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Oxygen scavenging and aroma affecting enzymes embedded in barrier coatings (ENZYCOAT)

- Need for effective and safe packaging materials that can keep the food fresh also after purchase
- An environmentally friendly technique due to easier recycling possibilities
- Barrier dispersion coatings can be applied on both plastic films and paper



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Oxygen scavenging and aroma affecting enzymes embedded in barrier coatings

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Final Report

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Fact-sheet

Title: Oxygen scavenging and aroma affecting enzymes embedded in barrier coatings		
Nordic Innovation Centre (NICE) project number: 06085		
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Abstract: The possibility to use enzymes as oxygen scavengers embedded in latex/TiO₂ dispersion coatings was demonstrated in a series of experiment where different grades of paperboard were coated. Simple draw-down coatings were used as application method. The enzyme system consisted of glucose oxidase in combination with an enzyme that removes peroxides (peroxidase/catalase). Glucose or glucose derivatives were used as substrates for the enzymatic reaction. The enzyme-containing coatings seemed to persist drying at elevated temperatures for limited period of times. The period of time with no dramatic decrease in enzyme activity was substantial shorter than drying times in real industrial coating and printing processes (on condition that the web will be cooled down before reeling up). The coatings could also be stored in air at room temperature for long period of times without any loss in enzyme activity. Oxygen absorption tests and rancidity tests revealed that an activation of the coated sheets with liquid water was needed in order to initiate the oxygen scavenging processes. The oxygen absorption tests showed that activated enzyme-containing coatings were able to decrease the oxygen level dramatically, also for a very high start concentration of oxygen (8.8% and atmosphere oxygen). The rancidity tests showed that minced ham can be kept at room temperature for extended period of times without any detectable onset of oxidation when packed in model packages containing enzyme-containing sheets, compared to reference packages without enzymes. However all rancidity tests were not consistent with this observation: Tests with an oatmeal "sponge cake" indicated no effects of the enzymes. The results clearly indicate that positive effects of the enzyme coatings did exist but further research is needed in order to understand the limitations with respect to end-use applications. Further research is also needed to enhance the window where the concept has the desired capacity to reduce oxygen concentration and prevent oxidative processes.		
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Executive summary

Main objectives

The ENZYCOAT project combined existing micro/nano technology and biotechnology in order to create innovative and commercializable solutions for food packaging. The micro/nano aspect comes from the latexes and fillers used in dispersion coatings and the biotechnology aspect comes from the enzymes (that also are materials in the nano size range). The driving force behind this development is to get more efficient packaging solutions optimized for the modern consumer pattern (i.e. prevent deteriorative and spoilage processes in food during storage and transportation).

The project has the objectives to (1) demonstrate the possibility to use dispersion coating technologies in order to create an active food packaging layer and (2) create a base for further European research application addressed to the topic of enzymes embedded in dispersion coatings for food packaging.

The ENZYCOAT project has been succeeded by the project ENZYCOAT II within the Nordic MINT II programme and the MNT ERA-Net programme. ENZYCOAT II became operative by February 2008 and will last for three years.

Background

There is a demand for more effective packaging in the European society. The driving forces behind this come from all parts of the packaging value chain. The producers need more effective materials and lean production methods in order to keep their competitiveness. Food producers need lean and effective packaging materials for food protection. The consumers need effective and safe packaging materials that can keep the food fresh also during the final storage time after purchase. An overall demand is to turn towards more environmentally friendly packaging materials and to use packaging solutions that cause less CO₂ emissions when the total emissions in the food packaging chain are summarized.

Barrier dispersion coating of packaging board is regarded as an environmentally friendly technique due to the fact that the material can be recycled without separating the coating from the paperboard. It is also a process that can be performed on site at the paper mill, adding extra value to the product. Barrier dispersion coatings can be applied on both plastic films and paper.

Enzymes for use as oxygen scavengers in order to prevent oxidation processes have been demonstrated in laboratory scale as well as in commercial products. Still the uses of enzymes in food packaging have not been optimized for large-scale production of packaging materials. In addition, the state-of-the-art concepts are in general based on enzyme-containing sachets or similar that should be placed into the package. A consumer may react negatively to any kind of foreign materials inside the food package.

Implementation

The project was divided into three different sub-projects:

SP 1: Development of a coated sheets and films.

SP 2: Tests of coated products and its ability to absorb oxygen exposed to different conditions essential to the actual food systems (prototypes of boxes and sheets).

SP 3: Clear evidences for longer shelf life.

The purpose of Sub Project 1 (SP 1) was to prepare functional coating and develop an efficient way to execute the coating process. Material to be coated was low density polyethylene (PE-LD) coated paperboard. From the production economy point of view, the most efficient solution would be the one, where coating process could be executed as on-line process. For example heat sealability of functional coatings can occasionally be poor. This often intimidates one to make certain compromises between production and effectiveness of the product.

An important task regarding the development of the sheets was the examination of the coating effectiveness. The main purpose of Sub Project 2 (SP 2) was to produce prototypes of material developed in SP1 to satisfy the packaging need of food products defined in Sub Project 3 (SP 3). One of the research tasks in SP 2 was to evaluate the sealability of enzyme-containing coated cardboard sheet against itself or against barrier polymeric material in order to use the coated sheet as part of a prototype packaging material.

In SP 3, the efficiency with which the developed prototype will retard oxidation in model foods was tested. This could be seen as a final phase in the development of an oxygen reducing packaging material, based on glucose oxidase coated on paper. Oxidative processes often determine the storage stability of foods. The development of rancidity with time is often very important even if other oxidative processes can be very relevant in some cases. We have thus chosen to follow the development of rancidity markers during storage experiments with two model foods, minced ham and oatmeal "sponge cake". Since it was not possible to produce sealable box-prototypes it was agreed to test the performance of the enzymatic preparations using enzyme coated paper sheets instead. Two different food systems were investigated in SP 3: (a) semi-dry food product with a short self life and (b) dry food product with a long self life.

Results and conclusions

It was possible to coat and dry paperboard with enzyme-containing aqueous dispersions by dispersion coating methods without substantial loss in enzymatic activity. The enzyme was active for at least three month after coating despite storage conditions. Even high temperatures for a short time did not substantially affect the activity.

Oxygen transmission rate (OTR) for the enzyme coated paperboard decreased substantially when the coatings were applied. When applied on PE-coated paperboard, the enzyme-containing coatings possessed significantly lower OTR-values than achieved by a corresponding enzyme-free reference dispersion coating. When the enzyme-containing coatings were applied on a paperboard already coated with a PE/EVOH barrier layer, the OTR values as low as $10 \text{ cm}^3/\text{m}^2 \text{ day}$ were obtained. However, these low OTR-values were also obtained for a reference dispersing coatings free of enzymes.

For reduction of oxygen in ambient atmosphere an activation process with liquid water was needed. The sample without activation with liquid water does not absorb oxygen and activation in 100% water vapour was not effective enough. The need for activation for oxygen absorption but not for oxygen transmission indicated different equilibrium conditions between absorption and transmission. The capacity of the enzyme could be sufficient for reduction of oxygen penetrating through the material also without activation, while this apparently not was the case for oxygen absorption.

When activated, the enzyme-containing coatings were proved to be able to decrease the oxygen level dramatically for a very high start concentration of oxygen (8.8% and atmosphere

oxygen). A problem appears regard different responses between samples of equal treatment. It might be related to differences of the enzyme-containing coatings, but stronger evidence point to the activation method. In practice it is not suitable to use liquid water for the activation, thus, the concept need improvement. However, the enzyme-containing coatings showed great potential for fast absorption of oxygen in conditions of room temperature and appropriate activation.

The enzyme-containing coatings did reduce the oxidation (rancidity) of packed minced pork meat. However, during the conditions used in the experiments of packed cakes, the coatings could not retard the oxidation taking place in the cakes more than control samples without enzymes.

The coatings without TiO₂ were heatsealable. However, even at high sealing temperatures, times and pressure, it produce what could be a hermetic seal, but too weak to be of practical packaging use. When TiO₂ was included no seal was obtainable.

Recommendations

Coating formulation

Only water-borne coating formulations were used in the project. The study showed that it is possible to add oxygen scavenging enzymes into a latex/mineral-based coating formulation with retained enzyme activity after the drying and film-forming process (the latex particles forms a continuous film during evaporation and drying). It was not only shown that the layer reduced the oxygen concentration in ambient liquids, it was also shown that the enzymes in the layer were active in reducing the transport of oxygen through the packaging materials.

Different layered structures were investigated. The main challenge for future research is to design concepts with (1) no migration to the food and (2) increased temperature/humidity window where the coated product still has an acceptable activity. Examples of companies that could be involved in such development work are producers of latexes, minerals and enzymes.

Production of packaging materials and boxes and pouches

In this study, the production was limited to laboratory-scaled techniques. We demonstrated that the layer could be applied on paper as well as on plastic films. The main problem in production of air-tight boxes and pouches was the poor heat sealability of the dispersion coated layers. This is a well-known problem and could be addressed by optimization of the filler (mineral) content or by changing the latex properties. Other alternatives were also discussed in the study, such as keeping the areas used for heat sealing free of the latex dispersing coating.

One key question is if the enzyme-containing layer will retain its enzyme activity after storage. In real production, there is a storage time between production of board and plastics and the formation of the packaging boxes/pouches. One has also to consider the time between production of the boxes/pouches and the filling operation in the food production chain. We could not detect any dramatic loss in activity with time. On the other hand, an activation step was needed in order to make the layers active. This could be regarded as positive or negative, the positive aspect is related to good storage stability as long as not activated.

