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From Lab to Industry – Enzymes from Undifferentiated Marine Rest Raw Material

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Master thesis in Marine Biotechnology (60stp)
May 2014

UNIVERSITY OF TROMSØ

Acknowledgements

This master thesis was conducted as a collaboration between the Norwegian College of Fishery Science (NFH) and Nofima AS in Tromsø, as a part of an ongoing project at Nofima. All practical work during the project was performed at Nofima's facilities in Tromsø, during the autumn and spring semester 2013/2014.

I would like to thank my main supervisor at NFH, Klara Stensvåg, for helping me find an exciting project, and my supervisors at Nofima, Diana Lindberg, for letting me take part of her project, and Bjørnar Myrnes, for help in the lab and the pilot scale plant. You have all given me the best guidance and support during my practical work and through the process of writing this thesis. I sincerely appreciate all the time you have spent, and all the knowledge you have given me. Also, I owe great thanks to Kersti Øverbø at the Marine Biotechnology department at Nofima, for helping me on many occasions, you know the lab like no other and have a solution for most problems. Thanks to the employees at NAMAB, for giving me knowledge about processes at a bioprocessing facility, and Jan Arne Arnesen, for your co-confusion and company during my time spent as a process operator.

Great thanks to all my friends at the University of Tromsø, you have all contributed to making these 5 years memorable. In addition, to my one and only classmate Ida, thank you for an awesome exchange semester in Tasmania, for many good times as master students, and for being you!

Finally, thanks to my family, for helping me get to where I am today.

Silje

Tromsø, May 2014

Abstract - English

Nowadays, there is much focus on the rest raw material created by the fishing industry. Rest raw material is defined as the parts of fish or other marine animals, that is not the main product. The world's population continue to increase, and the focus is no waste of resources that can be turned into products of nutritional value, or benefit the society in any other way. In 2011, 238 000 tons rest raw material was produced from herring fish, of this 7000 tons were discarded. Of the utilized raw material, most were used to produce fishmeal and ensilage, intended for animal feed.

The aim of this project was to optimize the extraction of esterase enzymes from undifferentiated herring rest raw material. This was conducted by comparing, in laboratory scale, the outcome of using homogenised and non-homogenised raw material, and by extracting with buffers of different pH. Further on, clarification experiments of flocculation were performed on the optimal extract. The focus was to define methods enabling a large-scale production, with regards to one of the esterases found, and also determined in a previous project, to be of particular commercial interest.

From the initial, optimisation experiments, it was decided to proceed with extracts from non-homogenised herring rest raw material, made at pH 5.0, in subsequent experiments. Further on, chitosan was found to be suitable for clarification of the pH 5.0 extract, as it resulted in a substantial decrease in turbidity, while much enzymatic activity remained. The methods for extraction and clarification yielded relatively good results in pilot-scale, and recommendations for a large-scale production at the *national facility for marine bioprocessing* in Tromsø was given according to these conditions. The facility provides the opportunity for bioprocessing a variety of raw material through its selection of equipment. However, an industrial scaled extraction is probably not possible to implement at the given facility in its current state, mainly because it is not adapted for processes requiring cold conditions during production.

Abstrakt - Norsk

I disse dager er det mye fokus på restråstoff produsert av fiskeindustrien. Restråstoff defineres som de delene av fisk eller andre marine dyr, som ikke er regnet som hovedproduktet. Verdensbefolkningen er i stadig økning, og det fokuseres på å ikke kaste bort ressurser som kan gi produkter av næringsverdi, eller være til fordel som samfunnet på andre måter. I 2011 ble det produsert 238 000 tonn restråstoff fra sildefiskindustrien. Av dette ble 7000 tonn kastet. Av det benyttede råstoffet gikk det meste til å produsere fiskemel og ensilasje til dyrefór.

Formålet med dette prosjektet var å optimalisere ekstraksjon av esterase-enzymer fra udifferensiert silderestråstoff. Dette ble gjort ved å sammenligne resultatene gitt av homogenisert og ikke-homogenisert råstoff, og ved å ekstrahere med buffere av forskjellig pH. Videre ble det ble forsøkt, ved hjelp av flokkulering, å klargjøre det optimale ekstraktet. Fokuset var å definere metoder som gjorde det mulig å foreta en storskala produksjon med hensyn på en av esterasene, som ble funnet i et tidligere prosjekt og viste seg å være av kommersiell interesse.

Fra de innledende optimaliseringsforsøkene ble det besluttet å gå videre med ekstraktene ikke-homogenisert silderestråstoff tillaget ved pH 5,0. Det ble funnet at kitosan var et godt alternativ for å klargjøre pH 5,0 ekstraktene, da dette resulterte i en substansiell nedgang i turbiditet, mens mye enzymatiske aktivitet ble gjenværende i ekstraktet. Metodene for ekstraksjon og klargjøring av ekstraktene ga relativt gode resultater i pilotskala, og det ble gitt anbefalinger for gjennomføring av storskalaproduksjon ved *nasjonalt anlegg for marin bioprosessering* i Tromsø. Anlegget gir muligheter for bioprosessering av mange typer råstoff gjennom dets utvalg av utstyr. Det er derimot sannsynligvis ikke mulig å implementere en storskala ekstraksjon slik anlegget foreligger i dag, da det ikke er tilpasset prosesser som krever kalde forhold under produksjon.

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Abbreviations

BSA	Bovine Serum Albumin
DMSO	Dimethyl sulfoxide
ExPASy	Expert Protein Analysis System
FAO	Food and Agriculture Organisation of the United Nations
IUBMB	International Union of Biochemistry and Molecular Biology
MeSH	Medical Subject Headings
mq-H_2O	Milli-Q water
MUF	4-methylumbelliferyl
NAMAB	National facility of marine bioprocessing (Norwegian: "Nasjonalt anlegg for marin bioprosessering)
NH	Non-Homogenized
NVG	Norwegian spring spawning (herring)
PAGE	Polyacrylamide gel electrophoresis
pNPb	*para*-Nitrophenyl butyrate
SH	Super-Homogenized
U	Units

1 Introduction

1.1 Utilization of rest raw material from the fishing industry

There is a need to fully exploit Norway's advantageous marine resources and the economical values that follow. The Norwegian government and marine sector has been aware of this for several years, and steps are continuously taken towards the goal for full utilization. This is stated in a report covering how Norway can be further developed as a seafood nation within year 2050 (Olafsen *et al.*, 2012), suggesting how far the sector should have advanced at that stage. The background for the report is the need to have strengths in the future other than gas and oil, and to be the foremost seafood nation in the world. Utilizing more of the fish, previously discarded, can be a contribution to achieve this. Exploiting the proteins available in the rest raw material to produce other, more processed food products, is an example of great importance. There is also a global responsibility to meet the increased requirements for the world's food production as a result of the population growth. The report from Olafsen and co-workers, mention the need to realize the full value of the rest raw material, and how better technology and competence is important for this to be implemented. It is presumed that by 2050 all rest raw materials will be utilized. The market is presumed to be willing to pay more than what they do nowadays, for marine products such as proteins, perhaps novel proteins that are yet to be discovered, and marine oil from this industry concerning marine products (Olafsen *et al.*, 2012).

1.1.1 Definition of rest raw material

Rest raw material can be defined as the parts of the fish, shellfish or other marine animals, that is not suitable for direct human consumption like filets, liver or roe from fish or the shrimp muscle. In Norway, the term refers to all of the material that is not considered the main product, but the EU has established a new definition. They define it as "all or parts of animals or products that is not intended for human consumption". Thus, rest raw material that is processed in a way that converts it further into foods for people is not a part of the definition, even though it is not the main product (Bekkevold & Olafsen, 2007). Rest raw material includes heads, intestines, trimmings, shell, etc. (some are illustrated in figure 1) but other terms are often used to define such materials. Previously *waste* was a common term, but it indicates products with no attached value. A term that is often used these days, perhaps even more than *rest raw material*, is *by-products*. However, the term *by-products* may also indicate something unfit for human

consumption for consumers, and therefor *rest raw material* is increasingly becoming the preferred term (Rustad *et al.*, 2011).

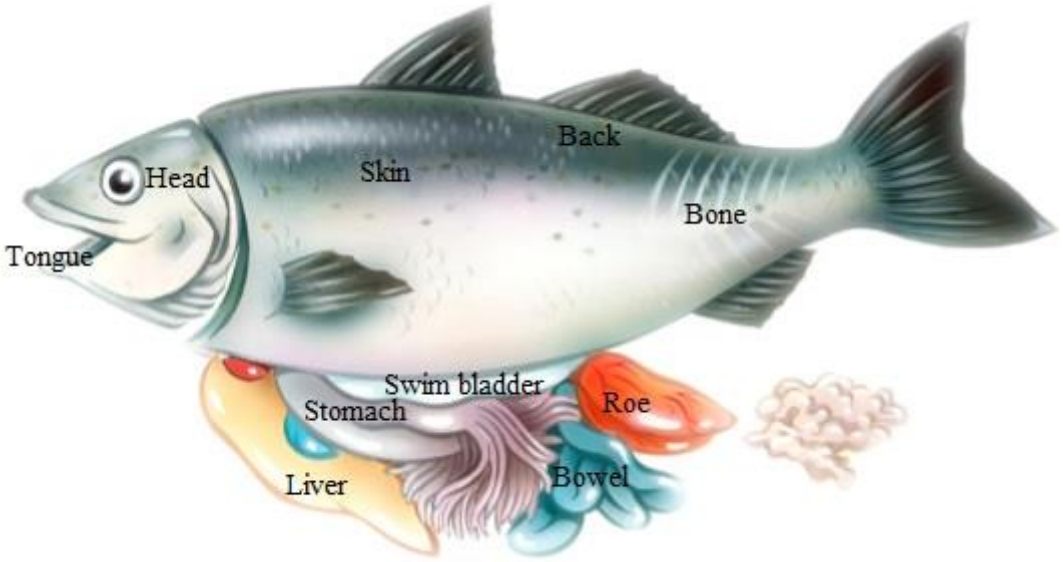

Figure 1**: Illustration of a fish with parts that are regarded as typical rest raw materials.** (Adapted from (RUBIN, 2012a))

1.1.2 The scope of rest raw material

The seafood industry in Norway gives rise to the largest export industry in Norway after oil and gas. An amount of 3.4 million tons seafood products were produced in 2012 from catch and aquaculture, but this was only a relatively small part of the raw material that were actually processed. (Regjeringen, 2013a;Regjeringen, 2013b). A large portion of the catch becomes rest raw material. In 2011, this amounted to approximately 815 000 tons of the catch. Of that 620 000 tons was exploited while the rest was discarded (figure 2) (RUBIN, 2012a).

According to the Food and Agriculture Organisation of the United Nations, the capture and aquaculture production had reached 148.5 million tons on world basis in 2010 (figure 3). Of this, 20.2 million tons was unsuitable for direct human consumption (FAO, 2010).

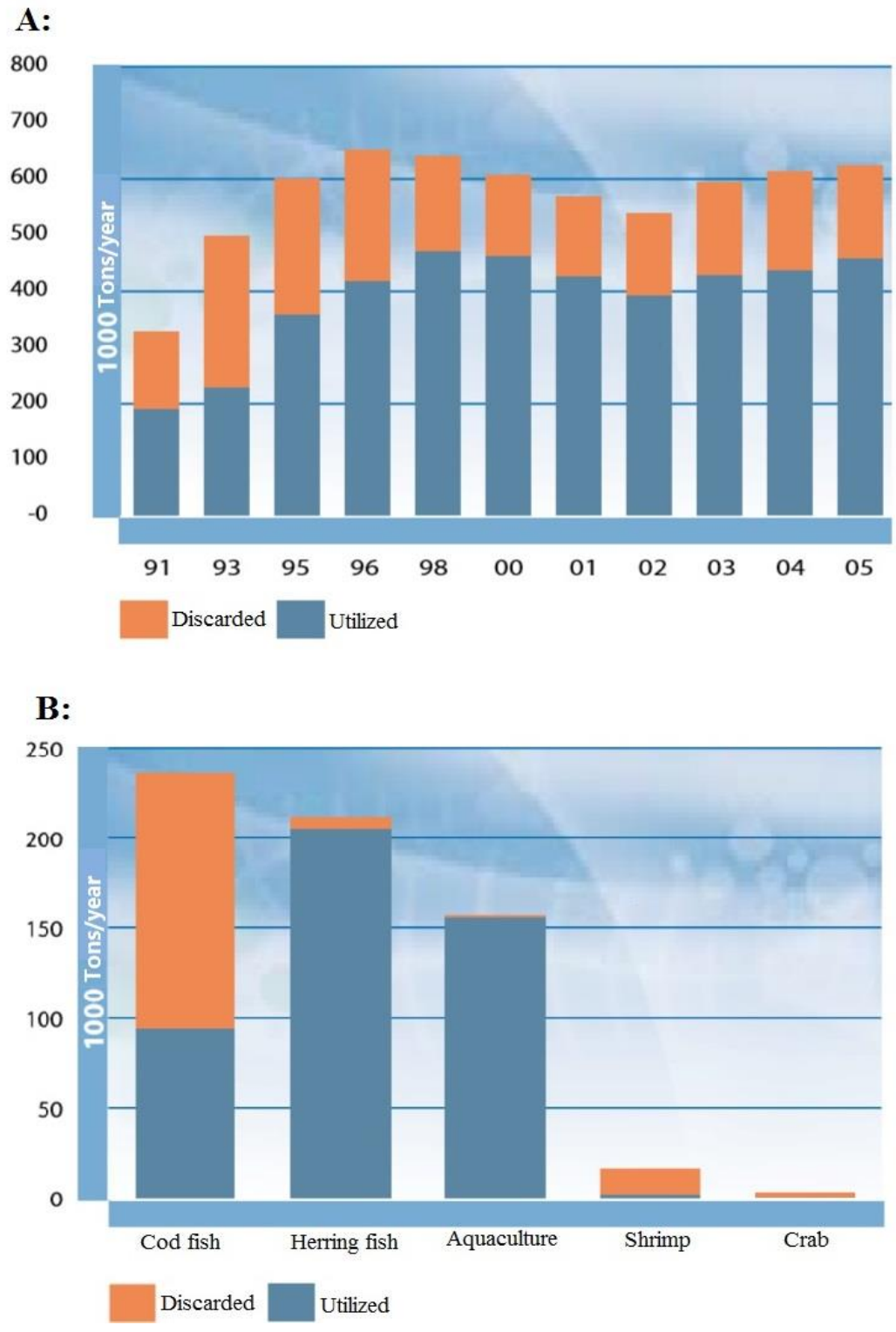

Figure 2: ***A:*** **The amount of rest raw material produced from 1991 to 2005**, and the distribution of utilized and discarded material. ***B:*** The distribution of rest raw material from 2005 in different marine sectors. Adapted from a RUBIN report (Bekkevold & Olafsen, 2007).

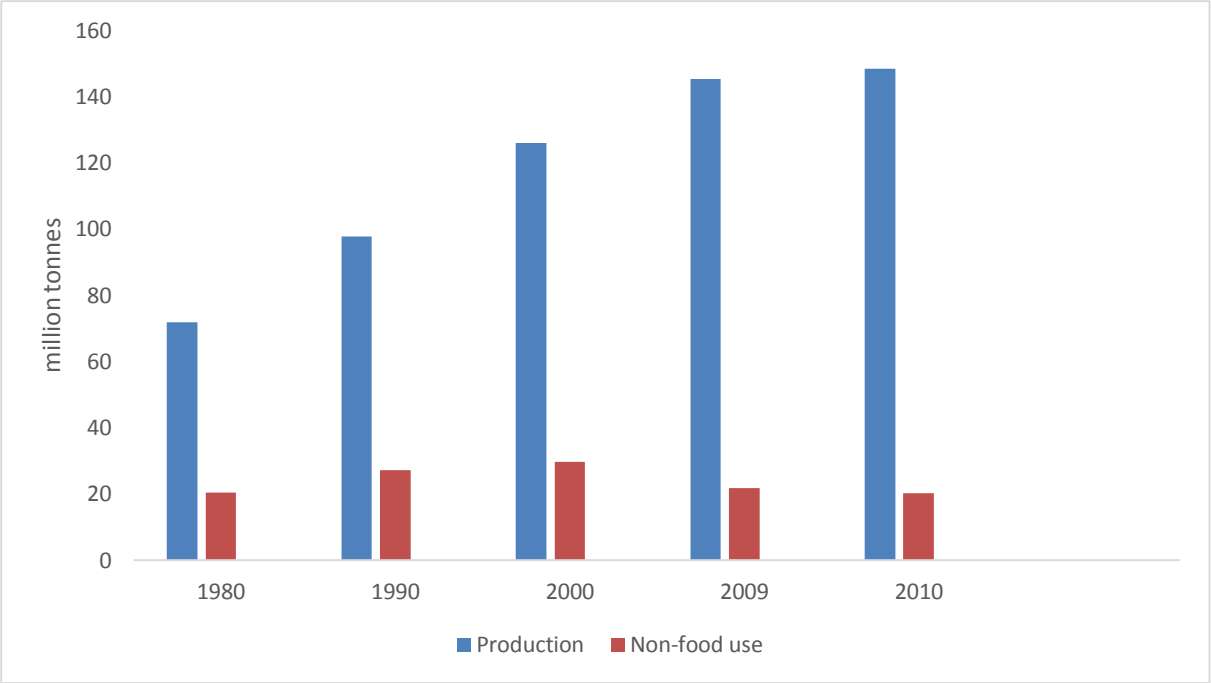

Figure 3: **The development of the world's fish production and utilization** over the last decades as stated by FAO in Fishery and aquaculture statistics from 2010. In the report, "Non-food use" is defined as material not destined for direct human consumption, but mainly for the manufacture of fishmeal and fish oil. The increase in production from 2001 is due to aquaculture as the proportion of captured fish has remain mostly the same (FAO, 2010).

In the Norwegian pelagic fish industry, in 2007, 660 000 tons herring were exported. This species was previously mostly sold as whole fish, but the amount of filleted fish on the market is increasing, thus the amount of rest raw material from each landing is increasing (Østvik *et al.*, 2009).

The rest raw material produced by the marine sector is often used in the production of fish meal, fish oil, protein concentrate, collagen, animal feed, chitin/chitosan and other relatively low and medium valued products (Fuentes, 2004). In the pelagic fish industry the rest raw material is mostly used for meal/oil (138 000 tons in 2007) and ensilage (60 000 tons in 2007). These products result in low financial gain for the parties involved (Østvik *et al.*, 2009). More recent numbers from the herring sector of the pelagic fish industry states that 143 000 tons of herring rest raw material were used to produce meal, while 84 500 tons became ensilage (RUBIN, 2012b). Searching for high-value components in the rest raw material may open for a more profitable business. Examples of such products can be native enzymes or other functional proteins. In a general, there is an objective to achieve an increased value and utilization of

resources in all sections of a process when it comes to rest raw material. The overall goal is to use the whole fish as cost efficient as possible.

According to the Norwegian herring sales team (Norwegian: *Norges sildesalgslag* [NSS]), the quota for Norwegian coastal spawning herring has decreased from 586 197 tons in 2011 to 376 757 tons in 2013 (sildesalgslag, 2013). With this in mind, there is a risk for lower volumes of rest raw material in the following years if the trend continues. This does not constitute a lesser need for better utilization of the rest raw material, but rather the opposite. If the fisheries are to have a smaller income because of the decreased landings of herring, the need to have other means of income until the quota can be raised becomes imperative. By increasing the value of all the material not suited for direct human consumption, all the parties involved in the industry will most likely benefit.

