Acid hydrolysation of starch and formulation of starch microspheres

Master Thesis in pharmaceutical technology

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Abstract

The aim of this diploma work is to investigate the acid hydrolyses of two types of starch, waxy barley and maize starch to be applied for further usage, in rheology studies and formulation of starch microspheres. Modified starch has always been essential in many areas, especially in tablets, where it could be used as a disintegrant. Starch has also been investigated for manufacturing microspheres. In difference from previous work in the pharmaceutical technology (Elfstrand,2006a) ‘’ from starch to starch microsphere’’, where starch microspheres were used for the encapsulation of protein drugs, starch is in this thesis was used to encapsulate yeast cells.

According to this diploma work the two starches that are acid hydrolysed have shown different rheological behaviour. The parameters that are found to affect the rheology behaviours of the starches are temperatures during acid hydrolysis, time, and type of starch. Light microscopy shows that waxy barley starch exhibited a regular spherical structure whereas maize starch has spheres with irregular structure and it also shows that morphology of the starches are intact after acid hydrolysis. Waxy barley starch consisting almost of amylopectin, is easy to gelatinize and solubilized in aqueous medium with the method used for heating in this work (microwave Owen).

Formulation of starch microspheres is investigated according to the methods described by Elfstrand.Light microscopy is used to discover if there are microspheres produced. Microspheres could be produced by acid hydrolysed waxy barley starch but not from the maize starch.Waxy barley starch is selected for further studies, where yeast cells are encapsulated.There are indications that yeast cells could be encapsulated or at least that the presence of yeast did not disrupt the formation of microspheres.

In conclusion, and regarded to this diploma work waxy barley is the more suitable type of starch to be used since granules are easy to swell and solubilize and do form microspheres.

Populärvetenskaplig sammanfattning

Syftet med examensarbetet är att undersöka två stärkelse-typer, waxy korn och majs stärkelse. Dessa behandlades med syra och de behandlade stärkelserna undersöktes vad avser deras reologiska egenskaper och sedan studerades om de kunde användas vid formulering av olika typer av stärkelsemikrosfärer. I denna studie skall stärkelse användas för att kapsla in jästceller. Tanken är att undersöka om mikroorganismer kan kapslas in i mikrosfärer. Om så är fallet skulle detta kunna användas för att leverera nyttiga bakterier till tarmen. Detta är baserat på det arbete som (Elfstrand ,2006a) gjorde i sin avhandling ''From starch to starch Microspheres'', där hon använder stärkelse för att kapsla in protein i läkemedlet. Det nya är att jag använder andra stärkelse sorter och studerar inkapsling av jäst.

För femtio år sedan användes stärkelse huvudsakligen inom hushållet. Idag produceras knappt 19 miljoner ton stärkelse om året. Stärkelse är en förnyelsebar råvara och utvinns ur stort antal växter runt omkring i världen. Den kan hittas i cerealier – exempel majs, vete, korn och ris – och i rotfrukter som potatis och tapioka (rötter från kassava). Stärkelse är en viktig ingrediens i vår mat.45 % av energibehovet som människan får, är av kolhydrater i maten. Nära 90 % av dessa kolhydrater utgörs av stärkelse.

Stärkelse används i många områden, särskilt inom medicin, där det kan användas som sprängmedel i tabletter, d.v.s. att när tabletten kommer i kontakt med det vätska t.ex. magsaft så underlättar det att tabletten löses upp och den aktiva substansen i läkemedlet(tabletten) frigörs.

Stärkelse är inte bara användbar inom läkemedelsbranschen. Det används i huvudsak inom livsmedelsbranschen. Stärkelsen har i huvudsak fyra funktioner som ingrediens i livsmedel. Stärkelsen ger konsistens i maten och ersätter fett. Den används som bindande medel, och binder till sig vatten. Modifierad stärkelsen fungerar även som emulgator. Ett stort område för majsstärkelse är att det bryts ner till stärkelsesirap, ett sötningsmedel som används i godis, glass och saft. Andra användningsområde är inom pappersindustrin, som ytlim. Potatisstärkelse kan användas för att öka styrkan i pappret. I kosmetika är även användning av stärkelse väsentlig. Risstärkelse används i kosmetika. Andra användningsområden kan vara inom glasfiberväv, gipsputsmassor och betong.

Det finns företag som utvecklar nya metoder och använder stärkelse som huvudkälla. Från Lyckeby stärkelse, har det utvecklats annan stärkelse som används som energikälla i sportdrycken Vitargo. Fördelen med denna sorts stärkelse, är att idrottsmännen får tillräckligt med energi som bevaras i kroppen, lätt och snabbt. Vidare används stärkelse för att producera etanol och även nedbrytbara material som ersätter plast.

Modifierad stärkelse erhåller betydelsefulla egenskaper som gör att den blir användbar inom ett visst område. Studie av (Bylund, 2011) har visat att modifikation

av stärkelse förändrar stärkelses gelatinisationstemperatur och viskositet. Detta leder till att resistensen av stärkelse ökas gentemot skjuvning, lågt pH, hög temperatur och retrogradering.

Detta examensarbete har visat att när två typer av stärkelse behandlas med syra hydrolys, olika kvaliteter i reologi tester erhålls. Kvaliteten påverkas av vilken typ av stärkelse som används. Vidare faktorer som kan påverka viskositeten är temperaturer under syrahydrolysen, tid, och reaktionshastigheter. Ljusmikroskopen har visat att waxy korn stärkelse erhåller runda regelbundna sfärer jämfört med majs stärkelse som är oregelbundna. Syra behandlingen ändrar inte utseendet på stärkelse vilket visar att den är ganska mild. Kornstärkelsen är lätt att arbeta med och dess viskositet minskar i huvudsak med tiden som den behandlas med syra. Kornstärkelse innehåller nästan bara amylopektin och är därför lätt nedbruten i mag-tarmkanalen. Waxy kornstärkelse kan vara bra alternativ att användas inom formulering av tabletter.

Formulering av stärkelsemikrosfärer erhåller olika egenskaper beroende på stärkelsetyper, polymeren som används för emulgering, buffert och inkubationstiden med enskild temperatur, kalla rumstemperatur och 40 °C. Ljusmikroskopi används för att upptäcka om mikrosfärer bildas av båda typerna av stärkelse. Endast korn stärkelsen visade sig kunna ge bra mikrosfärer. Kornstärkelse valdes därför ut för vidare studier för att kapsla in jästceller. Waxy korn påvisar egenskaper som inte majs stärkelse har. Det var lätt och upptäcka släta runda mikrosfärer i mikroskopin. Sfärerna har troligen kapaciteten att kapsla in jästceller.

1. Introduction

1.1. Aim

The aim of this diploma work is to study the acid hydrolysis of starch from two different botanical sources maize and waxy barley. The effect of time and temperature on the acid hydrolysis are studied. The degree of hydrolyse is followed indirectly using rheology. Furthermore the aim is to investigate if the acid modified starch could be used to produce placebo starch microspheres and if yeast cells could be encapsulated in the starch microspheres.

1.2. Background

Previously, Elfstrand has shown that proteins could be encapsulated in starch matrix (Elfstrand, 2006a). This is a formulation technology that could be used as a drug delivery system in pharmaceutical industry as well as in other areas such as encapsulation of probiotic bacteria. The inclusion of cells into these types of systems has to our knowledge not been studied previously.

