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ENZYCOAT II

- Enzymes embedded in barrier coatings for active packaging

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Executive summary

Main objectives

This project is about active packaging and reduction of oxygen and was based on results from a previous project funded by Nordic Innovation Center; a project within NordicMINT Phase I with acronym ENZYCOAT I. Since oxidation of lipids, vitamins, etc. is a big problem in the food industry, economically feasible methods to reduce the oxygen concentration in packed food are demanded. In the project ENZYCOAT I, it was demonstrated that it is in principle possible to use enzymes imbedded in a latex dispersion coating applied on paper, paperboard or plastics in order to reduce the oxygen content in packed food or in the headspace of the package.

The main objectives of the current project ENZTCOAT II was to demonstrate that it is possible to scale-up the technique of oxygen scavenging developed in ENZYCOAT I, but also to develop systems that do not need activation with liquid water (as was the case in ENZYCOAT I). Thus development of systems and investigations of enzyme preparations that are active also at relative humidity (RH)<100 % at low temperatures existing for storage of several fresh foods such as meat and fish products were part of the objectives. Our attempt to demonstrate the feasibility did not only include scaling-up (processability), but also improvements of important features such as no or very low migration and no or low toxicity. Thus also development of systems with low or no migration as well as low or no toxicity were parts of the objectives.

Method/implementation

The project was divided into four Work Packages (WPs) entitled as:

- WP1 Production (incl. immobilisation)
- WP2 To investigate the possibility to interface dispersion coated enzymes and actively control safety and display formation and degradation of sensory active compounds
- WP3 Optimized packaging material and optimized packaging solutions for food applications
- WP4 Toxicology, health aspects and organoleptic evaluations.

Most of the material development and some of the process studies took part in WP1.

Initially the work was addressed to finding latex grades that allows the enzyme-containing to be active also at semi-dry conditions without an activation step with liquid water. In WP1 we also investigated laccase enzyme systems in addition to the Glucose oxidase-catalase system already studied in ENZYCOAT I. Several different preparations of laccase were investigated together with different substrates. In order to find methods that minimize migration, two main strategies were followed: One was to were covalently bond (immobilize) the enzyme onto nano particles, the other was to coat the enzyme-containing latex layer with a extruded polymer layer, consisting of polyethylene, polypropylene or poly(lactic acid). None of these two strategies should hinder the oxygen scavenging process. In WP1 we also took actions to increase the enzyme activity at low RH by addition of clays (porous systems) and to use blends of latex and starch or blends of latex as gelatine as the continuous phase of the coating

A very important sub- task for WP1 was scaling up. Pilot dispersion coatings as well as pilot extrusion were performed for a wide range of substrates, both different grades of paperboard and polymer films were used. The influence of pre-treatment by Corona was studied. The formulation used was those that have been developed in other parts of WP1.

By an early decision in the project, we realised that we have to work quite focused. Thus we moved resources from WP2 to the other work packages. The tasks that remained in WP2 were those related to literature studies, identifying the type of oxidative reactions that will damage the food quality and also to identify food suitable for testing in the demonstrator part of the project (test of shelf life etc.).

WP3 was addressed to production of prototypes including filling and testing od food quality of selected foodstuff stored at different conditions.

WP4 was addressed to the important issue about toxicity and it layer can cause some off-odour. Both these aspects were investigated thoroughly. A novel protocol for analysing cytotoxicity was implemented.

Results and conclusions

The three most important results were:
It was demonstrated that oxygen scavenging coatings can be applied on paper and board by conventional coating machines used for high speed industrial production.

It was demonstrated that a plastic liner that will hinder direct contact between packed food and the active layer can be applied by a conventional extrusion processes without damage of the active properties.

It was demonstrated that the active coating can be used to hinder oxidation and rancidity reactions of packed food such as fish stored at chilled conditions.

This results indicates that our concept has been quite well developed and understood. Further development has to be performed by adjusting the concept to the conditions existing at a particular producer of packaging material, converter or at the food industry. The ability to extend the shelf life for e.g. fresh fish and other fresh products of high water activity may be of high importance for the food industry.

Recommendations

The ENZYCOAT II project clearly showed that it is possible to coat paper and plastic films in an industrial scale with a latex coating containing oxygen scavenging enzymes. Most of the existing drawbacks of the concept described in previous studies have been solved. However, it cannot be excluded that this technique is suitable to be used at a converter than at a paper mill. The trend today for producers of packaging materials is to more and more look into the full value chain, providing a service more than providing reels of paperboard and plastics.

In order to be implemented in a production line further optimization has to take place in order to optimize the formulations and processes to the particular type of unit operations used at the production unit.

The project has also shown that enzyme technology based on knowledge developed in this project can be used for innovative areas not covered by active packaging. Smart enzyme-based sensors can be developed for a lot of different applications and the possibilities to use laccases in order to increase the stiffness of a polymeric composite open up for the implementation in material development (environmentally friendly constructions, furniture, etc.). Some of the project partners also submitted an application to the 6th European Framework program. This application ended up as one of the very best applications, but not good enough in order to be approved.

Contents

1 Abstract

This project investigated the usage of oxygen scavenging enzymes in coating formulations that can be applied onto paper, paperboard or plastic films by ordinary coating and printing units existing in the industry. Test of food quality upon storage in boxes produced with the novel Packaging materials was performed. Methods to decrease migration and to reduce the direct contact with the packed food were developed. Tests of cytotoxicity were performed and testing protocols were developed. It was demonstrated that oxygen scavenging coatings can be applied on paper and board by conventional coating machines used for high speed industrial production. It was demonstrated that a plastic liner that will hinder direct contact between packed food and the active layer can be applied by a conventional extrusion processes without damage of the active properties. It was demonstrated that the active coating can be used to hinder oxidation and rancidity reactions of packed food such as fish stored at chilled conditions

2 Introduction

Oxygen scavengers are used in food packaging industry, mostly in modified atmosphere packaging in order to remove the last trace of oxygen from the headspace and packed food. Oxygen scavengers are normally added as sachet containing e.g. iron powder. Glucose oxidase (GOx) has also been proposed in literature as oxygen scavenging for food applications. In this work we are exploring the possibility to coat the packaging materials itself by latex-based coatings in which the enzyme and the substrate for the enzyme reaction are embedded. The project dealt with oxygen scavenging systems for food packaging application with the enzyme and substrate for the enzyme reaction embedded in polymer-based coatings. The enzyme can be free (physically entrapped) or immobilized on to a small organic or inorganic particle. The polymer matrix may also contain filler particles in order to create a porous matrix structure where air and water vapour may have easier to diffuse. In this study, platy barrier kaolin clays were used when effects of mineral fillers were studied.

Two different enzyme systems were studied: (1) the enzyme glucose oxidase (GOx) with glucose as substrate and (2) the enzyme laccase (Lac) with aromatic compounds as substrate.

The reaction catalysed by glucose oxidase produced hydrogen peroxide as the glucose is oxidized:

β-D-glucose + O2 → D-glucono-1,5-lactone + H2O2

To break down H2O2, catalase (Cat) was introduced into the system:

H2O2 → H2O + ½ O2

Normally, the GOx system when coated on paper and plastics also contains Cat in order to take care of H2O2 formed. Formation of H2O2 that may migrate from the packaging materials is not acceptable in food packaging at the same time as an increase in H2O2 activity may slow down the reaction rate of the GOx catalysed process. The reaction catalysed by GOx consumes both oxygen and glucose, and this means that glucose must also be present, either as a part of the food or as a component in the enzyme formulation. In the Lac system, the enzyme oxidizes phenolic hydroxyl groups to phenoxy radicals and reduces oxygen to water. The Lac system needs no additional enzyme to take care of reaction products since water and phenoxy radicals are formed. The radicals subsequently form quinones or polymerization products. The only reaction product formed with potential to migrate is water.

$$4\ PhOH + O2 \rightarrow \quad 4\ PhO\bullet + 2\ H2O\ (Lac)$$

Enzycoat II consisted of four Work Packages (WPs) entitled as:

- WP1 Production (incl. immobilisation)
- WP2 To investigate the possibility to interface dispersion coated enzymes and actively control safety and display formation and degradation of sensory active compounds
- WP3 Optimized packaging material and optimized packaging solutions for food applications
- WP4 Toxicology, health aspects and organoleptic evaluations.

The project produced 23 internal reports; whereof 15 reports were deliverables and 8 reports were other internal reports. A list of all reports from the project is given in Table 1. All 23 reports are possible to download for the project partners from the Internet-based communication platform BSCW.

Table 1 Summary of reports during the course of the project

Report No.	Work Package	Sub-trask	Delivery Date	Name
D0	2	1	Oct 2008	Selection of quality factors that can be monitored by enzyme systems
D1	4	1	Dec 2009	Identification of materials and components with/without toxic effects
D3-Part A	3	1	April 2010	Background of the decisions for production of flexible packaging and paperboard packaging prototypes based on enzymatic oxygen scavengers
D3-Part B	3	1	Feb 2011	Background of the decisions for production of flexible packaging and paperboard packaging prototypes based on enzymatic oxygen scavengers
D4	4	2	July 2010	Identification of materials and components with/without toxic effects
D5	3	2	May 2011	Production of packaging prototypes based on enzymatic oxygen scavengers
D6	3	2	Jan 2012	A report given the results obtained during measurements of the efficiency of the oxygen scavengers in the prototype
D7, D11, D17	4	3, 4, 6	Jan 2012	Toxicity and health risk analysis of functionalized polymers and materials , Penetration studies, Health risk analysis and resorption studies
D8	1	1	Jan 2012	Optimized application technique
D9	1	3	Dec 2011	Optimized immobilization of enzyme particles that are incorporable in the coating process
D10	1	2	Jan 2012	Proof of methods that make the coated layer active at dry conditions
D12	4	5	Jan 2012	Organoleptic evaluations
D14	1	4	Dec 2011	Scaling-up of preparation and application techniques
D16-Part A	3	3	Oct 2011	Oxidation in biscuits stored in different packaging materials
D16 - Part B	3	3	Nov 2011	Rancidity development of minced lumpfish during storage
IR1	3	1	June 2009	Oxygen scavengers
IR2	1	1	Dec 2009	Measurement of the efficiency of the oxygen scavenger of the developed coated sheets and films
IR3	1	2	Feb 2010	Effects of starch and gelatine on enzyme activity at different water activities
IR4	1	2	Feb 2011	Activation step
IR5	1	3	July 2012	First proof that immobilization of oxygen consuming enzymes is possible
IR7	3	1	Nov 2009	WP3 Phone meeting 18th of November 2009
IR8	1	4	Aug 2010	Cohesion Tests of Coating Color and Adhesion tests of PE coated PB255
IR9	3	2	Jan 2012	Migration test results

3 WP1 Production (incl. immobilisation)

In this Work Package, the research activities were divided into four sub-tasks:

- ST1-1 Optimization of coating recipes, mixing and application strategies, etc. and introduction of by-products from forestry industries as substrates for the enzymatic reaction.
- ST1-2 Enzymatic activity at low and high temperatures and at low relative humidity.
- ST1-3 Immobilisation
- ST1-4 Scaling-up

The work addressed to optimisation of formulations was simultaneously carried out in both ST1-1 and ST1-2. Those two sub-tasks became more or less merged into one during the course of event. Parts of the studies were addressed to systems that work well at low and high temperatures (ST1-2). For that reason both enzyme systems were also investigated at a wide range of different temperatures. The laboratory tests were extended to pilot-scaled coating trials, and it was successfully concluded that the coated board, paper and plastics can be produced in mass production. The most promising formulations were coated in pilot-scale operations onto selected substrates and delivered to other work-packages for further testing and prototype production. Also some heat-sealing experiments were performed within this Work Package (ST1-4). Immobilisation of enzymes by grafting on micron or sub-micron particles has been reported as one method to prevent unwanted migration of enzymes and also to increase the temperature stability of the enzymes. Grafting of enzymes on both inorganic and organic particles was reported in ST1-3.

3.1 ST1-1 Optimization of coating recipes, mixing and application strategies, etc. and introduction of by-products from forestry industries as substrates for the enzymatic reaction

3.1.1. Materials and methods

3.1.1.1. Coating formulation

Initially, the experimental work in this subtask was addressed to formulation of a suitable coating formulation for the GOx system. Based on results obtained within previous projects and on discussions within the current project, we decided to screen three latex products. The different lattices used in initial screening are shown in Table 2.

Table 2 Used lattices in the initial screening process (GOx system)

	Latex grade		
	HPU 69	HPU 70	Primal P308-AF
Neutralization	-	-	NaOH
Monomer	Styrene butadiene copolymer	Styrene butadiene copolymer	Styrene acrylic copolymer
Dry solids content as received (%)	52.2	47.6	49.1
pH as received	5.5	5.4	7.0
Tg (°C)	5	6	8
MFFT (°C)	6	9	
Manufacturer	Styron Europe, GmbH, Horgen, Switzerland	Styron Europe, GmbH, Horgen, Switzerland	Rohm and Haas, Valbonne, France[a]

a = Company name at the time of delivery.

The coating formulations were based on barrier kaolin clay together with a latex polymer. The kaolin clay, Barrisurf LX from Imerys Minerals Ltd, Cornwall, England, had a shape factor of 60 according to the manufacturer. The clay was dispersed in deionized water to a final solids content of 63% according to the manufacturer's protocol.

The enzymes used when formulating the GOx system were glucose oxidase (GOx, 11466 U/g, dry solids content of 99%) together with catalase (Cat, 26600 U/g, dry solids content of 52%), supplied by Novozymes A/S, Bagsvaerd, Denmark. The coating formulations also contained β-D-glucose (glucose) as substrate for the reaction.

The enzymes used in the Lac system was laccase from the basidiomycete Trametes versicolor (TvL), laccase from the ascomycete Myceliophthora thermophila (MtL), and laccase from the Japanese lacquer tree Rhus vernicifera (RvL). TvL was obtained from Jülich Fine Chemicals GmbH (Jülich, Germany), MyL was kindly donated from Novozymes A/S (Bagsvaerd, Denmark, and RvL was purified as described by Reinhammar . In one of the studies of the Lac system, a set of 17 aromatic compounds were used as substrates for the enzymatic reaction in order to find systems that are active also at low temperatures. In another study with the Lac system, lignosulfonate, supplied by Domsjö Fabriker, Örnsköldsvik, Sweden, was used as substrate.

The final Enzycoat II GOx recepie also contained starch in order to improve the mechanical properties (wet strength and adhesion). The starch used was hydroxypropylated and oxidized potato starch, Perlsoat 55 supplied by Lyckeby Industrial AB (now Solam), Kristianstad, Sweden. In some experiments hydroxypropylated and oxidized potato starch Pearlcoat 155 from the same supplier was used. The main difference between these two starch grades was that Pearlcoat 55 was slightly more oxidized than Pearlcoat 155. Thus, the viscosity of an aqueous solution of Pearlcoat 155 was slightly higher than that of a corresponding solution of Pearlcoat 55. The effects of starch and gelatine on the enzymatic activity and the water-holding capacity of the coatings at different relative humidity (RH) were also investigated. The starch used in these experiments was Pearlcoat 55 and the gelatine (Rousselot 160 LB) with a bloom strength of 145-175 g was supplied by Rousselot SAS (Courbevoie Cedex, France). These gelatine- or starch-containing coatings were also investigated with respect to disintegration in and migration to liquid water. Other chemicals used in the screening, optimization and characterization are described when the results are presented.

3.1.1.2. Paper and plastic substrates

Five different paper and paperboard grades were used in this work-package:

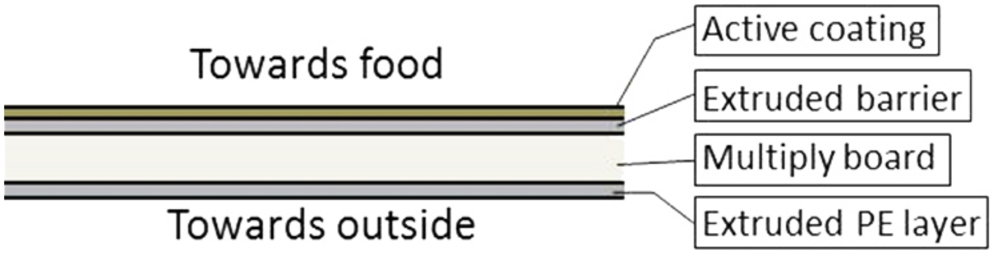
1. Performa Natura Barr (PNB), One side mineral coated CTMP board (GC2) with polymer coating on both sides, supplied by Stora Enso, Finland.
2. PE-coated PB255, One side mineral coated FBB (GC2) with polymer coating, supplied by Stora Enso, Finland.
3. Cupforma Classic, SBS board 350 g/m2, supplied by Stora Enso, Finland.
4. SBS board 310 g/m2 with PE-coating on top side and high barrier polymer multilayer extrusion coating on the back side, supplied by Stora Enso, Finland.
5. Gerbier HDS, a sized, coated and calendered 50 g/m2 flexible packaging paper, denoted “Paper A”, supplied by Ahlstrom Research and Services, Pont-Evêque, France.

Coating trials in pilot scale were also performed on plastic films consisting of 12 µm BOPET + 40 µm PE/EVOH/PE supplied Wipak. The coatings were applied on the PE side. Coatings in laboratory scale were also draw-down on polyester (Mylar) films and on the silicon-treated side of release papers. The release papers were used in the preparation of free-standing films, since the coatings could easily be peeled-off after drying.

3.1.1.3. Methods of preparation of free films and coated board and plastics in laboratory scale

The coating colours were draw-down onto the substrates using a K202 Control Coater (RK Print-Coat Instruments Ltd., Royston, UK) equipped with a series of different wire-wound rods. The coatings in this work-package was applied onto the side of the paperboard that was supposed to be facing towards the packed food, as indicated in Figure 1.

Figure 1. Example of a sandwiched packaging structure for a liquid-containing active packaging



3.1.1.4. Methods used in evaluation of properties of coating colours, free films and coated materials

The enzymatic activity was measured both in aqueous environment and in air. In aqueous environment, the enzymatic activity was evaluated by a evaluated by Hansatech oxygen electrode (Clark electrode). Both coating colours and coated layers were investigated by the oxygen electrode method. Coating colour (100 µl) or stripes of coated and uncoated (reference) papers were added to the reaction chamber containing deionised water or buffer solution. The total volume of liquid in the reaction chamber was 3 ml and the measurements were performed at 25°C.

Enzymatic activity of dry coated papers in air or O2/N2 gas mixtures of different RH was analysed by means of an O2/CO2 gas analyser (Checkmate II, PBI Dansensor A/S, Ringsted, Denmark). About 1 dm2 of coated board was placed in air-tight chambers with a volume of 128 ml at 23°C and either 50, 75 or 100% RH. The atmosphere inside the chambers was modified to about 1% oxygen by flushing the chambers for 1.5 min with a gas mixture consisting of 1% oxygen and 99% nitrogen (AGA Gas AB, Enköping, Sweden). The oxygen-scavenging capacity of the coated board was evaluated by measuring the decrease in oxygen concentration.

Water uptake of coated polyester films from gas phase were measured by keeping the samples at 23°C in desiccators at either 100% RH or 75% RH. The increase in weight was monitored for 24 h at 100% RH and for 72 h at 75% RH. The shorter time at the higher humidity was due to the condensation of water on the surface after 24 h. The water uptake was calculated as g water/g polymer. Water uptake of coated polyester films was also measured for films immersed in water for 30 or 60 min at different pH values.
Other analysis was SEM images, porosity measurements by oil absorption and mercury intrusion.

3.1.1.5. Overall Migration/Disintegration

The adhesive and cohesive properties of coatings congaing starch and gelatine when applied on polyester substrate were evaluated as overall migration. Coating were draw-down on polyester (Mylar) films using a wire-wound bar giving a nominal wet deposit of 50 μm. The coated films were immersed in buffered solutions (20 mM MOPS, pH 7.0) at 23ºC for 4 h and the overall migration/disintegration as mg/dm2 was calculated from the decrease in weight. The recipe given in Table 3 was used in these experiments. The biopolymer was gelatine or starch. When gelatine was used, polyvinyl alcohol (PVOH) was used for pre-treatment of the clay particles before gelatine addition in order to prevent flocculation of the clay particles. This was done by mixing the clay dispersion was mixed with a 20% dry solids content PVA solution in dry weight ratio of clay to PVA of 100:1 for 1 h.

Table 3. Typical coating colour formulation

Material	pph*	Density of dry material [g/cm3]
Latex, biopolymer and PVA	100	1
Glucose	13	0.7
GOx preparation	0.97	2.6
Cat preparation	0.88	1.54

* = Parts (by wt.) per hundred parts of dry polymers (pph)

The different levels of starch and gelatine levels used in the migration/disintegration tests are given by Table 4.

Table 4. Abbreviations used for the various coating colour formulations.

	Compound [pph]		Dry solids content [%]
Name	Gelatine	Starch	
Ref	0	0	49.9
St10	0	10	43.9
St20	0	20	38.5
Gel4	4	0	44.3
Gel8	8	0	39.5

$Cobb_{60}$ and $Cobb_{1800}$ (ISO standard ISO 535) were used in order to see if there were any differences in water absorptiveness and disintegration for:
Corona treated board (PNB) coated with

- Standard ECII recipe with and without gelatine
- Standard ECII recipe without Enzymes, with and without gelatine

The water added to the test cylinder was poured off after 60 or 1800 s contact time. In addition to the Cobb value, the turbidity of the test water indicated qualitatively the degree of disintegration. Residual water not taken up by the specimen was removed by blotting paper.

3.1.1.6. Heat Sealing

Heat sealing tests were made with Kopp Laboratory Sealer SGPE20. Testing conditions were 400N and 200N with temperatures 100 ºC – 300 ºC and sealing times 1 – 4 s. Heat sealing tests were made for all different latexes. In this report only results from the Standard ECII recipes (see section 2.1.2.1.1) with HPU70 will be reported.

3.1.1.7. Cross-sections

Microscopic pictures with 100× and 400× magnification of starch containing samples were taken with Carl Zeiss Axioskop 2 Plus microscope. Cross-sections were made with MICROM International GmbH Rotary Microtome HM325. Coating color layer thicknesses were measured from the pictures.

3.1.2. Results

3.1.2.1 Oxygen scavenging in GOx system

This part of the work was focused on development of the coating formulation for good mechanical properties, good adhesion to plastic substrates and good water resistance. The basic concept of the coating was embedded enzymes and substrates for enzyme reaction in latex films. The challenge was to find latex system open enough to allow enzyme, oxygen, substrate and reaction products to move in the coatings. The enzyme reaction takes place by diffusion of substrate and oxygen to the active site of the enzyme and it is likely that the diffusion of large enzyme molecules inside the coatings plays a minor role. Instead the types of movements of the enzyme required for the reaction to take place are likely to be more like orientational movements and reconfiguration.

3.1.2.1.1 Optimization of coating recipe

The as received latex samples were adjusted by NaOH to three different pH-levels and subsequently coated by the bench coater. The visual appearance after drying is shown in Table 5a-b. In Table 5a is also included the appearance after immersion in water. As seen in Table 5a, the coatings based on HPU 69 and HPU 70 were smoother compared to that based on Primal P308-AF. Particles were present in the dry films of Primal P308-

AF, probably due to some aggregation. At the highest pH (around 8.9) the viscosity of the lattices HPU 69 and HPU 70 increased dramatically. For HPU 70 the high viscosity resulted in large stripes in the coating layer. The high viscosity of HPU 69 made coatings impossible at pH 8.9. All of the lattices crept inwards at the edges when coated on PNB due to the low surface energy on board (Table 5b).

