Sveriges lantbruksuniversitet
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– An investigation for industrial use

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– En undersökning för industriell användning

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Abstract

Oat has been established as a useful crop hundreds of years ago all over the world. Oat contains starch which are compound polyhedral granules built out of amylose and amylopectin. Some of the starch can be resistant depending on type of crop, grade of maturation etc. Starch can be modified by heat and moisture treatment or enzymatic treatments to increase the yield of resistant starch. Resistant starch is defined as starch, or products from starch that is resistant towards digestion and absorption in the small intestine in healthy humans. Instead it can be fermented in the large intestine. Resistant starch occurs in four types depending on process or origin and these are known to have beneficial health effects. The food production enzyme pullulanase derived from microorganisms such as bacillus species has the function of breaking the 1,6-glyosidic linkage in amylopectin, debranching the molecule into straight chains that gives the starch larger opportunity to be converted to resistant starch. The aim of this study was to increase the yield of resistant starch in an oat flour from the company Lantmännen to be able to use it as a food product with a healthy approach. The method was performed on two different materials; oat starch and oat flour and the time setting was 60 minutes and the amount of material was 3 grams or 4 grams. The enzyme pullulanase was used at 50 µl or 100 µl. A rapid visco analyzer (RVA) was set at different temperature and time settings to figure out a good range to give a high resistant starch yield. The method design was divided into 3 programs, first the gelatinization program at 95°C for 15 minutes, second the enzymatic treatment, adding the enzyme pullulanase at 50°C. Finally, the last program was aimed to kill of the enzyme at 95°C. The samples were put in fridge for 12h and then into freezer before freeze-dried and then analyzed with "Megazyme resistant starch assay kit". The samples were analyzed in a light microscope and the results were calculated in excel. The results showed that the parameters for increasing resistant starch was not as clear as expected. The oat starch samples showed a decisive increase in resistant starch in comparison to oat flour. The results did not show any specific trend for amount of sample or amount of enzyme in the oat flour samples since the resistant starch content was too low to see any trend. In oat starch samples, there was higher yield of resistant starch with more enzyme added. The amount of material used did not show any specific trend. Further research is needed to see if it is possible to increase the resistant starch yield in an industrial process for oat flour.

keywords: oat, starch, amylose, amylopectin, resistant starch, pullulanase, rapid visco analyzer

Sammanfattning

Havre har varit en användbar gröda i flera hundra år i världen över. Havre innehåller stärkelse som består av polyhedralt formade granuler som är uppbyggda av amylopektin- och amylosmolekyler. En andel av stärkelsen är resistent, där mängden av resistent stärkelse beror på typ av gröda, mognadsfas osv. Stärkelse kan modifieras så att omvandlingen till resistent stärkelse ökar. Definitionen för resistent stärkelse är den stärkelse, eller den produkt från stärkelse, som är resistent mot digestionssystemet i tarmen. Den resistenta stärkelsen kan inte absorberas hos friska individer i tunntarmen utan fermenteras istället i tjocktarmen av mikroorganismer. Resistent stärkelse finns i fyra typer indelade efter framställningsprocess eller ursprung och dessa är kända för att ha hälsofrämjande effekter. Livsmedelsproduktionsenzymet pullulanase, framställt från mikroorganismer så som släktet bacillus, har funktionen att bryta ned 1,6-glykosidbindningar hos amylopektin. Pullulanaset avgrenar amylopektin till kortare, raka kedjor som i sin tur har lättare att bilda resistent stärkelse. Syftet med den här studien var att försöka öka halten resistent stärkelse i ett havremjöl från Lantmännen för att kunna använda detta mjöl i en livsmedelsprodukt med större hälsonytta. Metoden utfördes på två olika material; havrestärkelse och havremjöl och mängden var 3 gram eller 4 gram. Enzymet pullulanase tillsattes i 50 µl eller 100 µl. En rapid visco analyzer (RVA) användes och ställdes in på olika program med varierande temperatur- och tidsinställningar för att hitta ett optimum som ökar halten resistent stärkelse i havre. Metoddesignen delades in i 3 program, först ett gelatiniseringsprogram vid 95°C under 15 minuter, sedan ett enzymbehandlingsprogram där enzymet pullulanase adderades och var aktivt under 60 minuter. Slutligen avdödades enzymet genom ett program vid 95°C. Proverna kyldes därefter ned 12 timmar i kylskåp för att sedan läggas i frysen innan frystorkning. Proverna analyserades i ljusmikroskop och halten resistent stärkelse bestämdes med ett ”Megazyme resistant starch assay kit”. Resultatet visade att parametrarna som varierades för att öka halten resistent stärkelse var svårtolkade. Havrestärkelseproverna visade tydligt en högre halt resistent stärkelse än havremjölproverna. Resultaten visade inte någon som helst specifik trend för mängd prov eller mängd tillsatt enzym bland havremjölproverna eftersom halten resistent stärkelse var för låg för att kunna dra någon slutsats. För havrestärkelseproverna fanns en trend som visade att större mängd enzym gav större mängd resistent stärkelse. Mängden material visade ingen trend. Mer forskning behövs för att ta reda på om det finns en potential att öka halten resistent stärkelse under en industriellt hållbar process på ett havremjöl.

Nyckelord: havre, stärkelse, amylos, amylopektin, resistent stärkelse, pullulanase, rapid visco analyzer

Preface

This report has been written on request of Lantmännen. The work has been performed at the facilities of SLU.

I would like to thank Lantmännen for the experience and opportunity to learn about my subject. Thank you, Christian Malmberg, Food and R&D project manager at Lantmännen who has been my contact person.

I am very grateful to professor Roger Andersson, department of molecular sciences at SLU who has been my supervisor and helped to guide me through the project with his great knowledge.

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Table of contents

List of tables

List of figures

Abbreviations

ANN: Annealing
HMT: High moisture treatment
NSP: Non-starch polysaccharides
RS: Resistant starch
RVA: Rapid visco analyzer
SCFA: Short chained fatty acids

1 Introduction

Starch is a macromolecule that exists in a variety of cereal grains. The focus in this study is on oat, which has unique properties such as high fat and high protein content (Delcour & Hoseney, 2010). Oat is already used in products that are proven to have health beneficial effects where β-glucan is important for the healthiness (Kulp, 2000).

Resistant starch (RS) is a dietary fiber that is defined as the starch and/or products from starch that cannot get digested and absorbed in the small intestine. On the other hand, RS can be fermented in the large intestine by microorganisms creating several health beneficial short chain fatty acids (SCFA) (Eliasson & Gudmundsson, 2006). Examples of these SCFA are butyric acid, propionic acid and acetic acid, which are proved, due to several medical studies and in vitro studies, to lower the blood pressure and also to lower the bad cholesterol in the blood (Topping & Clifton, 2001). The RS, which is not absorbed is also responsible for a positive bulk effect in the intestines, in the same manner as several other dietary fibers such as non-starch polysaccharides (NSP) (Topping & Clifton, 2001).

There are four types of RS that have different origin. Treatments with heat and moisture together with storage where gelatinization and retrogradation can occur can increase the RS content. Also, enzymatic treatments are proved to have RS increasing effects (Milašinović *et al.*, 2010). Lantmännen has an interest in using flour residues for foods instead of for bioethanol industry. The aim is to increase the healthiness in a flour through increasing the yield of RS and therefore gain interest for the consumers and for use as ingredients in commercial food products.

The problem description is to investigate if there are possibilities to treat the product of interest, which is an oat flour residue from Lantmännen, so that its value may be enhanced through improving the RS yield. The aim of this study was to create a method using a Rapid Visco Analyzer (RVA) to be able to increase the yield of RS. The RVA can give controlled temperature programs and shear rates during controlled time periods and is suitable to translate to an industrial process.

Questions: Is it possible to increase RS value in oat starch and oat flours through heat and cooling cycles? Which parameters are of interest when increasing RS in an oat starch/oat flour? Is it possible to increase RS and not decreasing other substances of interest in the oat flour?

2 Background

2.1 Oat

In the early seventh century oat was established in western Europe as a cereal grain, and A.D. 1000-1500 oat became an important crop in northern Europe due to a new agricultural system with crop rotation and utilization of horses. Oat probably evolved and got established simultaneously in other regions over the world. Chinese historical records show the farming of oats in A.D. 1000. In the United states oats were first planted in 1602 and grew in importance over time (Kulp, 2000).

The breakthrough for oats rise with the development of milling in the 1850's, which reduced cooking time and increased the demand as a food for humans. This was therefore a starting point for the industrial development of milling oat products (Kulp, 2000).

The oat plant is a grass plant with leaf consisting of blade, sheath and ligule. The mature internode stems have hollow centers and the nodes are solid. The inflorescence is a panicle composed of rachis, rachis branches and spikelets, where each rachis branch is terminated in a pedicellate spikelet. The spikelets have two empty glumes and 1-3 fertile florets. A floret contains rachilla segments, lemma, palea and sexual organs, later on the mature caryopsis (Kulp, 2000).

The harvest of oat is similar to barley and rice, whereas the caryopsis enclosed in a floral envelope is harvested. The caryopsis, also called "groat" is similar to a kernel in other grains except it is covered with several hair-like structures called trichomes. The oat hull constitutes 25 % of the oat kernel total weight and the groat consist of seed coat, pericarp, nucellar epidermis, germ and endosperm (Delcour & Hoseney, 2010). Starch is the major component of groat and can approximately be 60 % of the dry weight (Zhu, 2017). Groat has higher fat and protein content than other cereal grains (MacArthur & D'appolonia, 1979), this promote oat to short shelf life due to oxidation of fatty acids (Delcour & Hoseney, 2010).

β-Glucan is a non-starch polysaccharide in oats which is viscous when dissolved in water, and is found in the subaleurone layer of oats. β-Glucan is linear, large, and consists of 1,4- and1,3-linked β-D-glucopyranosyl units. β-Glucan has beneficial health effects which makes it interesting for human consumption (Kulp, 2000; Beer *et al.*, 1996). The health effects are lowering serum blood cholesterol and moderating the glucose metabolism for diabetics (Kulp, 2000).

2.2 Starch

Starch molecules are polymers of glucose in an complex semi-crystalline structure, and occur in cereal grains (Smith, 2001). The glucose units of starch can be from 50 units up to several thousand (Hii *et al.*, 2012). The photosynthesis of crops creates sucrose which is synthesized into starch in the cytosol. The sucrose is then transported to the endosperm where it is stored as starch. The synthesis is occurring in the amyloplast organelle where the sucrose is converted to glucose 6-phosphate. Glucose 1-phosphate is developed and the enzyme ADPglucose pyrophosphorylase convert it to ADPglucose. ADPglucose is key substrate for starch synthases, which are enzymes that synthesize starch (Smith, 2001).

Starch is the second most abundant heterogeneous polysaccharide after cellulose and has the shape of water insoluble granule (Hii *et al.*, 2012). The granules are organized and have a great variety in size and shape. A granule consist of tightly packed chains of amylose and amylopectin that respectively consists of monomers of glucose (Hii *et al.*, 2012; Zavareze & Dias, 2011), where each starch molecule has a reducing end, a hemiacetal group (Delcour & Hoseney, 2010). The structure in which the amylose and amylopectin are ordered, regular or irregular, affects the shape and size of the granule, which depend on the plant origin of the starch. A cereal granule can vary within range 1 to 100 μm (Zavareze & Dias, 2011). In *figure 1* there is an overview of the structure and composition of starch granules.

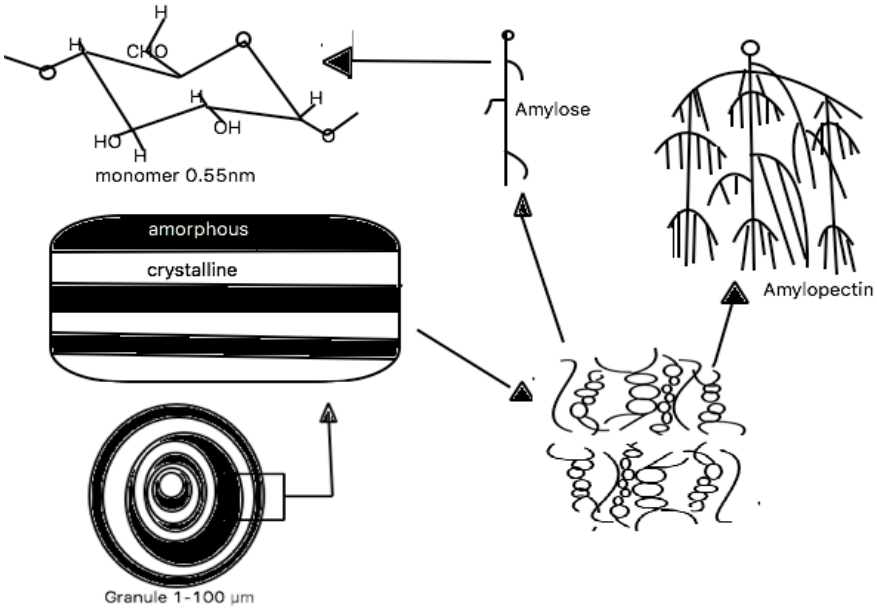


Figure 1. An overview of amylose and amylopectin ordered into starch granules, modified from: (Buléon *et al.*, 1998).

The amylose is mainly composed of 1,4-linked α-D-glucose (α-D-glucopyranosyl) units in a linear shape. Only small branches on amylose make the behaviour of the molecule dominantly linear (Hii *et al.*, 2012; Zavareze & Dias, 2011). The molecular weight differs depending on the maturation of the grain and also depending on species. For common starches it is estimated that amylose constitutes 18-33 % of the starch (Delcour & Hoseney, 2010).

Amylopectin is a branched molecule composed of short straight chains and longer sidechains of 1,4-linked α-D-glucose units. Some of these chains carries 1,6 linkages that create branching points (Hii *et al.*, 2012; Zavareze & Dias, 2011). There are 3 types of amylopectin chains (A-, B- and C-chains). The A-chains has only 1,4-linkages, B-chains have 1,4-linked chains attached by 1,6-linked branches, and C chains have a reducing end (Delcour & Hoseney, 2010).

The oat starch has compound granules, similar to rice, with several granules in an amyloplast in contrast to other cereal grain that have one granule in each amyloplast. The oat granules are small (3-10 µm) with a polygonal, irregular shape (Zavareze & Dias, 2011; Hoover & Vasanthan, 1992). Gelatinization temperature range at 53-59°C (Zavareze & Dias, 2011). According to a study on oat starch pastes, the pastes and gels of oat are more translucent and less susceptible to retrogradation compared to maize and wheat (Doublier *et al.*, 1987). Also, amylose and amylopectin were determined to be co-leached from the oat starch granules in some trials under the influence of internally bound starch lipids (Hoover & Vasanthan, 1992; Doublier *et al.*, 1987).

There are mainly four types of enzymes acting on starch; (i) endoamylases, (ii) exoamylases, (iii) debranching enzymes and (iv) transferases (Hii *et al.*, 2012). In general the amylases are acting on amylose and the debranching enzymes acting on amylopectin (Delcour & Hoseney, 2010). (i) Endoamylases, mainly found in micro-organisms, are endo-acting enzymes that cleave 1,4-glucosidic bonds in the inner parts of amylose or amylopectin chains. This randomized hydrolysis gives products of a mixture of oligosaccharides called α-limit dextrin. (ii) Exoamylases such as α-glucosidases and β-amylases are cleaving external glucose residues of amylase and amylopectin. α-Glucosidases cleave 1,4- and 1,6-bonds and produce only glucose (Hii *et al.*, 2012). β-Amylase attacks the non-reducing ends of the amylose in starch and create maltose units when breaking every second 1,4-bond. The β-amylase cannot pass an 1,6 branching point therefore it gives β-limit dextrin as product (Delcour & Hoseney, 2010). (iii) Debranching enzymes of starch hydrolyze the 1,6-glucosidic bonds in amylopectin and are divided into two major groups; indirect- and direct debranching enzymes. The indirect debranching enzymes such as amylo-1,6-glucosidase, can only release a single 1,6-linked glucose residue and thus need a modified starch substrate to be able to act. The direct debranching enzymes such as pullulanase and isoamylase can directly hydrolyse amylopectin (Hii *et al.*, 2012). Pullulanase is an enzyme of importance that is utilized to hydrolyze pullulan, oligosaccharides and amylopectin (Hii *et al.*, 2012). Amylopectin is hydrolyzed at the 1,6-bonds which results in products of free A- and B-chains with reducing ends making oligosaccharides as end products (Delcour & Hoseney, 2010). (iv) Transferases cleave 1,4-glucosidic linkage of a donor molecule and transfer this to a glucosidic acceptor molecule which forms a new bond (Hii *et al.*, 2012).

2.2.1 Gelatinization and pasting

The glass transition temperature, T_g, is when a substance changes from a glassy state into a rubbery state. This can be caused by several parameters, moisture, temperature and/or additives. Additives such as sugar binds water and lead to less water accessible for the granules. Granules reaches T_g before gelatinization, at which point the amorphous region of the granules changes and the crystallinity start to fade (Delcour & Hoseney, 2010).

Gelatinization state occur in an interval after the glass transition temperature is reached, when starch is exposed to water so that the granules swell due to uptake of water in the amorphous regions and the intercrystalline regions disrupting the crystallinity of the granule (Delcour & Hoseney, 2010). This swelling give increased molecular interaction and leads to leaking of amylose from the granules. When this occur, there is a loss in birefringence, T_0, in the granules which is followed by additional water uptake until the crystalline structure in all granules are lost, T_c. When

T_c is reached the gelatinization is finished. Pasting occurs during continued heating but after gelatinization, when the birefringence is lost and excess water give additional increase in viscosity and the starch continues to get solubilized. Not until 120°C all starch can be solubilized (Delcour & Hoseney, 2010).

