was used as pure samples for the measurements. Calibration curve from the Graph 18 was used for calculating the protein concentrations. As is shown in Table 16, the concentrations with filtered samples are unsuccessful, like also in the previous experiments.

TABLE 16. Absorbances and concentrations with ionized air

Time [min]	Absorbance [A] non-filtered	Concentration [µg/l]	Absorbance [A] filtered	Concentration [µg/l]
0	1505	37.29	502	1.45
10	2155	60.52	505	1.55
20	791	11.77	486	0.87
30	990	18.89	488	0.95
40	1382	32.89	496	1.23
50	1267	28.78	497	1.27
60	1463	35.79	500	1.37

TABLE 17. Absorbances and concentrations with non-ionized air

Time [min]	Absorbance [A] non-filtered	Concentration [µg/l]	Absorbance [A] filtered	Concentration [µg/l]
0	1450	35.32	483	0.77
10	1442	35.04	499	1.34
20	1268	28.82	489	0.98
30	1527	38.08	490	1.02
40	1832	48.98	503	1.48
50	1899	51.37	513	1.84
60	1849	49.58	492	1.09

In test 7, other kind of filters (MN 614 ¼) were used with 5 ml of the sample and centrifugation with 5 ml of the sample (see Table 18 and 19). Calibration curve from Graph 20 were used for calculating the protein concentrations. The results with this other filter are not successful either, because they fluctuate so much. It can be said now that there is no reason to use filters in the tests. Tables 18 and 19 show that the centrifugation was not worked either, because the results were also fluctuated very much. In non-ionized air the results in centrifuging increased slightly but centrifuging was not used anymore, because the effect from ionized air would have been needed.

TABLE 18. Absorbances and concentrations with ionized air

Time [min]	Absorbance [A] Filtered	Concentration [µg/l]	Absorbance [A] Centrifuged	Concentration [µg/l]
0	890	13.59	2113	61.99
10	969	16.72	1146	23.72
20	1244	27.60	919	14.74
30	995	17.75	1479	36.90
40	930	15.17	1188	25.38
50	1013	18.46	2041	59.14
60	1069	20.67	1274	28.79

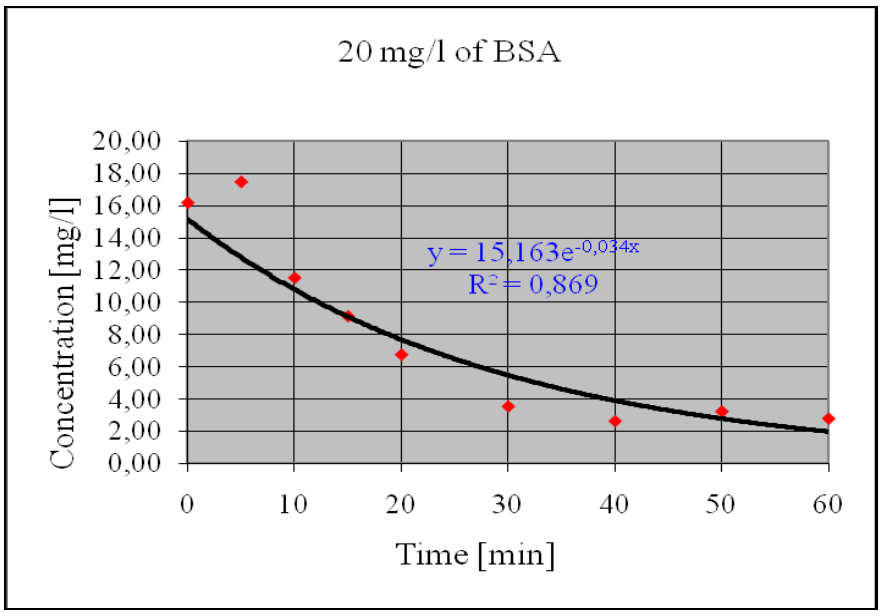
TABLE 19. Absorbances and concentrations with non-ionized air

Time [min]	Absorbance [A] Filtered	Concentration [µg/l]	Absorbance [A] Centrifuged	Concentration [µg/l]
0	1052	20.00	1057	20.20
10	1250	27.84	1043	19.65
20	1300	29.82	1068	20.63
30	1375	32.78	1106	22.14
40	1279	28.98	1329	30.96
50	1106	22.14	1260	28.23
60	1527	38.80	1351	31.83

The Bovine serum albumin (BSA) was used in test 8 beside the yeast, because the experiments with yeast were not worked. In the first test (see Table 20), using BSA with ionized air, approximately 9 g of NaCl and 20 mg/l of BSA were placed in to one liter of ion changed water. Yeast is not used in the tests anymore. Different amounts of BSA were used, to find which destroys the microorganisms best. Calibration 6 was used in the calculations. Concentrations, as shown in Table 20 were constantly decreasing, but more experiences were needed to do with different amounts of BSA.

TABLE 20. Absorbances and concentrations with ionized air by using BSA

Time [min]	Absorbance [A]	Concentration [µg/l]
0	955	16.16
5	869	17.45
10	719	11.52
15	659	9.14
20	599	6.77
30	518	3.56
40	495	2.65
50	510	3.25
60	499	2.81



GRAPH 21. Protein concentration with 20 mg/l of BSA

Test 9 included an experiment with BSA concentration 500 mg/l (see Table 21 and 22). In this test there appeared so much foam, in the beginning, that one drop of foam inhibitor was added to the solution but still some foam was appearing. Calibration 6 was used in the concentrations calculations. Table 21 and 22 show that the absorbances were unsuccessful so could be presumed that this was because of the foam inhibitor.

TABLE 21. Absorbances and concentrations to ionized air

Time [min]	Absorbance [A] Pure 1:1	Concentration [µg/l]	Absorbance [A] Diluted 1:10	Concentration [µg/l]	Absorbance [A] Diluted 1:100	Concentration [µg/l]
0	2121	62.31	1418	34.49	653	4.21
5	2114	62.03	1411	34.21	651	4.13
10	2114	62.03	1396	33.62	661	4.53
15	2121	62.31	1401	33.81	656	4.33
20	2114	62.03	1407	34.05	679	5.24
30	2098	61.40	1432	35.04	656	4.33
40	2106	61.71	1429	34.92	647	3.97
50	2114	62.03	1402	33.85	660	4.49
60	2130	62.66	1401	33.81	641	3.74