Table 5a
Visual inspection of pure latex coatings draw down on Mylar films, using bar No.3 at three different at 105°C. Dry films immersed in deionized water for 1 h at 23°C. Time between coating/drying and immersion was ca. 20 h

Latex	pH of wet latex dispersion		
	pH 5.45±0.05	pH 6.90±0.05	pH 8.90±0.10
HPU 69	After drying: Very few and small lumps, nice coating. After immersion: Flexible and slightly opaque film	After drying: Very few and small lumps, nice coating but thin stripes. After immersion: Inflexible, disintegrated and slightly opaque film.	Impossible to coat. (Immersion tests not performed)
HPU 70	After drying: Very few and small lumps, nice coating. After immersion: Flexible and opaque film after immersion.	After drying: Very few and small lumps, nice coating. After immersion: Flexible and opaque film.	After drying: A lot of large stripes. After immersion: Poor film (slightly like a mush)
Primal P308-AF	(not analysed)	After drying: A lot of very small particles (aggregates?), thin stripes. After immersion: Opaque film with relative strong adhesion to the Mylar film	After drying: A lot of very small particles (aggregates?), thin stripes. After immersion: Flexible and opaque film after immersion.

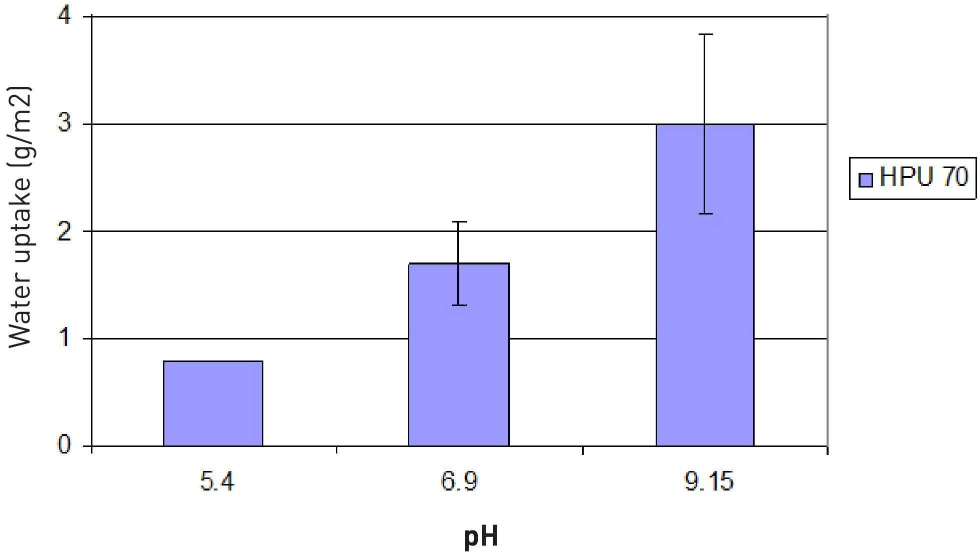
Table 5b
Visual inspection of pure latex coatings after drying, draw down on PNB using bar No.3 at three different at 105°C

Latex	pH 5.45±0.05	pH 6.90±0.05	pH 8.90±0.10
HPU 69	Nice coatings but crept inwards	Nice coatings but crept inwards	Impossible to coat
HPU 70	Nice coatings but crept inwards	Nice coatings but crept inwards	A lot of large stripes
Primal P308-AF	(not analysed)	Nice coatings but crept inwards	Nice coatings but crept inwards

A high opacity after immersion in water is indicative of swelling and water uptake. The most opaque films at the pH-levels were the GOx is most active (pH≤7) was observed for the HPU 70 grade, indicating that this latex may give the highest enzyme activity when the enzyme is imbedded in the dry coating and subsequently in contact with water vapour inside a package. At the same time, HPU 70 gave flexible films and films without aggregates and stripes at pH≤7. Based on this we selected HPU 70 as the latex used in the standard recipe. However, it cannot be excluded the styrene-acrylate latex would have perform equally well if used as active food packaging demonstrator, at least when in contact with liquid water.

The latex HPU70 was used for further testing to evaluate the influence of pH on water absorptiveness. The pH value was adjusted HPU70 (pH 5.4, 6.9 and 9.15) the formulation was draw down onto Mylar film using rod no 5 and speed 4. I addition, free films were prepared by coating the backside of release paper mounted onto a piece of cardboard using rod 6, i.e. the same technique as used in experiment with Lac-containing coatings reported in Section 2.2.2.2 (results only shown in this summary for the Lac-system). In Figure 2 the water uptake for HPU 70 at different pH are shown. The standard deviation for the water uptake increases as the uptake of water increases, showing that as the films became better at adsorbing water, the values also became less reliable, possible due to dissolution and release from the mylar film. This was also visually seen as the films with higher pH were looser and appeared to have less strength compared to those with lower pH.

Figure 2. Water uptake measurements for HPU 70 with different pH coated on mylar film. Standard deviation seen as error bars for 4 replicates



Of the tested latices, HPU 70 stood out as the one being most suitable. It gave smooth coatings for various pH without any formation of particles. It also has a good buffering ability in the pH span where the Barrisurf LX clay and most enzymes have good functions, reducing the need for buffers. HPU 70 was also found to be heat sealable by tests done at Tampere University, Finland for coatings with 33 and 55 pph Clay. By addition of 55 pph clay the coating did not give rise to blocking. The viscosity can also be kept almost constant over a pH range due to the buffering ability of HPU 70.

Based on several tests of flow properties and properties of the dry coated layer, the follow recipe was selected as the standard Enzycoat II recipe (see Table 6). However, in pilot coating trials the recipe was further developed by addition of starch in or der to improve the strength properties of the coatings.

Table 6. Standard ECII recipe. Water was added to a final solids content of 52 % (by wt.)

Material	Parts per hundred (pph)
Latex (HPU 70)	100
Clay (Barrisurf LX)	55
GOx 11200 units = 0,974 g dry	0.974
Catalase 44 800 units = 1.684 g suspension	0.876
Glucose	13

3.1.2.1.2 Optimization of drying strategy

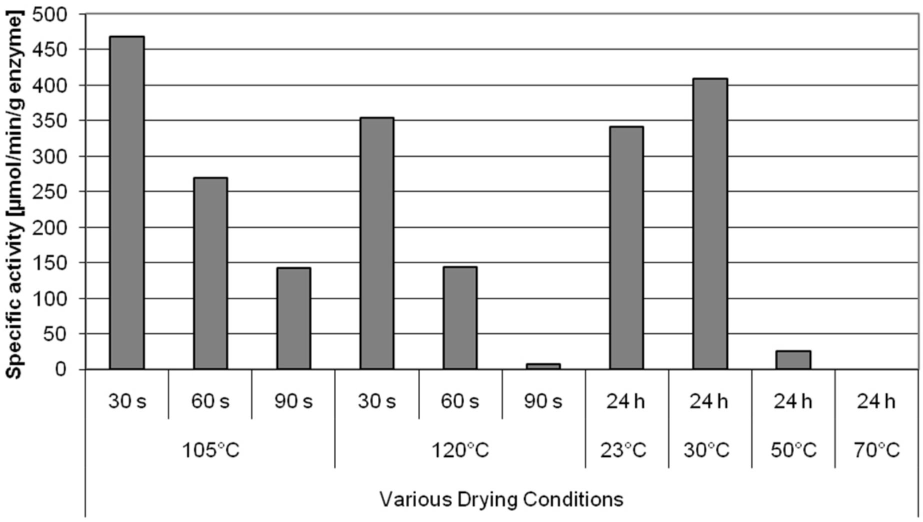
The optimum drying conditions was studied on free standing films prepared by draw-down coating on release paper using rod No. 6. The coated papers were dried in an oven at different temperatures. The enzymatic activity was evaluated spectrophotometrically by the ABTS method, this no catalase was added to the coating. The recipe used in this particular experiment is given by Table 7.

Table 7. Coating colour composition used in drying optimisation studies

Material	Parts per hundred (pph)
Latex (HPU 70)	100
Clay (Barrisurf LX)	33
GOx 11200 units = 0,974 g dry	0.974
Glucose	13

Figure 3 shows the effect of drying times. It is obvious that long drying times of several hours above 70°C reduce the activity to almost zero. On the other hand, short drying times at elevated temperatures seem not to be detrimental for the enzymes, even is slight reduction in activity could be observed at 120°C compared to 105°C.

Figure 3. The specific activity of samples dried in oven at different temperatures and times.



3.1.2.1.3. Loss on enzyme activity due to consumption of substrate

The possible correlation between the decrease in pH due to formation of gluconic acid and loss of activity was examined by preparing a coating colour with SB-latex containing final dry weight concentrations of 0.6 % GOx, 0.5 % Cat, 8.8 % glucose and 32.4 % clay corresponding to a pigment volume concentration of 16 %. The pH was adjusted to pH 6.9. The wet coating colour was kept at room temperature under stirring and the pH and activity were measured during four intervals, extended over a total time of four days, until no activity remained. Between each pair of intervals, the coating colour was stored at 4°C. The enzyme activity was estimated by adding 0.1 ml of coating colour to the reaction chamber containing 2.9 ml pre-tempered water, resulting in a final concentration of 7.74 mM glucose and 2.7 U/ml GOx. The activity of the free enzyme in a buffered solution at pH 4.5 (50 mM citrate buffer) was measured at the same final concentrations as above. The maximum enzyme rate was calculated as the rate of decrease in oxygen concentration per mass of enzyme preparation.

To find any possible correlation between the decrease in pH due to the formation of gluconic acid and loss of enzymatic activity, the pH and activity were measured for a coating colour prepared with SB-Latex. As can be seen in Figure 4, the pH in the coating colour decreased substantially over time as long as enzymatic activity was present, probably due to the generation of gluconic acid as a by-product. After approximately 40 h, both the decrease in pH and the enzymatic activity appeared to have levelled out. To validate that the loss of enzyme activity was not due to inactivation of the enzyme caused by the low pH, the enzyme activity was measured in a citrate buffer solution at pH 4.5. The enzyme activity at pH 4.5 was found to be 0.6 μmol oxygen/min which is the same activity as after 20 hours of reaction time i.e. the enzyme was still active at pH 4.5. The loss of activity in the coating colour at pH 4.5 was, at least in part, due to the consumption of all the glucose in the coating colour. This means that the enzyme-containing system probably does not need to be buffered, which also can be understood from the fact that the carboxylic acid groups on the latex surface give some buffering effect to the suspension.

Figure 4
The decrease in pH and enzyme activity in a coating colour with SB-Latex as a function of time

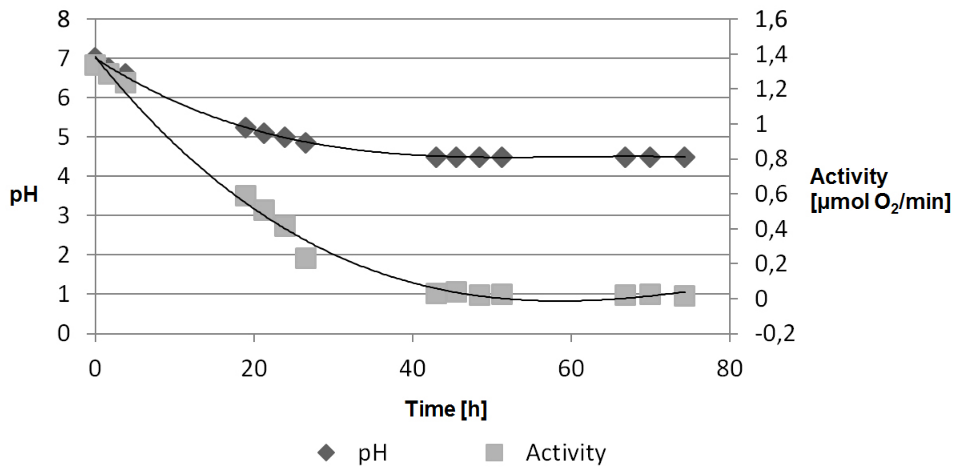
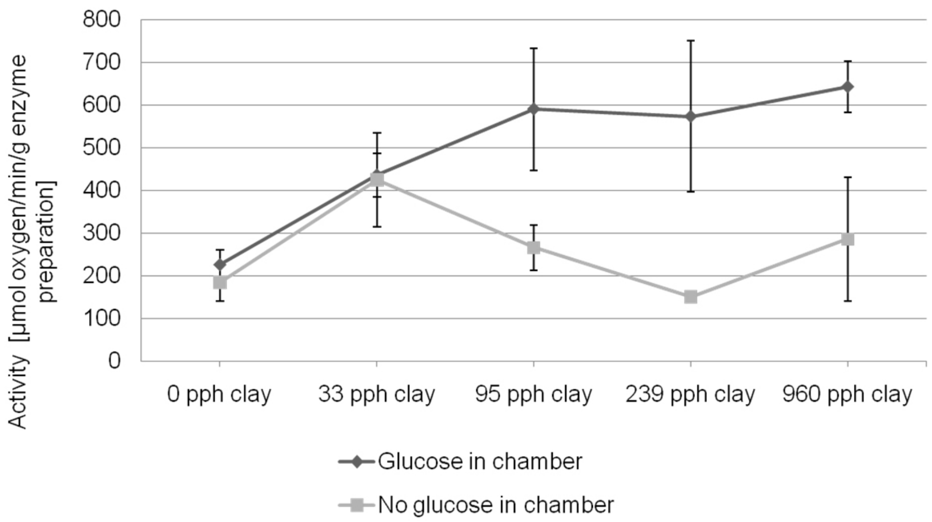


Figure 5 shows the variations in enzyme activity at different loading of the clay. The observed decrease in activity with increasing clay loading from 33 pph, corresponding to 10% pigment volume concentration (PVC), up to critical pigment volume concentration (CPVC) could, at least partly, be explained in terms of a hindrance to diffusion due to the plate-like clay particles. Experiments with the substrate for the enzymatic reaction (glucose) also present in the chamber (oxygen electrode) clearly showed that the decrease in activity at intermediate clay concentrations was due to restricted access to glucose, which further indicates that the hindrance to the diffusion of reagents was the main reason for the drop in activity. The porous structure could not compensate for the hindered diffusion, since the activity with no free glucose in the chamber was substantially higher than the corresponding activity with only water in the chamber. We conclude that the addition of clay has three (in some cases counteracting) main effects: (a) prevention of diffusion of oxygen and consumption of glucose in the coating process before the layer is dried, (b) slowing down the diffusion of oxygen and glucose when the layer should be active, and (c) creating porous structures at high clay loadings. Effects (a) and (c) will act to increase the activity and the capacity as oxygen scavenger, while effect (b) will decrease the activity and the capacity.

Figure 5
The enzymatic activity of layers with various pore structures, the activity was measured using an oxygen electrode at 25°C both with and without substrate (glucose) present



3.1.2.1.4. Disintegration of enzyme-containing latex colours
In parallel to experiments addressed to enzymatic activity in the GOx system, studies about disintegration, wet strength and migration to aqueous phase also were performed. The first part of the investigation was to add different amount of starch according to Table 8 different formulations tested are shown in Table 13. The coating colours were draw-down on board PB255, Corona pre-treated corresponding to 60 Wmin/m2.

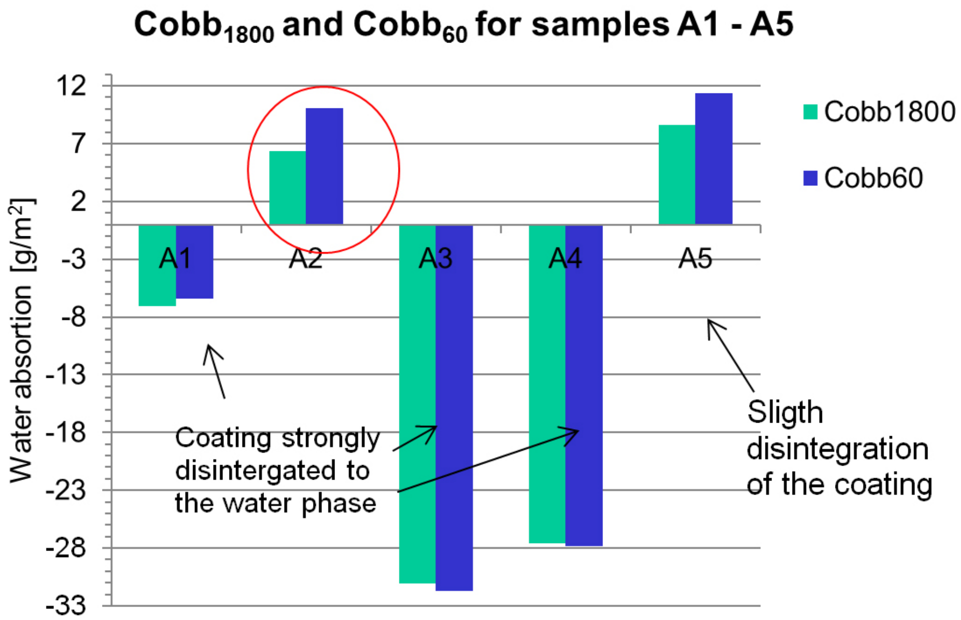
Table 8. Coating colour tested with Cobb tests

Sample	Latex HPU 70 [pph]	Clay BS XL [pph]	Glucose [pph]	Enzymes [pph]		Starch PC 155 [pph]
				Cat	GOx	
A1a)	100	55	13	0.876	0.974	0
A2	100	55	13	0.876	0.974	10
A3	100	55	39	0.876	0.974	0
A4	100	55	39	0.876	0.974	10
A5b)	100	55	13	0.876	0	10

a) Initial Standard ECII recipe
b) Reference sample without GOx

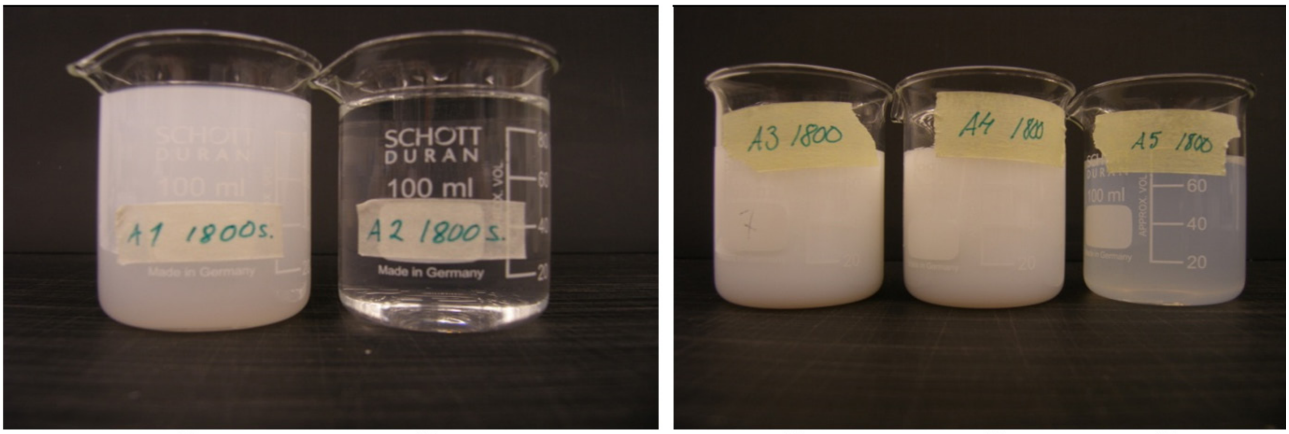
Figure 6 shows the Cobb 60 Cobb1800 values when the samples were in contact with water. The apparent negative water absorption can be explained by severe disintegration that led to reduced coat eight. It is clear that samples A2 and A5 are the best one. A comparison between the aqueous phases in the Cobb cell (Figure 7) reveals that sample A2 is more stable towards disintegration than the sample A5. Thus the formulation of A2 was taken as the modified standard ECII recipe.

Figure 6. Cobb60 and Cobb1800 measurement of coated PB255



The formulations are given in Table 8. Formulation A2 (indicating with the red circle, was the most stable coating according to the total assessment based on Cobb values and turbidity of test liquid (see Figure 7).

Figure 7. Visual comparison of cobb1800 test water phase

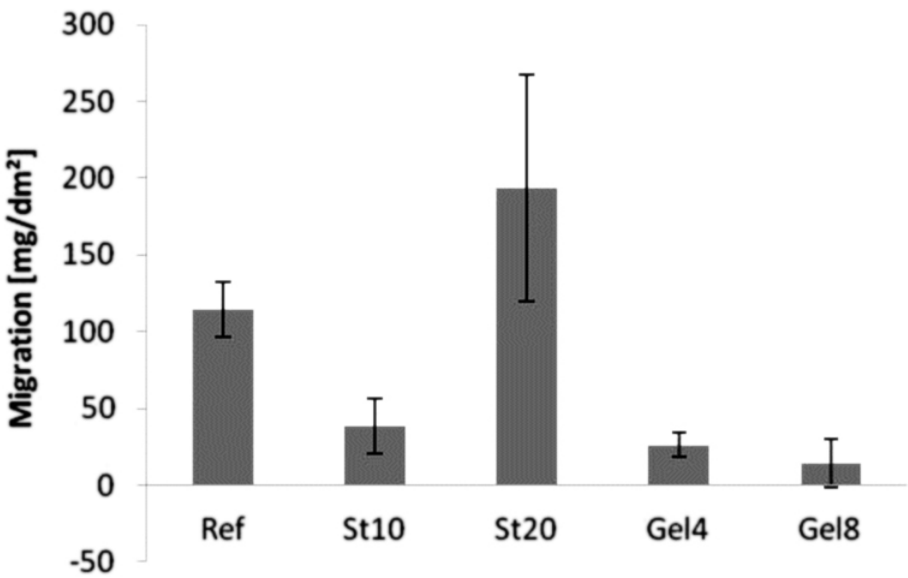


A1 A2 A3 A4 A5

(In the picture the A2 may seem like it has some cloudiness, but that is due to reflection during the photography, water phase was clear in this sample.)

It is clear from Figure 6 that starch prevented migration and disintegration of the coatings. Some experiments were also performed in order to investigate if addition of gelatine could have a similar effect. Coated polyester films (formulations are given in Table 4) were immersed in buffered solutions (20 mM MOPS, pH 7.0) at 23ºC for 4 h and the overall migration/disintegration as mg/dm2 was calculated from the decrease in weight (Figure 8). A comparison of gelatine and starch at similar level of addition revealed that gelatine was more effective than starch to prevent migration and disintegration.

Figure 8
Overall migration of coated Mylar films to a buffered solution (20 mM MOPS pH 7.0) at 23ºC. Error bars indicate standard deviations based on 5 replicates



3.1.2.1.5. Modified standard Enzycoat II recipe

The best recipe from the above mentioned results, i.e. the standard Enzycoat II recipe, was shown in Table 6. However, based on disintegration tests described in section

2.1.2.1.4., it was decided to add 10 pph of starch into the coating formulation, thus defining a modified standard recipe with improved mechanical properties (Table 9).

Table 9. Modified Enzycoat II recipe, including an addition of 10 pph starch

Material	Amount (pph)
Latex	100
Clay	55
GOx 11200 units	0.974
Catalase 44800 units	0.876
Glucose	13
Starch	10

3.2 ST1-2 Enzymatic activity at low and high temperatures and at low relative humidity

In this sub-task, additions of starch and gelatine were added to the formulation in order to investigate the possibility to get active coatings also at low RH. In order to find a way to improve the enzyme activity at low temperatures further, another strategy was chosen: to use different preparation of laccase (Lac) instead of the GOx system. The Lac-based coatings have also some other envisaged benefits compared to the GOx system, one is that the substrate for the reaction can be a polymer contributing to the strength of the coatings. In the GOx system, the substrate is glucose, a small molecule that does not enhance the strength of the layer.