Food producers and distribution chain

Further work is needed in order to adopt the concept to European food legalisation. This is an important issue before commercialisation. However, such investigations were beyond the scope of this project. In the project, the positive effect of preventing oxidation of minced meat was demonstrated in shelf life tests. However, the concept did not have the same positive effect on

other food products during storage. This indicates that the concept should be optimized for different classes of food and that the temperature/humidity window where the concept is working has to be expanded. Both the legalisation/safety issues and the temperature/humidity window have to be addressed in further investigations.

Consumer aspects

The consumers play a major role in development of packaging concepts. The developments in this field are to large extent pull-driven. The consumers are asking for cheap, strong, and effective packaging. Effective means very much that the packaging should do what it is expected to do. Improved capacities to prevent oxidation and food deterioration are properties that fit well into this. The future of the concept investigated in this study depends to large extent if the properties will meet these expectations.

Preface

This research report is the first report on active water-borne dispersion coatings based on nano/micro-sized mineral particles, latex and oxygen-scavenging enzymes. The project started in June 2006 and was finalized in January 2008. This report should be seen as the first report highlighting the potential of the concept. A continuation of the research will take place in the project ENZYCOAT II, which is a part of the transnational Nordic MINT Phase II programme organised by Nordic Innovation Centre in cooperation with MNT ERA-Net. We acknowledge the support from Nordic Innovation Centre and from all partners in the ENZYCOAT project.

Lars Järnström
Editor

January 2008

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1. Introduction

The main components of a paper coating colour are water, pigment and binder. The binder is usually a latex dispersion. In addition rheology modifiers, dispersing agents, and other additives are added to the colour in order to promote runnability (processability) and end-use properties. In this study enzyme (glucose oxidase) and a substrate for the enzymatic reaction were added to the wet coating colour before the colour was draw-down on the surface of liquid packaging board sheets. The enzyme should be compatible with the chemicals used in a coating mixture. Glucose oxidase is catalyzing glucose as substrate in an oxygen scavenging process. The most commonly used substrate for glucose oxidase is glucose. In addition, alkyl polyglucoside (APG) was investigated as substrate for the enzymatic reaction.

Experiments with dispersion coatings containing glucose oxidase and catalase have been made. The combined action of glucose oxidase and catalase results in the consumption of 0.5 mol of molecular oxygen for each mol of glucose consumed (*Figure 1*). Catalase prevents the accumulation of reactive hydrogen peroxide.

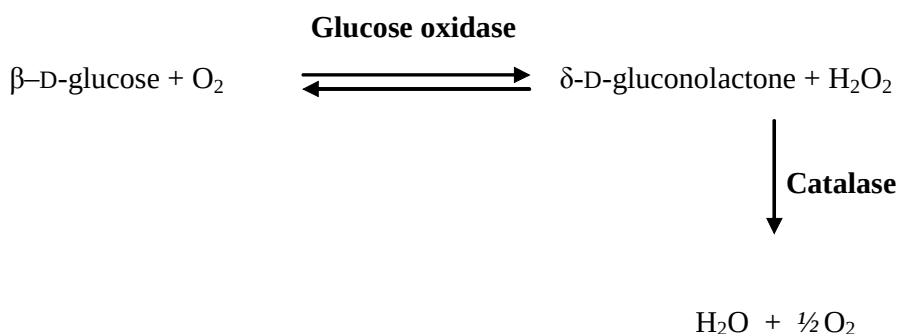


Figure 1. Reduction of oxygen to water by glucose oxidase and catalase.

Based on the reaction shown in Figure 1, this investigation has focused on enzymes as oxygen scavengers in dispersion coated packaging materials based on paperboard. The enzyme was still active also after drying of the coated board at high temperatures. However, prolonged drying was shown to cause a substantial reduction in enzymatic activity. In addition to the oxygen scavenging effects, the use of enzyme in paperboard coating resulted in improved barrier properties of the packaging material measured as lower (improved) oxygen transmission rates.

The project was divided into three different sub-projects:

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2. Materials

2.1. Enzymes

The enzymes used in the experiment had slightly different specific activities depending on the supply source and batch number. Slightly different enzymes were used in the initial optimization of coating formulation and in the laboratory scale production of coated sheets for prototype production and shelf life tests.

2.1.1. Enzymes used in initial optimization

Glucose oxidase (GOx) from *Aspergillus niger* was used in the coating material and was supplied by Novozymes A/S, Bagsvaerd, Denmark. The GOx had a specific activity of $1.15 \cdot 10^4$ U/g. Catalase from *bovine liver* with a specific activity of $1.70 \cdot 10^6$ U/g and horseradish peroxidase with a specific activity of $2.50 \cdot 10^4$ U/g, were purchased from Sigma, St Louis, MO, USA. In some parts in the investigation regarding alkyl polyglucoside, GOx from *Aspergillus niger* with a specific activity of $2.0 \cdot 10^5$ U/g was used (Sigma, St Louis, MO, USA).

2.1.2. Enzymes used in laboratory-scaled production

The coating contained two enzymes; Glucose Oxidase (GOx) and Catalase. Both enzymes were delivered by Novozymes, Denmark with following information:

- Gluzyme mono, batch OH100050 activity: $1.15 \cdot 10^4$ U/g.
- Catazyme 25 L, batch OKN03214 activity: $2.66 \cdot 10^4$ U/g.

The enzymes were stored at 2 °C when not used.

2.2. Chemicals

Titanium dioxide (TiO₂) AFDC was delivered by Kemira Pigments Oy, Finland. The TiO₂ delivered as dry powder (99.4 % solid content) was an uncoated anatase grade with particle size of 170 nm. The pH-value in aqueous suspension was 6.5-8.7. Styrene-butadiene latex (Latexia 302, denoted as L-302) was provided by Ciba Specialty Chemicals, Basel, Switzerland. The glass transition temperature (T_g) was 10°C, pH 4.5-6.5, and dry solids content in the as received latex ca. 50 % (by wt.). Sodium salt of polyacrylic acid (NaPA, trade name Dispex N40) was provided by Ciba Specialty Chemicals (Bradford, Great Britain). 2-

Amino-2-methyl-1-propanol (AMP) was obtained from KEBO Lab AB, Stockholm, Sweden. 2,2'-Azinobis(3-ethylbenzothiazolone-6-sulfonic) acid (ABTS) was purchased from Sigma. Anhydrous alpha-D(+) glucose was obtained from KEBO Lab AB (Stockholm, Sweden) and from Acros Organics (Geel, Belgium). Alkyl polyglucoside (APG, trade name ESTISURF GS40) was supplied by Esti Chem A/S, Gadstrup, Denmark..

2.3. Paperboard

2.3.1. Paperboard used in initial optimization

The paperboard sheets used in the coating initial optimization experiments were two different packaging boards delivered by Stora Enso Consumer Board. One was Performa Natura (PN), which is a one-side coated packaging board and the second one was Natura Barr (NB) which is a bleached PE/EVOH laminated liquid packaging board. Both grades of paperboard are described in *Table 1*.

Performa Natura (PN) was coated on the uncoated back side. Natura Barr (NB) was coated on the top side (i.e. the 20 g/m² PE side). Since NB has low surface tension, the top side of the board was corona-treated before coating. The board was cut into A4 sheets and corona treated by a laboratory-scaled corona equipment (Corona-Plus, Vetaphone, Kolding, Denmark), designed for the corona treatment of polymer films or paper and paperboard sheets. The corona power output was 35.2 Wmin/m² using ceramic electrodes and an aluminium roll with a perimeter speed of 1.65 m/s. The surface tension of the treated board was in the range 40 to 42 dynes/cm, as measured by Vetaphone's PRO-DYN test pens (Vetaphone, Denmark). All sheets were stored in a pile in a PE bag protected from dust, light and moisture. The time between corona treatment and subsequent coating was about 48 hours.

Table 1. The layout of the paperboards.

Layer	Performa Natura (PN)	Performa Natura 2PE (PN2PE)	Natura Barr (NB)
Top coating	Double mineral coating	Double mineral coating+ PE 12 g/m ²	PE coating 20 g/m ²
Top layer	Bleached sulphate pulp	Bleached sulphate pulp	Bleached sulphate pulp
Middle layer	Bleached sulphate pulp + bleached CTMP	Bleached sulphate pulp + bleached CTMP	Bleached sulphate pulp + bleached CTMP
Back layer	Bleached sulphate pulp	Bleached sulphate pulp	Bleached sulphate pulp
Back coating	None	PE 15 g/m ²	Multilayer extrusion coating, PE/EVOH 56 g/m ²

2.3.2. Paperboard used in laboratory-scaled production

The paperboard sheets used in the laboratory-scaled production of coated sheets for prototype production and shelf life tests were supplied by Stora Enso Consumer Board. The board has the trade name Performa Natura 2PE (here denoted as PN2PE), had a grammage of 295 g/m², and was a CTMP-based board coated on both sides with low density polyethylene (PE-LD) of coat weight 12 and 15 g/m². The PN2PE board used is described in Table 1. The board PN2PE was

cut into A4-sheets and the sheets were corona treated after production in order to achieve satisfactory adhesion between the board and the coating. All sheets were stored in piles in PE bags protected from dust, light and moisture.

3. Methods

3.1. Spectrophotometric assays for determination of substrate efficiency

These experiments were performed at Karlstad University.

Spectrophotometric assays were performed using a UV-2101PC spectrophotometer (Shimadzu, Kyoto, Japan) with a temperature control. The wavelength was 414 nm and the temperature was set to 25°C. The assay mixture is shown in *Table 2*. The total volume of assay mixture in the cuvette was 1000 μl .

Table 2. Assay mixture.

Volume (μl)	Component
250	Phosphate Buffer, 200 mM pH 5.9
584	ABTS, 0.685 mM
100	Glucose, 18 % /APG, 18 %
33	Horseradish peroxidase, 200 $\mu\text{g/ml}$
33	Glucose oxidase, 15 $\mu\text{g/ml}$

The first four assay components were added to the cuvette and allowed to temperature equilibrate in the spectrophotometer for two min before glucose oxidase was added.