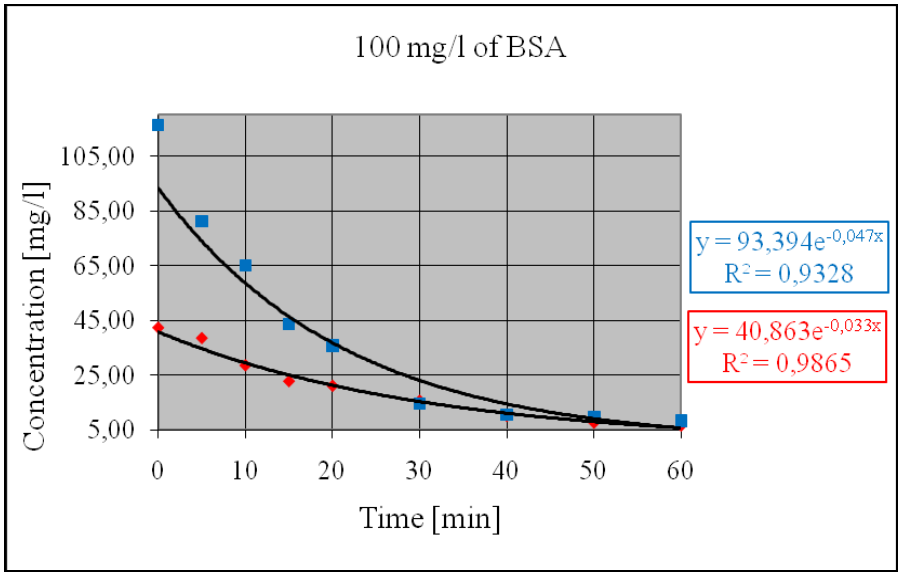
TABLE 22. Absorbances and concentrations to non-ionized air

Time [min]	Absorbance [A] Pure 1:1	Concentration [μg/l]	Absorbance [A] Diluted 1:10	Concentration [μg/l]	Absorbance [A] Diluted 1:100	Concentration [μg/l]
0	2146	63.30	1399	33.73	632	3.38
5	2164	64.01	1436	35.20	661	4.53
10	2146	63.30	1444	35.51	662	4.57
15	2130	62.66	1444	35.51	648	4.01
20	2121	62.31	1415	34.37	660	4.49
30	2098	61.40	1377	32.86	667	4.76
40	2106	61.71	1328	30.92	633	3.42
50	2015	58.11	1240	27.44	638	3.62
60	2034	58.86	1240	27.44	609	2.47

In test 10, 100 mg/l of BSA and ionized air was used (see Table 23), because 500 mg/l was very much. Because there was so much foam and lots of BSA effervesced from the vessel, the test was challenging to do. The foam inhibitor could not be used anymore because it affected the results very much. Calibration 6 was used in the concentrations calculations. Graph 22 shows the concentration from the pure samples and from the 1:10 diluted samples. From the results it could be presumed that the experiment worked. The BSA concentrations decreased but the experiment was challenging to do with such a large volume of BSA, because of the foam. That is why more experiments were made with smaller volumes of BSA.

TABLE 23. Absorbances and concentrations with ionized air

Time [min]	Absorbance [A] Pure 1:1	Concentration [µg/l]	Absorbance [A] Diluted 1:10	Concentration [µg/l]
0	1618	42.40	814	116.4
5	1522	38.60	733	81.1
10	1270	28.63	696	65.0
15	1123	22.81	647	43.7
20	1080	21.11	629	35.9
30	943	15.69	580	14.5
40	801	10.07	571	10.6
50	739	7.61	569	9.7
60	712	6.55	566	8.4



GRAPH 22. Protein concentration with 100 mg/l of BSA

Calibration 6 from Graph 19 was used in test 11 calculations (see Table 24 and 25). Tables 24 and 25 show the absorbances of pure solution. The absorbances increased with ionized air but not with non-ionized air. The same results were in diluted 1:10 samples, but not anymore in diluted 1:100 samples. Diluting the samples with 1:100 must be very punctual that it was not

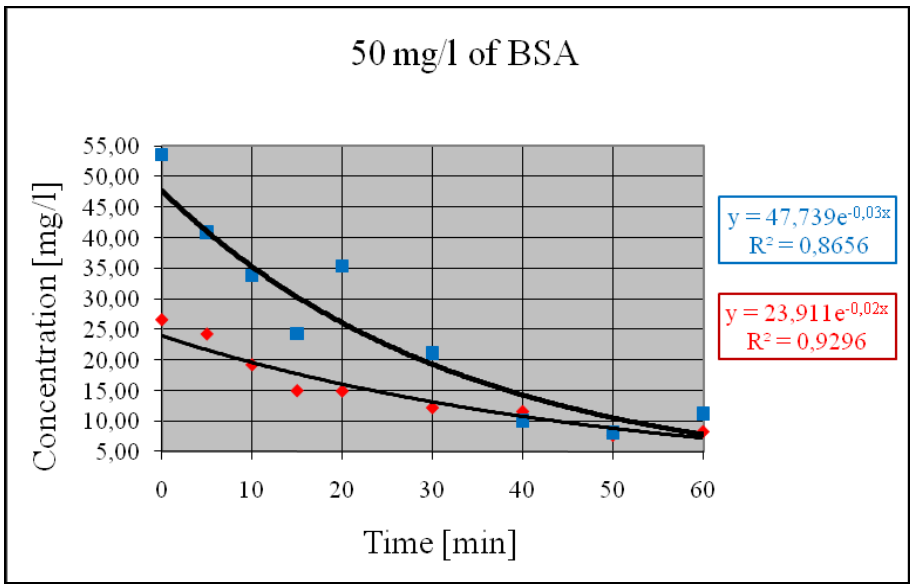
always possible to do it. The results decreased constantly, as was expected, but there appeared again slightly of foam.

TABLE 24. Absorbances and concentrations with ionized air

Time [min]	Absorbance [A] Pure 1:1	Concentration [mg/l]	Absorbance [A] Diluted 1:10	Concentration [mg/l]	Absorbance [A] Diluted 1:100	Concentration [mg/l]
0	1219	26.61	682	53.58	560	0.53
5	1160	24.28	650	40.92	547	0.02
10	1033	19.25	632	33.79	538	-0.34
15	926	15.01	608	24.30	544	-0.10
20	925	14.98	636	35.38	551	0.17
30	856	12.24	600	21.13	551	0.17
40	840	11.61	572	10.05	559	0.49
50	742	7.73	567	8.07	554	0.29
60	757	8.33	575	11.24	579	1.28

TABLE 25. Absorbances and concentrations with non-ionized air

Time [min]	Absorbance [A] Pure 1:1	Concentration [mg/l]	Absorbance [A] Diluted 1:10	Concentration [mg/l]	Absorbance [A] Diluted1:100	Concentration [mg/l]
0	1236	27.28	692	57,54	557	0.41
5	1151	23.92	662	45,67	545	-0.06
10	1171	24.71	673	50,02	557	0.41
15	1155	24.08	698	59,91	541	-0.22
20	1173	24.79	704	62,29	548	0.06
30	1185	25.26	717	67,43	558	0.45
40	1200	25.86	703	61,89	545	-0.06
50	1199	25.82	691	57,14	545	-0.06
60	1224	26.81	687	55,56	579	1.28