The technology investigated is based on the formation of starch microspheres, which are stabilised by the crystallisation of starch. The starch microspheres are prepared using an aqueous two-phase system, consisting of two structurally different polymers (poly (ethylene glycol) and dissolved starch).The starches used by (Elfstrand,2006a) were based on waxy maize, either acid hydrolysed or mechanically treated. In our study two types of starch for formation of micro-particles are investigated, maize (corn) and waxy barely. In difference from the work done by (Elfstrand 2006a), the acid hydrolysis is performed in house instead of using commercially available acid hydrolysed starch.

According to Elfstrand (2006a) it has been shown that microspheres can be produced of different qualities. The quality depends on starch type (Elfstrand, 2006a) and process conditions (Elfstrand, 2006b). This thesis investigates the differences between two starch types, maize starch and waxy barely in respect to acid hydrolysis and formulation of starch microspheres. Other parameters that could influence the quality of starch microspheres, such as buffer used, incubation times at different temperatures, are not investigated and the conditions for microsphere production is taken from the work done by (Elfstrand 2006ab) with optimized temperature 6 Cº and 37 Cº with individual incubation times.

1.3. Starch in medicine

The use of native or modified starch is very popular in pharmaceutical industries. This is because starch is biocompatible, nontoxic, biodegradable, eco-friendly and of low costs (Manek,2005).Native starch is used as an excipient in pharmaceutical products in tablets, here they are used as disintegrants and thus incorporated in tablets for easier immediate release formulations. This is in order to make the active drug available within short span of time to the absorptive area.

Starch can also be used as wet binder during granulation. Modified starches in general, have different physiochemical and functional characteristics compared to native starches such as cold swelling. They are also used in pharmaceutical product for example as an excipient in immediate release tablets (Shangraw, 1992). One example is sodium carboxymethyl starch, which is marketed as sodium starch glycolate. Modified starches could be used in other applications such as in food products as a thickening agent. Starches could furthermore be used as a binding agent in coated paper (Wikipedia, 2015). Modified starches are used as a thickening agent, stabilizer, emulsifier and as a binder in coated paper (Wikipedia, 2015). Starches are modified to increase their stability against the surroundings from chemicals and heat. Starch marketing requires production of high quality of starch granules, with shelf stability, desired viscosity, resistant to shear, low pH and high treated temperatures. Starch has also been used to replace gelatine capsules. That is done by formulation of starch capsules, where modified starch is combined with film forming polymers to produce a non-gelatine soft gel capsule with a high soluble property. This study has been done by the group members (Gillelland, 2000).

1.4. Starch structure

1.4.1. Starch components

Starch is a polysaccharide consisting of D-glucose units, called homoglucan or glucopyranose. Starch has been found in green plants and the purpose of starch in the plant is to be storage of energy. The main commercial source of starch is found mainly in seeds, roots and tubers, coming originally from maize, wheat, potato and rice. Depending on the botanical sources, granules of starch differ substantially in size and structures, However, the common native starch granules present a concentric 3D architecture figure with 15 % to 45 % crystallinity structure depending on the plant species (Zobel, 1988). The presence of hydroxyl groups in the polysaccharides makes the starch molecules hydrophilic (Xie, 2012). The two main molecules that starch is consisting of are amylose and amylopectin. The ratio of amylose and amylopectin are different for different botanic sources of starch. This can affect properties of the gelatinized or dissolved starch such as the crystallisation rate of dissolved starch and the rheology of starch solutions. Amylose is quite linear molecule based on α (1-4) bonds with a molecular weight of 10^5 to 10^6 with degree of polymerization DP, 600, (Xie, 2012).Amylopectin in particular is a highly

multiple branched polymer with a molecular weight of 10^7-10^9. Amylopectin is based on α (1-4) around 95% and α (1-6) with 5% links constituting of a branched structure. This huge structure has deep influence on the physical and biological properties of starch (Xie, 2012)

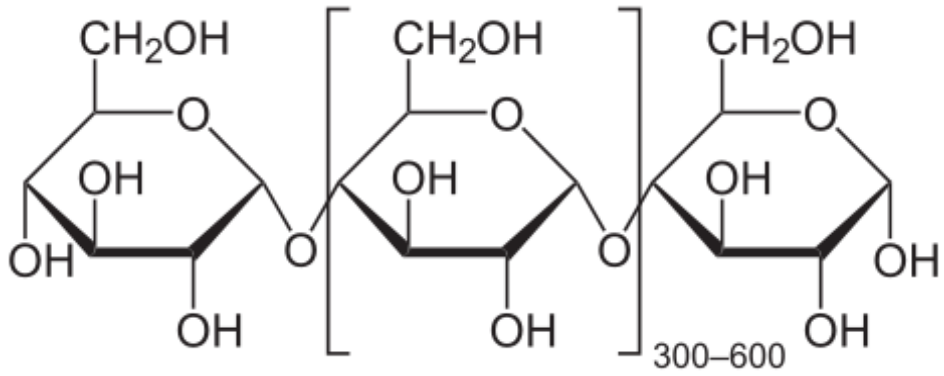


Figure 1: The structure of amylose

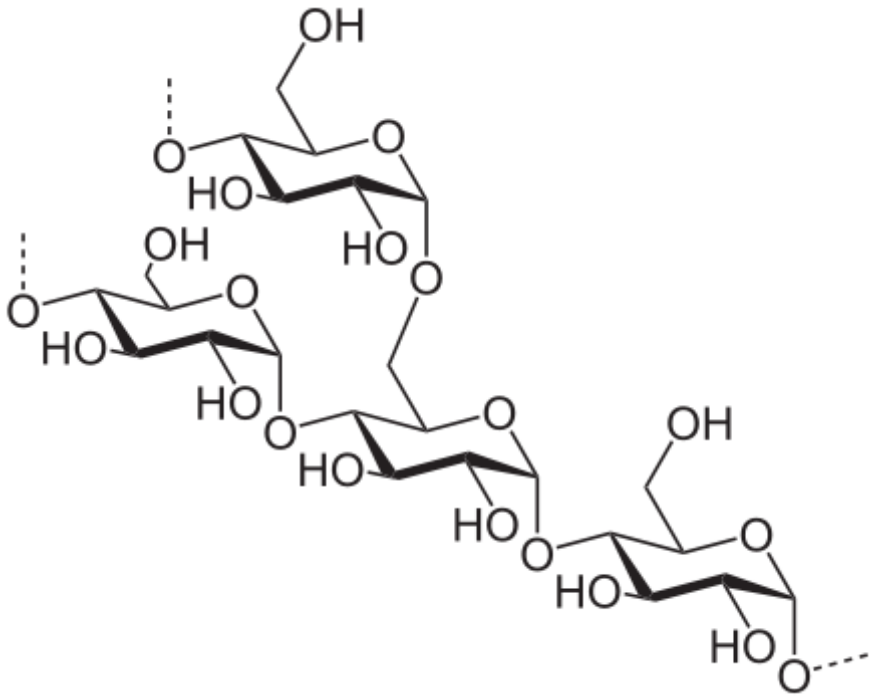


Figure 2: The structure of amylopectin

There are other substances included in starch granules, like proteins, lipids and phosphorus depending on the botanical resources. In conclusion starches with different amylose contents could in fact have profound impacts on starch properties and behaviours.

1.4.2. Gelatinization/melting of starch

When native starch granules are heated in presence of water, the semi crystalline structures are gradually destroyed, altering the phase transition from an ordered granular structure into a disordered state in water, the phenomena is called gelatinization (Ratnayake, 2008). Gelatinization occurs when starch is added to excess of water under heating. The amount of water needed to fully gelatinize waxy maize is above 63% (Sullivan, 1964). The heating causes starch granules to swell, losing their crystalline structures as seen by lose of birefringence in polarized light and molecules start to solubilize (Sullivan, 1964). Lack of water would lead to high melting temperatures and the swelling progress of starch granules are in that case slowed down. This is due to limited concentration of water that initiates high steric hindrance. In conclusion temperature is a very important factor in gelatinization causing changes in the structure of starch molecules. Starch molecules would become more flexible and soluble (Donovan, 1979).