3.2.1. Materials and methods

3.2.1.1. Effects of starch and gelatine

For the investigation where starch and glucose was added, the experimental design was based on an expanded version of the formulations shown in Tables 3 and 4. However, in this sub-task for the investigation at low RH, some of the formulations also contained clay in order to create porous layers. The typical formulation shown in Table 3 is valid also for the formulations used if no clay was used, while Table 10 shows a typical formulation for a clay-containing formulation. The full descriptions of the amount of starch or gelatine used in are shown in Table 11. When clay was added, the level of clays was always higher than the critical pigment volume concentration, i.e. a porous layer was created. The coatings were draw-down at SBS board using a wire-wound bar giving a nominal wet deposit of 50 µm.

Table 10. Typical clay-containing coating colour formulation.

Material	pph*	Density of dry material [g/cm3]
Latex, biopolymer and PVA	100	1
Clay	450	2.6
Glucose	13	0.7
GOx preparation	0.97	2.6
Cat preparation	0.88	1.54

* = *Parts (by wt.) per hundred parts of dry polymers (pph)*

Table 11. Abbreviations used for the various coating colour formulations. The letter "C" in the name indicates a clay-containing formulation

	Compound [pph]			Dry solids content [%]
Name	Gelatine	Starch	Clay	
Ref	0	0	0	49.9
St5	0	5	0	46.8
St10	0	10	0	43.9
St15	0	15	0	42.2
St20	0	20	0	38.5
Gel2	2	0	0	47
Gel4	4	0	0	44.3
Gel6	6	0	0	42.0
Gel8	8	0	0	39.5
RefC	0	0	450	60.0
St5C	0	5	450	59.6
St10C	0	10	450	58.7
St15C	0	15	450	58.0
St20C	0	20	450	57.1
Gel2C	2	0	450	58.8
Gel4C	4	0	450	57.7
Gel6C	6	0	450	57.6
Gel8C	8	0	450	56.2

The coated board was placed in an air-tight chamber at 23°C and either 100% RH or 75% RH. The atmosphere inside the chambers was modified to about 1% oxygen. The oxygen-scavenging capacity of the coated board was evaluated by measuring the decrease in oxygen concentration during one week at 100% RH and during two weeks at 75% RH using a Checkmate II (PBI Dansensor A/S, Ringsted, Denmark).

3.2.1.2. Oxygen scavenging in Laccase system

The enzymes used in the laccase (Lac) system was laccase from the basidiomycete Trametes versicolor (TvL), laccase from the ascomycete Myceliophthora thermophila (MtL), and laccase from the Japanese lacquer tree Rhus vernicifera (RvL). TvL was obtained from Jülich Fine Chemicals GmbH (Jülich, Germany), MyL was kindly donated from Novozymes A/S (Bagsvaerd, Denmark, and RvL was purified as described by Reinhammar . The investigation of the Lac system was split into two parts: In one of the studies of the Lac system, a set of 17 aromatic compounds were used as substrates for the enzymatic reaction in order to find systems that are active also at low temperatures. In another study with the Lac system, lignosulfonate, supplied by Domsjö Fabriker, Örnsköldsvik, Sweden, was used as substrate.

In the comparative study of TvL, MtL and RvL using 17 different substrates (small molecules containing aromatic groups), the enzyme was mixed with a latex/clay suspension as previously described for the GOx system, but no substrate was added since the substrate was added during the measurement of enzyme activity. Free standing films were prepared by coating the back of a release paper, attached to a smooth surfaced, card board. In cases when the coating colour displays poor adhesion to the release paper, PE coated alumina foil attached to a glass plate was used instead. Coating colour was coated as a single layer using nr 5 rod. Coated papers were immediately put in a ventilated oven for a specified temperature in the range from 75 to 105ºC and duration in the range from 30 to 50 s. The activity assay was performed with an oxygen electrode (Hansatech Instruments Ltd, Norfolk, England). The substrate used was added to the oxygen electrode reaction chamber. In the case that enzyme activity of free films were investigated, pyrogallol was always selected as the substrate present in the reaction chamber. The aqueous solution in the reaction chamber was buffered to pH 6.5 prior to the addition of the enzymes or free films.

In the investigation using lignosulfonate as substrate, Lac from Trametes versicolor was used. Lignosulfonate was provided by Domsjö Fabriker (Örnsköldsvik, Sweden). The coating colour containing both enzyme and substrate was draw-down on PB255 by a bench-coater using a rod that gave a nominal wet thickness of 24 μm (one-side double coated). The PE surface of the board was Corona-treated before the first coating operation. The oxygen scavenging capacity was measured in air-tight glass cambers with controlled RH. The RH was adjusted by a series of saturated salt solutions and pure water: magnesium chloride (34% RH), magnesium nitrate (52% RH), potassium chloride (84%), and pure water (100% RH). The atmosphere inside the chambers was modified to about 1% oxygen and 99% nitrogen. Strips of the board was placed in the air-tight camber and the decrease in oxygen concentration was measured by a Checkmate II (PBI Dansensor A/S, Ringsted, Denmark) in a procedure in which an aliquot of the head-space gas was taken out and analysed using a zirconium-based sensor. The oxygen concentration was analysed once every day during 3 days. To avoid a drop in gas pressure, fresh gas was subsequently refilled in the chambers and the head-space oxygen was again analysed once every day during 3 days.

The influence of the enzymatic reaction (crosslinking) was investigated on starch/clay coating colours without latex to which lignosulfonate, glycerol (plasticizer) and latex was added. A summary of the formulations used in the experiments with lignosulfonates and laccase is given in Table 12. Free standing films were prepared by casting in Petri dishes. The mechanical properties of the films were tested in a DMA (STDAA861, Mettler Toledo) operating in tension mode. The storage modulus (E') was measured at 23°C and at 50% RH.

Table 12. Coating colour formulations. Latex-based coatings were used in oxygen scavenging tests while starch-based coatings were used in mechanical testing

Component	Latex-based coatings, pph	Starch-based coatings, pph
latex	100	-
starch	10	100
clay	55	55
lignosulfonate	30	30
glycerol	-	30
enzyme preparation	6	6

3.2.2. Results

3.2.2.1. Effects of starch and gelatine

Figures 9a and 9b show the oxygen scavenging ability of coated SBS board measured at RH 75 % and 100 %, respectively. The scavenging capacity (measured oxygen decrease) was normalised per mass of polymer, since the enzyme and substrate are located in the polymer phase or at the interface between the polymer phase and some other phase and cannot be located inside the inorganic phase. Figure 9b reveals that a higher oxygen-scavenging capacity could be achieved both by (a) by introducing pores to the layer and (b) by introducing a high concentration of a water-retaining biopolymer.

However, it is much more effective to introduce a porous structure by addition of a mineral than by adding starch or gelatine. The porosity as such contributed to the higher oxygen scavenging capacity by promoting the diffusion of substrate and oxygen to the active site of the enzyme. The oxygen-scavenging capacity of the closed layer at 100 %RH was increased slightly with increasing amounts of water-holding biopolymer as a consequence of a higher molecular mobility. The addition of gelatine resulted in an improved adhesion to plastics polyester and a slightly higher oxygen-scavenging capacity at the highest humidity than with the corresponding addition of starch. The oxygen-scavenging capacity of the porous structures indicates that this system is suitable for both moist foods and intermediate-moist foods.

Figure 9a.
Oxygen-scavenging ability at 75% RH and 23°C of SBS boards coated with different amounts of gelatine, starch and clay. Error bars indicate standard deviations based on triplicate measurements.

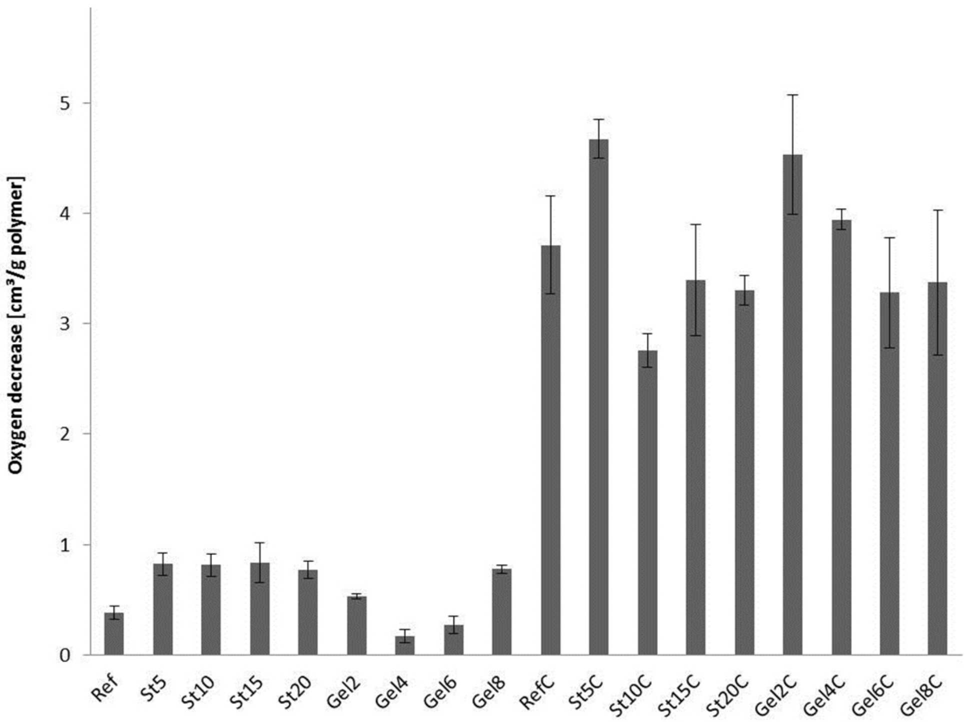
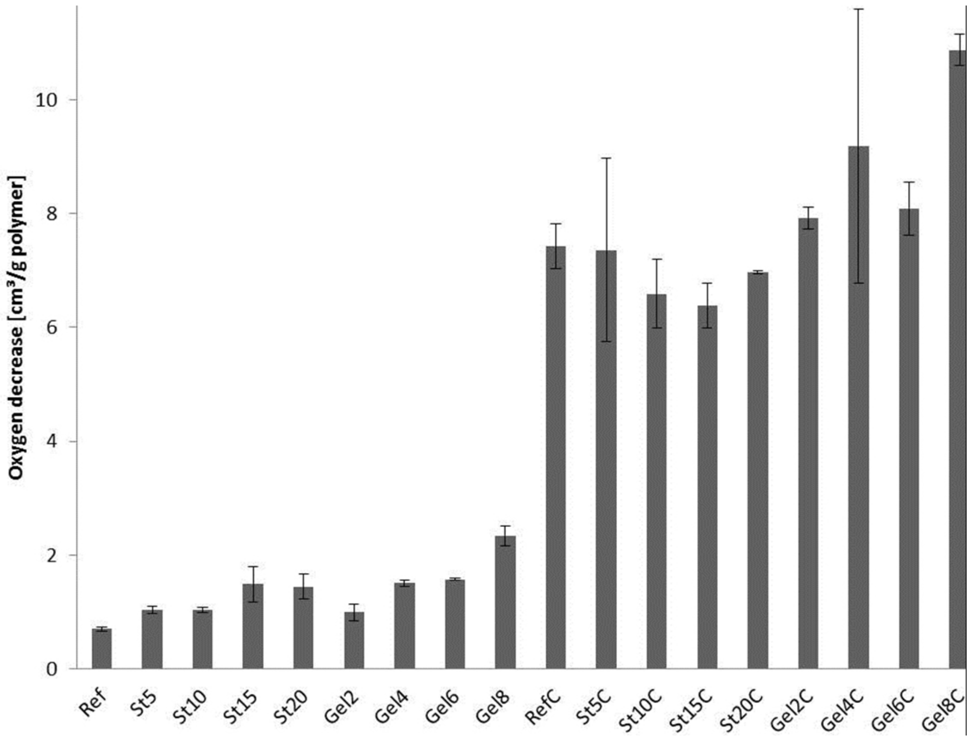


Figure 9b.
Oxygen-scavenging ability at 100% RH and 23°C of SBS boards coated with various amounts of gelatine, starch and clay. Error bars indicate standard deviations based on triplicate measurements.



3.2.2.2. Oxygen scavenging in Laccase system

The investigation of different laccases in combination with different substrates revealed high selectivity. Laccases from different sources have different ability to reduce a particular substrate. In this study TvL showed the most general behaviour with small differences in enzyme activity when the substrate was changed. On the other hand, RvL showed the highest selectivity with respect to the type of substrate.

The influence of the drying conditions on the enzyme activity of free Lac-containing film is shown in Figure 10. The remaining activity per g enzyme preparation is shown in Table 13, where the results are presented as % of the initial activity before drying. The results indicate that all three laccases can be successfully embedded/immobilized in the latex/clay matrix with sufficient remaining activity. The optimal temperature for the drying conditions varies between the three different laccases (Figure 10). Enzymes are sensitive to changes in temperature which can cause them to denature. Most enzymes show increased temperature stability upon immobilization, which could explain the dip in activity observed for TvL around drying temperature of 75°C and 40 to 50 s. A more rapid immobilization that occurs at higher temperatures may minimize the time where the enzymes still are free, i.e. more susceptible to temperature-induced inactivation.

Figure 10.
Activity of laccases from T. versicolor (TvL), M. thermophila (MtL) and R. vernicifera (RvL) after immobilization into latex/clay matrix and drying. The mean activity of three replicates is shown together with the standard deviation. Pyrogallol was used as substrate.

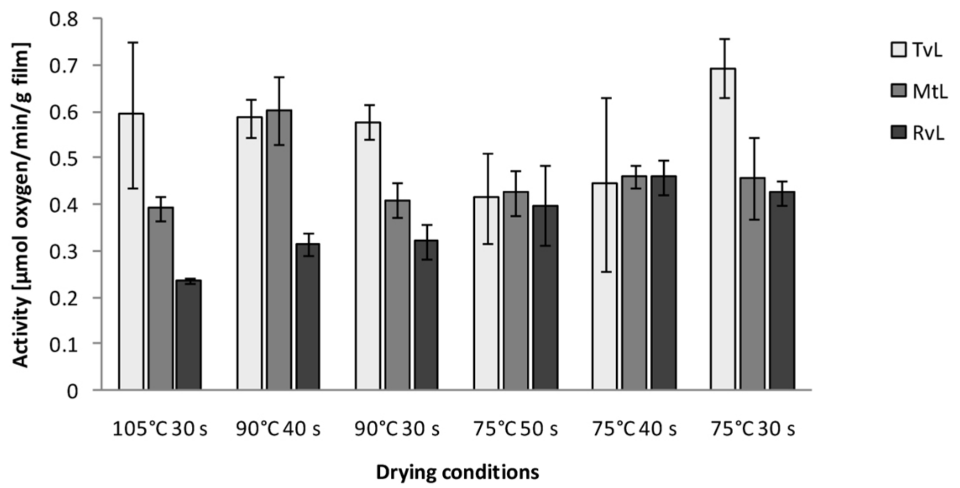
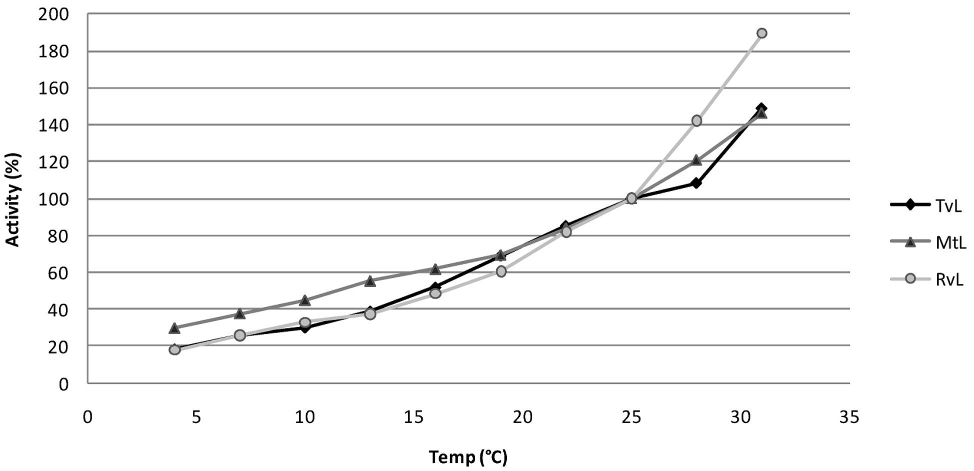


Table 13. Remaining laccase activity after immobilization in latex/clay matrix and drying

Name Drying conditions (°C/s)	Remaining activity (%)		
	T. versicolo laccase	M. thermophile laccase	R. vernicifera laccase
105/30	42	34	18
90/40	41	53	24
90/30	40	36	25
75/50	29	37	31
75/40	31	40	36
75/30	49	40	33

Figure 11 shows the relative activity for TvL, MtL and RvL where the activity for all kinds of Lac arbitrarily was set to 100 % at 25^{O}C, even if there was differences in the absolute values at that temperature. It is clear that all laccases retains a substantial part of enzyme activity also at 4°C. Thus the laccases seems to be promising oxygen scavenging enzymes to be used at chilled conditions.

Figure 11.
Activity of laccases from T. versicolor (TvL), M. thermophila (MtL) and R. vernicifera (RvL) in the temperature interval 4-31 °C. Pyrogallol was used as substrate and the activity at 25 °C was set to 100%.



The results show that TvL and MtL are less selective than RvL with regard to their reducing substrate, and have higher potential to be useful as oxygen scavengers in active-packaging applications. It is possible to produce coatings containing laccases with retained catalytic activity even after drying at temperatures as high as 105 °C. Furthermore, the relatively high activities observed at 4 °C suggest that laccases can serve as oxygen scavengers in packages with refrigerated food. Several factors including the high remaining activity after coating and drying, a wide variety of phenolic substrates to choose between, and no problem with hydrogen peroxide as by-product contribute to make laccases to attractive catalysts in future research on oxygen scavengers.

The experiments with lignosulfonate as substrate revealed that the active coatings reduced the oxygen concentration at 100 % RH, but not at 84 % RH or lower (Figure 12).

Figure 12.
Oxygen concentration in the headspace of airtight containers with latex-based coated boards containing both laccase and lignosulfonates. The temperature was 23 °C and the relative humidity ranged from 34 to 100%. The initial ratio of oxygen to nitrogen was 1:99. The arrow indicates refill of the chamber with a gas mixture consisting of 1% oxygen and 99% nitrogen. Error bars indicate standard deviations of three replicates.

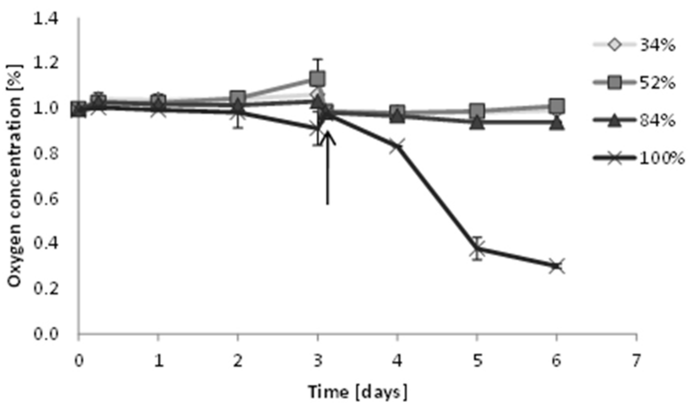
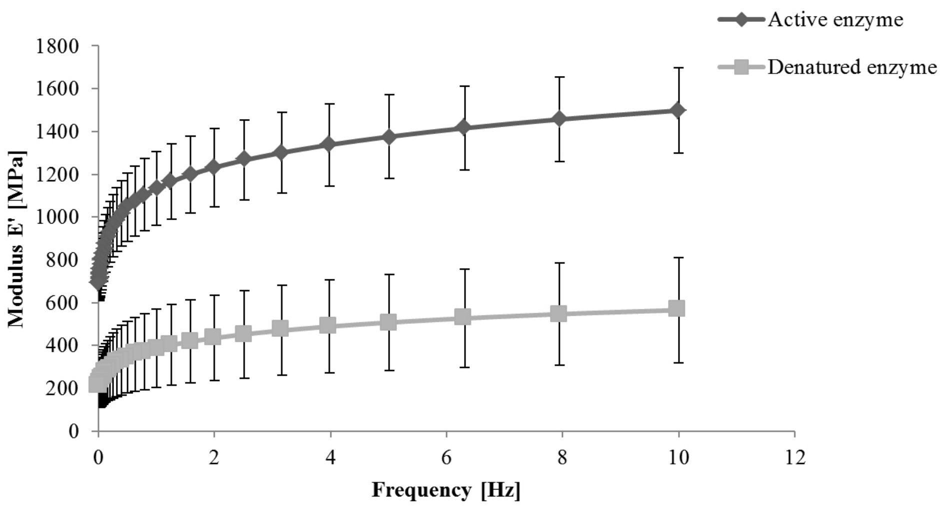


Figure 13 shows the mechanical properties, the E' storage modulus, of cast starch-based films with laccase and lignosulfonates. As a control, a film containing denatured enzyme was used. The E' modulus increased by 30% for the film containing active enzymes. The increase in modulus means that the material has become stiffer. This effect is most likely due to a more rigid structure resulting from cross-linking of lignosulfonate molecules after enzymatic oxidation and formation of radicals.

Figure 13.
Storage modulus (E') of cast starch-based films containing either active or denatured laccase. The measurements were performed at 23 °C and 50% RH. The figure shows mean values of six replicates with error bars representing standard deviations.



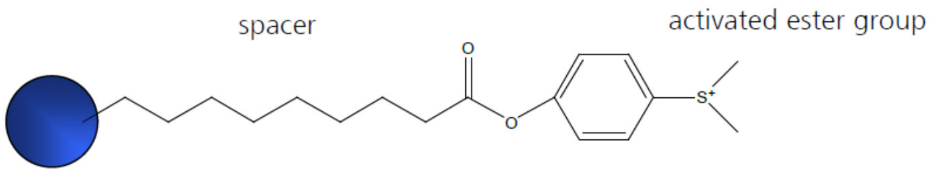
Coatings based on aqueous dispersions of latex, clay, lignosulfonates, starch, and laccase can successfully be applied on packaging board with active enzyme remaining after drying procedures involving temperatures >100°C. The coated board will be functional as an oxygen scavenger provided that the relative humidity is sufficiently high. The dependence on high relative humidity offers a way to control the enzymic activity. The results suggest that the system is useful for active packaging of high-moisture foods. Furthermore, laccase-catalyzed oxidation of lignosulfonates results in increased stiffness and increased water-resistance of starch-based films. Further research on coatings based on laccases and lignosulfonates is therefore of interest not only due to their potential in oxygen scavenging, but also due to their potential in improving the mechanical properties of packaging materials based on renewable bio-polymers. Investigations in this area may also lead to new applications for biorefining products derived from lignin.

3.3 ST1-3 Immobilisation

3.3.1. Materials and methods

For the immobilization of enzymes functional polymer nanoparticles were synthesized and co-polymerized with polymerizable surfactants, named "surfmers" and methacrylic acid methylester (MMA) and styrene in a one-stage reaction using an emulsion polymerization. Different surfmers that vary in spacer length and type of polymerizable groups bring out so-called surfmer-nanoparticles that carry an active-ester at their surface (see Figure 14). These active-ester groups act as binding sites for biomolecules, in this case three oxygen consuming enzymes, Glucose Oxidase, Catalase and Laccase.