2.2.2 Retrogradation and gelation

Retrogradation is when starch develops from an amorphous state to a more crystalline state. This will change the starch rheological properties into an increased firmness (Eliasson & Gudmundsson, 2006). More specific, retrogradation is when amylopectin forms a crystalline state after gelatinization. The concentration of starch, the shear rate and the temperature determine the grade of retrogradation. The crystalline state of amylopectin has a melting point at 50-60°C (Delcour & Hoseney, 2010).

Gelation of amylose is after gelatinization when the sample is cooled down. Amylose that is solubilized is forming helixes with another free amylose creating a continuous network. When the amylose has become crystalline after some hours, the amylose has a melting point at 150°C (Delcour & Hoseney, 2010).

The water content and storage temperature are important for the rate of retrogradation. Starch cannot retrograde without a certain amount of water. Lipids and surfactants are substances that can interfere with the retrogradation process (Eliasson & Gudmundsson, 2006).

2.2.3 Annealing/Heat Moisture Treatment and RVA

Annealing (ANN) and Heat moisture treatment (HMT) are methods that physically modify starch granules without gelatinizing or damaging the granules. This is done through controlled heat and moisture that is determined regarding the shape, size and birefringence of the starch granules (Stute, 1992). The amount of water needed for gelatinization to occur to starch depends on the temperature. Annealing is a process that increases the crystallinity of starch through treatment with high water conditions and low temperature (Delcour & Hoseney, 2010; Lehmann & Robin, 2007; Eliasson & Gudmundsson, 2006). The temperature is too low for gelatinization to occur. The annealing temperature T_A must be below the onset of gelation T_0 to counteract crystallites to melt at glass transition temperature T_g (Eliasson & Gudmundsson, 2006). Annealing will move gelatinization temperature to a higher and more narrow range (Eliasson & Gudmundsson, 2006; Krueger *et al.*, 1987).

HMT is done to change properties of starch. HMT is when the water content is lower than the content that is required for gelatinization to occur and the temperature is high (Delcour & Hoseney, 2010; Lehmann & Robin, 2007; Eliasson &

Gudmundsson, 2006). This semidry condition moves the onset of gelatinization (T_0) and the completion of gelatinization (T_c) to higher temperatures after treatment and also swelling power and solubility change (Eliasson & Gudmundsson, 2006).

Starch is a non-newtonian system meaning that it can exhibit both fluid characteristics and gel characteristics depending on shear rate and temperature. This can be measured with a RVA (Zhou *et al.*, 1998). The RVA measures the relative viscosity of starch in water when exposed to shear, controlled heating/cooling and holding periods at constant temperature (Delcour & Hoseney, 2010). The benefits of RVA are that it has a small sample size which can be set at several temperature profiles and shear rates. Also that it measures peak viscosity, peak area, time-to-peak, drop off and final viscosity (Zhou *et al.*, 1998). Autoclaving (140-145°C) is another heat treatment that is approved as a suitable process for increasing RS yield (Dundar & Gocmen, 2013; Sievert & Pomeranz, 1989).

2.2.4 Starch in food production

The starch content in a product may be problematic since starch is a non-stabile system over time. Starch is very sensitive to exposure of heat, moisture, cooling and shearing. After gelatinization have occurred, the crystalline structure of the amylopectin is destroyed, though the starch will recrystallize over time during the retrogradation phase (Zavareze & Dias, 2011). Crystalline amylose is a good source of thermally RS (type 3), which is useful for food applications (Haralampu, 2000).

Other factors affecting the stability of starch in food products are other components in the product, such as lipids and protein. These substances create physical modification and may form complexes with the starch and change the properties, such as a great decrease in stickiness. Since starch is a sensitive substance, chemically modifications can be needed in the food production systems where for example low pH, high shearing rates and temperature changes are common. Adding polar lipids such as mono-glycerides and/or proteins are possibilities to make starch less affected (Zavareze & Dias, 2011).

RS is small in particle size, has a low water holding capacity and has a mild flavor. RS has therefore potential to be incorporated into different foods and beverages (Jyothsna & Hymavathi, 2017; Sharma *et al.*, 2008). The high amylose starches are the most commonly used for the production of RS (Lehmann & Robin, 2007). Some studies have shown that RS has improved textural properties in foods. The problem with the research on RS is that many studies are different in method, dosage and/or sources (Sharma *et al.*, 2008).

2.3 Dietary fibers and Resistant starch

Dietary fiber (DF) is a material that is indigestible in the small intestine, therefore includes parts of foods that are not degraded in the stomach nor by the enzymes in the colon (Mudgil & Barak, 2013; Fuentes-Zaragoza *et al.*, 2010). DF can be classified according to their fermentability, solubility, source and physiological effect. DF includes non-starch polysaccharides, oligosaccharides, lignin and other plant substances. Resistant starch is included by some researchers as a DF since it is not digested in the small intestine (Mudgil & Barak, 2013; Sharma *et al.*, 2008). Others think that the health claims for DF is insufficient and that RS should be separated from DF and divided as a functional ingredient using specific health or function claims. This is to be able to properly inform consumers with labelling (Englyst *et al.*, 2007). Some of the health benefits gained from RS is likewise traditional DF, while some benefits are unique to RS (Haralampu, 2000).

The definition of RS is the starch, or the product from starch, that is not digested in the small intestine of healthy individuals and is instead fermented in the large intestine (Brumovsky *et al.*, 2009; Sharma *et al.*, 2008; Cummings & Englyst, 1991). RS was described in 1982 as starch that after gelatinization was not hydrolyzed by incubation with α-amylase and pullulanase (Cummings & Englyst, 1991). RS can be divided into subgroups after characteristics. RS type 1 (RS1) is starch that is physically inaccessible for the enzymes in the column, for example due to thick cell wall or protein matrices. RS1 is the type less resistant towards digestion in comparison to following types. RS type 2 (RS2) are starches that are protected from digestion through crystalline structure. RS type 3 (RS3) are starches that are retrograded, for example through have been cooked and then cooled down (Eliasson & Gudmundsson, 2006; Thompson, 2000). RS3 has the greatest potential for food industry among the RS types since it is thermostable through many food processing conditions (Milašinović *et al.*, 2010). RS type 4 (RS4) are chemically modified starches, for example by esterification or crosslinking (Eliasson & Gudmundsson, 2006; Thompson, 2000). Debranching of starch has been shown to produce linear chains that contribute to a higher RS yield. This has shown to be effective in a variety of starches (Milašinović *et al.*, 2010).

According to a study of RS content in Chinese diets, oat flour contains 1.82±0.63g RS/100g DM and oat meal flakes contain 4.76±0.50 RS g/100g DM (Chen *et al.*, 2010). RS has health promoting effects in the human body (Jyothsna & Hymavathi, 2017; Topping & Clifton, 2001). Colonic bacteria ferment RS and non-starch polysaccharides (NSP) into short chained fatty acids (SCFA) such as butyrate, propionate and acetate. These SCFA are proven to stimulate the blood flow in the colon, gives energy to the cells in the intestine and also stimulate the electrolyte uptake. Butyrate is the SCFA that is favoured by RS. RS may enhance stool

bulking, though not as effectively as NSP (Topping & Clifton, 2001). An important factor is that the increase in SCFA production in the intestine is significantly inter-individually varied in response (Lockyer & Nugent, 2017). One study on lipid oxidation as a result of RS consumption showed that a replacement of 5.4 % of the total dietary carbohydrate intake with RS could decrease fat accumulation in long term. This is due to that the study showed that a replacement significantly increased post-prandial lipid oxidation (Higgins *et al.*, 2004). Studies in mice also show that RS is positive for the gut health, producing SCFA and reduced abdominal fat and in human subjects the feeding with RS increased insulin sensitivity (Keenan *et al.*, 2015).

RS directly affects the large intestine in humans through decreasing pH value making hurdles for pathogenic microorganism growth. This also gives an increasing possibility for mineral absorption and other nutritional absorption. RS have also, through application tests showed to enhance flavor, crispiness, colour and mouthfeel in food products in comparison to products with traditional insoluble fibres (Milašinović *et al.*, 2010).

An in vitro study mimicking physiological conditions for starch digestion shows that different food processing techniques produce different amounts of RS. The study showed that the amount of RS decreased with increased chewing (Muir & O'dea, 1992). According to Eliasson & Gudmundsson, 2006 RS can be produced from starch during storage after going through gelatinization and retrogradation. To be able to increase RS further, the existing starch content can be enzymatically treated, hydrolyzing amylopectin into smaller molecules which through gelation and storage can create thermostable RS (Milašinović *et al.*, 2010).

According to one study where the thermostability of pullulanase derived from bacillus subtilis was tested, it was shown that the pullulanase is active up to temperature at approximately 60°C and has an optimum at 50°C (Silano *et al.*, 2017). Another study on maize starch showed that the RS yield after debranching with pullulanase at 50°C and retrogradation was 10.2 to 25.5 %. It took 5 hours for 70 % of the maize starch to be hydrolyzed (Milašinović *et al.*, 2010). The storage time and temperature are important since resistant starch is developed in different range varying these factors (Niba, 2003).

3 Materials and methods

3.1 Material

Barley from Swedish University of Agricultural Science (SLU) was used. A pullulanase "Diazyme P10" from the company Danisco produced by the microorganism Bacillus subtilis (*appendix 2*) was also used as well as another pullulanase from the company Novozyme produced by the microorganism Bacillus lichenformis (*appendix 2*). The oat starch used in this study had approximately 90 % pure oat starch and derived from Kristianstad (no specification). The oat flour used was from Lantmännen with 72 % pure starch content (*appendix 3*). The analyzing was done using a Megazyme Resistant Starch Assay Procedure KIT (Megazyme, Bray Buisness Park, Bray, Co. Wicklow, A98 YV29, Ireland) (AOAC Method 2002.02, AACC Method 32-40.01, Codex Type II Method) (*appendix 4*). Also a spectrophotometer was used for analyzation.

3.2 Enzymatic side effects

3.2.1 β-Glucan molecular weight analysis

High Performance Size Exclusion Chromatography (HPSEC) is used to estimate the β-glucan content and molecular size distribution of β-glucan. Solutions needed for the determination was calciumchlorid-dihydrat and $NaNO_3$ with 0.02 % NaN_3 and calcofluor.

$NaNO_3$ (0.1 M) with 0.02 % NaN_3, as well as 25 mg/l calcofluor in 0.1 M Tris-buffer, pH 8 was prepared. The calcofluor solution was put in a dark bottle and the solutions were inserted in the flow for the HPSEC. The analysis was performed essentially according to Rimsten et al. (2003) but with some modifications as described below.

Extraction with thermostable α-amylase was done in triplicates for each type of enzyme. Pullulanase from Novozymes and Diazyme P10 from Danisco were used for enzymatic treatment and control samples were also prepared. Barley flour (100 mg ± 5mg) was weighed and 7.5 ml aqueous ethanol (50 %) was added and incubated in a boiling water bath for 15 minutes. Another 5 ml 50 % ethanol was added and the samples were centrifuged (1000g 10 minutes). The supernatant was discarded carefully and additional 10 ml 50 % ethanol was added. The samples were mixed and centrifuged (1000g 10 min). The supernatant was discarded and the tubes were turned upside-down for 5 minutes. Distilled water (20 ml) with 0.30 mg/ml

$CaCl_2$ was added to the samples and also 50 µl α-amylase. The tubes were put directly in boiling water bath for 1.5 hour and the tubes were mixed 3 times during the time. The extract was cooled down to 50 degrees and 50 µl pullulanase was added. The tubes were incubated in room temperature for two hours then the tubes were put in boiling water bath for 30 minutes. The tubes were cooled down and centrifuged (1500 g, 15 min). The supernatants were filtrated (45 µm) into HPSEC vials and were run in the HPSEC overnight. The molecular weight and β-glucan content in the samples were calculated in MatLab.

This method was done to investigate if pullulanase had any enzymatic side effects such as β-glucanase that would degrade β-glucan. This would not be desirable due to the health effects of β-glucan.

3.3 RVA Oat Starch oat starch standard method

Different programs were set on the RVA to get a standard program (STD1) at different temperatures which can be seen in *figure 2*. The RVA was divided into three programs (i) gelatinization program, (ii) enzymatic treatment and (iii) kill off enzyme. The RVA treatments of oat starch for RS analysis can be seen in *Table 1*. Samples were run at a maximum temperature of respectively 95°C, 90°C, 88°C and 85°C. Oat starch (3.00 g) and 25 ml deionized water was used in each run. The samples were put in the fridge and after cooling down into the freezer. Additional one sample at 90°C was prepared the following day and was put into the freezer directly.

Table 1. *RVA standard treatments of oat starch for RS analysis. OS=Oat Starch, STD=Standard program*

Sample	Starch (g)	Deionized water (ml)	Temperature maximum (°C)	Enzymatic treatment	Fridge (hours)
OS STD95	3.00	25	95	None	12
OS STD90A	3.00	25	90	None	12
OS STD90B	3.00	25	90	None	None
OS STD88	3.00	25	88	None	12
OS STD85	3.00	25	85	None	12

Enzymatic treatment was carried out through preparing 3 samples which were run at maximum temperature 90°C before the enzyme was added. In *figure 3* there is an example of temperature program with enzymatic treatment over time. This temperature maximum at 90°C was estimated from the standard RVA results, estimated from pasting curve and the look of the sample, concerning viscosity. The

temperature program continued at 50°C which is the Pullulanase optimum temperature. The last part in the temperature program kills off the enzymatic effect when rising the temperature to 95°C. In *table 2* the enzymatic treatment of oat starch for RS analysis can be seen. The RVA was programed into three parts so that the enzyme could be added. In the first sample "Oat Starch Enzyme 1", 10 µl Diazyme was added between the first and second part of the program. In the second sample the double amount of Diazyme (20 µl) was added into the sample called "Oat Starch Enzyme 2". In the third sample the middle program was run two times to get double the time of enzymatic treatment. Diazyme (10 µl) was added and the sample was called "Oat Starch Enzyme 3". Oat starch Enzyme 1 was put directly into freezer. "Oat Starch Enzyme 2" and "Oat Starch Enzyme 3" were put in the fridge and after 12 hours into the freezer. When sufficiently frozen, all samples were put in a freeze dryer over weekend.

Table 2. *RVA enzymatic treatments of oat starch for resistant starch analysis. OS= Oat Starch*

Sample	Starch (g)	Deionized water (ml)	Temperature maximum (°C)	Enzymatic treatment concentration, time	Fridge (hours)
OS Enzyme 1	3.00	25	90	Diazyme 10µl, 30min	None
OS Enzyme 2	3.00	25	90	Diazyme 20µl, 30min	12
OS Enzyme 3	3.00	25	90	Diazyme 10µl, 60min	12

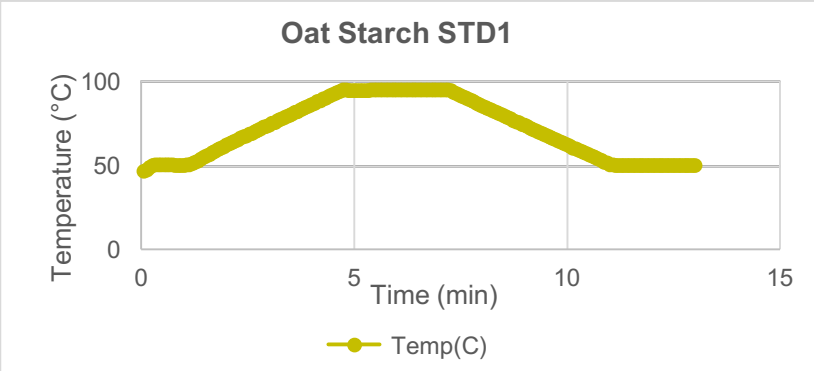


Figure 2. Example of oat starch standard temperature program STD1 over time.

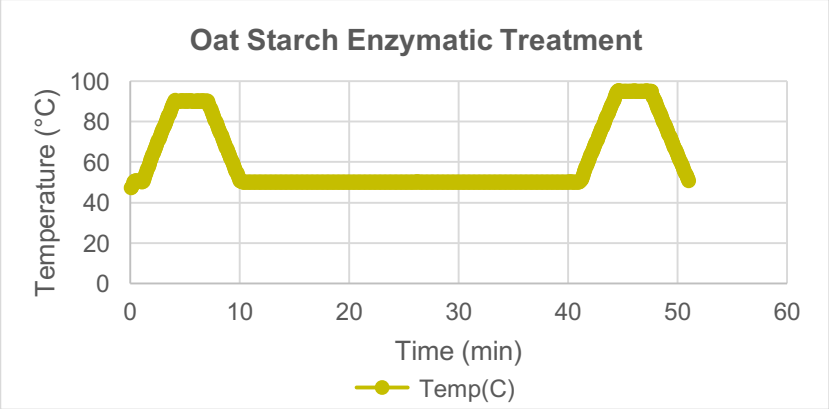


Figure 3. Example of temperature program enzymatic treatment over time.

3.4 RVA oat flour standard method

Different programs were set on the RVA to get a standard program at different temperatures, which can be seen in *table 3*. Samples were run at a maximum temperature of 95, 90, 88 and 85°C. Oat flour (3.00 g) and 25 ml deionized water was used in each run. The samples were put in the fridge and after cooling down into the freezer. When sufficiently frozen the samples were put in a freeze dryer over weekend.

Table 3. *RVA standard treatments of oat flour for RS analysis. OF= Oat Flour, STD=Standard program*

Sample	Flour (g)	Deionized water (ml)	Temperature maximum (°C)	Enzymatic treatment	Fridge (hours)
OF STD95	3.00	25	95	None	12
OF STD90A	3.00	25	90	None	12
OF STD90B	3.00	25	90	None	12
OF STD88	3.00	25	88	None	12
OF STD85	3.00	25	85	None	12

Enzymatic treatment on oat flour was carried out through preparing 3 samples which were run in similar way as the oat starch enzymatic treatment. In *figure 3* there is an example of temperature program for enzymatic treatment over time. The RVA was programed into three parts so that the enzyme could be added. The first program on the RVA was for gelatinization of the sample (85-95°C), the second RVA program was for enzymatic treatment (50°C) and the last RVA program was to kill off the enzymatic activity (95°C).