1.2 Herring

The herring is a relatively small fish species with an elongated body that is laterally compressed. The species can reach a length up to 49 cm, but rarely more than 40cm (Pethon, 1985). As a pelagic fish, it can be found at depths from 2 to 400 metres (FAO, 2013).

The Norwegian Spring-Spawning (NVG) herring has a migration pattern in the Norwegian Sea and coast. The contents of the fish, such as fat and water, varies in the different stages of the migration. During the feeding period from April to August, the stock is spread throughout most of the Norwegian Sea. From September to October, it migrates to several fjords in northern Norway and stays there until January. In mid-January, the fish starts migrating towards its spawning grounds. The spawning takes place from Lofoten to Lista, and lasts until March (Dragesund *et al.*, 1997). From the arrival at the wintering area (Sept-Oct) and until the end of spawning the spawning season (March-April) the herring does not feed (Slotte, 1999)

Research done by Slotte, et al., describes the changes in fat content of NVG herring in the different stages of their seasonal migration (Slotte, 1999). The herring store fat during their feeding period. In the study of Slotte and co-workers, individuals had up to 30% fat in July. When the herring reached the winter habitats the amount of fat in whole herring had decreased to 22% and further down to 8-10% after the spawning period (Slotte, 1999). The contents of the fish, especially the fat, can influence the downstream processing of the raw material, which is necessary if it is intended for other uses than whole fish sales.

1.3 Enzymes

1.3.1 Enzymes in general

Enzymes can in general be described as a biomolecule with a catalytic effect on a reaction (figure 4). An enzyme can be either protein based or RNA based, and it enhances the rate of its specific reaction by catalysing a reaction path which means lowering the activation energy needed for the reaction to occur (Nelson & Cox, 2008).

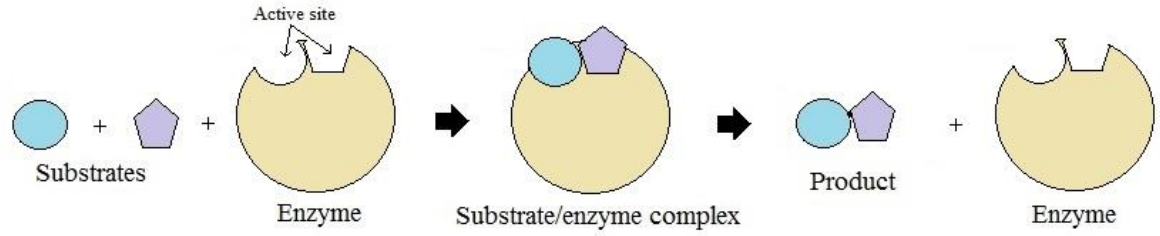

Figure 4: **Simplification of a general enzyme reaction where a substrate (two parts) are bound together by the assistance of an enzyme (catalysing the reaction) and a product is formed.** The enzyme reaction itself is happening in the active site of the enzyme when the specific substrate(s) are perfectly situated, and substrate-enzyme complex are formed. During the reaction, the product is formed and released, and the enzyme molecule is ready to catalyse a new reaction.

There is a vast number of enzymes in the world. They all have their specific substrates, though they can have several, and the reactions they catalyse are as varied as there are numbers of enzymes. To create a system to sort them all out they have been classified into group based on the type of reactions they catalyse (Gröger & Asano, 2012), as shown in table 1.

Table 1:**The international classification of enzymes** and the reactions they catalyse (Nelson & Cox, 2008). (As recommended by IUBMB. Class number correlates to its EC number)

International Classification of Enzymes		
Class no.	*Class name*	*Type of reaction catalysed*
1	Oxidoreductases	Transfer of electrons (hydride ions or H atoms)
2	Transferases	Group transfer reactions
3	Hydrolases	Hydrolysis reactions (transfer of functional groups to water)
4	Lyases	Addition of groups to double bonds, or formation of double bonds by removal of groups
5	Isomerases	Transfer of groups within molecules to yield isomeric forms
6	Ligases	Formation of C-C, C-S, C-O and C-N bonds by condensation reactions coupled to cleavage of ATP or similar cofactor

1.3.2 Hydrolases

Enzymes in the hydrolase class catalyse the cleavage of different bonds by conducting a reaction with H_2O. Hydrolases has several characteristics that make them most useful in organic chemistry and for some industrial applications. Most of these enzymes have wide substrate specificity, high stereospecificity and the ability to conduct reverse hydrolysis (condensation) (Bornscheuer & Kazlauskas, 2006). Hydrolases has been given the enzyme commission number 3 (EC 3) and there are several subgroups under this class that catalyse different varieties of hydrolysis reactions (NC-IUBMB, 2014).

1.3.3 Esterases

Esters are organic compounds with the functional group –COOR. For example in the presence of an acid or base, esters are hydrolysed at the expense of water, to from carboxylic acids or carboxylate anions. Triacylglycerol (TAG) is one of the most common type of ester found in nature, it is the main constituent of animal fat (Smith, 2008). However, different ester compounds are also often responsible for the pleasant smell of flowers and fruit. As they do not have hydrogen attached to their oxygen atoms, there are few intermolecular forces holding them together, thus they can easily diffuse into the surroundings (McMurry & Simanek, 2007).

Esterase is a large group of enzymes in the hydrolase family (EC 3.1) that is fairly well understood and structurally stable. The reactions that they catalyse are numerous and varied. These include, amongst others, the hydrolyzation of ester bonds (figure 5), esterification and transesterification (Bornscheuer & Kazlauskas, 2006). Esterification is when a carboxylic acid (ROOCH) and an alcohol (ROH) react to form an ester. If an ester is converted into another ester in the presence of alcohol and an acid catalyst it is called transesterification (Smith, 2008). These reactions can, and are catalysed by esterases. The enzymes can be a part of reactions including both specific and general types of esters (Dordick, 1989;Panda & Gowrishankar, 2005). Acetylcholinesterase (EC 3.1.1.7) is one example of an esterase. This enzyme cleaves acetylcholine, a neurotransmitter in many organisms, into acetyl and choline (MeSH, 2011) to end signal transmission. Carboxylesterase (often called “true” esterases) (EC 3.1.1.1) has wider specificity and reacts with a carboxylic ester and H_2O to form an alcohol and a carboxylate (Artimo *et al.*, 2012). Lipases (EC 3.1.1.3) cleaves triacylglycerol into diacylglycerol and a carboxylate with the help of water (Artimo *et al.*, 2012). If one is to separate lipases from “true”

esterases, an easy, simplified explanation is that the latter catalyse reactions with short fatty acid chains (1 to 10 C), while the former prefer longer substrates (Casas-Godoy *et al.*, 2012).

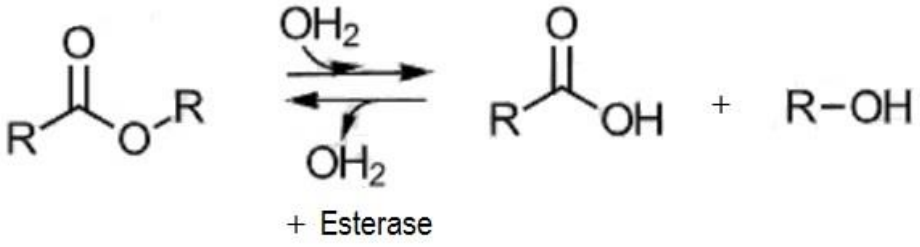

Figure 5: A general **esterase reaction** where an ester is converted into a carboxylic acid and an alcohol with the addition of H_2O. In the reverse reaction H_2O is removed and an esterase formed (Paravidino *et al.*, 2012).

Esterases can be applied for many industrial applications, and their sources are varied. They have been isolated from, for examples, different microbes, pig liver and horse liver (Faber, 2011). One example is the synthesis of terpenoid alcohol esters, which applied in, amongst others, food and pharmaceutical industries. This has proved possible to synthesize with the help of lipases, to avoid the use of adverse solvents in extractions from its natural source (Chaabouni *et al.*, 1996). An esterase produced from a *Pseudomonas* species has proved enantioselective for (S)-ketoprofen ethyl ester, and hydrolyses this to (S)-ketoprofen, the active enantiomer of an anti-inflammatory drug. Thus, preventing the formation of the inactive and negative acting (R)-ketoprofen (Kim *et al.*, 2002). To generalize it can be said that "true" esterases is mostly used in organic synthesis, while lipases are used in many different areas, such as food industry and detergents and cleaning agents (Casas-Godoy *et al.*, 2012). There is a large number of lipases, from microbial sources, available, but of "true" esterases there are only a few in use (Faber, 2011).

1.4 Project on rest raw material of herring at Nofima AS

The project *Gull fra havets sølv* (English: gold from the silver of the sea) commenced in 2010 by the marine biotechnology group at Nofima (Tromsø, Norway) in cooperation with SINTEF Fiskeri og havbruk AS (Trondheim, Norway). The objective, as stated in the project report (Myrnes *et al.*, 2014), was to identify new molecules and bioactivities in rest raw material from the herring, with commercial potential. The intention was to develop an industrial process for the recovery of bioactive molecules from the rest raw material at a later stage.

The reason for choosing herring was the lack of research preformed on wild fish of commercial interest, especially pelagic fish. There are some exceptions, but with herring, this is valid. Herring is one of Norway's most exported fish species, especially the Norwegian spring spawning (NVG) herring. However, even though, as mentioned earlier, the quota has decreased some, the landings of NVG herring is still high and expected to continue being so.

In addition to the objectives stated, there were also some other ambitions for the herring rest raw material project. To exploit the sustainable and nature given recourses, contribute to R & D results that can be commercialized and to build a national R & D expertise to an international leading level. Another goal of this project is that it may strengthen the academic awareness and expertise in the fish industry. This can be accomplished by increasing the interest of research in the rest raw material sector and developing methods that facilitate large-scale extraction from fish. The research in the project was divided between the two participating institutions. Nofima were responsible for the bioprospecting of enzymes and bioactive peptides, while SINTEF looked into the oil and functional proteins of the rest raw material.

At the initiation of the project at Nofima, extractions were made from herring delivered by Nergård Sild AS (Senjahopen, Norway). A variety of extractions, methods for clarification and determination of bioactivities were conducted. Hexane, a compound often used to remove grease and oil from solutions (TemaNord, 2003), and ammonium sulphate, commonly used in precipitation for clarification prior to proteomics, (Wingfield, 2001) were used to try to get more clear solutions. Enzyme assays were tested on all of the extractions, both with and without added $AmSO_4$ and hexane. These experiments proved that the alkaline extraction generally yielded a higher enzyme concentration and a higher activity level with the majority of the enzymes. At a later stage, the fat was tried to be separated fat from the rest of the solution by freezing the extract (from pH 9) and later thawing it at a low temperature. This resulted in a solution with two phases, a lower solid phase and an upper fluid phase. The fluid phase, subjected to filtration, yielded a fat and particle free solution. Based on findings in the project *Gull fra havets sølv*, a spinoff project was established (Lindberg *et al.*, 2014). The project *HerrZyme* focused on a partly purified enzyme with esterase activity from undifferentiated herring rest raw material. The objective of the project was to elucidate characteristics relevant for different, possible, applications of the enzyme. The enzyme of interest was found to be cold adapted and hydrolysed short-chained esterase substrates. In addition to other factors, this enzyme showed not to be a lipase. In conclusion, from these two projects in regards to the

mentioned enzyme, work remains before a complete characterisation and identification of the enzyme are revealed.

1.5 Flocculation as a method for clarification

An important part of working on extracts is to get a clear solution that it is possible to proceed with in further analysis and experiments. In addition, if one of the components of the extract is to be developed as a commercial product, it needs to be considerably pure to be fully characterised. Flocculation is a method that is widely used, and still experimented on, to remove suspended solids. This with the goal of collecting the solids for further utilization (Collingwood *et al.*, 1988;Taskaya & Jaczynski, 2009) or to remove contaminants in water purification (Amuda & Amoo, 2007;Silva *et al.*, 2009).

Flocculation is a process where particles that are dispersed in a substance aggregates and form larger flocs. The flocs can settle and form a sediment, thus forming a heterogeneous solution with a layer of solids that form the sediment, and a more clear substance above. The floc formation is generated by flocculants/coagulants. They can be separated into two main groups; inorganic and organic flocculants (Heitner, 1994). Examples of the former is ferric chloride and alum, and examples of the latter is chitosan and alginate.

The term flocculation is often used interchangeably with coagulation. However, these are different processes in the sedimentation of suspended solids. When reading literature in this field, the two terms flocculation and coagulation are often defined differently. One article in a book edited by Ives, K. J. distinguish the two as coagulation being aggregation of particles due to simple ions while flocculation is aggregation due to polymers (Lyklema, 1978). However, some describe coagulation as the process of destabilizing the suspended matter, or the colloids. These are kept stable in the solution by their charge (often negative) before addition of coagulant (Gregory, 1978). This causes the colloids to form smaller flocs through an initial aggregation, after which larger flocs are formed through the process of flocculation (Safferman, 2010).

The natural flocculant chitosan is a hydrolysed, deacetylated polysaccharide derived from chitin, a substance found e.g. in the exoskeleton of marine animals such as shrimps and crabs (Kawamura, 1991). The cost of chitosan varies depending on the quality and molecular weight of the compound, and is relatively expensive compared with for example ferric chloride (Sigma-

Aldrich, 2014). An advantage when using chitosan is its biodegradability and low toxicity towards animals. Several toxicity studies has been performed regarding the effect on different organs, were they conclude with negligible negative effects (Aspden *et al.*, 1996;Aspden *et al.*, 1997;Thanou *et al.*, 2001).

An inorganic flocculant that is widely used as a flocculant is ferric chloride ($FeCl_3$). Though the compound has proved effective in many liquid/solids separations, its environmental side effect is of concern. Experiments have shown that it is potentially harmful to the ecology of aquatic organisms (Sotero-Santos *et al.*, 2007). There are other disadvantages related to the use of metal salts as flocculants, such as the disposal of the sludge produced. Sharma *et al.* states that the use of these metal salt flocculants are decreasing, and is increasingly substituted with organic flocculants as chitosan (Sharma *et al.*, 2006).

The toxicity and environmental effect of different flocculants are of importance when deciding upon which flocculant to choose for any given process. Even though, it is beneficial to use flocculant/coagulant that gives the best result in the process at hand, the effect it has on the environment has to be taken into account. In addition, if a potential product is meant for human consumption in some way, the effect of residual flocculants in the product on humans should be well characterized and understood.

1.6 Large scale production and up-scaling related challenges

1.6.1 Up-scaling

Research conducted in a laboratory facility is usually carried out with an aim for further usage of the obtained results. For example, when laboratory research is performed on bioactive molecules isolated from an organism, it is most often with the intention of obtaining knowledge that can lead to a commercially interesting molecule/product (bioprospecting). In such a commercialisation process, there are many steps. One of them is to develop methods for production of the molecule in large scale, to achieve sufficient amounts. In the case of utilizing rest raw material, this is likely to require the processing of several tons of raw material to obtain a desired product. It is obvious that this is not feasible in a regular laboratory. To process tons of raw material, for instance in the extraction of a determined molecule, an industry scaled facility is needed. However, the process of up-scaling is not an easy task. There are several

challenges that arise when the same conditions are attempted created in an industry processing line, as was used for the methods in a laboratory.

Although the equipment available may vary from one industry plant to another, it is important to have knowledge about the equipment available and the possibilities that exists. Knowing this, one can try to simulate comparable industry conditions in the laboratory. This may ease the transition to large-scale production to some extent, and provide an increased comprehension of how the raw material and solutions of the process reacts to the treatment it undergo. Pilot-scale testing is an important element when transferring a method conducted in the lab to large-scale production. It is beneficial, if not necessary, to test how the process and methods work and identify potential challenges when larger volumes are processed, before large-scale production is initiated. Pilot-scale is conducted in volumes well above what is possible in the laboratory, but much smaller than the maximum capacity of an industry scaled production facility. In the former, it is possible to conduct trials at volumes around hundred litres, although volumes ranging from 5 – 100 kg is normal. In the latter several thousand litres can be processed at once.

1.6.2 Challenges

Even if factors related to up-scaling are considered during the research, the transition from one scale to another may still be problematic. One issue in when the transition is from laboratory-scale to pilot-scale is that the solutions may act differently when it is made in larger volumes. The temperatures may be difficult to match when there are larger volumes to either cool or heat which greatly influences the behaviour of solutions, especially if fat is involved. Although one strives to simulate the same conditions for the differently scaled processes, in reality, it is not possible to have the exact same conditions in every step. Everything from stirring mechanisms to filters will most likely vary and may influence any potential products. As an example, methods such as filtration by gravity using a funnel are not efficient when filtrating larger volumes. In the larger scaled processes, there are methods for filtration by using a motor driven filtration system and larger equipment for separation such as decanters and separators (see section 1.6.3) where solids, oils and stick water can be separated. In the laboratory, this can partly be obtained with centrifugation. It may be easier to compare and correlate the equipment and possibilities between large scaled facilities, with those of pilot scaled facilities, than the laboratory. This obviously depends on the layout of the large-scale facility, and the degree of investments in pilot-scale equipment.

1.6.3 National facility for marine bioprocessing – a description of the plant

The National Facility for Marine Bioprocessing (Nasjonalt anlegg for marin bioprosessering – NAMAB, Tromsø, Norway) will be used as a basis to describe the general layout of an industrial/large-scale facility. The plant recently opened for production and was designed by Alpha Laval (Lund, Sweden). Nofima describe it on their web page as a mini factory where companies can get assistance in transferring research results from a laboratorie-scale to produce advanced products in a larger scale (NOFIMA, 2013). The facility is over 1000 square metres and contains a process line able to manufacture products from a variety of marine raw materials.