The possibility to replace glucose with an alternative substrate, alkyl polyglucoside (APG), was investigated using the assay based on coupled reactions catalyzed by glucose oxidase and horseradish peroxidase (*Figure 2*). The hydrogen peroxide generated by glucose oxidase is reduced to water by the peroxidase. Concomitantly, the peroxidase oxidizes ABTS to the corresponding cation radical, $\text{ABTS}^{\bullet+}$, which has a green colour and can be quantified spectrophotometrically.

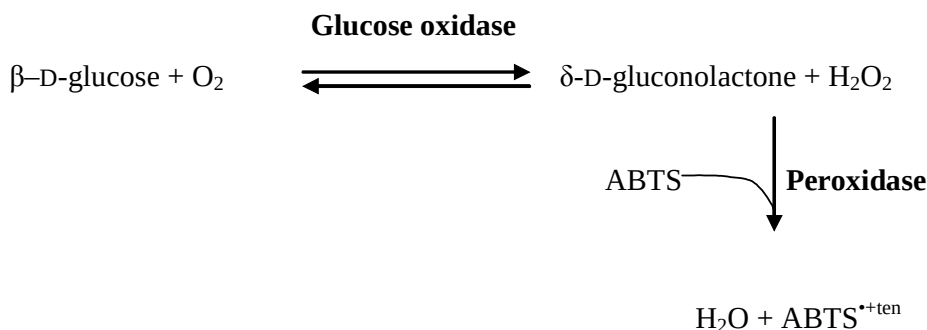


Figure 2. Reactions catalyzed by glucose oxidase and peroxidase When testing the efficiency of APG as substrate for the enzymatic reaction, $\beta\text{-D-glucose}$ was replaced by APG.

3.2. Coatings

3.2.1. Coating formulation used in initial optimization

These experiments were performed at Karlstad University.

Two different types of coating formulations were prepared based on the dispersing agent used, i.e. if NaPA or AMP was used. The first step of coating colour preparation was to prepare the TiO₂ dispersions.

In the preparation of the TiO₂ dispersion containing NaPA, the recipe shown in *Table 3* was used. The amount of water added during the dispersing action resulted in a dry solids content of ca 70 %. The added amount of NaOH corresponded to pH=6.9 in the final TiO₂ dispersion.

Table 3. Formulation of the NaPA-containing TiO₂ dispersions.

Component	Concentration pph ^a
TiO ₂	100
NaPA	0.36
NaOH	0.14

a) pph = parts (by wt.) per hundred per 100 parts of dry pigment

In the preparation of the TiO₂ dispersion containing AMP, the recipe is shown in *Table 4*. Instead of using pure water as the dispersing media a phosphate buffer (aqueous solution of Na₂HPO₄ and KH₂PO₄) of pH=4.6 was used, resulting in pH=7.1 in the final TiO₂ dispersion. The added phosphate buffer resulted in dry solids content of ca 65 %.

Table 4. Formulation of the AMP-containing TiO₂ dispersions.

Component	Concentration pph
TiO ₂	100
AMP	0.36

GOx and catalase were dissolved in 0.1 M potassium phosphate buffer (pH 6.9). The concentrations of GOx and catalase in the buffer solutions were 79 mg ml⁻¹ and 21 mg ml⁻¹, respectively. The TiO₂ dispersions were chilled on ice bath and the two enzyme solutions (GOx and catalase) were mixed with the cold TiO₂ dispersions before the latex and the substrate for the enzymatic reaction (glucose or APG) were added. The substrate was mixed with the latex and the latex/substrate mixture was subsequently added to the enzyme-containing TiO₂ dispersion. The composition of the final coating formulations are shown in *Table 5*. The TiO₂-latex dispersion was held on the ice bath until it was used for making free films or coating of paperboard. Coating formulations containing NaPA were used for coating on PN and for formations of free films. Coating formulations containing AMP were used for coating on NB.

Table 5. Final coating formulations. Added components given as % of total mass.

Amount of component % (by wt)	Components in coating formulations with NaPA	Components in coating formulations with AMP
32	TiO ₂ dispersion, (70 wt % TiO ₂ , 30 wt % H ₂ O, pH 6.9)	TiO ₂ dispersion, (65 wt % TiO ₂ , 35 wt % H ₂ O, pH 7.1)
0.3	GOx	GOx
0.1	Catalase ^a	Catalase ^a
7	Phosphate Buffer (pH 6.9) added as solvent for the enzymes	Phosphate Buffer (pH 6.9) added as solvent for the enzymes
57	Latex dispersion (50 wt % of dry latex, 50 wt % H ₂ O)	Latex dispersion (50 wt % of dry latex, 50 wt % H ₂ O)
3.5	Substrate for enzymatic reaction (glucose or APG)	Substrate for enzymatic reaction (glucose or APG)

a) Catalase from *bovine liver* with a specific activity of $1.70 \cdot 10^6$ U/g

A coating mixture without enzymes was prepared in the same way to use as a reference. The coated substrates were either dried under nitrogen atmosphere in a desiccator, or in room atmosphere.

3.2.2. Coating procedure used in initial optimization

These experiments were performed at Karlstad University.

Coatings were drawn down on the abovementioned paperboards by a bench coater (RK Control Coater, RK Print Coat Instrument Ltd., Litlington, UK). A wire-wound bar number 5 was used for the most of the coating experiments. Bars number 1 and 8 were also used for one series of experiments where the influence of coat weight on oxygen activity was investigated. In one experimental series the capacity of oxygen reduction of coated paperboard was compared to that of free films. The free films were draw down on Teflon-coated aluminium sheets and separated from the Teflon layer after film formation.

3.2.3. Coating formulation used in laboratory-scaled production

These experiments were performed at Tampere University of Technology.

The dispersing agent used in all laboratory-scaled production was AMP, i.e. no NaPA was used. In the preparation of TiO₂-containing AMP suspensions, the recipe used is shown in Table 4, i.e. the same recipe as used in the initial optimization tests. The amount of water resulted in dry solids content of ca 65 % (by wt.).

Phosphate buffer was prepared for mixing the enzymes. The composition of the buffer was 50 ml of 0.1 M potassium dihydrogen phosphate + 25.9 ml of 0.1 M NaOH (K₂HPO₄, pH 6.9). GOx and catalase were dissolved in the 0.1 M potassium phosphate buffer. TiO₂-dispersion was chilled in ice bath and the enzymes were added. Latex and glucose as a substrate for enzymatic reaction were then added. NaOH (10%) was added into acidic latex to increase the latex's pH value to ca. 7. The latex/glucose mixture was then added to the enzyme-containing TiO₂ dispersion. The composition of the final coating formulation is shown in Table 6.

Table 6. Coating formulations. Added components given as % of total mass.

Amount of component % (by wt)	Component
29 ± 1	TiO ₂ dispersion, (65 wt % TiO ₂ , 35 wt % H ₂ O, pH 7.1)
0.3	GOx
6.0	Catalase ^a
6.7	Phosphate Buffer (pH 6.9) added as solvent for the enzymes
54.4 ± 1.0	Latex dispersion (50 wt % of dry latex, 50 wt % H ₂ O)
4 ± 0.5	Substrate for enzymatic reaction (glucose or APG)

a) Catzyme 25 L, specific activity $2.66 \cdot 10^4$ U/g

Original plan was to use 0.1 wt % of catalase. Problems occurred however, because catalase previously used in the initial optimization tests (Table 5) had different activity ($1.70 \cdot 10^6$ U/g) compared to current catalase ($2.66 \cdot 10^4$ U/g). The new amount of catalase to be added to 100 g of coating colour was calculated as:

$$\text{Mass of catalase (g)} = 0.1 \times \frac{1.70 \cdot 10^6}{2.66 \cdot 10^4} = 6.4$$

Thus, the added amount of catalase to 100 g of coating colour was 6.4 g, corresponding to a concentration of 6.0 wt % of catalase in the final colour used in the laboratory-scaled production.

The final latex/TiO₂-dispersion was held in ice bath until coating was applied on the sheets. The adhesion between coating-dispersion and paperboard was on a satisfying level and no corona treatment was needed before coating process.

3.2.4. Coating procedure used in laboratory-scaled production

These experiments were performed at Tampere University of Technology.

Even that the main target is to develop a coating which scavenges oxygen inside of the package, the protection function is still not the only function that a coating has. It occurred that coated paperboard could not be heat sealed properly. It was concluded that the package produced must not contain any coating on the sealing areas. Roll-typed board would then require that in certain areas on sheets would not contain any coating. This kind of production would then require roller with covered areas. Also the size of the tray (which was original prototype plan) would have made its own limitations to the roller. As a solution, it was decided to use stripes cut from coated paper board sheets. These stripes would then be placed on pouches or trays, which then could be heat sealed properly.

The coating was done by using a bench coater (RK Control Coater, RK Print Coat Instruments Ltd, UK), which is shown in *Figure 3*.

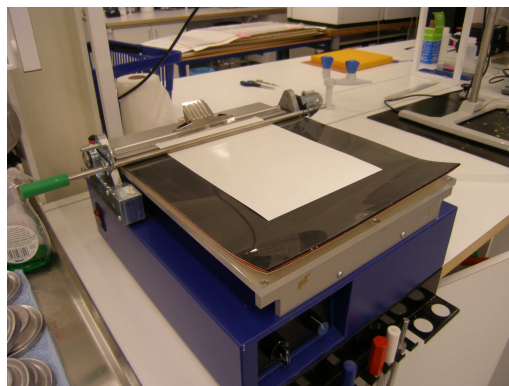


Figure 3. Coating was done by using RK Control Coater.