In *table 4* the enzymatic treatment of oat flour can be seen. In the first sample "Oat flour Enzyme 1", 20 µl Diazyme was added between the first and second part of the program. In the second sample 10 µl Diazyme was added into the sample called "Oat flour Enzyme 2" and the treatment was run for 60 minutes. In the third sample, 4 grams of oat flour together with 10 µl Diazyme was added for 30 minutes and the sample was called "Oat flour Enzyme 3". The samples were put in the fridge and after 12 hours into the freezer. When sufficiently frozen, all samples were put in a freeze dryer over weekend.

Table 4. *RVA enzymatic treatments of oat flour for RS analysis. OF= Oat Flour*

Sample	Flour (g)	Deionized water (ml)	Temperature maximum (°C)	Enzymatic treatment concentration, time	Fridge (hours)
OF Enzyme 1	3.00	25	90	Diazyme 20µl, 30min	12
OF Enzyme 2	3.00	25	90	Diazyme 10µl, 60min	12
OF Enzyme 3	4.00	25	90	Diazyme 10µl, 30min	12

3.5 Oat resistant starch analysis method

The samples were removed from the freeze-dryer and were carefully mashed into a powder with a spoon before weighed into analysis. The Megazyme Resistant Starch Assay Procedure (Megazyme, Bray Buisness Park, Bray, Co. Wicklow, A98 YV29, Ireland) was followed (AOAC Method 2002.02, AACC Method 32-40.01, Codex Type II Method). The spectrophotometer was set at 510 nm and the results were calculated in excel.

3.6 Enzyme treatment to increase RS content

RVA was run with 4.00 grams oat flour at 95°C during different time experiments to be able to see a complete gelatinization on the RVA viscosity curve, to use before enzymatic treatment. The time set 15 minutes was chosen as a suitable time period due to that it showed complete gelatinization and a decrease in viscosity that was steep in comparison to shorter time settings. The decision of time set was made regarding the percentage reduction in viscosity which showed that the longer time sets didn't show that much difference. It was also appreciated from the look of the viscosity curve, where the 15 minutes treatment looked completely gelatinized.

The results from the RVA standard treatments and the RVA enzymatic treatments were used to produce a new method focusing on parameters of interest to increase the level of RS in the samples. The RVA treatments applied on oat starch and oat flour for RS analysis can be seen in *table 5-6*, showing the RVA temperature maximum before enzymatic treatment, concentration of enzyme, amount of sample and fridge storage time.

Table 5. *RVA enzymatic treatments of oat starch for increase in RS. OS=Oat Starch*

Sample	Starch (g)	Deionized water (ml)	Temperature maximum (°C)	Enzymatic treatment concentration, time	Fridge (hours)
OS Enzyme 4	3.00	25	95	Diazyme 50µl, 60min	12
OS Enzyme 5	4.00	25	95	Diazyme 50µl, 60min	12
OS Enzyme 6	3.00	25	95	Diazyme 100µl, 60min	12
OS Enzyme 7	4.00	25	95	Diazyme 100µl, 60min	12

Table 6. *RVA enzymatic treatments of oat flour for increase in RS. OF=Oat Flour*

Sample	Flour (g)	Deionized water (ml)	Temperature maximum (°C)	Enzymatic treatment concentration, time	Fridge (hours)
OF Enzyme 4	3.00	25	95	Diazyme 50µl, 60min	12
OF Enzyme 5	4.00	25	95	Diazyme 50µl, 60min	12
OF Enzyme 6	3.00	25	95	Diazyme 100µl, 60min	12
OF Enzyme 7	4.00	25	95	Diazyme 100µl, 60min	12

The programs set on the RVA were changed with a prolonged heating period to make the viscosity to be lower when the enzyme is added. To conclude what time set that was suitable tests were made at 3, 12, 15 and 30 minutes. The tests were run at oat flour since it was proven earlier that oat flour gelatinized slower than the oat starch. When running tests the RVA viscosity diagrams showed a decrease in viscosity when the gelatinization heating period was longer. The temperature program for enzyme treatment can be seen in *figure 4*.

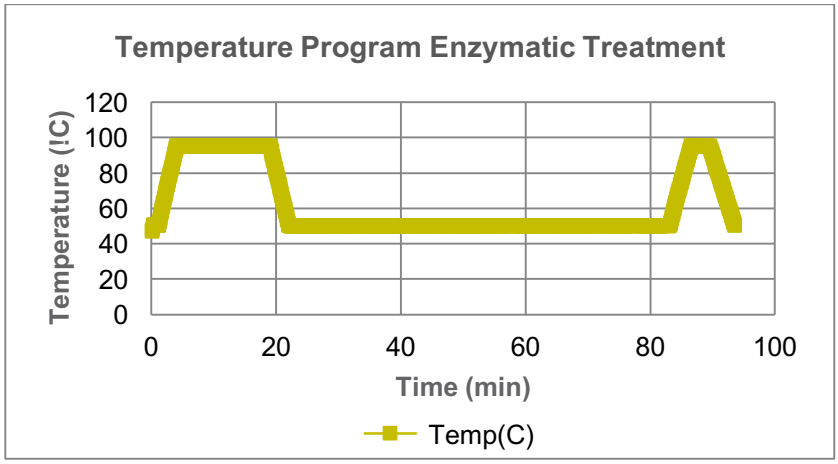


Figure 4. Example of temperature program for enzymatic treatment over time.

3.7 Microscopy

Microscopy was done on the samples “oat starch enzyme 6” and “oat flour enzyme 6” (see table 5 and table 6). The samples were analysed after a complete treatment and also before enzymatic treatment, after gelatinization program. Samples were suspended in diluted iodine solution and studied by light microscopy at 20X and 40X magnification. Scale bars were printed into the microscopy pictures.

3.8 Dietary fiber analysis

A dietary fiber determination test was done to measure RS type 3 in the sample to compare to the total RS analyzed by an AOAC method according to Theander *et al*. 1995.

3.9 Statistical analysis

The statistical analysis was performed using Minitab. A factorial design was made to see the effect on RS comparing the interaction between material, sample amount and enzyme concentration. An analysis of variance was made to see the relation between the parameters at 95 % significance level.

4 Results and Discussion

4.1 Enzymatic side activities

The results from the β-glucan analysis showed that the pullulanase from Novozyme have definitive enzymatic side activities breaking down β-glucan. The pullulanase Diazyme P10 from Danisco showed increased concentration in extractable β-glucan compared to the control. This can be seen in *table 7*, which show the average mean value of β-glucan (%), calculated from HPSEC results on barley flour treated with the different enzymes.

Table 7. *Average mean value of β-glucan content (%) calculated from HPSEC results on barley flour sample treated with enzymes*

Sample	β-glucan %
Blank	3,39
Diazyme	3,91
Novozyme	0,20

The increased concentration in β-glucan may be due to that the enzyme had a small side effect increasing the extractability. Diazyme P10 had a limited effect on the β-glucan molecular weight, while the enzyme preparation from Novozyme degraded the majority of β-glucan to fragments smaller than 10000Da.

4.2 RVA viscosity

The results from the RVA run without enzymatic treatment showed that the oat starch had in general a higher viscosity than oat flour. The results also showed that higher temperatures on the standard treatments (STD1) showed higher final viscosity in comparison to lower temperatures. The high final viscosity may be due to that the granules are gelatinized at a greater extent at the higher temperatures, though the

short period of time treated at this temperature may have left some granules unaffected. The maximum temperature and the final viscosity in the oat starch and oat flour standard treatments can be seen in *table 8*.

Table 8. *Treatment of oat starch standard samples and oat flour standard samples. OS: oat starch, OF: oat flour, STD1: standard treatment at different temperatures*

Sample	Maximum temp (°C)	Final viscosity (cP)
OS STD1 95	95	3303
OS STD1 90	90	1721
OS STD1 88	88	950
OS STD1 85	85	319
OF STD1 95	95	2704
OF STD1 90	90	1736
OF STD1 88	88	782
OF STD1 85	85	80

Enzymatic treatments 1-3 were run at 90°C estimated from the gelatinization temperature interval and look of the standard samples. The results showed that the enzymatic treated samples had lower RS content than standard samples and therefore a new maximum temperature at 95°C was set for following enzymatic treated samples. The hypothesis was at start that the granules shouldn't be heated too much and be swelled but not ruptured to give the enzyme highest affinity. Without evidence, it was supposed that the enzyme has higher affinity to the long chains rather than the short crystalline chains. This would lead to that, when the temperature rise, the short chains take more place and gets more active and gets prioritized by the enzyme. Therefore, the temperature shouldn't be too high so that the longer chains will be prioritized by the enzyme. In the second hypothesis, the temperature wasn't high enough for the first hypothesis to work. The granules did need to rupture completely and the enzyme should be added after the viscosity decreased. In *table 9* and *table 10* the enzymatic treatments and the effect on viscosity can be seen on oat starch and oat flour. The viscosity decreased in all samples which indicate that the enzyme was active. The tables also suggest that more enzyme, higher temperature and longer treatment time give larger decrease in viscosity. Though, if all of these parameters take part in the decrease in viscosity is hard to tell. The amount of sample doesn't suggest any effect on the viscosity. The oat starch samples suggest more stable trends that oat flour.

Figures of the RVA treatment in the method design can be seen below in *figures* 5-8, which shows viscosity before adding enzyme after 95°C gelatinization treatment at different time sets. This test was done to conclude the parameters before the method design was set.

Table 9. *Treatment of oat starch samples, showing viscosity decrease during enzymatic treatment at different conditions. OS: oat starch, n.d: not detected*

Sample	Amount sample (g)	Maximum temp (°C)	Enzyme treatment time (min)	Diazyme (µl)	Viscosity decrease (cP) during enzymatic treatment
OS Enzyme 1	3	90	30	10	n.d.
OS Enzyme 2	3	90	30	20	459-235
OS Enzyme 3	3	90	60	10	1339-213
OS Enzyme 4	3	95	60	50	1124-51
OS Enzyme 5	4	95	60	50	1454-221
OS Enzyme 6	3	95	60	100	1817-56
OS Enzyme 7	4	95	60	100	4544-195

Table 10. *Treatment of oat flour samples, showing viscosity decrease during enzymatic treatment at different conditions. OF: oat flour*

Sample	Amount sample (g)	Maximum temp (°C)	Enzyme treatment time (min)	Diazyme (µl)	Viscosity decrease (cP) during enzymatic treatment
OF Enzyme 1	3	90	30	20	1390-120
OF Enzyme 2	3	90	60	10	1599-117
OF Enzyme 3	4	90	30	10	3281-767
OF Enzyme 4	3	95	60	50	1594-4
OF Enzyme 5	4	95	60	50	1509-36
OF Enzyme 6	3	95	60	100	1579-18
OF Enzyme 7	4	95	60	100	1223-52

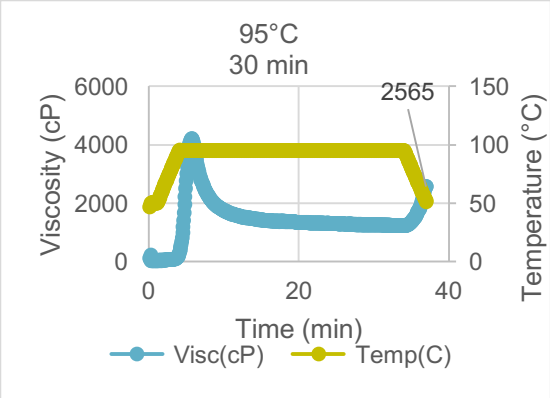


Figure 5. Temperature program at 95°C for 30 minutes, part 1 showing viscosity before adding enzyme in oat flour. The number 2565 is the final viscosity before adding enzyme.

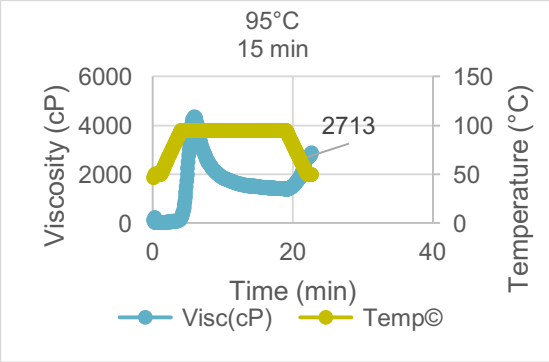


Figure 6. Temperature program at 95°C for 15 minutes, part 1 showing viscosity before adding enzyme in oat flour. The number 2713 is the final viscosity before adding enzyme.

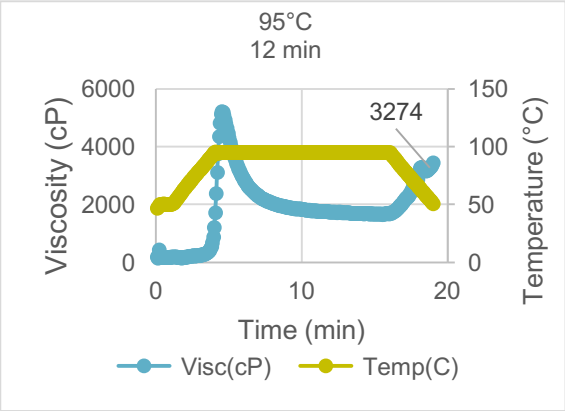


Figure 7. Temperature program at 95°C for 12 minutes, part 1 showing viscosity before adding enzyme in oat flour. The number 3274 is the final viscosity before adding enzyme.

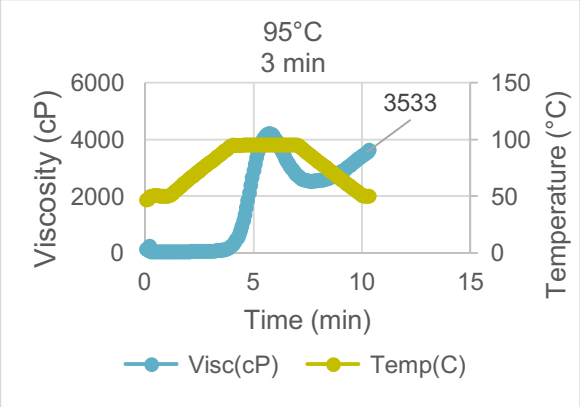


Figure 8. Temperature program at 95°C for 3 minutes, part 1 showing viscosity before adding enzyme in oat flour. The number 3533 is the final viscosity before adding enzyme.

When analyzing the *figures 5-8* it is clear that the treatment of 30 minutes gave a lower viscosity (2565 cP) in comparison to 15, 12 and 3 minutes (2713, 3274 and 3533 cP respectively). Though, the percentage reduction in relation to time was regarded and therefore the decision was taken that 15 minutes treatment was more suitable for further experiments.

4.3 RS determination for the standard method

The results from RS determination on oat starch samples showed an increase in concentration with increased temperature. The oat starch had a higher concentration of RS than oat flour. In *table 11*, the average value of RS calculated from absorbance gained from spectrophotometric measurements on samples without enzymatic treatment can be seen.

Table 11. *Average value of RS (%) calculated from absorbance gained from spectrophotometric measurement on oat starch samples and oat flour samples without enzymatic treatment. OS: oat starch, OF: oat flour*

Sample	RS%
OS STD1 95°C	2,86
OS STD1 90°C (A)	1,57
OS STD1 90°C (B)	1,52
OS STD1 88°C	0,92
OS STD1 85°C	0,31
OF STD1 95°C	0,18
OF STD1 90°C	0,11
OF STD1 88°C	0,14
OF STD1 85°C	0,13

For oat starch, it increased from 0.31% at 85°C to 2.86% at 95°C, and for oat flour from 0.13% at 85°C to 0.18% at 95°C.

In *table 12* the average value of RS calculated from absorbance gained from spectrophotometric measurement on samples with enzymatic treatment can be seen. According to these results it is indicating that OS Enzyme 2 with higher enzyme concentration and OS Enzyme 3 with longer time treatment give higher RS values. Also, OF Enzyme 1 with higher enzyme concentration and OF Enzyme 2 with the longest time treatment gave the highest results. Though, the difference is too small between samples to draw any conclusions. The RS contents are very low which proposes that something in the treatment didn't work.

Table 12. *Average value of RS (%) calculated from absorbance gained from spectrophotometric measurement on oat starch samples and oat flour samples with enzymatic treatment. OS:oat starch, OF:oat flour*

Sample	RS%
OS Enzyme 1 a	1,47
OS Enzyme 1 b	1,42
OS Enzyme 2 a	1,71
OS Enzyme 2 b	1,65
OS Enzyme 3	1,99
OF Enzyme 1	0,28
OF Enzyme 2	0,24
OF Enzyme 3	0,23

4.4 Method design for increased RS content

A method design was estimated from earlier results. The gelatinization was completed to a greater extent at high temperature and longer time of treatment. Since the enzymatic affinity was poor after gelatinization at 90°C, it is suggested that the enzyme will have higher affinity if the sample is fully gelatinized at 95°C. Also, a longer gelatinization treatment gave lower viscosity when the enzyme was added which was expected to be good for the enzymatic affinity.

In *table 13* you can see the average value of RS calculated from absorbance gained from spectrophotometric measurement on oat flour and oat starch samples with enzymatic treatment is shown.

Table 13. *Average value of RS (%) calculated from Absorbance gained from spectrophotometric measurement on oat starch samples and oat flour samples with enzymatic treatment. OS:oat starch, OF:oat flour*

Sample	RS%
OS Enzyme 4	4,42
OS Enzyme 5	4,02
OS Enzyme 6	4,62
OS Enzyme 7	5,77
OF Enzyme 4	0,31
OF Enzyme 5	0,44
OF Enzyme 6	0,20
OF Enzyme 7	0,51

As can be seen the results from the method design was more successful for the oat starch samples than in oat flour samples. The oat flour sample results are indicating that the treatment did not work. The oat flour samples have less starch and more

fatty acids and protein than the oat starch samples, which may have interfered with the results when analyzing the RS content. One theory is that the treatment did work, though not the analysis since the amylose might be encapsulated between other substances such as proteins and fatty acids. Therefore, it would be hard for the amylose to form complexes with another free amylose, forming helixes, which can be measured as RS. The literature supports the formation of RS in this way, that free amylose creates helixes, which are thermostable (Eerlingen & Delcour, 1995).