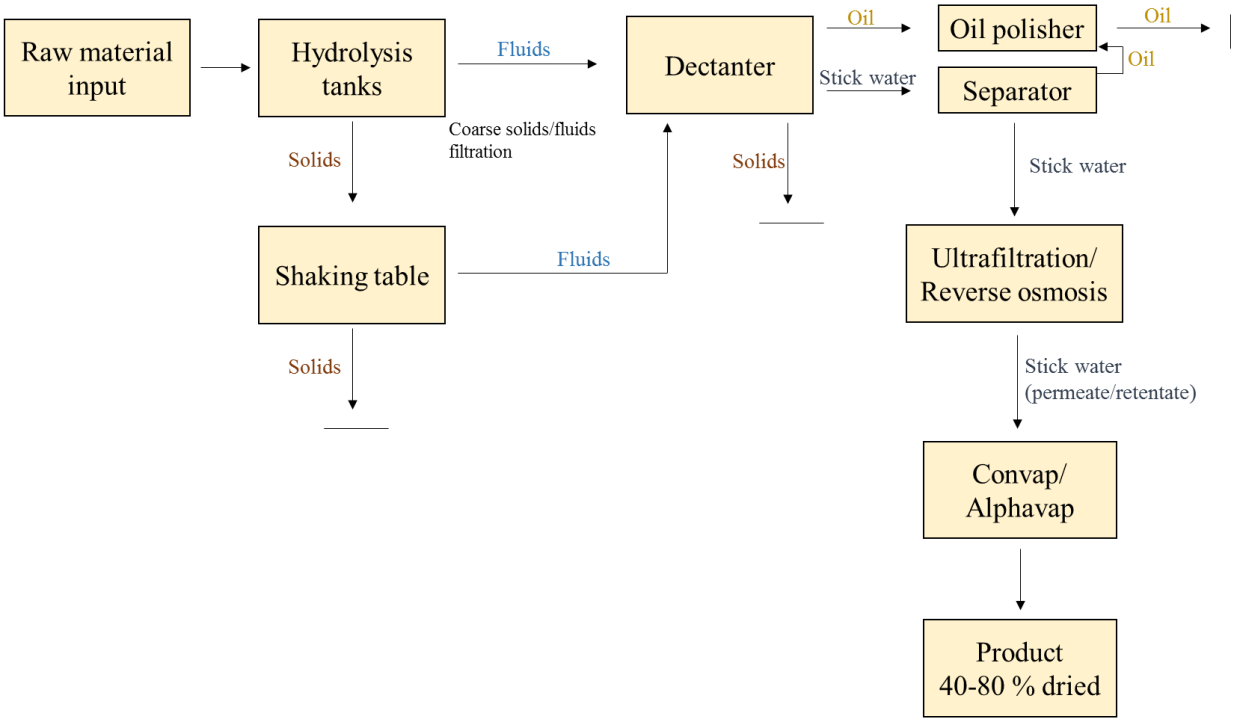

Figure 6 **Flow chart of a possible process line at NAMAB.**

Figure 6 illustrates a process line with some of the main components of the facility as it is today. It is partially automatic, in such a way that computers can control a large part of the production, but there are elements that need manually activation and setup. Flow plates (figure 7) has to be set up for the individual processes to lead the product to the different elements of the facility depending on the customers' needs.

Figure 7: **Flow plate** at NAMAB that controls the product line through the facility.

The factory is divided into two main parts, to separate the feed in of raw material and hydrolysis/reaction tanks, from the hydrolysed products as they go to their designated separation steps. This is particularly important if the products are to be used in human food industry, where there are high requirements for hygienic production.

The following list gives a short explanation of how components from figure 6 work (Alfa-Laval, 2014):

- Reaction tanks: There are two reaction tanks that both can hold up to 2000 l. They have a stirring device and are connected to a steamer for heating of solutions. The agitation of the stirring and the steam output can be controlled manually or be automatically controlled by decided set points for production (figure 8A).
- Shaking table: Coarse separation of liquid and solids by a shaking sieve where the liquid go through and gets pumped back into the product line while the solids are dropped into a container and removed from the product line (figure 8B).
- Decanter centrifuge: A three-phase separation systems that separates solids, stick water and oil. This occurs by centrifugal force that pushes the solids towards the walls of a cylindrical bowl. A screw conveyor, which rotates in the same direction as the bowl, but at a different speed, causes the solids to move along the wall and to the solids outlet. The light phase (oil) floats in the middle while the water remains in the middle, and can thus be separated (figure 8C).
- Separator: As the liquid is fed into the bowl, it accelerates and is fed from a distributor to a disc stack where the liquid-liquid-solids separation occurs. The oil phase moves through the disc stack to the centre of the bowl where it is discharged to a container. The stick water and remaining solids are separated from the oil as they move towards

the outer side of the discs and the stick water flows via channels to an outlet and is pumped to a container. The solids are discharged from the separator at regular intervals to a drain. Water is used as a safety feature, and will put pressure on the rotating part of the bowl to cause a stop if need be (figure 8D).

- Ultra filtration system: The stick water can be filtered based on a molecular size cut-off value (range from 1000 to 100,000 Da in molecular weight (MWCO)). The separation of the molecules occurs through a semipermeable membrane when pressure is applied. Fluid and molecules with a size below the cut-off value go through the membrane, and is called *permeate*, while fluid and molecules that remain on the outside is called *retentate*. Both can be routed to a suitable container or go to the drain. The system has the ability to heat the liquid as it circulates (figure 8E).
- Reverse osmosis filtration system: Can remove ions and very small molecules from the stick water. This happens when the system is applied pressure to overcome the osmotic pressure (figure 8F).
- Evaporators (Alfavap/Convap): Concentrates the peptides in the stick water by evaporating a certain amount of the excess water in the solution. The alfavap (figure 8G) can evaporate water so to reach up to 40% dry matter, while the Convap (not shown) up to 80%.

Figure 8: **Components of the NAMAB facility.** The raw material is fed into a grinder and then pushed up to the reaction tanks by a large conveyor screw (A), and then into the tanks by another conveyor screw which controls whether it goes into tank 1 or 2. From the reaction tank the hydrolysate, or possibly extract, is dropped into a container with a screw (not shown) where a coarse filtration of fluids and solids happen. The fluids are drained into a tank while the solids are pushed onto a shaking table (B) with a sieve that separates more fluids from the solids. The fluid continues to the decanter (C) where the material is separated into oil and stick water. In addition, smaller solid particulates are sorted out and dropped from the decanter into a mobile container. The oil, which floats on top of the stick water, is drained into one container and the stick water into another, similar container. The oil is pumped into an oil polisher/separator (not shown) where the last of proteins and other impurities are removed with the help of water. The stick water is pumped into a separator (D) where the last oil residues are removed. The oil can be transported back to the polisher. Both the clean oil and stick water is pumped into holding tanks. After going through the separator, the stick water can be ultra-filtered (E) and if need be, reverse osmosis filtered (F). The ultra-filtration system have three cylinders for the membrane filters (with a cut-off value of choice), which gives the possibility for relatively high throughput. The reverse osmosis system can remove molecules and ions from the solution by applying pressure to the system and forcing them through a semi-permeable membrane. Evaporators (G) can also be applied to concentrate the peptides in the stick water.

1.7 Aim of the project

1.7.1 The main aim

The main aim of this project is to optimize the extraction and purification of a selected enzyme with esterase activity in undifferentiated herring rest raw material in such a way that it is feasible in large-scale production.

The project will be performed at Nofima AS and carried out as a side project to the ongoing project *Gold from the silver of the sea* (Norwegian: *Gull fra havets sølv*). One of the objectives in the mentioned project is to search for commercially interesting enzymes in undifferentiated herring rest raw material.

1.7.2 The sub goals

- Lab scale
  - To conduct extraction of enzymes at different conditions (pH and using raw material with different pre-treatment) to obtain the maximum enzyme yield.
  - To define the optimal conditions for extraction of enzyme(s) based mainly on the yield of the enzymatic activity. Chose this condition as the standard extraction method in subsequent experiments.
  - To perform initial studies of clarification using different flocculation methods (that might be feasible in the large-scale production facility NAMAB).
- Pilot scale
  - To test the chosen methods for extraction and clarification in a pilot scaled plant.
  - To evaluate how the process of extraction/clarification work in this scale to obtain a greater understanding of how it might function when up-scaled to industry-scale.
- Industrial/large scale
  - To make recommendations for the implementation of the extraction process in the industrial/large scale facility NAMAB.
  - To define the potential challenges related to a large-scale extraction, based on the experiences from observing different processes at NAMAB and the experiments performed in pilot-scale and lab-scale.

2 Material and Methods

2.1 Material

2.1.1 Undifferentiated herring rest raw material

Undifferentiated herring rest raw material was kindly obtained from Norway Pelagic (Sommarøy, Troms, Norway) at several occasions. In the initial optimization experiments, raw material obtained 4.12.2012 was used. The raw material had been grinded in a Stephan homogeniser and frozen at -20°C in 0.5 kg portions for later use. In the pilot-scale and first flocculation experiments, raw material obtained 24.10.2013 was used. This was frozen in blocks of ~15 kg (for the pilot-scale experiment) or grinded and frozen at -20°C in 0.5 kg portions. For the ultra-fresh raw material experiment the raw material was obtained 15.01.2014, grinded with an Industrial bowl cutter (Kilia Vertrieb und Engineering, Neumünster, Germany) and used the same day. Some samples of the fresh raw material were grinded in a Stephan homogeniser and frozen in portions of 0.5 kg at -30°C for comparative experiments later.

2.1.2 Chemicals equipment and buffers

All chemicals (appendix 1, table A1), equipment (appendix 1, table A2) and different buffers used (appendix 2), are listed in the appendix and tables indicated. Buffers at pH 3.0 to 6.0 were sodium acetate (stock of 0.5 M), buffers of pH 7.0 to 9.0 were Tris/HCl (stock of 0.5 M), buffer at pH 10.0 was ammonium carbonate (stock of 0.5 M) and buffer at pH 7.4 was 0.1 M phosphate buffer.

2.2 Extraction of esterases from herring rest raw material

Extracts were made from undifferentiated herring rest raw material with buffers at pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 (see appendix 1). The raw material was prepared in to ways, both extracted from at all pH. The raw material, coarsely grinded, was either applied directly for extraction, named Non-homogenized (NH), or homogenized in a blender (Waring commercial blender 7011S, Connecticut, U.S.A.) with buffer for one minute at maximum speed before extraction was performed, named super-homogenized (SH). The latter resulted in a completely homogenised solution of raw material and buffer, while the NH solution had visible pieces of raw material in it.

2.2.1 Extraction

Coarsely grinded undifferentiated herring rest raw material was thawed at 4°C. Buffers with the ionic strength of 50 mM was added to the raw material in a 1/5 ratio (raw material/buffer) to a total volume of 500 ml. The extraction occurred under stirring for one hour at 12°C. Sodium acetate/HCl buffer was used for pH 3.0 to 6.0, Tris/HCl for pH 7.0 to 9.0 and ammonium carbonate/NH_3 for pH 10.0 (see appendix 2). The extract were frozen at -20°C until analysed further (see 2.3, 2.4 and 2.5). All extractions were conducted in triplicates, giving 48 extracts in total with all the eight different buffer/pH and with NH and SH material.

2.2.2 Clarification of extract through centrifugation and filtration

The extracts were stored at -20°C for different lengths of time (8−28 days) as only five extracts could be processed in one day. They were thawed overnight at 4°C, giving a semi-separated solution with a solid and a liquid phase that were separated by decanting. The liquid phase of the extracts were centrifuged at 20 000 x g for 20 min (Sorvall Instruments RC5C, GMI, Minnesota, U.S.A.). The solid fractions, that still contained some fluid, were centrifuged at 3000 rpm for 20 min (Eppendorf centrifuge 5810R, Eppendorf, Hamburg, Germany). The pellets were discarded and the supernatants centrifuged at 20 000 x *g* for 20 min. All the supernatants were filtered through a Whatman 41 filter. In addition, the supernatants that resulted from the solid phase centrifugation at 3000 rpm and then at 20 000 x *g*, were filtered through a GF/C filter after Whatman 41 filtration. Both pellets and filters were weighed to obtain the weight of the extracted material. Esterase activity was measured the same day and aliquots of the extract were frozen for later analyses of protein concentration (section 2.4) and native PAGE (section 2.5).

2.3 Esterase activity measurements

The enzyme activity Unit (U) is defined as the amount of enzyme that is needed to hydrolyse 1 µmol of pNP (*para*-Nitrophenol) from the substrate pNPb (*para*-Nitrophenyl bytyrate) per min (figure 9).

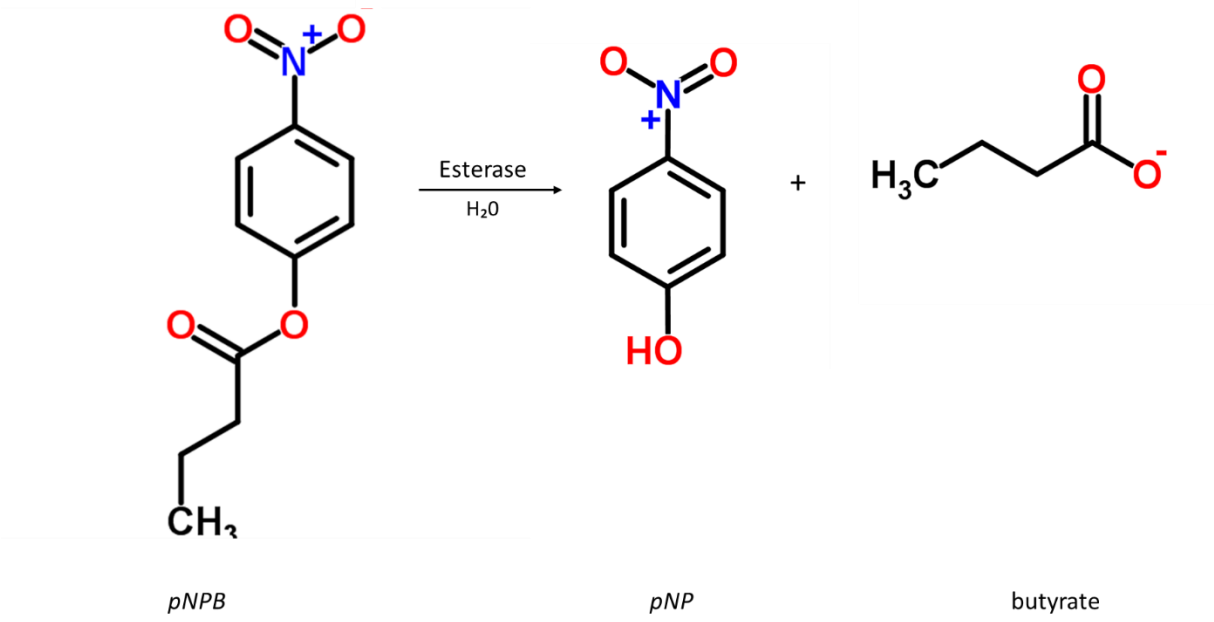

Figure 9: **The hydrolysation of pNPb to pNP and butyrate**

The specific activity (U/mg) refers to the enzyme activity per mg of the total protein contents of the extracts. The specific activities in the extracts were calculated by dividing the amount of pNP produced per minute per ml by the concentration of proteins in the extracts ((U/ml)/(mg/ml)). The total esterase activity of the extractions were calculated by multiplying the units per ml of the samples with the total ml obtained for each if the extractions ((U/ml)*ml).

Beer-Lamberts law (A=εlc) was used to calculate the concentration of pNP released per minute from pNPb, where A is absorbance, ε is molar extinction coefficient of pNP in units of mol^{-1} *cm^{-1}, l is the path length of the sample in cm and c is the molar concentration of the compound in solution in mol*1^{-1}.

Significant differences (significance level of 0.05) between enzymatic activities of some of the extracts were calculated (see 2.10).

2.3.1 Cuvette assay

An amount of 890 µl of 0.1 M sodium phosphate buffer, pH 7.4 (see appendix 2) was mixed ("end over end") with 100 µl 8 mM pNPb in dimethyl sulfoxide (DMSO) in a cuvette. Ten µl of extract was added, and the enzyme activity was measured at 405 nm for one minute (UV1800 Shimadzu UV spectrophotometer, Kyoto, Japan). Background reactions was measured with

corresponding extraction buffer (pH 3.0−10.0). All measurements were performed in triplicates. The enzymatic activity data was presented as activity per ml extract (U/ml) and total activity (U) depending on the experiment.

2.3.2 Microplate assay

An aliquot (10 µl) of each extract to be measured was added to three wells (for triplicate) in a 96 well plate (Tissue culture testplate 96F, TPP, Trasadingen, Switzerland). An amount of 190 µl stock solution was added (0.1 M sodium phosphate pH 7.4 and 8mM pNPb in DMSO, ratio 1/10) and inserted in the plate reader (Molecular devices, Versamax microplate reader, California, U.S.A.). The plate was shaken once, before reading commenced at 405 nm for 20 min with 61 reads of 20 sec intervals at room temperature (~ 25°C).

2.3.3 Determination of extinction coefficient of pNP

The extinction coefficient of pNP was measured photospectrometrically. The absorbance at 405 nm for pNP was measured in triplicate with 0, $1*10^{-1}$, $1*10^{-2}$ and $1*10^{-3}$ mM pNP in DMSO. The molar extinction coefficient of pNP at 405 nm was calculated and refers to how much light pNP absorbs at the given wavelength (Pavia *et al.*, 2009). This value is needed to calculate U from absorbance.

2.4 Protein quantification

All protein measurements were done in triplicates.

2.4.1 Pierce Protein Assay

The protein assay kit of Pierce (Thermo scientific, Illinois, USA) was used according to the procedure of the producer. In short, bovine serum albumin (BSA) was used as standard (pre-diluted protein assay standards, Thermo scientific, Illinois, USA). The assay linear range stated in the protocol for the microplate procedure was 50 − 2000 µg/ml, thus the concentrations applied for the standard curve were 2000, 1000, 750, 500, 250 and 125 µg/ml. Ten µl of sample was added 150 µl reagent in a microplate. . The microplate was shaken for several seconds, and incubated at room temperature for 10 minutes. Absorbance was measured at 660 nm with the Versamax microplate reader.

All the triplicates of the samples (pH 3.0 − 10.0 of both NH and SH) were tested with the Pierce reagent to determine if dilution was necessary, by adding 150 µl reagent to 10 µl of undiluted samples

The required dilutions were prepared and 10 µl of each were added in triplicates to the 96 wells plate together with the BSA standards for the standard curve. One plate was needed for the homogenised extracts and another for the non-homogenised extracts. The extraction buffers (appendix 1, table A1) were used as group blanks, while water was used as plate blank.

2.4.2 Bio-Rad Protein Assay

Protein concentration was determined according to Bradford (Bradford, 1976) by using the micro method of the Bio-Rad protein assay (#500-0006, Bio-Rad, California, USA). The Bio-Rad dye reagent was prepared by diluting it 1/5 with milli-Q water (mq-H_2O) and filtered through a Whatman 589/1 filter. An amount of 200 µl Bio-Rad reagent was added to 10 µl of sample in the wells (in triplicate). The plates were shaken for several seconds, and then incubated at room temperature for 10 min. Absorbance was measured at 595 nm with the Versamax microplate reader.

BSA concentrations of 500, 250 and 125 µg/ml were used for the standard curve. The dilutions needed were calculated from the results given by the Pierce assay.

For measuring protein concentration in herring extracts, the respective buffers of the extracts were used as group blanks, and water was used as plate blank. The dilutions needed were calculated from the Pierce assay (see 2.4.1). One plate was used for the NH extracts, and one for the SH extracts.

2.5 Determination of in-gel enzymes with esterase activity

2.5.1 Native PAGE

Native Page was performed according to the protocol from Invitrogen (California, USA), that is based on a method of Schägger and Jagow (Schagger & Vonjagow, 1991)

In preparation for the running buffers, the 20X anode and cathode buffers were diluted to 1X. The anode buffer was diluted with mq-H_2O, and the cathode buffer was diluted with the anode buffer.

Preparation of the samples where done by diluting the pH 3.0 and pH 4.0 extracts were diluted with mq-H_2O in a 1/2 ratio, pH 5.0 – 9.0 were diluted 1/3, and the pH 10.0 1/4 and 1/10 based on preliminary protein content measurements (see 2.4.1).

Native PAGE Novex 4-16% Bis-Tris gels (10 wells type gels) were used. According to the procedure from the producer, the gels were prepared before the electrophoresis by being cleansed for storage solution, and the sample well filled with 1X cathode buffer. The gel cassettes were placed in the cathode chamber, and the chamber filled with 1X cathode buffer up to the point where the sample wells were just visible. An amount of 21µl extract (NH pH 3.0 – 10.0, SH pH 3.0 – 10.0) were mixed with 7 µl 4X sample buffer. Of each sample, 25 µl of extract/sample buffer mixture were added to the wells. Samples from SH extracts were added to one cassette and the ones from the NH extract to the other. In total nine wells in each cassette were used for extract samples, while one was used for the NativeMark unstained protein standard (15 µl). The electrophoresis was run at 150 V (constant) until the samples reached the end of the gel (~1h 50min).