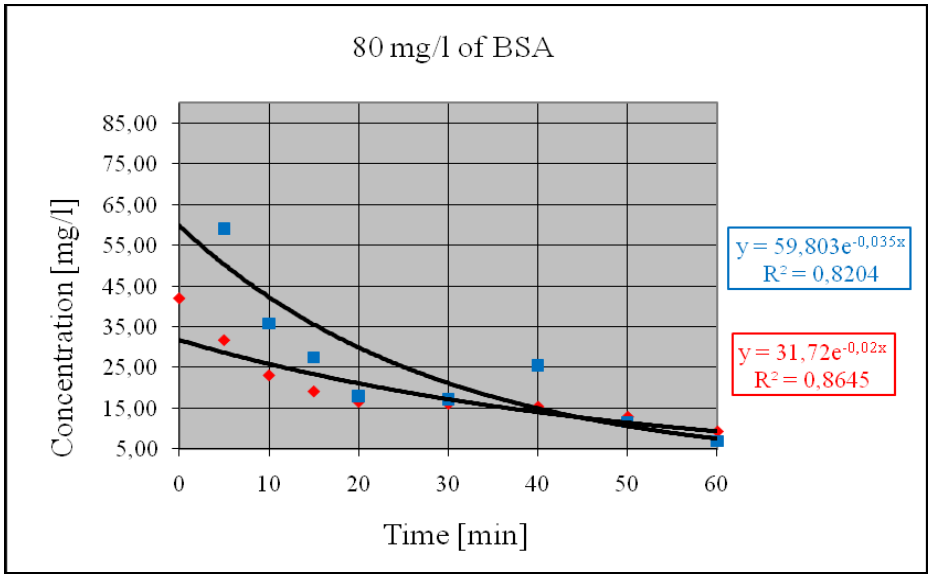


GRAPH 23. Protein concentration with 50 mg/l of BSA

To get more information how ionized air destroys the BSA, a higher amount of BSA concentration was used. In test 12, 80 mg/l of BSA was used (see Table 26). Calibration 6 in Graph 19 was used in the calculations. Table 26 indicates that the absorbances increased with pure solution. But dilution samples 1:10 was not successful anymore. In the vessel appeared lots of foam and it effervesced, so the experiment with also this high BSA concentration was challenging to do. Comparing the results from tests 12 and 8 shows that the results were successful enough with less than 80 mg/l of BSA concentration. Some experiments were made with 30 mg/l of BSA concentration.

TABLE 26. Absorbances and concentrations wit ionized air

Time [min]	Absorbance [A] Pure 1:1	Concentration [mg/l]	Absorbance [A] Diluted 1:10	Concentration [mg/l]
0	1610	42.08	780	92.4
5	1350	31.79	696	59.1
10	1131	23.13	637	35.8
15	1031	19.17	616	27.5
20	966	16.60	592	18.0
30	953	16.08	590	17.2
40	936	15.41	611	25.5
50	873	12.92	576	11.6
60	782	9.32	564	6.9



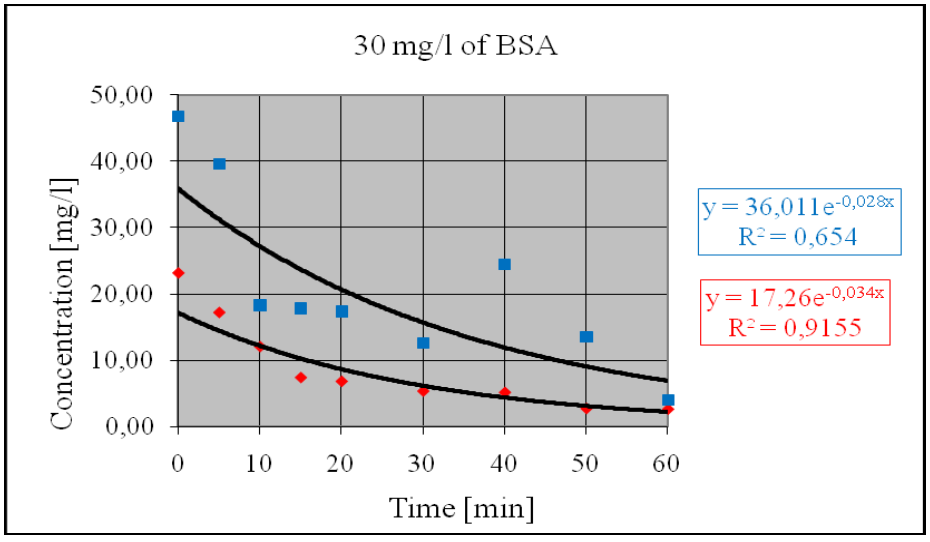
GRAPH 24. Protein concentration with 80 mg/l of BSA

In test 13 (see Table 27), 30 mg/l BSA concentration was used for seeing if the results are as successful as in the previous experiments with higher concentration, because with 50 mg/l was shown some foam. Calibration 7 from the Graph 20 was used in the concentrations calculations. Table 27 indicates that the BSA concentration in the 0 sample was 23,12 mg/l when it should be 30 mg/l. The results decreased still with the pure solution samples, as can be seen from Table 27, where the protein concentration decreased constantly.

The first dilution 1:10 samples results were unsuccessful. The BSA concentration was very high in the first samples and the points scattered a lot around the curve in Graph 25 and from the results in pure samples. The dilution 1:100 samples were difficult to interpret and more than 1:10 dilutions were not necessary to do.

TABLE 27. Absorbances and concentrations with ionized air

Time [min]	Absorbance [A] Pure 1:1	Conc. [mg/l]	Absorbance [A] Diluted 1:10	Conc. [mg/l]	Absorbance [A] Diluted 1:100	Conc. [mg/l]
0	1029	23.12	640	46.72	542	0.024
5	904	17.19	625	39.61	554	0.593
10	796	12.07	580	18.26	560	0.877
15	698	7.42	579	17.79	543	0.071
20	686	6.85	578	17.31	539	-0.119
30	655	5.38	568	12.57	541	-0.024
40	651	5.19	593	24.43	542	0.024
50	600	2.77	570	13.52	553	0.545
60	598	2.68	550	4.03	558	0.783



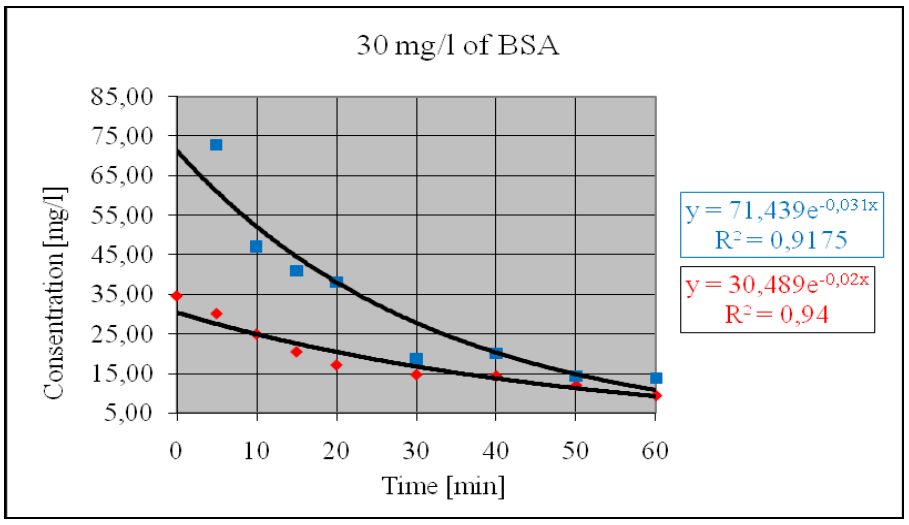
GRAPH 25. Protein concentration with 30 mg/l of BSA

The results of test 14 indicate that the BSA concentration in the 0 sample was 34,7 mg/l when it should be 30 mg/l (see Table 28). This could have been caused by a mistake in weighting the BSA. The results decreased still constantly. Calibration 7 from Graph 20 was used in the concentrations calculations. The ionized air destroyed the BSA concentration the longer ionized air was added. In the first dilution 1:10 samples the results decreased also constantly. The third dilution 1:100 samples did not decrease constantly anymore and could have been caused by a mistake in dilution, because the exact dilutions were challenging to do.