After determining statistical analyses significant results could be seen only for different materials used (oat starch and oat flour). The p-values for enzyme level and sample amount showed that there was no evidence that these factors affect the formation of RS (*figure 9*).

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	76.6219	12.7703	58.82	0.000
Linear	3	75.1719	25.0573	115.41	0.000
Material	1	73.8318	73.8318	340.05	0.000
Enzyme	1	1.0877	1.0877	5.01	0.052
Amount	1	0.2524	0.2524	1.16	0.309
2-Way Interactions	3	1.4500	0.4833	2.23	0.155
Material*Enzyme	1	0.7961	0.7961	3.67	0.088
Material*Amount	1	0.0626	0.0626	0.29	0.604
Enzyme*Amount	1	0.5912	0.5912	2.72	0.133
Error	9	1.9541	0.2171		
Lack-of-Fit	1	0.6116	0.6116	3.64	0.093
Pure Error	8	1.3424	0.1678		
Total	15	78.5760			

Figure 9. Analysis of variance in RS% content gained from RS analysis, showing p-value and significance level of the samples.

The results from the statistical analysis showed insignificant results for most parameters. The p-values were high and insignificant at a 95 % significance level. None of the relationships between parameters has an effect when relying on the statistics. Though, when the experimental was done, there was a trend showing that high enzyme concentration at complete gelatinized sample did show an increasing RS content in comparison to the other tests. As can be seen the p-value for enzyme is almost

significant. Also, the interaction between material*enzyme is quite close to the significance level, though it is not significant enough to draw a conclusion. The experimental was done at a limited time span, so the analysis and experiments was only done in duplicates. The p-value should be lowered if the analysis was done in more replicates and the relation between enzyme and material could in that case be significant.

Concerning material, it is clear that the experiment did work on oat starch, though not on oat flour. The reason for this is not clear and can only be hypothetically discussed. The literature show that oat flour has high amounts of components such as proteins, fats and minerals (Delcour & Hoseney, 2010). These components may have interfered with the enzyme or hurdled the retrogradation process in some way. It may be that these substances are preventing amylose to create helices with another free amylose.

The reason for the in general low RS in oat starch, which doesn't have that many components other than pure starch, may be that the method is not suitable for this type of starch. The literature show that oat has small granular starch granules which also may take part in the difficulty of transforming regular starch into RS.

The time of treatment is another factor that is important for the development of RS, according to studies (Milašinović *et al.*, 2010). The time of the enzymatic treatment and/or the time in storage may be insufficient for the RS to develop during the current method.

The Megazyme Resistant Starch Assay Kit is not a precise analysis method which is more statistical reliable for high RS contents rather than low RS. The low values of RS in the oat flour were not therefore the ultimate sample for analyzing with this method. Even though the work was thorough there are always sources of error in the method. In the analysis of RS in the control samples it differed between every run which indicates that the analysis method isn't that precise. Another source of variability between RS determinations could be inhomogeneties in the freezedried samples.

4.5 Microscope analysis

The pictures gained from the microscope analysis were difficult to comprehend. The oat starch show much less structure than many other starch types do after similar treatment (Paes *et al.*, 2008). This is indicating that the granules are broken and the starch is evenly suspended in the mixture. The oat flour samples show some particles that are difficult to identify but there are no obvious swollen granules visible. There is a clear difference in color between the oat flour samples and oat starch

samples. In *figure 10* there are gelatinized oat starch samples. In *figure 11* there are gelatinized oat flour samples.

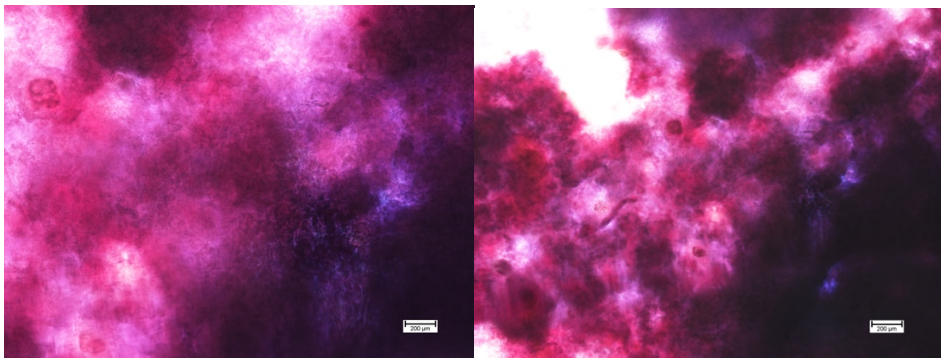


Figure 10. microscopy pictures of gelatinized oat starch.

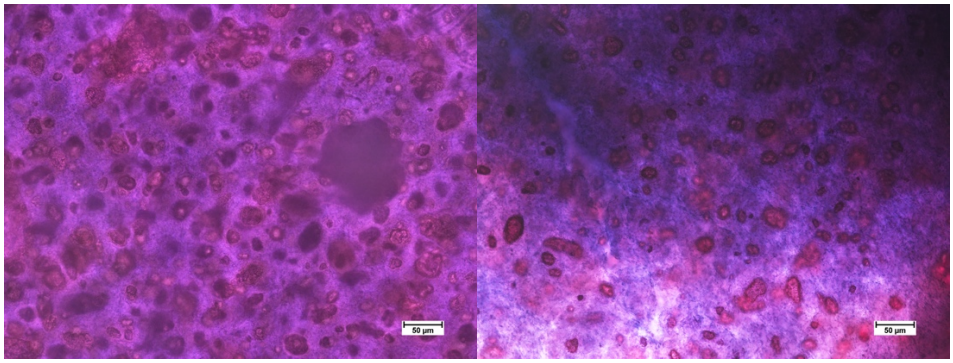


Figure 11. microscopy pictures of gelatinized oat flour.

Not much can be seen in the gelatinized oat starch and oat flour samples in *figure 10* and *figure 11,* although, there are more particles visible in the oat flour samples than in oat starch samples as expected. Otherwise, both samples look broken down, which indicate gelatinized starch granules. The color differs between oat starch and oat flour where oat starch is redder and oat flour more purple. It is hard to know the reason for this though it may be due to more leaked amylose in the oat starch samples. Since the color is not clearly separated in either of the samples this may be due to that the amylose and amylopectin have co-leached from the granules due to internally bound lipids. In *figure 12* there are pictures of oat starch samples treated with enzymatic treatment 6. In *figure 13* there are pictures of oat flour samples treated with enzymatic treatment 6 (see method in *table 5* and *table 6*).

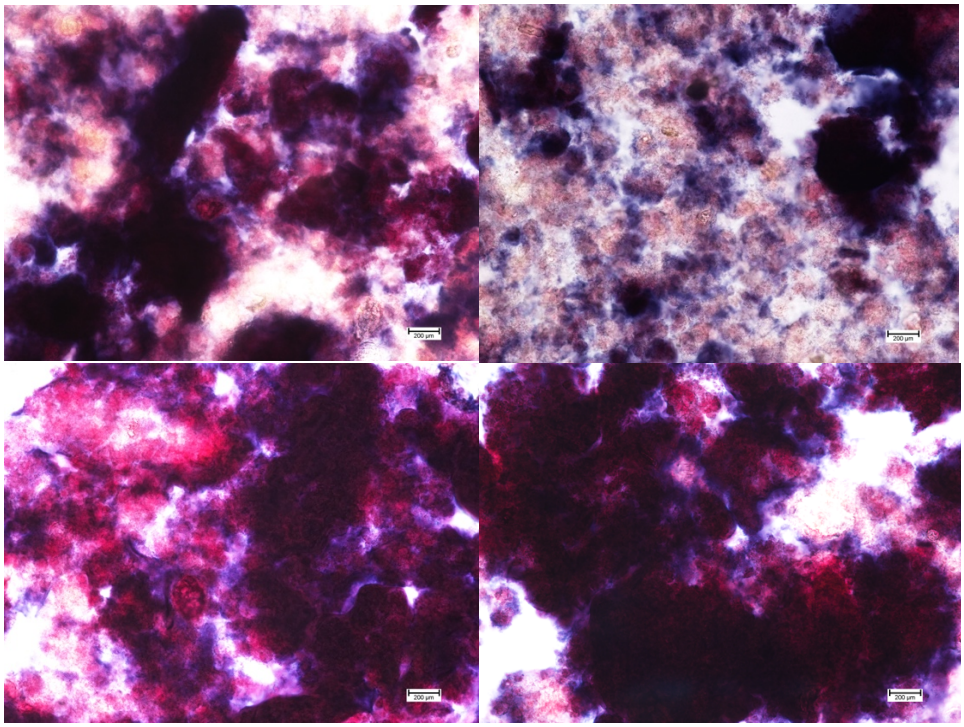
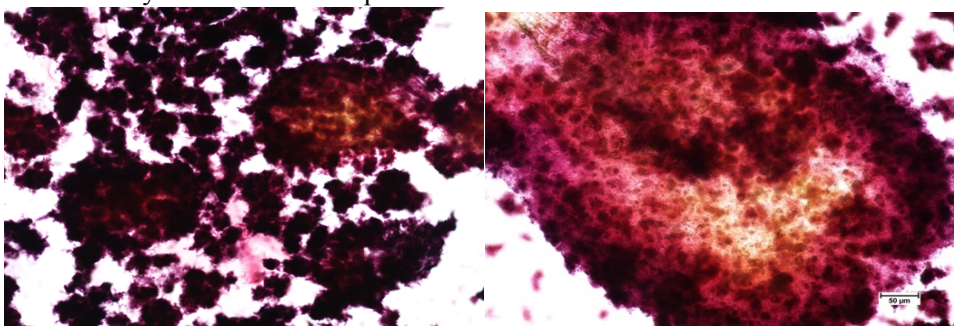


Figure 12. Enzymatic treatment 6 on oat starch samples showing sample directly taken from the RVA of starch granules colored with iodine.

In the enzymatic treated samples in *figure 12* and *figure 13* it is difficult to actually understand what you see. It is a clear difference in color where the oat starch samples are red in contrast to dark purple oat flour samples. Also, other particles such as fiber and/or protein can be seen in the oat flour samples. There is no clear explanation for the results in the enzymatic treated samples more than that the microscopy could have been performed with more phase separation. It is also strange that the samples didn't get more homogenized during the longer treatment in comparison to the non-enzymatic treated samples.



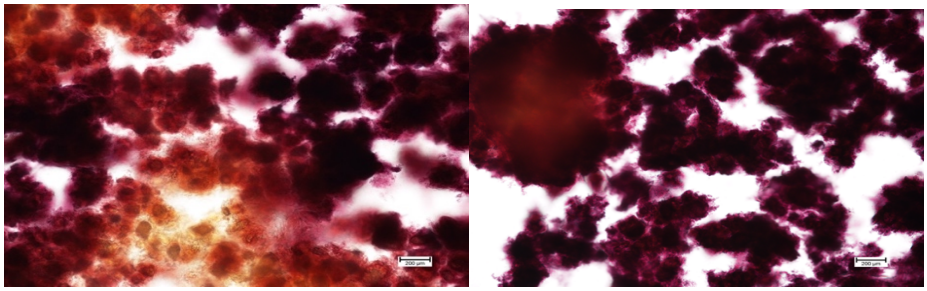


Figure 13. Enzymatic treatment 6 on oat flour samples showing sample directly taken from the RVA of starch granules colored with iodine.

It appears that even though a lot of iodine was added to the samples, it seems to be hard for the samples to absorb it. This may be due to the great viscosity of the samples. Considerably deeper investigation for these mysterious pictures and the behavior of RS formation is needed.

4.6 Dietary fiber analysis

The results from the DF analysis showed that the RS3 (seen as glucose) in the oat starch sample is 0.4 % as can be seen in *table 14*. It shows the oat flour and oat starch samples treated with enzymatic treatment 6 (see method *table 5 and table 6*).

Table 14. *Results from dietary fibre analysis in oat flour (OF) and oat starch (OS) sample treated with enzymatic treatment 6 (sugar residues as % of DM). Rha:D-rhamnose, fuc:D-fucose, ara:L-arabinose, xyl:D-xylose, gal:D-galactose, glc:D-glucose, n.d:not detected*

	Rha	fuc	ara	xyl	man	gal	glc
OF Enz 6	n.d.	n.d.	0.36	0.39	0.31	0.20	1.45
	n.d.	n.d.	0.36	0.41	0.31	0.20	1.44
OS Enz 6	n.d.	n.d.	n.d.	n.d.	0.24	n.d.	0.40
	n.d.	n.d.	n.d.	n.d.	0.23	n.d.	0.41
OF	n.a.	n.a.	0.33	0.36	0.28	0.19	1.34
	n.a.	n.a.	0.33	0.36	0.30	0.20	1.32

In the oat flour, it is impossible to grasp what value that is RS3 in an enzymatic treated oat flour sample (OF Enz 6) until a control oat flour is analysed to compare with (OF). This is due to that oat flour has some cell walls with β-glucan and cellulose that can be confused for RS3. In pure oat starch these components does not exist, therefore the glucose residues in the enzyme treated sample (OS Enz 6) show only pure RS3. There is no reason to believe that the actual content of RS3 is higher in the oat flour than in the oat starch, which is confirmed by the results above. The pure oat flour analysed for dietary fibre show that the RS3 in the oat flour is approximately 0.1 % while the RS3 in the oat starch is approximately 0.4 %. These values were calculated from the table, where *OS Enz 6* has a value at ~0.4 %. The *OF Enz*

6 with a D-glucose value at ~1.4 % was subtracted from the pure *OF* sample with a value at ~1.3 %, resulting in a value at approximately 0.1 %. This means that the RS3 is not developed in the oat flour in the same extent as in oat flour and since the results only show the RS3 that can stand high temperatures, the rest of the amount of RS can be assumed to be other types of RS.

5 Final Discussion

To increase the RS yield it is possible to use autoclaving and the temperature for autoclaving that is most optimal depends on the material (Eerlingen & Delcour, 1995). The aim in this study is to treat the flour in an industrial process and this is the reason for not using autoclaving at 120°C for 20 minutes since it is an expensive method to use. In this method, no color defect or other defect could be visualized in the flour which makes it worthwhile to study further, trying the parameters time, material amount and enzyme concentration.

The RVA method in this study shows that the yield is possible to increase when using higher temperatures, in this case 95°C is more successful than 85, 88, and 90 °C. The parameters of interest for increasing RS yield are definitely temperature, enzymatic treatment time and also storage time. When it comes to material, this study and the literature confirms, that the higher concentration of pure starch makes it easier to increase the RS yield. Also, enzyme concentration is suggesting better results with increased amount. The downside with using a lot of enzyme is that it is too expensive to use in an industrial process. Therefore, it is good to use as small amount of enzyme as possible that still shows a positive increasing effect on the RS yield. Water is another parameter that is expensive and needs to be minimized as much as possible. In this study, the water amount was high in relation to sample which is not suitable for the industry.

The mixing while using the RVA was hypothesized to be positive for the enzyme affinity, to be able to reach all material. It may be that the mixing was in a too great extent for the RS to be able to form. In a study trying to form RS by a twin-screw extruder, they could see that the RS formation had a negative correlation with increased screw speed (Unlu & Faller, 1998).

Another important factor to keep in mind is enzymatic treatment time. In one study 70 % of the RS formation was developed during the first 7h of enzymatic treatment (Milašinović *et al.*, 2010). In this study only one hour enzymatic treatment was performed. The enzymatic treatment can be prolonged for further studies, even

though the results should have shown the largest increase in the start of the treatment showing a trend for this to be possible.

The dietary fiber analysis showed a low amount of RS type 3 in comparison to the total RS. This may be due to that it is true in this case or that it is hard to get a representative sample from the freeze-drying method that was used.

The oat flour needs to be studied to recognize the preventing factors in this method. In further studies, it is possible to use proteases acting on protein in the flour to see if this can be a possible hurdle for RS development.

It is important to keep in mind that a risk using an enzyme is that some have side effects on other substances in the flour. In this study, the Novozyme enzyme had a degrading side effect on β-glucan, while the Diazyme enzyme only had a very small degrading side effect. Since it was a difference between Novozyme and Diazyme pullulanase while acting on β-glucan there may be differences in the action on the starch as well. Other pullulanases derived from other microorganism can be compared in further studies to see if the action on oat starch differs.

The pure starch content in the oat starch samples (~90 %) was higher than in the oat flour samples (~72 %). Even though the RS difference between materials is too large to explain. Repeatability and preparation of samples differs too much and therefore more trials need to be performed to get more exact results of RS, even though the aim in this study was to develop a method and not to get exact values of RS.

Oat is a promising crop to the use for resistant starch production, though the methodology to develop RS is strongly dependent varying between botanical origin of the grain. According to studies oat starch does not differ much from other cereal starches when it comes to thermal properties but oat starch retrogrades faster though not in the same extent as other cereals (Chu, 2013). When doing further studies it would be useful to do treatments on wheat starch and oat starch simultaneously to be able to compare. In that case it would be possible to analyze if the results are depending on the complexity of oat or if it depends on the method.

6 Conclusion

It is possible to increase the RS value in oat starch and oat flour through using moisture, heat and cooling cycles according to this study and according to literature. The parameters of interests are temperature, enzymatic treatment time and storage time. It is important to try the enzyme for side effect since it may breakdown other substances of importance.

It is clear to conclude that there is a need for more studies on oat starch and oat flour to increase the yield of RS. Enzymatic treatment with RVA gave oat flour unreliable results, therefore more studies are suggested on pure oat starch and when these show stabile results, the oat flour can be tested in regard that the values will be much lower. The prevention factors in oat flour should be tested further to figure out the mechanism behind why the RS yield was much lower than in oat starch.