When the protein concentration of the extracts were known, an additional Native PAGE was performed with the same method, but the extract was diluted to 1000 and 2000 µg protein/ml. The extracts made at pH 5, 6, 8 and 9 were added in both concentrations, and pH 7 only in protein concentration of 1000 µg/ml.

2.5.2 MUF-butyrate treatment of native PAGE gels

The Native PAGE gels with the separated samples when through a 4-methylumbelliferyl butyrate (MUF-butyrate) tagging process to detect bands with esterase activity by fluorescence. When MUF-butyrate is subjected to esterases, the compound is hydrolysed into butyric acid and the fluorescent compound 4-methylumbelliferone (figure 10) (Roberts, 1985).

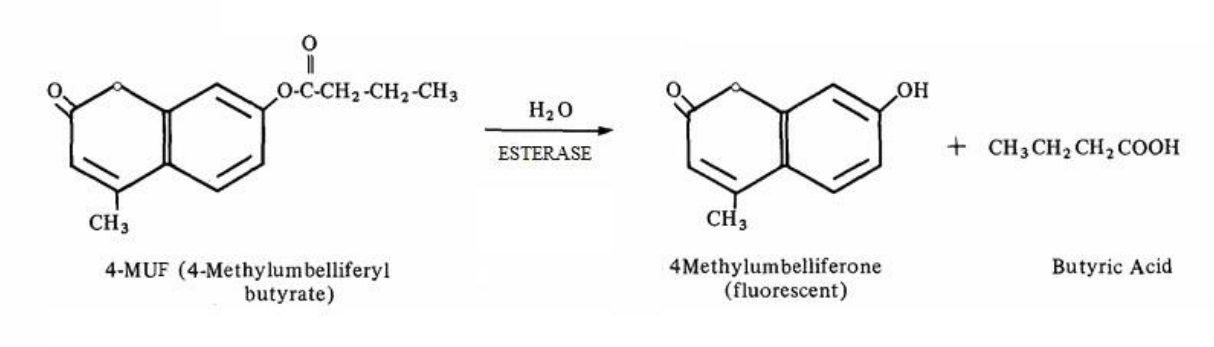

Figure 10: **The hydrolysation of MUF-butyrate into MUF and butyrate by esterase activity. (**Adapted from Roberts, 1985)

The electrophoresis gels were rinsed twice in 50 mM sodium phosphate buffer, pH 7 (containing 0.1% gum Arabic and 0.4% triton X-100) for 5 min under tilting. The substrate, 100 µM MUF-butyrate (40 µl 25 mM MUF-butyrate in 10 ml 50 mM sodium phosphate buffer containing 0.1% gum Arabic and 0.4% triton X-100), were added to the gels. Then the gels were placed in UV light (UV transillumination), revealing where the substrate were cleaved by enzymes of esterase activity, as the fluorescent product molecules (MUF) became apparent after some min. These fluorescent bands were marked by punching holes next to the bands with a glass Pasteur pipette. Pictures were taken were taken of the gels with a BioDoc-It imaging system (UVP, California, USA) to document the fluorescent bands. The gels were then transferred to a 50 mM Tris/HCl, pH 9.0, buffer for stronger fluorescence signal. New pictures were taken.

2.5.3 Coomassie staining

The NativePAGE gels were rinsed three times in 100 ml mq-H_2O for five minutes under tilting. Simply Blue Stain Reagent (modified Coomassie G-250) (Invitrogen) was added and the gels left overnight under tilting.

The next day the Coomassie stain was washed off the gels with mq-H_2O under tilting. When the excessive stain was removed and distinct blue protein bands could be seen, pictures were taken with a VersaDoc imaging system (Model 100, Bio-Rad)

2.6 Flocculation to clarify extracts

To obtain a relatively clear extract without centrifugation, flocculation was tested with chitosan and ferric chloride ($FeCl_3$). The flocculation experiments were based on the jar test, which is

used to determine the dosage required of a chemical to reduce the turbidity of a chemical (Clark & Stephenson, 1999).

Measurements of enzymatic activity in the flocculated extract were done in accordance to section 2.3.2. The flocculation experiments were conducted on NH, pH 5.0 extracts (see 3.3)

2.6.1 Ferric chloride flocculation

Flocculation experiments were performed on extracts (filtered through a cheesecloth) of both 1/5 and 1/10 raw material/buffer ratios (see 2.2.1 for extraction method), with a total volume of 80 ml in the flocculant/extract mixtures. $FeCl_3$ concentrations of 0.2, 0.4, 0.6 and 0.8 g/l were added the extract samples from a $FeCl_3$ stock solution of 50 g/l. Decrease in turbidity was compared to a control with natural sedimentation, i.e., no added flocculant. After addition of flocculant the pH was adjusted back to pH 5.0, as the flocculant caused a decrease in the extract's pH level. The extracts were set to stir hard for 1 minute, then gently for 3 min, and set to flocculate for 1 h 40 min. Samples were taken of the filtrate before flocculation, and of the supernatants after flocculation/sedimentation for analysis of turbidity (duplicate) and enzyme activity (triplicate) of all samples.

Another flocculation experiment was conducted to try to counteract the drop in pH brought forth by the flocculant, $FeCl_3$. Thus, the stock solution of 50 g/l was diluted to 10 g/l with the 50 mM sodium acetate, pH 5.0 extraction buffer. Flocculation was conducted in duplicate with 0.01, 0.05, 0.1, 0.15 and 0.2 g/l $FeCl_3$. Turbidity (duplicate) and enzyme activity (triplicate) were measured in aliquots of all samples.

2.6.2 Chitosan flocculation

The method for flocculation with chitosan was based on research described in an article by Riske *et al.* (2007). A 1 % chitosan solution (w/v) was made in 50 mM sodium acetate buffer, pH 5.0, and added 1 % acetic acid to solubilize the chitosan.

Expriment I: Method with same concentration of acetic acid in flocculation reactions

A 1/10 raw material/buffer, pH 5, extract was made in accordance to part 2.2.1. and filtered through a cheesecloth. A 1% chitosan (1.6, 3.2, 4.8 and 6.4 ml) was added to the extract samples (~74 ml) to make final chitosan concentrations of 0.02, 0.04, 0.06 and 0.08% respectively. The

samples where then added additional 1% acetic acid in 50 mM sodium acetate buffer, pH 5.0, to a total volume of 80 ml, to eliminate differences in acetic acid concentrations in the reactions. Along with the control with no added flocculant (only the acetic acid/buffer solution), all reactions were set to stir for 30 min, and flocculate for 1 h, after which turbidity (duplicate) and enzymatic activity (triplicate) was measured.

Experiment II: Detection of the effect of acetic acid on the flocculation reaction

A chitosan flocculation experiment was done to see whether the additional acetic acid (se section above) had any impact on the flocculation reaction. Duplicates of control, 0.01 and 0.02% chitosan in the flocculation reaction were performed with and without the additional acetic acid. Turbidity (duplicate) and enzymatic activity (triplicate) were measured of all samples, including filtrate.

Experiment III: Flocculation without additional acetic acid

Another flocculation experiment was performed, with chitosan concentrations of 0.001, 0.005, 0.01, 0.015, 0.02% and control (0%) in duplicate. The extract was made as described above, but filtered through a sieve (0.5 mm pores), and the 1% chitosan stock solution was made with 0.3% acetic acid. Total volume of the extract/chitosan samples was 200 ml. Turbidity (duplicate) and enzyme activity (triplicate) were measured of all samples, including filtrate.

Experiment IV: Flocculation on extracts of ultra-fresh raw material

The method described in experiment III was used for flocculation with ultra-fresh raw material. However, the extract was then made in pilot-scale with a volume of 150 l, but the flocculation volumes were the same.

Experiment V: Flocculation on extracts of raw material stored 2 months at -30°C

Flocculation was performed on the raw material from experiment IV after 2 months storage at -30°C, with the same method as described in experiment III.

2.6.3 Optimal pH for chitosan flocculation

Flocculation was performed with 0.01% chitosan (from a 1% chitosan stock solution with 0.3% acetic acid) at pH 5.0, 6.0 and 7.0 to investigate if there was a considerable difference in the outcome of flocculation at nearby pH. The extracts was made in accordance to section 2.2.1, but with a 1/10 raw material/buffer ratio. The experiment was performed in duplicate with total volumes of 200 ml. Turbidity (duplicate) and enzyme activity (triplicate) were measured of all samples.

2.6.4 Native PAGE of flocculated extracts

Native PAGE was run on some of the flocculated pH 5.0 extracts. A pH 5.0 extract from the initial freeze/thawed experiment was used as control. All samples were diluted 1/3 with mq-H_2O. The following flocculated extracts were added the wells in accordance to the method presented in section 2.5:

- Extracts flocculated with 0.001, 0.005, 0.01% chitosan and their control (0%) described in section 2.6.2, experiment III.
- 0.01% chitosan flocculated extract from ultra-fresh raw material described in section 2.6.2, experiment IV.
- 0.01% chitosan flocculated extracts from freeze/thawed ultra-fresh raw material described in section 2.6.2, experiment V.
- 0.4 g/L $FeCl_3$ flocculated extract described in section 2.6.1.

2.7 Pilot scale

Pilot scaled extraction and flocculation experiment was conducted. The rest raw material was processed in an industrial bowl cutter (Kilia Vertrieb und Engineering, Neumünster, Germany) and added to a large tank that holds 200 l. Extraction was done with pH 5.0 buffer in accordance to part 2.2.1, but with a 1/10 raw material/buffer ratio and a total volume of about 100 l. The extract was filtered through a metal sieve (0.5 mm pores) to remove the larger bits of raw material from the extract. Chitosan (0.01%) was added and stirred in manually for some minutes. The extract was set to flocculate in the process hall (~15°C) and the gradual sedimentation was observed. At the end of the day, it was set in a 1°C storage room overnight. The clarified fluid was ultra-filtered through a membrane with a cut-off value of 10 kDa (Model R Membrane Filtration Pilot Plant, GEA liquid processing, Søborg, Denmark). Enzyme activity

(triplicate) and approximate protein concentration was measured by a photospectrometer (UV-1800 UV-Vis spectrophotometer) at 280 nm (duplicate).

2.8 Enzyme temperature tolerance

A sample from the initial pH5-NH extract was used to measure how the enzymes tolerated different temperatures over a length of time. Duplicates incubated at room temperature, 37°C, 45°C and 60°C were measured after incubation at 0, 2.5, 5, 7.5, 10, 15 20 and 60 min. The activity (mAbs) was measured photospectrometric at 405 nm for one minute with pNPb as substrate. Of the substrate, 100 µl was added to 10 µl enzyme, and 890 µl 0.1 M sodium phosphate buffer, pH 7.4.

2.9 A theoretical approach to large-scale extraction

Prior to evaluating a potential large-scale extraction from the herring rest raw material, bioprocesses performed on different types of raw material were observed at NAMAB. Knowledge of the facility and its equipment was acquired through this observation, and also through participating in the production as a process operator.

2.10 Statistics

Significant differences between the parallels (3) were calculated using students T-test in Microsoft Excel 2013.

3 Results

3.1 Enzymatic activity, protein and volume yield of herring rest raw material extracts

3.1.1 Enzyme activity of the extracts

To get an indication of what extract contained most of the studied enzyme, esterase activity measurements were conducted with pNPb as substrate.

The molar extinction coefficient of pNP at 405 nm was calculated to 15.3 +/- 0.3 mM^{-1} cm^{-1}, and refers to how much light pNP absorbs at the given wavelength.

The results showed that there was an increasing activity, both calculated in total U and U/ml, with increasing pH in the extracts, from pH 3.0 to pH 9.0 (table 2). In the pH 10.0 extracts however, the activity detected was much lower than at pH 9.0. This trend applied to both the NH and the SH extracts. For the NH extracts, there was no significant difference in total U (significance level of 0.05) between the extracts containing the highest enzyme content (pH 9.0), and those (above pH 7.0) with the lowest active material. For the SH extracts there was a significant difference in enzyme active material detected in the pH 9.0 extract, the pH 5.0 extract and those of lower activity than the pH 5.0.

For the NH extractions, the specific activity increased from extracts of pH 3.0 to 4.0, and then further on decreased until the pH 10.0 extract that had the lowest specific activity. It was similar for the SH extractions, but there the specific activity was highest at pH 3.0 and then decreased for each extract with increasing pH to pH 10.0 (table 2). For the NH extracts, the highest specific enzymatic activity was found in the pH 4.0 extract. With a significance level of 0.05, there was no significant difference in activity between the pH 4.0 and the pH 3.0 extracts, the latter with second highest specific activity.

Between the pH 5.0 extract with the third highest specific activity, and the pH 4.0 extract, there was a significant difference. For the SH extracts, the pH 3.0 extract showed the highest specific activity. There was no significant difference in the activity of the pH 3.0 extract and the pH 4.0 extract, with the second highest activity. However, the difference in specific activity between the pH 3.0 and pH 5.0 extracts was significant.

3.1.2 Protein contents and volume of initial extracts

The protein content of the extracts was measured to be able to calculate the specific activity. The extracts showed an overall increase in the amount of protein with increasing pH values (table 2). The SH extracts contained slightly less protein per ml than the NH group. Thus, the trend of increasing protein content with increasing pH value, still applied.

The extractions were conducted at different pH values, and resulted in various volumes of extract solution after centrifugation and filtration (table 2). Some of the pellet fractions seemed to contain fluid that did not separate well from the pellet during centrifugation. Particularly pH 10.0 extracts deviated from the other ones, as it was quite viscous and the pellet had a gel like texture. Thus, the fluid contained in the pellet of the pH 10.0 extracts, resulted in relatively low volume of filtered extract at the end compared to the other extractions of lower pH-conditions. This was valid for both extract types, with non-homogenised and homogenised starting material.

Table 2: **Volume, protein and activity yield of extracts** made from NH and SH herring rest raw material at pH 3.0 to pH10.0. Protein contents is given in mg/ml and the total protein (g) in each of the extracts. The specific esterase activity is given in U/mg while the enzyme content isgiven in U. The highest calculated value in each activity category are highlighted in yellow.

Extracts		Volume	Protein		Activity		
		ml	mg/ml	Total (g)	U/ml	U/mg*10^{-3}	Total U
NH	pH 3.0	176	16	3	0.14	9.0	25
	pH 4.0	180	21	4	0.22	11.0	40
	pH 5.0	173	43	7	0.26	6.1	45
	pH 6.0	159	66	11	0.30	4.6	47
	pH 7.0	166	117	19	0.32	2.8	53
	pH 8.0	165	158	26	0.40	2.6	67
	pH 9.0	163	227	37	0.41	1.9	68
	pH 10.0	97	359	35	0.26	0.7	26
SH	pH 3.0	174	11	2	0.16	15.2	28
	pH 4.0	186	16	3	0.23	14.5	44
	pH 5.0	174	37	6	0.29	7.7	50
	pH 6.0	159	57	9	0.35	6.2	55
	pH 7.0	162	108	18	0.37	3.4	60
	pH 8.0	158	161	26	0.37	2.3	59
	pH 9.0	158	216	34	0.41	1.9	65
	pH 10.0	122	329	40	0.24	0.7	29

Sub-conclusion extract yield: Extracts made at pH 9.0 had the highest total enzymatic activity. Extracts made at pH 3.0 and 4.0 had the highest specific activity. However, these activity results is a combined value of all the enzymes of esterase activity in the extracts, and cannot solely determine the best extract to proceed with in up-scaled experiments.

3.2 MUF-butyrate cleaving enzymes in the extracts

To get a better understanding of what enzymes that were responsible for the enzymatic activity measured in the extracts, native PAGE was run on all the extracts and the resulting gels was subjected to the substrate MUF-butyrate.

The MUF-butyrate treatment of the Native PAGE gels showed that the extractions at different pH resulted in a variety of fluorescent bands that corresponds to the MUF product and thus indicated presence of esterase activity in the samples. At pH 3.0 and 4.0 it appeared that only two small esterases was extracted, and at pH 5.0 an additional, presumably larger one. At pH 6.0 and 7.0 it was possible to see another, weak band relatively high up in the lanes. In the lane for pH 8.0 and 9.0, the same fluorescent band appeared visible, but at much higher intensity, which indicate higher concentrations. The extraction performed at pH 10.0 resulted in detection of fewer fluorescent bands of esterase activity in the gels, compared to extractions done at pH 6.0-9.0. Three weak bands were observed in the lanes after the gel was subjected to MUF-butyrate. The result is in accordance with the content detected in extracts made from both SH and NH rest raw material. In the SH extractions however, there generally seemed to be more enzyme present in all the bands detected in the gels, as they were slightly more fluorescent (figure 11 and figure12).

All gels were colored with Coomassie staining after the MUF-butyrate treatment. The two fluorescent bands visualized by the MUF-butyrate method in all the lanes, at the bottom of the gels, and the fluorescent upper bands detected in the lanes of extract of pH 6.0 to pH 10.0, did not correspond to any bands stained in the gel by Coomassie blue. For the Coomassie stained bands in the middle of the lanes (~300 kDa), there seemed to be a smear on the stained gels where it was fluorescent with substrate present (figure 11 and figure 12).

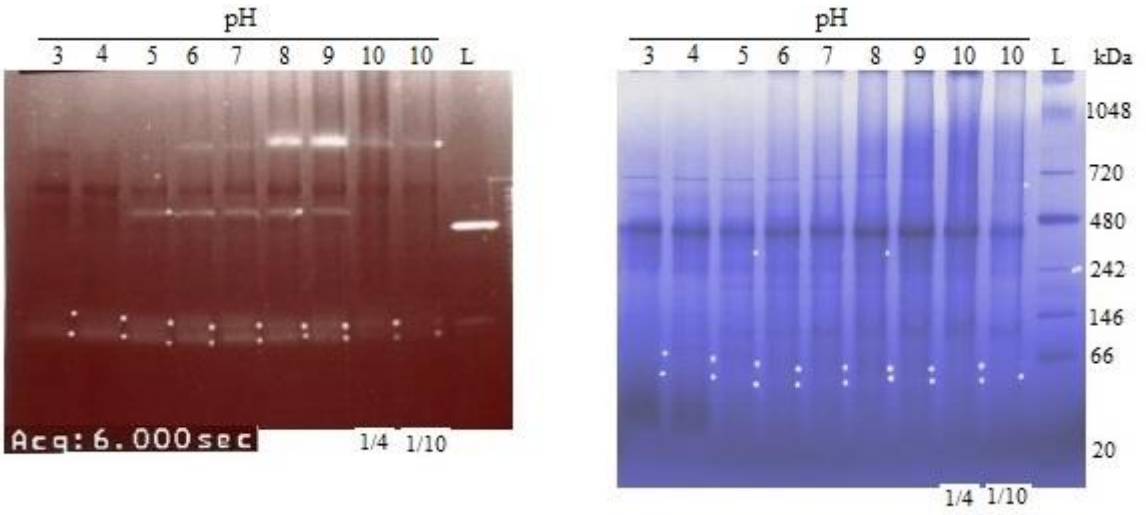

Figure 11: **Native PAGE and esterase active material from homogenized herring rest raw material made at pH 3 to 10.** The pH 10 extract was run with samples diluted 1/4 and 1/10. The other samples was diluted 1/3. Left: Gel with MUF-butyrate causing bands containing enzymes with esterase activity to fluoresce (marked with a small hole) when subjected to UV-light. The pH 3 and 4 extracts have two bands with esterase activity, pH 5 has three bands, pH 6, 7, 8 and 9 have four bands. pH 10 has three bands that is relatively weak. Right: Same gel stained with Simply Blue Stain Reagent (modified Coomassie G-250). There is generally little correlation between the visible bands with MUF-butyrate and with Coomassie stain. The ladder (L) has one fluorescent band (242 kDa), which is stated by the supplier.

