PALACKÝ UNIVERSITY OLOMOUC

FACULTY OF SCIENCE

DEPARTMENT OF BOTANY

OPTIMIZING THE METHOD OF DETERMENING THE YIELD OF HYALURONIC ACID IN SMALL VOLUMES OF THE FERMENTATION CULTURE

bachelor thesis

Anesa Suljic

Biology – Geology and Environmental Protection for Education

full-time

Supervisors: doc. RNDr.Vladan Ondřej, Ph.D., Ing. Zbyněk Černý

august 2019

Bibliographical identification

Author's first name and surname: Anesa Suljic

Title: Optimizing the method of determining the yield of hyaluronic acid in small samples of the fermentation culture

Institution: Palacký University in Olomouc, Faculty of Science, Department of Biology

Supervisors: doc. RNDr.Vladan Ondřej, Ph.D., Ing.Zbyněk Černý

The year of presentation: 2019

Abstract:

The present research done within a scope of my bachelor thesis describes an optimization of fast, affordable and reliable method of hyaluronic acid (HA) determination in small volumes of fermentation culture of *Streptococcus equi* subsp. *zooepidemicus*. The research includes several inverstigated aims. The first aim was to determine the yield of HA obtained using standardized precipitation procedure with isopropyl alcohol. The average yield of HA obtained using isopropyl precipitation method is 3.63 HA (g/l). The second aim involved optimization of sodium dodecyl sulfate concentration used to separate HA from bacterial capsule. The optimal concentration that did not have a denaturation effect on hyaluronan lyase (SpHyl) was estimated at 0.015% SDS. The aim of the third step was to evaluate the effect of three different factors (H_2O, SDS and chelaton III + SDS solution) that samples were treated with on the yields of HA obtained by SpHyl-MBTH method. It was shown that the yield of HA obtained using SpHyl-MBTH method is 2.60 HA [g/l] from samples which are treated with H_2O, 3.30 HA [g/l] from samples in which SDS is added and 3.46 HA [g/l] from samples in which chelaton III + SDS solution is added. The final aim of the research was the comparison of the yield of HA obtained by SpHyl-MBTH method to the yield of HA obtained by precipitation method using isopropyl alcohol. The yield of HA calculated on the basis of 10 samples obtained by SpHyl-MBTH method is 3.46 HA [g/l], whereas the yield of HA obtained by precipitation method using isopropyl alcohol is 3.63 HA [g/l].

Key words: hyaluronic acid, MBTH, Streptococcus, hyaluronan lyase, precipitation, isopropyl alcohol, sodium dodecyl sulfate

Number of pages: 43

Nuber of appendices: 0

Language: English

Abstrakt

Současný výzkum provedený v rámci mé bakalářské práce popisuje optimalizaci rychlé, dostupné a spolehlivé metody stanovení kyseliny hyaluronové (HA) v malých objemech fermentační kultury *Streptococcus equi* subsp. *zooepidemicus*. Výzkum zahrnuje několik zkoumaných cílů. Prvním cílem bylo stanovit výtěžek HA získaný pomocí standardizovaného postupu srážení pomocí isopropanolu. Průměrný výtěžek HA získaný metodou srážení pomocí isopropanolu je 3.63 HA (g/l). Durhým cílem byla optimalizace koncentrace dodecylsulfátu sodného (SDS) použitého k oddělení HA od bakteriální kapsle. Optimální koncentrace, která neměla denaturační účinek na hyaluronan lyázu (SpHyl) byla odhadnuta na 0.015% SDS. Cílem třetího kroku bylo vyhodnotit účinek tří různých faktorů (H_2O, SDS a roztok chelaton III + SDS), se kterými byly vzorky ošetřeny, na výtěžky HA získané metodou SpHyl-MBTH. Ukázalo se, že výtěžek HA získaný metodou SpHyl-MBTH je 2.60 HA (g/l) ze vzorků které byly ošetřeny H_2O, 3.30 HA (g/l) ze vzorků do nichž byl přidán SDS a 3.46 HA (g/l) ze vzorků do kterých je přidán roztok chelaton III + SDS. Konečným cílem výzkumu bylo srovnání výtěžku HA získaného metodou SpHyl-MBTH s výtěžkem HA ziskáného srážecí metodou pomocí isopropanolu. Výtěžek HA vypočtený na základě 10 vzorků získaných metodou SpHyl-MBTH je 3.46 HA (g/l), zatímco výtěžek HA získaný srážecí metodou pomocí isopropanolu je 3.63 HA (g/l).

Klíčová slova: kyselina hyaluronová, MBTH, Streptococcus, hyauronan lyáza, srážení, isopropanol, dodecylsulfát sodný

Declaration

I declare that I have worked on present bachelor thesis independently using only the sources listed in the bibliography. All resources, sources, and literature, which I used in preparing or I drew on them, I quote in the thesis properly with stating the full reference to the source.

Olomouc, 31st of July, 2019

_____

Anesa Suljic

Acknowledgement

I want to thank Contipro A.S. for giving me an opportunity to work on my bachelor thesis in their laboratories providing me with all the equipment and sources needed. I would extend special acknwoledgement and gratitude to my supervisor doc. RNDr.Vladan Ondřej, Ph.D, firstly for enabling me to work on my bachelor thesis at Contipro A.S. and secodnly for providing me with constant help and advices on my work. Furthermore, special thanks also goes to Ing. Zbyněk Černý, who helped me to overcome any obstacles I had to cope with during my research at Contipro A.S. Above all, I am so grateful to my family and friends for enourmous support and unconditional love.

Contents

Introduction

Hyaluronic acid (hyaluronan, hyaluronate, HA) is a linear glycosaminoglycan polymer with specific rheological and biological features. It is present in tissues of all vertebrates including epithelial, neural and conncective tissues. It is estimated that in a body of person of 70 kg, 15 g of hyaluronan can be found in various type of tissues including human skin that contains over 50% of the hyaluronan in the whole body (Stern, 2003). Apart from vertebrates, portions of hyaluronan are also present in the capsule of pathogenic bacteria including group A and C streptococci. In living orgamisms hyaluronan is produced by enzyme called hyalornan synthase (Boeriu et al. 2013).

Due to its viscoelasticity and high moisture properties hyaluronic acid has a wide range of medical, pharmaceutical and cosmetic applications. High molecular weight HA is used in cosmetics, tissue engineering, ophtalmology and orthopaedic (Kogan et al. 2007), whereas low molecular weight HA is used for production of substances to inhibit tumor progession or to induce heat shock proteins (Tammi et al. 2008).

Two main ways of industrial manufacturing of hyaluronan are extraction from animal tissues and using bacterial strains through microbial fermentation (Shiedlin et al. 2004). Since my bachelor thesis was done within a scope of my internship program at Contipro A.S., my research will be based on working with bacterial fermentation culture, hence using bacterial strains of *Streptococcus equi* subsp. *zooepidemicus* (SEZ) as a main way of HA production since it is a manufacturing method used in Contipro A.S.

The increasing interest in hyaluronic acid has led to demand for a development of simple, fast, low cost and reliable method of hyaluronan determination in a wide range of hyaluronan-containing substances. One of the standardized methods used for HA isolation and purification that was applied and evaluated within my research is precipitation method using isopropyl alcohol. Precipitation method works with large volumes of fermentation culture and it is suitable for large scale production of hyaluronic acid. It is widely used in pharmaceutical and cosmetic production (Cavalcanti et al. 2018). However, a recent trend of using small reactors, so-called microbioreactors, that can work with small volumes of sample has became a popular and

preferable way for research analysis. Working with large volume samples using large reactors is time-consuming since it has low capacity of possible cultivations and the costs of production and analysis are higher since it uses large amounts of fermentation culture. The main advantage of using microbioreactors is that it enables the increase in capacity of parallel cultivation while it uses small amounts of fermentation culture. However, the problems related to small volumes of fermentation culture should be taken into consideration.

Since the standardized precipitation method used within the research I have done has shown to be effective when working with large volumes of fermentation culture and not suitable for microbioreactors it was necessary to find the method that works with thousand times smaller sample volume. It was assumed that new colorimetric method that was proposed by Pepeliaev et al. (2017) was able to cover all the requirments with minimum of limitations. The new colorimetric method was based on the digestion of hyaluronan by hyaluronan lyase from Streptococcus pneumoniae (SpHyl) and the following reaction between 4,5-unsaturated dissacharides with 3-methyl-2-benothiazolinonehydrazone (MBTH). The result is highly colored product (blue color) detected using spectrophotometer at 654 nm.

The main goal of my bachelor thesis is to apply the SpHyl-MBTH method using small volumes of fermentation culture of SEZ and to test its reliability by comparing the yield of HA obtained using SpHyl-MBTH method to the yield of HA obtained using isopropyl precipitation method.

Before applying the SpHyl-MBTH method on the fermentation culture of SEZ it was necessary to separate the hyaluronic acid from the capsule of bacteria, hence bacterial cell wall. According to study done by Schiraldi et al. (2010) several methods are used to accomplish the separation of hyauronic acid from bacterial cell wall including the usage of different organic solvents, detergents, proteolytic enzymes (pepsin, pronase, trypsin) etc. For my research I will be using sodium dodecyl sulfate (SDS) and chelaton III + SDS solution to accomplish the release of hyaluronan from bacterial capsule. Furhtermore, the effect of H_2O added to sample will be tested with the expectancy of lower amount of hyaluronan to be released from bacterial cell wall hence resulting in lower yield of HA after applying the SpHyl-MBTH method.

However, since sodium dodecyl sulfate has a role as a detergent and protein denaturant (Farrell, 2010) it was essential to optimize the concentration of SDS in that way to be efficient to release

the hyaluronic acid from bacterial capsule but at the same time not to have a denaturation effect on the activity of hyaluronan lyase (SpHyl). In order to optimize the concentration of SDS, the effect of different concentration of SDS (0%, 0.2%, 0.1%, 0.05%, 0.015%) on the activity of SpHyl will be tested and an optimal concentration will be used.

To sum up, the first aim of my reserch is to determine the yield of hyaluronic acid (HA) by precipitation method with isopropyl alcohol which works with large volume of the fermentation culture. Second aim is to optimize the concentration of sodium dodecyl sulfate (SDS) in a such way that the activity of hyaluronan lyase (SpHyl) is not affected. Third aim was to evaluate the effect of three different factors (H_2O, SDS and chelaton III + SDS solution) that samples were treated with on the yields of HA obtained by SpHyl-MBTH method. The final aim is to evaluate the reliability of SpHyl-MBTH method used for determining the yield of HA in small volumes of fermentation culture by comparing the yield of HA obtained using precipitation method with isopropyl alcohol to the yield of HA obtained by SpHyl-MBTH method.

It is assumed that the yield of HA obtained by SpHyl-MBTH using small volumes of fermentation culture will be similar to the yield of HA obtained using isopropyl alcohol precipitation method which works with large volumes of fermentation culture. Furthermore, it is expected that the yield of HA obtained from samples that are treated with H_2O will be lower in comparison to the yield of HA obtained from samples treated with SDS and chelaton III + SDS solution.

Properties of hyaluronic acid (HA)

Hyaluronic aicd (hyaluronan, hyaluronate, HA) is a high molecular weight linear polysaccharide composed of a repeating disaccharides of glucuronic and N-acetylglucosamine. Unlike other glycosaminoglycans such as chondroitin/dermatan sulfate, keratan sulfate and heparin/heparan sulfate, hyaluronan is the only one that is neither sulfated nor covalently bound to proteoglycan core protein. (Boeriu et al. 2013). Hyaluronic acid can be found in all vertebrates. It is a structural element in cartilage, synovial fluid between joints, skin of vertebrates and the vitreous humor of the eye (Fraser et al. 1997). Hyaluronan is also present in the capsule of microbial pathogens such as *Pasteurella multocida* and group A and C streptoccoci (Boeriu et al. 2013). Due to its specific rheological and biological features, for instance pseudoplasticity, water-holding capacity, HA has a wide range of commercial applications in different fields. Speaking of that it is important to mention that HA plays an important role in maintaining of intact architecture in normal tissues as it absorbs large volume of water and creates a gel-like environment (Fraser et al. 1997). Nowadays, hyaluronan is widely used in pharmaceutical industry. It has been extensively utilized in cosmetic products due to its biocompatibility and viscoelastic properties. It is reported that cosmetic products containing HA demonstrates an antiwrinkle effect providing the skin with moisture and restoring its elasticity. Apart from its role in aesthetic field, HA is an important component of pharmaceutical ingredients that are used for treatment of arthritis and osteoarthritis. The HA-iodine complex has shown to be effective in the treatment of different types of complicated wounds including diabetic foot ulcers, leg ulcers, bed sores, acute wounds and superficial burns (type II a-b) (Frankova et al. 2006).

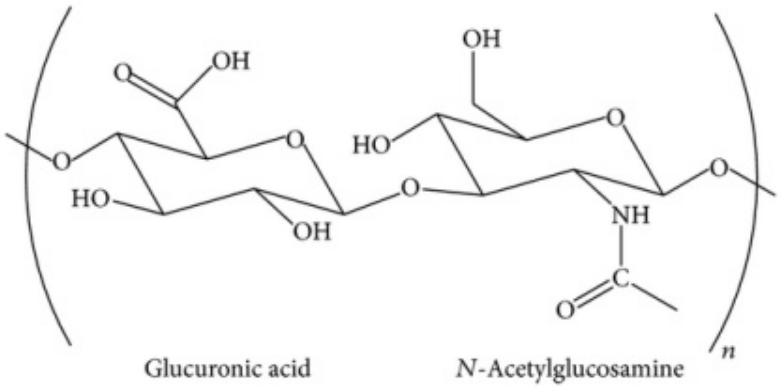


Figure 1: Repeating unit of hyaluronan (www1)

Speaking about wide range of hyaluronan applications, it is important to mention that the size of hyaluronan polymers has an influence on its biological functions. High molecular weight hyaluronan polymers are antiangiogenic, space filling and immunosuppressive, medium size hyaluronan polymers have function in wound repair and ovulation, whereas small hyaluronan oligomers are antiapoptotic and inducers of heat shock proteins. (Kogan et al.2007, Tammi et al. 2008).