When using enzymatic treatment with pullulanase on oat flour, the starch granules in the samples needs to be fully gelatinized for the RS yield to increase. High enzyme concentration gives high RS yield in pure oat starch. More replicates in this study would have given more reliable results. Oat flour samples needs to be studied further regarding the preventing factors that hurdles the enzyme to degrade the regular starch and create more RS.

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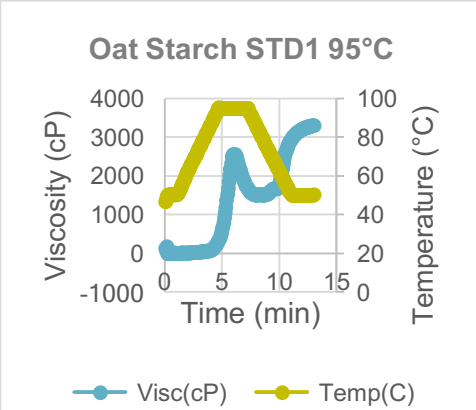
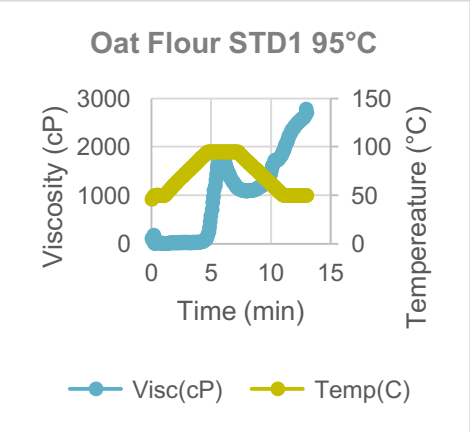
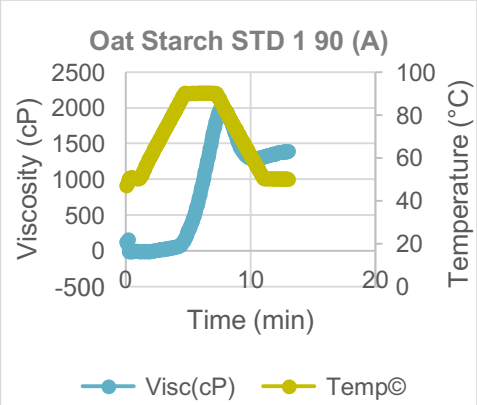
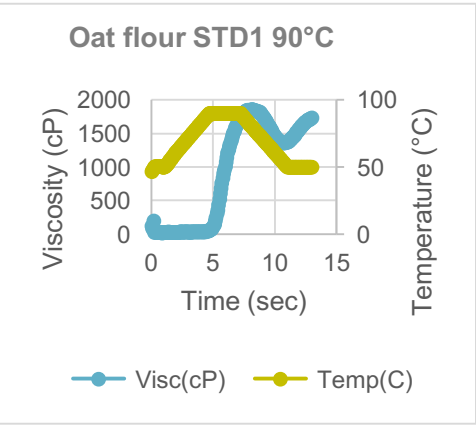
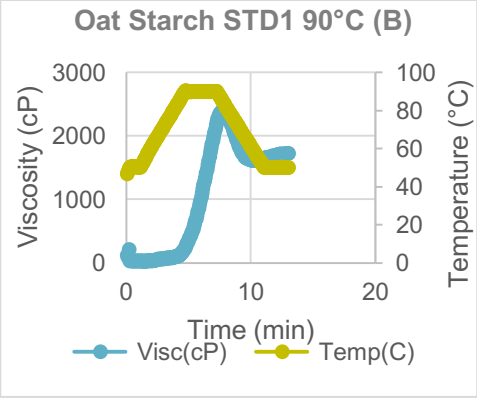
Appendix 1: RVA treatment figures

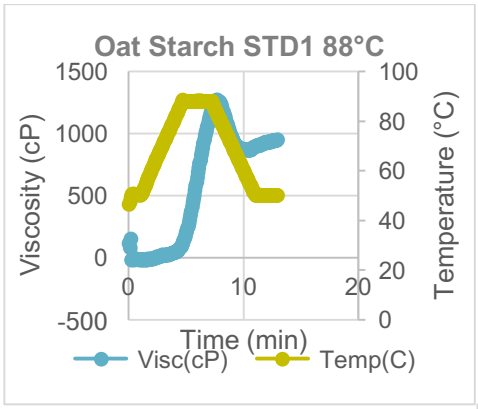








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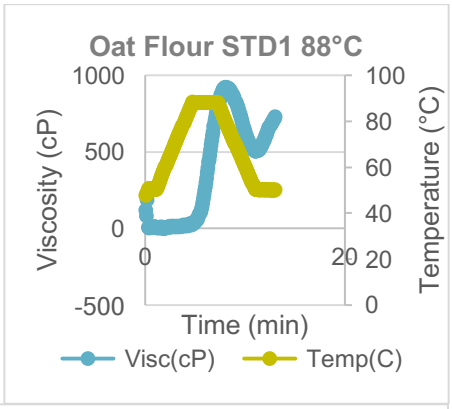
Time (min)
Visc(cP)
Temp(C)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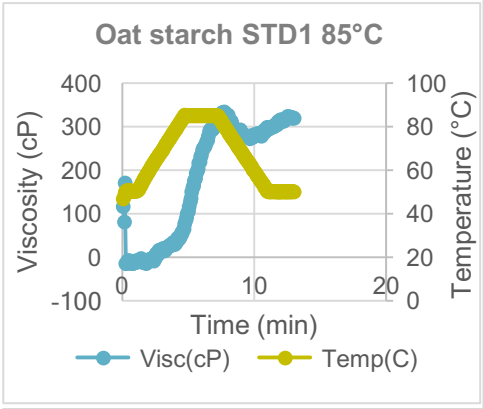
Temperature (°C)
Time (min)
Visc(cP)
Temp(C)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Time (min)
Visc(cP)
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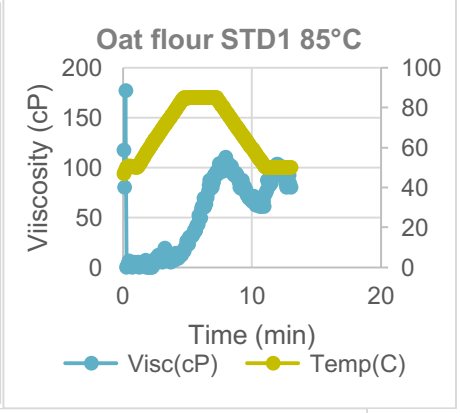
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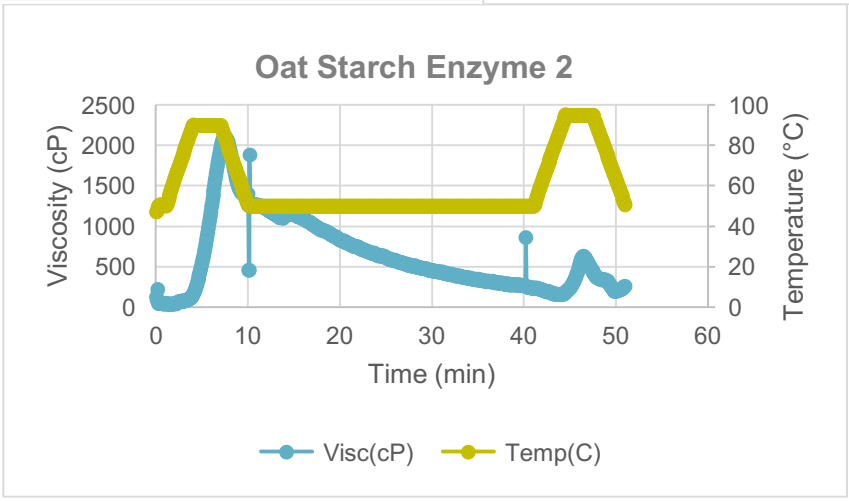
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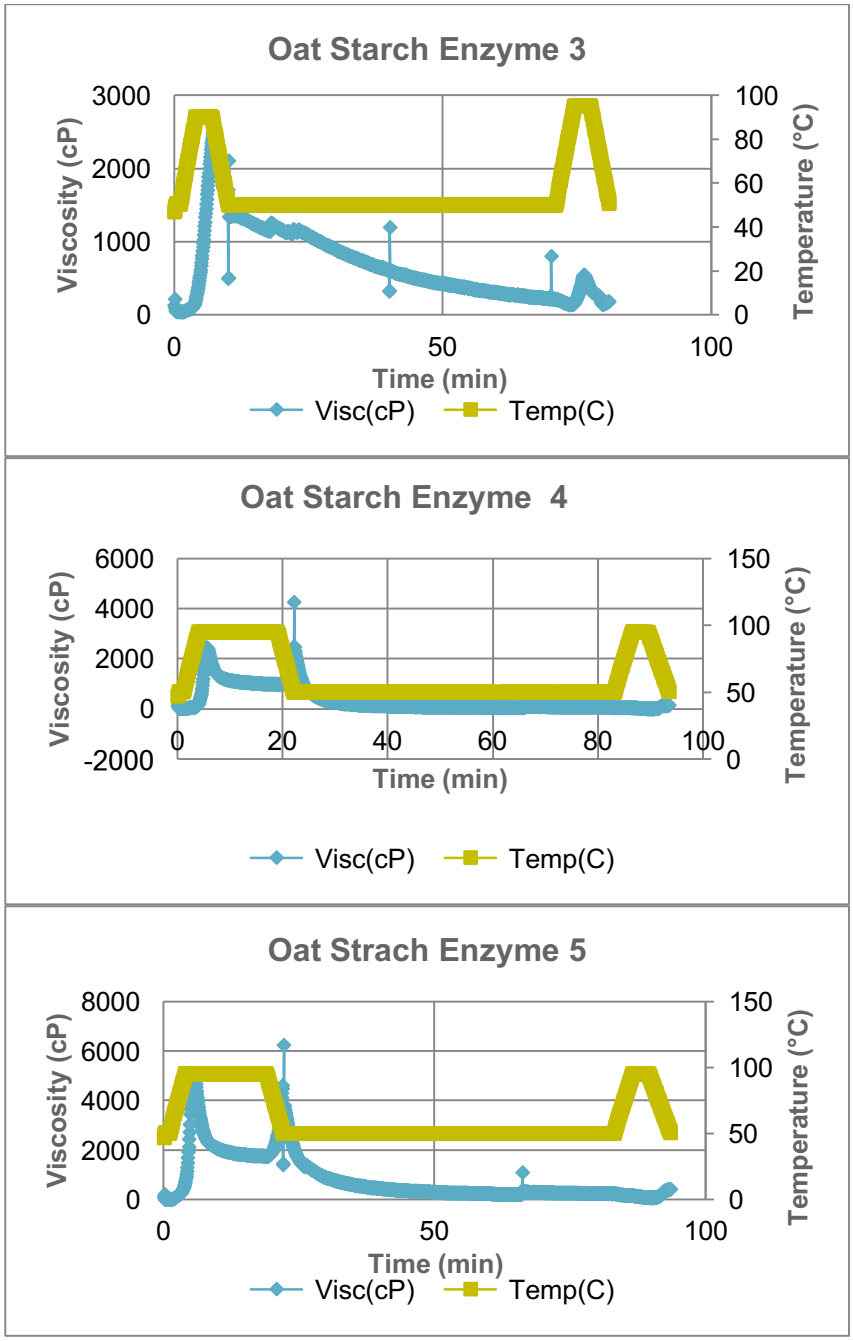
Time (min)
Visc(cP)
Temp(C)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2000
1000
0
0
50
100
Time (min)
100
80
60
40
20
0
Temperature (°C)
Visc(cP)
Temp(C)
Oat Starch Enzyme 4
Viscosity (cP)
6000
4000
2000
0
-2000
0
20
40
60
80
100
Time (min)
150
100
50
0
Temperature (°C)
Visc(cP)
Temp(C)
Oat Strach Enzyme 5
Viscosity (cP)
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0
50
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100
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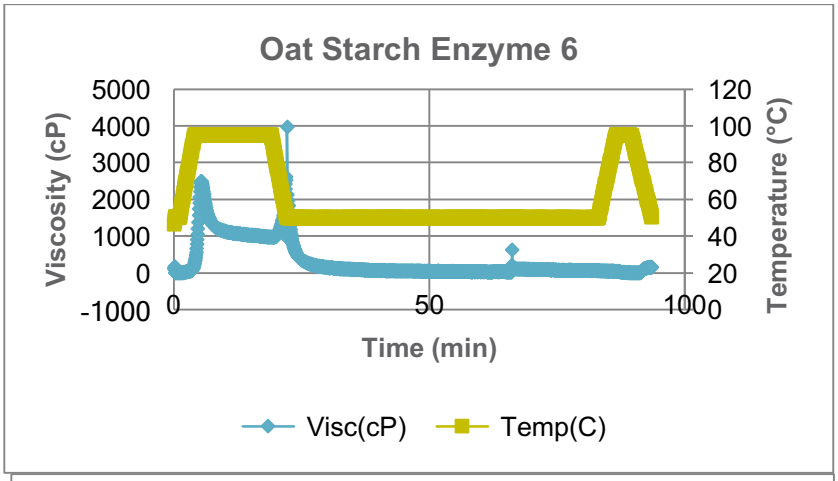
Visc(cP)
Temp(C)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2000
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0
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Time (min)
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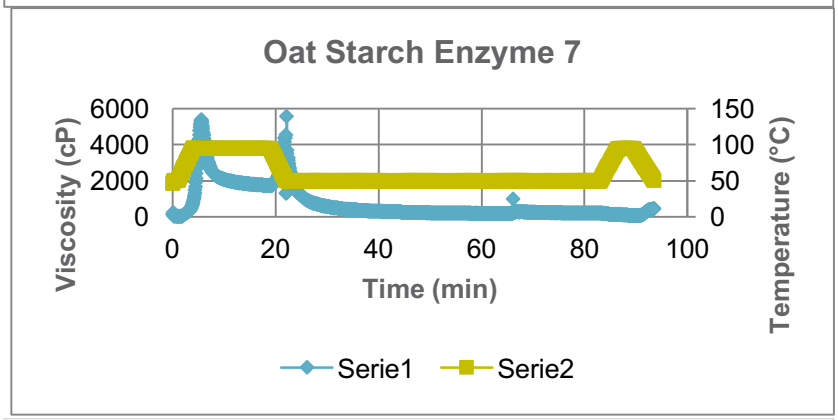
100
80
60
40
20
0
Temperature (°C)
Visc(cP)
Temp(C)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2000
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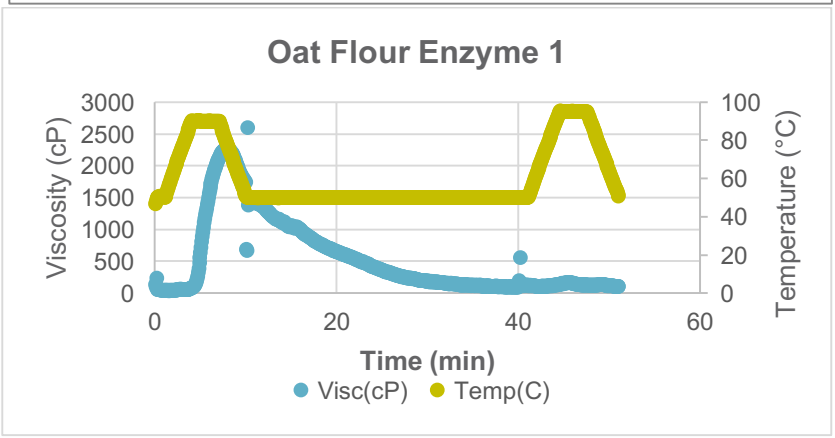
20
40
60
80
100
Time (min)
150
100
50
0
Temperature (°C)
Serie1
Serie2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1500
1000
500
0
0
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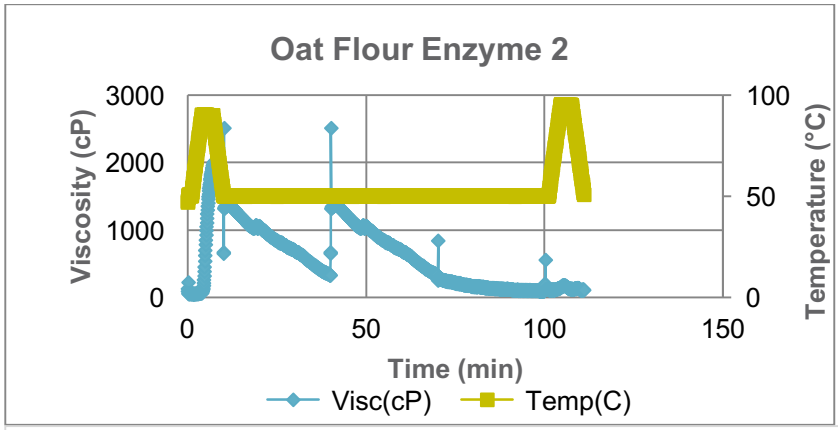
60
Time (min)
100
80
60
40
20
0
Temperature (°C)
Visc(cP)
Temp(C)