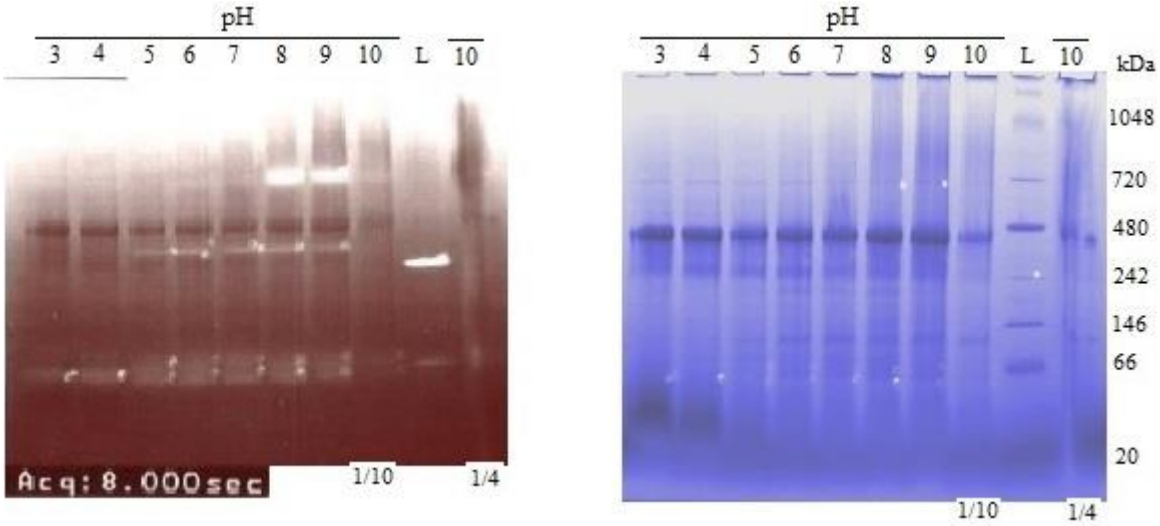

Figure 12: **Native PAGE and esterase active material in extracts from non-homogenized herring rest raw material made at pH 3 to 10.** The pH 10 extract was run with samples diluted 1/4 and 1/10. The others were diluted 1/3. Left: Gel with MUF-butyrate causing bands containing enzymes with esterase activity to fluoresce (marked with small holes) when subjected to UV-light. The pH 3 and 4 extracts have two bands with esterase activity, pH 5 has three bands, pH 6, 7, 8 and 9 have four bands. pH 10 has three bands that is relatively weak. Right: Same gel stained with Simply Blue Stain Reagent (modified Coomassie G-250). There is generally little correlation between the visible bands with MUF-butyrate and with Coomassie stain content. The ladder (L) has one illuminating band (242 kDa), which is stated by the supplier.

When Native PAGE was run with extracts adjusted to the same protein concentrations (NH extracts), the strong bonds that were visible at pH 8.0 and 9.0 did not appear as strong when no account was taken of the protein contents in the extracts. However, at 1000 and 2000 µg/ml these bands are not visible at all in the pH 6.0 and 7.0 extracts (figure 13) as it was in the previous runs (figure 11 and figure 12). Again, the bands that were fluorescent around 60 kDa when substrate was added did not appear visible when the gel was stained with Coomassie. Due to an accident, the pH 5 extract was added in a slightly higher concentration.

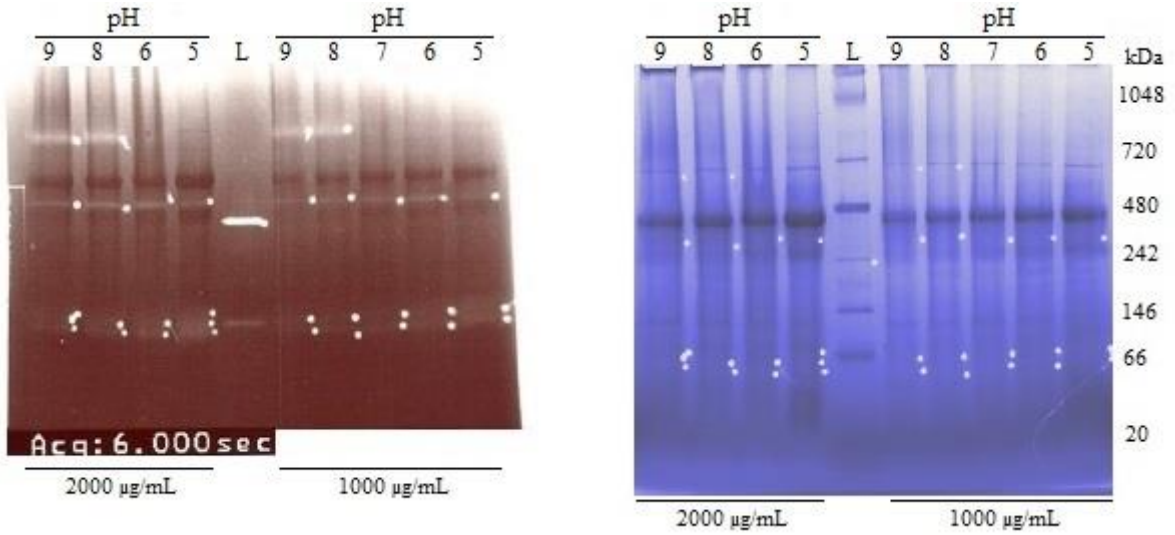

Figure 13: **Native PAGE and esterase active material in extracts made from non-homogenized herring rest raw material**. Left: Gel with MUF-butyrate substrate, causing bands containing enzymes with esterase activity to illuminate when subjected to UV-light. The pH 8 and 9 wells gives four bands with esterase activity. The pH 5, 6 and 7 gives three bands. Right: Same gel stained with Simply Blue Stain Reagent (modified Coomassie G-250). The samples was added in two concentrations: 1000 and 2000 µg/ml (the well of sample pH 5.0 added slightly higher concentration). The 1000 µg/ml samples consists of extracts made at pH 7 to 9, while the 2000 µg/ml samples consists of extract made at pH 5, 6, 8 and 9. The difference in concentration does not provide much difference in the strength of the bands when treated with MUF-butyrate. There is generally little correlation between the visible bands with MUF-butyrate and with Coomassie stained material. The ladder (L) has one illuminating band (242 kDa), which is stated by the supplier.

To observe how flocculation influenced the different enzymes with esterase activity in the extract, some samples of flocculated pH 5.0 extracts were subjected to native PAGE. Only the ~ 60 kDa bands were visible when subjected to MUF-butyrate (figure 14).

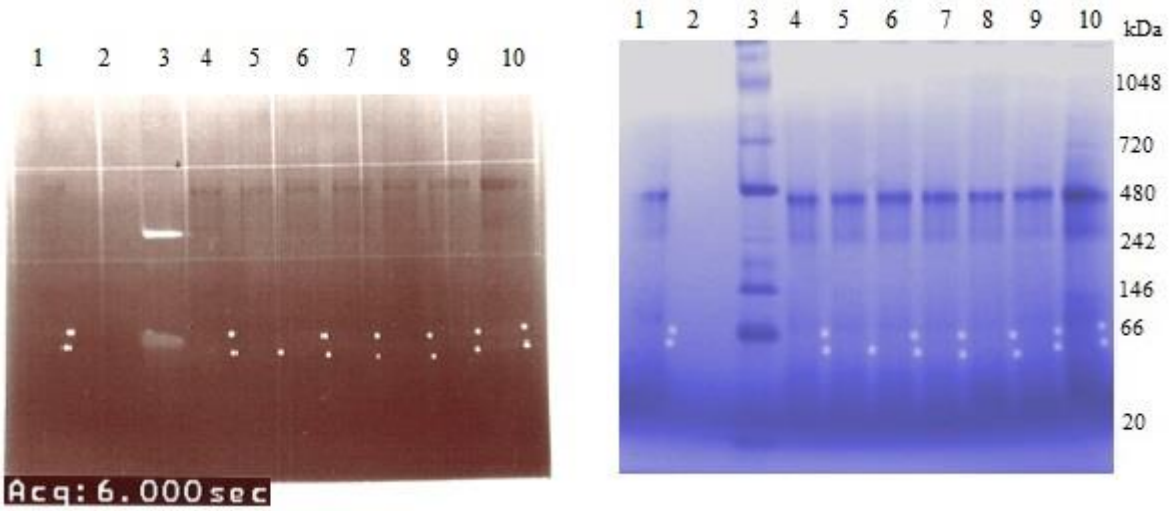

Figure 14: **Native PAGE and esterase active material in flocculated pH 5 extracts from undifferentiated herring rest raw material.** . Left: Gel with MUF-butyrate substrate, causing bands containing enzymes with esterase activity to illuminate when subjected to UV-light. Right: Same gel stained with Simply Blue Stain Reagent (modified Coomassie G-250). Contents of the lanes: ***1:*** 0.4 g/L $FeCl_3$ flocculated extract. ***2:*** empty. ***3:*** Ladder. ***4:*** 0.01% chitosan flocculated extract. ***5:*** 0.005% chitosan flocculated extract. ***6:*** 0.001% chitosan flocculated raw material. ***7:*** Control (0.00%) a chitosan flocculation experiment. ***8:*** 0.01% chitosan flocculated ultra-fresh raw material extract. ***9:*** 0.01% chitosan flocculated frozen/thawed ultra-fresh raw material extract. ***10:*** pH 5 extract from initial frozen/thawed raw material experiments.

Sub-conclusion MUF-butyrate binding enzymes: The higher pH-valued extracts contain a larger number of enzymes that binds to MUF-butyrate than those of lower pH-value. This means that the enzyme activity of the low pH extracts (table 2) is exerted from a smaller number of enzymes.

3.3 Defining the optimal conditions for extraction

The results already shown (table 2, figure 11 – 14) and other observations, were taken into consideration and a standard protocol of pH 5.0 extracts were defined based on the following factors: enzyme activity of the extracts, behaviour of the extracts during the procedure and a possibility of implementation of this protocol in an industrial scaled facility.

Sub-conclusion of defining a standard extraction method: The extraction procedure at pH 5.0 from non-homogenised herring rest raw material was defined as the optimal standard extraction method, and was decided to be the extraction method to be applied in further studies of this project.

3.4 Clarity of extract when flocculated with various flocculants

In the attempt to clarify the extracts by methods feasible in an industrial scaled production, flocculation was conducted on pH 5.0 extracts as defined in 3.3.

3.4.1 Chitosan

The first flocculation with chitosan concentrations between 0.2 and 0.8% gave the highest degree of floc formation at 0.2% chitosan (results not shown). When lower concentrations were used it proved that of these, 0.01% chitosan resulted in the best flocculation and thus the clearest extract evident by the turbidity results presented in figure 15a. Figure 15b illustrates the clarity of the extract flocculated with 0.01% chitosan compared to the control and the extract flocculated with 0.02% chitosan. First at a concentration of 0.005% chitosan, did the turbidity decrease noteworthy compared to the control where a natural sedimentation happened without flocculant present. The activity measurements of the flocculated extracts showed some decrease compared with the control. When compared with the other flocculated extracts, there were little variation in activity. From the filtrate to the control, there were a substantial drop in both activity and turbidity, 70.5 and 27.5% respectively.

It was found that if acetic acid added to solubilize chitosan, it did not affect the results of the flocculation reaction (data not shown).

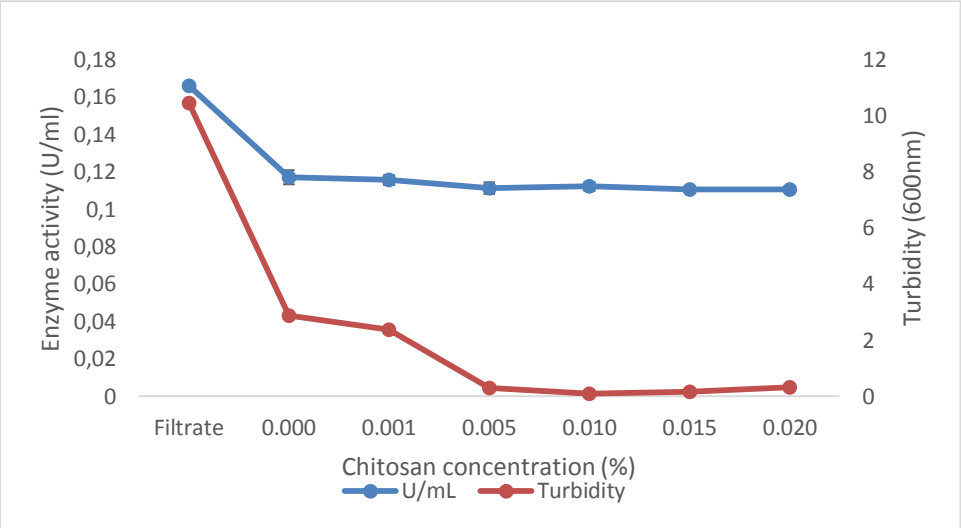

Figure 15a: **Enzyme activity (U/ml) and turbidity of pH 5 extracts from herring rest raw material flocculated with chitosan.** Activity and turbidity measurements are from filtered, unsedimented extract, controls with natural sedimentation (no added flocculant) and extract samples flocculated with 0.001, 0.005, 0.01, 0.015 and 0.02% chitosan (200 ml total volume). Enzyme activity is a measurement of pNP formed from pNPb (substrate). Because of low standard deviation values, some were excluded from the figure. Standard deviation ranges from 0.001 to 0.003 U/ml and turbidity from 0.01 to 0.04.

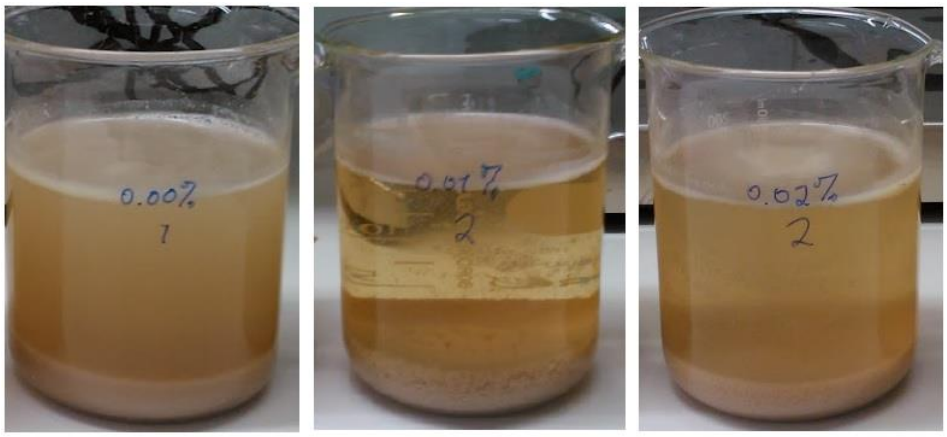

Figure 15b: **An excerpt of pictures of chitosan-flocculated pH 5.0 extracts**. The beaker marked 0% is a control without flocculant, thus the observable sedimentation is of natural nature (1 h). The other beakers are flocculated with 0.01% and 0.02% chitosan. Only 3% of the turbidity remained in the 0.01% chitosan flocculated extract compared to the control, while 11% remained in the 0.02% chitosan flocculated extract.

3.4.2 $FeCl_3$

The first attempt to use $FeCl_3$ as a flocculant showed that increasing concentrations of $FeCl_3$ led to decreasing enzyme activity (figure 16). At 0.4 g/l, the drop in activity stagnated some but at this point relatively low activity was left (46%). Also at this point, the turbidity decreased visibly compared to the control with natural sedimentation, but still over 30% of the control turbidity remained.

The second attempt to use a buffer-diluted $FeCl_3$ solution as a flocculant failed to decrease the turbidity of the extracts (data not shown).

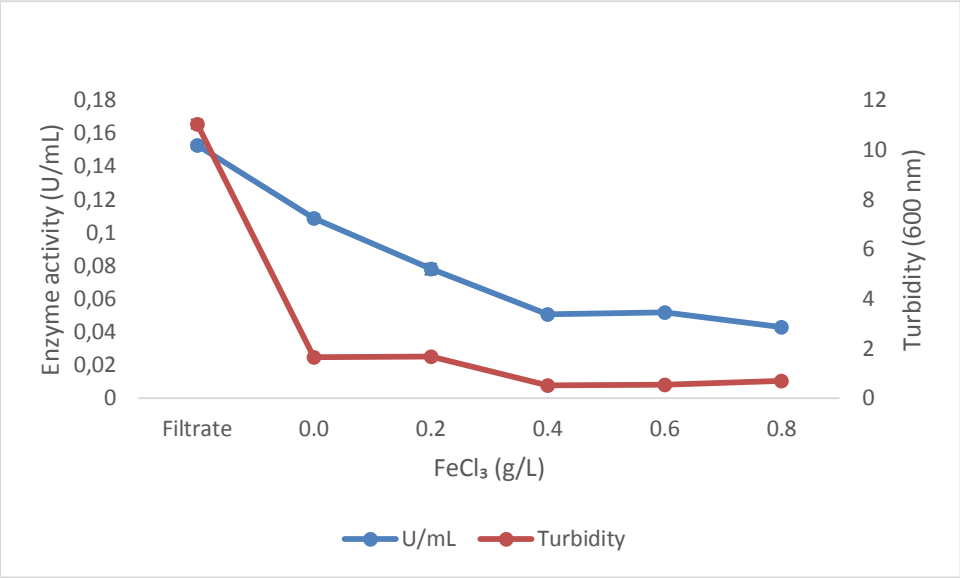

Figure 16: **Enzyme activity (U/ml) and turbidity of pH 5 extracts from herring rest raw material flocculated with $FeCl_3$.** Activity and turbidity measurements are from filtered, unsedimented extract, controls with natural sedimentation (no added flocculant) and extract samples flocculated with 0.2, 0.4, 0.6 and 0.8 g/l $FeCl_3$ (80 ml total volume). Enzyme activity is a measurement of pNP formed from pNPb (substrate). Because of low standard deviation values, some were excluded from the figure. Standard deviation ranges from $4.4*10^{-4}$ to $3.2*10^{-3}$ U/ml and turbidity from $7.0*10^{-3}$ to $1.7*10^{-1}$.

3.4.3 Flocculation with set flocculant concentration at different pH

When the optimal concentrations of chitosan as flocculant were decided, an experiment was conducted to see how the flocculation process worked on extracts made at nearby pHs. It was found that for chitosan, pH 5.0 was indeed the best condition for the process. Although the turbidity was lower at pH 7.0, there was a greater loss in enzymatic activity, than in the pH 5.0 flocculated extracts (figure 17).