A wire-wound bar number 3 was used (wire diameter 0,31 mm). Coating process was executed with constant speed and pressure, so layer achieved has quite constant thickness. The thickness (dry) on the samples varied between 16 and 18 micrometers. The samples were studied with a microscope (Zeiss Axioskop 40) to make sure that coating layer is constant all over the sheets.

150 sheets were coated with dispersion which contained enzymes. In addition to that, 150 reference samples were made. Reference samples were coated by using similar dispersion, only without using any enzymes. Reference sheets were dried and cooled also similarly.

The sheets were dried in an oven by using temperature of 120°C. Every sheet was dried approximately 20 seconds and cooled off on a table into room temperature. After cooling off, the sheets were packed in bags. The effect of oxygen on the enzymes was known to be critical, so oxygen contamination had to be minimized during the transportation. As a solution, metallized (aluminium) bags with sealing mechanism (Armeka engineering, Finland) were used in transportation. Cooled sheets were packed in bags, by placing 10 sheets per bag. The bags were then flushed with nitrogen, which replaced most of the oxygen. In addition to the sealing mechanism, the openings of the bags were sealed with tape. It is presumable that oxygen did not have nearly any affect to the sheets during transportation.

The enzyme-containing board coated in laboratory-scaled production is denoted as Enzysheets and the process is denoted as enzycoating.

3.3. Critical pigment volume concentration

These experiments were performed at Tampere University of Technology.

Critical pigment volume concentration (CPVC) is the point where there is just enough binder to surround all the pigment particles. Above CPVC coating becomes porous and permeability of coating increases dramatically. It may be needed to create porous films in order to increase the availability of the enzymes as oxygen scavengers.

Permeability of coating was tested by means of Water vapour transmission rate (WVTR). The test was done by the cup method (ASTM E-96) where CaCl_2 is put inside the aluminium cup and the sample of 50 cm² is placed on top of cup and sealed with wax. The cups are placed in controlled conditions and weight gain is measured. Conditions were 75% RH and 25°C.

In CPVC tests the bar number 3 was used and coated sheets were dried for 20 seconds at 120°C.

3.4. Oxygen consumption

These experiments were performed at Karlstad University.

The enzyme activity in the coating material was measured by using a Hansatech oxygen electrode (Hansatech Instruments Limited, Norfolk, England). The temperature of the sample was kept at 25°C. A sample of 1 cm times 2 cm was cut and added to 3 ml of phosphate buffer (pH 7) containing 100 mM glucose. In experiments where effects of APG were investigated, APG was used instead of glucose in the buffer-substrate mixture. Dithionite was used for measuring the concentration of O₂ in the buffer. Dithionite is consuming all the O₂ present and a rapid decrease in the signal occurs. The decrease in signal is proportional to the decrease in O₂-concentration. The saturated oxygen content in water in contact with air is 0.253 µmol/ml at 25 °C and standard atmosphere pressure [System Manual Hansatech, 2000]. This value was used to calculate the activity of the samples. The decrease in oxygen concentration per minute was calculated.

3.5. Oxygen transmission rate

These experiments were performed at Karlstad University.

The oxygen transmission rate on coated board was measured at 50% RH and 23°C and 760 mm Hg according to ASTM D3985-05, using an Ox Tran oxygen permeability tester (MOCON OX-TRAN 2/21, Minneapolis, Minnesota, USA). All the samples were masked with aluminium foil to decrease the sample area and also to prevent leakage of oxygen at the edges due to porous material. The sample area was 5 cm². The sample was conditioned at 23°C and 50%RH for four hours before the testing started. Two replicates of each coating were investigated.

3.6. Sealability tests

These experiments were performed at Norconserv.

Enzycoated A4 sheets were produced with or without 38% TiO₂ with Latexia 302 styrene-butadiene coating on paperboard NB (supplied by Stora Enso Consumer Board) as described in chapters 3.2.3 and 3.2.4, and oriented polypropylene (OPP) coated samples with or without corona treatment.

Sealability tests were carried out using laboratory sealing equipment (Brugger Feinmechanik HSC-C, Munich, DE) with the ability to control seal time, temperature, pressure, one or two sided heating, and seal bar profiles.

Sealability against the coated material and against polypropylene (PET12 AlOX/OPA15/OPP 100µ, Elag, Kirchberg, CH) was tested at sealing temperatures from 150 to 230 °C, sealing times from 0.5 to 6 s, sealing pressures from 200 to 600 N (sealing area 10 * 120 mm). Seal strength evaluated according to ASTM F88/05 with tensile strength/texture analyzer, and seal quality by airborne ultrasonic imaging (Seal-Scan PTI-525 Airborne Ultrasonic Inspection, PTI, Tuckahoe, NY).

3.7. OxySense® 4000B

These experiments were performed at Danish Technological Institute.

The OxySense® 4000B is a non-invasive oxygen determination instrument for the partial pressure of (dissolved) oxygen that is based on the effect of oxygen on fluorescence lifetime of an optical excited Ruthenium complex immobilized in a highly stable polymer.

The OxySense utilizes an optical methodology that determines the oxygen concentration within a sealed package by measuring the fluorescence generated upon illumination of an oxygen-sensitive film (OxyDot[®]). The OxyDot is manually attached to the inside the barrier coating of the packaging material.

Specification of the OxySense technique: Measurement range oxygen 0-300 mbar partial pressure; Detection limits 0.03% in air; Accuracy 5% of reading; and Temperature range 0°C - 90°C.

Each sample was placed in separate glass jars of 100 ml. The oxygen concentration of the headspace of the glasses was measured with the OxySense system (The glass jars were tightened with a rubber ring between the lid and the container. The lid contain two valves.

3.8. Design of oxygen absorption experiments

These experiments were performed at Danish Technological Institute.

The coated board prepared as described in Sections 3.2.3 and 3.2.4 is here denoted as Enzy sheets. In order to investigate their ability of absorbing oxygen, the following experimental design was carried out:

- a. Variation in humidity; 100% water vapour and liquid water
- b. Variation in amount of Enzy sheet material; 70 cm² and 100 cm²
- c. Variation of temperature; 5°C and 23°C
- d. Variation in start oxygen level; 1%, 8.8% oxygen and atmosphere

Before the experiment started, the OxyDot was placed in the glass jar with use of silicone glue and dried for 24 hours.

The Enzy sheet was cut in the strips of 6cm x 11.7 cm (in three pieces) for an area of 70 cm² and 6 cm x 16.7 cm (in three pieces) to provide an area of 100 cm². Each strip of material was cut in three pieces in order to fit the glass jar of 100 ml volume.

The 100% water vapour was produced by filling a small container with water inside the sample, without direct contact between the Enzy sheet and the water.

The sample with direct contact with liquid water was produced by dipping the Enzy sheet for approximate 1 second in liquid water before placed in the glass jar. Approximately 5 ml water was added in the bottom of the glass jar with a pipette.

When the Enzy sheet was placed in the glass jar it was properly fastened and the air was replaced with the chosen gas mixture (1%, 8.8% oxygen or atmosphere) through the valves. The oxygen level inside the glass jar was controlled before storage at the chosen temperature (5°C or 23°C).

3.9. Rancidity tests

3.9.1. Cake

These experiments were performed at SIK and Norconserv.

Oatmeal cakes were baked according to the following recipe for 5 cakes/1.5L trays;

0.5 L oatmeal supplied directly from the mill by Nordmills.

1 spoon of baking powder

0.5 L of sugar

11 eggs
0.5 spoon of sodium benzoate

Eggs and sugar was mixed and battered. After mixing with oatmeal and baking powder the cake mixture was put in 1.5 L trays and baked at 175 °C for 30 minutes.

After baking, and cooling the cakes were withdrawn from the trays and kept over night until cutting and packaging.

A piece of cake (38 – 42g) was placed in a plastic tray together with strips of enzyme coated paper. The strips of paper were placed around the edges of the cake piece, either in the tray or just outside, with the coated side facing the cake. The total area of Enzysheet was 350 cm². Before being placed in the trays the Enzysheet strips were activated by quickly immersing them in water. Cake pieces with uncoated paper were used as reference.

Cakes, trays and paper strips were placed in plastic bags (with an OTR of 15 cc/m² day atm at 23°C). The bags were evacuated and filled with nitrogen before being sealed.

The exact weight of the cakes and the concentration of residual oxygen were measured for each sample before the storage experiment was started. Oxygen concentration was also measured at each sampling time using Dansensor equipment.

The cake samples were stored under constant light at 23°C (at SIK) and 37°C (at Norconserv) and triplicate samples of enzymed and reference samples were withdrawn at intervals during storage. The 37°C samples were stored for a total of 27 days and the 23°C samples were stored for a total of 69 days.

Dynamic headspace analysis of hexanal concentration was chosen to measure lipid oxidation in the cake samples.

At regular intervals triplicate samples of enzymed and reference samples were withdrawn from storage and the oxygen concentration within the packages was measured. The samples were stored at -70°C until determination of hexanal concentration. The samples stored at 37°C by Norconserv were shipped to SIK and frozen in -70°C before analysis of hexanal.

Hexanal concentration was determined by placing the cake material in closable 500 ml vessels. After equilibration of volatiles was established (30 minutes at 35°C) a total gas (helium) volume of 1 L was passed through the vessels onto Tenax material where the volatiles were collected. Thermal desorption from the absorbent material was at 250 °C. The volatiles were separated by gas chromatography and detection was with both FID and mass spectrometry. The peak area of hexanal was quantified using the mass fragment 44 with samples of known hexanal concentration as reference to calculate the concentration in ng hexanal per L of sampled gas.

3.9.2. Minced ham

These experiments were performed at the Innovation Centre of Iceland

The experiments were done on fresh minced pork meat that was approximately 50% fat.