Oat Flour Enzyme 2
Viscosity (cP)
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100
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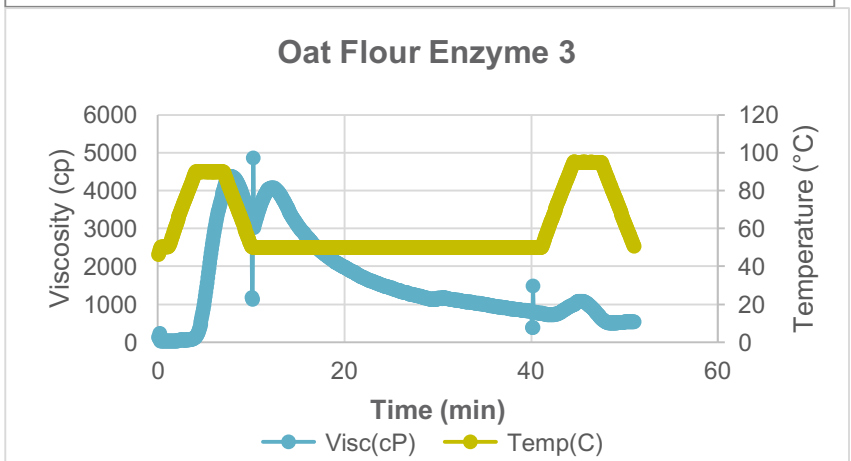
Temperature (°C)
0
50
100
150
Time (min)
Visc(cP)
Temp(C)


Oat Flour Enzyme 3
Viscosity (cp)
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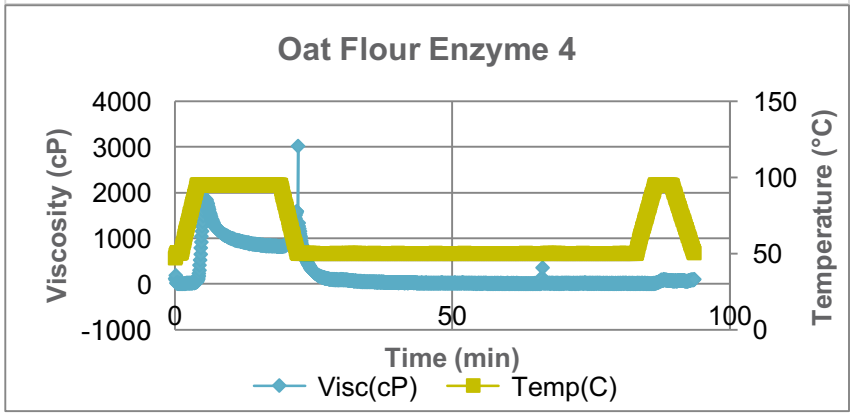
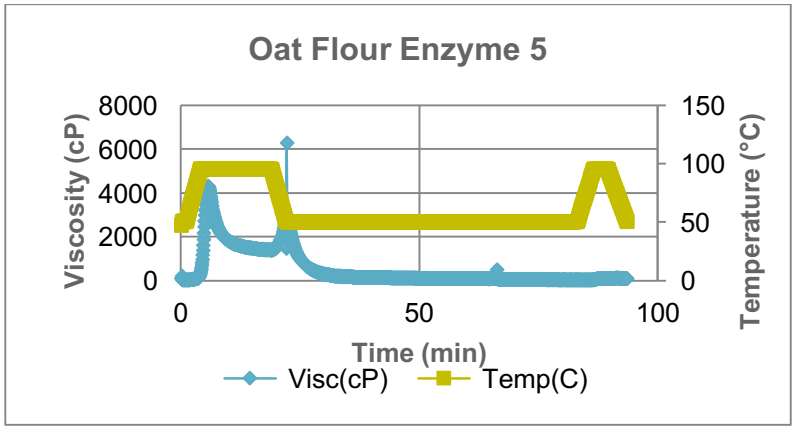
5000
4000
3000
2000
1000
0
120
100
80
60
40
20
0
Temperature (°C)
0
20
40
60
Time (min)
Visc(cP)
Temp(C)


Oat Flour Enzyme 4
Viscosity (cP)
4000
3000
2000
1000
0
-1000
150
100
50
0
Temperature (°C)
0
50
100
Time (min)
Visc(cP)
Temp(C)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Viscosity (cP)
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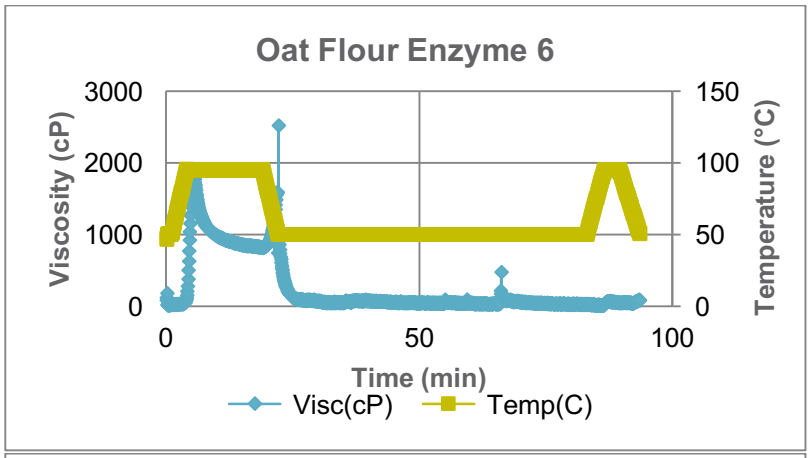
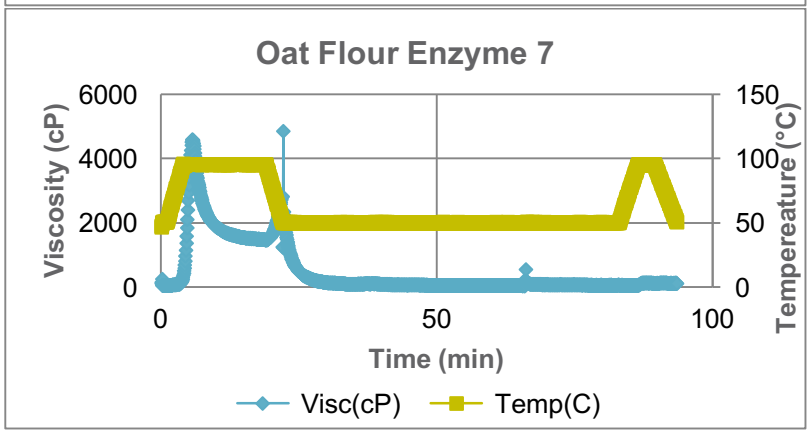
6000
4000
2000
0
150
100
50
0
Temperature (°C)
0
50
100
Time (min)
Visc(cP)
Temp(C)


Oat Flour Enzyme 6
Viscosity (cP)
3000
2000
1000
0
150
100
50
0
Temperature (°C)
0
50
100
Time (min)
Visc(cP)
Temp(C)


Oat Flour Enzyme 7
Viscosity (cP)
6000
4000
2000
0
150
100
50
0
Tempereature (°C)
0
50
100
Time (min)
Visc(cP)
Temp(C)

Appendix 2: Pullulanase enzymes

DuPont Industrial Biosciences
Bioactives, Food Enzymes
foodenzymes@dupont.com
www.food.dupont.com

Valid from: February 18, 2016

DANISCO
First you add knowledge...

PRODUCT DESCRIPTION - PD 215916-10.0EN

DIAZYME® P10

Description

DIAZYME® P10 is a pullulanase enzyme preparation derived from Bacillus lichenformis.

Application areas

Brewing

Potential benefits

- Debranching of liquefied starch
- Increases level of fermentable carbohydrates with low impact on sugar composition (in the mash)
- Improves rate of fermentation
- Is typically inactivated at beer pasteurisation

Usage levels

Typical dosage rate	
In the mash	0.5-2.0 kg/MT of grist
In the fermentor	0.03-0.12 kg/MT of grist

Directions for use

It is recommended to add DIAZYME® P10 to the mash at or right after mashing-in for adjustment of attenuation. DIAZYME® P10 is heat labile and recommended to be used < 65 °C.
For fermentor usage, DIAZYME® P10 is beneficially added in combination with DIAZYME® FA to improve RDF level.

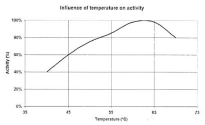
Composition

DIAZYME® P10 is composed of:

• Water	57 - 89 % (w/w)
• Dextrose	10.0 - 38.0 % (w/w)
• Pullulanase	1 - 5 % (w/w)
• Sodium benzoate	0.26 - 0.35 % (w/w)
• Potassium sorbate	0.09 - 0.13 % (w/w)

Physical/chemical specifications

Physical form	liquid
Specific gravity	1.15 +/- 0.03 kg/l
Activity	min. 1000 ASPU/g



The data for the graphs are generated under laboratory conditions and may not reflect performance in the application. It is therefore recommended to evaluate the performance under the specific local conditions.
Pasteurisation units (PU): PU requirement for inactivation is 30 as measured in an all malt beer, 4.6 ABV at pH = 4.5. Validation should be performed under local conditions.

Microbiological specifications

Total viable count	less than 10000 /ml
Coliforms	less than 15 /ml
E. coli	absent in 25 ml
Salmonella species	absent in 25 ml
Lactic acid bacteria	less than 5 /ml
Yeast	less than 10 /ml
Mould	less than 10 /ml
Antibiotic activity	negative by test

Heavy metal specifications

Arsenic	less than 3 mg/kg
Lead	less than 5 mg/kg
Heavy metals (as Pb)	less than 30 mg/kg

Nutritional data

Calculated values per 100 g

Energy	105/440 kcal/kJ
Protein	less than 5 g
Fat	less than 1 g
Carbohydrates	20-30 g
Moisture	70-80 g
Ash	less than 5 g

Storage

DIAZYME® P10 should be stored dry and cool (max. 10°C/50°F) and sheltered against direct sunlight

Packaging

28 kg plastic can
225 kg plastic drum
1125 kg transparent container

Purity and legal status

DIAZYME® P10 meets the specifications laid down by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC) and is GRAS (Generally Recognized as Safe) in the US. When used as a processing aid under 21 CFR 101.00, it may exempt from FDA labelling requirements and is typically not labelled.

DIAZYME® P10 is approved by most countries for use in food. However, as legislation regarding its use in food may vary from country to country, local food regulations should always be consulted concerning the status of this product. Advice regarding the legal status of this product may be obtained on request.

Safety and handling

Avoid unnecessary contact with enzyme preparations during handling. In case of spillage, rinse with water. Additional information can be found in the Material Safety Data Sheet.

Kosher status

DIAZYME® P10 is certified kosher pareve by Union of Orthodox Jewish Congregations of America (OU).

GMO status

The microorganisms used for production of DIAZYME® P10 are developed by recombinant DNA technique according to the definition of Directive 2009/41/EC on the contained use of genetically modified micro-organism.

DuPont Industrial Biosciences
Bioactives, Food Enzymes
foodenzymes@dupont.com
www.food.dupont.com

Valid from: February 18, 2016

DANISCO
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PRODUCT DESCRIPTION - PD 215916-10.0EN

DIAZYME® P10

The information contained in this publication is based on our own research and development work and is to the best of our knowledge reliable. Users should, however, conduct their own tests to determine the suitability of our products for their own specific purposes and the legal status for their intended use of the product. Statements contained herein should not be considered as a warranty of any kind, expressed or implied, and no liability is accepted for the infringement of any patents.

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Bioactives, Food Enzymes
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Valid from: February 18, 2016

DANISCO
First you add knowledge...

PRODUCT DESCRIPTION - PD 215916-10.0EN

DIAZYME® P10

Allergens

The table below indicates the presence (as added component) of the following allergens and products thereof (according to US Food Allergen and Consumer Protection act (FALCPA), 2004 and Directive 2000/13/EU as amended).

Yes	No	Allergens	Description of components
	X	Wheat	
(X)		Other cereals containing gluten	Glucose (used in fermentation)* Glucose: This level was below quantification level of 5 ppm, based on ELISA analysis. This component is exempted from allergen labeling in the EU.
	X	Crustaceans	
	X	Eggs	
	X	Fish	
	X	Peanuts	
	(X)	Soybeans	Soy flour (used in fermentation)* Soy hydrolysate (used in fermentation)*
	X	Milk (incl. lactose)	
	X	Nuts	
	X	Celery	
	X	Mustard	
	X	Sesame seeds	
	X	Sulphur dioxide and sulphites (>10mg/kg)	
	X	Lupin	
	X	Molluscs	

*Danisco has determined that fermentation nutrients are outside the scope of US and EU food allergen labeling requirements † , ‡ †
Position paper sent by ETA to the FDA on September 12, 2005 (www.enzymetechnicalassoc.org/Allergen%20per%20paper-2.pdf).
‡ Summarized in the position paper of the Association of Manufacturers and Formulators of Enzyme products: http://www.amfep.org/documents/AmfepstatementScopeAllergyLabellingDrfinal_000.pdf

The information contained in this publication is based on our own research and development work and is to the best of our knowledge reliable. Users should, however, conduct their own tests to determine the suitability of our products for their own specific purposes and the legal status for their intended use of the product. Statements contained herein should not be considered as a warranty of any kind, expressed or implied, and no liability is accepted for the infringement of any patents.

DANISCO

MATERIAL SAFETY DATA SHEET

1. CHEMICAL PRODUCT AND COMPANY IDENTIFICATION

Trade name:	**DIAZYME P10**
Product code:	-
Supplier:	Danisco A/S Edwin Rahrs Vej 38, DK-8220 Brabrand, Denmark. Tel.: +45 89 43 50 00 Fax.: +45 86 25 10 77
Emergency telephone:	+45 79 72 56 00

2. COMPOSITION/INFORMATION ON INGREDIENTS

Description: DIAZYME P10 is an enzymatic preparation.

3. HAZARDS IDENTIFICATION

Repeated inhalation of enzyme aerosols may cause sensitization and will cause allergic type reactions in sensitized individuals.
Prolonged skin contact may cause minor irritation.

4. FIRST-AID MEASURES

Eye contact:	Rinse with plenty of water.
Skin contact:	Rinse with plenty of water.
Inhalation:	Fresh air if discomfort is felt. If irritation or allergic response occur consult a doctor.
Ingestion:	Rinse mouth and throat. Drink water, milk or juice. If symptoms occur seek medical advice.
First aid facilities:	Not applicable.
Advice to doctor:	None.

5. FIRE-FIGHTING MEASURES

DIAZYME P10 is not flammable.

6. ACCIDENTAL RELEASE MEASURES

Avoid raising aerosols.
Clean up spillage with a towel.
Wash the soiled area with water.
Never use high pressure water jet.
Wear personal protective equipment as described under section 8.
Wash contaminated clothing.

7. HANDLING AND STORAGE

Handling:	Avoid the formation of aerosols.
Storage:	Store container in a dry and cool place.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Exposure standards:	Not available.
Engineering controls:	Any equipment used to handle this product, should be designed to minimize the escape of aerosols, dust and vapours.
Protective equipment:	Wear protective clothing, protective gloves and safety goggles, when there is a risk of getting in contact with the substance.
Educational demands:	Instruction in the use of this product and knowledge of this Material Safety Data Sheet.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance:	A liquid.		
Solubility:	Miscible with water.		
Boiling point:	Not available.	**Melting point:**	Not available.
Flash point:	Not applicable.	**Flammability limits:**	Not applicable.
Vapour pressure:	Not available.	**Density:**	Not available.

10. STABILITY AND REACTIVITY

DIAZYME P10 is stable under normal conditions of use.

Conditions to avoid:	None.
Materials to avoid:	None.
Hazardous decomposition products:	None.

11. TOXICOLOGICAL INFORMATION

Repeated inhalation of enzyme aerosols may cause sensitization and will cause allergic type reactions in sensitized individuals.
Prolonged skin contact may cause minor irritation.

Oral rat LD50:	> 2000 mg/kg b.w..
Symptoms:	Coughing, difficulty in breathing.

12. ECOLOGICAL INFORMATION

DIAZYME P10 is believed not to be dangerous to the environment with respect to mobility, persistency and degradability, bioaccumulative potential, aquatic toxicity and other data relating to ecotoxicity.

13. DISPOSAL CONSIDERATIONS

Small quantities of waste are disposed of as domestic refuse.
Greater quantities are disposed of in accordance with the local regulations.

14. TRANSPORT INFORMATION

DIAZYME P10 is not considered dangerous according to ADR, RID, IMO and IATA.

HermLJ

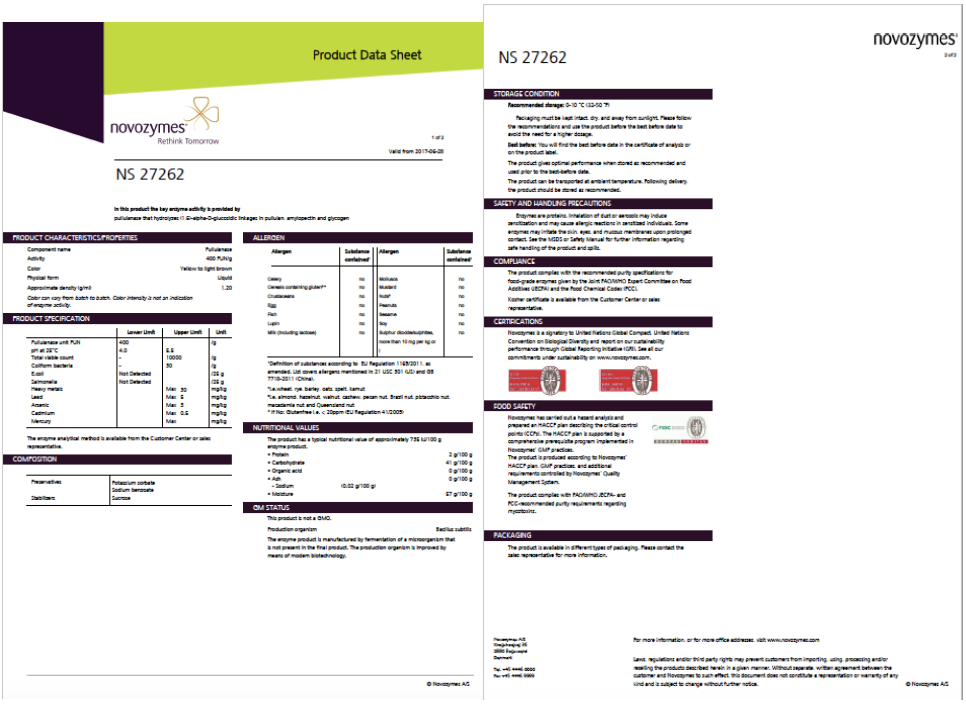
15. REGULATORY INFORMATION

According to EU regulations, DIAZYME P10 must be labelled as follows:

R 42	May cause sensitization by inhalation.
S 23:	Do not breathe vapour.
S 24:	Avoid contact with skin.
S 36/37:	Wear suitable protective clothing and gloves.