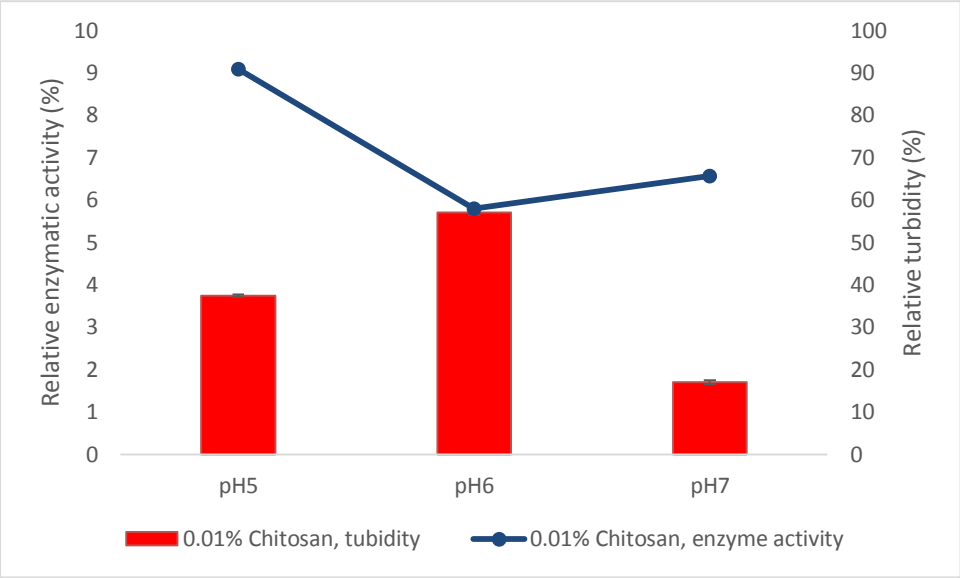

Figure 17: **Enzyme activity and turbidity measurements of herring rest raw material extract flocculated with 0.01% chitosan at different pH.** The flocculant concentrations were used on extracts made at pH5, pH6 and pH7 (200 ml total volume). Values (%) given are relative to controls (natural sedimentation). Enzyme activity is a measurement of pNP formed from pNPb per min.

Sub-conclusion for clarity of extract when flocculated: At this point, chitosan seemed to be the best option for flocculation as it resulted in low turbidity, while the enzymatic activity in the extracts remained relative high, compared to $FeCl_3$, which resulted in a higher loss of enzymatic activity.

3.5 Enzyme activity of flocculated and ultra-filtered pilot scale extracts

As a standard extraction method had been chosen and an optimal concentration of the flocculant chitosan had been found, a pilot-scale experiment was conducted to evaluate how these methods, developed in the laboratory, worked when applied in larger volumes. In addition, ultrafiltration was tested to see if the extract could be further purified, if an industrial scaled production were initiated.

The enzyme activity measurements of the extract through the extraction and clarification process, showed enzymatic activity in the extract throughout the entire process (figure 18). However, the activity measurement of the supernatant after flocculation indicated that a substantial portion of the enzymes bound to the flocs and were lost to the sediment. During the flocculation with chitosan in pilot-scale, over 64% of the activity found in the crude extract remained in the supernatant (U/ml). The membrane giving a cut-off value of 10 kDa, resulted in a permeate with no enzymatic activity, but considering that the retentate was about 3 X concentrated supernatant, the enzymatic activity yield was relatively low. Table 3 shows that

the flocculation process resulted in a 3-fold purification, which was not increased by ultrafiltration. However, the ultrafiltration did cause a substantial decrease in yield.

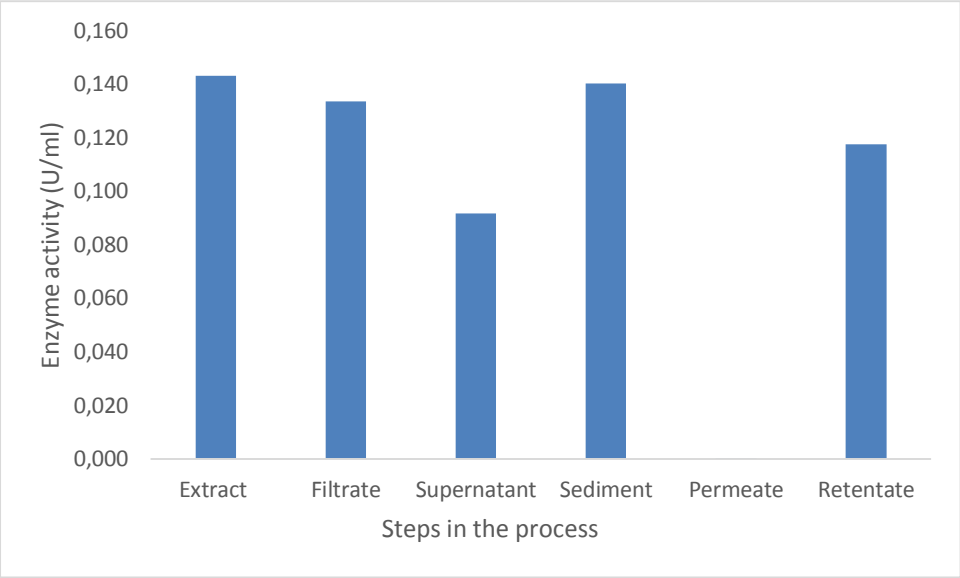

Figure 18: **Enzyme activity (U/ml) of the herring rest raw material extracted in pilot-scale.** Enzymes were lost in some of the steps in the process, particularly during flocculation (sediment) and ultra-filtration.

Table 3: **Outcome and yield of pilot-scale extraction and purification of enzymes with esterase activity from undifferentiated herring rest raw material.**

Purification step	Total protein g	Total activity Units	Specific activity (Units/mg) $*10^{-3}$	Purification (-fold)	Yield (%)
Extract	6300	14316	2.3	1	100
Supernatant	762	5814	7.6	3	41
Retentate	296	2172	7.3	3	15

Sub-conclusion pilot-scale extraction and flocculation: The extraction in pilot-scale resulted in an extract with approximately half the specific activity (U/mg) from that of the initial pH 5.0 extracts made in the laboratory in enzymatic activity. In addition, a large fraction of the enzymes was lost during the flocculation and ultrafiltration procedures.

3.6 Extraction and flocculation with ultra-fresh rest raw material

Ultra-fresh rest raw material was used to evaluate how the quality/freshness of the rest raw material influenced the enzymatically active content of the extracts from herring rest raw material.

Extraction from ultra-fresh rest raw material was done at pH 5.0 in pilot scale in accordance to section 2.2.1, but with 1/10 raw material/buffer ratio. The extract was used to test multiple flocculation methods in lab scale. The activity measurement of the filtrate showed that this raw material contained material with a lower activity than material used earlier, and thus resulted in lower enzymatic active material in the flocculated samples (figure 19).

The enzymatic activity of the control was 0.06 U/mL, which means that 64% of the enzymatic active content remained from the filtrate sample, after which there was no substantial decrease in enzyme activity. In the 0.001% chitosan flocculated sample, 72% of the control turbidity remained. The lowest turbidity was found at 0.01% chitosan where only 6% of the control sample debris remained.

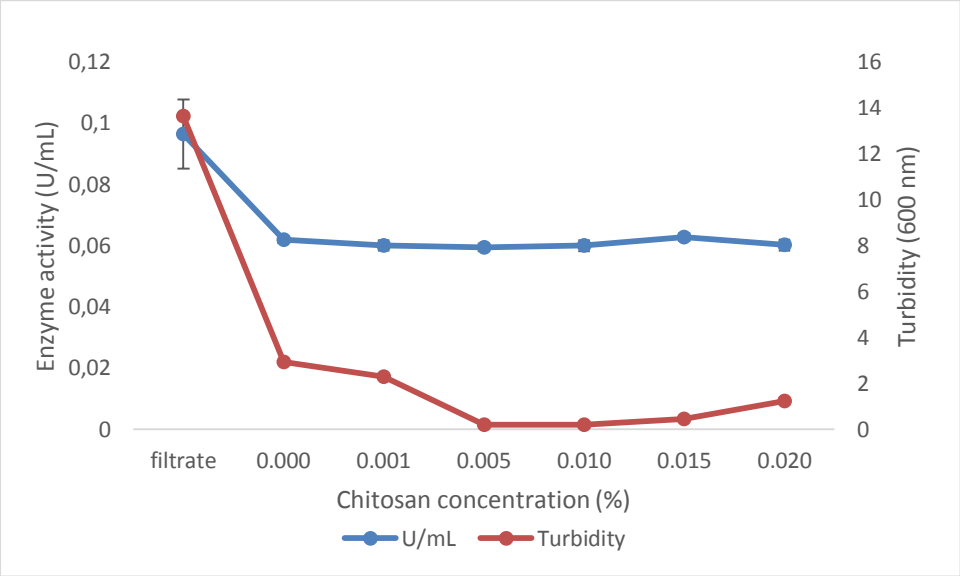

Figure 19: **Enzyme activity and turbidity measurements of flocculated extracts from ultra-fresh herring rest raw material.** Activity and turbidity measurements are from filtered, unsedimented extract, controls with natural sedimentation (no added flocculant) and extract samples flocculated with 0.001, 0.005, 0.01, 0.015 and 0.02% chitosan (200 ml total volume). Enzyme activity is a measurement of product formation (pNP) from pNPb, per min. (Note that the primary y-axis' range is from 0 to 0.12, not 0 to 0.18 as the other chitosan flocculation charts).

To get an indication of how freezing the raw material influenced the enzymatic activity of extract, the raw material used for ultra-fresh extraction was stored at -30°C for approximately two months before another extraction was performed.

The enzymatic activity of the filtrate sample was 0.15 U/ml and dropped to 0.08 U/ml in the control. This means that 53% remained, and the flocculation caused little further loss of activity.

The turbidity was at its lowest when 0.01% chitosan was added. Only 4% of the turbidity in the control remained, but there was an overall low turbidity at all samples with concentrations of chitosan between 0.005 to 0.02% (figure 20 a and b).

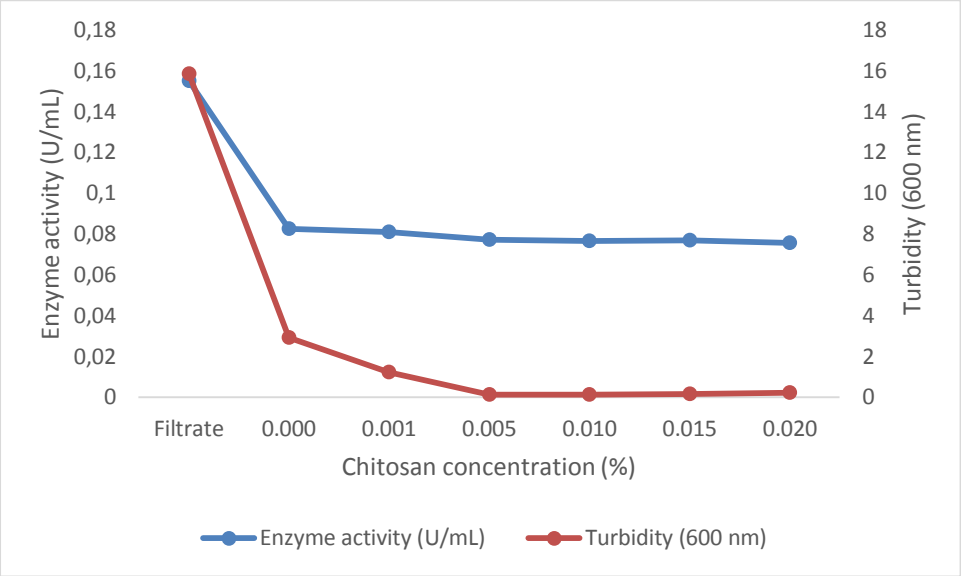

Figure 20a: **Enzyme activity and turbidity measurements of extracts from frozen and thawed ultra-fresh herring rest raw material.** Activity and turbidity measurements are from filtered, unsedimented extract, controls with natural sedimentation (no added flocculant) and extract samples flocculated with 0.001, 0.005, 0.01, 0.015 and 0.02% chitosan (200 ml total volume). Enzyme activity is a measurement of pNP formed from pNPb per min (substrate).

Figure 20b: **Extract flocculated with 0.01% chitosan** (left) **compared with control (0%)** (Right)**.** The extracts are from raw material stored at -30°C for two months. The flocculated extract had a turbidity of 0.12, which was a 96% decrease in turbidity from the control with 2.91.

There was a difference between enzymatic activity of extracts from the rest raw material when it was ultra-fresh and after two months storage at -30°C. In general, the activity was higher in extracts made from the frozen rest raw material. There was a 62% increase in the enzymatic activity in the filtrate of the extracts made from frozen raw material, compared to that of ultra-fresh raw material. However, the decrease in activity from the filtrate to the unflocculated, but sedimented, control was higher in the extract from frozen raw material, 47% compared to 36% in activity in the ultra-fresh extracts, respectively (figure 21).

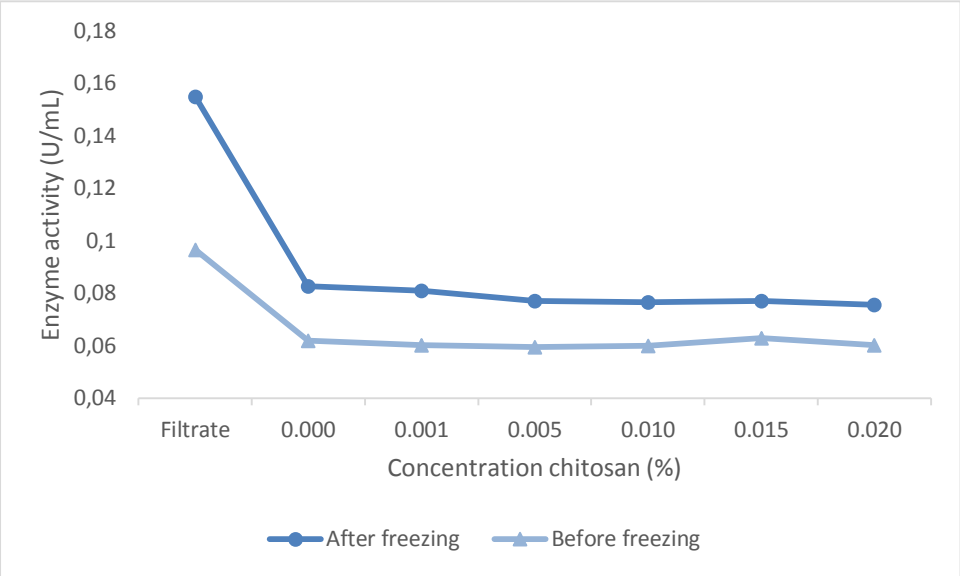

Figure 21: **Difference in enzyme activity (U/ml)** of extracts made from ultra-fresh herring rest raw material before and after it had been frozen and thawed. The ultra-fresh material had 61% higher enzyme activity after freezing/thawing.

As a little additional experiment, the stored, previously ultra-fresh raw material was also flocculated with 0.4 g/L $FeCl_3$ in duplicate (figure 22). The resulting supernatant had an average enzyme activity of 0.06 U/ml and turbidity of 1.96. Compared with the results presented in part 3.4.2, where over 30% of the turbidity remained after flocculation with 0.4 g/L, this experiment resulted in less debris removal. Here 67% of the turbidity remained, though there was more loss of colour in the extract.

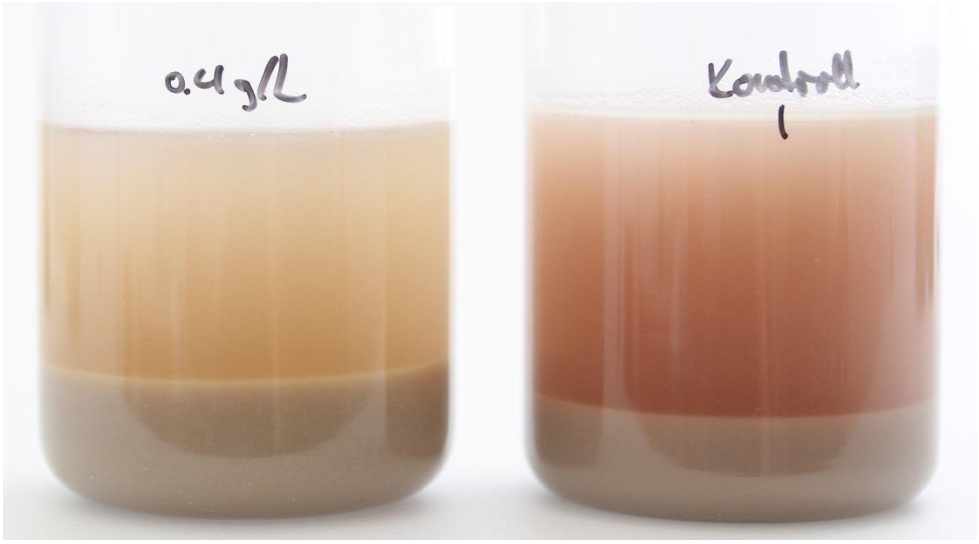

Figure 22: **Herring rest raw material extract flocculated with 0.4 g/L $FeCl_3$** (left) compared with control (right) containing no flocculant. The flocculated extract sample had a turbidity of 1.96 while the control had 2.91. In addition, a decrease in the colour of the extract can be observed.

Sub-conclusion, the use of ultra-fresh rest raw material for extraction: The process of freezing/thawing the rest raw material before extraction appears to have a noteworthy effect on the composition of the extract, causing higher enzymatic activity of esterases in raw material after its been frozen.

3.7 Temperature tolerance of the enzymes

To get an indication of how the enzymes would tolerate the working conditions at NAMAB, where there is no possibility of keeping the temperature down (< 12°C) during production, a temperature tolerance experiment was performed.

The measurement of enzyme temperature tolerance in the pH5-NH extracts indicated a relatively stable activity over time at room temperature and 37°C. At 45°C, the activity seemed to decrease to some extent, whereas it at 60°C showed a significant drop in activity after only 2.5 minutes (figure 23).

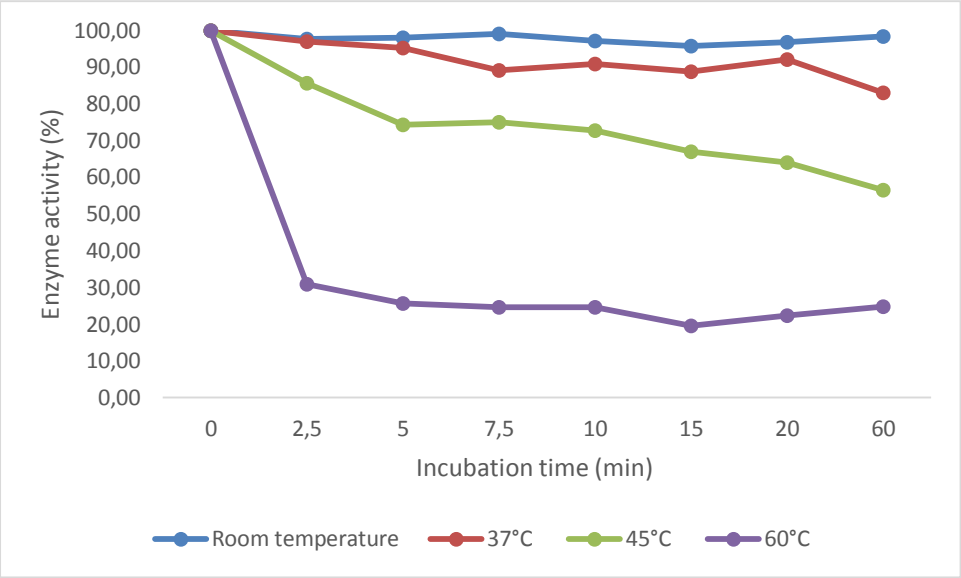

Figure 23: **Temperature tolerance of enzymes** in the pH5-NH extract measured over time incubated at room temperature (RT), 37, 45 and 60°C. The enzyme activity is relatively stable at RT and 37°C during one hour of incubation. At 45°C, 56% of the measured enzymatic activity is left after one hour incubation. Only 31% enzymatic activity is left after 2.5 minutes at 60°C.