1.5. Starch microspheres

The usage of starch microspheres has gained some interest during the last decades. The main area where the usage of starch microspheres could be of interest is in pharmaceutical industries, where microspheres are investigated as dosage form for the administration of an active substance. Starch is used, as it is a biodegradable polymer. It is biocompatible, biodegradable and bio-adhesive. The concepts of using biodegradable starch microspheres (DMS) in drug delivery is introduced in pharmaceutical studies, especially for nasal delivery and are described more in detail in the review article '' Development of Biodegradable Starch Microspheres for intra nasal Delivery''(Yadav, 2008). When starch microspheres are approaching the nasal mucosa, it forms a gel like system, with prolonged residence time in the nose. That makes starch promising for future use especially in nasal administration. One important key property for this application of starch microspheres is that starch microspheres do not produce immune response as a drug excipient in nasal administration.

Starch microspheres are not only beneficial when it comes to nasal delivery formulation but also in wound healing, which has been developed by Magle Life Sciences in Lund Sweden. Magle produces microspheres particles, which have the purpose to concentrate platelets and other blood constituents, where blood becomes dehydrated, holding pure blood flow. This kind of application is used for example in treatment of liver cancer patients (Magle AB, 2012).

Furthermore starch microspheres have been developed to be used as a core for PLGA coated controlled release formulations aimed for controlled delivery (Reslow,2002).

1.6. Starch hydrolysis/modification

There are several methods to change starch structure to obtain starch derivatives. This includes methods based on physically, enzymatically or chemically treating native starch to change its properties (Wikipedia, 2015). One method of modification of starches is acid treatment. In this method starch is heated with HCl. This will lead to partial acid hydrolysis of the glucosidic bonds in the amorphous regions of the starch granules. The degree of hydrolysis will be dependent on the time and temperature used for the acid treatment. According to (Sivak, 1998), already Lintner and Naegeli studied this in the late 1800s, and found that the crystalline areas remain intact. They are not freely available to the acids. Second step after hydrolysis, is when starch is filtered and freeze-dried for further usage. One reason to modifications is that gelatinization processes are affected by hydrolysis. The modified starch has decrease molecular weight but with parts of its crystalline structure intact (Sivak, 1998). However when modified starch is heated with water, the granules fragment more and swell less. This would lead to increase temperature range of gelatinization and the starch becomes more soluble, which could be important for applications, such as microsphere production. Starch structure is changing constantly during the hydrolysis, which can be followed by for example change in rheological properties. There are many parameters effecting starch hydrolysis such as, pH, hydrolysis time, concentrations, buffers and other additives.

1.7. Rheology

Rheology is the study of flow and deformation of materials under applied forces. The measurement of rheological properties is applied to all materials from fluids, polymer melts to solid polymers, but the instruments could be different. For most fluids and gels traditional rotational rheometers are the instruments most commonly used. For low viscosities using a microcapillary viscometer are usually giving more accurate measurements while new methods such as optical technique and microrheology are current under development (Malvern Instruments Ltd, Worcestershire England). For polymeric material rheological properties are impacted first of all by their formulation (botanical source, plasticizer and contents included). Other parameters that could be of interest are in processing conditions, temperature, mechanical energy, etc. To get a better understanding of the rheology of gelatinized polymer in production scale, the relation between shear rate and shear viscosity is explained further. This newtonian-power-law model were η is viscosity and g is shear rate.

$$\eta = k\, g^{n-1}$$

According to this model when n value is lower than 1 that corresponds to shear thinning behaviour. Shear thinning behaviour is when reduction of molecular entanglement is occurred by increased shear rate. The nearer n approaches 1, the

more the solution behaves like a Newtonian fluid, which means that the fluid is less sensitive to shear rate (Xie, 2012). Based on the relation equation between shear viscosity and shear rate, changing of parameters like plasticizer type, content or processing temperatures, obtains different behaviours of the polymer melts. In this work we aim to investigate the effect of acid hydrolyse on solutions of heat gelatinized starches.

2. Materials and Methods

2.1. Materials

The study has been divided into three parts, production of acid hydrolysis starch, **rheology test and production of microspheres**

Table of chemicals used in studies presented in table 2.1

Material	**Manufacturer**
Hydrochloric acid	Titrisol, 1.09956.0001, Merck
maize starch	**Sigma-Aldrich S4126-2kg (Starch from Corn)**
Waxy Barley	From Lyckeby starch, Large fraction after sedimentation
PEG	Sigma-Aldrich, No.P-2263 polyethylene glycol
Distillated water	**-----**
Sodium bicarbonate	Sigma-Aldrich, S-8875
Yeast	Svensk Torrjäst för matbrödsdegar, jästbolaget AB
Sodium hydroxide	Titrisol, 1.09956.0001, Merck

2.2. Methods

2.2.1. Method for acid hydrolysis of starch

300 g of either maize starch or barley starch is placed in a beaker. The beaker is placed in a water bath with a stirrer. The temperatures, studied are 25□C, 40□C and 50□C. 1000ml of 1 M HCl solution is gradually added the starch during stirring. During the hydrolysis process 100 ml of starch solution is taken out at (60, 120, 240, and 300) min respectively and putted in a separate container. These starch solutions are immediately neutralized with 1 M NaOH without specific amount. The samples are then centrifuged at 1700 RPM for 10min and the supernatant is discarded. The starch is then washed three times with water by resuspending the starch pellets with deionized water and removing the added water by centrifugation. The starch is freeze-dried in aluminium pans for at least three days. Prior to freeze drying they are frozen at -20°C for at 16 hours to be frozen completely.

2.2.2. Viscosity measurements

0,25g of hydrolysed starch is added to separate beaker and weighted. 4.75 ml of a buffer solution 50 mM, consisting of 50 mM is added to each beaker. The starch complex is mixed with a stirrer in about 5min. Solutions are then put into a microwave oven and heated 3 periods of 6 s, for complete homogenous solutions. This step requires high temperature and sealed containers to completely dissolve the starch. Next step is cooling them down by washing with ice/cold water. Viscosity measurements are set up, by adding 4 ml of each sample to the rheometer. The viscosity is measured with a rheometer Viscotech, Reologica AB Sweden, using bob and cup setup. Viscosity measurements where done with shear rates between 0.03-20 s^{-1} measuring ten points per decay. Three consecutive measurements are done on the same sample. The main purpose is to observe the viscosity, which is done by plotting the mean viscosity at the semi-plateau of the shear thinning solutions versus the time for acid, hydrolyses.

2.2.3.Preparation of the microspheres

Selected batches of acid hydrolysed barley and maize starch are used for to produce microspheres. 18% of a starch/ buffer solutions are mixed separately and then heated in sealed containers in a microwave for 3 periods of 6 s. If the solution is not clear indicating fully gelatinized samples, the starch solution is heated again. Afterwards solutions are cooled down to 40°C. 2.9g of starch / buffer solution is mixed with 1ml carbonate buffer (50 mM). The starch solution is now added to 25g of a 38% PEG buffer solution (50 mM). The solutions are emulsified using an ULTRA TURAX, and running in 45 s at highest speed. The emulsions are stirring for about 17 h in at 8°C. Light microscopy is used to observe the formed microspheres.