The size of the polymers ($M\omega$ from 5,000 Da to 20 million Da) of isolated hyaluronan depends on the type of tissues. For instance, in human umbilical cord molecular mass of hyaluronan is estimated to be 3-4 million Da, while the one in human synovial fuid is 6 million Da (Porsch et al. 2008). However it, should be taken into consideration that molecular weight of isolated hyaluronan can be influenced by isolation and analysis method used. Along with the hyaluronan concentration, the molecular weight and the type of tissue are exactly factors influencing the cell response when it comes to healing processes, cancer, embryonic development etc.

Synthesis of hyaluronic acid (HA) in living organisms

In living organisms, hyaluronan is produced by hyaluronan synthase enzymes. These enyzmes synthesize linear polymers of the repeating disacharide of glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) by addition of GlcUA and GlcNAc to the growing chain using their nucleotid sugars as a substrate (Boeriu et al. 2013). Hyaluronan molecules are synthesized at the plasma membrane and then expelled into the extracellular matrix where they can exist in different forms. For instance, hyaluronan can be bound to the membrane receptors on the surface of the cell or it can be embaded into glycocalyx. Apart from being present extracellulary, hyaluronan is present intracelullary as well where it can react with binding proteins (Prehm 1984 in Boeriu et al. 2013).

The overall reaction of hyaluronan synthesis is following:

$n\ UDP - GlcUA + n\ UDP - GlcNAc \rightarrow 2n\ UDP + [GlcUA + GlcNAc]_n$ (DeAngelis, 2012)

where UDP represents uridine diphosphate, $GlcUA$ represents glucuronic acid, $GlcNAc$ is N-acetylglucosamine and n is the number of repeat disaccharides.

In 1993 DeAngelis et. al (1993) reported the identification of gene encoding the enzyme responsible for hyaluronan synthesis. It was identified from group A streptococci. This gene is part of an operon containing the *has*A gen encoding hyaluronan synthase, the *has*B gene encoding UDP-Glucose dehydrogenase, and the *has*C gene encoding UDP-Glucose pyrophosphorylase. Further studies done by cloning of the group A as well as the group C streptoccocal hyaluronan synthaes genes showed that only one gene product (the HA-synthase protein) is required for hyaluronan biosynthesis (Kumari & Weigel 1997 in Boeriu et al. 2013).

The first vertebrate gene encoding HA synthase was identified in 1996 and it was the *Xenopus laevis* gene DG42 (DeAngelis & Achyuthan 1996 in Boeriu et al. 2013). Couple of years later an HA synthase from an algal virus was discovered (DeAngelis, 1999). The streptoccocal, viral and bacterial HA synthase has shown a similar protein sequence and all of them had a single glycosyl transferase 2 (GT2) family module (Coutinho et al.2003). However, HA synthase that differs in protein sequence and in mentioned family module was identified in type A *P.multocida,* an animal pathogen. It was found that *P.multocida* HA synthase differs from other HA synthases in

a way that has a membrane attachment domain near the carboxyl terminus, has two GT2 modules and elongates the HA chains at the nonreducing end, while other HA synthases elongate HA chains at the reducing end (DeAngelis et al.1998). According to Weigel and DeAngelis (2007) who proposed hyaluronan classification system, *P.multocida* HA synthase is the member of Class II HA synthases, while other HA synthases which are integral proteins are members of Class I HA synthases.

However it is important to say that the regulation of HA synthase in vertebrates is much more complex than in bacteria because hyaluronan performs various functions in the mammalian body depending on the type of tissue and the size of the polymers required. Speaking of complexity, it is shown that several regulatory factors such as cytokinesis, morphogenesis, growth factors and antisense mRNA are responsible for controlling HA snythase transcription (Suzuki et al.1995, Chao & Spicer 2005). Furthermore, hyaluronan synthesis is also controlled through translation regulations, since the latent pool of HA synthase in the cell interior upon insertion in cell membrane becomes activated (Rilla et al. 2005).

The metabolic pathway of hyaluronan formation is also invastigated in *S. zooepidemicus*. The streptococcal HA snythase appears to be found in operons encoding one or more enzymes involved in biosynthesis of activated sugars (Blank et al.2008). It was discovered that *has* operon in *S. zooepidemicus* encodes for five genes: hyaluronan synthase (*has*A), UDP-Glucose dehydrogenase (*has*B), UDP-glucose pyrophosphorylase (*has*C), a glmU paralog encoding for a dual function enzyme acetyltransferase and pyrophoshorylase (*has*D) and a pgi paralog encoding for phosphoglucosiomerase (*has*E). *has*B and *has*C are involved in UDP-GlcUA synthesis, whereas *has*D and *has*E are responsible for the synthesis of UDP-GlcNAc. However, other bacterial strains have *has* operons that apart from hyaluronan synthase, contain only *has*B and *has*C, suggesting that hyaluronan snythesis observed in *S.zooepidemicus* is related to the availability of UDP-sugar precursors (Chen et al. 2009).

Production of hyaluronic acid (HA)

Industrial production of hyaluronic acid is generally based on two main processes. One of them is the extraction of hyaluronan from animal tissue and the other is microbial fermentation using bacterial strains (Shiedlin et al. 2004). Recently, a new in vitro method of hyaluronan production using isolated HA synthase has been discovered and tested, but large scale production was not achieved yet (DeAngelis et al. 2003).

4a. Extraction from animal tissue

Method of extraction of hyaluronan from animal tissue was the first method to be used at the industrial scale. Hyaluronan from almost all tissues of vertebrates, including the umbilical cord, synovial fluid, vitreous body of the eye, pig skin, cartilage of sharks etc., was isolated and investigated (Ignatova & Gurov, 1990). Marine sources of H, such as fish vitreous humor, have also been investigated and reported (Amagai et al. 2009). However, it was reported that for large scale production of high weight hyaluronan the most reliable source are rooster combs, since it contain the highest concentration of hyaluronan ($7.5\ mg\ g^{-1}$) that has been discovered so far for animal tissues (Laurent & Fraser 1992 in Boeriu et al. 2013). Apart from rooster combs, sources such as human umbilical cord, the vitreous humor of cattle and bovine synovial fluid are also used.

Balazs (1979), reported development of an ultimate method for isolation and purification of hyaluronic acid from rooster combs and human umbilical cord that has proven to be efficient and reliable for pharmaceutical purposes. Furthermore, the new procedure set the basis of the industrial production of hyaluronan for medical applications.

Although the method of hyaluronan production by extraction from animal tissue is still an important technology for commerical products, some potential limitations should be taken into consideration. Extraction of highly pure, high molecular weight hyaluronan from animal tissues is difficult since it is in a biological materials present in a complex along with the other biopolymers including proteoglycans (O'Regan et al. 1994). In order to release hyaluronan from these complexes several methods have been tested and applied, such as the use of proteolytic enzymes (pepsin, pronase, trypsin), hyaluronan ion-pair percipitation, percipitation with organic

solvents, detergents, etc. (Schiraldi et al. 2010). Furthermore, ultrafiltration and chromatography are used to remove the degradation products and finally sterile filtration is used to remove all the microbial cells before the alcohol precipitation, drying and conditioning of the end product. However, the risk of contamination of animal hyaluronan with proteins, viruses and nucleic acids is still high despite extensive purification which is apart from being complex also expensive. It has been reported that hyaluronan isolates from human umbilical cord and bovine vitreous humor has a higher level of contamination compared to those from rooster comb and bacterial capsule isolates (Shiedlin et al. 2004). This limitation can be minimized by using healthy animal tissues and by conducting an extensive purification. Apart from contamination risk, another disadvantage related to this method is a degradation of hyaluronan caused by endogenous hyaluronidase activity in animal tissues which causes the break down of the polymer chain through enzymatic hydrolysis. Even though the extraction methods have been improved over the years, it still suffer from low yields due to the low concetration of hyaluronan in tissues. Nevertheless, available raw material at low costs and well-established technology has made an animal waste the most important source for the industrial manufacturing of hyaluronan for medical application.

4b. Poduction of hyaluronan through bacterial fermentation

As already mentioned hyaluronan is also present in the capsule of microbial pathogenes such as *Pasteurella multocida* and group A and C streptoccoci among which are human pathogen *Streptococcus pyogenes* and the animal pathogenes *Streptococcus equi* and *Streptococcus uberis*. Due to high risks of contamintion of animal derived hyaluronan with proteins that can cause undisered allergic reaction (Thonard et al. 1964 in Boeriu et al. 2013), in the last two decades a method of hyaluronan production through bacterial fermentation has become more preferable.

These microorganisms use hyaluronan to enclose their cells forming a disguise against the animal defense system and facilitating the adhesion and colonisation of bacterial cells (DeAngelis, 1999). Since the hyaluronan polymer derived from animal tissue and bacterial one are identical, the host immune defense is not triggered to repel the pathogenic bacteria contrary to the other bacteria with a different strucutre of capsule. Therefore, bacterial hyaluronan is not immunogenic and it is a great source of hyaluronan that can be used for medical purposes. The production of hyaluronan though bacterial fermentation is relatively simple process which results in high yields of hyaluronan with high molecular weight (1-4 Da) (Boeriu et al. 2013). Both advantage and disadvantage of the mentioned method is the ability to manipulate and adapt microbial cells, culture media and cultivation conditions in oder to produce larger amounts of high molecular weight hyaluronan with better quality. Although, it is relatively mature technology and nowadays it is more preferred with using either pathogenic streptococci or safe recombinant hosts, it is important to mention that this method as well rises some questions when it comes to the risks of contamination with bacterial endotoxins, nucleic acids and heavy metals.

Kendall et al. (1937) in Boeriu et al. (2013) reported first attempt to isolate hyaluronic acid from group A hemolytic streptococci which resulted in $60 - 140\, mg\, L^{-1}$ hyaluronan. Group C streptococci, described as non-human pathogenes with high hyaluronan productivity are frequently used instead of group A streptococci and the animal pathogenic bacterium *P.multocida*. The most frequent used strains are *S.equi* subsp. *equi* and *S.equi* subsp. *zooepidemicus*. During past years, many attempts have been made to increase the hyaluronan production by means of optimizing the extraction method, adapting the culture media and improving and selecting the strains with high hyaluronan productivity. These attempts resulted in

the increase of hyaluronan yields using batch fermentation from $300 - 400\, mg\, L^{-1}$ (Holmstrom & Ricica, 1967 in Kim et al. 1996) to $6 - 7\, g\, L^{-1}$ (Kim et al. 1996).

Streptococci strains for hyaluronan production generally use glucose as carbon soruce. However, some other carbon sources like starch, lactose, sucrose and dextrin which are available at lower costs can also be used (Zhang et al. 2006). Streptococcal fermentation to produce hyaluronan is influenced by several factors such as temperature, pH, medium composition, agitation, carbon resources, energy.

Hyaluronan biosynthesis in streptococci requires a large amount of energy and at the same time competes with bacterial cell growth for glucose. When unlimited amount of glucose is present, the highest bacterial growth was observed at optimal cultivation conditions, whilst the highest hyaluronan productivity and molecular weight was achieved at suboptimal growth conditions, since when cells are growing slowly, the carbon and other energy sources are available for other processes (Armstrong et al. 1997). On the other hand, when the amount of glucose decreases it leads to a decline in the hyaluronan productivity and molecular weight (Chong et al. 2005).

Another factor influencing the production of hyaluronan is culturing condition. Under aerobic fermentation conditions, streptococci change their metabolism from producing lactate into producing acetate, formate and ethanol. This results in increased levels of ATP and NADH oxidase which removes the excess levels of NADH in the presence of oxygen. These events are assumed to be related to the increase of hyaluronan production (Chong & Nielsen, 2003). Several studies have shown that aerobic fermentation conditions increase the production of hyauronan by 50% as well as molecular weight, while cell growth stays unaffected (Armstrong et al. 1997). Duan et al. (2009) have reported that the expression of HA synthase in *S.zooepidemicus* is nine times higher under aerobic conditions than under anaerobic conditions. Furtherore, in the presence of oxygen, enzymes involved in the production of UDP-GlcNAc (*has*D) are induced resulting in the further increase of ATP and acetyl-CoA that can be used in the hyaluronan production (Wu et al. 2009). However, the increase in hyaluronan yield under both aerobic and anaerobic fermentation conditions is achieved when the agitation is increased. This happens due to an enhanced mass tranfer induced by the reduced viscosity of the broth (Huang et al. 2006). In relation to that, Zhang et al. (2010) reported that the molecular weight of hyaluronan increases at moderate impeller speed due to enhanced mass transfer, but decreases at

high impeller speed due to degradation caused by oxygen species that are formed under aerobic conditions. They also suggest that this can be avoid by adding oxygen scavengers such as salicylic acid in the culture media.

In order to avoid risks of contamination with exotoxins from pathogenic streptococci stains, a method of hyaluronan production using nonpathogenic microorganisms is also technique that is widely used. For this purpose, nonpathogenic microorganisms are genetically modified into hyaluronan producers by introducing the HA synthase enzymes from either streptococci or *P.multocida*. Applying this protocol on microorganisms such as *Enterococcus faecalis*, *E.coli*, *Agrobacterium sp*., *Bacillus subtilis* and *L.lactis*, hyaluronan producing strains were obtained (DeAngelis et al. 1993, Mao & Chen 2007).

In order to improve the mentioned method and increase the hyaluronan production the hyaluronan producing strians with improved intracellular availability of sugar precursors were obtained by coexpression of the HA synthase *has*A gene derived from *S.equi* or *P.multocida* with *has*B homologue (UDP-glucose dehydrogenase) from *E.coli*. A recombinant E.coli strain produced $2\ g\ L^{-1}$ hyaluronan and the yield increased to $3.8\ g\ L^{-1}$ as the culture media was supplemented with glucosamine (Mao & Chen, 2007).