Sub-conclusion temperature tolerance of enzymes: The experiment show that at least one of the enzymes is fairly temperature stable up to 45°C.

3.8 Suggested method for extraction at NAMAB

NB! This part of the results is a theoretical presentation of how a large-scale extraction could be performed at NAMAB. It has not physically been executed, thus, it is presented in the results section.

An amount of 800 l water heated up to 15°C is transferred into one of the reaction tanks. An amount of 200 kg cold undifferentiated herring rest raw material gets grinded and transported into the tank by conveyor screws. An additional 970 l of water is added, and 30 l of 3M sodium acetate, pH5 buffer is manually poured into the tank. The solution is set to stir for 1 h at 50-70% agitation with a temperature set point at 12°C.

After the extraction is completed, the extract is dropped down to the conveyor screw which pushes the larger solids onto the shaking table where it is removed from the product line. The fluid sieved through the table is pumped to the drop tank. There it reunites with the main fluid, which is sieved into the drop-tank as it exits the reaction tanks, and is pumped to the decanter. The decanter separates the extract into oil and stick water, while smaller debris from the extract is removed from the product line.

The stick water is pumped to a large holding tank (2 m^3) where 0.01% chitosan is added and left to flocculate over night. The sediment is drained until clear extract is visible, which is collected into suitable containers. A flow chart containing the elements that would be used is presented in figure 24.

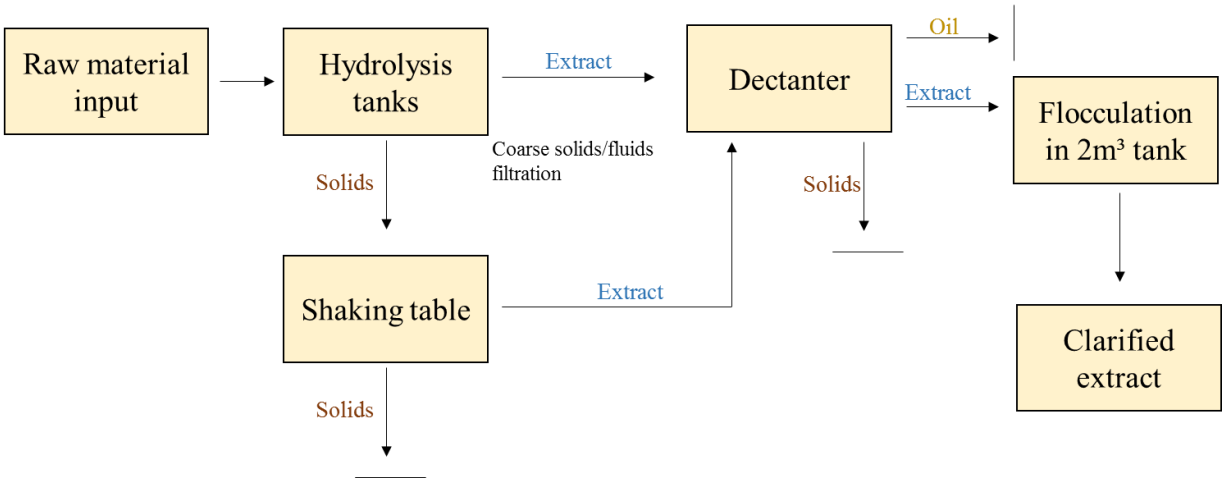

Figure 24: **Suggested flow chart of the process line for extraction of enzymes with esterase activity from herring rest raw material at NAMAB.**

4 Discussion

In this project, enzymes with esterase activity were extracted from undifferentiated herring rest raw material, both homogenized and non-homogenized, at different pH conditions ranging from pH 3.0 to 10.0. In previous research on herring rest raw material at Nofima AS it was found that one of the enzymes with esterase activity could be of commercial interest (Lindberg *et al.*, 2014;Myrnes *et al.*, 2014). In their study, a method of pH 9.0 was chosen to isolate esterases, but in this master project, an optimal standard extraction procedure at pH 5.0 from non-homogenised herring rest raw material was defined and analysed more closely. Flocculation with a polymer and a metal salt was tested, as methods to clarify the extracts. Extraction and clarification was performed in a pilot scaled plant. A protocol that could be used in an industrial scaled production was proposed. It is not in the scope of this project to elucidate the characteristics of the enzymes extracted, other than determining the number of enzymes with esterase activity present, or to purify the enzymes to any extent beyond the flocculated extracts.

Other studies describes how enzymes are extracted from fish processing waste, but few offers any detailed solution for up scaling (Esposito *et al.*, 2009;Daboor *et al.*, 2012). There are also a very recent paper that focuses on the utilization of herring rest raw material, but through hydrolysis of the material (Slizyte *et al.*, 2014).

4.1 Defining the optimal protocol for extraction of enzymes with esterase activity

There were several factors to consider when deciding upon the best protocol. First, the measured enzyme activity of the extracts was important. Secondly, it was essential to observe how the different extracts behaved during the extraction, and thirdly, the possibility of implementation of the protocol in an industry scaled facility.

The initial extraction experiments yielded different volumes of extracts at the different conditions. Thus, to get the most comprehensive insight into the enzymatic activity of these extracts, the total enzymatic activity (U) was mostly used when comparing the different extracts.

4.1.1 Homogenized or non-homogenized rest raw material for extraction

Two types of herring rest raw material, SH and NH, were initially used in the conducted extraction experiment. Though both types of treated material were grinded to some extent, the NH material had visible bits of fish skin and bone. There were some differences in the results

from the enzyme activity measurements between the SH and NH extracts. When comparing the corresponding NH and SH extracts, only the pH 6.0 extracts gave a significant difference in total U, with a significance level of 0.05. Thus, the pre-treatment of the raw material had relatively low influence on the enzyme activity outcome. However, as the process step of homogenizing might cause a substantially higher workload in the up-scaled experiment, especially in large-scale, it was initially deemed more beneficial to proceed with the NH raw material as the starting material for the extractions.

4.1.2 The optimal extraction protocol based on enzyme activity of the extracts

Extractions performed at different pH resulted in extracts showing varying degrees of enzymatic activity. The extracts are referred to in accordance with the pH of the buffers added to the raw material, but the pH was not calibrated after mixing the two. Thus, because of the natural buffer capacity of the raw material, the final pH of the extracts deviates from the pH of the added buffer. The main reason for not calibrating the final pH of the extracts, was that this would be difficult to do during a large-scale extraction, so it was more beneficial to get the results given when a more large-scale feasible method was used.

Extracts at pH 9.0 showed the highest total activity (U). The highest specific activity (U/mg) was found in the pH 3.0 and 4.0 extracts. If the only concern in choosing upon a protocol for extraction were the given enzymatic activity, the extract made at pH 9.0 would have been used in to the subsequent experiments for clarification and up-scaling.

In regards to the extract outcome of the different conditions for extraction, no previous work could be found to compare it to. However, the enzymes with esterase activity found earlier in the herring rest raw material project at Nofima (pH 4.5 and 9.0 extracts), correlates to the findings in this, current, project. This can be detected in in-gel detection of enzyme after native PAGE (see section 4.1.3), but the characteristics and optimal extraction conditions for each enzyme is still unknown. It is unclear how each distinct enzyme detected in the native PAGE, affect the enzymatic activity reads, as the results of the enzymatic assay performed on the extracts gives the combined activity of all the enzymes with esterase activity. It is important to be aware of this, as only one of the enzymes has so far been found to be of potential commercial value, based on research in the project "gold from the silver of the sea" at Nofima. Very little is known of the other enzymes of esterase activity in the extract, thus focusing in those at this stage could be a waste of resources, as they might not have any of the qualifications required

for commercial use, such as stability under different conditions. However, this should be verified by isolation and characterisation of the different enzymes.

In regards to the enzyme activity, it is important to consider that a generic substrate was used. There are both advantages and disadvantages in use of such. As an example in esterase research, one article states that the hydrolytic activity of their lipase towards pNPb was two times higher than towards its natural lipid substrate (Holm & Østerlund, 1999). Therefore, even though the enzyme exerts much activity with pNPb, it is not certain that it would give the same activity reads with its natural substrate or when applied for some commercial process. The mentioned article explains how the non-specificity of the substrate (can be hydrolysed by almost all eserases) is a disadvantage. However, when little is known of the esterase in question, also mentioned earlier in the case of this project, generic substrates can be an advantage in preliminary research. The generic substrate gives an overview of all the esterases in the solution, even though little is known of what the natural substrate of the enzyme is.

In research regarding bioactive compounds extracted from organisms it is important to be aware of the possibility that molecules found can originate from the microflora of the organism. It is not certain whether this is the case or not for the enzymes detected in this project.

4.1.3 Variation in MUF-butyrate cleaving enzymes of the extracts

Native PAGE was carried out on an aliquot of all the extracts (SH and NH, pH 3.0 to 10.0) and then the gels were subsequently subjected to a solution containing MUF-butyrate resulting in a fluorescent product, when hydrolysed by esterases present in the gels. From the results presented in figure 11 and 12 it is clear that the pH of the extraction buffers influence the resulting enzymatic material in the extracts. At most, four distinct bands with esterase activity were detected. Two fluorescent bands around 60 kDa appeared common for all the extracts. Previously in the herring rest raw material project at Nofima, they found that one of the enzymes constituting these ~ 60 kDa bands with esterase activity were commercially interesting, as one of them proved to be stable under several conditions, such as temperature and pH. Though the bands were present in all the wells of different extracts, they were relatively weak compared to the other observable bands. One of them was barely visible in the pH 3.0 and 4.0 extracts, but it became stronger in the pH 5.0 extract, and continued with the same fluorescent strength up to the pH 9.0 extract, after which it was weaker in the pH 10.0 extract. Although the fluorescent bands are referred to in accordance to their position relative to that of the ladder, it is important

to state that their placement in the lanes are not just based on their size. As the enzymes are in their native form, their hydrodynamic size and charge influence their progression through the gel during electrophoresis (Wittig *et al.*, 2006).

The bands that were marked as fluorescent under UV-light were barely visible after Coomassie staining. The ~60 kDa bands could not be observed at all. This might be because of very low concentrations of the enzymes, and thus be below the detection limit of the Coomassie staining method, though the reagent manufacturer states that as little as 7 ng protein (reduced BSA) can be detected (lifeTechnologies^{TM}, 2014). There is also the possibility that the enzymes are glycosylated, as such proteins are known to not stain properly with Coomassie (Møller & Poulsen, 2002). Glycosylated esterases have been found in other teleost fish species, so it is not unlikely that such enzymes are present in the herring as well (Leibel, 1988).

Increasing pH in the buffers used for extraction, resulted in higher numbers of bands with esterase activity observed in the gels, with the exception of the pH 10.0 extracts. This coincides with the increasing activity measured with increasing pH (table 2). The highest number of fluorescent bands were found in pH 6.0 to 9.0 extracts, with four bands of esterase activity detected. As mentioned previously, these extracts showed the highest total esterase activity, and with four different enzymes with esterase activity present in the extracts, a larger number of different enzymes constitutes the activity obtained. However, attention should be given to the largest bands (~700 kDa) present in the gels presented in figure 11 and 12. A schematic overview of the enzymes present in the gels is given in table 4. There are some indications that these enzymes does not contribute to the enzymatic activity measured with pNPb as substrate. In the pH 8.0 and 9.0 extracts, these bands are extremely fluorescent compared to all the others. Although these two extracts have a little higher activity per ml than the other extracts, it does not seem to be a difference of such a magnitude to explain the much stronger fluorescence. It might be that those enzymes are more efficient in hydrolysing MUF-butyrate. Since the enzyme constituting these bands has not yet been found to be of commercial interest, it is not discussed any further, but one should be aware of it when interpreting the data.

If an industrial production was to be realized, the enzyme of interest would have to be further purified after flocculation, and it is likely that the other enzymes that contributes to the enzymatic activity of the extracts would be removed. This may give a completely different enzymatic activity. Thus, solely focusing on the given enzymatic activity in the current,

relatively crude extracts, that combines the activity outcome of all the enzymes present, would be wrong when deciding upon the optimal extraction protocol.

Table 4: **The distribution of bands with esterase activity after Native PAGE runs with extracts made from undifferentiated herring rest raw material.**

Extract		Band I ca. 700 kDa	Band II ca. 300 kDa	Band III ca. 60 kDa	Band IV ca. 60 kDa
SH/NH	pH3			x	x
	pH4			x	x
	pH5		x	x	x
	pH6	x	x	x	x
	pH7	x	x	x	x
	pH8	x	x	x	x
	pH9	x	x	x	x
	pH10	x		x	x

Studies on esterase from herring have been conducted previously, though mostly as a genetic tool in population studies, some results can be compared with this study. Ridgway *et al.* proposes that there are at least four sets of esterases in herring and that all can be considered classified as aliesterases (carboxylesterase). They found a set of small enzymes in the gel that they named set 1, and these might be the same as the ~60 kDa esterases found in the research for this thesis (Ridgway *et al.*, 1970). Nævdal *et al.* also found four groups of esterases in herring, this in fish from the Northeast Atlantic, and state that there was variations in the phenotype from spring to autumn spawning herring (Nævdal *et al.*, 1997). Although this was done with the purpose of elucidating variation and genetic background between herring populations, this can be considered to support this project's findings of four distinct bands with esterase activity.

4.1.4 Characteristics of standard extract made at pH 5.0 from non-homogenised herring rest raw material

As already mentioned, it was decided to proceed with the pH 5.0 extract from the non-homogenized raw material. It is not the extract of highest enzymatic activity, however, we planned to use chitosan as a flocculant to purify the extract. Riske *et al.* has stated that it provides the best decrease in turbidity at a pH range from 5.5 to 7.5 (Riske *et al.*, 2007). This might imply that the pH 6.0 or 7.0 extracts were preferable, as samples produced under these conditions showed a higher total enzyme content (total U). Nevertheless, in these pH 6.0 and

7.0 extracts, there is an additional enzyme constituting the enzymatic activity, compared to the pH 5.0 extract, and the pH 6.0 extract proved to be a little difficult to work with, because of its behaviour during extraction and clarification. The outcome of enzymatically active material, detection of the ~ 60 kDa bands after electrophoresis, and handling ability of the various extracts all influenced the choice of the pH 5.0 extract.

4.2 Flocculation for purification of herring extracts in regards to up-scaling

At a larger scale, the initial freezing/thawing process ahead of the extraction procedures would not be possible to conduct (described more detailed in 4.4), thus another method had to be applied. It was decided to try some common flocculants for the removal of debris from herring rest raw material extracts in a purification step. Chitosan and $FeCl_3$ were used, so that both a polymer and a metal salt was tested.

As the flocculation experiment started, both chitosan and $FeCl_3$ were in focus and were tested on extracts made at same conditions. The pH of the flocculant added extracts were not calibrated back to their original pH, other than in the first $FeCl_3$ experiment, as this would be difficult to achieve in a large-scale production. The results indicated that chitosan might work better at concentration lower than 0.02% while $FeCl_3$ gave the lowest turbidity around 0.4 g/l. However, already at this point it was apparent that the iron-based flocculant caused a substantial decrease in the enzyme activity of the extract, which might be due to a relatively high drop in pH. In addition, traces of $FeCl_3$ in the enzyme product may limit its use to industries with products not intended for human use. Thus, the focus on chitosan as flocculant increased and little effort was made to further optimize and follow up the use of $FeCl_3$ as flocculant. Others have also reported decreased enzyme activity when using inorganic flocculants, such as $FeCl_3$, for example the decrease of α-amylase activity after flocculation in an attempt to clarify a bacterial broth (Bajpai *et al.*, 1990). However, successful experiments with this flocculant have also been reported, with one example of enzyme recovery from shrimp waste where the clarification was achieved with ferric chloride and ultrafiltration (Olsen *et al.*, 1990).

Though the flocculation resulted in rather clear extracts, some problems were evident. First, not all of the fat was removed from the solution and a thin layer of it could usually be observed in the surface. In the event that further analysis were to be conducted on the extracts, the fat might interfere with the analysis methods. Secondly, and of greater concern, was the drop in enzymatic activity that was observed in the samples (figure 15 and 16). It is likely to speculate

that some of the enzymes bound to the floc and fell down in the sediment along with the unwanted debris. However, this is probably not only because of the flocculant present. The main activity drop may be caused by the natural sedimentation in the samples. This is indicated by the decrease in enzymatic activity from the extract filtrate, to the sedimented control sample. The amount of activity left in the control without flocculant, compared with the filtrate, varied between the different flocculation experiments, but usually there were about 70% activity left in the control supernatant. However, when Native PAGE was run on some of the flocculated extracts, only the two ~60 kDa bands were visible when subjected to MUF-butyrate. In the initial pH 5 extracts there were three bands present. This could indicate that the enzymes constituting the third band was removed in the flocculation process, and all of the enzymatic activity measured in the extracts after flocculation were from the enzymes of ~60 kDa. There is one problem with this reasoning though. The third fluorescent band was not present in the 0% chitosan or the pH 5.0 extract from the initial experiment where freezing/thawing was a step in the clarification process. If the flocculants were the cause for the removal of the enzymes that constitutes the third band, it should have been present in the two mentioned extracts. It can be postulated that the samples had been frozen for too long, and that the missing enzyme were not stable under such condition over a longer period, but this was not the case for all of the extract samples. The extracts made from ultra-fresh raw material, had only been stored for few weeks. All of these uncertainties regarding the effect of the flocculation on the enzymes in the extracts, makes it difficult to make any sound conclusions about this topic at this stage.

If flocculation is to be implemented in an industrial process it would be preferable to perform further analysis for optimization before this commences. Based on the results obtained until now, the loss of enzymes to the sediment can be regarded as too high. However, it is not certain if only one of the enzymes are removed from the extract during flocculation, or if a fraction is lost of all the enzymes with esterase activity, causing the reduced enzymatic activity in the flocculated extracts. It would be beneficial to have a better understanding of how the flocculation influences the contents of the extracts.

4.3 Ultra-fresh rest raw material for esterase extraction

In processes where raw materials are used the quality of the material is usually of great importance (Gildberg, 2002). Because of this, ultra-fresh rest raw material is regarded as optimal. This is defined as raw material that has not been frozen, and preferably produced the same day as it is utilized. It was assumed that by using such ultra-fresh raw material in this

project, it would provide insight into how the freshness of the raw material influences the enzyme yield and enzymatic activity of the material in the extracts.