2.2.4. Encapsulation of yeast

Two batches of barley starch that has been hydrolysed for, 60 and 300 min, respectively are used in encapsulation of yeast cells. 2g of starch is mixed with 8.5g

of buffer solution (50 mM) and heated in microwave as described previously. The starch solution is cooled to 40°C. 0.5g of buffer added to starch solution to make reference placebo starches. The step of encapsulating yeast follows the same procedure but with 0.5g of yeast solution instead of buffer. Starch/buffer /yeast dispersion are added to 25g of 38% PEG solution, and emulsions are formed using Ultra Turax for 45 s at the highest speed. The emulsions are placed at 8°C under stirring in 5 h. After 5 h, samples putted at room temperature about (16-17) h. The formation of microspheres is followed using light microscopy. Samples are taken at different time intervals of the production.

3. Result and discussion

The results presented in this chapter are divided into two sections: The acid hydrolysis of starch and formulation of starch microspheres.

3.1. Observations during acid hydrolysis

In developing the method for acid hydrolyse the following observations are made, the neutralisation after acid hydrolysis is critical and addition of excess NaOH to the starch solution leads to gelatinization of the starch. This is seen at high pH around 11, as measured with pH indicators. The result is a rigid gel complex of starch solution. Another observation during the hydrolysation of starch was that when neutralizing starch solutions to a pH in a range between 6-7, the solutions are clear and have yellow colour afterwards. At pH above 7.5 -10, the starch solutions become progressively more brown in colour with hydrolysation time. The colour change is seen when hydroxide is added and this could be an indication that low molecular weight carbohydrates react at high pH forming coloured entities. In contrast white starch powder is obtained when to high pH is avoided during neutralisation and the pH value ends up around 7. Couples of tests from barley starch solutions have been treated the same way when neutralizing. It is noted that for barley samples prior to centrifugation that the solutions have slightly brown colour and are somewhat cloudy. However the final samples of starch are seldom miscoloured.

3.1 Acid hydrolyse of starch

3.1.1. Acid treated starch granules

Figure 3.B, shows the morphology of native maize starch compared to figure 3.A, with acid treated maize starch. According to the light microscopy result, the external granule structure of each type demonstrated no clear differences between acid treated starch and native starch. As discussed later there are however changes in the rheological properties of the starch. The result means that acid hydrolysis could have an impact inside the granules, which make them swell easier when heating is applied. The same result was obtained by (Vataire, 2014). In figure 4.B, native barley starch is compared to barly starch that has been treated with acid at 40°C for 240 minutes, figure 4.A . As can be seen in figure 4.A, barley starch are swelling more and are more aggregated due to acid hydrolysis, forming a large compact area that is

easy to rupture when treated at high temperature. This result is quite similar to the results observed by (Vataire 2014) .In conclusion when starches of any type, are treated with acids, the external structure is not changed to any larger extent. It still obtains the same spherical sharp shape, only aggregation of particles occurs (Sandhu 2007, Vataire 2014),the same observation is made in this diploma work.

A. B.

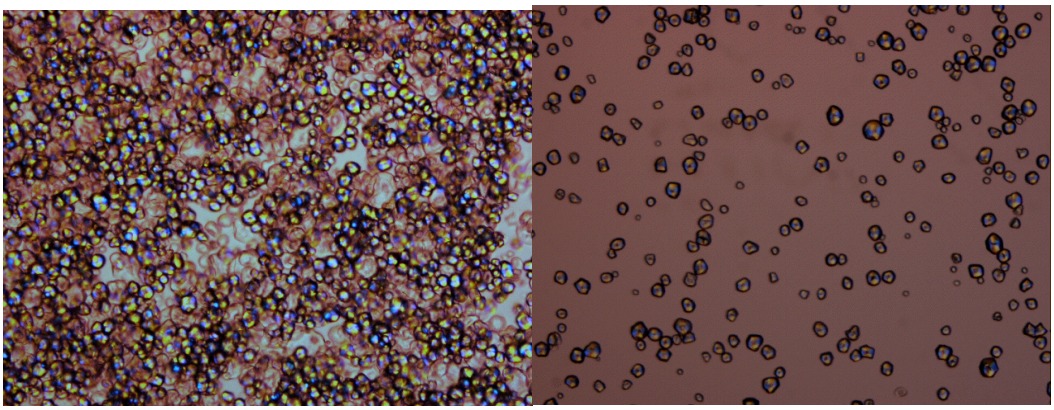


Figure 3: A. acid treated maize starch at 40°C for 240min 100X and B. unmodified maize starch 100X

A. B

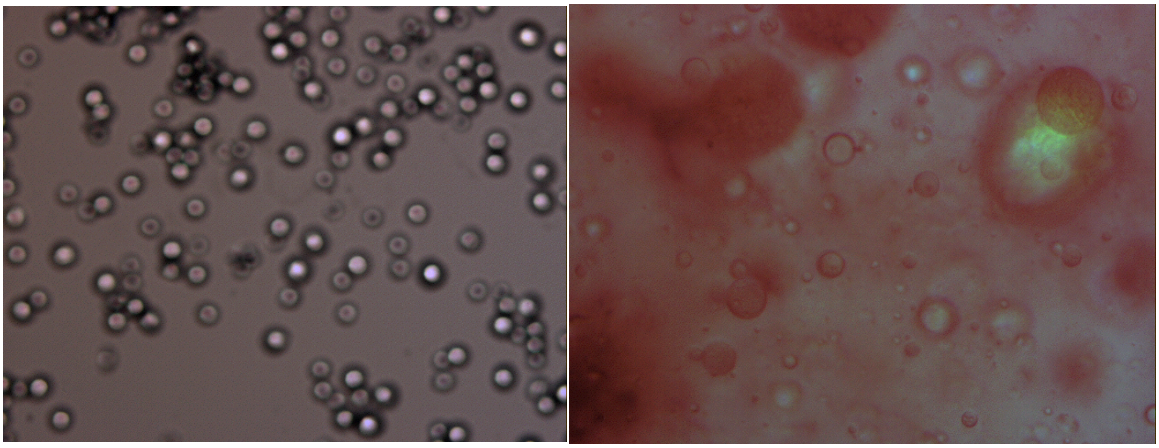


Figure 4: A. acid modified barley starch at 40°C for 240min 200X and B. unmodified barley starch 100X

3.1.2. Rheology study of acid hydrolysed starches

Figure 5 shows a typical result for the rheology measurements. As can be seen the viscosity decreases with shear rate until a semi plateau is reached. At the starch concentrations used here the gelatinized starch solutions are shear thinning. Three measurements are done in sequence and as can be seen from figure 5, the plateau value is more or less the same for all measurements and only minor differences are seen at the lower shear rates. This indicates that the structure of the starch solution has not been permanently affected by the shear rates.