Figure 14. Schematic illustration of a surfmer-anoparticle with an active ester on its outer shell.



Particle size measurements have been done with photon correlation spectroscopy; particle charge detection was used for the determination of the surface charge. Seven different surfmers were produced, with yield of reaction ranging from 23 to 97 %. The hydrodymanic ddiameters was in the range of 130 to 170 nm and the zeta-potential in the range from 16 to 24 mV. The two surfmer-nanopartices that had the higest yield was selectd for coupling with GOx (see Table 14).

Table 14.
Molecular composition of two surfmer-nanoparticles. The table shows values for different surfmer contents, the hydrodynamic diameter of the nanoparticles and their surface charge.

Surfmer	Co-monomer	Surfmer content [%]	Yield [%]	Hydrodynamic diameter [nm]	Zeta-potential [mV]
AUPDS p-(11-(Acrylamido)-undecanoyloxy) phenyl- dimethyl-sulfonium- methyl sulphate	MMA	1	72	145.8±1.5	23.5±0.9
AUPDS p-(11-(Acrylamido)-undecanoyloxy) phenyl- dimethyl-sulfonium- methyl sulphate	MMA	3	88	132.4±1.0	23.0±0.9
MUPDS p-(11-(Methacrylamido)-undecanoyloxy) phenyl- dimethyl-sulfonium-methylsulfate	MMA	1	97	130.4±1.1	19.6±0.5
MUPDS p-(11-(Methacrylamido)-undecanoyloxy) phenyl- dimethyl-sulfonium-methylsulfate	MMA	3	84	124.2±1.2	22.1±1.0

For differentiating between specific and non-specific binding all measurements have been done with hydrolysed nanoparticles as well that do not contain active-ester functionalities at their surface anymore.

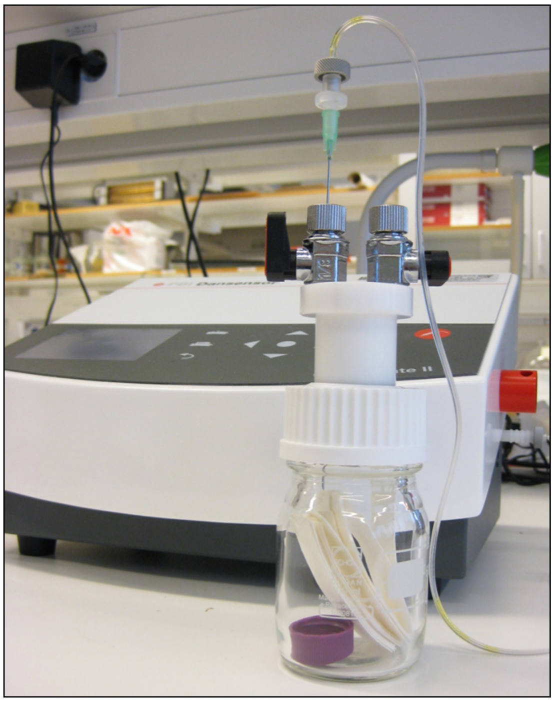
Coating colurs were prepared according to the formulation given in Table 15. The surfmer-nanoparticles uses was the p(MMA-co-MUPDS) at 3% surfmer content. The colours were draw-down on packaging board and dried in an oven. The surfmer-nanoparticles used in the coating of packaging board were labelled with GOx. In some experiments the GOx was labelled non-specifically as described above. The coated board was placed in the air-tight camber and the decrease in oxygen concentration was measured by a Checkmate II (PBI Dansensor A/S, Ringsted, Denmark) in a procedure in which an aliquot of the head-space gas was taken out and analysed using a zirconium-based sensor (see Figure 15). The atmosphere inside the chambers was modified to about 1% oxygen and 99% nitrogen and 100% RH. The samples of coated board tested were denoted:

- a1, a3 and a4 (GOx labelled specifically)
- b1 (GOx labelled non-specifically)
- b0 (Reference unlabelled particles)
- p1 (Pure GOx)

Table 15. Coating colour formulation

Material	Composition [Parts per hundred]
Latex pH 7	100
Starch	10
Clay	55
Glucose	13

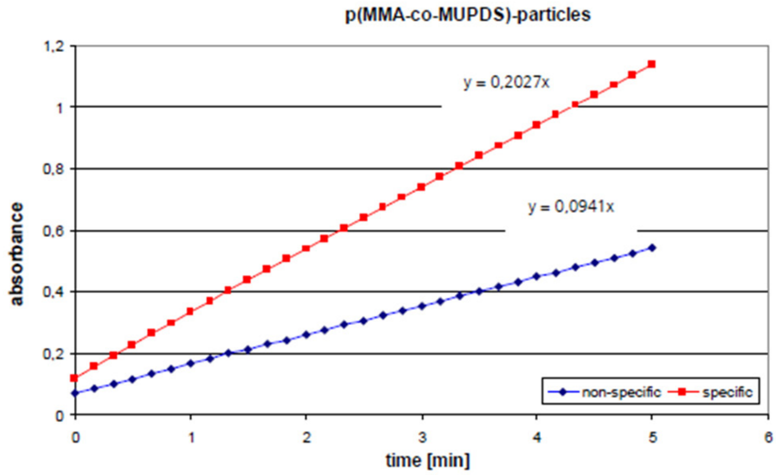
Figure 15. Test equipment for oxygen scavenging activity.



3.3.2. Results

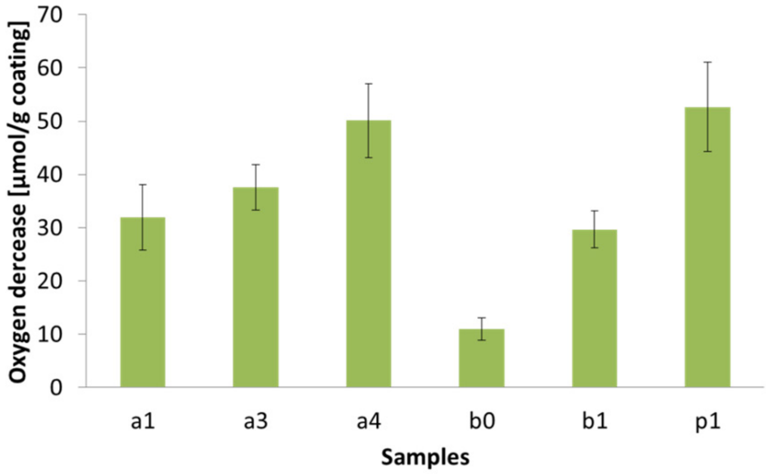
An enzyme coupling protocol could be successfully developed for binding Glucose Oxidase and Catalase to the surface of surfmer-nanoparticles. For the coupling trials a given amount of enzyme solution was added to the particle suspension. Reaction time was 4 hours at room temperature. After washing of the bioconjugates the enzyme activity of was determined (Figure 16)

Figure 16. Measurement of enzymatic activity of particle-bound Glucose Oxidase.
Both active-ester functionalised and hydrolysed particles have been investigated to differentiate between specific und non-specific binding.



As it can be seen in Figure 17 there is a significant difference between all the samples and the reference b0. There is no significant difference between the pure GOx (p1) and the specifically labelled sample a4. The results clearly indicate that it is possible to incorporate the optimized nanoparticle coupled enzymes in standard latex coatings with retained oxygen scavenging ability.

Figure 17. Results oxygen scavenging capacity.
For all samples, the total addition of enzymes to corresponded to 1.25 U/ml of GOx and 5U/ml of Cat.



3.4 ST1-4 Scaling-up

3.4.1. PE, PP, or PLA liner on top of the latex/clay coating

The aim of this study was to investigate the possibility to sandwich the Enzycoat sheets with an extruded plastic in order to overcome the cytotoxicity issue reported in Work Package 4. Three different plastics were used; polylactic acid (PLA), polypropene (PP) and low density polyethylene (LDPE). Plastic films from these polymers differ in their water vapour barrier (WVB) properties as well as in their oxygen barrier (O2-barrier) properties. The order of OTR values is PLA < PP < PE.

The SBS board Cupforma Classic was used in the coating and in lamination tests. The coating of the board was performed by draw-down coating using Standard recipe ECII with 10 pph starch. The coated sheets were dried in an oven at 105°C for 30 s. The coated sheets were stored at ambient conditions before extrusion coating. Before the extrusion coatings the sheets was attached to the web material by tape. The appropriate line and screw speeds for approximately 20 g/m2 coat weight of the plastic were determined for all plastics before coating the sheets. NatureWorks 3051D (PLA) was extrusion coated onto the Enzycoated paperboard with melt temperature 239 °C, air gap 170 mm and line speed of 60 m/min resulting in a coat weight of 23 g/m2. Borealis WF420HMS (PP) was coated onto the Enzycoated board with melt temperature 277 °C, air gap 175 mm and line speed of 40 m/min resulting in a coat weight of 22 g/m2. Borealis CA7230 (LDPE) was coated onto the Enzycoated board with melt temperature 292 °C, air gap 270 mm and line speed of 40 m/min resulting in a coat weight of 16.5 g/m2.

Prior to extrusion coating the sheets were corona treated at 3400 W. This had in a pre-study shown to not affect the enzyme activity but to be necessary for the plastic adhesion on the Enzycoat coating.

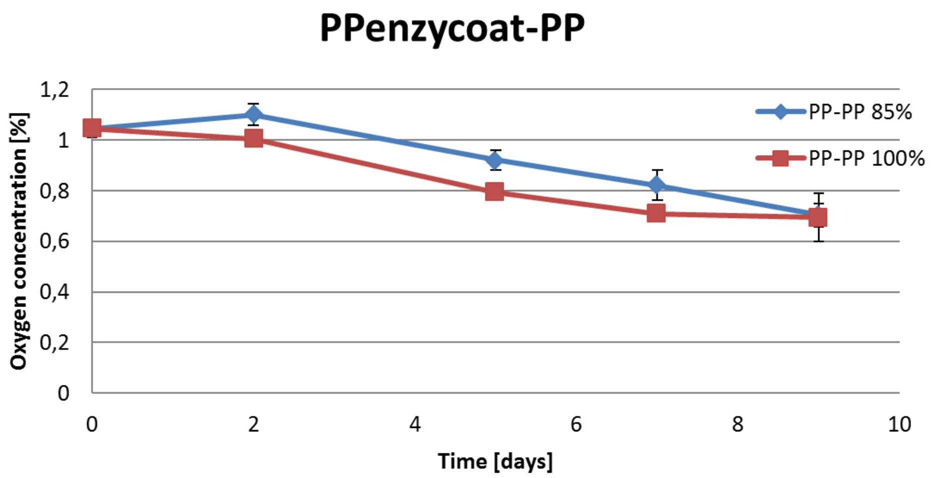
Before testing the oxygen scavenging capacity of the coated and laminated board, the sample was cut in stripes and the edges were sealed in order to prevent oxygen diffusion through the exposed edges. The stripes were placed in the air-tight camber and the decrease in oxygen concentration was measured by a Checkmate II (PBI Dansensor A/S, Ringsted, Denmark) in a procedure in which an aliquot of the head-space gas was taken out and analysed using a zirconium-based sensor. The atmosphere inside the chambers was modified to about 1% oxygen and 99% nitrogen and ca. 85% and 100% RH.
The Enzycoat coated structure of coatings is shown in Table 16.

Table 16. The combination of plastic materials used to extrusion coat the Cupforma.

Back side	Enzycoat side
PP	PP
PP	PE
PLA	PP
PLA	PE
PLA	PLA

All combinations shown in Table Q had the capacity to reduce the oxygen concentration, see Figure 18 as one example for PP extruded on both sides. Figure 18 indicates that there is no substantial difference between 85% and 100% RH, indicating that diffusion through the extrusion coated layer may be the rate- determining step.

Figure 18.
Oxygen scavenging in N2/O2 gas mixture containing 1% O2 vs. time. Room temperature at ca. 85% RH and 100% RH.



3.4.2. Pilot coating and related scaling-up of preparation and application techniques

3.4.2.1. Introduction

Scaling-up experiments were done with three different compositions on three different line materials with two different coating lines. Dispersions used were standard recipe with starch and enzymes, the same without enzymes and standard recipe with enzymes and triple amount of glucose. Dispersion coating was done on uncoated paperboard, PE coated paperboard and on a plastic film, which had PE surface layer. The first coating was done in a pilot line scale and coating parameters were adjusted between coatings. The second coating was done on a smaller line, which required some of its own parameters. Generally in the pilot line the coating part went well and main problems rose later in sheet cutting. With the smaller line rewinding did not work very well.

3.4.2.2. Experimental

3.4.2.2.1 Dispersion materials and preparation

Starch was Perlcoat 155 from Lyckeby Stärkelsen, kaolin clay Barrisurf LX from Imerys Minerals Ltd, glucose α-D(+) glucose or D(+) glucose from Acros or D(+) glucose monohydrate from Merck (water taken into account in the calculation), latex HPU 70 made in Livorno latex plant and delivered by Styron Europe GmbH, glucose oxidase Gluzyme Mono 10000 BG from Novozymes and catalase suspension Catazyme 25 L from Novozymes. NaOH was used as 10 % solution and water was distilled.

First starch was boiled in water about 30 min to make approximately 20 % solution. Accurate values were used in adding the starch solution into the dispersion. Then clay was added to water for 63 % dry solids content. Glucose (single or triple amount) was added to the latex, and this solution was then poured to the clay dispersion. Next starch solution was added and after that pH was adjusted to 6.8–6.9 (temperature of the dispersion was near room temperature) with NaOH solution. Finally, in two cases, the enzymes were added. Strong mixing was required at all stages, especially after adding starch. 10–15 litre containers were used in preparing the dispersions. This size could still be mixed efficiently with a laboratory motor mixer and no new methods had to be developed in this stage. Dispersions were kept in fridge until used the next day. Table 17 summarises dry solids contents of different dispersions used.

Table 17. Dry solids contents of the used dispersions.
Latex amount is fixed at 100 pph and other material amounts are compared to it.

Dispersion	Latex HPU 70 (pph)	Clay BS LX (pph)	Glucose Oxidase (pph)	Catalase (pph)	Glucose (pph)	Starch PC 155 (pph)
No enzymes	100	55	0	0	13	10
With enzymes	100	55	0.974	0.876	13	10
3x glucose	100	55	0.974	0.876	39	10

3.4.2.2.2 Coating general information

Dispersion coatings were performed on the pilot line and the smaller line of Paper and Packaging Technology, Tampere University of Technology (technical information of pilot line can be found at http://www.tut.fi/idcprod/groups/public/@l602/@web/@p/documents/liit/p011877.pdf and of the smaller line at http://www.tut.fi/idcprod/groups/public/@l602/@web/@p/documents/liit/p011879.pdf or through navigation from www.tut.fi/epr in Finnish pages). In dispersion coating line material width up to 40 cm can be used with both coating lines. Uncoated paperboard was StoraEnso Cupforma Classic 350 g/m2 and PE coated paperboard StoraEnso 310 g/m2 + 42EB56, which had a PE top layer on the other side and pure paperboard on the other. Plastic film was Wipak 12 μm BOPET + 40 μm

PE/EVOH/PE. Dispersion was coated on the PE side of materials if present. PE sides were pre-treated with corona (1500 W, 50 m/min = 3600 J/m2) one day before coating. This made the plastic film roll quite electric and during coating it gave some electric shocks when touched.

3.4.2.2.3 Coating with pilot line

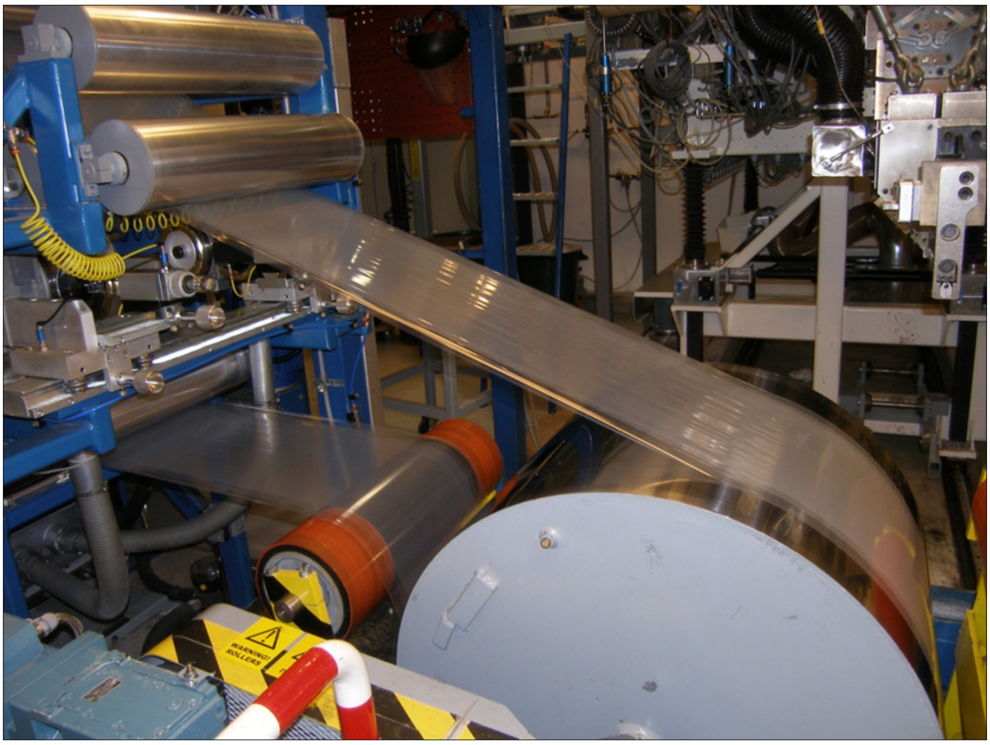
With pilot line only lines having a PE surface were used. A rod giving as thick layer as possible was used to wipe extra dispersion from the line material before drying (Figure 19). Extra dispersion was led back to use. A roll system was used in delivering the dispersion to the line and this provided continuous mixing of the dispersion. All dispersions were in room temperature when used.

Figure 19.
Dispersion coating of plastic film in pilot line: extra dispersion wiped from the line material with a rod. The glow comes from IR heater right above.

Coating parameters are collected in Table 18. Four IR heaters and three hot air dryers were used. With the first paperboard coating (no enzymes) IR heaters setting was 18 kW and hot air at 110 °C. Temperature of the coating right after the last heating unit was 63 °C and line speed 40 m/min. With the plastic film the temperature of hot air had to be dropped to 90 °C because at 105 °C PE layer apparently shrank and thus bent the whole film from sides towards the centre. Hot air temperature could not be increased after beginning of the dispersion coating because the whole width of the line was not coated to keep rolls inside the system clean. However, maximum usable temperature was not searched. IR power was increased to 21.6 kW. Temperature of the coating right after the last heating unit and line speed remained the same. The coating felt dry after the chill roll (Figure 20) in all cases. After measuring the thickness after the first coating another layer was coated onto it to get the final thickness as near as possible of the handmade coatings. The second layer proved problematic with the plastic film as the roll blocked (film layers next to each other stuck together). Higher heat power should have been used.

With the next coating material (with enzymes) hot air temperature with paperboard was increased to 120 °C (IR at 18 kW, temperature of the coating right after the last heating unit 66 °C) and with the second coating layer IR power was also increased (21.6 kW, T = 71 °C). Settings with plastic film were 90 °C and 25.2 kW (T right after drying = 64 °C). This roll also blocked after the second coating.

Figure 20. Dried coating on the plastic film (pilot line).



The third material (3x glucose) had higher solids content than the previous ones. This showed itself in slower drying. With plastic film the same settings were used as above with the same result but with paperboard IR power was increased to 19.2 kW in the first coating (T = 69 °C) as the surface felt a little wet. In the second coating 21.6 kW and 35 m/min line speed were used (T = 76 °C) but still the roll blocked.

3.4.2.2.4 Coating with the smaller line

The smaller line was used later because only a narrower plastic film was available, and its coating parameters are also collected in Table 18. This time uncoated paperboard was used instead of the PE coated one. Only hot air drying was used, and the temperature was set at 90 °C. Line speed was as low as possible (10 m/min). Extra dispersion was removed with a blade from the gravure roll delivering the dispersion to the line. This method produced an uneven coating due to too viscous dispersion. Dispersions were diluted with water before coating but apparently not enough. Tilted stripes could be seen on the plastic film. All coatings felt dry and were at about room temperature after hot air drying and cooling with a small chill roll. Unwinding did not use as much pressure as in the pilot line, which led to wrinkles. Some wrinkles formed even to thick paperboard. On the other hand blocking was not so severe. In the second coating unevenness was still more clearly visible (Figure 21).

Figure 21. Undried second coating layer in the smaller line.
Wrinkles (dark) and white tilted stripes of the coating are clearly visible.

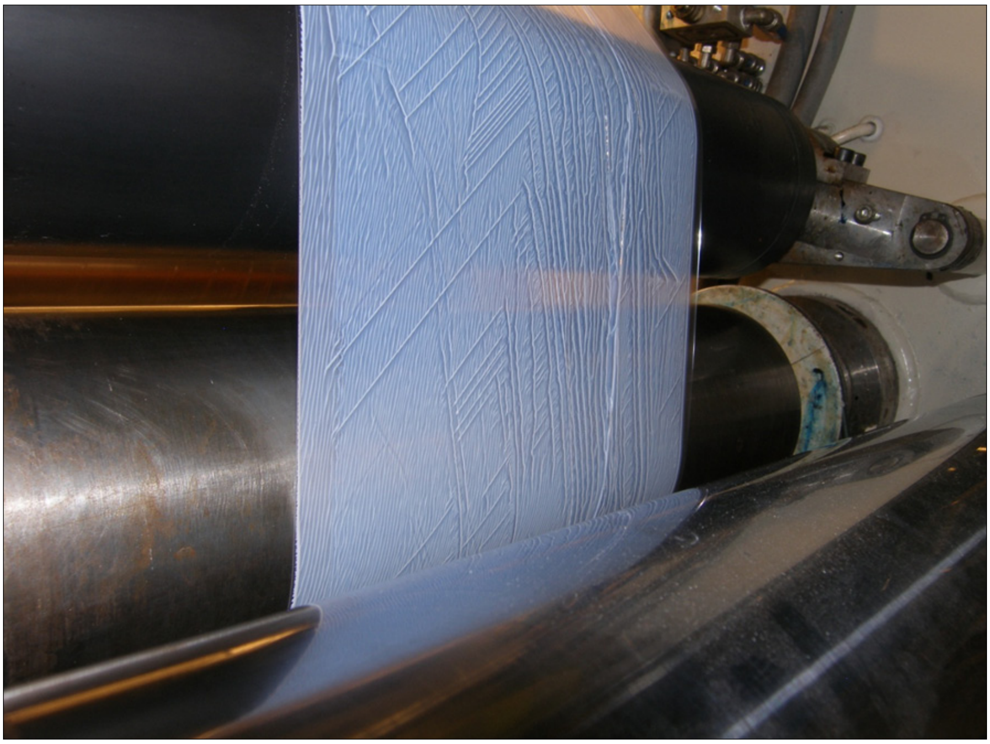


Table 18. Coating parameters used in pilot line and the smaller line dispersion coatings.