The resulting enzymatic activity yield from extracts of ultra-fresh rest raw material deviated from the earlier flocculated extracts, from frozen and stored raw material. The filtrate showed relatively low enzymatic activity; it was measured to 0.09 U/ml. In the earlier flocculation experiments, the enzymatic activity was between 0.15 to 0.18 U/ml. To examine whether this difference in activity detected was because of the quality of the raw material itself or if there might be some other factor that had an impact on the enzymatic activity, another extraction was performed after two months storage (at -30°C) on the same raw material. The filtrate sample from the second extraction (on frozen and stored, previously ultra-fresh material) showed higher enzymatic activity, 0.15 U/ml, compared to the filtrate from ultra-fresh raw material extract. Although the enzymatic activity was higher in the extract from frozen and thawed ultra-fresh material, the decrease in activity between the samples when comparing the activity in the filtrate to the control sample that was set to sediment without flocculant, were even higher. When the material was ultra-fresh, 64% of the activity remained in the liquid phase of the control, while only 53% did after freezing. However, the activity of the flocculated samples from ultra-fresh material were still generally lower than in extracts from frozen material. The reason for this difference in activity (figure 21) can only be speculated on at this stage. It may be possible that there are some enzyme inhibitors present is in the raw material that no longer function after being frozen. Another possibility is that the freezing of the raw material, which does not happen at a very fast rate, creates large water crystals inside the cells causing them to burst and thus release intracellular enzymes. Further on, when thawed, more enzymes will be dispersed in the solution.

Moreover, it should be stressed that these experiments were not performed optimal for completely comparable studies since the first extraction was done in pilot scale (150 l), while the second one was done in lab scale (3 l). The reason for this was a restricted supply of raw material, and the work capacity required for pilot scaled experiments when larger quantities were available. Considering this non optimal way of comparing these experiments, the results can still be used to interpret differences in outcome of unlike pre-treatment (fresh or frozen) of the raw material. These different extraction methods may have influenced the difference in enzymatic activity, but the first pilot scale extraction performed on thawed raw material still

had an activity measured to 0.13 U/ml, which is higher than the 0.09 U/ml of the ultra-fresh raw material.

A possible explanation for the different enzymatic activity yield of the extracts from the raw material, or the different behaviour when flocculated, is the fact that the fish was landed at different times of the year. The ultra-fresh raw material came from fish landed in January, while the other was landed in November. Though both landings happened during the wintering period of the fish, it is likely that there is some variations of the contents of the fish, especially the amount of fat, which is presumably lower in January than in November as the fish barely feeds while overwintering (Huse & Ona, 1996;Slotte, 1999). Thus, it is likely that different enzymes are expressed in the fish in these periods, as some enzymes may be of no use when there is no food present that need to be digested.

4.4 Pilot scale extraction and flocculation

At the beginning in the pilot-scale part of this project, it was known that the process line in the pilot scale plant was limited compared to what was available in the large-scale facility, NAMAB. Equipment that is common in a production line for bioprocessing was lacking. No decanter or centrifugation separator was available. However, as it was planned to use flocculation as a step in the purification process, this lack of equipment was not considered a major issue, although it would be beneficial to observe how the extract and the following enzymatic activity are influenced by the decanter separation step. In the proposed large-scale extraction process the decanter is involved without any knowledge of the outcome such a process step will give.

In the pilot-scale experiment, only a single flocculation process was tested, with 0.01% chitosan. When comparing the supernatant, or clear phase, with the filtrate, almost 70% of the enzymatic activity remained in the supernatant (U/ml). This correlates with the results from the lab scale experiments mentioned in section 4.2, which indicate that, concerning the enzymatic activity, the flocculation process gave the same enzyme yield applied in pilot-scale. In this experiment where the enzymatic activity of the sediment after flocculation was also measured, and it is apparent that a substantial amount of enzyme is lost in the floc formation. The resulting clear phase was then ultra-filtered with a 10 kDa cut-off value. Thus, all enzymes with esterase activity should have been contained in the retentate as the smallest one detected had a size around 60 kDa. This is supported by the fact that no enzymatic activity towards pNPb was

measured in the permeate after ultrafiltration. However, as a large fraction of the extract fluid went into the permeate (the retentate was concentrated about 3 times), it was expected that the retentate would hold a higher amount of enzymatic activity in per ml. This was not the case. The enzymatic activity of the retentate was measured to 0.12 U/ml, which is only a little higher than the 0.09 U/ml measured in the supernatant of the flocculated extract. When the volume of the extracts at these steps in the process are taken into consideration, only 37% of the total enzymatic activity remained in the supernatant after ultrafiltration. Thus, the process of ultrafiltration does not seem to agree with the enzymes of esterase activity, as it is likely that they are deactivated. Initially it was thought that ultrafiltration would be successful in concentrating the enzymes, as diafiltation had shown good results in the laboratory during the two projects *gold from the silver of the sea* and *HerrZyme*. The ultrafiltration did cause a lot of foam formation, which could contribute to the deactivation of enzymes, or some of the enzymes could have been stuck to the membrane, preventing them to go in the retentate (personal communication). However, others have described methods to stabilize enzymes during membrane filtration (Esterbauer *et al.*, 1991;Woltinger *et al.*, 2001), though it is not esterases that is mentioned, it does open up for consideration of ultrafiltration as a step in potential further purification of the enzymes in this project, when more is known of the enzymes at hand.

Before any industrial production is initiated, it is recommended that additional pilot-scale experiments should be performed and perhaps to a larger extent than accomplished in this project. Particularly considering variable conditions in the plant, such as the method for stirring in the flocculant. In addition, further insight into how the temperature of the flocculation process affected the enzymatic activity and the degree of flocculation, would be preferable in the evaluation of the theoretical large-scale process.

4.5 Large-scale extraction of herring rest raw material at NAMAB, and identified challenges in the process of up-scaling and industrial production

Upon evaluation of the opportunities present at NAMAB, a method for extraction from undifferentiated herring rest raw material was proposed in section 3.8. Although this method is assumed to be the best option for extraction with the layout of the factory as it is today, there are several issues that cannot be bypassed without further development of the facility. This includes, e.g., that of the feed-in processes of raw material to the reaction tanks, no possibility of cooling throughout the process and lack of stirring equipment in holding tanks.

There are many equipment units available at NAMAB for the separation of liquids and solids, such as a three-phase decanter, centrifugation separator, membrane filtration systems and a variety of sieves. For the large-scale process, it is suggested not to use the separator, as the flocculation process should remove the little debris left after going through the decanter. In addition, excluding the separator could lower the expenses related to use of equipment. The flow chart in figure 24 shows the components that is suggested to be used in an extraction process at NAMAB.

4.5.1 Aspects related to production time

In the laboratory scaled process, the extractions were frozen and thawed to initiate a separation of solids, fat and liquid. However, this was mostly to elucidate the contents of the various extractions, as the freezing thawing method would not be applicable in larger scale. This process would take days to finish with hundreds of litres extract and degradation by endogenous enzymes would likely occur in those parts of the extract first thawed.

By experience from observing production at NAMAB it is clear that the bottleneck of productions is the ultrafiltration system and the evaporators if those are used. As neither of these are included in the suggested product line for this project (see flow chart, figure 24), it does not pose an issue in time consumption, however, there is one step in the large-scale production that causes uncertainties in the time aspect for the production line. The flocculation method showed potential, at least at pilot scale, but when it comes to large scale, it is more uncertain. It proved to be more time consuming to reach an acceptable amount of sedimentation in about 90 l of extract than in the laboratory though the ratios were the same. Although it is not certain, it would presumably require considerable more time than one night to produce the same level of sediment in extracts that might constitute more than 1000 l. One option to decrease the time span of production is to use the decanter to remove the flocs, instead of waiting for them to for a sediment. However, if this is to be possible, the flocs will need to be stable enough, so they do not disperse during transportation through piping and in the decanter. This would require further research into the stability of the extract flocs.

4.5.2 Temperature control throughout the process

Although there are numerous issues related to the large-scale production that are not negligible, I would say that the main obstacle for this large-scale process at NAMAB is temperature

control. When built, it is obvious that hydrolysis and other processes that require high temperatures and heating were in focus. The facility does not have any methods for keeping the fluid cool during the process. In theory, although not tried in production, it should be possible to start the process with water at the temperature it acquires at the communal water source, which is relatively low. During the extractions in lab and pilot scale it was attempted to keep the temperature around 12°C, thus with a low heating of the process water such a temperature can be achieved. However, once the extracts is in production, as mentioned, there are no opportunities to maintain a low temperature. The temperature tolerance experiment of the pH 5.0 extract showed that the enzymatic activity of the extract is stable at room temperature for 1h. However, the industrial production will far exceed 1 h when flocculation is included. In the *HerrZyme* project, they performed a temperature experiment with the one enzyme of commercial interest, with incubation times over 1 h, where they found that 75% of the enzymatic activity remained after 3.5 h incubation at 37°C (Lindberg *et al.*, 2014). Thus, it is likely that some of the remaining activity presented in figure 23 comes from the enzyme of interest. Although the temperature stability found gives reason to hope that the enzyme can endure the whole process of extraction and flocculation in large-scale production.

4.5.3 Fat contents of the material

A potential problem arises with the fat present in the raw material. Other processes conducted at NAMAB have been performed under high temperatures, in contrast to this extraction of enzymes, as mentioned in the previous section. It is uncertain how the apparatus used would react to the cold, fat containing solutions, if the extraction process was tried in large-scale. The fat would likely start to clot due to the low temperature. This because of the lack of heating that will decrease water-lipid interaction (Cevc, 1993).

4.5.4 Options for optimal flow of the process

The temperature issue is an important one, but there are other potential problems at hand. First, there is the input of the raw material. There are two possible methods of feeding the reaction tanks, and both gives the rise of issues that need to be evaluated.

Method 1:
In the first method, the raw material can either be dropped it into a funnel, which drops the raw material directly down to the conveyor screw that pushes it up to the reaction tanks (figure 8A).

By using this method the raw material will not be grinded or such before extraction, therefore, if the material is to be partly homogenized as in the lab and pilot scaled trials, it will have to be done beforehand.

Method 2:
In the other in-feed method, a conveyor would transport the raw material to a grinder that feeds the processed material into a container where it is mixed with water before it is pumped up to the reaction tanks. By using this method, the raw material would be minced before extraction. However, this method does requires that some of the water binge added together with the raw material to make it possible to pump it through the piping leading to the reaction tanks. This does not necessarily pose a problem, other than perhaps even less control of the reaction temperature. As cold water would be used for the process, the raw material should be thawed if either method is used. If not, it is more likely to cause problems such as blocking the pipes and less control of the solution's properties. Previously this grinder has only been used on frozen raw material, and of a completely different nature, so whether this is feasible or not is unsure.

There is a possibility of combining the two methods. The raw material can be grinded into a container and dropped into the funnel mentioned for method 1. One could argue that the screw might not be able to push the grinded material up to the reaction tanks (there is a small gap between the screw and the bottom), but it have been successful previously with raw material of different nature, though this was frozen. This combination of the two in-feed methods is believed to give extracts that correlates best with the ones made in the laboratory.

As a conclusion for the in-feed issue it is proposed to use thawed, cold raw material through the grinder, and transport it with the conveyor screws into the reaction tanks.

4.5.5 Other challanges

As mentioned, it is uncertain what state the raw material will be in after the grinder, but most likely, there will be some larger bits of fish in it. It is possible that this would cause problems when emptying the reaction tanks. The consistency of the solids is different from that of raw material already tested on the facility. It is stickier than for example the exoskeleton of shrimps. This, and the fact that there probably will be larger pieces of skin in the extract, is likely to cause the first sieve to clog during the coarse separation of fluid and solids. This is also likely

to happen when the solids reach the shaking table (figure 8B), causing fluid to go in to the container for solids, as the clogged sieve prevents the fluid to go through.

When the extract reaches the decanter, this part will contribute a great deal to removing the fat in the extract before it goes to flocculation. However, how the decanter will work with the extract of low temperature is uncertain. Several issues might cause problems during the process of flocculation. Mainly the temperature (see section 4.5.2). There is currently no possibilities available to keep the holding tank cool, so an increase in temperature is not preventable, though one can make an effort to keep the ambient temperature down. In the methods part, it was suggested to drain the sediment until the clear part of the solution is visible. This might be possible by opening the drain valve carefully and keep a low flow, so that the sediment is not disturbed. Another issue is that the holding tanks is of metal, so it is not possible to observe the degree of sedimentation at any time. There is a hatch on top of the tank, but this only gives a view of the uppermost part of the extract, thus poorly contributes to observing the process.

Another issue is the getting the flocculant evenly distributed throughout the extract. There is one tank meant for oil products that can circulate the contents, while heating it. It might be possible to use this, without heating. Another possibility is to use the tank meant for condensate products after evaporation, as it contains an agitator. However, the best solution would be to insert a stirring apparatus in a holding tank intended for stick water.

For NAMAB to be capable of conducting cold bioprocesses, such as enzyme extraction, additional construction work is recommended, to enable cooling of the solutions during production. A more suited holding tank, with an agitation mechanism and opportunity of observing its contents, should be acquired for the possibility of conduction a flocculation process.

4.6 Some perspectives on industrial utilization of marine rest raw material

In regards to utilizing enzymes from marine rest raw material, there are some successful examples, shrimp alkaline phosphatase (SAP) being one of them. Although first isolated from effluents from shrimp processing plants, it is now produced recombinant, giving it more stability and consistent purity (Olsen *et al.*, 1990;ArcticZymes, 2014). With this in mind, if the enzyme of esterase activity in this project is found to be of use in some industry, it is likely to

be produced recombinant by microorganisms when sufficient information of its origin and sequence is obtained.

Although the focus of this project was the extraction of enzymes from the herring rest raw material, it should be mentioned that also the other components, or co-products of the material, can also be utilized, such as the oil and solids removed during clarification of the extract. The quality of the material may not be of such a standard that it can be used for products meant for human utilization, but it might be suited for use in the fish food industry. An article by Olsen *et al.* concludes that products such as fish meal and oil are in great demand these days that they should not be considered as low-value products (Olsen *et al.*, 2014).

The herring fishery has seasonal landings, from November to February (Fiskeri.no, 2014). Thus, the supply of rest raw material will be restricted to these periods. However, in this project it was found that the highest esterase activity was obtained after freezing the rest raw material. Thus, in regards to the enzymatic yield, freezing seems to be beneficial. This opens up for the possibility of obtaining large amounts of raw material during the herring season and store it, enabling production after the season ends. This would require large storage facilities, which increases expenses related to production. Thus, the final product would have to be of substantial value, if any financial gain is to be achieved. One aspect to consider if stock storing the rest raw material was realized, is that long time storage might negatively influence the quality of any potential co-products, particularly the marine oil (Sikorski & Kolakowska, 1990).

5 Concluding remarks

The experiments performed in this project showed that it was possible to extract esterases from homogenized and coarsely grinded undifferentiated herring rest raw material at different pH conditions ranging from 3.0 to 10.0. Undifferentiated grinded herring rest raw material extracted at pH 5.0 was defined as a standard for further experiments. Flocculation with 0.01% chitosan on extracts made at pH 5.0 resulted in clear solutions with much remaining enzymatic activity in laboratory-scale, but also in pilot-scale. Based on the experience from the laboratory and the pilot scaled plant, a method for large-scale production at NAMAB was suggested. The facility provides the opportunity for the bioprocessing of a variety of raw material through its selection of equipment. However, an industry scaled extraction is probably not possible to implement at the given facility in its current state, mainly because it is not adapted for processes requiring cold conditions during production.

This project have given insight into challenges present and factors to consider when up-scaling from laboratory experiments to large scaled production. A lot of work is required in the process of taking finding in the laboratory to a commercially available product.

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7 Appendix

Appendix 1

Table A1: **List of chemicals used in the experiments**

Chemicals	Producer
4-methyl umbelliferyl(MUF)-byturate	Sigma-Aldrich, Missoury, U.S.A.
4-Nitrophenyl butyrate (pNPb)	Sigma-Aldrich, Missoury, U.S.A.
Alginic acid sodium salt (Alginate)	Sigma-Aldrich, Missoury, U.S.A.
Ammonium carbonate	Merck, Darmstadt, Germany
Bio-Rad Protein Assay reagent #500-0006	Bio-Rad, California, U.S.A.
Bovine Serum Albumin	Thermo scientific, Illinois, U.S.A.
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich, Missoury, U.S.A.
Gum arabic	Sigma-Aldrich, Missoury, U.S.A.
Hydamer CMFP (Chitosan)	Chitinor, Haugesund, Norway
Iron (III) chloride tetrahydrate ($FeCl_3$)	Merck, Darmstadt, Germany
Native PAGE reagents and gel	Invitrogen, California, U.S.A.
Pierce 660 nm protein assay reagent	Thermo scientific, Illinois, U.S.A.
Simply Blue Safe Stain (Coomassie G-250)	Invitrogen, California, U.S.A.
Sodium acetate	Merck, Darmstadt, Germany
Sodium phosphate	Sigma-Aldrich, Missoury, U.S.A.
Tris ((hydroxymethyl) –aminomethan)	Merck, Darmstadt, Germany
Triton X-100	Sigma-Aldrich, Missoury, U.S.A.

Table A2: **List of equipment used in the experiments.**

Equipment	Producer
BioDoc-It imaging system	UVP, California, U.S.A.
pH-meter (PHM 210)	Radiometer analytical, Villeurbanne, France
Model R Membran Filtration Pilot Plant	GEA liquid processing, Søborg, Denmark
Centrifuge 5810R	eppendorf, Hamburg, Germany
Industrial Bowl cutter, TK 20Ltr	Kilia Vertrieb und Engineering, Neumünster, Germany
Sorvall Instruments RC5C	GMI, Minnesota, U.S.A.
Tissue culture testplate 96F	TPP, Trasadingen, Switzerland
UV-1800 UV-Vis spectrophotometer	Shimadzu Scientific Instruments, Kyoto, Japan
VersaDoc imaging system, Model 100	Bio-Rad, California, U.S.A.
Versamax microplate reader	Molecular devices, California, U.S.A.
Waring commercial blender 7011S	Waring, Connecticut, U.S.A.
Whatman 41 filter paper	GE Healthcare, Pollards Woods, U.K.
Whatman 589/1 filter paper	GE Healthcare, Pollards Woods, U.K.
Whatman GF/C filter paper	GE Healthcare, Pollards Woods, U.K.

Appendix 2

Buffer recipes

0.5 M Sodium acetate buffer, pH 3, 4, 5 and 6.

- 34.02 g of sodium acetate was weighed out.
- 400 ml dH_2O was added.
- The pH was calibrated (PHM 210, Radiometer analytical, Villeurbanne, France) with glacial acetic acid.
- dH_2O was added to a total volume of 500 ml.

0.5 M Tris buffer, pH 7, 8 and 9

- 30.29 g of Tris was weighed out.
- 400 ml dH_2O was added.
- The pH was calibrated with HCl.
- dH_2O was added to a total volume of 500 ml.

0.5 M Ammonium carbonate buffer, pH 10

- 19.52 g of Ammonium carbonate was weighed out.
- 400 mL dH_2O was added.
- The pH was calibrated with 33 % ammonia
- dH_2O was added to a total volume of 500 ml.

0.1 M Sodium phosphate buffer, pH 7.4

- 0.2 M Na_2HPO_4 were added to 0.2 M NaH_2PO_4 until a pH of 7.4 (in room temperature) were reached.
- The solution was diluted to 0.1 M.