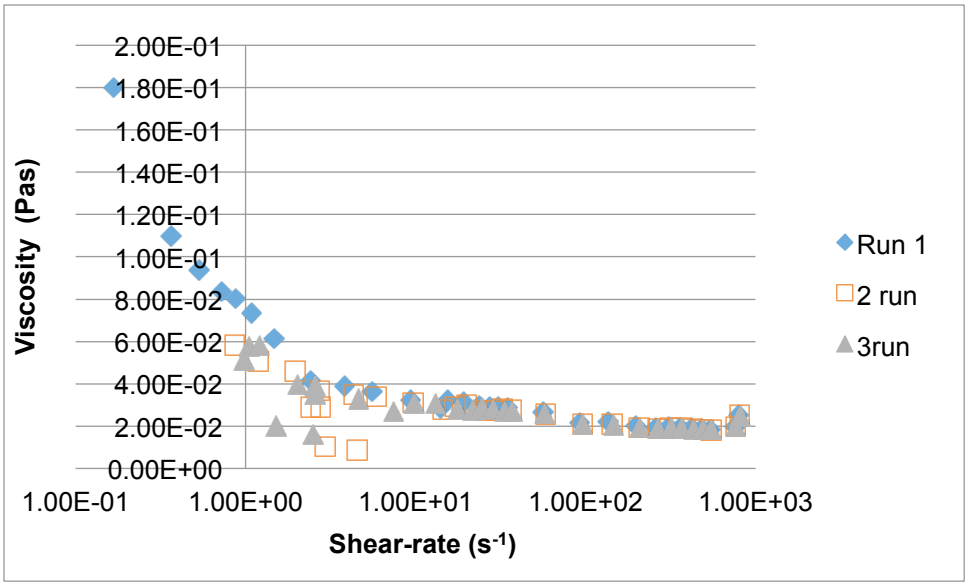


*Figure 5: Viscosity versus shear rate for barley starch hydrolysed at 40°C for 300 minutes. The concentration of the starch is 0.05g/ml and the dispersion has been heated 3*6s in a microwave oven.*

Figure 5, presents the shear rate applied on hydrolysed barely starch with temperature 40 C°. Reduced viscosities, is because of the movements between starch inter and intra molecular chains, which reduce the viscosity with increasing shear rate, since it can decrease the polymer entanglement density and increase the ease of disentanglement (Xie, 2012). Most polymeric solutions have their viscosity reduced upon shearing (Sunthar, 2010). Shear thinning behaviour is seen, causing decreased viscosity as shear rate is increasing (Paolucci, 2012), which is also the same result in figure 5.

Figure 6-10 shows how the plateau value of the rheology measurements vary with starch source and time of acid hydrolysis.

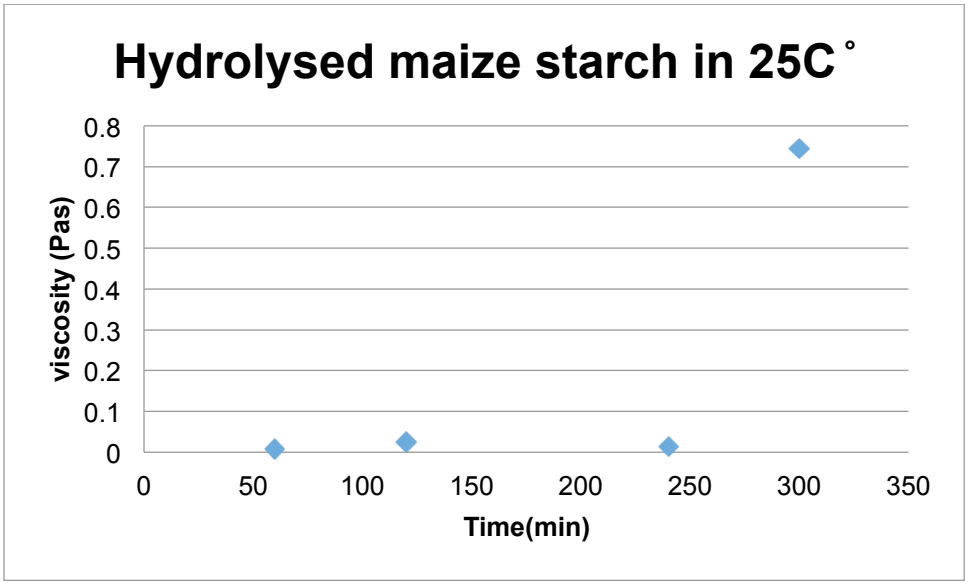


Figure 6: Average viscosity of acid treated maize starch at different time intervals of hydrolysis at 25°C, the concentration of the starch is 0.05g/ml and it has been heated in a microwave oven for 3x6 s.

A B

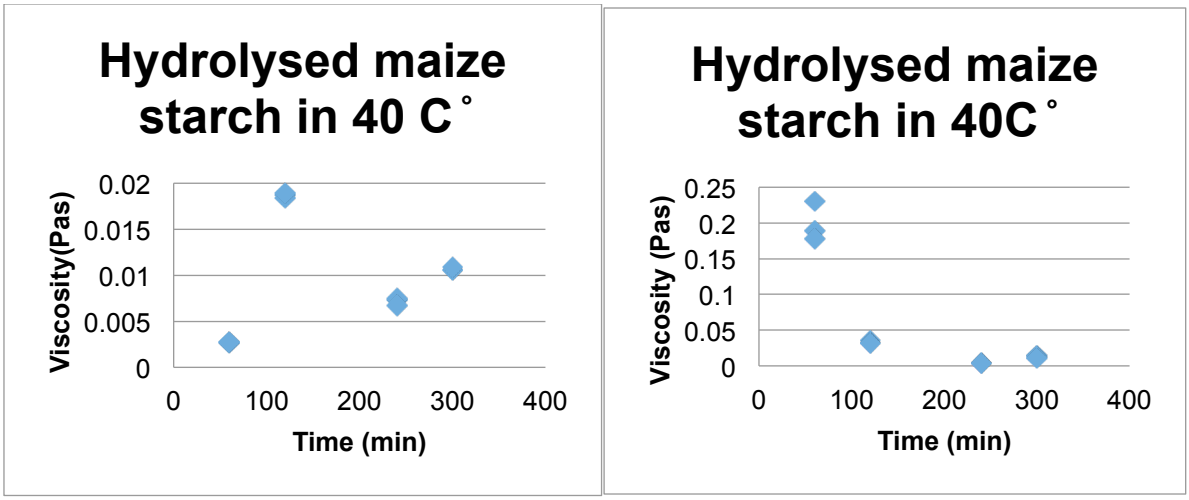


Figure 7: Two rounds of experiments of Average viscosity of acid treated maize starch at different time intervals of hydrolysis at 40°C, the concentration of the starch is 0.05g/ml and it has been heated in a microwave oven for 3x6 s. Result from three sequential measurements are shown