	Line speed m/min	Hot air temperature °C	IR power kW	Temperature right after drying °C	Roll blocking	Extra coating removal
No enzymes/ paperboard	40	110	18	63	-	Rod after coating
No enzymes/ plastic	40	90	21.6	63	2. coating	
With enzymes/ paperboard	40	120	18/21.6	66/71	-	
With enzymes/ plastic	40	90	25.2	64	2. coating	
3x glucose/ paperboard	40/35	120	19.2/21.6	69/76	2. coating	
3x glucose/ plastic	40	90	25.2	64	2. coating	
Smaller line coatings/ paperboard	10	90	-	-	-	Blade from gravure roll before coating
Smaller line coatings/ plastic	10	90	-	-	Mild after 2. coating	

3.4.2.3. Results and Discussion

All coatings in pilot line proceeded well even though the dry solids content varied between samples. Line speed was only 40 m/min but if heating power were raised the line speed could also be increased. In the second coatings there were no difficulties in unwinding the roll. Only paperboard coatings with enzymes and no enzymes did not block (coated layers did not stuck to each other) after the second coating, which is partly because water could absorb to the next paperboard layer in the roll. Higher heating power should have been used with all the other second layer coatings. Based on the measured temperatures right after the last heating unit (the highest temperature the coating rises to) and the short heating time enzymes should not be damaged even with a higher heating power.

With the smaller line roll and coating quality was not as good. Lower rewinding pressure guaranteed lower blocking and only 3x enzymes on plastic showed some blocking tendency.

Pilot line rolls were first cut into long sheets and then several long sheets at a time to A4 or A3 sheets. Paperboard roll with 3x glucose and especially plastic rolls were difficult to cut to the long sheets because of blocking. A bunch of blocked plastic sheets was easy to cut to A4 size but single or several loose ones were too flexible for manual cutting without wrapping them first with paperboard. A bunch of plastic sheets proved to be very time consuming to take apart to single sheets. However, cutting to A4 sheets with a machine that takes material directly from a roll should not be a problem, as long as the roll unwinds easily. As all plastic sheets appeared wet (= they had blocked) they were allowed to dry in oven at 30 °C in normal atmosphere in loose stacks. Drying period ended up to be about 60 h, which may have consumed enzyme activity. Plastic sheets were quite curled after drying. All paperboard long sheets remained untouched the first night, after which they were cut to A4 sheets and stored in oven. Paperboard with 3x glucose dried enough during the first night so that sheets were easy to detach from each other although they remained a little sticky still after all drying. Overall time in contact with normal atmosphere was the same with plastic sheets.

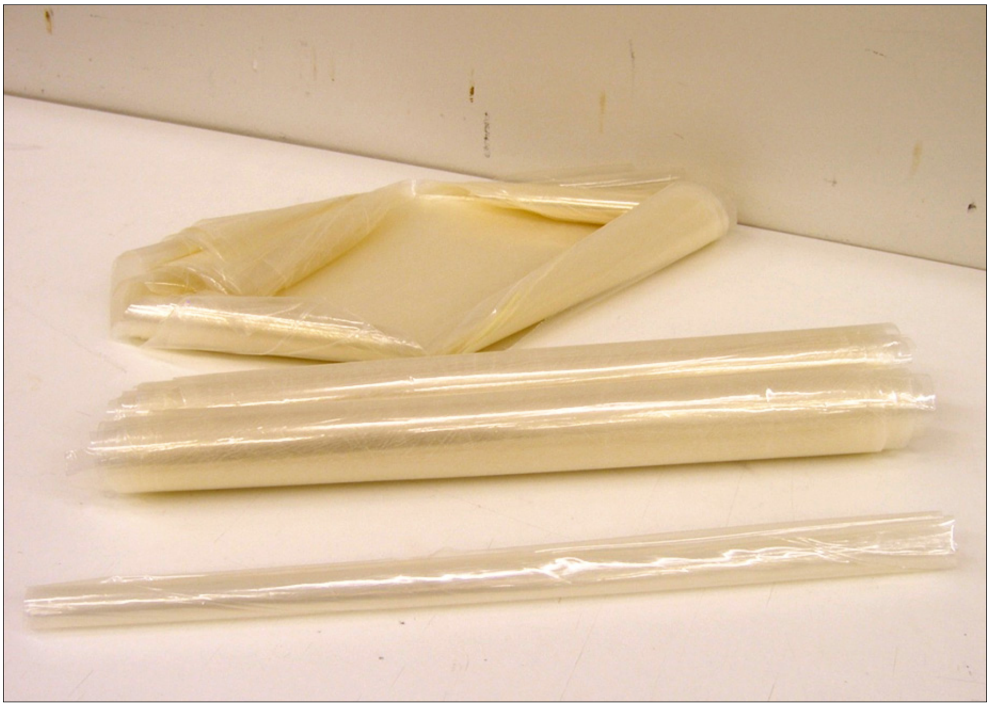
With smaller line rolls no exact A4 cutting was done as the coating width was not quite as wide due to equipment and narrower plastic roll. Approximately the correct length sheets were cut directly from a roll. This method further diminished the effects of blocking.

Cross-section of paperboards coated with dispersion revealed unevenness of the dispersion coating due to uneven paperboard surface, even with the PE coating. Plastic film surface was even and thus the dispersion coatings had also even thickness. On average these thicknesses corresponded well to the handmade samples.

PE-paperboard samples with 3x glucose and no enzymes were hot air heat sealed towards itself and uncoated paperboard. 3x glucose dispersion coating, which still felt a little sticky, heat sealed at 330 °C towards itself and at 350 °C towards uncoated paperboard. These values are near LDPE heat sealing temperature against paperboard. No enzymes dispersion coating heat sealed properly in both cases at 440 °C but it showed a strong sealing without fibre tear already at lower temperatures. 440 °C was enough to cause small brown burned areas around the seal. Glucose may act as glue.

Smaller line coated sheets were further PP extrusion coated. Corona was used to improve adhesion (3400 W, 50 m/min = 8160 J/m2). 20 g/m2 coating attached on the paperboard coated sheets better (adhesion 3/5) than on the plastic film coated sheets (adhesion 2/5). Even with PP coating the sheets curled strongly (Figure 22). With 3x glucose curling was strongest.

Figure 22. 20 g/m2 PP coated dispersion coated plastic sheets.
In front 3x glucose, back 1x glucose.



3.4.2.4. Conclusions

Dispersion coating of different Enzycoat dispersions can be done in pilot line scale. Hundreds of metres of coating were produced at 40 m/min coating speed. A rod wiping extra dispersion from the line after coating worked well, and other methods are recommended to be avoided with this viscous dispersion. For proper rolls high enough pressure at rewinding should be used, which though increases blocking. Drying parameters should be optimised for each machine individually. Pure plastic does not absorb water so it should be completely dry before rewinding. Wet coatings stick to the next layer causing difficulties in sheet detaching. Cutting machine is recommended for making large amounts of sheets directly from a roll, or if sheets are cut manually it should be done by unwinding the roll. If dispersion is further coated with extrusion the same roll should be used instead of sheets. Two coating layers produce about the same thickness as handmade sheets. Thickness varied in the paperboard samples but not in the plastic film samples. Extra glucose improved sealability but the coating can feel a little sticky and curling increased.

4 WP2 To investigate the possibility to interface dispersion coated enzymes and actively control safety and display formation and degradation of sensory active compounds

4.1 Lipid oxidation

4.1.1. Introduction

Lipid oxidation is one of the major causes of food spoilage. It is of great concern to the food industry because it leads to deleterious changes in foods such as loss of flavour, development of off-flavours, loss of colour, nutrient losses and even generation of toxic compounds.

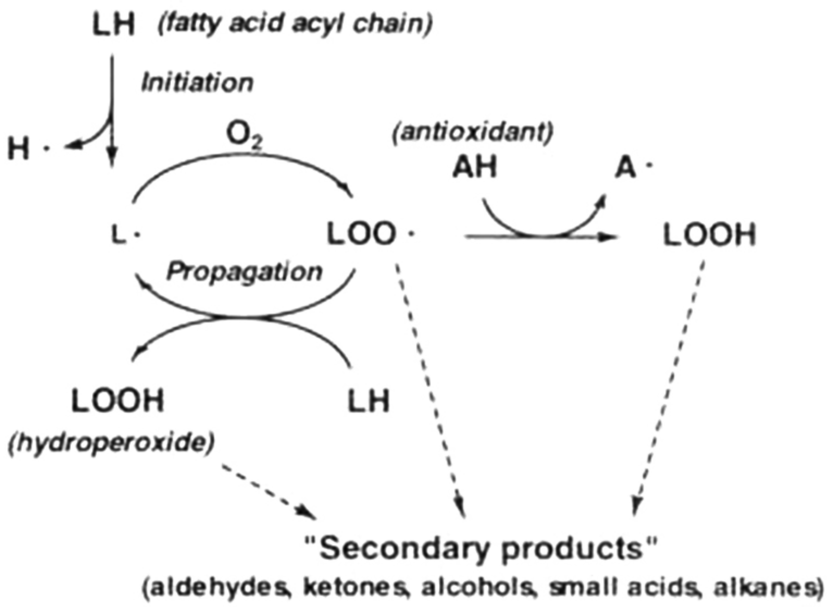
Oxidation of lipids can occur in foods containing substantial amounts of fat, e.g. dairy products, meat products, oils and nuts but also in those that only have low lipid concentrations such as vegetable products. Practically all quality attributes can be negatively affected by this process. Thus, aroma changes result from volatile odorous compounds being formed, flavour modifications are caused by hydroxyl acids, colour can darken as a result of condensation reactions between oxidation products and proteins and even the texture might be altered by oxidative induction of protein cross-links. In addition to the resulting loss of consumer acceptance because of sensory changes, the nutritive value and safety of the food can be impaired.

4.1.1. Autoxidation

The main reaction involved in oxidative deterioration of lipids is termed autoxidation. The mechanism of this process is a free radical chain reaction which consists of the three stages of initiation, propagation and termination. The primary radicals that are formed in the initiation step can be generated through presence of oxidation initiators, such as transition metals, oxidants and enzymes. Light can also act as an initiator. A foodstuff is a complex chemical matrix and no one mechanism can be held exclusively responsible for the initiation of lipid oxidation. While in the raw foods the enzymatic oxidation plays a most significant role, the chemical initiation is probably determinant in processed foods.

Once a free radical is generated, the chain reaction of oxidation is initiated, new free radicals, carbon- and oxygen-centered, are formed and the process is easily propagated. The net chemical result of lipid oxidation is very complex. Multiple initial products result from one starting material and many more are generated by the decomposition of the unstable hydroperoxides, which are the primary initial products of lipid autoxidation. This decomposition proceeds by homolytic cleavage of a peroxy bond to form alkoxy radicals. These radicals undergo carbon-carbon cleavage to form a myriad of compounds of various molecular weights, flavour thresholds and biological significance, such as aldehydes, ketones, alcohols, hydrocarbons, esters, furan and lactones. Lipid hydroperoxides can react again with oxygen to form such secondary products as epoxyhydroperoxides, oxohydroperoxides, bihydroperoxides, cyclic peroxides and bicyclic endoperoxides. These secondary products can in turn decompose in similarity to monohydroperoxides and generate volatile breakdown products. Alternatively, the hydroperoxides can condense into dimers and polymers. A general scheme that outlines the most important steps of lipid autoxidation is presented in Figure 23.

Figure 23. Generalised scheme for autoxidation of lipids.



4.1.2. Factors influencing lipid oxidation in foods

Food lipids contain a variety of fatty acids that differ in chemical and physical properties and also their susceptibility to oxidation. In addition, foods contain numerous non-lipid components that may co-oxidize and/or interact with the oxidizing lipids and their oxidation products. Thus, oxidation of lipids in foods is a dynamic, multifaceted series of events, often overlapping and continually interacting, which makes accurate description of oxidation kinetics almost impossible. Innumerable factors influence the rate of oxidation and also the relative amounts of reaction products formed by lipid oxidation. They include, amongst others, fatty acid composition, the ratio between free fatty acids and the corresponding acylglycerols, oxygen concentration, temperature, surface area of the lipid exposed to air, moisture content, physical state of the lipid, presence of prooxidants, presence of antioxidants and exposure to radiation. It is obvious that many factors combined determine the route, rate and final effect of lipid deterioration in foods.

4.1.3. Assessment of lipid oxidation

Oxidative decomposition of lipids is of major significance in regard to both the acceptability and nutritional quality of food products and many methods have therefore been devised for assessing this occurrence. Some of the most frequently used methods for assessing lipid oxidation include analyses of peroxide value, thiobarbituric acid test, determination of volatile carbonyl compounds, measurement of anisidine value, spectrophotometric analyses and sensory evaluations. In spite of the multitude of assays, a universal method which correlates well with the extent of food deterioration throughout the entire course of autoxidation is not available. The present methods give information about particular stages of oxidation process and some are more applicable to certain lipid systems than others. This situation should not be unexpected in view of the chemical diversity of the food matrices and of the oxidation pathways.

When the process of oxidation is followed comparatively in the course of time, the loss of lipid substrate or the amount of oxygen uptake can serve as general, though non-specific and usually not sensitive enough, indexes for peroxidation of lipids.

Since the primary products of lipid oxidation are hydroperoxides, it is reasonable to determine their concentration as a measure of oxidation. The peroxide value test reflects the total concentration of peroxides and hydroperoxides present at a certain time. However, this approach is restricted by the chemical instability of these compounds – after their concentration reaches a maximum level it decays as a function of temperature, the presence of other food components etc.

An alternative approach to the determination of the extent of oxidation is the measurement of products of hydroperoxide degradation. In contradiction to the peroxide determination, such as an assay is not limited to the early stages of oxidation and may reflect the formation of products, like carbonyl compounds, which actually contribute to rancid and other objectionable flavours. Among these methods, thiobarbituric acid (TBA) test is one of the most commonly used. The test is based on the colour product resulting from the condensation of TBA with malonaldehyde which is presumably generated in the oxidized fats. However, a large body of evidence suggests that other food components can also react with TBA to form the same chromophore and even the formation of malonaldehyde is dependent on the composition of the initial lipid. Hence, it is a controversial method because of concerns about its reproducibility and even reliability.

Methods for determining total carbonyl compounds are usually based on measurement of hydrazones that arise from reaction of aldehydes and ketones (carbonyl oxidation products) with 2,4-dinitrophenylhydrazine. However, under the experimental conditions used for these tests, carbonyl compounds may be simultaneously generated by decomposition of unstable intermediates, such as hydroperoxides, thus detracting from the accuracy of the results.

Because the carbonyl compounds in oxidized fats are of relatively high molecular weight, they can be individually separated by a variety of techniques. The volatile carbonyls can be recovered by distillation at atmospheric or reduced pressure or enriched on adsorbent material after headspace sampling. They can then be analysed by chromatographic methods that enables identification and quantification of the different substances. The lower molecular weight carbonyl compounds are of interest because of their influence on flavour. Aliphatic aldehydes are of specific interest since they are the most abundant volatile breakdown products and they also exhibit low flavour thresholds. In foods they may cause flavours described as rancid, oily, fishy, green, cucumbery etc. Normally such aromas result from the presence of a number of compounds in certain ratios but in some foods a single aldehyde plays a leading role in the generation of the characteristic flavour. Hexanal is the single aldehyde that is generally acknowledged as the most important one, and quantitative measurement of hexanal by headspace analysis followed by gas chromatography is a common technique for determining the extent of lipid oxidation.

As indicated before, the resulting mixture of oxidation breakdown products is very complex and it is also dependent on a number of factors. However, relatively large amounts of hexanal are almost always produced, regardless of the initial lipid composition and other contributing factors, and it must therefore be considered as an ideal marker substance. Furthermore, hexanal itself contributes to the oxidized off-flavour and it has a low flavour threshold value. The hexanal level that can be considered to cause rancidity problems depends on the food matrix but it is generally acknowledged that off-flavour problems occur when the hexanal concentration reaches somewhere between 10 and 100 nanograms per liter headspace.

4.1.4. Selection of foodstuff

All fatty foods can undergo lipid oxidation. In the current project it has been requested to use a solid foodstuff as the model in order to facilitate oxygen scavenging and also to enable the indicator system to function properly. A range of food products can be suggested for the coming experiments, including nuts, potato crisps and cereals. It is desirable to use a food with known composition and is therefore preferable to use a man-made food, where the recipe is known, instead of a food produced by nature. A suggestion is therefore to select some type of biscuit for the storage tests. This is a product that contains high fat levels and is also known to suffer from off-flavour problems caused by lipid oxidation.

4.1.5. Conclusion

Hexanal is the substance that has been selected as a marker substance for lipid oxidation. A sensor should indicate that the concentration has exceeded a value somewhere in the range between 10 and 100 nanograms hexanal per liter headspace.

4.1.6. References

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4.2 Fish quality and spoilage

4.2.1. Quality aspects associated with seafood

Fish tissue is characterized in being rich in protein and non-protein-nitrogen (e.g. amino acids, trimethylamine-oxide (TMAO) and creatinine), but low in carbohydrate, that results in a high post mortem pH (>6.0). Further, the pelagic, fatty fishes have a high content of lipids consisting mainly of triglycerides with long-chain fatty acids which are highly unsaturated. Also the phospholipids are highly unsaturated that have important consequences for spoilage processes under aerobic storage conditions. Fish spoilage is not clearly defined in objective terms. Obvious signs of spoilage are (http://www.fao.org/DOCREP/003/T1768E/T1768E03.htm):

- detection of off-odors and off-flavors
- slime formation

- gas production
- discoloration
- changes in texture

The development of these spoilage conditions in fish and fish products is due to a combination of microbiological, chemical and autolytic phenomena. In Table 19 the causes of fish spoilage are summed up.

Table 19. Causes of fish spoilage

	Causes of fish spoilage			
Signs of spoilage	Micro-biological	Chemical (oxidation)	Autolytic	Physical
Off odors/ off flavors	+	+	+	-
Slime formation	+	-	-	-
Gas formation	+	-	-	-
Discoloration	(+)	+	+	+
Change of texture	(+)	-	+	+

4.2.2. Effect of TMAO and microbiological and other spoilage

4.2.2.1. TMAO

TMAO (trimethylamine oxide) is a common compound in most marine organisms, where it is used to help maintain water balance against the high salinity of the sea. TMAO and its breakdown product, TMA, are what make marine animals smell fishy (http://people.whitman.edu/~yancey/deepsearesearch.html). TMAO is also a stabilizer of proteins to help them remain functional when perturbed, and there is some evidence that it may be very high in these animals in order to help pressure-sensitive proteins overcome pressure inhibition (perhaps by helping to remove dense water from charged molecules). TMA is recognized as the characteristic 'fishy' odour of spoiled fish. When the oxygen level is depleted, many of the spoilage bacteria utilize TMAO as a terminal hydrogen acceptor, thus allowing them to grow under anaerobic conditions. Towards the end of shelf-life of fresh fish various odorous low molecular weight sulfur compounds such as H2S and CH3SH, together with volatile fatty acids and ammonia are produced because of bacterial growth (Sivertsvik et al. 2002). The major bacterial species causing ultimate spoilage of normally iced fish are probably the pseudomonads and altermonas putrefaciens. These spoilage organisms can use the non-protein nitrogen present in the fish, particularly compounds such as trimethylamine oxide (TMAO) to produce various volatile aromatic compounds. Specifically, these bacteria produce trimethylamine (TMA), a volatile and odoriferous compound, from TMAO. TMA is produced by the bacterial enzyme TMA-oxidase (Ashie and Simpson 1996).

The TMA is believed to react with fish fats to produce the typical spoilage odor associated with fish beyond their prime. White fish muscle is generally lower in TMAO, but usually produces more TMA than dark muscle. In Table 20 one can see the usual spoilage compounds in fish.

Table 20. Spoilage compounds of fish (a)

Compound	Odor	Probable source
H2S (hydrogen sulfide)		Cysteine
(CH3)2S	Cabbage	Methionine
CH3SH	Musty	Methionine
Acetic, butyric, propionic and hexanoic acid esters	Fruity	Glycine
Serine, TMA, DMA	Fishy, ammonia	Leucine, TMAO
NH3 (ammonia)		Amino acids, urea

a) from Regenstein and Regenstein 1991

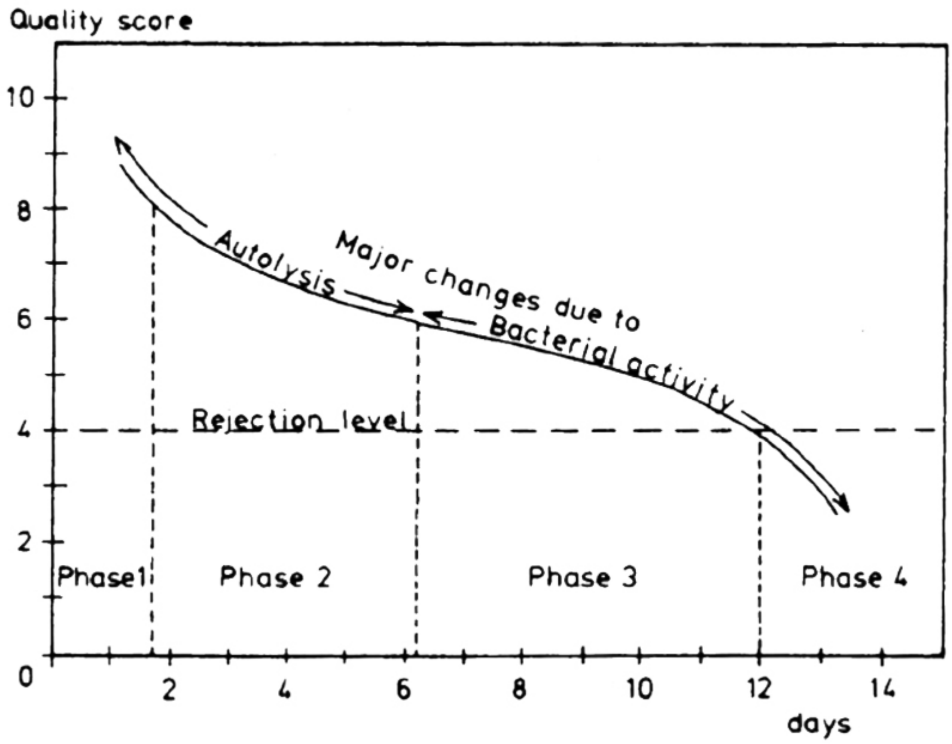
Gadoids constitute an important class of commercial fish including the cods, haddock, the whitings, the hakes, and the pollocks. These fish and many other species obtain the TMAO from their own diets. TMAO is higher in fish from cold waters and from fish that are more active and larger fish tends to have more TMAO then a smaller fish. Sharks usually have 200-250 mg TMAO/100 g of flesh but the gadoids have between 50 and 100 mg TMAO/100 g. TMA is the normal bacterial breakdown product of TMAO. Levels above 15 mg /100g TMA-N (measured as nitrogen) which equals 1.08mmoles TMA/100g of flesh is considered to be a signal of spoilage in fish. One of the routine ways to measure these breakdown products is to measure the total volatile base (TVB). Other compounds such as ammonia are included in the measurement, but the most rapid change in the TVB is attributed to bacterially produced TMA. Spoilage generally occurs at 30mg N/100g as measured by the TVB method that is the spoilage level with TVB is about twice the level for TMA alone. The fishy odor of TMA is generally detectable when TMA level reaches 4-6 mg N/100 g that is before the actual spoilage point.

The presence of ammonia in fish is quite natural. It comes and goes during the postmortem storage period and is thus not a good indicator of the quality of the fish.
The production of DMA (dimethylamine) and FA (formaldehyde) from TMAO is a separate biochemical reaction that takes place in some species of fishes, particularly the gadoids. It is much slower than the TMA reaction, but occurs during frozen storage at temperatures above -29°C.

4.2.2.2. Microbiological spoilage

Initial loss of quality of fresh (non-preserved) lean or non-fatty fish species, chilled or not chilled, is caused by autolytic changes, while spoilage is mainly due to the action of bacteria (see Figure 24).