16. OTHER INFORMATION

This data sheet complies with EU Directive 91/155 as amended.
A Product Description is available on request.



Product Data Sheet

novozymes
Rethink Tomorrow

1 of 2

Valid from 2017-06-28

NS 27262

In this product the key enzyme activity is provided by
pullulanase that hydrolyses (1 6)-alpha-D-glucosidic linkages in pullulan, amylopectin and glycogen

PRODUCT CHARACTERISTICS/PROPERTIES

Component name	Pullulanase
Activity	400 PUN/g
Color	Yellow to light brown
Physical form	Liquid
Approximate density (g/ml)	1.20

Color can vary from batch to batch. Color intensity is not an indication of enzyme activity.

PRODUCT SPECIFICATION

	Lower Limit	Upper Limit	Unit
Pullulanase unit PUN	400		/g
pH at 25°C	4.0	6.0	
Total viable count	-	10000	/g
Coliform bacteria	-	30	/g
E.coli	Not Detected		/25 g
Salmonella	Not Detected		/25 g
Heavy metals:			
Lead		Max. 30	mg/kg
Arsenic		Max. 3	mg/kg
Cadmium		Max. 0.5	mg/kg
Mercury		Max.	mg/kg

The enzyme analytical method is available from the Customer Center or sales representative.

COMPOSITION

Preservatives	Potassium sorbate Sodium benzoate
Stabilizers	Sucrose

ALLERGEN

Allergen	Substance contained	Allergen	Substance contained
Celery	no	Molluscs	no
Cereals containing gluten*1	no	Mustard	no
Crustaceans	no	Nuts*	no
Eggs	no	Peanuts	no
Fish	no	Sesame	no
Lupin	no	Soy	no
Milk (including lactose)	no	Sulphur dioxide/sulphites, more than 10 mg per kg or l	no

*Definition of substances according to EU Regulation 1169/2011, as amended. List covers allergens mentioned in 21 USC 301 (US) and GB 7718-2011 (China).

*1 as wheat, rye, barley, oats, spelt, kamut.
*2 e. almond, hazelnut, walnut, cashew, pecan nut, Brazil nut, pistachio nut, macadamia nut and Queensland nut.
* If No: Glutenfree i.e. < 20ppm (EU Regulation 41/2009)

NUTRITIONAL VALUES

The product has a typical nutritional value of approximately 736 kJ/100 g enzyme product.

• Protein	2 g/100 g
• Carbohydrate	41 g/100 g
• Organic acid	0 g/100 g
• Ash	0 g/100 g
- Sodium (0.02 g/100 g)	
• Moisture	57 g/100 g

GM STATUS

This product is not a GMO.

Production organism: Bacillus subtilis

The enzyme product is manufactured by fermentation of a microorganism that is not present in the final product. The production organism is improved by means of modern biotechnology.

novozymes
2 of 2

NS 27262

STORAGE CONDITION

Recommended storage: 0–10 °C (32–50 °F)

Packaging must be kept intact, dry, and away from sunlight. Please follow the recommendations and use the product before the best before date to avoid the need for a higher dosage.

Best before: You will find the best before date in the certificate of analysis or on the product label.

The product gives optimal performance when stored as recommended and used prior to the best-before date.

The product can be transported at ambient temperature. Following delivery, the product should be stored as recommended.

SAFETY AND HANDLING PRECAUTIONS

Enzymes are proteins. Inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals. Some enzymes may irritate the skin, eyes, and mucous membranes upon prolonged contact. See the MSDS or Safety Manual for further information regarding safe handling of the product and spills.

COMPLIANCE

The product complies with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemical Codex (FCC).

Kosher certificate is available from the Customer Center or sales representative.

CERTIFICATIONS

Novozymes is a signatory to United Nations Global Compact. United Nations Convention on Biological Diversity and report on our sustainability performance through Global Reporting Initiative (GRI). See all our commitments under sustainability on www.novozymes.com.

FOOD SAFETY

Novozymes has carried out a hazard analysis and prepared an HACCP plan describing the critical control points (CCPs). The HACCP plan is supported by a comprehensive prerequisite program implemented in Novozymes' GMP practices.
The product is produced according to Novozymes' HACCP plan, GMP practices, and additional requirements controlled by Novozymes' Quality Management System.

The product complies with FAO/WHO JECFA- and FCC-recommended purity requirements regarding mycotoxins.

PACKAGING

The product is available in different types of packaging. Please contact the sales representative for more information.

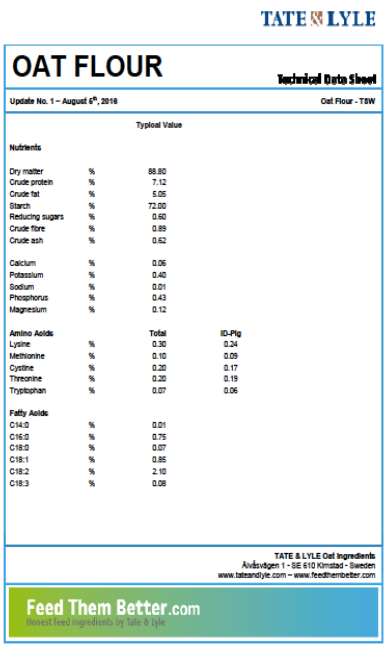
Novozymes A/S
Krogshøjvej 36
2880 Bagsværd
Denmark

Tel.: +45 4446 0000
Fax: +45 4446 0999

For more information, or for more office addresses, visit www.novozymes.com

Laws, regulations and/or third party rights may prevent customers from importing, using, processing and/or reselling the products described herein in a given manner. Without separate, written agreement between the customer and Novozymes to such effect, this document does not constitute a representation or warranty of any kind and is subject to change without further notice.

Appendix 3: Oat flour composition



TATE & LYLE

OAT FLOUR

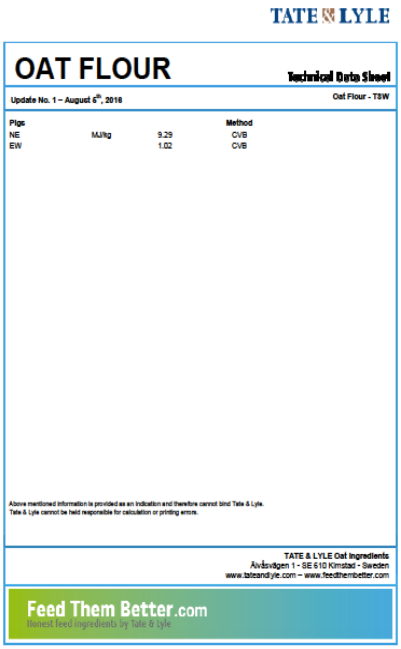
Technical Data Sheet

Update No. 1 – August 6th, 2016 — Oat Flour - TSW

Nutrients		Typical Value	
Dry matter	%	88.80	
Crude protein	%	7.12	
Crude fat	%	5.05	
Starch	%	72.00	
Reducing sugars	%	0.60	
Crude fibre	%	0.89	
Crude ash	%	0.62	
Calcium	%	0.06	
Potassium	%	0.40	
Sodium	%	0.01	
Phosphorus	%	0.43	
Magnesium	%	0.12	
Amino Acids		**Total**	**ID-Pig**
Lysine	%	0.30	0.24
Methionine	%	0.10	0.09
Cystine	%	0.20	0.17
Threonine	%	0.20	0.19
Tryptophan	%	0.07	0.06
Fatty Acids			
C14:0	%	0.01	
C16:0	%	0.75	
C18:0	%	0.07	
C18:1	%	0.85	
C18:2	%	2.10	
C18:3	%	0.08	

TATE & LYLE Oat Ingredients
Åhåsvägen 1 - SE 610 Kimstad - Sweden
www.tateandlyle.com – www.feedthembetter.com

Feed Them Better.com
Honest feed ingredients by Tate & Lyle



TATE & LYLE

OAT FLOUR

Technical Data Sheet

Update No. 1 – August 6th, 2016 — Oat Flour - TSW

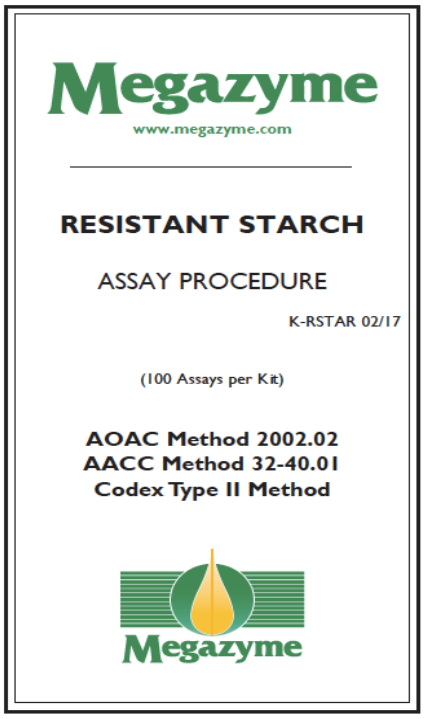
Pigs			Method
NE	MJ/kg	9.29	CVB
EW		1.02	CVB

Above mentioned information is provided as an indication and therefore cannot bind Tate & Lyle.
Tate & Lyle cannot be held responsible for calculation or printing errors.

TATE & LYLE Oat Ingredients
Åhåsvägen 1 - SE 610 Kimstad - Sweden
www.tateandlyle.com – www.feedthembetter.com

Feed Them Better.com
Honest feed ingredients by Tate & Lyle

Appendix 4: Megazyme resistant starch assay procedure



Megazyme

www.megazyme.com

RESISTANT STARCH

ASSAY PROCEDURE

K-RSTAR 02/17

(100 Assays per Kit)

AOAC Method 2002.02
AACC Method 32-40.01
Codex Type II Method

Megazyme

INTRODUCTION:

By definition, resistant starch (RS) is that portion of the starch that is not broken down by human enzymes in the small intestine. It enters the large intestine where it is partially or wholly fermented. RS is generally considered to be one of the components that make up total dietary fibre (TDF).

The presence of a starch fraction resistant to enzymic hydrolysis was first recognised by Englyst *et al.* in 1982 during their research on the measurement of non-starch polysaccharides.[1] This work was extended by Berry[2] who developed a procedure for the measurement of RS incorporating the α-amylase/pullulanase treatment employed by Englyst *et al.*[1] but omitting the initial heating step at 100°C, so as to more closely mimic physiological conditions. Under these conditions, the measured resistant starch contents of samples were much higher. This finding was subsequently confirmed by Englyst *et al.*[3-5] through studies with healthy ileostomy subjects.

By the early 1990's the physiological significance of RS was fully realised. Several new/modified methods were developed during the European Research Program EURESTA.[6, 7] The Champ[7] method was based on modifications to the method of Berry[2] and gave a direct measurement of RS. Basically, sample size was increased from 10 mg to 100 mg, the sample was digested with pancreatic α-amylase only (not pancreatic α-amylase plus pullulanase, as used by Englyst[1] and Berry[2]) and incubations were performed at pH 6.9 (pH 5.2 was used by Englyst[1] and Berry[2]). RS determinations were performed directly on the pellet. Muir and O'Dea[8] developed a procedure in which samples were chewed, treated with pepsin and then with a mixture of pancreatic α-amylase and amyloglucosidase in a shaking water bath at pH 5.0, 37°C for 15 h. The residual pellet (containing RS) was recovered by centrifugation, washed with acetate buffer by centrifugation and the RS was digested by a combination of heat, DMSO and thermostable α-amylase treatments.

More recently, these methods have been modified by Fausant *et al.*,[9] Goni *et al.*,[10] Akerberg *et al.*[11] and Champ *et al.*[12] These modifications included changes in enzyme concentrations employed, types of enzymes used (all used pancreatic α-amylase, but pullulanase was removed and, in some cases, replaced by amyloglucosidase), sample pre-treatment (chewing), pH of incubation and the addition (or not) of ethanol after the α-amylase incubation step. All of these modifications will have some effect on the determined level of RS.

In developing the current modified method for the measurement of RS, our aim was to provide a robust and reliable method which (as much as feasible) reflected *in vivo* conditions, and which yielded values that were physiologically significant (see Table 1, page 12). To do this, we[13] studied the effect of concentration of pancreatic α-amylase, the pH of the incubation, the importance of maltose inhibition of α-amylase and the need, or otherwise, of amyloglucosidase inclusion, the effect of shaking and stirring on the determined values, and problems in recovering and analysing the resistant starch containing pellet.

The method that we developed, as described in this booklet, allows the measurement of resistant starch, solubilised starch and total starch content of samples. Twenty four samples can be analysed within a 24 h period. The procedure has been subjected to interlaboratory evaluation (see Table 2, page 13) under the auspices of AOAC International and AACC International[14] and accepted by both associations (AOAC Official Method 2002.02; AACC Method 32-40.01).

PRINCIPLE OF THE CURRENT METHOD:

Samples are incubated in a shaking water bath with pancreatic α-amylase and amyloglucosidase (AMG) for 16 h at 37°C, during which time non-resistant starch is solubilised and hydrolysed to D-glucose by the combined action of the two enzymes. The reaction is terminated by the addition of an equal volume of ethanol or industrial methylated spirits (IMS, denatured ethanol) and the RS is recovered as a pellet on centrifugation. This is then washed twice by suspension in aqueous IMS or ethanol (50% v/v), followed by centrifugation. Free liquid is removed by decantation. RS in the pellet is dissolved in 2 M KOH by vigorously stirring in an ice-water bath over a magnetic stirrer. This solution is neutralised with acetate buffer and the starch is quantitatively hydrolysed to glucose with AMG. D-Glucose is measured with glucose oxidase/peroxidase reagent (GOPOD) and this is a measure of the RS content of the sample. Non-resistant starch (solubilised starch) is determined by pooling the original supernatant and the washings, adjusting the volume to 100 mL and measuring D-glucose content with GOPOD.

APPLICABILITY AND ACCURACY:

The method is applicable to samples containing more than 2% w/w RS. With such samples, standard errors of ± 5% are achieved routinely. Higher errors are obtained for samples with RS contents < 2% w/w.

KITS:

Kits suitable for performing 100 determinations of resistant starch are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: **Amyloglucosidase** [12 mL, 3,300 U/mL on soluble starch (or 200 U/mL on *p*-nitrophenyl β-maltoside*)] at pH 4.5 and 40°C. AMG solution should be essentially free of detectable levels of free D-glucose.
Stable for > 3 years at 4°C.

*Full assay procedure is available at "www.megazyme.com" - Product Code: R-AMGR3.

Bottle 2: **Pancreatic α-amylase** (Pancreatin, 10 g, 3 Ceralpha Units/mg).
Stable for > 3 years at -20°C.

Bottle 3: **GOPOD Reagent Buffer.** Buffer (50 mL, pH 7.4), *p*-hydroxybenzoic acid and sodium azide (0.095% w/v).
Stable for > 4 years at 4°C.

Bottle 4: **GOPOD Reagent Enzymes.** Glucose oxidase plus peroxidase and 4-aminoantipyrine. Freeze-dried powder.
Stable for > 5 years at -20°C.

Bottle 5: **D-Glucose standard solution** (5 mL, 1.0 mg/mL) in 0.2% (w/v) benzoic acid.
Stable for > 5 years at room temperature.

Bottle 6: **Resistant starch control.** Resistant starch content shown on the label.
Stable for > 5 years at room temperature.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use the contents of bottle 1 (AMG; Solution 1) as supplied. This solution is viscous and thus should be dispensed with a positive displacement dispenser, e.g. Eppendorf Multipette® with 5.0 mL Combitip® (to dispense 0.1 mL aliquots).
Stable for > 3 years at 4°C.

   Dilute AMG (300 U/mL). Dilute 2 mL of concentrated AMG solution (bottle 1) to 22 mL with 0.1 M sodium maleate buffer (0.1 M, pH 6.0; Reagent 1; not supplied). Divide into 5 mL aliquots and store frozen in polypropylene containers between use. Stable to repeated freeze/thaw cycles and for 5 years at -20°C.

2. ***Immediately before use***, suspend 1 g of the contents of bottle 2 (pancreatic α-amylase) in 100 mL of sodium maleate buffer (100 mM, pH 6.0; Reagent 1; not supplied) and stir for 5 min. Add 1.0 mL of **Dilute AMG (300 U/mL)** and mix well. Centrifuge at > 1,500 *g* for 10 min and carefully decant the supernatant solution. Use this solution (Solution 2) on the day of preparation.

3. Dilute the contents of bottle 3 (GOPOD Reagent Buffer) to 1 L with distilled water (this is solution 3). Use immediately.

NOTE:
1. On storage, salt crystals may form in the concentrated buffer. These must be completely dissolved when this buffer is diluted to 1 L with distilled water.
2. This buffer contains 0.095% (w/v) sodium azide. This is a poisonous chemical and should be treated accordingly.

4. Dissolve the contents of bottle 4 in 20 mL of Solution 3 and quantitatively transfer this to the bottle containing the remainder of Solution 3. Cover this bottle with aluminium foil to protect the enclosed reagent from light. This is Glucose Determination Reagent **(GOPOD Reagent)**.
Stable for ~ 3 months at 2-5°C or > 12 months at -20°C.

   If this reagent is to be stored in the frozen state, preferably it should be divided into aliquots. Do not freeze/thaw more than once.

   When the reagent is freshly prepared it may be light yellow or light pink in colour. It will develop a stronger pink colour over 2-3 months at 4°C. The absorbance of this solution should be less than 0.05 when read against distilled water.

5 & 6. Use the contents of bottles 5 and 6 as supplied.
Stable for > 5 years at room temperature.