Graph 26 shows that the points in this case were not also in the curve. The curve with the results from the dilution 1:10 samples did not decrease constantly and scattered around the pure samples curve.

TABLE 28. Absorbances and concentrations with ionized air

Time [min]	Absorbance [A] Pure 1:1	Conc. [mg/l]	Absorbance [A] Diluted 1:10	Conc. [mg/l]	Absorbance [A] Diluted 1:100	Conc. [mg/l]
0	1273	34.70	724	86.56	545	0.166
5	1179	30.24	695	72.81	588	2.206
10	1070	25.07	641	47.19	570	1.352
15	976	20.61	628	41.03	542	0.024
20	906	17.29	622	38.18	549	0.356
30	855	14.87	581	18.74	550	0.403
40	848	14.54	584	20.16	573	1.494
50	798	12.17	572	14.47	545	0.166
60	744	9.60	571	13.99	559	0.830

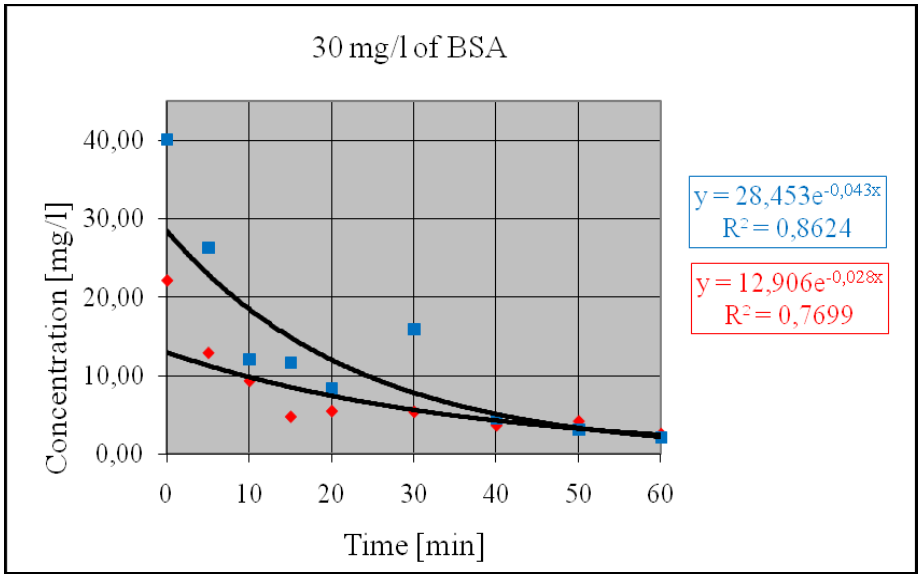


GRAPH 26. Protein concentration with 30 mg/l of BSA

In test 15 the concentration in the 0 sample was very low (see Table 29). The concentration was 22,08 mg/l when it supposed to be 30 mg/l. Calibration 7 in Graph 20 was used in the concentrations calculations. Graph 27 shows that the BSA concentration decreases but not linearly, based on which it could be presumed that the ionized air worked the way it was supposed to. The exponent from the y value (straight curve equation) is expected 0,028 when it should be 0,03. When the value is multiplied with 1000 it should be the same BSA concentration that has been placed in the beginning of the experiment. The dilutions in 1:10 samples worked but the y exponent is very high. The dilution 1:100 samples did not work again so it could be presumed that too much diluted samples do not give successful results.

TABLE 29. Absorbances and concentrations with ionized air

Time [min]	Absorbance [A] Pure 1:1	Conc. [mg/l]	Absorbance [A] Diluted 1:10	Conc. [mg/l]	Absorbance [A] Diluted 1:100	Conc. [mg/l]
0	1007	22.08	626	40.08	566	1.162
5	813	12.88	597	26.32	556	0.688
10	738	9.32	567	12.10	561	0.925
15	642	4.77	566	11.62	562	0.972
20	657	5.48	559	8.30	556	0.688
30	655	5.38	575	15.89	557	0.735
40	619	3.68	551	4.51	570	1.352
50	630	4.20	548	3.08	552	0.498
60	596	2.59	546	2.13	553	0.545

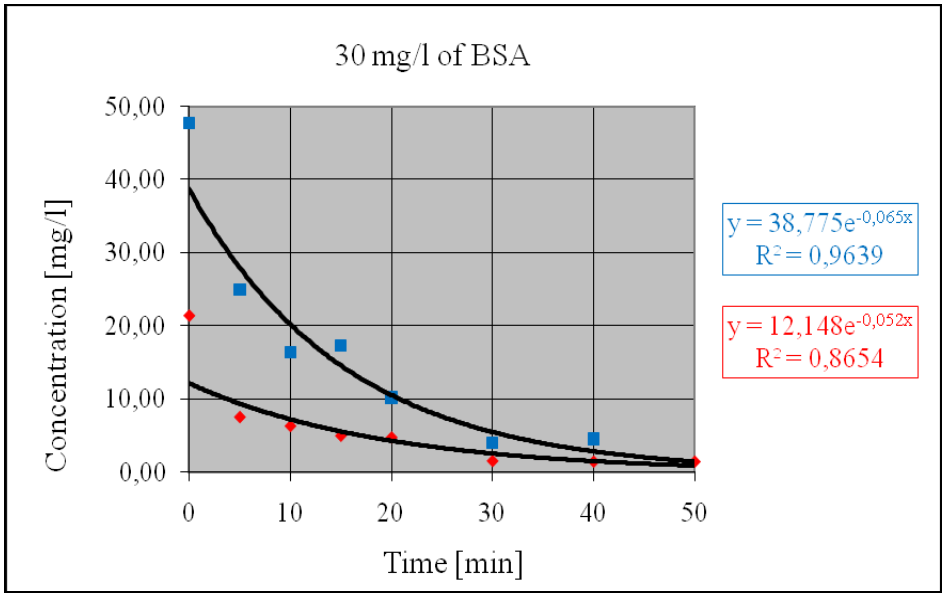


GRAPH 27. Protein concentration with 30 mg/l of BSA

Calibration 7 from Graph 20 was used in the concentrations calculations in test 16 (see Table 30). The results were quite similar comparing to those found in test 15. The BSA concentration in sample 0 is 21,42 mg/l when it supposed to be 30 mg/l, so it seems possible that the weighting scale was not calibrated. In both tests 15 and 16 the pressure in the ionized air flow was perhaps too much in the beginning, because the protein concentration came down rapidly in the first minutes and stabilised after that.