Figure 24. Changes in sensory quality of iced cod (0°C) (Huss, 1976).



The initial flora on fish is very diverse, although most often dominated by Gram-negative psychrotrophic bacteria. In Table 21 one can see typical spoilage compounds of several types of spoilage bacteria.

Table 21. Typical spoilage compounds during spoilage of fresh fish stored aerobically or packed in ice or at ambient temperature
(The bacterial flora on live fish: http://www.fao.org/docrep/v7180e/V7180E0b.htm)

Specific spoilage organism	Typical spoilage compounds
Shewanella putrefaciens	TMA, H2S, CH3SH, (CH3)2S, Hx
Photobacterium phosphoreum	TMA, Hx
Pseudomonas spp.	ketones, aldehydes, esters, non-H2S sulfides
Vibrionaceae	TMA, H2S
Anaerobic spoilers	NH3, acetic, butyric and propionic acid

During storage a characteristic flora develops, but only a part of this flora contributes to spoilage (see Table 22). The specific spoilage organisms (SSO) are producers of the metabolites responsible for the off odours and off flavours associated with spoilage, especially Pseudomonads and Alteromonads putrfaciens as has been mentioned.

Table 22. Dominating microflora and specific spoilage bacteria at spoilage of fresh white fish (cod).

Storage temperature	Packaging atmosphere	Dominating microflora	Specific spoilage organisms (SSO)
0°C	Aerobic	Gram-negative psychrotrophic, nonfermentative	S. putrefaciens
		(Pseudomonas sp.,S. putrefaciens, Moraxella, Acinetobacter)	Pseudomonas (3)
	Vacuum	Gram-negative rods; psychrotrophic or with psychrophilic character (S.putrefaciens, Photobacterium)	S. putrefaciens
			P. phosphoreum
	MAP1	Gram-negative fermentative rods with psychrophilic character	P.phosphoreum
		(Photobacterium)	
		Gram-negative non-fermentative psychrotrophic rods	
		(1–10% of flora; Pseudomonas, S. putrefaciens)	
		Gram-positive rods (LAB (2))	
5°C	Aerobic	Gram-negative psychrotrophic rods	Aeromonas sp.
		(Vibrionaceae, S. putrefaciens)	S. putrefaciens
	Vacuum	Gram-negative psychrotrophic rods	Aeromonas sp.
		(Vibrionaceae, S. putrefaciens)	S. putrefaciens
	MAP	Gram-negative psychrotrophic rods	Aeromonas sp.
		(Vibrionaceae)	
20 – 30°C	Aerobic	Gram-negative mesophilic fermentative rods	Motile Aeromonassp.
		(Vibrionaceae, Enterobacteriaceae)	(A. hydrophila)

1) Modified Atmosphere Packaging (CO2 containing)
2) LAB: Lactic Acid Bacteria
3) Fish caught in tropical waters or fresh waters tend to have a spoilage dominated by Pseudomonas sp

Another bacterium is Shewanella putrefaciens which is typical for the aerobic chill spoilage of many fish from temperate waters and produces trimethylamine (TMA), hydrogen sulfide (H_2S) and other volatile sulfides which give rise to the fishy, sulfide cabbage like off-odours and - flavours. Similar metabolites are formed by Vibrionaceae and Enterobacteriaceae during spoilage at higher temperatures. During storage in modified atmosphere (CO_2-containing), a psychrophilic Photobacterium producing large amounts of TMA is one of the major spoilage bacteria. Some fresh water fish and many fish from tropical waters are during iced, aerobic storage characterized by a Pseudomonas type of spoilage. Several volatile sulfides (e.g. methylmercaptan (CH_3SH) and dimethylsulfide ($(CH_3)_2S$), ketones, esters and aldehydes but not hydrogen sulfide are produced by Pseudomonas as are several ketones, esters and aldehydes. For fresh, non-preserved fish, the SSO which have been identified are shown in Table 22. Putrefaction or spoilage proceeds very rapidly once the load of SSO exceeds approximately 107 CFU/g. Microbiological activity is also the cause of spoilage of many preserved fish products stored at temperatures >0°C. However, in most cases the specific spoilage bacteria are not known.

The addition of small amounts of salt and acid, as in lightly preserved fish products, changes the dominating microflora to consist mainly of Gram positive bacterial species (Lactic acid bacteria, Brochotrix) and some of these may act as SSO under certain conditions as shown in Table 23. However, also some Enterobacteriaceae and Vibrionaceae may act as SSO for these products. In products with low levels of preservation, Shewanella putrefaciens may also play a role. Also more strongly preserved fish products such as salt cured or fermented products spoil due to the action of certain microorganisms. The dominating flora on these products are Gram positive, halophilic or halotolerant micrococci, yeasts, spore formers, lactic acid bacteria and moulds. A number of SSO are known such as the extremely halophilic, anaerobic Gram negative rods and halophilic yeasts identified by Knochel and Huss (1984) as specific spoilage organisms by causing off-odors and -flavors (sulfide-like, fruity) in wet salted herring. Extreme halophile spoilage bacteria are known to cause a condition called “pink”. These bacteria (Halococcus and Halobacterium) cause pink discoloration of salt, brines and salted fish as well as off odors and -flavors normally associated with spoilage (hydrogen sulfide and indole). Some halophilic moulds (Sporendonema, Oospora) are also classified as spoilers. They do not produce off-odours but their presence detracts from the value of the product because of their undesirable appearance.

Table 23. Spoilage of lightly preserved fish products
(salt content in water phase 3 - 6%, pH > 5, temperature > 5 °C).

Product	Packaging atmosphere	Other preserva-tives than NaCl	Signs of spoilage	Dominating microflora	Specific spoilage organisms (SSO)1
Cold smoked fish	Vacuum		Off-odor / off-flavor	Gram-negative rods	
			(putrid, sickly, sulfurous)	(Entero-bacteriaceae, Vibrionaceae) occasionally LAB (2)	
			Off-flavor	LAB	
			(sourness, acrid)		
			Loss of aroma	LAB	
Shrimps	In brine	Benzoic acid and/ or sorbic acid; citric acid; pH 5.5 – 5.8	Slime	LAB	Leuconostoc sp.
			Gas production occasionally yeasty off-odor / off-flavor	LAB	Hetero-fermentative LAB, occasionally yeasts
			Diacety1	LAB	LAB
			Off-odor / off-flavor	LAB, Brochothrix	
Sugar-salted fish	Vacuum		Off-odor / off-flavor	LAB, Brochothrix	
			Mackerel: rancid		
			Salmon: sour, acrid	(Entero-bacteriaceae,	
			Greenland Halibut:	Vibrionaceae,	
			putrid	S. putrefaciens)	
	MAP		Off-odor / Off-flavor (sour)	Gram-positive bacteria (LAB)	

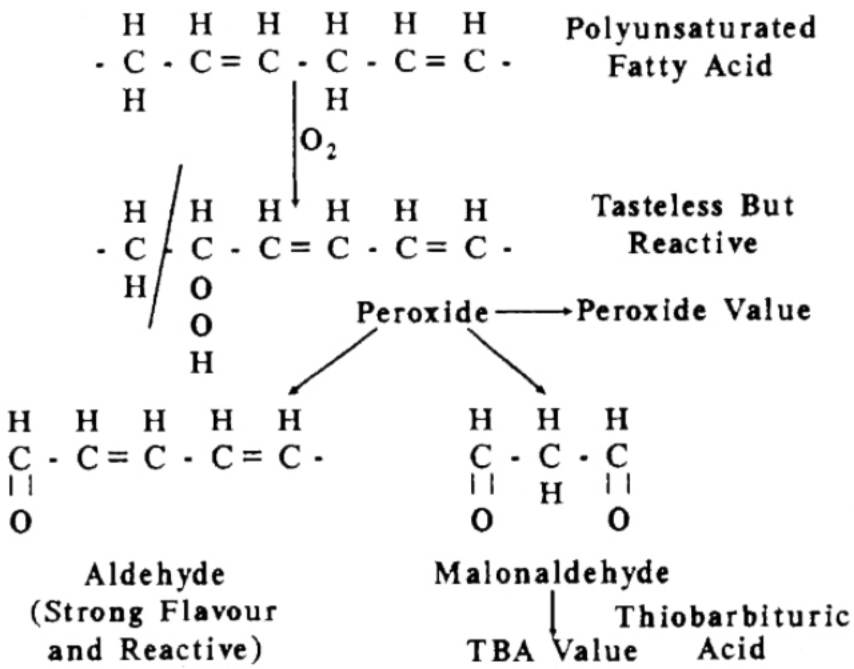
1) i.e. specific spoilage organisms which have been related to the spoilage of the product
2) LAB = Lactic Acid Bacteria

4.2.2.3. Chemical spoilage (Oxidation)

The most important chemical spoilage processes are changes taking place in the lipid fraction of the fish.

Oxidative processes, autoxidation, are a reaction involving only oxygen and unsaturated lipid. At first step it leads to formation of hydroperoxides, which are tasteless but can cause brown and yellow discoloration of the fish tissue. The degradation of hydroperoxides gives rise to formation of aldehydes and ketones as shown in Figure 25. These compounds have a strong rancid odor. Oxidation may be initiated and accelerated by heat, light (especially UV-light) and several organic and inorganic substances (e.g. Cu and Fe). Also a number of antioxidants with the opposite effect are known (alpha-tocopherol, ascorbic acid, citric acid, carotenoids).

Figure 25. Basic processes for oxidation of polyunsaturated fatty acids found in fish tissue



Using TBA (thiobarbituric acid) test to measure the malondialdehyde in a colorimetric procedure is the most common way to measure lipid oxidation of fish. Since the TBA values reaches maximum at one point and then decreases, it is very important to measure samples which are known. TBA test cannot be used to measure unknown samples.

It can be mentioned in this context that the presence of oxygen in modified atmosphere packing is not detrimental to low-fat fish. In fact, the presence of oxygen might be desirable for the frozen storage of gadoid and other white-fleshed fish (Regenstein 1991).

4.2.2.4. Autolytic spoilage

Autolytic spoilage or autolytic changes are responsible for early quality loss in fresh fish but contribute little to spoilage of chilled fish and fish products. An exception from this statement is the rapid development of off-odors and discolorations due to action of gut enzymes in un-gutted fish e.g. capelin.

However, in frozen fish the autolytic changes are of great importance. One example is the reduction of trimethylamine-oxide (TMAO), which in chilled fish is a bacterial process with formation of trimethylamine (TMA) as has been mentioned. In frozen fish, however, bacterial action is inhibited and TMAO is broken down by autolytic enzymes to dimethylamine (DMA) and formaldehyde (FA). The effect of the FA formed in frozen fish is increased denaturation of fish tissue, changes in texture and loss of water binding capacity. Other enzymatic reactions such as formation of free fatty acids are also believed to greatly influence the sensory quality of frozen fish. Autolytic enzymes are active down to -29°C, but are proceeding at a much faster rate at high, sub-zero temperatures.

4.2.2.5. Nucleotide breakdown

The breakdown of the nucleotides is often used as an indicator of fresh fish quality (Regenstein 1991). As with all flesh foods, the decrease in ATP (adenosine triphosphate) is part of the process leading to rigor mortis. First, ATP is broken down to ADP (adenosine diphosphate) and then re-made using stored high-energy phosphates, e.g. creatine phosphate. When these are used up, the ADP itself is used to produce one mole each of ATP and AMP (adenosine monophosphate). Further breakdown of AMP leads to IMP (inosine monophosphate) and I (inosine), and eventually to Hx (hypoxanthine). Subsequently the Hx may be metabolized further. Depending on the species of fish, the rate limiting enzyme step in this breakdown sequence may be either the IMP to I step, in which case IMP tend to accumulate, or the I to Hx step, in which case inosine will tend to accumulate.

IMP is actually sweet and may be an important part of the flavor associated with seafood. Hx is mildly bitter compound whose build-up can make the taste unacceptable in the later stages of shelf-life. Various chemical methods of determining the different nucleotide breakdown compounds have been proposed so that the change in these compounds might be used as a freshness indicator. One test involves a rapid color reaction using an enzyme-impregnated piece of filter paper and an extract from the fish (Jahns et al. 1976) but this technique has not been made available commercially. High pressure liquid chromatography (HPLC) has been used to detect the nucleotide breakdown compounds and their ratios in hope to evaluate fish quality accurately.

However, the question is how well these compounds can be evaluated organoleptically i.e. how well their scores correlate with sensory tests but that has not been done yet.

Hx is produced at various rates in different fishes, fastest in redfish and slowest in swordfish. Two enzymes are involved, a hydrolase that probably is autolytic and phosphorylase that probably is of bacterial origin. It has been proposed to use a test called K ratio ((I+ Hx)/total nucleotide) or K1 ratio ((I + Hx)/(IMP +I +Hx)) could be more accurate than measurements of only Hx and I (Regenstein 1991, Botta 1994). Enzyme reactor system based on K-value has been developed for measuring the quality of chicken meat (Suzuki et al. 2001). The enzymes were immobilized and the measuring reaction took 5 minutes.

4.2.2.6. Histamine

Histamine can develop during spoilage of some fish, particularly scromboid fish such as tuna and mackerel. Histamine is a breakdown product of the amino acid histidine, and fish normally have measurable amounts of this free amino acid. Histamine can cause an allergic response in some humans. The bacteria that produce histamine do not grow at proper cold-storage temperatures. The presence of histamine is a sign that the fish have been handled improperly.

4.2.3. Modified atmosphere packaging (MAP)

The main spoilage bacteria in MAP is P. phosphoreum limiting shelf-life of fresh MAP seafood's (Dalgaard (2004). The result indicates that the concentration of TMA can be used as a single compound quality index in fresh MAP cod.

CO2 is the most important gas used in MAP of fish, because of its bacteriostatic and fungistatic properties.

CO2 is highly soluble in water and fat, and the solubility increases greatly with decreased temperature. Four activity mechanisms of CO2 on micro-organisms have been identified (Dixon & Kell, 1989):

1. Alteration of cell membrane functions including effects on nutrient uptake and absorption;
2. Direct inhibition of enzymes or decreases in the rate of enzyme reactions;
3. Penetration of bacterial membranes, leading to intracellular pH changes;
4. Direct changes in the physico-chemical properties of proteins.

Probably a combination of all these activities account for the bacteriostatic effect.
Nitrogen (N2) is an inert and tasteless gas, and is mostly used as a filler gas in MAP.
Oxygen (O2) in MAP-packages of fresh fish will inhibit reduction of TMAO to TMA (Boskou & Debevere, 1997).

Photobacterium phosphoreum is widespread in the marine environment and it seems likely that this organism or other highly CO_2 resistant micro-organisms are responsible for spoilage of packed fish products (Dalgaard et al., 1993).

Increased solubility of CO_2 at lower temperatures will relatively increase the effect of MAP. The freezing points of foods are dependent on the water content in the products. Freezing points for fishery products vary from about -1 to -2.5°C, e.g. salmon, shrimp, and mackerel -2.2°C, carp -1.0°C (Rahman & Driscoll, 1994). A typical shelf-life extension of about 7 days is obtained on super-chilling fish as compared with traditional ice stored fish of the same type.

The extended shelf-life will depend on the species, fat content, initial microbial load, gas mixture, the ratio of Gas/Product, and most importantly temperature of storage. The SSO of MAP cod at 0°C has been found to be P.phosphoreum. Whether this bacterium is the general SSO for all marine fishes at different storage temperatures and under various $CO_2/N_2/O_2$ mixtures is not known.

4.2.4. Film sensors for detecting TMA vapours

Several film sensors have been reported for detecting TMA vapors like In_2O_3, TiO_2, SnO_2 and ZnO (Roy and Basu, 2004). However the sensors may not be able to detect food deterioration at room temperature because the food molecules, which are normally large in size, need higher activation energy for adsorption and for splitting leading to some chemical reactions as a result of which some free electrons are generated. These free electrons are responsible for change in resistivity or conductivity. And this change is the measure of sensitivity. High temperature is thus required. At room temperature this sensor can work as hydrogen detector.

4.2.5. Markers and concentration of markers as limit of detection and choice of seafood

Any lean white fish can be the chosen as the product to measure or shrimp could also be used as candidate for testing. Shrimp is a possible candidate as seafood for testing oxygen scavenging enzyme system, mainly because of ease to handle. The main substances that could serve as markers are TMA (trimethylamine) from degradation of TMAO and three compounds from degradation of ATP i.e. inosine monophosphate (IMP), inosine and hypoxanthine.

4.2.6. TMA

TMA in shrimp is dependent on temperature as it increases with higher storage temperature. Increased temperature means increased number of spoilage bacteria which means increased production of TMA. The production of TMA is also related to some extent the initial amount of TMAO in the fish.

Sensory score of 6 (of 10 points) for shrimps responds to 360 μmoles/100 g which equals 21 mg/100g or 5 mg TMA-N/100 g (measured as nitrogen). Usually the limit of 360 μmoles TMA or 5 mg TMA-N is used.

The level of TMA found in fresh fish rejected by sensory panels varies between fish species, but is typically around 10-15 mg TMA-N/100 g in aerobically stored fish and at a level of 30 mg TMA-N/100 g in packed cod (Dalgaard et al., 1993).

TMA has been measured with methods like colorimetric method by reaction with picric acid that gives a colored complex. The use of so called steam distillation has also been used as well as Conway micro-diffusion procedure. These methods according to Timm and Jörgensen (2002) are not accurate or specific enough. They used an electrophoresis method with a better result.

Other methods that have been used are gas chromatography, HPLC and chemiluminecence (Garcia-Garrido and Castro 1997). Wong and Gill (1987) used the enzyme TMA dehydrogenase from the bacterium Hyphomicrobium X to measure TMA with good results. Also the enzyme monooxygenase isoform 3 (FMO3) is known to change TMA and can be used to measure it (Li etal 2007). Pacquit etal (2005) developed a sensor for real-time monitoring of fish freshness. The sensor is an on-package sensor that contains a pH sensitive dye, bromocresol green, that responds through visible color change to basic volatile spoilage compounds, such as trimethylamine (TMA), ammonia (NH3) and dimethylamine (DMA) collectively known as Total Volatile Basic Nitrogen (TVB-N). Latest development in TMA detection is the use of QCM (quartz crystal microbalance) sensor (Li et al.2007) in a polyaniline plastic film.

When measuring TMA, other volatile bases in TVB (all bases are measured with TVB (total volatile base) method) must be excluded otherwise the measurement is too high. TMA measurements are most useful at later stages of spoilage when the concentration begins to rise considerably (Oehlenschläger 1998, Baixas-Nogueras 2003).

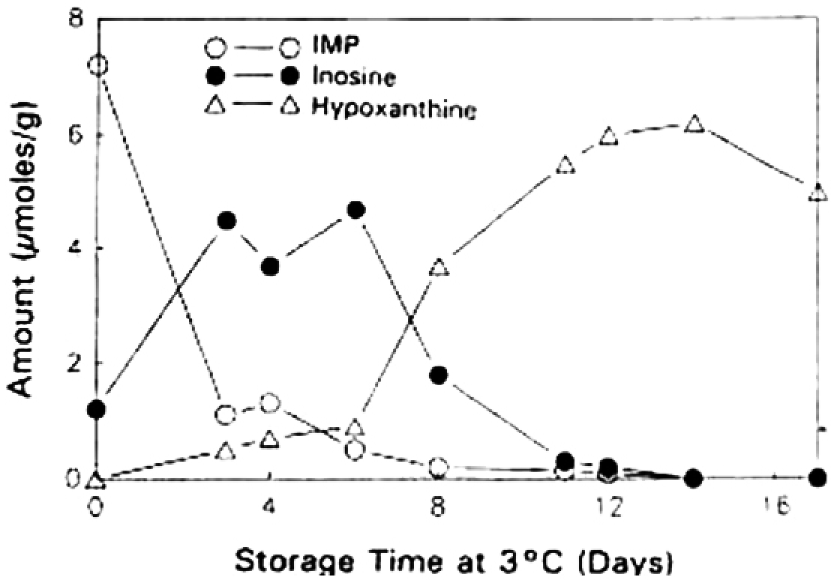
4.2.7. Nucleotides

The nucleotides inosine 5'monophosphate (IMP), inosine (INO) and hypoxantine (Hx) that are breakdown products of ATP could also be used as freshness indicators for fish or shrimps. For most species it is necessary to measure all three compounds as the variation in breakdown is great. This means that the K1 index is measured which is the ratio (INO+HX/INO+HX+IMP *100) for many species but for species like cod the K1 ratio (Hx/INO+Hx+IMP*100) is more appropriate (Park and Kim 1999). In the case of cod measuring Hx could be enough. Paterson et al (2000) has shown that to measure only IMP is not enough to estimate freshness of shrimps. According to Fatima etal (2006) there is a good correlation of IMP concentration and sensory panel score for shrimp in the beginning of storage. However hypoxanthine needs also to be measured as when the IMP is absent and the concentration of hypoxanthine is higher than two μmole/g in the shrimp muscle the quality is not good.

Variations in nucleotides depends on the storage conditions and packaging methods (fresh, iced, vacuum-packed, MAP etc.) (Özogul etal 2007).

Fish with K1 value below 20 is considered very fresh, values between 20-40 are not as fresh and the product need to be cooked and values above 40 are considered not fit for human consumption (Volpe and Mascini 1995). Usually IMP is at maximum within 24 hours after death and declines after that. Then the inosine starts to increase and after a while hypoxanthine starts to increase and inosine to decrease, as shown in Figure 26.

Figure 26. Sterile cod at 3°C; measurements of IMP, inosine and hypoxanthine during storage. (Gill 1990)



As shown in the figure for sterile cod at 3°C the IMP is almost zero at day 8, inosine is at maximum from day 3 to day 7 and hypoxanthine is at maximum at day 12-15. The start concentration (IMP, INO, HX) where 7.5 μmole/g, 1.5 μmole/g and zero μmole/g and after 8 days it was 0.2, 2 and 4 μmole/g and after 14days it was 0, 0 and 6 μmole/g. Non-sterile cod changes more quickly (Gill 1990). Another species Common carp has the initial values of 4.9, 1.4 and 0.29 μmole/g (IMP, INO, Hx). Özogul etal (2005) reported that sardines kept in MAP at 4°C had storage time of 12 days and at that time the IMP, INO and Hx were 0.2, 0.3 and 0.7 μmole/g respectively. They also reported that the hypoxanthine level increased linearly with time and thus a good indicator for freshness of vacuum and MAP packed sardines.

The detection range of hypoxanthine is 2*10-6 to 1.85 10-4 mole/L (Nakatani etal 2005) using the enzyme xanthine oxidase. Volpe and Mascini (1995) found the detection range of the three compounds IMP, INO and Hx to be in the range 1*10-6 to 2*10-5 mole/L with the detection limit of 5*10-7 mole/L (or 1-100 μmole/L with detection limit of 0,5 μmole/L). They used the enzymes nucleoside phospholylase, 5'-nucleotidase and alkaline phosphatase for IMP, INO and Hx respectively. Watanabe etal (2005) developed a sensor system based on oxidation reaction of hypoxanthine with xanthine oxidase accompanying redox reaction of thiazole blue with good results.

4.2.8. References

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control based on KI value. Biosensors and Bioelectronics 21, 534–538.

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http://www.fao.org/docrep/v7180e/V7180E0b.htm

http://people.whitman.edu/~yancey/deepsearesearch.html

4.3 Oxidation of vitamins

Vitamins undergo various degradations during storage of food. The stability of vitamins can be generalized as presented in Table 24.