Similar strategy was applied when food-grade microorganisms were used. Genetically engineered strins of *Agrobacterium* sp. were able to produce $0.3\ g\ L^{-1}$, while the strains of recombinant *L.lactis* were able to produce up to $0.65\ g\ L^{-1}$ of hyaluronan (Mao & Chen, 2007).

Sloma et al. (2003) in Marcelli et al. (2010) developed a method of hyaluronan production by using nonpathogenic recombinant strains of *B.subtilis*. A major advantages of using the strains of *B.subtilis* are that this microorganism is cultivable at a large scale and it does not produce exo- and endotoxins. Findings have shown that recombinant strians of *B.subtilis* were able to produce up to $5\ g\ L^{-1}$ of hyaluronan with a molecular weight of $1 - 1.2\ million\ Da$, when cultivated on a minimal medium based on sucrose at pH 7 and 37 C° (Marcellin et al. 2010).

However, as an alternative to prokaryotic HA production, hyaluronan can be produced by infecting green algae cells of the genus Chlorella with a virus, although the reported yields were low, resulting in $0.5 - 1\ g\ L^{-1}$ (Graves et al. 1999).

Methods of determination of hyaluronic acid (HA)

The increasing interest in hyaluronic acid has led to the demand for development of a simple, fast and reliable method of hyaluronan determination in a wide range of hyaluronan-containing substances including fermentation media, down-stream flows, biological samples, pharmaceutical, veterinary and cosmetic products.

Elson-Morgan method and carbazole assay are one of the methods of HA quantification derived from colorimetric methods developed for specific monosaccharide detection, especially for N-acetylglucosamine and D-glucuronic acid (Morgan et al. 1934 in Pepeliaev et al. 2017). Before proceeding to quantification, decomposition of HA is essential due to its polymetric properties. The carbazole assay relays on the acidic hydrolysis of HA in 80 % sulfuric acid at 60 °C, while in the Elson – Morgan method the decomposition of HA is performed by hyrolysis in 2 N HCl at 100 °C. In case of Elson – Morgan method hydrolysis can be replaced by enzymatic digestion with hyaluronidase from Streptomyces hyalurolyticus. However, the activity of the enzyme is unsatisfactory, as the full HA digestion takes at least 20 hours (Greiling, 1965 in Pepeliaev et al. 2017). Since carbazole assay was primarly developed for galacturonic acid quantification, the specificity of the method is questionable as the extinction coefficient of galacturonic acid is $11,200\ mol^{-1}$, while that of glucuronic acid is only $1750\ mol^{-1}$. Furthermore, in the analyzed sample other glycosaminoglycans that are present also undergo acidic hydrolysis and participate in a color reaction as well (Pepeliaev et al. 2017). On the other hand, Elson – Morgan method is more specific because it is aimed for N-acetylhexosamine detection. It is also more sensitive than carbazole assay with the extinction coefficient of the colored product of $18{,}000 - 21{,}000\ mol^{-1}$ (Greiling, 1965 in Pepeliaev et al. 2017). Still, one of the limitation is that it interferes with proteins, which contribute to sample turbidity. Furthermore, it is more dangerous and more demanding, since it uses glacial acetic acid and requires strict temperature and time regulation.

Another method for HA quantification worth mentioning is thiobarbituric assay. The essence of this method is the reaction between thiobarbituric acid and formyl pyruvic acid which is the product of the oxidation of 4,5-unsaturated oligosaccharides by periodic acid. On the other hand, unsaturated oligosaccharides are products of HA digestion by the lyase from S.hyalurolyticus (Jourdian et al. 1979 in Pepeliaev et al. 2017). However this method as well has some advantages and limitations. The advantages are high specificity, applicability and sensitivity comparable to that of the Elson – Morgan method, absence of strict time control and easier manipulation. As limitations, a very slow analysis and the usage of arsenic toxic compounds are considered.

All the methods discussed above despite being complex offers accuracy, while the following group of methods offer simplicity and speed. One of these methods is based on the discovery, that aliphatic ammonium salts having at least one long paraffin chain (cetyltrimethylammonium bromide (CTAB), cetylpyridinium chloride (CPC)) percipitate polyanionic polysaccharides including HA. Under certain conditions a colloidal solution is formed and in that way a fondation of a simple and fast turbidimetric method of HA determination was made (Oueslati et al. 2014). However, the problem with percipitation using aliphatic ammonium salts is that it also percipitates sulfated glycosaminoglycans and strach. Furthermore, the CTAB or CPC percipitation is used only for rough estimations of HA in well-defined samples, since the percipitate formation depends on the ionic composition and strength (Pepeliaev et al.2017).

Since the concentraion of HA in most evaluated substances, such as serums, is tens of nanograms per milliliter, it was necessary to develop a method for hyauronan quantification in nanogram scale. One such method that fulfill these requirments is enyzme-linked immunosorbent assay (ELISA). It is a sensitive assay using a microtiter plate coated with the HA binding protein (HABP) and horse radish peroxidase (HRP) conjugated HABP for detection. HRP conjugated HABP binds to HA and is followed by a substrate reaction. The color product is analyzed denepding on the amount of HA present in the samples (Haserodt et al. 2011). Despite being very sensitive and specific, ELISA assay has some limitations such as associated high costs, long analysis that requires special skills and the need of microplate reader, which are making this method short in use.

Recent studies conducted by Pepeliaev et al. (2017) have reported a new colorimetric enzyme-coupled assay that is based firstly on the digestion of HA by hyaluronan lyase from S.pneumonia (SpHyl) and secondly on the reaction between 4,5-unsaturated dissacharides with 3-methyl-2-benothiazolinonehydrazone (MBTH). The result is highly colored product with A_{max} at 620 nm (blue color). For the detection a spectrophotometry is used, which is considered to be a highly accurate instrument due to its sensitivity. Enzyme-coupled assay is considered to be relatively simple process with low costs of analysis. However, in order to maintain sensitivity and keep low costs of analysis, the assay should be based on a specific HA derivatization that yields a highly colored product absorbing at longer wavelengths so that any backgound signals are eliminated. Due to its accuracy, simpicity and low costs of analysis I have chosen to test the efficiency of SpHyl-MBTH method when appling it to small volume samples of fermentation culture of *Streptococcus equii* subsp. *zooepidemicus*. Furthermore, by comparing the yield of HA obtained using this method to the yield of HA obtained using standard precipitation method with isopropyl alcohol the reliability of SpHyl-MBTH method will be tested.

Materials and methods

Unless otherwise specified, all chemicals were reagent grade or higher obtained from Sigma-Aldrich (St.Louis, MO) or Fisher Scientific (Pittsburgh, PA) and used without further purification. The hyaluronic acid in different molecular weights, fermentation culre of bacteria Streptococcus equii sub.zooepidemicus and processive hyaluronan lyase from Streptococcus pneumoniae (SpHyl) are from Contipro A.S. Absorbance readings were performed on a Shimadzu UV-1800 spectrophotometer or a Rayleigh VIS723G.

SpHyl activity assay

The effect of different concentrations of sodium dodecyl sulfate (SDS) on the activity of SpHyl enzyme was tested. Different concentrations (0%, 0.2%, 0.1%, 0.05%, 0.015%) of SDS were added in microtubes containing 3 $g(l)$ HA dissolved in water. Immediatelly after adding 1 μl of SpHyl, the solution was mixed and the activity of enzyme (absorbance) was measured using Shimadzu UV-1800 spectrophotometer at 232 nm. The slope of the linear part of kinetic curve corresponded to enzyme activity. The obtained results were presented using graphs.

Isopropy alcohol precipitation assay

- In analyzed samples (10 samples were analyzed, one sample was taken each day for 10 days) optical density (OD) to determine the growth of the culture and residual sucrose were measured.
- OD (optical density – OD_{640}) – at 640 nm using spectrophotometer Helios Epsilon (Thermo Fisher). As a blank destilled water was used. Before mesuring OD, analyzed sample was diluted using destilled water (1:9).
- After measuring all the parameters, chelaton III + SDS solution (10 ml/l) was added in order to release HA from bacteria capsule, mixed and left for 30 min
- 250 g of sample was diluted with H_2O - 1:3 (250 g of sample + 750 ml H_2O)
- 15 g NaCl is added to reduce viscosity and filtrated using filter plate, HS800 (PALL) is used
- pH of the sample is optimized at 5 using concetrated acetic acid (CH_3COOH)

- firstly 1500 ml of 86% isopropyl alcohol (RIPA2) is added and then 500 ml of 100% isopropyl alcohol is added while mixing for 20 min using shaft mixer
- the precipitated product is left to sediment
- using Ultra-Turrax the settled product was washed 2x in 300 ml of 86% isopropyl alcohol (RIPA2) and 2x in 300 ml of 100% isopropyl alcohol without turraxing in order to remove any residuals of endotoxins left at filtration stage
- The product is left to dry over night (15 – 16 h) at 60 °C
- Dry matter is estimated using drying scales HR73 (Metteler Toledo)
- Yield of hyauronic acid is expressed as HA (g/l)
- Procedure is repeated 3x for each sample and the average values were calculated.

SpHyl-MBTH assay

Preparation of reagents (40 ml)

Reagent A: 100 mM acetate buffer with pH 6 and 160 mM Na_2SO_4

- 908.8 mg Na_2SO_4
- Dissolve in 40 ml of 0.1M acetate buffer with 6 pH

0.1M acetate buffer (100 ml):

- 100 ml 0.1M $CH_3COONA*3H_2O$ – 1.36 g dissolve in 100 ml H_2O
- 10 ml 0.1M CH_3COOH (density: 1.05 g/cm^3) – 57 μl CH_3COOH add up to 10 ml H_2O

➔ Mix 91 ml of $CH_3COONa*3H_2O$ solution and 9 ml of CH_3COOH solution

➔ optimize the pH at 6 using NaOH

Reagent C: 1 M NaOH

- 1600 mg NaOH

Reagent D: 0.2% MBTH and 10 mM DTT

- 80 mg MBTH
- 400 μl 1M DTT

Reagent E: 50 mM $FeCl_3$, 1 M HCl, 1% sulfamic acid, 0.2 M citric acid

- 324 mg $FeCl_3$
- 3880 μl 32% HCl (density: 1.16 g/cm^3)
- 400 mg sulfamic acid
- 1680 mg citric acid monohydrate

Process of HA determination

- Three microtubes were filled with 970 μl of analyzed sample (the same samples that were used in precipitation method) and marked.
- In first microtube 30 μl of 10% SDS was added, in second microtube 30 μl of chelaton III + SDS and in third one 30 μl of H_2O were added and vortexed for 5 s.
- Samples were diluted 10x using water (900 μl of water + 100 μl of sample) and vortexed for 5 s.
- Sediment was removed by centrifugation done at 14 000 G for 10 min.
- The remaining supernatant was transfered to the new microtubes.
- 200 μl of sample was mixed with 200 μl of reagent A (100 mM acetate buffer with pH 6 + 160 mM Na_2SO_4) and placed into microtube marked as "sample" and the same amount into microtube marked as "background"
- 1 μl of SpHyl (2.000 U/mg, 2 mg/ml) was added to the microtube marked as "sample" and incubated for 10 min at 37 °C.
- 200 μl of reagent C (1 M NaOH) and 400 μl of solution D (0.2% MBTH and 10 mM DTT) were added to both "sample" and "background", vortexed and then incubated at 75 °C for 30 min.
- 500 μl of reagent E (50 mM $FeCl_3$, 1 M HCl, 1% sulfamic acid, 0.2 M citric acid) was added to "sample" and "background". It is left to cool to the laboratory temperature for 5 – 10 min.
- Absorbance at 654 nm was measured using spectrofotometry. The product is diluted if the optical absorbance was exceeding 1.
- The same procedure was repeated 3x for each sample.

- Hyaluronan concentration was calculated in mg/ml, or g/l:

$$\mathrm{C(HA)} = \mathrm{k} * (\mathrm{As} - \mathrm{Ab})$$

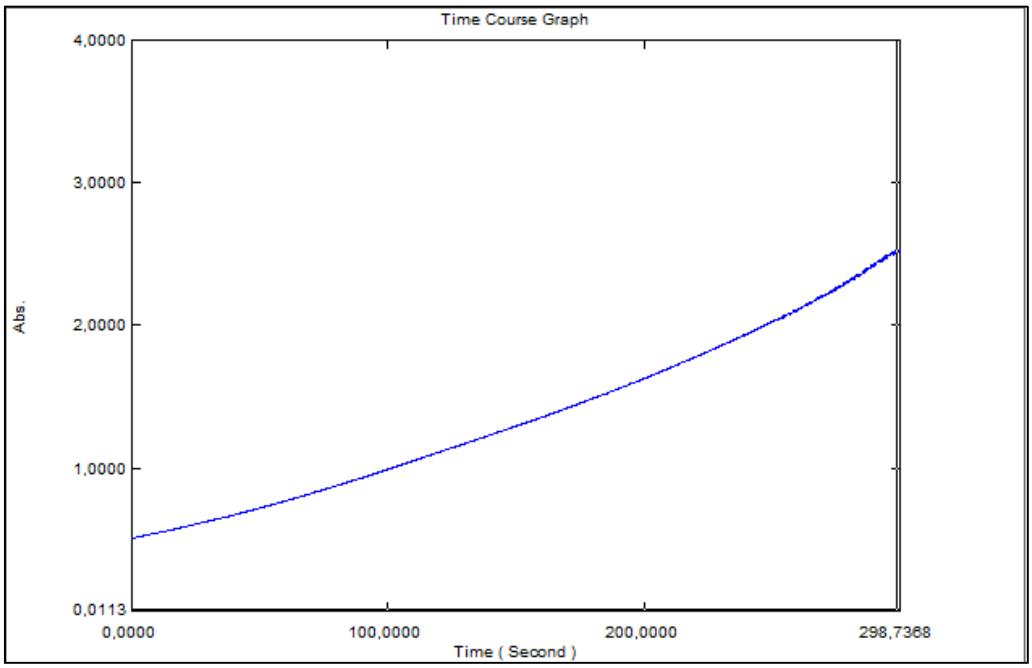
  Where A_s and Ab are absorbances of "sample" and "background" respectively taking into account dilution after color development; k is a coefficient that should be calculated for each measurment of HA concentration
- Coefficient k is calculated on the basis of five calibration solutions of HA with different concentrations (1 g/l, 2 g/l, 3 g/l, 4 g/l, 5 g/l) which are used as a control trial – "sample" and "background" for each concetration is calculated using described method above
- Background values were substracted from sample values and the obtained results were presented using a calibration curve and the value of coefficient k is calculated.
- Since the whole procedure was repeated 3x for each sample the average amount of HA concentration for each condition (H_2O, 10% SDS and chelaton III + SDS) was calculated and presented using tables and graphs.
- The standard deviation from the mean is calculated and presented on the graphs.