TABLE 30. Absorbances and concentrations with ionized air

Time [min]	Absorbance [A] Pure 1:1	Conc. [mg/l]	Absorbance [A] Diluted 1:10	Conc. [mg/l]	Absorbance [A] Diluted 1:100	Conc. [mg/l]
0	993	21.42	642	47.67	548	0.308
5	700	7.52	594	24.90	588	2.206
10	674	6.28	576	16.36	548	0.308
15	646	4.96	578	17.31	545	0.166
20	641	4.72	563	10.20	574	1.542
30	573	1.49	550	4.03	548	0.308
40	571	1.40	551	4.51	553	0.545
50	571	1.40	541	-0.24	559	0.830
60	541	-0.02	543	0.71	574	1.542



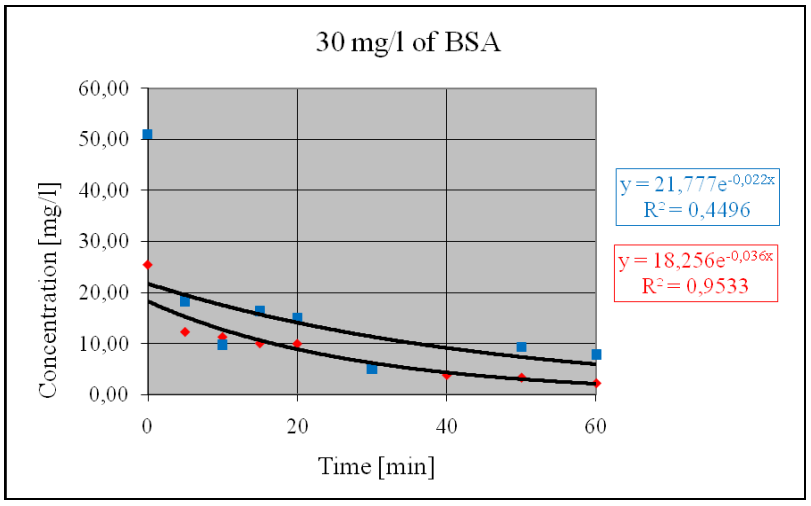
GRAPH 28. Protein concentration with 30 mg/l of BSA

Calibration 7 from Graph 20 was used in the concentrations calculations in test 17. The dilution did not work in this test, because the calculated concentration was two times higher than the real one in the pure 0 sample, when they have to be the same amount (see Table 31). This can also be seen from the exponential curve from the diluted sample results the dilution went wrong because the points are not on the curve. The BSA concentration in the pure 0 sample was also too low but better than the concentrations in tests 9 and 10. The protein

concentration dropped rapidly in the first minutes also in this test and slowed down to become more stable after that. From in could be presumed that the ionized air affected strongly in the beginning and came weaker after that. But the results decreased constantly and the points were in the exponential curve.

TABLE 31. Absorbances and concentrations with ionized air

Time [min]	Absorbance [A] Pure 1:1	Conc. [mg/l]	Absorbance [A] Diluted 1:10	Conc. [mg/l]	Absorbance [A] Diluted 1:100	Conc. [mg/l]
0	1078	25.45	649	50.99	554	0.59
5	800	12.26	580	18.26	578	1.73
10	779	11.27	562	9.72	550	0.40
15	753	10.03	576	16.36	549	0.36
20	751	9.94	573	14.94	565	1.11
30	661	5.67	552	4.98	564	1.07
40	622	3.82	541	-0.24	553	0.55
50	611	3.30	561	9.25	559	0.83
60	588	2.21	558	7.83	555	0,64



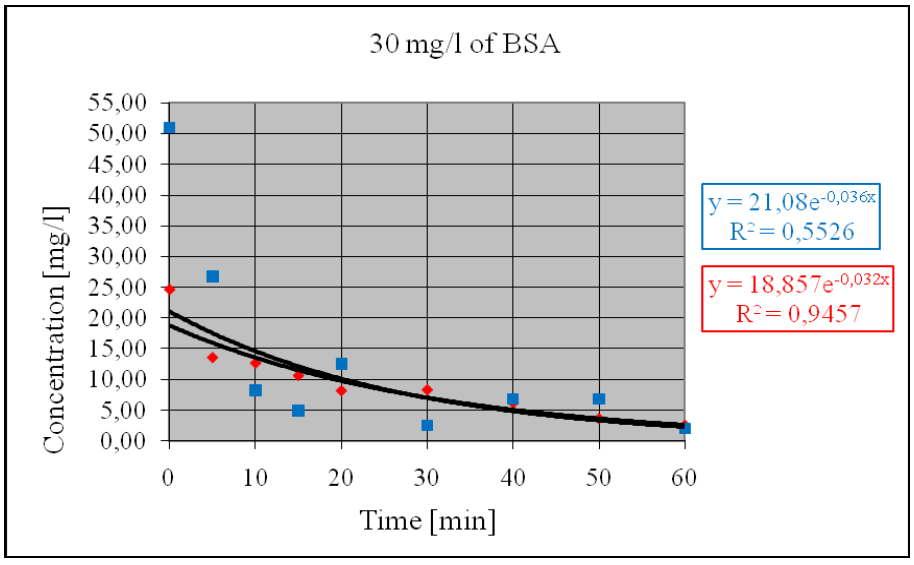
GRAPH 29. Protein concentration with 30 mg/l of BSA

In test 18 the results were again very similar compared to test 17 results (see Table 32). Calibration 7 in Graph 20 was used in concentrations calculation. The BSA concentrations were very low again in the beginning but still the same affects could be seen that in previous

expermients. The ionized air affected very strongly in the first minutes and stabilised after this. But the pure samples results in test 18 were successful, because the points are on the exponential curve.