The minced meat samples were put in aluminium trays containing about 50g samples filled to the rim. The samples were vacuum packed, in thick (0.16mm) PA/LDPE bags, but were filled with nitrogen gas before they were vacuumed. Half of the samples contained about 75 cm² of Enzysheet each and half of the samples contained reference sheet without enzymes. There were two samples for each day of measurement and three measurements for each sample. The

samples were stored at room temperature and with a 100W light shining on the samples the whole time.

The rancidity was tested by TBARS method where malon dialdehyde, an oxidation product in meat, reacts with 2-thiobarbituric acid to form a reddish compound that is measured at 538 nm in a spectrophotometer.

4. Results and discussions

4.1. Critical pigment volume concentration

Determination of CPVC was done in two phases. The first phase was for rough determination and was done with next concentrations: 25, 30, 35, 40, 45 and 50 V/V. Rough determination indicated that CPVC was between 35 and 40 V/V. The second phase was done with concentrations between 35 and 40 and just in case with 41 V/V. All coating compositions of two phases are shown in *Table 7*. 0.1% of AMP of TiO₂ (m/m) was used as dispersing agent.

Table 7. Coating compositions for CPVC tests and corresponding WVTR values.

Sample	L-302 (volume/mass)	TiO ₂ (AFDC) (Dry) (volume/mass)	Water ml	WVTR g/m ² /d
(L-302)	100%	-	-	51.1
(25%V/V)	1000ml/1030g	161.8ml/631.1g	270.5	55.6
(30%V/V)	1000ml/1030g	208.0ml/811.4g	347.7	50.0
(35%V/V)	1000ml/1030g	261.4ml/1019.4g	436.9	53.9
(36%V/V)	1000ml/1030g	273.1ml/631.1g	453.4	54.5
(37%V/V)	1000ml/1030g	285.1ml/631.1g	476.5	56.2
(38%V/V)	1000ml/1030g	297.5ml/811.4g	497.3	57.5
(39%V/V)	1000ml/1030g	310.4ml/1019.4g	518.7	74.1
(40%V/V)	1000ml/1030g	323.6ml/1262.1g	540.9	87.0
(41%V/V)	1000ml/1030g	337.3ml/1549.0g	563.8	103.6
(45%V/V)	1000ml/1030g	397.2ml/1549.0g	963.9	181.0
(50%V/V)	1000ml/1030g	485.4ml/1893.2g	1211.4	538.9

Between 38% and 39% is the first dramatic change in permeability as shown in *Figure4*. According to these results CPVC is 38 %.

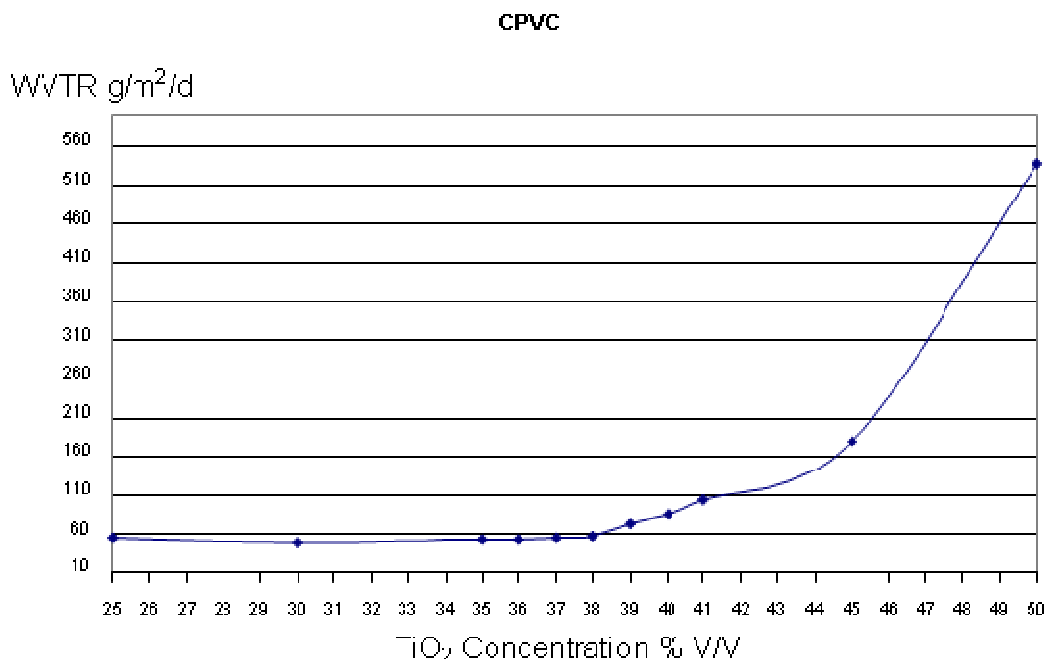


Figure 4. WVTR vs. TiO₂ concentration.

4.2. Enzyme activity

An important parameter for the enzymatic activity is the time scale for retained activity. A experimental design with two different storage procedures was organised, see *Figure 5*. One coated paperboard was stored in room atmosphere and one sample was stored in a desiccator with N₂ atmosphere. The average coat weight of all samples was 24.1 ± 2 g/m². The enzyme was still active after about three month for both samples. The sample stored in room atmosphere seemed to have a retained activity after three months. The sample stored in N₂ seemed to still have a retained activity after three months and more. Both samples indicated the possibility of a slight increase in enzymatic activity with time. One explanation could be that the enzyme becomes more available with time due to diffusion to surface. Another explanation is aging effects of the film itself. In particular films stored above the minimum film forming temperature (MFFT) changes the film structure over time [Zohrehvand S., te Nijenhuis K, 2006]. Figure 5 indicates that it may be beneficial for the enzyme-containing sample to be stored in an oxygen free atmosphere. The differences between the samples stored at the two storage conditions did fluctuate and no strong conclusion can be made about the optimum storage conditions.

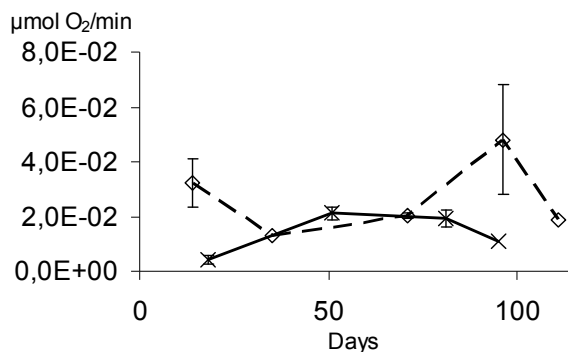


Figure 5. Oxygen consumption vs. days after application of NaPA-containing colours draw down on PN. The solid line shows samples that have been stored in room atmosphere and the dashed line shows samples that have been stored in N₂ atmosphere. Error bars indicate standard deviation.

One other important factor is the drying conditions. During a paper coating operation the paper surface can attain rather high temperatures in the drying section. The relative humidity is also rather high for a short time. The enzyme is denaturing over 35°C. Therefore different drying conditions were investigated (temperature, relative humidity and time), see Figure 6. A temperature of 23°C and a RH of 30% give the highest enzyme activity of $8.5 \cdot 10^{-2}$ μmol O₂/min. If the temperature is 50°C and RH 50% or 23°C and RH 80% or even 105°C and RH 0% they all gave the same enzyme activity of about $5 \cdot 10^{-2}$ μmol O₂/min. Drying at 85°C, 50 % RH and 0.5 h destroyed the enzyme (the enzyme has been denaturated) and the oxygen consumption dropped to zero. The retained activity at even higher temperatures but very short times indicates that the drying time may be more severe than the temperature. In the production process of coated paperboard at modern paper mill the time in the drying section is in the order of a few seconds and the maximum temperature of the coating layer is below 100°C. However, the temperature in the jumbo reel after the winding section may be high (temperatures up to 75°C is not unrealistic) for several hours if not the paper has passed over a chilling role or similar. The coat weights in Figure 6 were 29.7 ± 2.9 g/m².

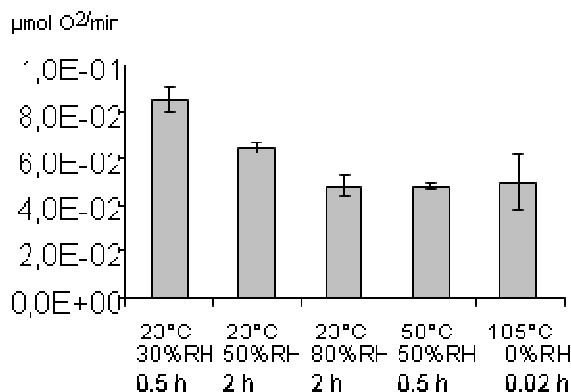


Figure 6. Enzyme activity at different drying conditions. Error bars indicate standard deviation of 3-4 samples. NaPA-containing colours coated on PN.

The results from coating on NB were compared with those obtained from coating on PN. When the samples were dried in room atmosphere or at 105°C they showed a higher enzyme activity for NB than for PN, see Figure 7. All samples were single coated and the average coat weight was $27.7 \pm 2.3 \text{ g/m}^2$.

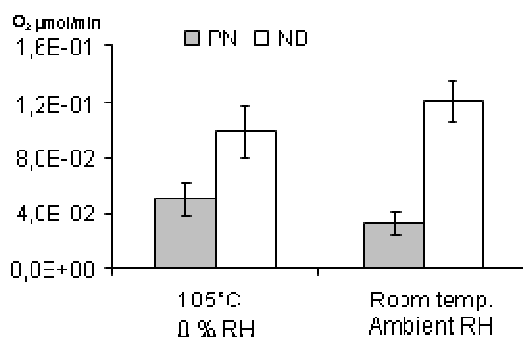


Figure 7. Enzyme activity at two different drying conditions and on two different paperboards. NaPA-containing coating on PN and AMP-containing coating on NB.