A *B*

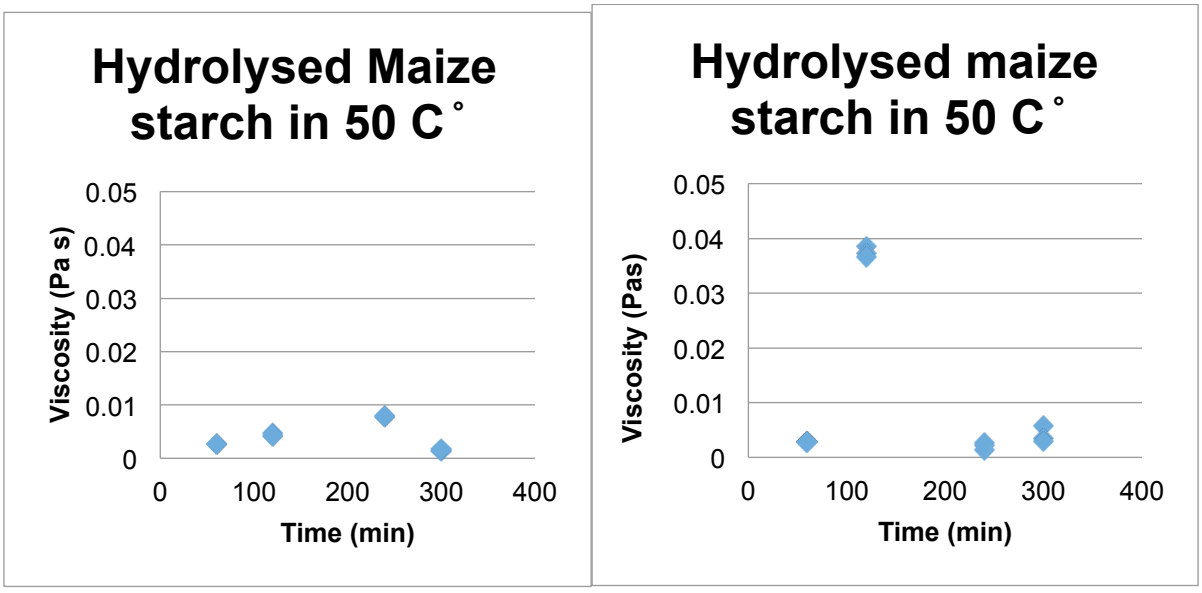
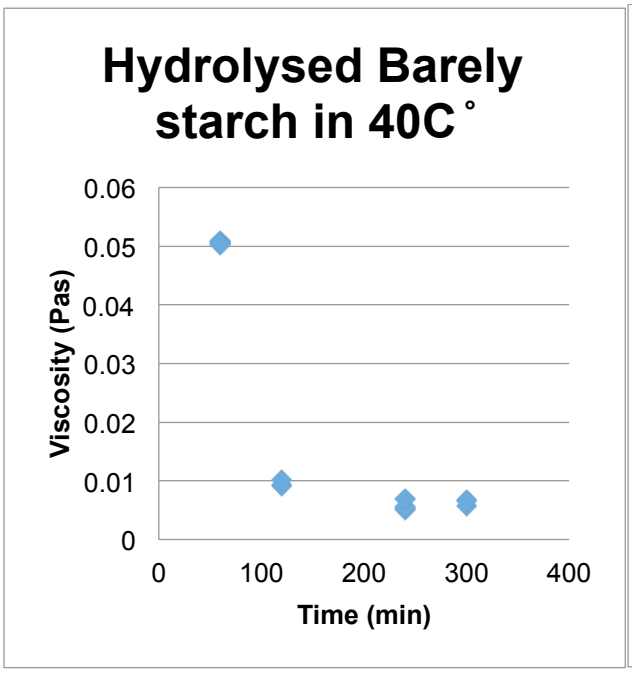


Figure 8. Two rounds of experiments. Average viscosity of acid treated maize starch at different time intervals of hydrolysis at 50°C, the concentration of the starch is 0.05g/ml and it has been heated in a microwave oven for 3x6 s. Result from three sequential measurements are shown

A



B

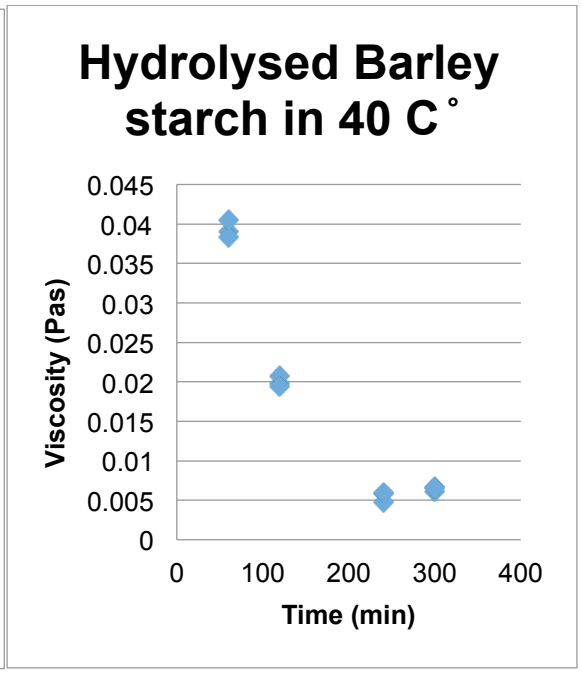
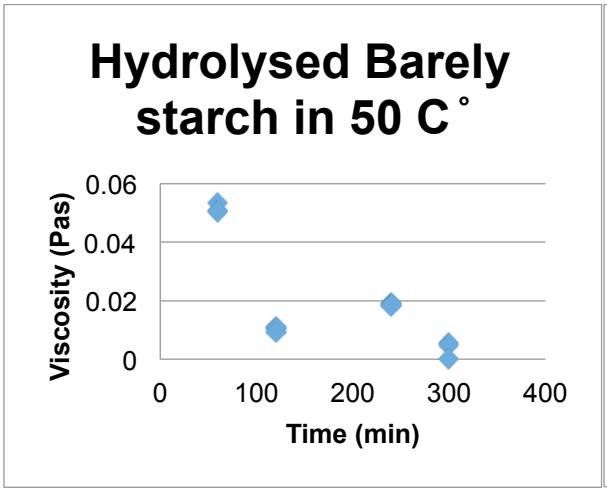


Figure 9: Two rounds of experiments. Average viscosity of acid treated waxy barley starch at different time intervals of hydrolysis at 40°C, the concentration of the starch is 0.05g/ml and it has been heated in a microwave oven for 3x6 s Result from three sequential measurements are shown

A



B

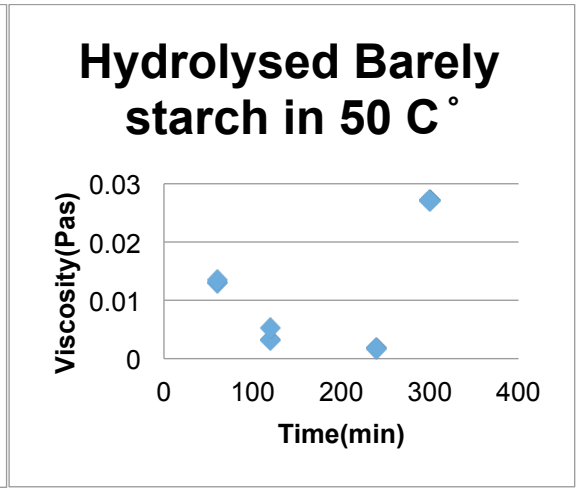


Figure 10: Two rounds of experiments. Average viscosity of acid treated waxy barley starch at different time intervals of hydrolysis at 50°C, the concentration of the starch is 0.05g/ml and it has been heated in a microwave oven for 3x6 s. Result from three sequential measurements are shown

The measurements of the average viscosity versus hydrolysed time give some information about the behaviour of starch polymer solutions after different hydrolysis time. The expected results are that average viscosity with the acid hydrolysed times would decrease (Abodrreza, 2012). The results of this diploma work show that this was not always the case. The method used for gelatinization of the starch is the same that will be used for the production of microspheres. The advantage of using this method is that it allows for small samples and is easy to do. The major disadvantage is that it does not in all occasions lead to fully dissolved starch.