Table 24. General stability (U=unstable, S=stable) of vitamins to environmental effects

Nutrient	Oxygen	Light	Temperature
Vitamin A	U	U	U
Vitamin B6	S	U	U
Vitamin B12	U	U	S
Biotin	S	S	U
Vitamin C	U	U	U
Cholin	U	S	S
Vitamin D	U	U	U
Folic acid	U	U	U
Inositol	S	S	U
Vitamin K	S	U	S
Niacin	S	S	S
Pantothenic acid	S	S	U
Riboflavin B2	S	U	U
Thiamin B1	U	S	U
Tocopherols	U	U	U

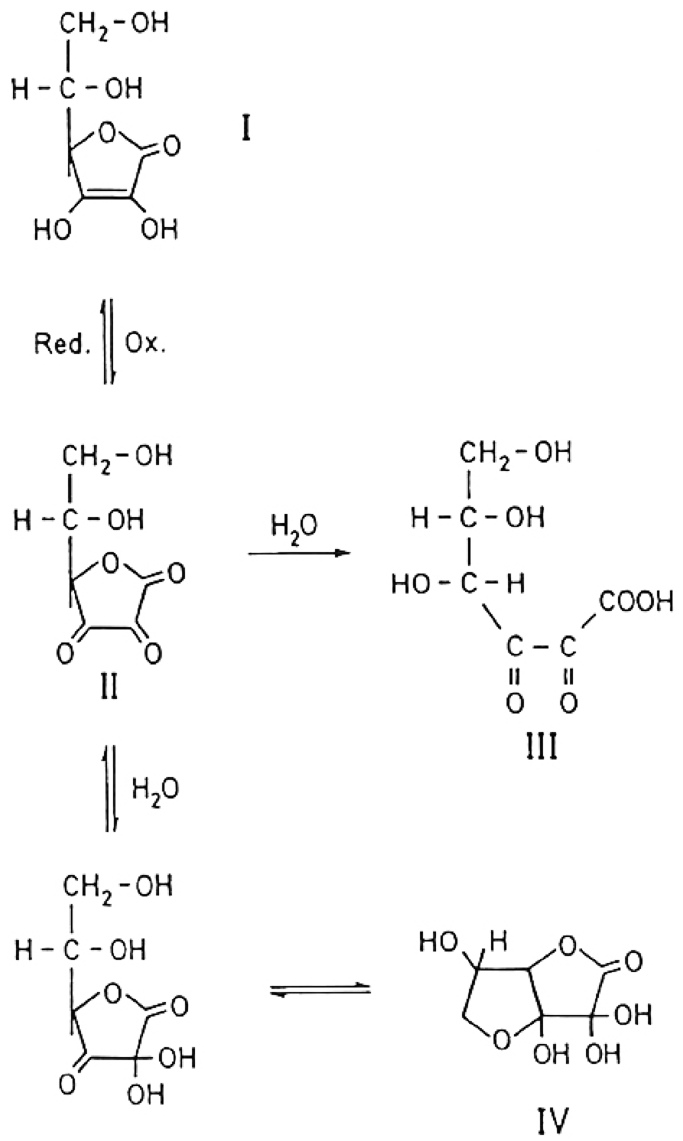
Point must be made that exceptions exist and invalid conclusions could be reached on the basis of this generalizations.

As shown in Table 24, nine vitamins are unstable in contact with oxygen. In the following documents, one of the main fat soluble vitamins and one of the main water soluble vitamins, that is vitamin A and C will be studied.

4.3.1. Vitamin C (Ascorbic acid)

Vitamin C is present in all animal and plant cells, mostly in free form. It is one of the main antioxidant in the water phase. Ascorbic acid is readily and reversibly oxidized to dehytdroascorbic acid (II), which is present in aqueous media as hydrated hemiketal (IV) (see Figure 27). The biological activity decreases by oxidation from I to II. The activity is lost when the dehydroascorbic acid lactone ring is irreversibly opened, giving rise to 2,3-diketogulonic acid (III). The oxidation of ascorbic acid depends on a number of parameters; oxygen partial pressure, pH, temperature and presence of heavy metal ions.

Figure 27. Oxidation of ascorbic acid.



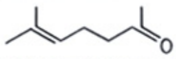
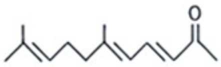
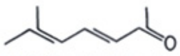
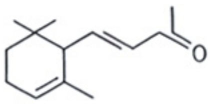
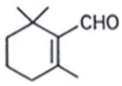
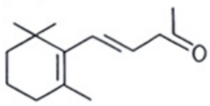
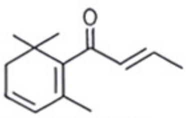
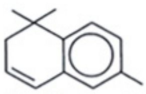
A wealth of data is available on ascorbic acid losses during preservation, storage and processing of food. Ascorbic acid degradation is often used as a general indicator of changes occurring in food, but it's the loss of ascorbic acid and not the production of degradations products that is monitored.

4.3.2. Vitamin A

This vitamin also includes pro-vitamins as carotenoids. Vitamin A occurs in animal tissue, while carotenoids occur in plants. Vitamin A and carotenoids are fat soluble. Food processing and storage can lead to 5-40 % destruction of Vitamin A and carotenoids.

In the presence of oxygen, oxidative degradation leads to a series of products (Table 25), some of which are volatile. This oxidation often parallels lipid oxidation. The rate of oxidation is influenced by oxygen partial pressure, water activity, temperature, etc. Dehydrated products are particularly sensitive to oxidative degradation.

Table 25. Aroma compounds formed in oxidative degradation of carotenoids.

Precursor[a]	Aroma compound	Odor threshold (µg/l, water)	Occurrence
Lycopene (I)	 6-Methyl-5-hepten-2-one	50	Tomato
	 Pseudo ionone	800	Tomato
Dehydrolycopene	 6-Methyl-3,5-heptadien-2-one	380	Tomato
α-Carotene (VI)	 α-Ionone	R(+): 0.5–5 S(–): 20–40	Raspberry, black tea carrots, vanilla
β-Carotene (VII)	 β-Cyclocitral	5	Tomato
	 β-Ionone	0.007	Tomato, raspberry, blackberry, passion fruit, black tea
Neoxanthin (XX)	 β-Damascenone	0.002	Tomato, coffee, black tea, wine, beer, honey, apple
	 1,2-Dihydro-1,1,6-trimethylnaphthalene	2	Wine, peach, strawberry

4.3.3. Conclusion

Oxidative degradation of vitamins is very complex. The degradation is influenced by a wide variety of factors, making prediction of degradation product to be produced very difficult. In addition, oxidation of fat soluble vitamins parallels the oxidation of fat,

which makes it more convenient to monitor fat oxidation.
This makes it difficult to find a degradation product that is suitable to use as a marker for food degradation.

4.3.4. References

Belitz, H.-D., Grosch, W. and Schieberle, P. Chapter VI, In: Food Chemistry 3rd revised version. Springer-Verlag, Berlin, 409-426

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5 WP3 Optimized packaging material and optimized packaging solutions for food applications

5.1 Introduction

Work package WP3 "Optimized packaging material and optimized packaging solution for food applications" had the purpose to make selected prototypes of packaging material with enzymatic oxygen scavengers, based on the knowledge from WP1. Furthermore shelf life test on selected food packed in packaging prototypes with enzymatic oxygen scavengers are made, and the influence of the developed oxygen scavengers on the quality of the selected foods is evaluated.

The WP3 consist of 3 sub tasks:

- ST3-1 Initial developments of packaging material. Delivery report D3
- ST3-2 Production of prototypes of packaging. Delivery reports D5 and D6
- ST3-3 Self life test of selected foods in prototypes of packaging. Delivery report D16

5.2 Materials

The composition of the cardboard and coating is given in the delivery report D3, Table 1 and Table 2 (recipe 2) in this deliverable.

The film material used was BIAXER 55 XX, a composite film of oriented polyester and barrier -polyethylene as sealant (EVOH as barrier layer) provided by WIPAK with OTR below 1 cc/m2*day.

The procedure of coating is described in the internal report by Sami Kotkamo, TUT (Dispersion coating on PE coated paperboard and PE surfaced plastic film, 25.01.2011). Coating colour for test was as follow:

1. Standard recipe (13pph glucose)
2. Recipe with 39pph glucose
3. Reference - coating without enzyme
4. Plain – no coating

5.3 Results

5.3.1. Delivery report D3

The scavenger is based on an enzymatic reaction which mean, that the reaction is dependent on humidity and temperature inside the package system. A rule-of-thumb says that the enzyme activity increases 2-3 times when the temperature rises with 10° within the interval of use and this corresponds well with the experimental measurements.

The dependency of relative humidity has been evaluated in the internal report, IR4 and shows that the relative humidity must not drop below 85% or the food may not have water activity below 0.85, in order to make the enzyme function at a reasonable speed. The OTR of the card board material has been measured. The OTR is dependent on temperature, and increases with increasing temperature for all known cases. Humidity also influences the OTR for some polymers. The OTR of card board at 5°C was measured to 2 cc/(m2*24h*1bar) and 4.7 cc/(m2*24h*1bar) at 23°C. In a brought manner the temperature dependency of the material OTR corresponds well with the lowering of the activity of the enzyme for identical temperature change.

The glucose of the coating mixture provides the enzyme with substrate to convert the oxygen. The amount of glucose will therefore settle the theoretically limit of the removal of oxygen.

Experiments showed identical results both for coating of cardboard and film material. The capacity for a glucose concentration of 1.1 g glucose /m2 was measured to be approximately 100 cc O2 NTP/m2 (internal report, Peter Togeskov, DTI). In the same experiment the conversion rate is measured to maximum 4 cc/m2*24h*bar at 23°C.

5.3.2. Delivery report D5

The film material used was BIAXER 55 XX, provided by WIPAK. OTR of plain film material was measured by MOCON OX-TRAN® 2/21 at 23°C. The results obtained were 0.5-0.7 cc/m2*day and OTR of a pack (0,014m2) was 0.09 cc/pack*day; estimated to 0.9 cc/m2 *day. This shows that the transformation of material to a pack reduces the barrier properties of the material with about 40%.

It was decided in delivery rapport D3 to work with pouch and pouch combined with a tray/paper box. The dimensions of the final box are 6cm x 12cm fitting into a pouch with dimensions 8cm x 18cm. The pouch was produced by folding 1 A4 sheet of BIAXER 55 XX one time and then sealing the material on two sides.

In order to separate liquid water (for activation of enzyme) from the biscuit the pouch contains an inner bag. This was necessary because the biscuit itself had a water activity below 0.85 as reported as critical in IR4. For the bag-in-bag prototype the inner bag was made of PE, 50μm (transparent) produced by Polyprint with no. 54456.

During the production of prototypes problems with the sealing ability was recognized. This may be caused by improper binding between the coating and the film material. Thus, the layers separated during sealing. In order to solve this problem, another bag with identical oxygen barrier as BIAXER 55 XX was used to secure the systems from leaking.

5.3.3. Delivery report D6

The efficiency of the oxygen scavengers in the prototypes was evaluated. The tested prototypes were pouch and pouch combined with a tray/paper box produced for the food experiments (as described in D5).

The enzyme was activated with liquid water to raise the RH. 0.25 g liquid water was added to the samples before sealing as a substitute for a food component.

The result of the pouch system at 23°C shows that the activity of difference coating colour differs as expected: Plane material do not remove any oxygen, the samples with glucose may remove some oxygen due to spontaneous oxidation and the samples with the most glucose remove more of the oxygen and faster.

At maximum converting rate the sample containing 39pph glucose was removing 9.5cc/day at 23°C at day 4 (for 0.047m2 material). The available glucose for this material had a capacity of removing 10.3cc oxygen in total based on calculation with complete catalase reaction. In practice the capacity is higher.

The result of the box-in-bag prototype at 5°C was almost as expected as described above. However, both samples with enzyme are performing very alike, even though one of the samples contains more glucose. The rate is not as fast as for the bag-in-bag system mainly because the storage temperature is much lower. At maximum converting rate the sample containing 13pph glucose is removing 1.2cc/day at 5°C at day 4 (for 0.0225m2 material).

For the bag-in-bag system the glucose content is the limiting factor while the temperature is the limiting factor for the box-in-bag system. Unfortunately, the humidity was not high enough though out the entire experiment, which is the explanation for the dropping activity of the enzyme after only 4 days.

5.3.4. Delivery report D16

Two foodstuffs, biscuits and fresh white minced fish, were selected in subtask 2-1 as model foods to be used in the final evaluation of the effects of the packaging materials on the quality of real food products.

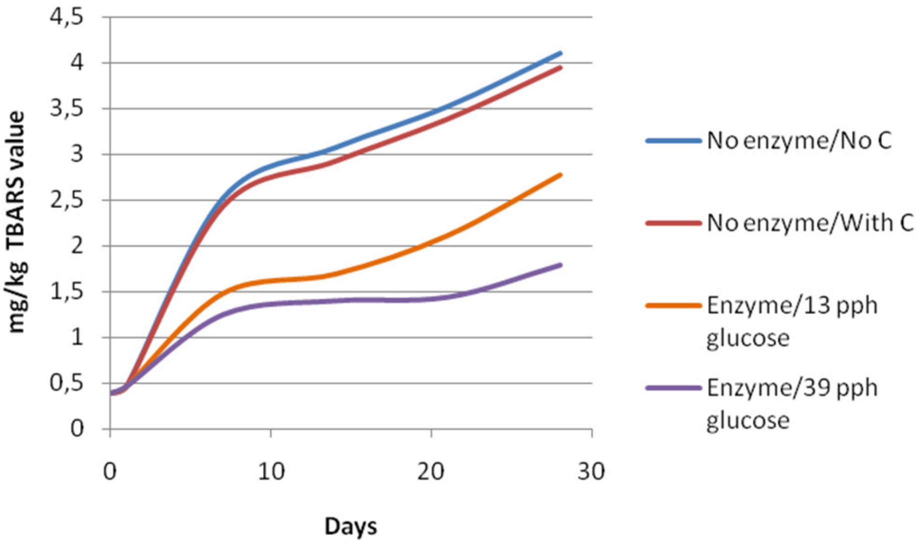
The experiment with biscuits was performed at SIK, the Swedish Institute for Food and Biotechnology. Biscuits were obtained from Göteborgs Kex AB (Kungälv, Sweden). The water activity of the biscuits was 0.32 at 23°C. In order to activate the enzymes, 0.3 ml of water was added between the inner and outer bag for raising the relative humidity in the headspace of the packaging. The samples were stored at 23°C in the dark for up to 27 weeks. Hexanal was used as a marker substance for oxidation.

The result of the experiment shows no reduction in oxygen level. A steady increase of the hexanal levels, and thereby the oxidation in the biscuits, was observed throughout the storage period. There was no significant difference between the sample types. Samples that were exposed to oxygen levels ranging from 1 to 3% became just as rancid as those that were exposed to air.

The experiment with fresh minced lumpfish was performed at ICI, Innovation Center Iceland. Lumpfish is a fish with high lipid content and therefore the TBARS method was suitable to measure rancidity. About 100 g of minced lumpfish were put in each tray and filled to the rim. The samples were vacuum packed with the bags from DTI and also with extra vacuum bag to secure good sealing. The water activity of the fish is estimated to above 0.9, therefore, no activation steps are needed to start the enzyme reaction. The samples were stored in a refrigerator at 7°C for up to 28 days.

The results of the experiment show values at 2.7-3.0 mg malondialdehyde per kg sample at day 14 for plane material and material without enzyme. The material with enzyme and 13pph glucose had 1.7 mg/kg and material with enzyme and 39 pph glucose had 1.4 mg/kg at day 14. It has been considered acceptable to have values up to 1.4 mg/kg and below 0.6 mg/kg is considered excellent. According to the TBARS measurements the packaging material is affecting the oxidation process. An extended shelf time when stored at 7°C is clearly indicated (see Figure 28). The enzyme and glucose content of the packaging material contribute to slowing down the oxidation process compared to no-enzyme material. The higher glucose content seems to make the reaction of the enzyme more effective.

Figure 28. The TBARS rancidity of samples during 28 days is shown.
The curves show the effect of trays without enzyme and trays with enzyme but with different glucose level marked as shown in the legends. The boxes were of four types; 1) no enzyme or coating material, 2) no enzyme but with coating material, 3) with coating containing enzyme and 13 pph of glucose and 4) with coating containing enzyme and 39 pph of glucose.



5.4 Discussion

The enzyme based scavenger has been proven to function. The results in D6 show promising oxygen scavenger efficiency of the material for storage temperature at 23°C. This was, however, not shown in the food experiment with biscuit mainly because the material is highly dependent on the humidity of the food to be above 0.85 aw (water activity). The rancidity of fish was reduced and shelf life extended despite the low storage temperature at 7°C. The reason for this was the very small headspace inside the pack, given small amount of oxygen to remove, as the fish was vacuum packaged.
The migration test, Internal report IR9, showed that the only material passing the migration limit of 10 mg/dm2 for aqueous simulants was the material containing a liner or top layer (of PP), as the coating colour is highly water soluble. The material passed for fatty food simulant also without top layer of PP.

6 WP4 Toxicology, health aspects and organoleptic evaluations.

6.1 Introduction

Work package WP4 "Toxicology, health aspects and organoleptic evaluations" had the purpose to test cytotoxic potential of single components, functionalized polymers or fully equipped prototypes of packaging material with enzymatic oxygen scavengers, based on the knowledge from WP1. Furthermore tests with selected packaging materials on human skin models were made to evaluate the influence on assembling and differentiation of epidermal layers of the skin. Penetration studies on the skin model and resorption studies on a vascularized small intestine model should exclude any health risk caused by the developed material. Organoleptic evaluations were carried out to investigate possible effects on taste and odour caused by the material.

The WP4 consist of 6 sub tasks:

- ST4-1 (Cyto)toxicity tests of single components. (Delivery report D1)
- ST4-2 (Cyto)toxicity tests of functionalized polymers and materials. (Delivery report D4)
- ST4-3 Toxicity and health risk analysis of selected materials (AP1 and AP2) in human skin model. (Delivery report D7)
- ST4-4 Penetration studies. (within Delivery report D7)
- ST4-5 Organoleptic evaluations. (Delivery report D12)
- ST4-6 Health risk analysis and resorption studies. (Delivery report D17 / within Delivery report D7)

6.2 ST4-1 (Cyto)toxicity tests of single components (Delivery report D1) and ST4-2 (Cyto)toxicity tests of functionalized polymers and materials (Delivery report D4)

6.2.1. Materials

During the project 57 materials, including untreated reference materials (paperboard or plastic film), single components as well as pretreated paperboards and fully equipped paperboards with linkeage of enzymes were tested for their cytotoxic potential according to DIN ISO 10993-5. Only selected materials were then investigated in the human skin model. Materials have been supplied by different cooperation partners. See the list of the tested materials in the attachment.

1. Sterilization of the samples

Samples were cut into pieces and were gamma sterilized by BBF Streilisationsservice GmbH, Kernen with 25kGy.

2. Material extraction according to DIN ISO 10993-12

Extraction was performed according to DIN ISO 10993-12 for 72h±2h, at 37°C±1°C and 5% CO2. The extraction-relationship was 6cm2/mL with material-thickness of <0.5 mm. Culture medium without serum was used as extraction medium. For each material three identical extractions were carried out. After 72h±2h of extraction time, medium was harvested, supplemented with 10% fetal calf serum (FSC) and used for the cytotoxicity test according to DIN ISO 10993-5.

3. MTS-Proliferation assay according to 10993-5

The proliferation assay was performed with the epidermal cell line HaCaT, which is derived from human skin. A defined cell number of 20.000 HaCaT cells was seeded out in a microtiterplate and a total volume of 200 µL. After the incubation time of 24h±2h, when cells adhered to the plastic, medium was replaced with the harvested extract, supplemented with 10% FCS. Cells were then incubated for another 24h±2h at 37°C±1°C and 5% CO2. Wells without cells were run as a background control. The proliferation assay with MTS solution was carried out as described in Delivery report D1 and D4.

4. Evaluation of cytotoxic potential

The cytotoxic potential of the tested materials was evaluated via controls and a cytotoxicity scale Measured absorbance is calculated as percentage of the proliferation with respect to negative control (100 % proliferation) and positive control (of 0 % proliferation).

A linear relationship of cell number and proliferation is presupposed.
The damaging effect of the test substances is assessed on the basis of the impairment of cell proliferation by the classification scale of cytotoxicity (Table 26).

Table 26. classification scale of cytotoxicity

scale of cytotoxicity	proliferation in % (rel. to control)	interpretation
0	100 - 81 %	non-cytotoxic
1	80 - 71 %	weakly cytotoxic
2	70 - 61 %	moderately cytotoxic
3	60 - 0 %	strongly cytotoxic

Cytotoxicity testing of nanoparticles or laccase on paperboard was not possible, because materials interfered with the MTS-proliferation assay. All reference materials (untreated paperboard / plastic film), free latex film, copolymer films (styrene acrylic / styrene butadiene) free or on paperboard, corona treated or PP-treated (alone or in combination) showed non cytotoxic or weakly cytotoxic potential. Enzyme-linkage to paperboards (with or without pretreatments) always resulted in strong cytotoxicity, due to the fact, that the enzymes act as oxygen-scavangers and hydrogen peroxide is formed as a product. Hydrogen peroxide itself is strongly cytotoxic for cells.

6.2.2. Discussion

The cytotoxicity test according DIN ISO 10993-5 showed that there are some pretreatments of paperboards, which are not cytotoxic and could be used as a basis for the linkage of different enzymes. Unfortunately none of the tested materials, which have been fully equipped with pretreatment and linked enzymes, could pass the cytotoxicity test.

Testing of nanoparticles might be possible, if they are coated on paperboards. The absorption measurement via MTS-proliferation assay is then not disturbed by any particles left in the extract. Extraction of Laccase-coated paperboards resulted in a dark-brown coloured extract, which could not be analysed, due to the high background. But optimization of this coating might be very promising, due to the fact of the absence of hydrogen peroxide within the system.

6.3 Toxicity and health risk analysis of selected materials (AP1 and AP2) in human skin model (Delivery report D7)

The object was to test, if package material influences the differentiation of keratinocytes and assembling of the three-dimensional, organoid skin model.

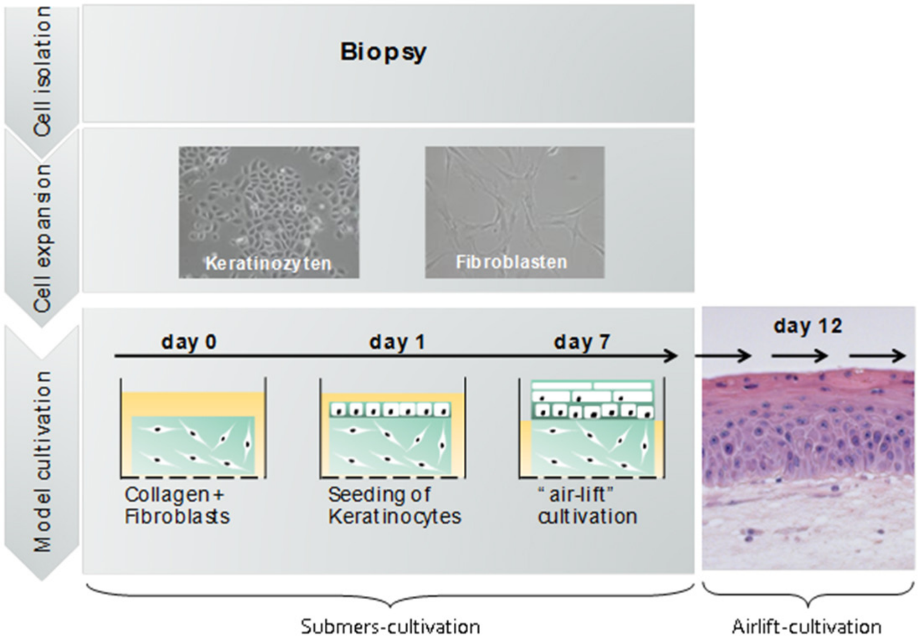
1. Sterilization of samples

   Samples of selected materials were cut into pieces with a diameter of 0.8 cm and were gamma-sterilized by BBF Sterilisationsservice GmbH, Kernen with 25 kGy.