This observation in Figure 7 may be explained as an effect of the different paper board. Since NB is precoated with PE the coating holdout was higher for NB than for PN. This probably gave the enzyme higher exposure to the atmosphere and by that increased enzyme activity. Coating on the porous surface of the uncoated side of PN could involve some degree of penetration of coating material into the paperboard. The differences observed in Figure 7 may also indicate that the enzyme has higher activity when AMP was used as dispersing agent compared to when Dispex N40 was used.

In order to investigate further if the enzyme could be affected by the paperboard, an additional series of experiment was performed where the paper was coated up to three times (Figure 8 a-b). In Figure 8a, PN board was used and three different coating bars were used. The symbol *E* in the figure stands for coating layer with enzyme and *P* for coating layer without enzyme (enzyme-free phosphate buffer was used). The first letter in the name of the multilayer structure symbolizes the first coating layer and the last letter symbolizes the top layer. As expected, the lowest enzyme activity (in the range from $3 \cdot 10^{-2} \text{ μmol O}_2/\text{min}$ to $5 \cdot 10^{-2} \text{ μmol O}_2/\text{min}$ for bars No. 1 to 8) was observed when *E* was the first layer in direct contact with the paperboard and covered by a top coating of *P* (this coating sequence is denoted as *EP*). When the enzyme was imbedded in *P* (as in *PEP*), both bar No. 1 and bar No. 5 showed a higher activity than for (*EP*), bar 8 gave almost the same activity as for *EP*. When bar 8 was used it was difficult to achieve an even coating layer since the layers became thick. The results indicated that the oxygen absorbing rate was higher when a protective layer was applied between the board and the enzyme-containing layer. Figure 8a clearly shows that the activity is highest when the enzyme-containing layer was the outer layer.

Figure 8b indicates that the enzymatic activity was not depending on the thickness of the coatings. This implies that the enzymatic activity comes from the very top region of the coatings and the higher the surface area, the higher the oxygen consumption. Figure 8a indicates that a higher rod number should give increase activity. However, this was probably not due to the increased thickness but instead most likely to the increase in roughness and specific surface area that took place as an effect of the larger diameter of the wire on the rods. In addition, the increase in coat weight when going from single to double or triple coating on NB did not result in a corresponding

increase in enzymatic activity (Figure 8b). This indicated that the bulk of the coating is merely acting as a reservoir for enzymes and substrates that in a long-term process can migrate to the surface and that the enzyme activity comes from the uppermost surface.

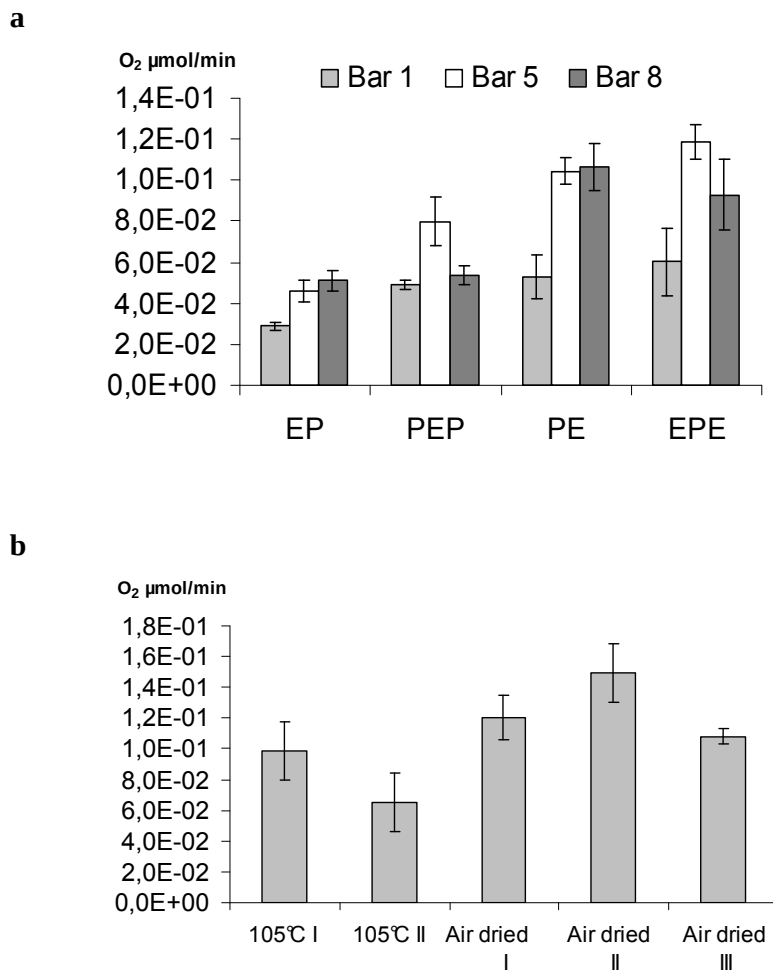


Figure 8. Enzymatic activity of samples with different coat weights.

a) Oxygen activity of PN with layered coatings of enzyme-containing coatings (E) and enzyme-free coatings (P). The coatings sequences were repeated with three different bars, where the highest number gave the thickest layer. The coating bar was the same for each layer in a particular coating sequence. NaPA-containing coatings.

b) NB coated with different amount of layers of enzyme containing coatings and prepared at different temperatures. I indicates single coating, II indicates double coating, and III indicate triple coating. AMP-containing layers.

4.3. Oxygen transmission rate

The oxygen transmission rate of the samples varied with type of base paper and drying temperature during coating process. In Figure 9, OTR for different strategies of the manufacturing of coating colour and drying of the coatings are shown. All samples were coated on PN. OTR for uncoated PN was $> 16.000 \text{ cm}^3/\text{m}^2 \text{ day}$. If the paper board was coated without any enzyme, OTR was decreased to about $10.000 \text{ cm}^3/\text{m}^2 \text{ day}$ (Samples D and E). OTR decreased if enzyme was added to the coating colour and if the coating was dried at room

temperature and room humidity (Sample C). OTR was further decreased when the coating colour was held at an ice bath before coating and then dried in N_2 atmosphere at $23^\circ C$ (Sample A). However, the lowest OTR value ($300\text{ cm}^3/\text{m}^2\text{ day}$) was observed when the coating was dried in an oven at $105^\circ C$ (Sample B). Samples D and E show the barrier improvement by the dispersing coating itself. It is obvious that the enzyme coating gave an extra improvement of the barrier properties.

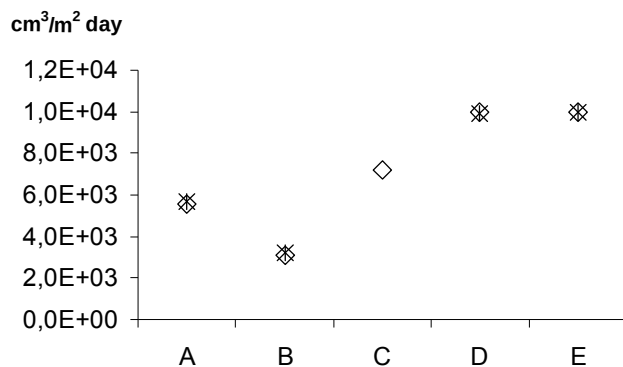


Figure 9. OTR for different manufacture principles of NaPA-containing coating colours and drying temperature coated on PN. A) with enzyme, ice bath, dried N_2 , B) with enzyme dried $105^\circ C$ one minute, C) with enzyme dried in air at room temperature, D) without enzyme dried in N_2 at $23^\circ C$, and E) without enzyme dried in $105^\circ C$ one minute. OTR measured at 50 % RH and $23^\circ C$.

When the relative humidity was held constant, slightly decreasing OTR-values were observed with increasing temperatures. In Figure 10 it is shown that OTR was lowest for $85^\circ C$ of the three drying temperatures investigated. OTR values of about 300 and $850\text{ cm}^3/\text{m}^2\text{ day}$ was obtained at the highest temperature, indicating the same trend as Figure 9 above. In general, the OTR values was higher when the layers were dried at 50 % RH compared to when dried at lower relative humidity (room temperature at ambient conditions or in air at $105^\circ C$). This may be an effect of the latex film formation process resulting in less efficient barriers formed at high relative humidity.

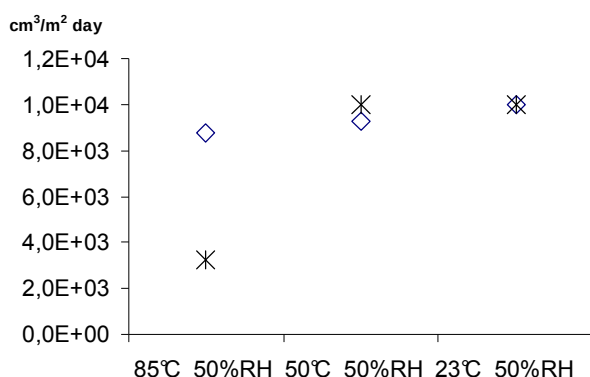


Figure 10. OTR obtained at different drying strategies. The relative humidity was held at 50% and the temperature was varied from 23°C to 85 °C. NaPA-containing colours coated on PN. There were two replicas of the experimental points. OTR measured at 50 % RH and 23°C.

In Figure 10, the samples dried at 85°C also had a rather long drying time. Probably the conditions were quite precise at the point when the enzyme started to denaturize. In Figure 9, sample E was dried in 105 °C at low relative humidity and consequently also at very short drying time.