Comparison of the yield of HA obtained using isopropyl precipitaton method to the yield of HA obtained using SpHyl-MBTH method

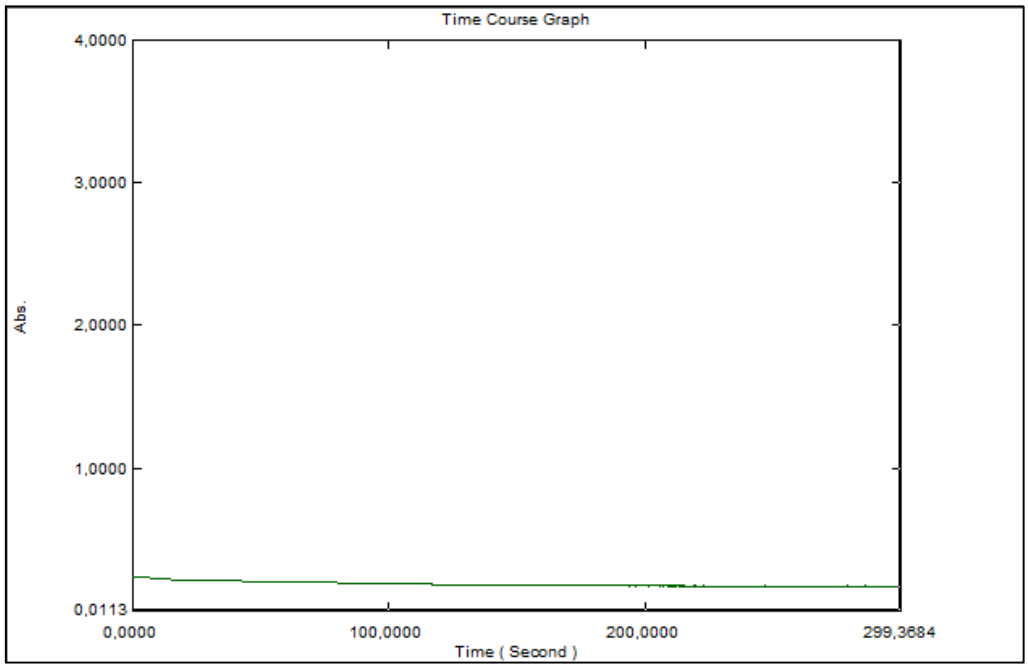
The yield of HA obtained by precipitation method with isopropyl alcohol was compared to the yield of HA obtained using SpHyl-MBTH method. Since at the begining of isoproyl precipitation method, chelaton III + SDS solution was used to release hyaluronic aid from bacterial capsule the results were compared to those values obtained by SpHyl-MBTH method where samples were also treated with chelaton III + SDS solution. The results were presented using tables and graphs.

Results

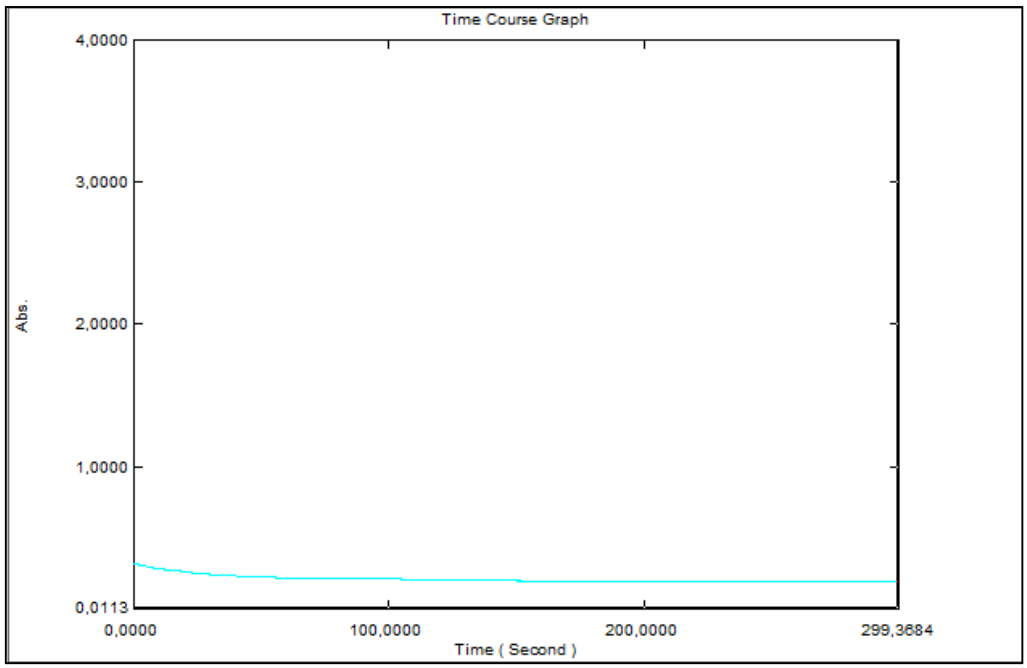
The following graphs are showing the effect of different concentrations of SDS (0%, 0.2%, 0.1%, 0.05% and 0.015%) on the activity of SpHyl enyzme. The absorbance presented with y-axis was measured at 232 nm. Each measure was repeated 3 times. The x-axis is showing the time presented in seconds.



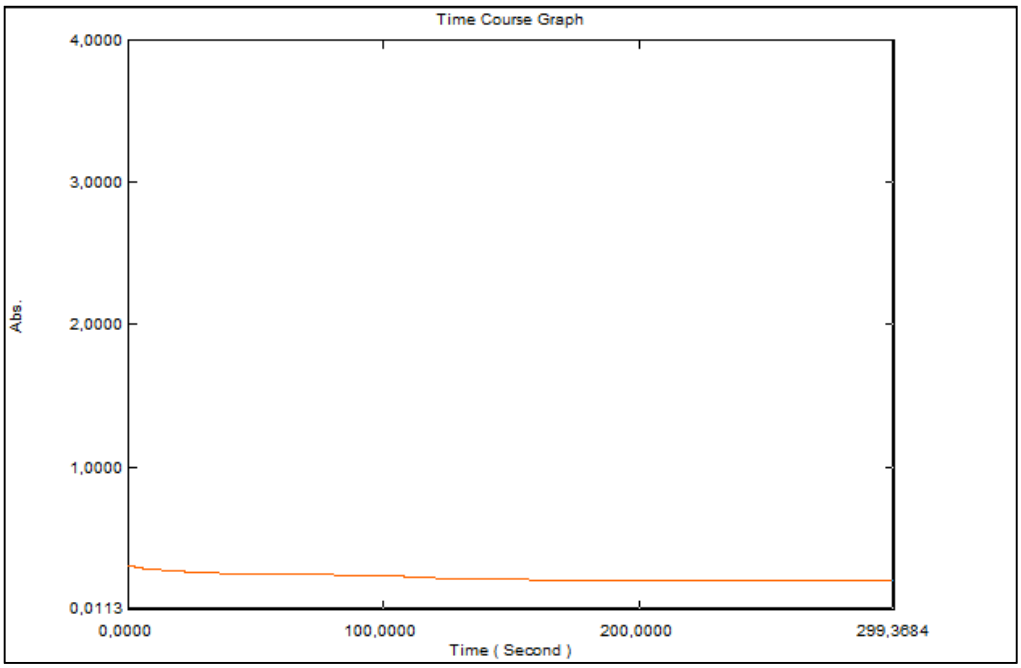
Graph 1: The effect of 0% SDS on the activity of SpHyl enzyme



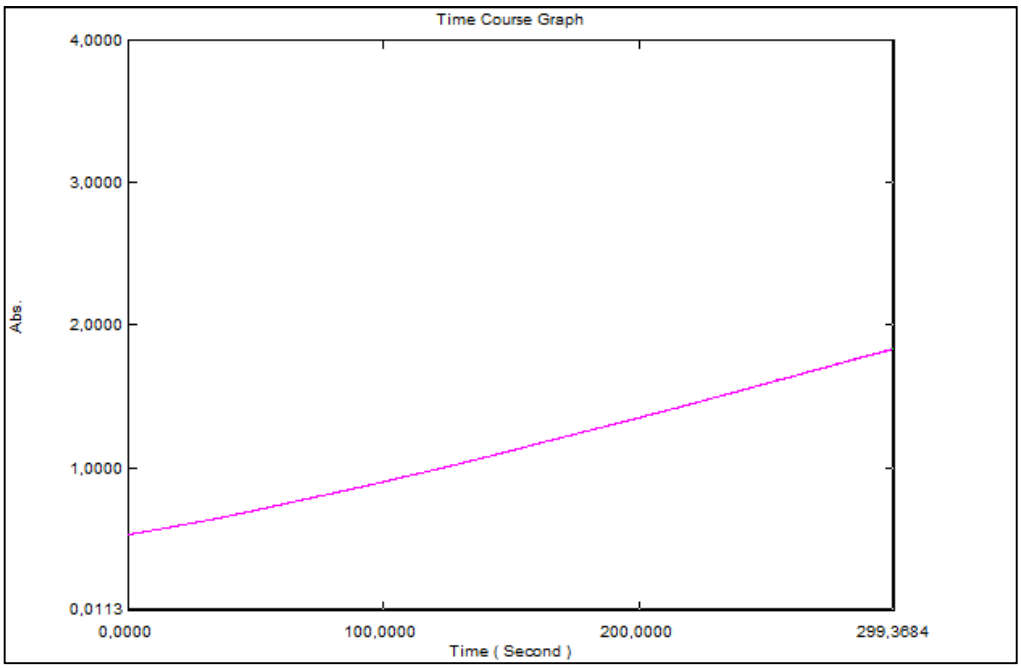
Graph 2: The effect of 0.2% SDS on the activity of SpHyl enzyme



Graph 3: The effect of 0.1% SDS on the activity of SpHyl enzyme



Graph 4: The effect of 0.05% SDS on the activity of SpHyl enzyme



Graph 5: The effect of 0.015% SDS on the activity of SpHyl enzyme

The following table (Table 1) is showing the average values of the yield of HA (g/l) obtained by applying precipitation method with isopropyl alcohol for each sample. Final average value of the yield of HA (g/l) obtained using isopropyl precipitation method on the basis of 10 examined samples is shown. Calculated optical density (OD) and the name of reactor used for cultivation are also presented.

Table 1: The yield of HA (g/l) obtained using isopropyl precipitation method

Sample number	Reactor	OD	HA (g/l)
1	BR15	0.141	3.98
2	BR15	0.409	3.31
3	BR15	0.481	3.65
4	BR15	0.412	3.74
5	BR15	0.368	3.90
6	BR15	0.410	3.10
7	BR15	0.402	3.61
8	BR15	0.315	4.07
9	BR15	0.470	3.40
10	BR15	0.405	3.58
Average HA (g/l)			3.63

Table 2 is showing the yield of HA (g/l) obtained using SpHyl-MBTH method where H_2O is added to samples. Table 3 and Table 4 represents the yield of HA (g/l) obtained using SpHyl-MBTH method where SDS (Table 3) and chelaton III + SDS (Table 4) are added to samples. Three trials were conducted for each sample , average value and standard deviation (CV) were calculated.