TABLE 32. Absorbances and concentrations with ionized air

Time [min]	Absorbance [A] Pure 1:1	Conc. [mg/l]	Absorbance [A] Diluted 1:10	Conc. [mg/l]	Absorbance [A] Diluted 1:100	Conc. [mg/l]
0	1062	24.69	649	50.99	580	1.83
5	828	13.59	598	26.80	551	0.45
10	809	12.69	559	8.30	547	0.26
15	766	10.65	552	4.98	550	0.40
20	714	8.18	568	12.57	561	0.92
30	717	8.32	547	2.61	537	-0.21
40	672	6.19	556	6.88	542	0.02
50	618	3.63	556	6.88	534	-0.36
60	595	2.54	546	2.13	553	0.55



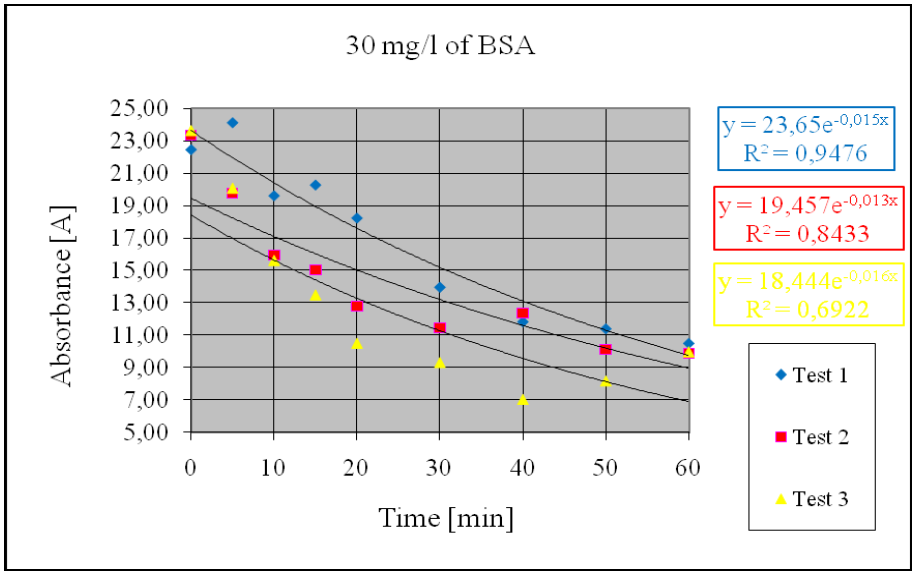
GRAPH 30. Protein concentration with 50 mg/l of BSA

The three last experiments were made with 30 mg/l of BSA (see Table 33) but only the pure samples were measured. No dilutions were done anymore, because it could be seen from the results of the previous experiments that the dilutions did not work and were not relevant to compare the results of pure samples and diluted samples.

Calibration 7 in Graph 20 was used in concentrations calculations. In test 1 (see Table 33) can be seen that something was not working in sample 0, because the concentration was lower than in sample at 5 minutes. Otherwise it could be seen that the experiments were successful and the results are comparable with the previous experiments. An exception in these experiments is test number 3 (see Table 33), where the two last sample concentration are rising again.

TABLE 33. Three 30 mg/l BSA concentration

Test no 1			**Test no 2**		**Test no 3**	
Time [min]	Absorbance [A] Pure 1:1	Concentration [mg/l]	Absorbance [A] Pure 1:1	Concentration [mg/l]	Absorbance [A] Pure 1:1	Concentration [mg/l]
0	1015	22.46	1034	23.36	1040	23.64
5	1050	24.12	959	19.80	965	20.09
10	955	19.61	878	15.96	871	15.63
15	969	20.28	859	15.06	826	13.49
20	926	18.24	811	12.78	763	10.51
30	836	13.97	783	11.45	738	9.32
40	791	11.83	802	12.36	690	7.04
50	782	11.41	755	10.13	714	8.18
60	763	10.51	750	9.89	752	9.98



GRAPH 31. Three 30 mg/l BSA concentration

Placing all the results from 30 mg/l of BSA in the same graph (see Graph 32), it can be seen that, if test concentration 2 is left out, the results remind each other well. From this could be made an assumption, that the effect of ionized air is strongest in the first minutes and constantly slows down after that.

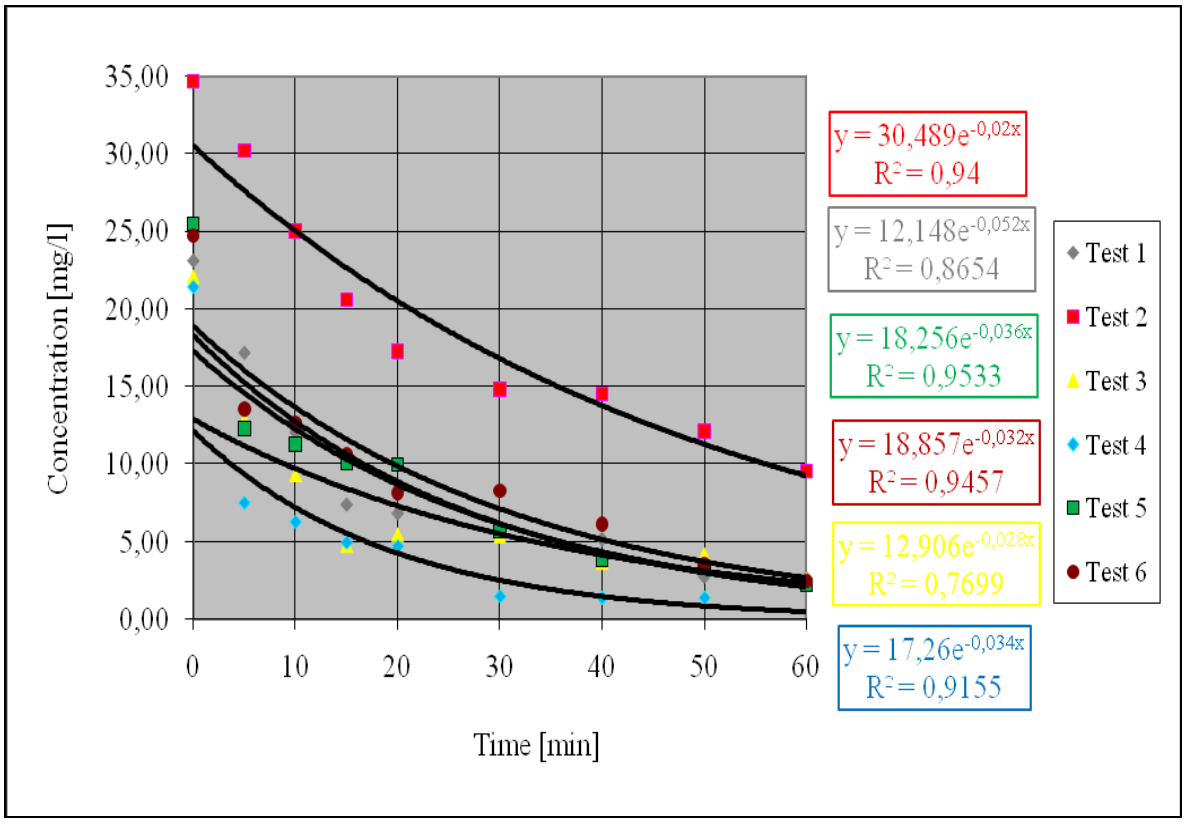
TABLE 34. All BSA 30 mg/l absorbances and concentrations with ionized air

Time [min]	Absorbance 1	Conc.	Absorbance 2	Conc.	Absorbance 3	Conc.
0	1029	23.12	1273	34.70	1007	22.08
5	904	17.19	1179	30.24	813	12.88
10	796	12.07	1070	25.07	738	9.32
15	698	7.42	976	20.61	642	4.77
20	686	6.85	906	17.29	657	5.48
30	655	5.38	855	14.87	655	5.38
40	651	5.19	848	14.54	619	3.68
50	600	2.77	798	12.17	630	4.20
60	598	2.68	744	9.60	596	2.59

(cont.)