When NB was used as substrate, OTR was lower then when PN was used. NB without any dispersion barrier coating had an OTR of about 300 cm³/m² day. When NB was coated with enzyme-containing coating colours, the OTR-value decreased to values in the range from 11 to 26 cm³/m² day (see Figure 11). The enzyme-containing coatings are denoted by E in Figure 11. The enzyme-free coatings are indicated by P. Also the enzyme free-coating gave a low OTR-value, which probably can be understood in terms of the relative good barrier properties of the as-received NB.

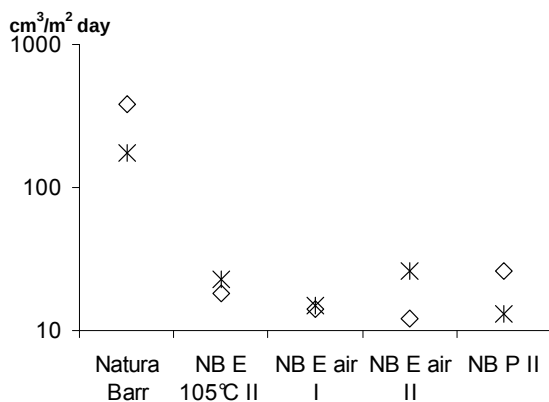


Figure 11. OTR of NB board without dispersion coating and coated with different layers of dispersion coating and dried at different conditions. E indicates enzyme-containing coating and P indicates enzyme-free coating. I and II indicates single and double coating, respectively. “150°C” indicates drying in air at 150°C and “air” indicates drying in air at room temperature. There were two replicas of the experimental points. OTR measured at 50 % RH and 23°C.

There was no significant difference between the coated samples in Figure 11 in OTR, despite differences in drying temperature and the number of coating layers or if the coatings contained enzymes or not. The barrier dispersion coating itself seemed to have the most important effect on the OTR values for the coated NB samples, contradictory to results obtained for the coated PN samples (see Figure 9). The observed differences between the effects of enzyme coating on NB and PN cannot be explained in detail from this study, more experiments are needed.

However, the results in Figure 11 indicated a very good barrier to oxygen of dispersion coated NB compared to High Density PE which has an OTR of about $600 \text{ cm}^3/\text{m}^2 \text{ d bar}$ at 23°C , 50%RH and Low Density PE which has an OTR of about $2000 \text{ cm}^3/\text{m}^2 \text{ d bar}$ at 23°C , 50%RH of a $100\mu\text{m}$ thick film [Noller (2005) and Jonhed et al. (2006)].

4.4. APG as potential substrate

When the substrate β – D glucose in the reaction shown in Figure 2 was replaced by APG, the same green colour was developed, indicating that also APG act as a substrate for the enzymatic reaction.

The potential in using APG as substrate for GOx was first studied in a spectrophotometric assay. The increase in absorbance was measured for two min and the slope of the initial part of the curve was used to calculate the activity of GOx. The activity was calculated as the mean value of four replicates. The activities of GOx with glucose and APG as substrates were fairly similar at the conditions used in this test (Table 8).

Table 8. Activity of glucose oxidase.

Substrate	U/g
Glucose	38000 ± 1300
APG	35000 ± 360

Samples coated with coating mixture containing APG instead of glucose was measured with the oxygen electrode. The enzyme activity of the coated board (PN) containing APG was compared with the enzyme activity of the coated board containing glucose, see Figure 12. Three different drying conditions were measured and there were no significant difference between the samples with APG or glucose.

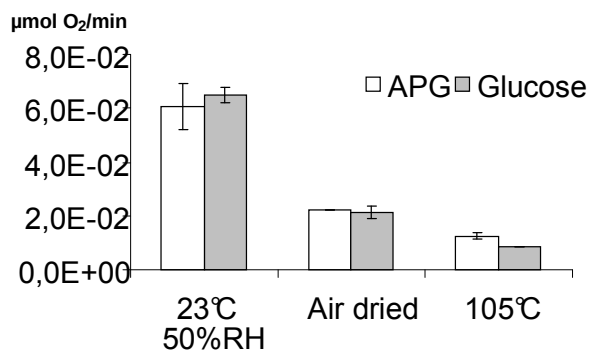


Figure 12. Oxygen consumption of coated board (PN). NaPA-containing colours.

APG is based on glucose synthesized with fatty alcohol. This could be the explanation to the similarity between the substrates. When comparing OTR-values of double coated layers on NB (see Figure 13) the same trend was observed, i.e. no significant difference between the samples coated with the coating colours containing APG or glucose.

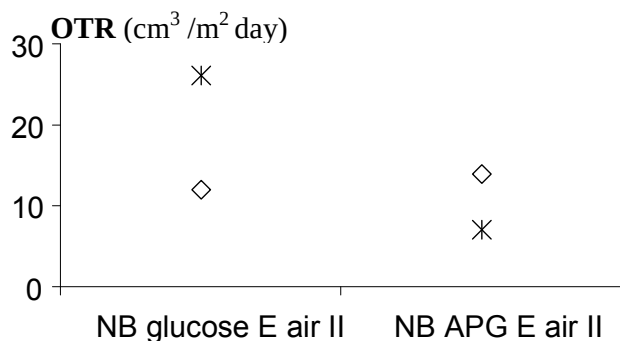


Figure 13. OTR of paperboard NB coated with glucose or APG, two replicates of each sample. Double coating with AMP-containing coating colour. “E” indicates enzyme-containing colours and “II” indicates double coating. The coatings were dried in air at 23°C.

4.5. Free films

Free enzyme-containing latex films were also tested to investigate if they could reduce oxygen in aqueous solutions. The result is shown in Figure 14 as reduced oxygen in percent vs. time. The reference sample, i.e. board coated with enzyme-free coatings, showed a reduction of oxygen from 100% oxygen in the buffer to 95%. When board coated with enzyme-containing coatings was examined, the oxygen content in the buffered aqueous solution was reduced to about 85% of its initial value (Figure 14). However, the enzyme-containing free film showed a reduction from 100% to about 66% oxygen after 420 seconds. The amounts of GOx immersed in the buffer solution were $15 \pm 2.3 \mu\text{g}$ for the coated paperboards and $90 \pm 11 \mu\text{g}$ for the free films. The faster reduction of oxygen in the case that free films were investigated could be due to the differences in amount of enzyme between the free films and the coated paperboards. It could also be due to the tendency of the enzyme to dissolve into the buffer. When the coating material is coated on a substrate, the enzyme could be more fixed to the material. The oxygen consumption values in Figure 14 are relative values and not absolute values.

Oxygen (%)

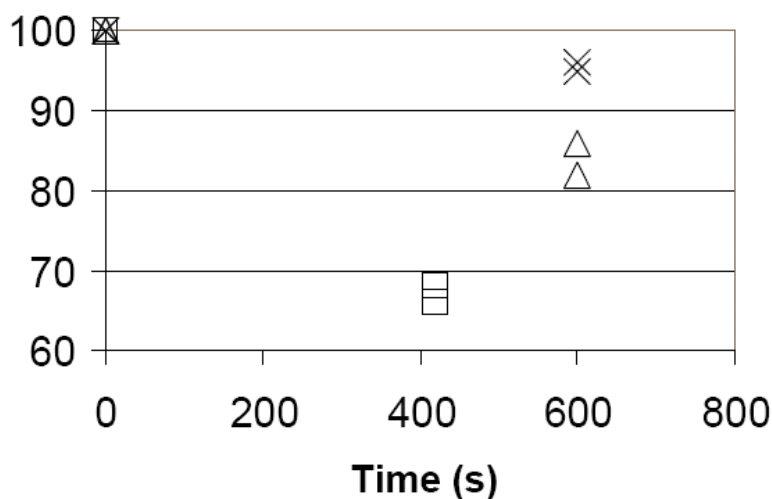


Figure 14. Oxygen reduction of board with enzyme-containing coating (Δ), board with enzyme-free coating as a blank sample ($\times$), and free enzyme-containing latex film ($\square$). Two replicates were measured.

4.6. Sealability tests of enzyme-coated samples

4.6.1. Heat sealing bags versus bags

TUT have coated bags made of OPP laminates with Latex and with Latex added TiO_2 . OPP bags coated with pure latex is transparent but after TiO_2 addition its white (not transparent). The sealing strength of bags coated with latex without TiO_2 to itself seems OK. The sealing strength of bags coated with latex with TiO_2 is to low.

4.6.2. Heat sealing cartons versus cartons

Carton coated with latex without TiO_2 , seems to have a good heat seal, when it is heat sealed against it self. Cartons coated with latex containing TiO_2 have a very low heat sealing strength, when it is heat sealed against it self.

Due to trouble sealing the coatings with high content of inorganic material (38% TiO_2) rinsing in water prior to heat-sealing was applied without success.

4.6.3. Cartons versus bags

It was not possible to obtain a good seal for latex coated bags versus latex coated carton for latex both with and without TiO_2 . When seal is obtained, the seal strength is much lower than for usual polymer heatseals. With a limited force it is possible to open the seal and it looks like the latex coating follows the OPP and not the board. I.e. the adhesion to the uncoated OPP is stronger than the latex-board adhesion. This might indicate that the coating of board itself should be looked upon also, i.e. replacing the PE layer of the carton with a PP layer could be a future solution. This was however not easily applicable and a solution with enzymoated strips within barrier polymeric bags was chosen for the testing the effect of enzymoating during storage of the end products, oatmeal cakes (chapter 4.8.1) and cooked ham (chapter 4.8.2). Another possible solution is to exclude enzymoating from the sealing areas in the final packaging, since TiO_2 should be included in the latex coating.

4.7. Oxygen absorption in Enzysheets

4.7.1. Variation in humidity

The experiment with 100% water vapour showed no absorption of oxygen within 4 days (Appendix 1). 6 samples were prepared: 3 with 70cm² material and 3 samples with 100cm² and no difference appeared. No reference material was provided.