In figure 6-8 the plateau values for maize starch, treated with acid at different temperatures are shown. At each temperature the viscosity is plotted against the time for acid treatment. The average viscosities at the time range [120-300] min are 0.26 Pas, 0.02 Pas, 0.07 Pas for hydrolysed maize starch, at respectively 25, 40, 50 °C. Maize starch has been acid treated with temperature 25 °C exhibited quite high average viscosity compared to acid treated maize with temperatures 40 °C and 50 °C respectively. The reason could probably be due to low degree of acid hydrolysis when acid treated with temperature 25 °C. The amylose content could still be high in starch solutions at constant temperature; viscosity is rapidly increasing due to hydrogen interactions between maize starch molecules, which leads to a more firm gel (Sivak, 1998). The higher the amylose content in maize starches (Della Valle, 1996 and Xie, 2009a), the higher is the viscosity, which is also a likely explanation for the results seen in this diploma work. Another explanation could be that the molecular weight of both amylose and amylopectin only is reduced to a minor degree at this temperature. As can be seen the higher viscosity is mainly due to high viscosity after 300 min, as can be seen in figure 6, that the viscosity at shorter times than 300 minutes are low whereas viscosity is increasing after 300 min. This is contra intuitive if one only considers the effect of chain length on the viscosity. However, the low amount of acid hydrolyse could either lead to that the method used to dissolve the starch granules are not functioning well for maize starch at short time of acid hydrolyse or that the amount of amylose is still so high that recrystallization of released amylose occur. The latter is quite likely as the sample is seen to cloud reasonably fast after heating. Both these phenomena will lead to less amount of free starch molecules in the solution, which most likely will reduce the observed viscosity. In figure 7, the acid hydrolyse at 40°C is shown. The plot in figure 7B is considered to be more correct compared to plot in figure 7A, since viscosity is dropping with hydrolysation time, this is also the later of the two duplicates. In the beginning high viscosity, because of the unchanged amount of amyloses. It could be speculated that the amylose content in the sample decreases with acid hydrolysation time and that this leads to decrease in viscosity but this could also be an affect of reduction in molecular weight of both amylose and amylopectin. Plot in figure 7A shows quite strange result, because of the lower viscosity at the beginning of the hydrolysis process than in the end. This could be due to a difference in hydrolysation rate between the two experiments and that at the early points in figure 7A the hydrolysation is not sufficient enough. Thus as for hydrolysation at 25°C it could be speculated that the low viscosity observed either is due to a lack of swelling and solubilisation of the starch granules during the heating of the sample or that amylose has recrystallised prior to the rheology measurements. In figure 8 the results for 50°C is shown. One measurement at 300 minutes in figure 8 A is not shown. This value is considerably higher than the other measured values at this temperature and is considered to be an out-layer. In general all values for maize at hydrolysation 50°C are low and one could expect that after the first reduction of molecular weight the

effects on rheology had levelled out. All in all, one can conclude that there is an effect of acid hydrolysis on maize starch but the results are not straightforward and show high variations probably due to incomplete dissolution of the starch when conducting the viscosity measurements.

In figure 9-10 the plateau values for waxy barley starch treated with acid at different temperatures are shown. The temperatures are 40 °C and 50 °C with quite low viscosities of 0.007 Pas and 0.01 Pas, respectively for the average viscosities at time range [120-300] min. As can be observed in the figures the viscosity in most cases also decreases with increasing time of acid hydrolysis. Waxy barley starch is close to 100% amylopectin (Eriksson, 2012). Amylopectin as a molecule has high molecular weight and short chain branches, but despite its high molecular weight, it has a compact ellipsoidal conformation, which reduces the ability to create entanglements, resulting lower viscosity (Della Valle, 1996 and Xie, 2009a). When acid hydrolysing barley starch, the chemical modification weakens the starch intra-and inter molecular interactions, resulting in lower viscosities and higher n value thereby showing more Newtonian behaviour (Thunwall, 2008). These explanations could be the reason for why such behaviour was obtained when investigating the viscosity of waxy barley starch.

3.1.3. Discussion acid hydrolysis

In table 1 the yield after hydrolysation is shown.

Table 1: Yield from acid hydrolysing of two types of starches, waxy barley and maize

Time(min)	Temperature °C	**Yield barley %**	**Yield maize %**
60	**25**	**----**	**30**
120	**25**	**----**	**51**
240	**25**	**----**	**54**
300	**25**	**----**	**60**
60	**40**	**63**	**81**
120	**40**	**57**	**66**
240	**40**	**45**	**48**
300	**40**	**45**	**72**
60	**50**	**39**	**60**
120	**50**	**48**	**45**
240	**50**	**45**	**51**
300	**50**	**42**	**99**

Table 1 shows acid treated starches of types barley and maize are subjected to heating with different temperatures and at a certain time range. Yield of each of them are calculated. Yield in theory should be 30 % that is when hydrolysis is not taking place at all and no loss of fragments. According to the result in table 1, the “yield maize” and “yield waxy barley” are in the same range although the loss for barley is slightly higher. We also see a decrease in the amount of starch retained with time of acid hydrolysis as could be expected if starch is hydrolysed to low molecular entities

that are dissolved in the acid solution and removed during washing. The lower values of “yield barley” is indicating a higher amount of acid hydrolysis compared to maize.

When starch is modified, both types change their molecular structure as evident by the rheological measurements.Maize starch consists of 25% amylose and 75% amylopectin.The amylopectin (not amylose) is the component responsible for the crystalline structure of the starch granule. This portion isn’t freely accessible to the acid and remains intact according to (Sivak,1998).

Waxy barley consisting mostly of amylopectin, where there have been discoveries of barley varieties containing 100 % amylopectin called ‘’Waxy barley’’ (Eriksson, 2012). Since amylopectin is the component responsible for the crystalline structure of starch granules, one could have expected the acid hydrolyses to be more incomplete, but this has not been seen according to this diploma work. Instead the viscosity does decrease with time and is lower than for waxy maize which could indicate that degradation has occurred during hydrolysis of waxy barley starch. This is most likely due to the fact that although amylopectin is part of the crystalline structure of the granule also in waxy variety of starch, there will be amorphous areas available for hydrolysis.

3.3. Formulation of starch microspheres and encapsulation of yeast cells

3.3.1. Placebo microspheres

Microspheres of different particle sizes are produced during the so called emulsified aqueous two phase system which is based on two immiscible polymer solutions, hydrolysed starch and polyethylene glycol PEG Mw=20 000 g/mol with added buffer solution of $NaHCO_3$.

In figure 11, formulation based on maize starch with solution concentration of 3g of starch and 7 g of buffer (50mM $NaHCO_3$) according to (Elfstrand, 2006a) recipe on the production of starch microspheres are shown, Figure 11 shows how granules are swelled and aggregated with high concentration, which is 30% by weight. There are no microspheres formed. Due to the high viscosity the starch/buffer solution, the concentration of starch solution is decreased to 18 % by weight in the following experiments. However no proper starch microspheres are obtained with maize starch at any of the experiments conducted.

In figure 12 .E, barley starch microspheres are compared to figure F. maize starch microspheres, where microspheres with maize starch show aggregated and swelled granules with black bobbles of air. Barley starch microspheres on the other hand are regular circular surfaces in cold room temperature .Light microscopy shows stable microspheres during incubation in cold room temperature due probably to the crystalline structure pattern. This result with forming crystalline structures in starch microspheres was also observed in (Elfstrand,2006a) thesis work, ‘’from starch to starch microspheres’’.The results indicated that waxy barley starch is a better

candidate than maize starch for microsphere production and it is thus chosen to be used in the final test with yeast incorporation.