TABLE 34 (cont.)

Time [min]	Absorbance 4	Conc.	Absorbance 5	Conc.	A.bsorbance 6	Conc.
0	993	21.42	1078	25.45	1062	24.69
5	700	7.52	800	12.26	828	13.59
10	674	6.28	779	11.27	809	12.69
15	646	4.96	753	10.03	766	10.65
20	641	4.72	751	9.94	714	8.18
30	573	1.49	661	5.67	717	8.32
40	571	1.40	622	3.82	672	6.19
50	571	1.40	611	3.30	618	3.63
60	541	-0.02	588	2.21	595	2.54



GRAPH 32. All 30 mg/l BSA concentration curves

6.3 Determining the microorganisms with the Koch method

With the Koch method was made the research, how ionized air affects to the microorganisms in NaCl-yeast solution. The same solution and the samples were made with the same procedure in the Koch method as in the Bradford method with NaCl-yeast solution. The Koch method was also made with non-ionized and ionized air to compare them. In this method the samples were first cultivated on a Petri dish containing agar gel and next grown on the plate for about 24 hours and they were finally counted after that. The line in Tables 35–37 means that there were more than 600 microorganisms in the plate and they could not be counted.

In the first experiment (as shown in Table 35) the pure solution, diluted samples 1:10 and 1:100 had very much microorganisms, because in most time points there were more than 600 microorganisms on the plate. If dilution 1:1000 or 1:10 000 had been made, there could have been seen how the ionized air is affecting the solution in very beginning. The amount of microorganisms was not under 500 until 20 minutes of adding the ionized air to the solution. After adding ionized air for 20 minutes the amount of microorganisms decreased constantly (see Table 35). From these results it can be presumed that the ionized air disintegrated the microorganisms in the expected way. Meaning that the longer the solution was treated with the ionized air the fewer microorganisms appeared on the plates. From the samples dilutions could be calculated how many microorganisms one liter solution contained. This was done with multiplying the amount of microorganisms, for example, the 1:100 dilutions with 1×10^8.

TABLE 35. The amount of microorganisms with ionized air

Time [min]	Pure 1:1	Diluted 1:10	Diluted 1:100	In 1 liter of solution
0	-	-	-	-
5	-	-	-	-
10	-	-	-	-
15	-	-	>500	-
20	-	-	504	5.04×10^{10}
30	-	-	276	2.76×10^{10}
40	-	>500	120	1.20×10^{10}
50	-	>500	133	1.33×10^{10}
60	-	389	25	2.50×10^{9}

Test 2 was made with non-ionized and ionized air (see Table 36 and 37) to find out how non-ionized air affected the microorganisms comparing to ionized air. Dilution 1:1000 was needed to get fewer than 600 microorganisms, because usually in samples dilutions 1:10 and 1:100 were more than 600 microorganisms and they could not be counted. Table 36 indicates that the non-ionized air did not disintegrate the microorganisms, because the amount of microorganisms fluctuates in every sample.

With ionized air (see Table 37) were used 1:1000 dilutions, because in test 1 the 1:100 dilutions were not enough. From Table 37 it can be seen that the ionized air affected to the amount of microorganisms in the solution and decreased constantly after every minute. The more ionized air was added the fewer microorganisms appeared. The amount of microorganisms was also counted in one liter solution, which can be seen from Table 37.

TABLE 36. The amount of microorganisms with non-ionized air

Time [min]	Diluted 1:10	Diluted 1:100	Diluted 1:1000	In 1 liter of solution
0	-	-	372	3.72×10^{10}
5	-	-	552	5.52×10^{10}
10	-	-	475	4.75×10^{10}
15	-	-	-	-
20	-	-	60	6.00×10^{9}
30	-	-	-	-
40	-	-	-	-
50	-	-	-	-
60	-	-	-	-

TABLE 37. The amount of microorganisms with ionized air

Time [min]	Diluted 1:10	Diluted 1:100	Diluted 1:1000	In 1 liter of solution
0	-	-	679	6.79×10^{10}
5	-	-	534	5.34×10^{10}
10	-	-	359	3.59×10^{10}
15	-	>600	222	2.22×10^{10}
20	-	>600	348	3.48×10^{10}
30	-	>600	109	1.09×10^{10}
40	>600	259	21	2.10×10^{9}
50	>600	140	7	7.00×10^{8}
60	-	182	7	7.00×10^{8}

Test 3 was made with non-ionized air (see Table 38) once more to be sure that it was not working for disintegration of microorganisms. Because in previous test the amount of microorganisms in smaller dilutions were over 600, only 1:10 000 samples dilutions were used in this test. Table 38 indicates the same as was presumed from test 2, in other words that the non-ionized air did not affect for disintegration of microorganisms. The amount of microorganisms fluctuated in every minute and from this it could be presumed that it was not useful to make this experiment with non-ionized air anymore. With ionized air (see Table 39) the results fluctuated in every minute with samples dilutions 1:1000 and 1:10 000 and it can be

presumed that it was because the samples were in the fridge for one day before culturing the plates. It was also possible that there had been made other errors, because the experiment was very sensitive in every step and it was hard to know if something went wrong at some step before seeing the results.

TABLE 38. The amount of microorganisms with non-ionized air

Time [min]	Diluted 1:10 000	In 1 liter of solution
0	16	1.60×10^9
5	49	4.90×10^9
10	59	5.90×10^9
15	64	6.40×10^9
20	67	6.70×10^9
30	198	1.98×10^{10}
40	61	6.10×10^9
50	92	9.20×10^9
60	100	1.00×10^{10}

TABLE 39. The amount of microorganisms with ionized air

Time [min]	Diluted 1:1000	Diluted 1:10 000	In 1 liter of solution
0	195	15	1.5×10^9
5	252	39	3.9×10^9
10	588	76	7.6×10^9
15	203	31	3.1×10^9
20	211	28	2.8×10^9
30	172	43	4.3×10^9
40	438	62	6.2×10^9
50	213	21	2.1×10^9
60	310	19	1.9×10^9

7 CONCLUSION AND DISCUSSION

In this study were determined the effect of ionized air for protein content and disintegration of microorganisms in a Baker's yeast-sodium chloride-solution. The Bradford method was used for determining the protein content and the Koch method was used for determining the microorganisms. Non-ionized air and ionized air was used in both methods, so the effects of ionized air could be compared with the non-ionized air. The samples in all experiments were taken in different time points.

The second part of experiments was determining protein contents in NaCl-yeast solution with Bradford method. Approximately the same amount of yeast (4 g) and NaCl (9 g) in one liter solution were used in NaCl-yeast solution. The pressure changed between 0,1–1 bar to see how different pressures affected in experiments. One bar pressure was too high, because the absorbances in the samples did not change much. Lower air pressure was used in other experiments but the results were still not successful, because they fluctuated very much. Other reason for fluctuating results could be a cause of varying air flow, because the pressure of the air flow in the compressor did not stay stable all the time. The successful pressure for disintegration of protein content and microorganisms was 0,1–0,2 bars.