2. Preparation of three-dimensional skin models

   Fibroblasts and keratinocytes were isolated from a human skin biopsy. Cultivation of both cell types was performed until the necessary cell number was reached. Fibroblasts were embedded in a collagen type I matrix to form the dermal part of the three-dimensional, organoid skin model. After one day of cultivation keratinocytes were seeded onto the dermis. Submers-cultivation for 7 days with reduction of serum level from 5% to 0% was followed by an airlift-cultivation of 12 days to form a well stratified epidermis (see Figure 29).

Figure 29. Generation of a three-dimensional, organoid skin model.



3. Quality control, histological staining and immuno-histological staining of skin models

Before skin models were used for the topical application of material the quality of the models was checked by histological sectioning (3 μm thickness) of one to two samples between day 7 and day 10 of airlift-cultivation.

Hematoxilin-eosin staining of the models showed a well stratified epidermis with stratum basale, stratum spinosum, stratum granulosum and stratum corneum.

The different epidermal layers, as detected in normal human skin, could be stained by using diverse antibodies, specific for the differentiation state of the epidermis. See the results of the histological staining in

All skin models, which have been used for the evaluation, had a well stratified epidermal layer proven by histological and immuno-histological staining. (Shown in Delivery report D7, figure 2A-2D and Figure 31)

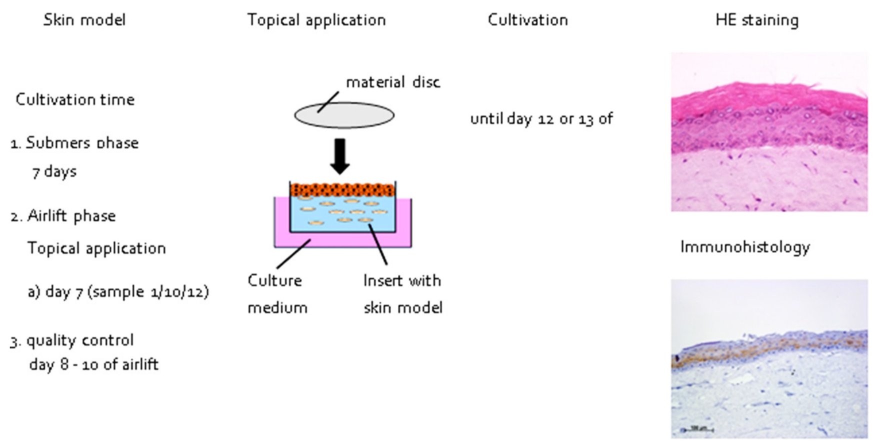
4. Topical application of selected materials

Influence of package material was tested via topical application onto the skin model. Tested materials are listed in Table 27. Figure 30 gives an overview of the testing procedure. (See as well Delivery report D7)

Table 27. Materials chosen for topical application with indication of cytotoxic potential

Sample	Modification	Cytotoxic potential	Corresponding Quality Control
Sample 1	Performa natura Barr 20+270+42EB58 (reference)	non cytotoxic (ST4-1)	Figure 2A and 2B (Delivery report D7)
Sample 10	Primal P308 AF Styrene acrylic copolymer onto board	non cytotoxic (ST4-1)	
Sample 12	HPU 70 Styrene butadiene copolymer onto board	non cytotoxic (ST4-1)	
Sample 47	no enzyme (reference)	non cytotoxic (ST4-2)	Figure 2C and 2D (Delivery report D7)
Sample 48	1:4 GOx:catalase (10.08.2011)	weakly cytotoxic (ST4-2)	

Figure 30. procedure of topical application and further cultivation of the skin model



Sample 1 was the unmodified paperboard and served as non-treated reference. Sample 10 and 12, both copolymer-treated paperboards without enzyme-linkeage, passed the cytotoxicity evaluation (ST4-2) as non cytotoxic and were therefore selected for the topical application test.

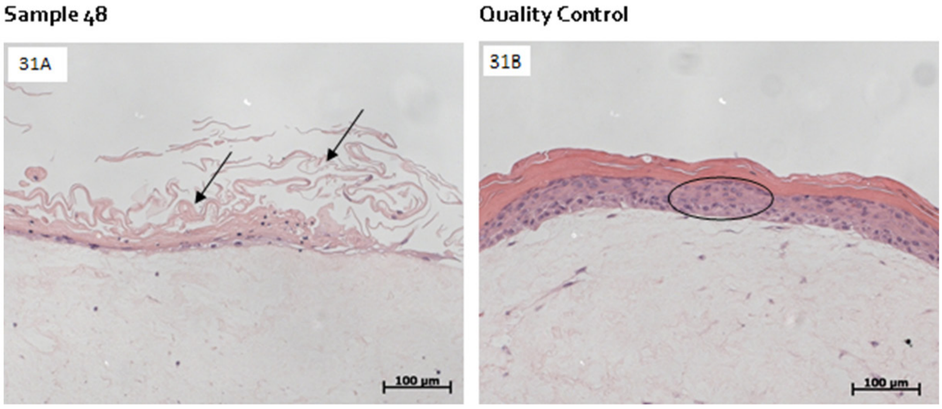
None of these materials resulted in a disturbance of keratinocyte stratification and differentiation while skin model generation.

Sample 47 was a non cytotoxic reference and sample 48 was an enzyme-linked paperboard (1:4 GOx:catalase / preparation 10.08.2011) with a weak cytotoxic potential.

5. Evaluation of material influence the differentiation of keratinocytes and assembling of the three-dimensional, organoid skin model

   Only the non treated paperboards (references / sample 1 and 47) previously determined as non cytotoxic, showed no influence on the skin model. In contrast sample 48, which was fully equipped (with enzyme-linkeage) and a weak cytotoxic potential, hardly influenced the differentiation process of keratinocytes (Figure 31A). Cells did not connect to each other as they should (indicated by arrows). In comparison to the corresponding quality control (Figure 31B), the epidermal stratification was poorly developed.

Figure 31:
HE-staining of skin model after removal of topically applicated material discs of sample 48 (31A) in comparison to HE-staining of the corresponding quality control (31B).



6.3.1. Discussion

In every case keratinocytes were able to form a perfect skin model, when no material disc was applied. This was proven by the quality controls run parallel to the material test. The epidermis was well stratified.

The paperboard without treatment (sample 1) and modifications with styrene acrylic copolymer (sample 10) or styrene butadiene copolymer (sample 12) showed no influence. Epidermis formation was as good as in the quality control. These results correspond with the findings of cytotoxic potential. The materials are suitable for further modifications like binding of enzymes, but were not investigated for penetration and resorption, because they were not fully equipped.

Sample 48, the enzyme-linked paper board (1:4 GOx:catalase / preparation 10.08.2011 was the only material which was fully equipped, but showed a weak cytotoxic potential. This cytotoxic effect can be seen in the results of the topical application test. Keratinocyte differentiation and stratification were hardly affected, that the generation of a well stratified, three-dimensional, organoid skin model was not possible.

None of the tested materials was evaluated positive in this test. Further investigations could not be carried out.

6.4 Penetration studies. (within Delivery report D7)

The object of this subtask was to investigate the penetration potential of selected package materials (non critical tested materials of ST4-2) on three-dimensional, organoid skin model in comparison with well-known test substances.

No modifications of paperboard, where enzymes have been linked to, were identified as non cytotoxic via the cytotoxicity testing according to DIN ISO 10993-5 (ST4-2). Penetration studies were not meaningful and therefore not carried out.

As discussed with the consortium cytotoxic investigations of further materials have been continued until the end of project to find a non cytotoxic, but fully equipped material to run these tests.

6.5 Organoleptic evaluations. (Delivery report D12)

Investigations of this sub task focus on, how the coated materials affect taste and odour, and to control the total volatiles, partition coefficients between different media, and other parameters. A link of the organoleptic properties to barrier properties and enzymatic activity is made.

6.5.1. Materials and methods

The evaluations were applied to two different sets of samples, which are given in Table 28.

Table 28. Materials chosen for organoleptic studies

Set 1, produced week 48, 2010			
Base	Coating	Enzymes	Sample-ID (see Attachment)
board[1]	double, 10 pph starch	yes	
		no	
board[1]	none	no	1

1 = PE coated Performa

Set 2, produced November 2nd – 8th, 2011					
Base	Coating	Enzymes	Top plastic	Abb.	Sample-ID (see Attachment)
board[2]	no	no	no	B	23
	yes	no	no	B+C	24
	yes	yes	no	B+CE	25
	yes	no	yes	B+C+PP	
	yes	yes	yes	B+CE+PP	
plastic[3]	no	no	no	P	27
	yes	no	no	P+C	28
	yes	yes	no	P+CE	29
	yes	no	yes	P+C+PP	
	yes	yes	yes	P+CE+PP	

2 = Cupforma Classic, 350 g/m2

3 = Wipak 12 μm BOPET + 40 μm PE/EVOH/PE

6.5.2. Analysis

a) Evaluation of emitted organic compounds

Sample to be analysed was cut into strips and 2.0 g were put into a 22 mL vial aimed at headspace analyses after which the vial was sealed with a gas tight septum.

For measuring volatile organic compounds emitted by the samples, the vial was heated to 100°C for 25 min, after which an aliquot of the headspace was injected into a gas chromatograph (He carrier gas) equipped with a flame ionisation detector (FID) and a mass spectrometric detector. The samples of set 1 were analysed with addition of water (wet) as well as without such an addition (dry). When applying these wet conditions, 0.3g of water and thereafter a detail of aluminium was inserted into the vial. Subsequently the board sample was placed on top of the detail in such a way that the samples strips did not come into direct contact with the water.

b) Evaluation of enzymatic activity
Evaluation was performed at Karlstad University. The sample preparation and headspace handling was analogue to the wet conditions applied at Iggesund, when measuring emitted compounds. Since the vials used at Karlstad were of another size (110 mL) the amounts of water and board sample were simply scaled up (1.5 g and 10 g respectively), to match the vial size. The concentration of oxygen was measured continuously for a time period of seven days, by applying an oxygen probe.

Duplicates of coated board samples with and without enzymes, respectively, were evaluated by applying this procedure.

c) Sensory analysis
Evaluation of off-flavour induced into test food
Off-flavour was evaluated by applying a slight modification of the EN1230-2 method according to accredited procedures. Two different test foods (ground milk chocolate and water) were used as food simulants when testing the samples of the first set, whereas only water was applied analysing the set 2 samples. In this way, the sample and water were brought into indirect contact for 48h. After this time period, the jar was opened and the test food was portioned into encoded plastic cups (water) or plastic plates (chocolate). The cups were subsequently sealed with a lid and the plates covered with Al-foil until the assessments performed by 10-14 assessors. The 4 grade scale of the method was applied and the character of the off-flavour was described when possible.

6.5.3. Results and discussion

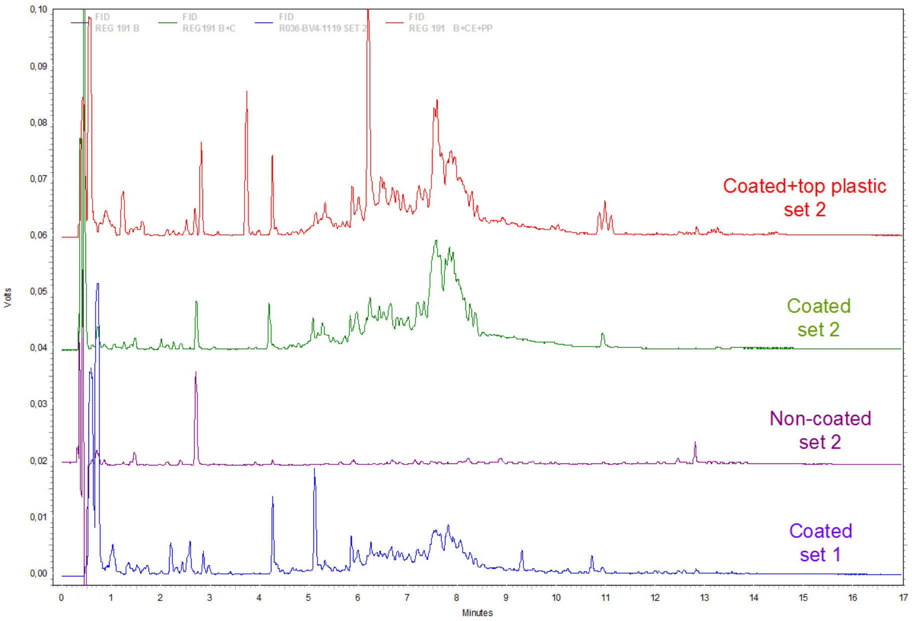
a) Emission of volatile organic compounds

Set 1
The main difference, when it comes to emitted volatile organic compounds, was attributed to the coating, whereas the water addition hardly influenced the headspace composition at all. The water addition did not effect the headspace composition of the enzyme samples. This means that the processes taking place in the enzyme activated samples, did neither produce nor consume any clearly measurable amounts of the organic volatile compounds measured by this analysis.

Set 2

The non-coated base board emitted quite a few organic volatile compounds. (Figure 32).

Figure 32. Chromatograms samples of set 1 and set 2



Worth noticing, however, is that the base board emitted considerable amounts of hexanal. The headspace composition of the coated set 2 samples, however, resembles that of the set 1 samples. The addition of the upper polypropylene (PP) layer did not change the headspace composition dramatically, and the volatiles added by the PP did probably not influence the taint and odour related properties to any larger extent.

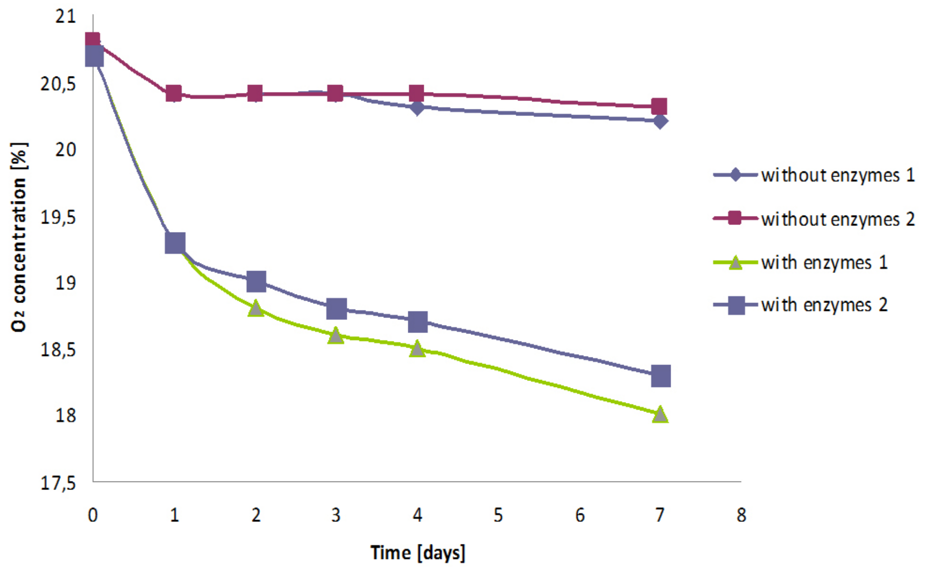
The coated (B+C) and coated + plastic (B+C+PP) samples emitted lower amounts of hexanal which might be attributed to a change of hexanal distribution due to the coating and polypropylene.

No clear alteration of the headspace composition related to the enzyme was found.

b) Enzymatic activity

Oxygen was consumed considerably upon storage, clearly verifying that the enzymes were activated at these conditions, since the comparable samples, without any enzyme activity did not consume any oxygen (Figure 33).

Figure 33: Measurement of oxygen consumption during storage



c) Sensory analysis

Sensory analysis was carried out with samples listed in Table 29.

Table 29. Set 1 samples.

Sample	Chocolate*		Water*	
	Intensity*	Description	Intensity*	Description
Base board	0,1		1,1	musty, bitter, plastic(2), sweet
With enzymes	0,7	oil, cat urine	2,0	musty, old, saw dust, plastic, varnish, ink, oil, metal
Without enzymes	0,7	cat urine	1,7	musty, plastic, oily, ink, metal

** = mean value of 11 assessors, accredited lab. 1740.*
0 - no, 1 - just noticeable, 2 - weak, 3 - clear, 4 - strong deviation from reference

It is very clear that the off-flavour found in water is more intense than that found in chocolate (Table 29). This was rather expected, since the very weak own flavour of the water makes foreign off-flavour easy to detect. Chocolate has quite a rich flavour and off-flavours are therefore harder to detect. In the same time, this also means that the odorants related to the off-flavour cannot be completely lipophilic, but also show some affinity to water.

The results indicate that the coating contributed to the off-flavour whereas no clear effects could be attributed to the enzyme activity, although a small tendency may be suggested by the water test. Due to the higher sensitivity of the water to this type of samples, water was applied as food simulant also to the set 2 samples (Table 30).

Table 30. Set 2 samples.

Sample	Without enzymes		With enzymes	
	Intensity*	Description	Intensity*	Description
Base board (B)	2,2c	plastic, ink, board(2), musty	1,1	-
Coated (B+C)	0,9c	plastic, sweet, caramell	2,0	plastic(3), shoe polish, leather, board(2), paper, musty
Coated+ plastic (B+C+PP)	1,0c	plastic, board	1,7	plastic, sweet, leather, board, paper, musty
	2,3a	plastic(6), skin, gum, board(3), ink(3), oil		plastic(4), skin, ink(2), oil, board(2), sugar
Coated+ plastic (B+C+PP)	0,1b		-	-
	0,5b		1,0b	
Coated+ plastic (P+C+PP)	0,5b		0,8b	plastic
	0,9a	plastic, iron, skin, ink	0,1a	

*accredited lab. 1740, *= mean value:*
a 14 assessors, assessed 2011-12-16
b 10 assessors, assessed 2011-12-30
c 11 assessors, assessed 2012-01-04
0 - no, 1 - just noticeable, 2 - weak, 3 - clear, 4 - strong deviation from reference.

The results were a little bit contradictory where one of the replicates of B+C and the B+C+PP sample showed unexpected low intensity of off-flavour.

Besides from these irregularities the board samples generally induced an off flavour assessed as weak by the assessors. It could possibly be related to processes indicated by the high hexanal level. In contrast, the off-flavour of the plastic based samples was all assessed as being just noticeable or lower.

Although not completely consistent, the results suggest that the enzyme activity did not induce any clear off-flavour effects.

6.6 Penetration studies. (Health risk analysis and resorption studies. (Delivery report D17)

Health risk and resorption studies in the vascularized intestine model should be carried out with selected materials, which have been positively tested in ST4-3 and ST4-4.

Because of the remaining problems of cytotoxicity (ST4-2), the lacking of materials, which do not disturb the epidermal differentiation of the skin model (ST4-3) and do not harm the skin model via penetration (ST4-4), no resorption studies in the vascularized intestine model could be accomplished.

As discussed with the consortium cytotoxic investigations of further materials have been continued until the end of project.

6.7 Attachments

Sample		cytotoxic potential
No.	name	
1	Performa natura Barr 20+270+42EB58	non cytotoxic
2	Performa natura Barr 20+270+42EB56, recipe with H2O	strongly cytotoxic
3	Performa natura Barr 20+270+42EB56, recipe negative	non cytotoxic
4	Performa natura Barr 20+270+42EB56, recipe with PBS 10mmol	strongly cytotoxic
5	Performa natura Barr 20+270+42EB56, recipe with Corona pretreatment	strongly cytotoxic
6	Performa natura Barr with Latex without enzymes (Latex=Primal P-322 AF)	weakly cytotoxic
7	free film of Latex (Primal P-322 AF)	non cytotoxic
8	Performa natura Barr 20+270+42EB56, recipe with double amount of catalase	non cytotoxic
9	Performa natura Barr 20+270+42EB56, corona pretreatment, coating ECII with enzymes and (+) D-Glucose (mixture of alpha and beta-forms)	strongly cytotoxic
10	Primal P308 AF Styrene acrylic copolymer onto board	non cytotoxic

Sample		cytotoxic potential
No.	name	
16	P.G. Laccase on board	not possible
17	Particles - NH2	not possible
18	Particles - COOH	not possible
19	Particles - surfmere	not possible
20	HPU and double amount of catalase	strongly cytotoxic
21	101 = ECII stardard recipe e.i, 100pph latex, 55pph clay, 13pph glucose, 0,974pph GOx and 0,876pph Catalase	moderately cytotoxic
22	102 = ECII stardard recipe with double amount catalase e.i. 100pph latex, 55pph clay, 13pph glucose, 0,974pph GOx and 1,751pph Catalase	strongly cytotoxic
23	reference	non-cytotoxic
24	without enzyme	non-cytotoxic
25	with enzyme	strongly cytotoxic
26	3 x Glucose	strongly cytotoxic
27	plastic reference	non-cytotoxic
28	plastic without enzyme	non-cytotoxic
29	plastic with enzyme	strongly cytotoxic
30	plastic 3 x glucose	strongly cytotoxic
31	reference	non-cytotoxic
32	1 x catalase fresh	strongly cytotoxic
33	1 x catalase over night	strongly cytotoxic
34	5 x catalase	strongly cytotoxic
35	10 x glucose	strongly cytotoxic
36	20 x glucose	strongly cytotoxic
37	no treatment	non-cytotoxic
38	no enzyme / no corona	weakly cytotoxic
39	no enzyme / corona	weakly cytotoxic
40	no enzyme / PP / no corona	weakly cytotoxic
41	no enzyme / PP / corona	weakly cytotoxic
42	with enzyme / no corona	strongly cytotoxic
43	with enzyme / PP / no corona	strongly cytotoxic
44	with enzyme / corona	strongly cytotoxic
45	with enzyme / PP / corona	strongly cytotoxic
46	corona treated paperboard	non cytotoxic
47	no enzyme	non cytotoxic
48	1:4 GOx:catalase (10.08.2011)	weakly cytotoxic
49	1:50 GOx:catalase (10.08.2011)	strongly cytotoxic

Sample		cytotoxic potential
No.	name	
50	no treatment	non cytotoxic
51	no starch	strongly cytotoxic
52	no enzyme	non cytotoxic
53	with enzyme (Sami)	strongly cytotoxic
54	50x catalase (Sami)	strongly cytotoxic
55	no enzyme (Kristin)	non cytotoxic
56	1:4 GOx:catalase (Kristin)	strongly cytotoxic
57	1:50 GOx:catalase (Kristin)	strongly cytotoxic

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ENZYCOAT II

- Enzymes embedded in barrier coatings for active packaging

This project investigated the usage of oxygen scavenging enzymes in coating formulations that can be applied onto paper, paperboard or plastic films by ordinary coating and printing units existing in the industry. Test of food quality upon storage in boxes produced with the novel Packaging materials was performed. Methods to decrease migration and to reduce the direct contact with the packed food were developed. Tests of cytotoxicity were performed and testing protocols were developed. It was demonstrated that oxygen scavenging coatings can be applied on paper and board by conventional coating machines used for high speed industrial production. It was demonstrated that a plastic liner that will hinder direct contact between packed food and the active layer can be applied by a conventional extrusion processes without damage of the active properties. It was demonstrated that the active coating can be used to hinder oxidation and rancidity reactions of packed food such as fish stored at chilled conditions.

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