REAGENTS (NOT SUPPLIED):

Reagents should be analytical purity grade.

1. **Sodium maleate buffer** (100 mM, pH 6.0) plus 5 mM calcium chloride dihydrate and sodium azide (0.02% w/v).

   Dissolve 23.2 g of maleic acid (Sigma cat. no. M0375) in 1600 mL of distilled water and adjust the pH to 6.0 with 4 M (160 g/L) sodium hydroxide. Add 1.47 g of calcium chloride dihydrate ($CaCl_2.2H_2O$) and 0.4 g of sodium azide and dissolve. Adjust the volume to 2 L.
   Stable for 12 months at 4°C.

2. **Sodium acetate buffer** (1.2 M, pH 3.8).

   Add 69.6 mL of glacial acetic acid (1.05 g/mL) to 800 mL of distilled water and adjust to pH 3.8 using 4 M sodium hydroxide. Adjust the volume to 1 L with distilled water.
   Stable for 12 months at room temperature.

3. **Sodium acetate buffer** (100 mM, pH 4.5).

   Add 5.8 mL of glacial acetic acid to 900 mL of distilled water and adjust to pH 4.5 using 4 M sodium hydroxide. Adjust the volume to 1 L with distilled water.
   Stable for 2 months at 4°C.

4. **Potassium hydroxide solution** (2 M).

   Add 112.2 g KOH to 900 mL of deionised water and dissolve by stirring. Adjust volume to 1 L. Store in a sealed container.
   Stable for > 2 years at room temperature.

5. **Aqueous ethanol (or IMS)** (approx. 50% v/v).

   Add 500 mL of ethanol (95% v/v or 99% v/v) or industrial methylated spirits (IMS; denatured ethanol; ~ 95% v/v ethanol plus 5% v/v methanol) to 500 mL of H_2O. Store in a well-sealed bottle.
   Stable for > 2 years at room temperature.

NOTE:
A set of control samples containing RS levels from 0.6 to 78% w/w is available from Megazyme International Ireland (Cat. no. K-RSTCL).

EQUIPMENT (RECOMMENDED):

1. Grinding mill - Centrifugal, equipped with 12-tooth rotor and a 1.0 mm sieve, or similar device. Alternatively, a cyclone mill can be used for small samples.
2. Meat mincer - Hand operated or electric, fitted with a 4.5 mm screen.
3. Bench centrifuge - Capable of holding 16 x 120 mm glass test tubes, with rating of approx. 1,500 *g* (~ 3,000 rpm).
4. Shaking water bath (Grant OLS 200) (Grant Instruments Cambridge Ltd.) (or similar) set in linear motion at 100 revolutions per min on the dial (equivalent to a shake speed of 200 strokes/min), a stroke length of 35 mm and 37°C.
5. Water bath - Capable of maintaining 50 +/- 0.1°C.
6. Vortex mixer.
7. Magnetic stirrer.
8. Magnetic stirrer bars – 5 x 15 mm.
9. pH Meter.
10. Stop-watch/timer (digital).
11. Analytical balance (correct to 0.1 mg).
12. Spectrophotometer - capable of operating at 510 nm, preferably fitted with flow-through cell (10 mm path length).
13. Pipettor - capable of delivering 100 µL; with disposable tips. Alternatively, motorised hand-held dispenser can be used.
14. Positive displacement pipettor - Equipped with 50 mL tips capable of delivering 2.0 mL, 3.0 mL and 4.0 mL.
15. Corning® Culture Tubes - screw cap, 16 x 125 mm [Fisher Scientific Cat No. TKV-173-030B (tubes); TKV-178-020V (caps)], Fisher Scientific, interact@fisher.co.uk.
16. Glass test tubes - 16 x 100 mm, 14 mL capacity.
17. Plastic "lunch box", large enough to hold test-tube rack and serve as an ice-water bath (see Figure 1, page 9).
18. Thermometer - Capable of reading 37 +/- 0.1°C and 50 +/- 0.1°C.
19. Volumetric flasks - 100 mL, 200 mL, 500 mL, 1 L and 2 L capacity.

SAMPLE PREPARATION:

Grind approx. 50 g of sample of grain or lyophilised plant or food product in grinding mill to pass a 1.0 mm sieve. Transfer all material to a wide-mouthed plastic jar and mix well by shaking and inversion. Industrial starch preparations are usually supplied as a fine powder, so grinding is not required. Mince fresh samples (e.g. canned beans, banana, potatoes) in a hand operated or electric meat mincer to pass an ~ 4.5 mm screen. Determine moisture content of dry samples by AOAC Method 925.10 (15) and of fresh samples by lyophilisation followed by oven drying according to AOAC Method 925.10.

ASSAY PROCEDURE:

(a) Hydrolysis and solubilisation of non-resistant starch.

i. Accurately weigh a 100 ± 5 mg sample directly into each screw cap tube (Corning® culture tube; 16 x 125 mm) and gently tap the tube to ensure that the sample falls to the bottom.

> **NOTE:** For wet samples such as minced canned beans or food product, the sample size is approx. 0.5 g (weighed accurately). With such materials, the moisture content is usually 60-80%.

ii. Add 4.0 mL of pancreatic α-amylase (10 mg/mL) containing AMG (3 U/mL) (Solution 2) to each tube.

iii. Tightly cap the tubes, mix them on a vortex mixer and attach them horizontally in a shaking water bath, aligned in the direction of motion (see Figures 2 and 3, pages 11 and 12).

iv. Incubate tubes at 37°C with continuous shaking (200 strokes /min) for **exactly 16 h** (**Note**: for linear motion, a setting of 100 on the water bath is equivalent to 200 strokes/min; 100 forward and 100 reverse).

v. Remove the tubes from the water bath and remove excess surface water with paper towel. Remove the tube caps and treat the contents with 4.0 mL of ethanol (99% v/v) or IMS (99% v/v) with vigorous stirring on a vortex mixer.

vi. Centrifuge the tubes at 1,500 *g* (approx. 3,000 rpm) for 10 min (non-capped).

vii. Carefully decant the supernatants and re-suspend the pellets in 2 mL of 50% ethanol or 50% IMS with vigorous stirring on a vortex mixer. Add a further 6 mL of 50% IMS, mix the tubes and centrifuge again at 1,500 *g* for 10 min.

viii. Decant the supernatants and **repeat** this suspension and centrifugation step once more.

ix. Carefully decant the supernatants and invert the tubes on absorbent paper to drain excess liquid.

(b) Measurement of Resistant Starch.

i. Add a magnetic stirrer bar (5 x 15 mm) and 2 mL of 2 M KOH to each tube and re-suspend the pellets (and dissolve the RS) by stirring for approx. 20 min in an ice/water bath over a magnetic stirrer (Figure 1, page 9).

> **NOTE:**
> 1. Do not mix on a vortex mixer as this may cause the starch to emulsify.
> 2. Ensure that the tube contents are vigorously stirring as the KOH solution is added. This will avoid the formation of a lump of starch material that will then be difficult to dissolve.

ii. Add 8 mL of 1.2 M sodium acetate buffer (pH 3.8) to each tube with stirring on the magnetic stirrer. Immediately add 0.1 mL of AMG (solution 1; 3,300 U/mL), mix well and place the tubes in a water bath at 50°C.

iii. Incubate the tubes for 30 min with intermittent mixing on a vortex mixer.

iv. ***For samples containing > 10% RS***; quantitatively transfer the contents of the tube to a 100 mL volumetric flask (using a water wash bottle). Use an external magnet to retain the stirrer bar in the tube while washing the solution from the tube with the water wash bottle. Adjust to 100 mL with distilled water and mix well. Centrifuge an aliquot of the solution at 1,500 *g* for 10 min.

v. ***For samples containing < 10% RS***; directly centrifuge the tubes at 1,500 *g* for 10 min (no dilution). For such samples, the final volume in the tube is approx. 10.3 mL (however, this volume will vary particularly if wet samples are analysed, and appropriate allowance for volume should be made in the calculations).

vi. Transfer 0.1 mL aliquots (in duplicate) of either the diluted (step iv) or the undiluted (step v) supernatants into glass test tubes (16 x 100 mm), add 3.0 mL of GOPOD reagent (solution 4) and incubate at 50°C for 20 min.

vii. Measure the absorbance of each solution at 510 nm against the reagent blank.

> **Prepare reagent blank** solutions by mixing 0.1 mL of 100 mM sodium acetate buffer (pH 4.5) and 3.0 mL of GOPOD reagent.
>
> **Prepare D-glucose standards** (in quadruplicate) by mixing 0.1 mL of D-glucose (1 mg/mL) and 3.0 mL of GOPOD reagent.

(c) Measurement of Non-Resistant (Solubilised) Starch.

i. Combine the supernatant solutions obtained on centrifugation of the initial incubation [*(a)*vii, page 7] with the supernatants obtained from the subsequent two 50% ethanol washings [*(a)*viii and *(a)*ix, page 8] and adjust the volume to 100 mL with 100 mM sodium acetate buffer (pH 4.5) in a volumetric flask. **Mix well.**

ii. Incubate 0.1 mL aliquots of this solution (in duplicate) with 10 µL of **dilute AMG solution** (300 U/mL) in 100 mM sodium maleate buffer (pH 6.0) for 20 min at 50°C. Add 3.0 mL of GOPOD reagent (Solution 4) and incubate the tubes for a further 20 min at 50°C.

iii. Measure the absorbance at 510 nm against a reagent blank.

iv. Calculate the content of non-resistant (solubilised) starch.

Total starch content is the sum of resistant starch and non-resistant (solubilised) starch.

Figure 1. Arrangement of ice-water bath over a magnetic stirrer for treatment of samples with 2 M KOH and dissolution of RS.

CALCULATIONS:

Calculate resistant starch, non-resistant (solubilised) starch and total starch content (%, on a dry weight basis) in test samples as follows:

Resistant Starch (g/100 g sample)(samples containing > 10% RS):
= ΔE x F x 100/0.1 x 1/1000 x 100/W x 162/180
= ΔE x F/W x 90

Resistant Starch (g/100 g sample)(samples containing < 10% RS):
= ΔE x F x 10.3/0.1 x 1/1000 x 100/W x 162/180
= ΔE x F/W x 9.27

Non-Resistant (Solubilised) Starch (g/100 g sample):
= ΔE x F x 100/0.1 x 1/1000 x 100/W x 162/180
= ΔE x F/W x 90

Total Starch = Resistant Starch + Non-Resistant Starch

where:

ΔE = absorbance (reaction) read against the reagent blank.

F = conversion from absorbance to micrograms (the absorbance obtained for 100 µg of D-glucose in the GOPOD reaction is determined and F = 100 (µg of D-glucose) divided by the GOPOD absorbance for this 100 µg of D-glucose.

100/0.1 = volume correction (0.1 mL taken from 100 mL).

1/1000 = conversion from micrograms to milligrams.

W = dry weight of sample analysed
= "as is" weight x [(100-moisture content)/100].

100/W = factor to present RS as a percentage of sample weight.

162/180 = factor to convert from free D-glucose, as determined, to anhydro-D-glucose as occurs in starch.

10.3/0.1 = volume correction (0.1 mL taken from 10.3 mL) for samples containing 0-10% RS where the incubation solution is not diluted and the final volume is ~ 10.3 mL. **When wet samples are analysed, this volume will be larger and this should be allowed for in the calculations.**

NOTE: These calculations can be simplified by using the Megazyme *Mega-Calc*™, downloadable from where the product appears in the Megazyme web site (www.megazyme.com).

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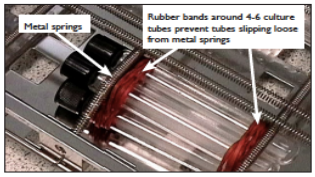

Figure 2. Attachment of Corning® culture tubes to shaking tray in Grant shaking water bath (close view).

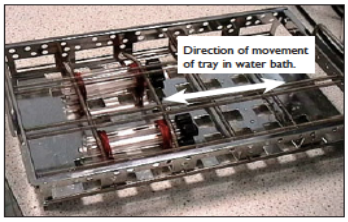

Figure 3. Attachment of Corning® culture tubes to shaking tray in Grant shaking water bath.

Table 1. **Comparison of RS values obtained using several *in vitro* analytical methods to *in vivo* results.**

Source of starch	RS (*in vitro* method/results)[a]					RS (*in vivo*)
	Englyst	Faisant	Champ	McCleary	Goni[b]	
Potato starch (native)	66.5	83.0	77.7	77.0	-	78.8
Amylomaize starch (native)	71.4	72.2	52.8	51.7	-	50.3
Amylomaize starch (retrograded)	30.5	36.4	29.6	42.0	37.8[b]	30.1
Bean flakes	10.6	12.4	11.2	14.3	15.3[c]	9-10.9
Corn flakes	3.9	4.9	4.3	4.0	4.7[c]	3.1-5.0
Canned beans	17.1	-	17.1	16.5	-	16.5
ActiStar[d]	63[d]	-	57[d]	58.0	57[d]	54[d]

[a] Values are presented as a percentage of the total starch content of the sample. All data except that of McCleary, Goni *et al.* (10) and values for ActiStar®, are from Champ *et al.* (16).
[b] From Goni *et al.* (10).
[c] From Goni *et al.* (10), calculating RS as a percentage of total starch, assuming a starch content for bean flakes of 40%, and for corn flakes of 70% (based on in house results for similar materials).
[d] Results kindly provided by Bernd Kettlitz, Cerestar, Vilvoorde, Belgium, except for values by McCleary, which were produced in-house. The "Englyst" data was produced by Englyst Carbohydrate Services; "Champ" data at INRA, Nantes, and "Goni" data at Cerestar, Vilvoorde.

Table 2. Method performance for measurement of resistant starch by enzymic digestion in starch samples and selected plant materials (*AOAC/AACC interlaboratory study results*).

Sample	Mean RS%, %	No. of labs [n(x)]	S_r	S_R	RSD_r, %	RSD_R, %	r^d	R^e	Horrat
Hylon VII [HAMS][f]	46.29	37(0)	1.91	3.87	4.12	8.37	5.34	10.84	3.72
Green banana	43.56	36(1)	1.39	3.69	3.18	8.47	3.88	10.34	3.74
Native potato starch	63.39	35(2)	2.66	3.77	4.20	5.94	7.45	10.54	2.77
CrystaLean®, (Retrograded HAMS)	39.04	34(3)	0.77	2.00	1.97	5.13	2.15	5.61	2.23
ActiStar®, RS	48.28	36(1)	1.12	2.81	2.32	5.83	3.14	7.87	2.61
Kidney beans (canned)	4.66	35(2)	0.11	0.21	2.42	4.58	0.32	0.60	1.44
Corn flakes	2.20	34(3)	0.08	0.24	3.43	10.9	0.21	0.67	3.08

[a] Calculated on "as is" basis ("as is" for banana, kidney beans and corn flakes means on a lyophilised basis)
[b] n = number of collaborating labs (number of outlier labs)
[c] $r = 2.8 \times S_r$
[d] $R = 2.8 \times S_R$
[e] High amylose maize starch

DETERMINATION OF RESISTANT STARCH

Date.....

(100 ± 5 mg sample: pancreatic α-amylase (4 mL, 10 mg/mL, pH 6.0) containing AMG (3 U/mL); shaking (200 strokes/min; 35 mm stroke length); 16 h, 37°C.

Sample	Weight (mg)	Moisture content (%)	Corrected weight (mg)	Final vol. (mL)	Absorbance values (510 nm)	Average absorbance (510 nm)	Resistant Starch (% w/w)
1							
2							
3							
4							
5							
6							
7							
8							

D-Glucose/100 µg =.....; Average =.....; F =.....;

Starch, % = ΔE x F x 100/0.1 x 1/1000 x 100/W x 162/180 = ΔE x F/W x 90 (for samples with > 10% RS).
= ΔE x F x 10.3/0.1 x 1/1000 x 100/W x 162/180 = ΔE x F/W x 9.27 (for samples with < 10% RS).

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Appendix 5: Popular scientific summary

Enzymatic treatment to increase resistant starch in oat flour – an investigation for industrial use

Oat has been established as a useful crop hundreds of years ago all over the world. Oat contains starch which consists of compound shaped like balls built out of amylose and amylopectin. Some of the starch can be resistant depending on grain, grade of maturation etc. Starch can be modified by heat and moisture treatment or enzymatic treatments to increase the yield of resistant starch. The food production enzyme pullulanase has the function of debranching the starch molecules into straight chains that have larger opportunity to be transformed into resistant starch. Resistant starch is defined as starch, or products from starch that is resistant towards digestion and absorption in the small intestine in healthy humans and instead it can be fermented in the large intestine. Resistant starch occurs in 4 types depending on process or origin and these are known to have beneficial health effects. When resistant starch is fermented in the large intestine short chain fatty acids, such as butyrate, propionate and acetate are created as a product. These short chained fatty acids are proved to stimulate the blood flow in the colon, gives energy to the cells in the intestine and also stimulate electrolyte uptake etc.

The aim of this study was to increase the yield of resistant starch in an oat flour residue from the company Lantmännen to be able to use it as a food product with a healthy approach. To be able to adapt the method in an industrial scale, a Rapid visco analyzer (RVA) was used since it is suitable for experiments on small sample size that can be translated into larger scale. The RVA measures the relative viscosity of starch in water when exposed to controlled shear rates, controlled heating/cooling and controlled time settings.

The results were analyzed in a light microscope and the RS content was analyzed with a Megazyme Resistant Starch Assay Procedure. The results showed that the parameters for increasing resistant starch using a RVA was difficult to comprehend. The oat starch samples showed a decisive increase in resistant starch in comparison to oat flour. The method did not show any specific trend in the oat flour samples since the resistant starch content was too low to conclude anything. In oat starch samples, it was indicated a trend that the more enzyme added the higher yield of resistant starch you get. The amount of material didn't show any trend. Further research is needed to see if it is potential to increase the resistant starch yield in oat flour.