Table 2: The yield of HA (g/l) obtained by SpHyl-MBTH method – sample + H_2O

Sample number	sample + H_2O HA $[g/l]$			Average HA $[g/l]$	CV ($p \leq 0.5$)
	Trial 1	Trial 2	Trial 3		
1	2.39	2.45	2.84	2.56	0.24
2	1.87	2.77	2.56	2.40	0.46
3	2.67	3.00	2.98	2.88	0.18
4	2.66	2.4	2.85	2.64	0.22
5	3.00	2.54	2.00	2.51	0.49
6	2.11	2.03	1.96	2.03	0.07
7	2.63	2.97	2.60	2.73	0.21
8	2.63	2.47	2.77	2.63	0.14
9	2.55	2.77	3.02	2.78	0.23
10	3.02	2.88	2.75	2.88	0.13

Table 3: The yield of HA (g/l) obtained by SpHyl-MBTH method – sample + SDS

Sample number	sample + SDS HA $[g/l]$			Average HA $[g/l]$	CV ($p \leq 0.5$)
	Trial 1	Trial 2	Trial 3		
1	3.21	3.50	2.99	3.23	0.25
2	3.02	3.55	3.41	3.33	0.27
3	2.81	3.14	2.75	2.90	0.20
4	2.78	3.56	3.63	3.32	0.46
5	3.20	2.51	3.45	3.05	0.48
6	3.45	3.77	3.46	3.56	0.18
7	3.45	2.99	3.37	3.27	0.24
8	3.98	3.12	3.55	3.55	0.43
9	3.66	3.93	3.00	3.53	0.47
10	3.45	2.99	3.44	3.29	0.26

Table 4: The yield of HA (g/l) obtained by SpHyl-MBTH method – sample + (chelaton III + SDS)

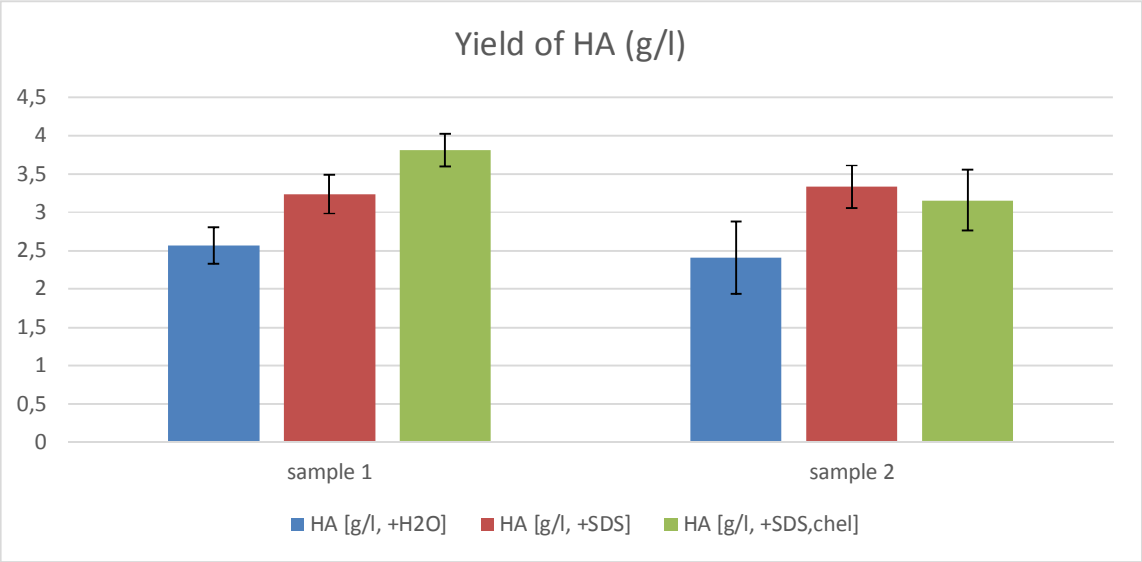
Sample number	sample + (chelaton III +SDS) HA $[g/l]$			Average HA $[g/l]$	CV ($p \leq 0.5$)
	Trial 1	Trial 2	Trial 3		
1	3.57	4.00	3.85	3.81	0.21
2	3.12	3.57	2.77	3.15	0.39
3	3.61	3.99	3.23	3.61	0.38
4	3.55	3.08	3.44	3.36	0.24
5	4.11	3.48	3.72	3.77	0.31
6	3.60	3.00	3.39	3.33	0.30
7	3.16	3.92	3.56	3.55	0.37
8	3.42	4.00	3.36	3.59	0.35
9	3.00	3.21	3.36	3.19	0.17
10	3.62	3.30	3.00	3.30	0.30

Table 5 represents the yield of HA (g/l) (averages) obtained by applying the SpHyl-MBTH method on samples in which H_2O, SDS and chelaton III + SDS were added. Final average value of the yield of HA (g/l) obtained for each condition on the basis of 10 samples is also presented.

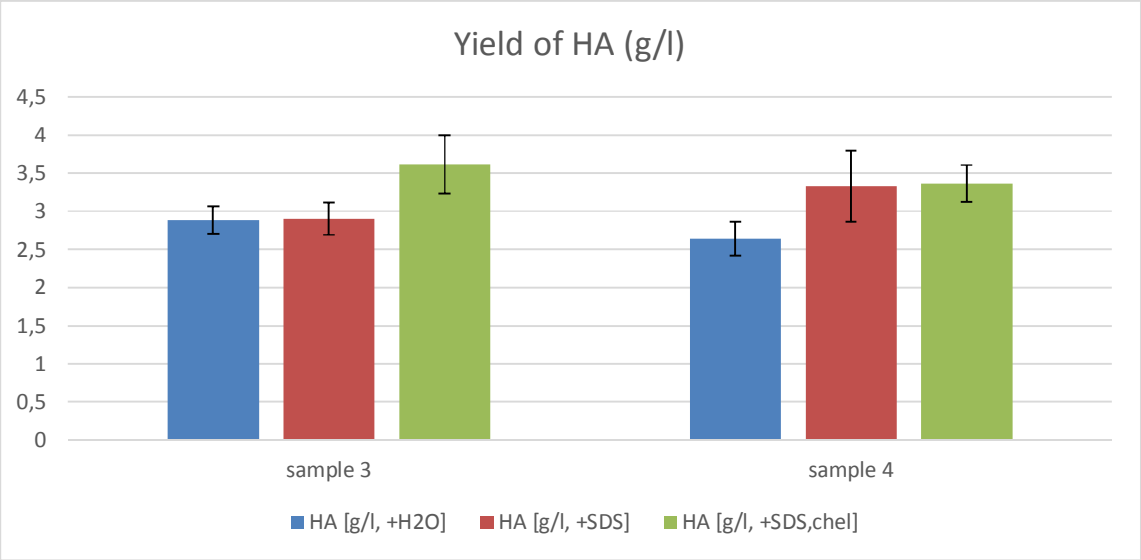
Table 5: The yield of HA (g/l) obtained by SpHyl-MBTH method – sample + H_2O, sample + SDS, sample + (chelaton III + SDS)

Sample number	Sample + H_2O HA [g/l]	Sample + SDS HA [g/l]	Sample + (chelaton III + SDS) HA [g/l]
1	2.56	3.23	3.81
2	2.40	3.33	3.15
3	2.88	2.90	3.61
4	2.64	3.32	3.36
5	2.51	3.05	3.77
6	2.03	3.56	3.33
7	2.73	3.27	3.55
8	2.63	3.55	3.59
9	2.78	3.53	3.19
10	2.88	3.29	3.30
Average HA [g/l]	2.60	3.30	3.46

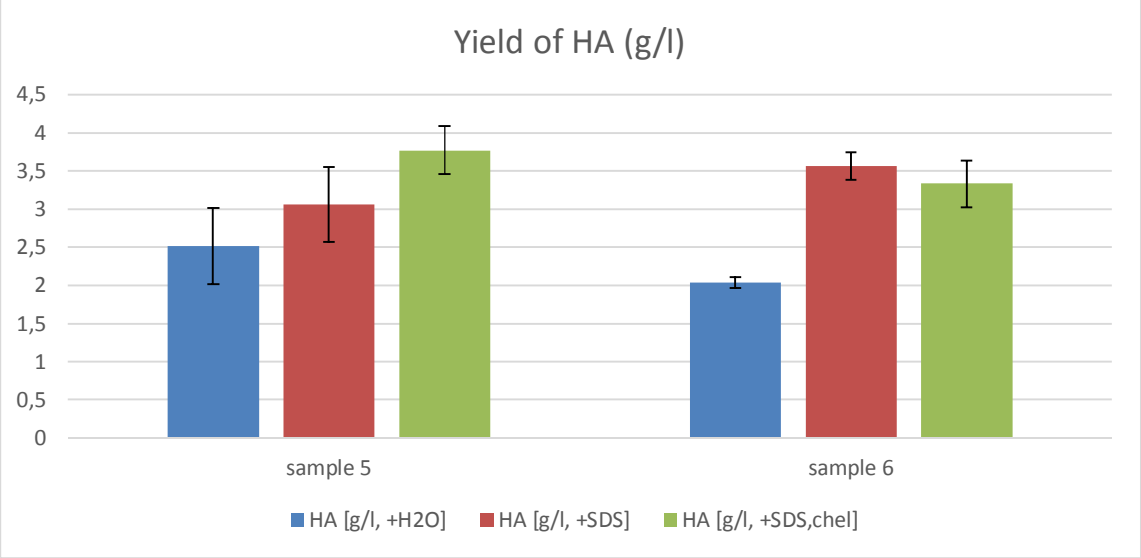
The following graphs (Graph 6, Graph 7, Graph 8, Graph 9, Graph 10) are showing the yield of HA (g/l) obtained by SpHyl-MBTH after H_2O, SDS and chelaton III + SDS solution were added to samples. Error bars (statistical significance) were included in graphs. The graph 11 is showing the average yield of HA (g/l) calculated on the basis of 10 samples for each condition (H_2O, SDS, chelaton III + SDS solution).



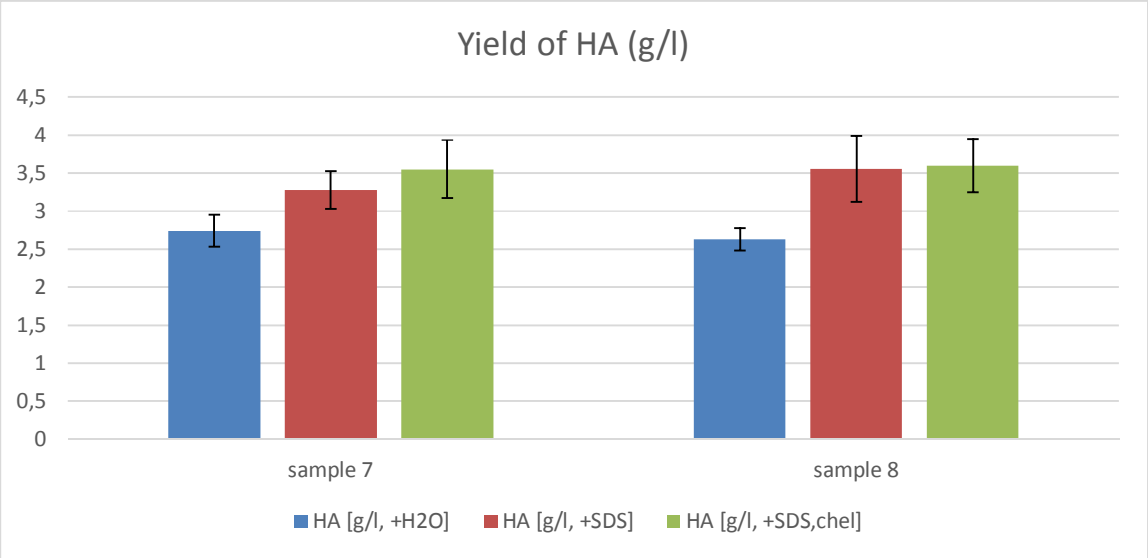
Graph 6: The yield of HA (g/l) obtained by SpHyl-MBTH (sample 1, sample 2)



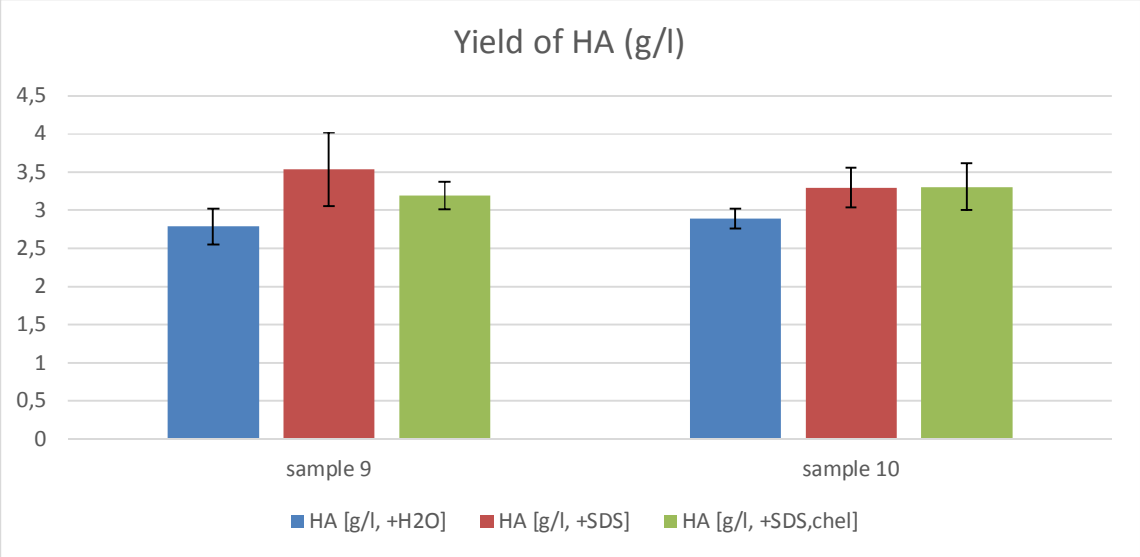
Graph 7: The yield of HA (g/l) obtained by SpHyl-MBTH (sample 3, sample 4)



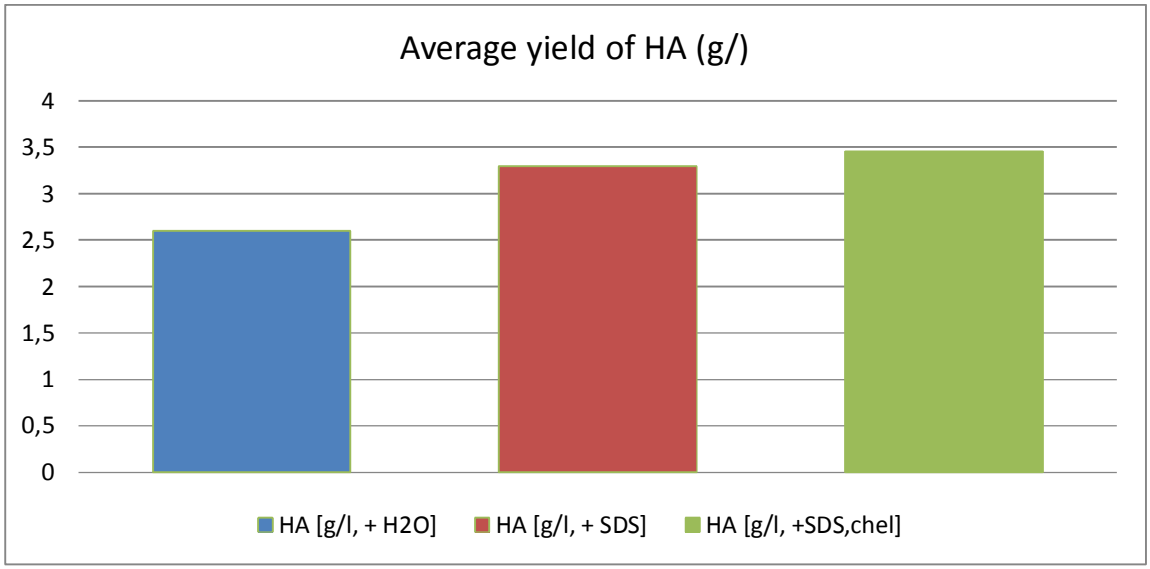
Graph 8: The yield of HA (g/l) obtained by SpHyl-MBTH (sample 5, sample 6)