It could be presumed that the Bradford method was not suitable for yeast, because only one result was received which diverged from the others. In this result, which diverged from others, the protein content increased constantly. The results between ionized air and non-ionized air had no difference between each other. The Bradford method did not give the expected results with yeast, so an attempt was made to apply the Bradford method by adding BSA standard beside yeast. Non-ionized and ionized air was used in the experiments to see how it affected to BSA concentration.

Different amounts of BSA concentrations were used between 20–500 mg/l. A foam inhibitor was needed to use with 500 mg/l, otherwise it was too challenging to do the experiment. The experiment was not successful, because the results were not changing much and it could be presumed that it was because of the foam inhibitor, which slowed down the reactions. So

much foam was created in the experiment that it came out from the vessel and it was very difficult to handle it. The protein could be also on the vessel wall and then the results were not very strict. In other BSA concentrations the results increased constantly (between 20–100 mg/l). The best concentration, which was 30 mg/l, was chosen from these results, where was not appearing very much foam and was the easiest to do. Several experiments were made with this concentration to get as much information about disintegrations as possible.

Successful results were got with pure solution in every experiment. The dilution 1:10 was also quite successful, but the more diluted the samples the more inaccurate the results. Dilutions were very strict that it was very difficult to make them. In earlier laboratory work the dilutions have always been done only with ion changed water, so it was not known that would be better to do the dilutions with 0.9% NaCl. Other errors could be that magnetic mixer was not used in the first experiments (only in the beginning mixing the substances). So the solutions were not so homogeneous, but it was used through the whole last experiments. With non-ionized air there was no impact to the results. Based on the results of experiments with ionized air can be assumed that it destroys the protein content, because the protein concentration decreased after every minute of adding ionized air.

The third part of the experiments determined the amount of microorganisms with the Koch method. The Koch method had to be done in very sterile conditions to get successful results. It was very punctual that everything, the substances and equipment, were very sterile. Making the dilutions was difficult in high dilutions, because it needed to be exact. With the pipettes were needed to be very careful. There was very big risk to make mistakes with this. The first samples were made with the pure solution so there were large amounts of microorganisms and was not possible to be counted. The first dilution from where the microorganisms could be counted was diluted 1:100 but the best results were got from the dilution 1:1000. It could be presumed that the Koch method can be successful in disintegration of microorganisms with using of ionized air. Non-ionized air had not affects to the disintegration of microorganisms.

The results for the impact of ionized air were as same as for ozone, but only with doing disintegration with Bradford method and using bovine serum albumin (BSA) solution. The

results of yeast-sodium chloride solution were not successful. The Koch method gave only one expected result, so they are not enough for saying that the method worked. Because of ozone's disadvantage of being dangerous for people and environment, ionized air is better alternative for disintegrating of microorganisms from the wastewater. Ionized air has no disadvantage for them at all and it has only healthy affects for people. Ozone kills effectively bacteria (Zhang, Yang, Liu & Zhang 2009) and the same kind of results was obtained with ionized air. Ionized air is very much cheaper than ozone. It can be presumed that ionized air is an better alternative for small amounts and ozone with higher amounts of disintegration of microorganisms.

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Roti® – Nanoquant manual

Protein-assay

A. Introduction

Roti® - Nanoquant solution has resulted from a modification of Bradford's protein assay (1,2). By using the required amount of solution, reproducible protein amounts from 200 ng onwards (c = 1 ng/µl, V = 200 µl) can be analyzed in aqueous solutions.
Each sample is measured at 590 nm and 450 nm.
Linearity results from the quotient $OD_{590/450}$.

B. Formulas

Roti® - Nanoquant working solution:
20 ml Roti® - Nanoquant (5X–Conc.) + 80 ml H_2O_{dd} Roti® - Nanoquant working solution need to be filtered

BSA-concentrations for calibration series
200 ng/200 µl = 1 µg/ml to
20 µg/200 µl = 100 µg/ml
We recommend using BSA in flakes (Albumin Fraktion V), Art. No. T844.1.

C. Literature

(1) Bradford, M., 1976, Anal. Biochem. 72, 248–254.
(2) Zor, T. und Selinger, Z., 1996, Anal. Biochem. 236, 305–308.

D. Instructions: Measuring range 200 ng–20 µg

1. Pipette the following volumes into clean cuvettes:
   - For the zero value (Sample 1) of the calibration line: 200 µl H_2O_{dd} + 800 µl Roti® - Nanoquant working solution
   - For the calibration series (Sample 2 to Sample S): 200 µl each of standard + 800 µl Roti® - Nanoquant working solution
   - For the actual analysis (Sample T to Sample Z): 200 µl of each sample + 800 µl Roti® - Nanoquant working solution
2. Mix by inverting repeatedly.
3. Pipette H_2O_{dd} in your reference cuvette.
4. Determine OD_{590} of all your samples (Sample 1 to Sample Z) with H_2O_{dd} as a reference (Figure 1)
5. Determine OD_{450} of all your samples (Sample 1 to Sample Z) with H_2O_{dd} as a reference (Figure 1)
6. Plot quotient OD_{590}/OD_{450} and compare to amount of protein used (Figure 2). The protein amount in your sample corresponds to a certain value of calibration line.

E. Tips for better pipetting accuracy

1. Submerge the tip only few mm into liquid.
2. Hold pipette vertically.
3. Wet tip with protein solution prior to sample removal.
4. Lean tip against the tube when releasing liquid.
5. Only use high-quality tips. We recommend Mµlti® - standard tips 1–200 µl, (Art. No. 7058.1) and 100–1000 µl (Art. No. 8163.1).

F. Protein assay in 96-well culture plates

1. Required equipment
   - Roti®-Nanoquant
   - 96-well culture plate
   - ELISA reader
   - BSA stock solution (400 µg/ml); We recommend using our BSA in flakes (Albumin Fraktion V), Art. No. T844.1
2. Preparation

Please prepare your calibration standards. We recommend making the solution with a concentration of 100 µg BSA/ml first, because thereafter the remaining solutions can easily be prepared by dilution.

3. Procedure
   - Please dilute the samples to be measured, e.g. cell extract (ce).
   - If avaible: Make a copy of your pipetting schedule with Procalc and lay it under your culture plate.
   - According to your schedule pipette into the wells of your culture plate 50 µl of each calibration standard and of the cell extract dilutions. We recommend pipetting each solution at least two times to verify your results within a double assay.
   - Please dilute 1 volume Roti® - Nanoquant (5x) in 4 volumes H2Odd and 200 µl of this solution to the standards and the samples on your plate.
   - Incubate the culture plate for 5 min at room temperature and thereafter measure the OD_{590} and OD_{450}.
   - For automated calculation: Store your data as an ASCII file and import them into Protcalc. Manual calculation: Plot and compare the quotient OD_{590}/OD_{450} of each sample to the calibration curve.

G. Safety information:

Roti® - Nanoquant:	R-codes: 11–20/21/22–33–34
	S-codes: 16–26–45

Failed calibration curves