3.3.2. Yeast microspheres

As can be seen in figure 13 and 14, it is possible to form microspheres in the presence of yeast cells. Figure 13. A, shows the starch emulsion after storage at 8 °C. As can be seen the droplets are not completely round which is most likely due to shearing when the cover slip is applied. This indicates that the microspheres are not fully hardened during this step. Comparing with 13.B, where barley starch that has been acid treated for different length, we see that acid hydrolysed barley starch treated in 60 min and 40 °C shows irregular surfaces of swollen aggregated granules similar to the ones for maize starch in the placebo experiments and for barley starch treated for 300 min 40 °C, we see as in the placebo case that droplets of starch solution has been formed at cold room temperature. The result of the irregular swollen aggregated surfaces is because of the incomplete acid hydrolysis. Better results are obtained if the hydrolysis time is extended. In figure 14. we show the microspheres after storage at room temperature. The microspheres are now solid spheres not droplets. The time in room temperature has thus been enough to crystalize the starch and form microspheres, the larger surface area of microspheres, the more is the encapsulation efficiency. The emulsion process supposed to be forming a stable crystalline structure of starch micro particles (Elfstrand, 2006), which is the same result found here. There is similarity in shape between placebo spheres and spheres with yeast cells incorporated as seen in figure 14.B. There have also been irregular and small spheres observed with and without yeast cells incorporated. The similarity in both spheres with/without yeast cells could indicate that yeast cells are successfully incorporated.

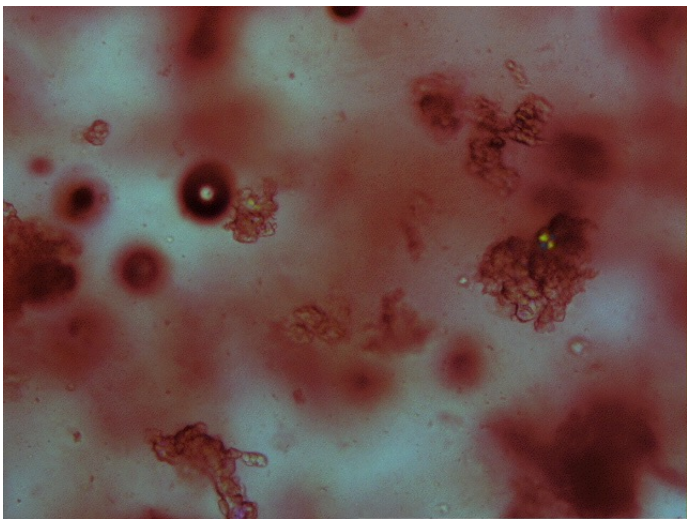


Figure11: Maize starch microspheres with 40 °C, 240 min in room temperature 100X with high concentration of starch –Peg solution

A. B.

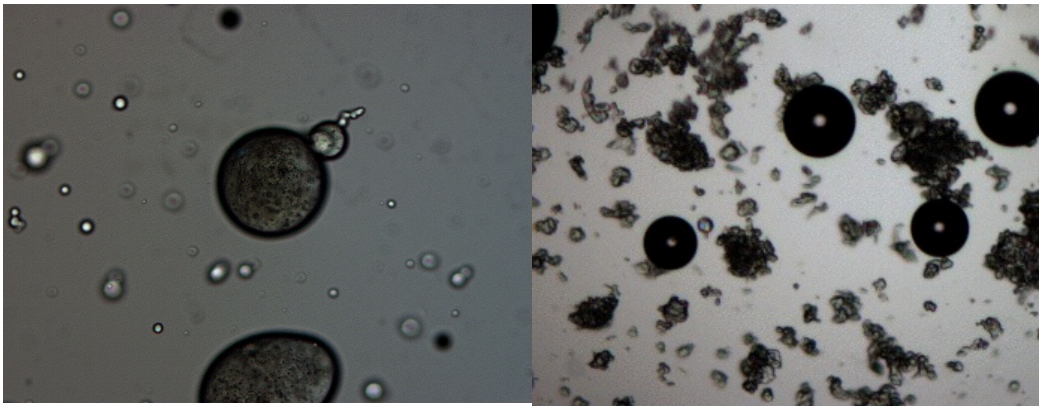


Figure 12: A. Barley starch microspheres with 40° C and 240 min in cold room temperature 100X vs B. maize starch microspheres with 40 °C and 240 min in room temperature 100 X

A. B.

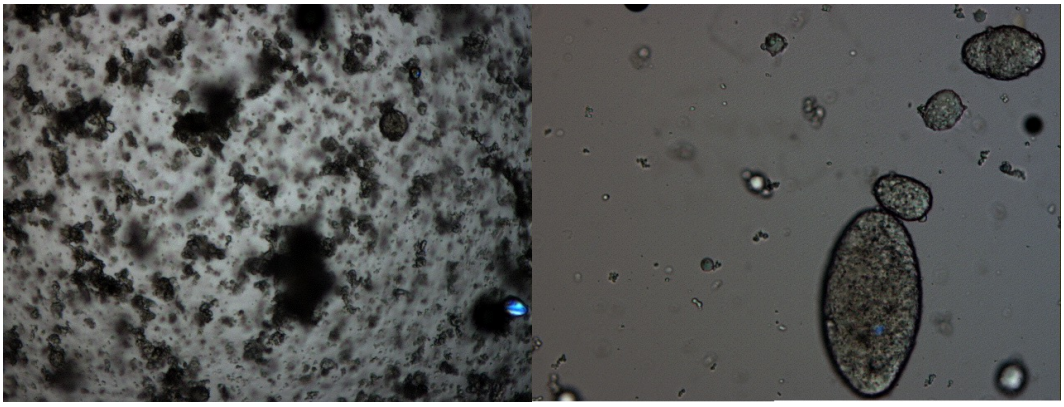


Figure 13: A. Encapsulation of yeast cells with barley starch in 60 min and 40°C in room temperature 50 X vs B. yeast cell in barley starch with 40 °C and 300 min in cold room temperature 100X

A. B.

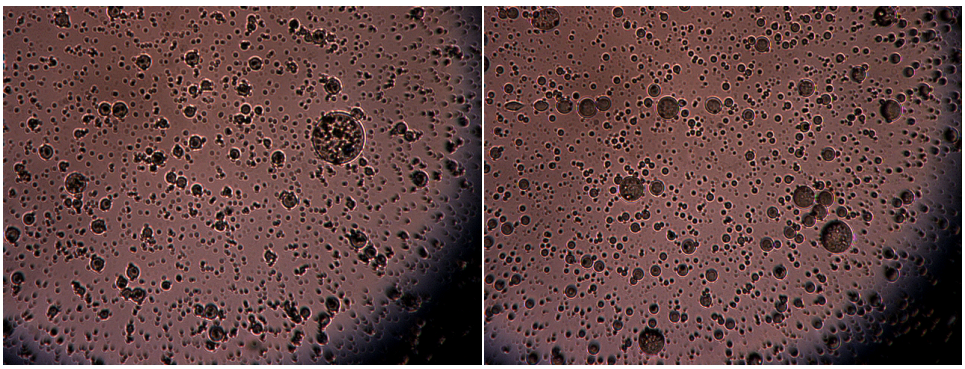


Figure 14: A. Barley starch with yeast cells in 40 °C and 300 min in room temperature 50X vs B. barely starch microspheres without yeast cells in 40 °C and 300 min in room temperature 50X

4. Future work

To investigate varied properties of hydrolysed starches that are of importance, more types should be hydrolysed and studied such as rheology and granular structure should be examined.

Changing the hydrolysis time and prolonging the process should lead to different results compared to this diploma work. Other parameters that should be considered are, temperature of the acid hydrolysation process, or the amount of starch used. These changes have an impact on rheology of starch as well. An interesting issue to investigate further is the formulation of starch microspheres, where microspheres with yeast could be used in control release. That is to observe, how the intended amount of yeast is released in aqueous medium.

5. Acknowledgements

First of all I thank God for helping me through this diploma work, and secondly I really want to thank my supervisor Marie Wahlgren for helping and supporting all the time. I would also like to thank everyone in food Technology department for giving me enough help.

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