Graph 9: The yield of HA (g/l) obtained by SpHyl-MBTH (sample 7, sample 8)



Graph 10: The yield of HA (g/l) obtained by SpHyl-MBTH (sample 9, sample 10)



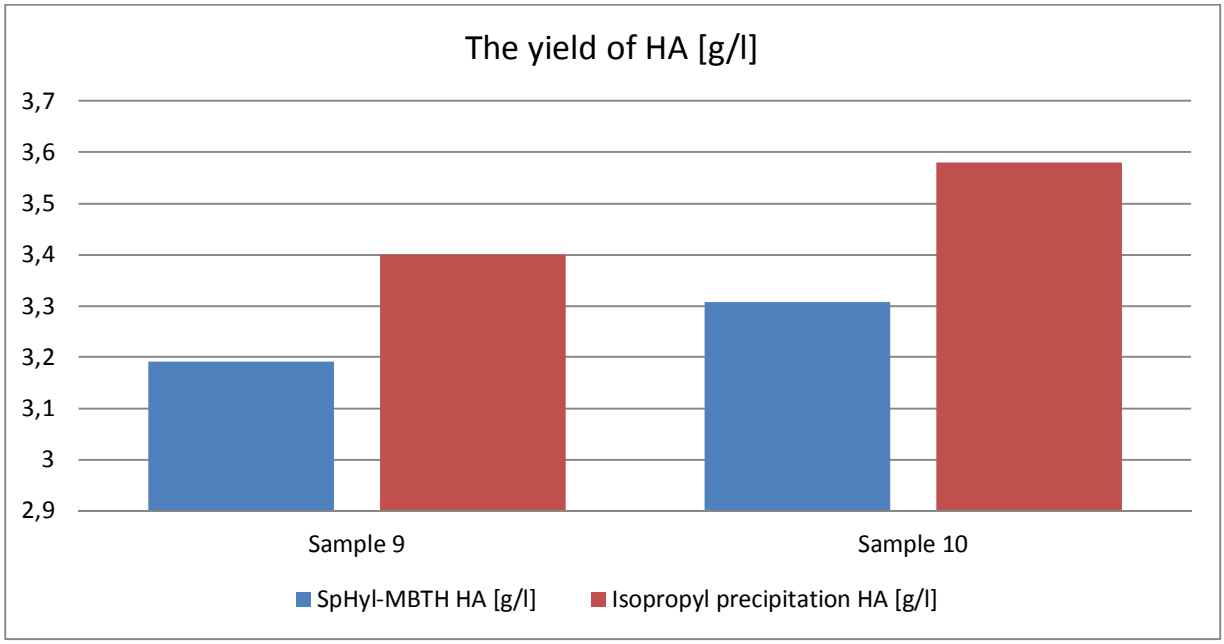
Graph 11: The average yield of HA (g/l) obtained by SpHyl-MBTH

The following table (Table 6) is showing the yield of HA $[g/l]$ obtained by SpHyl-MBTH method using small volumes of fermentation culture (values of samples treated with chelaton III + SDS solution) and the yield of HA $[g/l]$ obtained by applying the isopropyl precipitation method where large volumes of fermentation culture were used. The average values of the yield of HA (g/l) calculated on the basis of 10 samples for both methods are also presented.

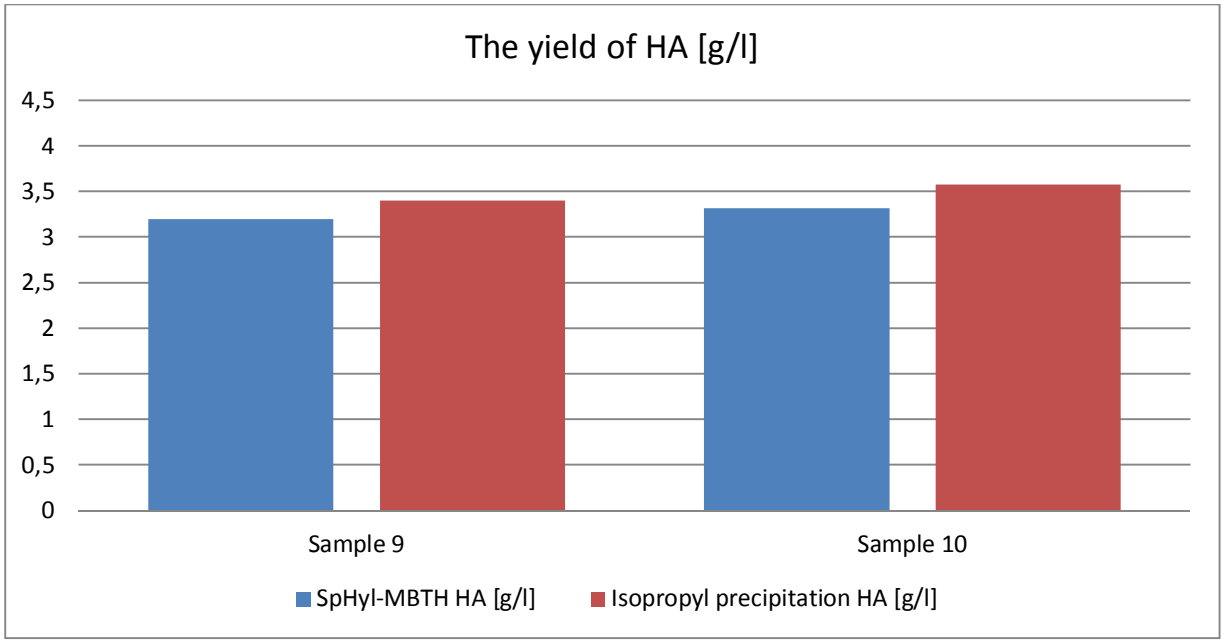
Table 6: The yield of HA $[g/l]$ obtained by SpHyl-MBTH method and precipitation method using isopropyl alcohol

Sample number	SpHyl-MBTH HA $[g/l]$	Isopropanol precipitation HA $[g/l]$
1	3.81	3.98
2	3.15	3.31
3	3.61	3.65
4	3.36	3.74
5	3.77	3.90
6	3.33	3.10
7	3.55	3.61
8	3.59	4.07
9	3.19	3.40
10	3.30	3.58
Average HA $[g/l]$	3.46	3.63

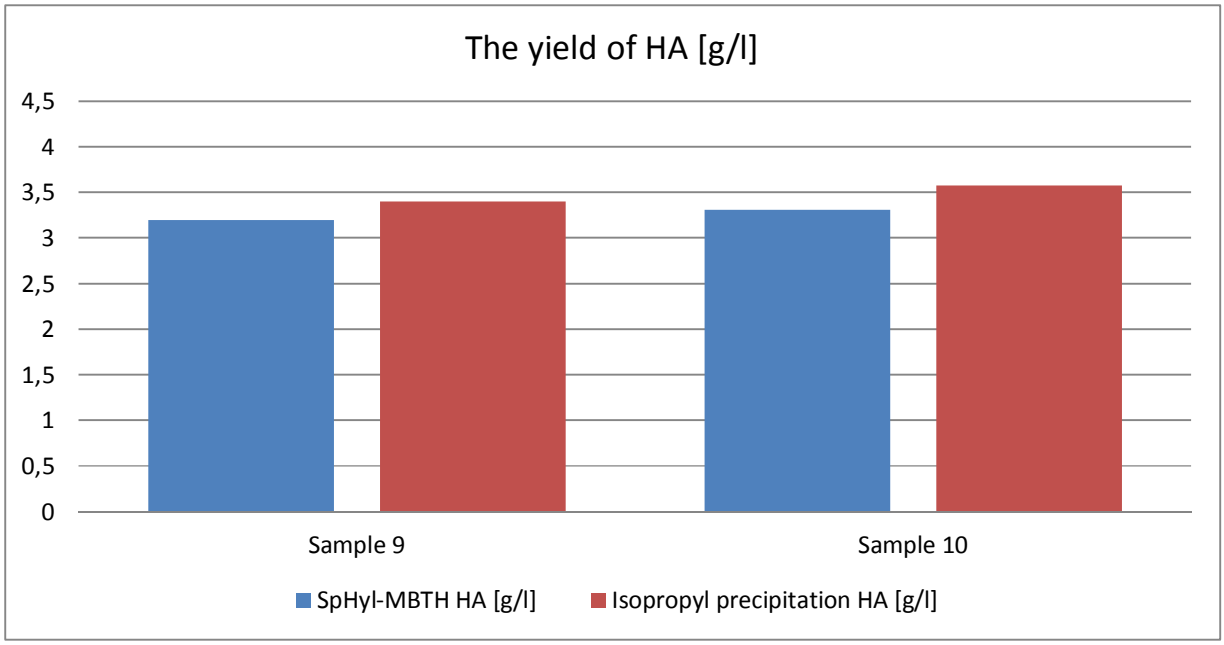
The following graphs (Graph 12, Graph 13, Graph 14, Graph 15 and Graph 16) are showing the obtained yield values of HA (g/l) using SpHyl-MBTH method and Isopropyl precipitation method in 10 samples. The graph 17 shows the average yield of HA (g/l) obtained on the basis of 10 samples.



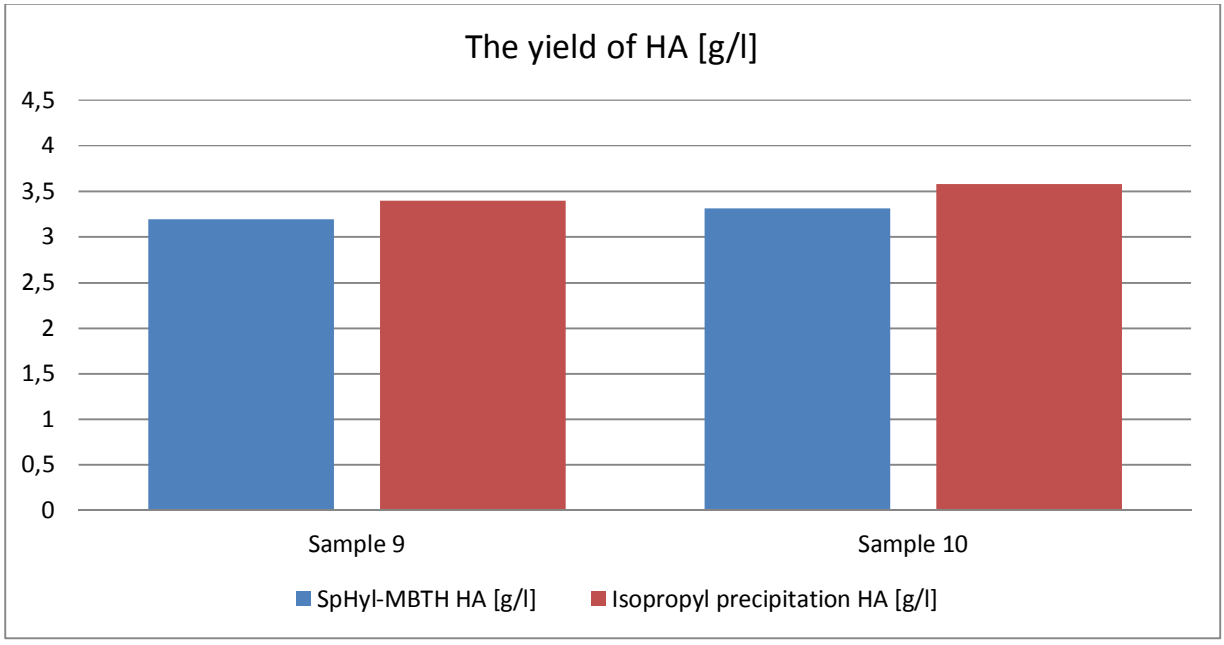
Graph 12: The yield of HA $[g/l]$ obtained by SpHyl-MBTH and Isopropyl precipitation (sample 1, sample 2)



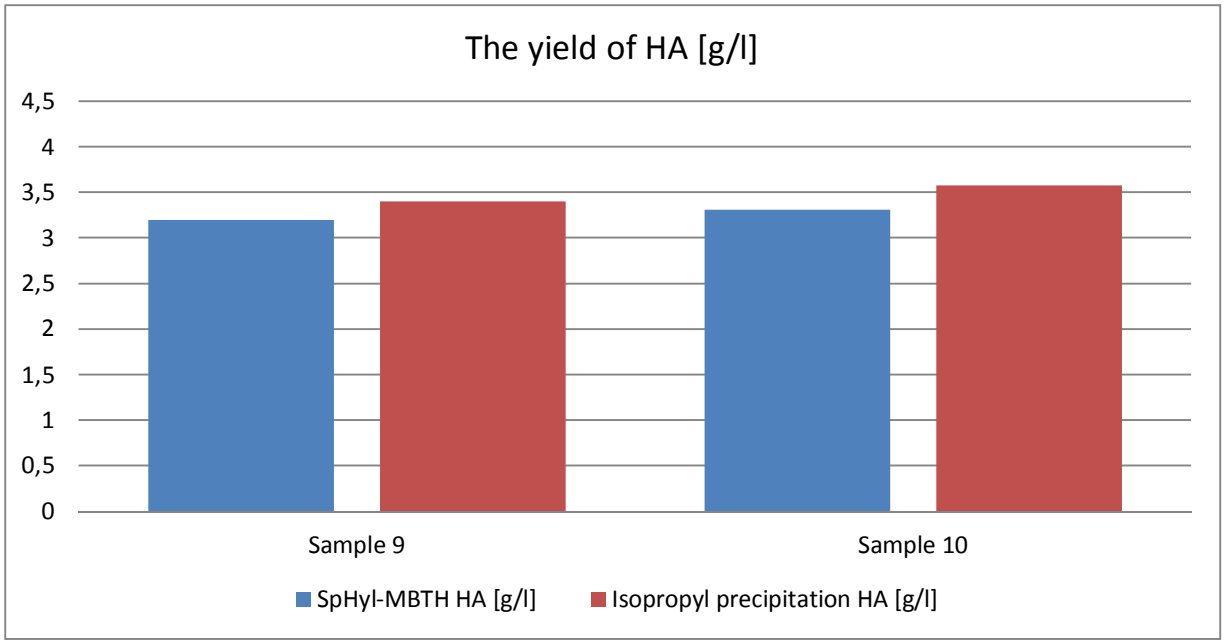
Graph 13: The yield of HA $[g/l]$ obtained by SpHyl-MBTH and Isopropyl percipitation (sample 3, sample 4)



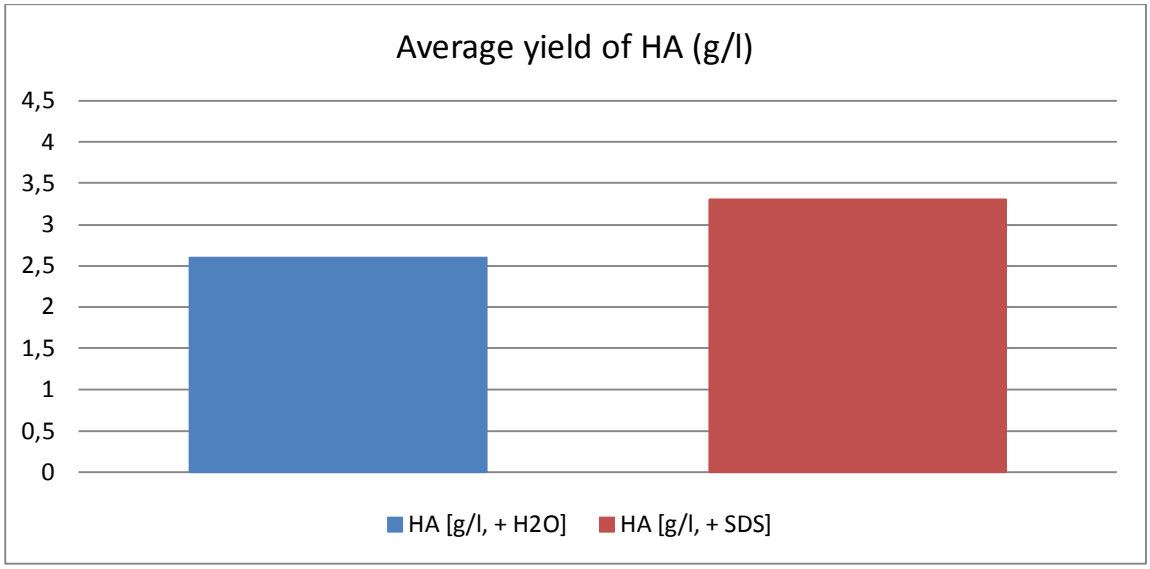
Graph 14: The yield of HA $[g/l]$ obtained by SpHyl-MBTH and Isopropyl percipitation (sample 5, sample 6)



Graph 15: The yield of HA $[g/l]$ obtained by SpHyl-MBTH and Isopropyl percipitation (sample 7, sample 8)



Graph 16: The yield of HA $[g/l]$ obtained by SpHyl-MBTH and Isopropyl percipitation (sample 9, sample 10)



Graph 17: The average yield of HA $[g/l]$ obtained by SpHyl-MBTH and Isopropyl precipitation

Discussion

As it is already been mentioned, extraction of HA from animal tissues such as rooster combs and bovine vitreous humor is a method that was firstly used for large scale production in industrial manufacturing of HA (Ignatova & Gurov, 1990). However due to difficulties to isolate high molecular weight HA from these sources since it forms complex with other proteoglycans and due to high costs of complex purification procedure it is believed that this method does not suit the best the mass production of HA. Furthermore, the risks of contamination and possibility of pathogenic material derived from animal tissue to retain in product are of a great concern for this method to be the best solution for the production of HA used for medical applications. However, an alternative that reflects in using microbial pathogens such as group A and C streptococci which naturally produce a capsule containing HA has shown to be effective. Obtaining HA from bacterial strains through microbial fermentation has resulted in high yields of HA having high molecular weight with simple purification methods at low costs. Furthermore, low risks of contamination have made this method more suitable for medical use.

Since HA used in cosmetic industry and for medical applications has to be highly pure with preferable high molecular weight, nowadays studies have been focusing on testing various methods of purification and isolation of hyaluronic acid produced by microorganisms. Since the main goal of my research was to optimize the method for determination of HA in small volumes of the fermentation culture, in order to test its reliability and compare yield values of HA obtained I have decided to preform also a method of HA isolation and quantification that works with large volumes of fermentation culture and is used for mass production. The method of purification and isolation of HA used for my research and generally used within Contipro A.S. for large scale production is precipitation method using isopropyl alcohol which works with large volume of fermentation culture. Hyaluronic acid from ten samples of fermentation culture of *Streptococcus equi* subsp. *zooepidemicus* has been isolated and quantified. The growth of the culture was determined by measuring optical density (OD) at 640 nm using spectrophotometer Helios Epsilon (Thermo Fisher). The values of OD for each sample are presented in Table 1. Before precipitation has taken place, the separation of hyaluronic acid from bacterial capsule was done using chelaton III + SDS (sodium dodecyl sulfate) solution. Overall yield of HA obtained using isopropyl precipitation method is 3.63 HA $[g/l]$.

However, it is important to say that since each sample was collected from a reactor on a different day hence prepared and cultivated under different conditions, different values of the yield of HA has been obtained for each sample. The results are presented in Table 1 along with the average concentration value of HA obtained. Although, the process of separation of HA from the complexes with other polysaccharides and proteins is usually achieved by using enzymes, organic solvents and detergents which was the case in my research, it is argued that these methods are related to higher production costs. Furthermore, it is believed that the method is sensitive since it becomes difficult to completely remove proteins, exothermic material, nucleic acids etc. Reddy & Karunakaran (2013) have conducted a research in which they have optimized cost-effective purification process for obtaining highly pure HA. The optimized purification process has included trichloroacetic acid (0.1%) and activated charcoal treatment (1-2%) followed by centrifugation. The procedure resulted in a recovery of 72.2 % of clinical grade HA with molecular weight of 2.5×10^6 Da. Furthermore, Warren & Gray (1959) developed a semisynthetic medium for the cultivation of group A streptococci. Their method for rapid isolation of partially purified HA consisted of acetone, acetate and ethanolic precipitation resulting in yields of 250 mg per liter of culture medium. As a suggestion for further studies, in order to make results more convinient an additional measurment of molecular weight by measuring intrinsic viscosity $[\eta]$ can be conducted. Furthermore, it is importat to emphisize that isolation and purification of HA using precipitation method with isopropyl alcohol is a process that should be done with high precision if product is to be used for cosmetic and medical application as it is the case with HA produced by Contipro A.S. Therefore, for production needs an additional steps of purification should be done, which I have not done since the results of the yield of HA obtained by using this method in my research were only used for comparison and research purposes.

Precipitation method using isopropyl alcohol that is used in Contipro A.S. for the production of hyaluronic acid works on large volumes of fermentation culture and is used for large scale production. However, due to high costs and low capacity of possible parallel cultivation it is not suitable for research purposes. The advantage of using microbioreactors and working with small volumes of fermentation culture is higher capacity of possible parallel cultivations while using small volumes of sample along with the lower costs of analysis.

Pepeliaev et al. (2017) have proposed a new colorimetric enzyme-coupled method that is suitable for work with small volumes of fermentation culture and which enables detection of HA in hyaluronic acid-containing substances. The essence of the method relies on the digestion of HA by hyaluronan lyase from S.pneumonia (SpHyl) following the reaction between 4,5-unsaturated dissacharides with 3-methyl-2-benothiazolinonehydrazone (MBTH). The result is highly colored product with A_{max} at 620 nm (blue color) with a minor peak at 645 nm also being detected. Spectrophotometry is considered to be a highly accurate instrument that is also very sensitive hence extremely precise. This method is also convenient for use in laboratory experiments because is simple and the costs of analysis are low. However in order to minimize any background signals and maintain high sensitivity the assay should be based on a specific HA derivatization yielding a highly colored product absorbing at longer wavelengths. MBTH is a reagent that is used for aldehyde detection. The reducing sugars in the open chain form have an active aldehyde group that condensate with MBTH. The product of condensation of sugar and MBTH has at A_{max} 390 nm (yellow color). According to Anthon and Barret (2002) the MBTH adduct of sugars can further undergo the oxidative addition of a second molecule of MBTH yielding a highly colored final product with A_{max} at 620 nm (blue color). In the research conducted by Pepeliaev et al. (2017) it is shown that using unmodified Anthon-Barret method HA is able to react with MBTH resulting in a blue-colored product. However, since HA is a polymer and contains just negligible amount of the reducing ends it is impossible to directly determine HA due to its weak signal. Therefore, in order to increase analytical signal and make MBTH method more convenient the HA should be decomposed to lower-molecular-weight elements. For that purpose, the processive hyaluronan lyase from *S.pneumoniae* is used in order to digest HA resulting in unsaturated dissacharides allowing direct spectrophotometry quantification of hyaluronan. However, in the procedure done by Pepeliaev et al. (2017), cetyl-trimethylammonium bromide fractioning was used to separate hyaluronic acid from glycosaminoglycans and other mono- and oligosaccharides. In my research for the purpose of separation of HA from bacterial capsule I have tested the effect of sodium dodecyl sulfate (SDS), chelaton III + SDS solution and H_2O. Chelaton III is the trivial name for disodium salt of ethylenediaminetetraacetic acid (EDTA) used for titration analysis. Since sodium dodecyl sulfate acts as a detergent and protein denaturant firstly it was necessary to find the concentration of SDS that is being effective enough to release the HA from bacterial cell wall, but not causing the

SpHyl denaturation. The absorbance which was used to indicated activity of SpHyl was measured at 232 nm. Graph 1 is showing the activity of SpHyl when no SDS is added to 3 g(l) HA dissolved in water which is used as a control trial. Graph 2 is showing the activity of SpHyl when 0.2% SDS is added to 3 g(l) HA dissolved in water. Since the kinetic curve started to decrease at this point indicating the enzyme denaturation it was necessary to test another concetrations of SDS that would be suitable. The following concentrations of SDS that were evaluated are 0.1% (Graph 3) and 0.5% SDS (Graph 4), both causing the denaturation of SpHyl enzyme. The concentration of SDS that did not have a denaturation effect on the acitivity of SpHyl was 0.015% SDS and as a result the kinetic curve presented on Graph 5 is increasing. 0.015% SDS was the concentration of SDS used in further analysis.

In order to be able to compare the yield of HA obtained using isopropyl precipitation method to yields of HA obtained using SpHyl-MBTH method same samples of fermentation culture were used. For instance, sample 1 of fermentation culture was cultivated under specific conditions, therefore both methods were applied on that same sample. The same procedure was done with 9 other samples. Therefore, each sample was tested three times for each condition (H_2O, SDS, chelaton III + SDS solution) and the average values along with the standard deviations are calculated and presented in Table 2 (sample + H_2O), Table 3 (sample + SDS) and Table 4 (sample + chelaton III + SDS solution). Graphical presentations of the yields of HA obtained from each sample treated with three different conditions was also presented (Graph 6-10) including standard deviations from the mean values with estimated $p \leq 0.5$ as a level of significance. Table 5 is showing the average value of the yield of HA obtained using SpHyl-MBTH which is 2.60 HA $[g/l]$ from samples which are treated with H_2O, 3.30 HA $[g/l]$ from samples in which SDS is added and 3.46 HA $[g/l]$ from samples in which chelaton III + SDS solution is added. It is noticable that the average value of the yield of HA obtained from samples which are treated with H_2O is lower than average value of the yield of HA obtained from samples which are treated with SDS and chelaton III + SDS solution. The possible explanation of lower value of obtained HA in case where samples were treated with H_2O is that not enough HA was able to separate from bacterial capsule, resulting in lower yields of HA. On the other hand sodium dodecyl sulfate is an anionic detergent that breaks down fatty acids and lipids associated with cell membrane thus enabling the release of HA from bacterial cell wall. Therefore the results of yield of HA obtained from samples which are treated with SDS and

chelaton III + SDS solution were similar to each other and higher from the results of the yield of HA obtained from samples treated only with H_2O. The obtained results are also presented graphically on Graph 11. However, since the SpHyl-MBTH method is really sensitive and requires extreme precision the errors that could have happened during each measurment should be taken into consideration as a possible way to affect the results obtained from each sample and also the final average value of the yield of HA. These errors could have been made mostly during pipetting, measuring the amount of substances used for reagents, difficulties to work with small sample of fermentation culture as well as under the effect of other factors such as temperature of the laboratory and other human mistakes.

Table 6 represents the comparison between the values of the yield of HA (g/l) obtained using two different methods, precipitation method with isopropyl alcohol and SpHyl-MBTH method. Since chelaton III + SDS solution was used in precipitation method in order to release HA from bacterial capsule, the values of the yield of HA (g/l) obtained from samples that were also treated with chelaton III + SDS solution in SpHyl-MBTH method were used for comparison. The results obtained from each sample in both methods were presented in Table 6 and graphically (Graph 11-15) due to different cultivation conditions each sample was exposed. The final average value of the yield of HA (g/l) calculated on the basis of 10 samples is 3.46 HA (g/l) for SpHyl-MBTH method and 3.63 HA (g/l) for isopropyl precipitation method. The results are presented on Graph 16. On the basis of obtained results it can be said that the yield of HA (g/l) obtained by applying SpHyl-MBTH method from the small volumes of the fermentation culture is similar to the yield of HA (g/l) obtained using precipitation method that was working with large volumes of fermentation culture. Similarity in results confirms that reliability of SpHyl-MBTH method indicating that the estimated optimal concentration of chelaton III + SDS solution (0.015%) that was used in all samples was able to release HA from bacterial capsule and that at the same time did not have a denaturation effect on the activity of SpHyl. Furthermore, hyaluronan lyase from S. pneumoniae has shown to be effective in the digestion of HA resulting in unsaturated dissacharides which were able to react with 3-methyl-2-benothiazolinonehydrazone (MBTH) and form colored product detected and quantify at 654 nm. However, it is also suitable to mention that since the yields of HA obtained using both methods were similar but not the same all the possible mistakes done during appliance of both methods such as pipetting, estimation of concentration of substances and reagens used, any left impurities

that were not removed by isopropyl alcohol and centrifugation, lab temperature, pH, and other human errors should be taken into consideration as a possible way of influencing the final results. MBTH method is widely used nowadays for various types of analysis. Van Wychen et al. (2017) have used MBTH method for carbohydrate determination in microalgae. Furthermore, De Oliveira et al. (2005) also applied MBTH method for total aldehyde determination in automative fuel ethanol samples.

Conclusion

The main goal of my bachelor thesis was optimizing the method of determining the yield of hyaluronic acid (HA) in small volumes of fermentation culture of *Streptococcus equi* subsp. *zooepidemicus*. On the basis of obtained results it can be concluded that SpHyl-MBTH method that I have chosen to use in my research for determination of HA in small volumes of fermentation culture is reliable and effective as the results of the yield of HA from each sample were similar to results obtained using standardized precipitation method with isopropyl alcohol. The results obtained from each sample using SpHyl-MBTH method have showed to be statistically significant. Furthermore, it can be concluded that the yield of HA obtained by applying SpHyl-MBTH method was higher from samples treated with SDS and chelaton III + SDS solution than from samples treated only with H_2O.

However, for further research an influence of other detergents such as Sodium Lauroylsarcosine, Triton or TWEEN on the separation of HA from bacterial capsule and the activity of SpHyl can be analyzed. Furthermore, the usage of other enyzmes such as *S. hyalurolyticus* hyaluronidase for the purpose of HA digestion can also be tested. Since a method of hyaluronan production using nonpathogenic microorganisms is widely used and popular, a suggestion for further analysis could be the applience of SpHyl-MBTH method using genetically modified nonpathogenic microorganisms instead of fermentation culture of *Streptococcus equi* subsp. *zooepidemicus*. The applications of spectrophotometry are wide mostly because it is inexpensive and simple process. It is very frequent method used in biochemistry for DNA, RNA and protein isolation, measurment of bacterial cells growth. Since it is used to measure absorbance at various wavelengths, within a scope of teaching program it can be used for measuring concentration of photosynthetic pigments such as chlorophylls, carotenoids, etc. Furthermore, the property of hyaluronic acid to absorb a large volume of water and create a gel-like environment gives an opportunity to observe its effect while studying tissue organization and morphogenesis. Due to its viscoelastic properties and excellent biocompatibility the effect of hyaluronic acid can be examined in teaching activities while studying skin tissues.

Literature

- Amagai I., Tashiro Y, Ogawa H. (2009): Improvement of the extraction procedure for hyaluronan from fish eyeball and the molecular characterization. - Fisheries Science, vol. 75, 3, pp. 805–810.
- Armstrong D.C., Cooney M.J., Johns M.R. (1997): Growth and amino acid requirements of hyaluronic-acid producing Streptococcus zooepidemicus. - Applied Microbiology and Biotechnology, vol. 47, 3, pp. 309–312.
- Balazs A. (1979): Ultrapure hyaluronic acid and the use thereof. - U.S. Patent, US4141973.
- Blank L.M., Hugenholtz P., Nielsen L.K. (2008): Evolution of the hyaluronic acid synthesis (has) operon in Streptococcus zooepidemicus and other pathogenic streptococci. - Journal of Molecular Evolution, vol. 67, 1, pp. 13–22
- Boeriu C.G., Springer J., Kooy F.K., van den Broek L.A.M., Eggink G. (2013): Production method of hyaluronan. – Intern. J. of Carbohydr. Chem., vol. 2013, pp. 1-3.
- Cavalanti A.D.D., Melo B.A.G., Oliviera R.C. Santana M.H.A. (2019): Recovery and Purity of High Molar Mass Bio-hyaluronic Acid Via Precipitation Strategies Modulated by pH and Sodium Chloride. – Applies Biochem and Biotech, vol. 188, 2, pp. 527-539
- Chao H., Spicer A.P. (2005): Natural antisense mRNAs to hyaluronan synthase 2 inhibit hyaluronan biosynthesis and cell proliferation. - The Journal of Biological Chemistry, vol. 280, 30, pp. 27513–27522.
- Chen W.Y., Marcellin E., Hung J., Nielsen L.K. (2009): Hyaluronan molecular weight is controlled by UDP-N-acetylglucosamine concentration in Streptococcus zooepidemicus. - The Journal of Biological Chemistry, vol. 284, 27, pp. 18007–18014.
- Chong B.F., Blank L.M., Mclaughlin R., and L. K. Nielsen L.K. (2005): Microbial hyaluronic acid production. - Applied Microbiology and Biotechnology, vol. 66, 4, pp. 341–351.
- Chong B.F., Nielsen L.K. (2003): Aerobic cultivation of Streptococcus zooepidemicus and the role of NADH oxidase. - Biochemical Engineering Journal, vol. 16, 2, pp. 153–162.

- Coutinho P.M., Deleury E., Davies G.J., Henrissat B. (2003): An evolving hierarchical family classification for glycosyltransferases. - Journal of Molecular Biology, vol. 328, 2, pp. 307–317.
- DeAngelis P.L. (1999): Hyaluronan synthases: Fascinating glycosyltransferases from vertebrates, bacterial pathogens, and algal viruses. - Cellular and Molecular Life Sciences, vol. 56, 7-8, pp. 670–682.
- DeAngelis P.L., Jing W., Drake R.R., Achyuthan A.M. (1998): Identification and molecular cloning of a unique hyaluronan synthase from Pasteurella multocida. - The Journal of Biological Chemistry, vol. 273, 14, pp. 8454–8458.
- DeAngelis P.L., (1993): Glycosaminoglycan polysaccharide biosynthesis and production: today and tomorrow. - Applied Microbiology and Biotechnology, vol. 94, 2, pp. 295–305
- DeAngelis P.L., Oatman L.C., Gay D.F. (2003): Rapid chemoenzymatic synthesis of monodisperse hyaluronan oligosaccharides with immobilized enzyme reactors. - The Journal of Biological Chemistry, vol. 278, 37, pp. 35199–35203.
- De Oliveira F.S., Leite B.C.O., De Andrade M.V.A.S., Korn M. (2005): Determination of total aldehydes in fuel ethanol by MBTH method – sequential injection analysis. – J. Braz. Chem. Soc., vol. 16, 1.
- Duan X.J., Niu H.X., Tan W.S., Zhang X. (2009): Mechanism analysis of effect of oxygen on molecular weight of hyaluronic acid produced by Streptococcus zooepidemicus. - Journal of Microbiology and Biotechnology, vol. 19, 3, pp. 299–306.
- Farrell Jr. R.E. (2010): RNA Methodologies (4th Edition) – A Laboratory Guide for Isolation and Characterization. – Academic Press, pp. 155-172
- Frankova J., Kubala L., Velebny V., Ciz M., Lojek A. (2006): The effect of hyaluronan combined with KI3 complex (Hyiodine wound dressing) on keratinocytes and immune cells. – J. Mater. Sci. Mater. Med., vol. 17, pp. 891-898
- Fraser J.R., Laurent T.C., Laurent U.B. (1997): Hyaluronan: its nature, distribution, functions and turnover. – J. Intern. Med., vol. 242, pp. 27-33
- Graves M.V., Burbank D.E., Roth R., Heuser J., Deangelis P.L., Van Etten J.L. (1999): Hyaluronan synthesis in virus PBCV-1-infected chlorella-like green algae. - Virology, vol. 257, 1, pp. 15–23.

- Haserodt S., Metin A., Dweik R.A. (2011): A comparison of the sensitivity, specificity, and molecular weight accuracy of three different commercially available hyaluronan elisa-like assay. – Glycobiology, vol. 21, pp. 175-183
- Huang W.C., Chen S.J., Chen T.L. (2006): The role of dissolved oxygen and function of agitation in hyaluronic acid fermentation. - Biochemical Engineering Journal, vol. 32, 3, pp. 239–243.
- Ignatova E.U., Gurov A.N. (1990): Principles of extraction and purification of hyaluronic acid. - Khimiko-Farmatsevticheskii Zhurnal, vol. 24, 3, pp. 42–46, 1990.
- Kim J.H., Yoo S.J., Oh D.K. (1996): Selection of a Streptococcus equi mutant and optimization of culture conditions for the production of high molecular weight hyaluronic acid. - Enzyme and Microbial Technology, vol. 19, 6, pp. 440–445.
- Kogan G., Soltes L., Stern R., Gemeiner P. (2007): Hyaluronic acid: a natural biopolymer with a broad range of biomedical and industrial applications. – Biotechnol Lett, volume 29, pp. 17-25.
- Mao Z., Chen R.R. (2007): Recombinant synthesis of hyaluronan by Agrobacterium sp. - Biotechnology Progress, vol. 23, 5, pp. 1038–1042.
- Marcellin E., Chen W.Y., Nielsen L.K. (2010): Understanding plasmid effect on hyaluronic acid molecular weight produced by Streptococcus equi subsp. Zooepidemicus. - Metabolic Engineering, vol. 12, 1, pp. 62–69.
- O'Regan M., Martini I., Crescenzi F., De Luca C., Lansing M. (1994): Molecular mechanisms and genetics of hyaluronan biosynthesis. - International Journal of Biological Macromolecules, vol. 16, 6, pp. 283–286.
- Oueslati N., Leblanc P., Harscoat-Schiavo C., Rondags E., Meunier S., Kapel R., Marc I. (2014): CTAB turbidimetric method for assaying hyaluronic acid in complex enivironments and under cross-linked form. – Carbohydr. Polym., vol. 112, pp. 102-108
- Pepeliaev S., Hrudíková R., Jíková J., Pavlík J., Smirnou D., Černý Z., Franke L. (2017): Colorimetric enzyme-coupled assay for hyaluronic acid determination in complex samples. – European Polymer Journal, vol. 94, pp. 460-470
- Porsch B., Laga R., and Konak C. (2008): Batch and size-exclusion chromatographic characterization of ultra-high molar mass sodium hyaluronate containing low amounts of strongly scattering impurities by dual low angle light scattering/refractometric detection.

- Journal of Liquid Chromatography and Related Technologies, vol. 31, 20, pp. 3077–3093
- Reddy K.J., Karunakaran K.T. (2013): Purification and characterization of hyaluronic acid produced by Streptococcus zooepidemicus strain 3523-7. – J. BioSci. Biotech., vol. 2, pp. 173-179.
- Rilla K., Siiskonen H., Spicer A.P., Hyttinen J.M.T., Tammi M.I., Tammi R.H. (2005): Plasma membrane residence of hyaluronan synthase is coupled to its enzymatic activity. - The Journal of Biological Chemistry, vol. 280, 36, pp. 31890–31897.
- Shiedlin A., Bigelow R., Christopher W. (2004): Evaluation of hyaluronan from different sources: streptococcus zooepidemicus, rooster comb, bovine vitreous, and human umbilical cord - Biomacromolecules, vol. 5, 6, pp. 2122–2127.
- Suzuki M., Asplund T., Yamashita H., Heldin C.H., Heldin P. (1995): Stimulation of hyaluronan biosynthesis by platelet-derived growth factor-BB and transforming growth factor-β1 involves activation of protein kinase C. - Biochemical Journal, vol. 307, 3, pp. 817–821.
- Tammi R.H., Kultti A., Kosma V.M., Pirinen R., Auvinen P., Tammi M.I. (2008): Hyaluronan in human tumors: pathobiological and prognostic messages from cell-associated and stromal hyaluronan. – Seminars in Cancer Biology, vol. 18, 4, pp. 288-295.
- Van Wychen S., Long W., Black S.K., Lauren L.M. (2017): MBTH: A novel approach to rapid, spectrophotometric quantitation of total algal carbohydrates. – Anal Biochem., vol. 518, pp. 90-93
- Weigel P.H., DeAngelis P.L. (2007): Hyaluronan synthases: a decade-plus of novel glycosyltransferases. - The Journal of Biological Chemistry, vol. 282, no. 51, pp. 36777–36781.
- Wu T.F., Huang W.C., Chen Y.C., Tsay Y.G., Chang C.S. (2009): Proteomic investigation of the impact of oxygen on the protein profiles of hyaluronic acid-producing Streptococcus zooepidemicus. - Proteomics, vol. 9, 19, pp. 4507–4518.
- Zhang J., Ding X., Yang L., Kong Z. (2006): A serum-free medium for colony growth and hyaluronic acid production by Streptococcus zooepidemicus NJUST01. - Applied Microbiology and Biotechnology, vol. 72, 1, pp. 168–172.

- Zhang X., Duan X.J., Tan W.S. (2010): Mechanism for the effect of agitation on the molecular weight of hyaluronic acid produced by Streptococcus zooepidemicus. - Food Chemistry, vol. 119, 4, pp. 1643–1646.

Internet source:

- www1: Journal. Hindawi. Derived from: https://www.hindawi.com/journals/ijcc/2013/624967/fig1/