The experiment with activation of Enzysheet with liquid water showed ability of absorbing oxygen within 2-3 days (Figure 15 and Appendix 1). The mean value of the oxygen level is 0.8 of 70 cm² material and 0.6 for 100 cm² within 3 days, however the best sample has only a level of 0.1 % oxygen for 100 cm² within 3 days. The increasing level of oxygen at day 1 can not be explained.

The larger standard deviation between the 100 cm² samples compared to 70 cm² samples is properly due to better expose to the headspace of the glass jar. 100 cm² is considered as the absolute maximum amount of material for the specific glass size of 100 ml volume.

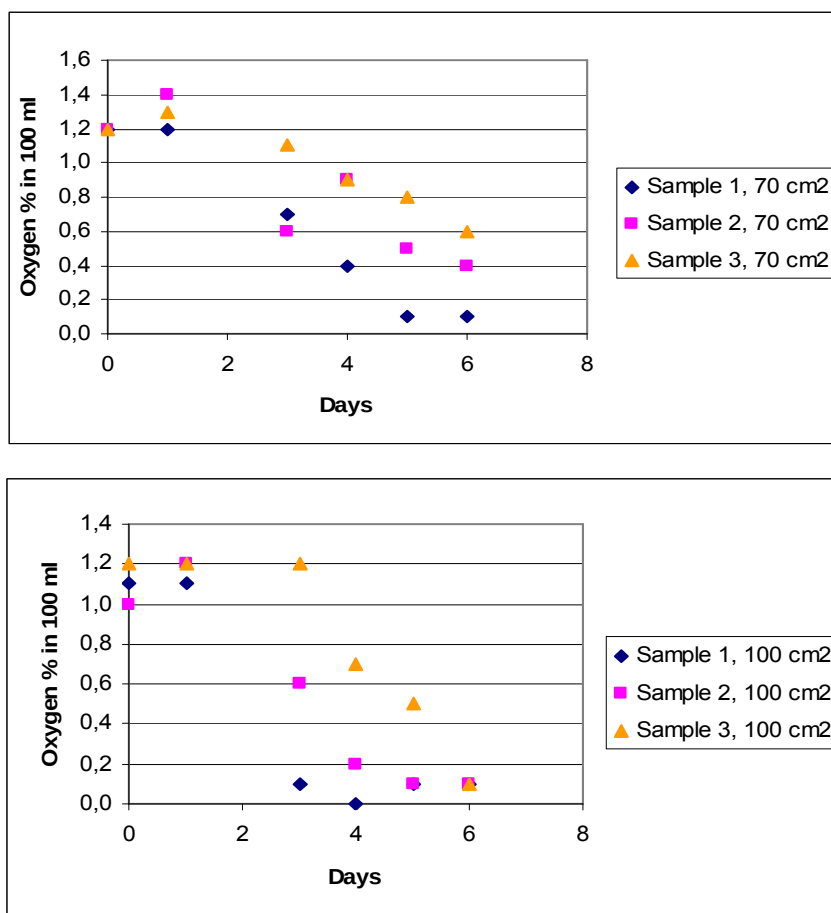


Figure 15. Enzysheet activated with liquid water at 23 °C for 70cm² and for 100cm² of material. The glass jar has a volume of 100 ml. The oxygen level is measured with OxySense.

4.7.2. Variation of temperature

10 samples were prepared: 5 samples for 5°C and 5 samples for 23°C and all samples with 100 cm² material. No reference material was provided. One sample storage in 23°C was leaking and do not appear in the result. The list of measurements is given in *Appendix 2*. The results obtained at 5°C and 23°C are summarized in *Figure 16*.

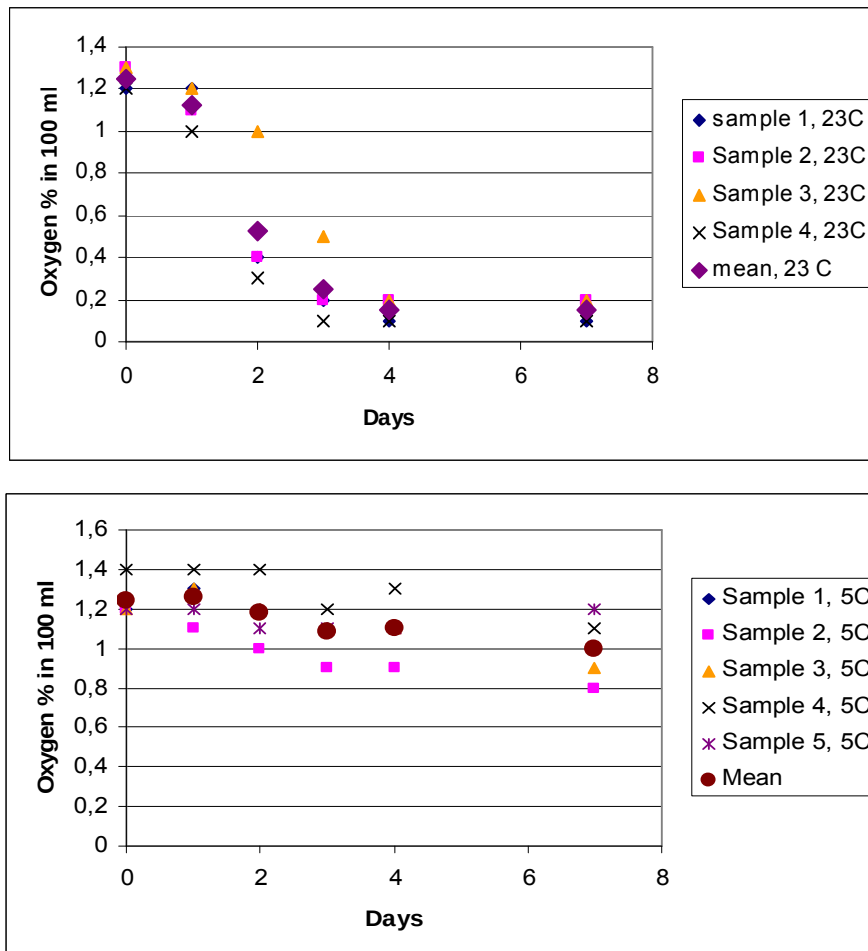


Figure 16. EnzySheet activated with liquid water at 23 °C and 5 °C for 100cm² of material. The glass jar has a volume of 100 ml. The oxygen level is measured with OxySense.

Based on 7 days measurements it appears that the enzymes at a temperature of 5°C are very slow (*Figure 16*). Most food product kept at this temperature does not have shelf life more than 1-2 weeks, thus, the speed is considered to low to be useful; approximately 0.2 % was absorbed in 7 days.

For 23°C the enzymes decrease the oxygen lever from 1.3 to 0.5 in mean level within 2 days (1 sample of 1%, 2 samples of 0.4 % and 1 sample of 0.3% oxygen).

4.7.3. Variation in start oxygen level

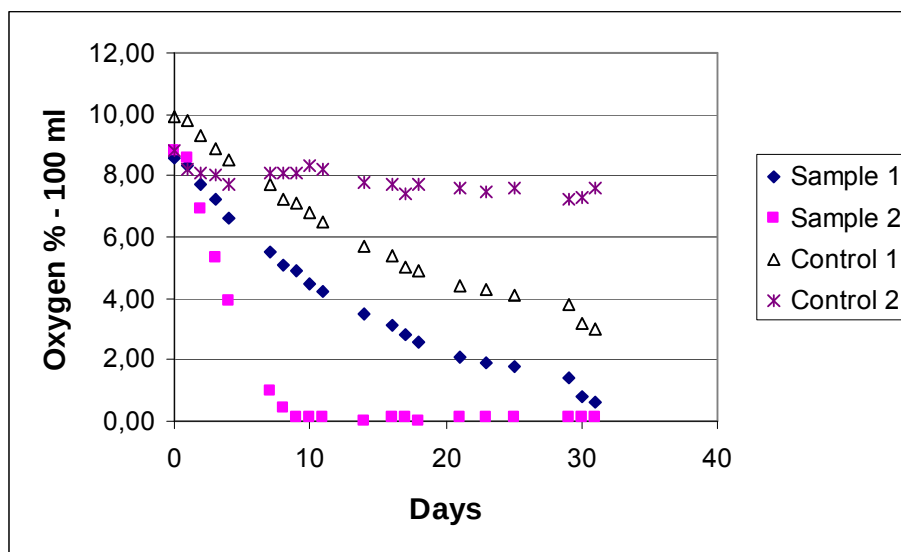
Three start level of oxygen is varied to 8.8% oxygen and atmosphere oxygen was approximately 22 % oxygen. 100 cm² of material is used and the storage condition was 23°C. 3

samples of each oxygen level and 2 control samples equally treated as the samples but without enzymes in the coating. One sample at start oxygen level at 8.8% was leaking and will not appear in the result.

It is quit obvious that something is wrong with the control samples (*Figure 17*); for samples with 22 % oxygen start level there is no difference between samples and control samples and for samples with 8.8 % oxygen start level only a single sample seems like a true control. The reason why is not clear, but by mistake the sheets must have been misplaced some how. For high oxygen levels the accuracy of the reading of 5% is noticeable. The list of measurements is given in *Appendix 3*.

The enzymes of the Enzysheet are able of absorbing the oxygen from a high oxygen stat level and decrease the oxygen level dramatically. Within 32 days the oxygen level was decreased from 8.8 % in star level and below 3% oxygen. A single sample reached 0.1% oxygen within 9 days. For 22% oxygen in start level the oxygen level was decreased until approximately 14 % for 4 samples and until 3% for a single sample.

One sample at each start oxygen level is much faster to absorb the oxygen than the rest of the samples. Two possible explanations are suggested. 1) The sheet for the different samples is different 2) Activation has been done differently. It may be both, but it may be interesting to investigate the activation time more precise.



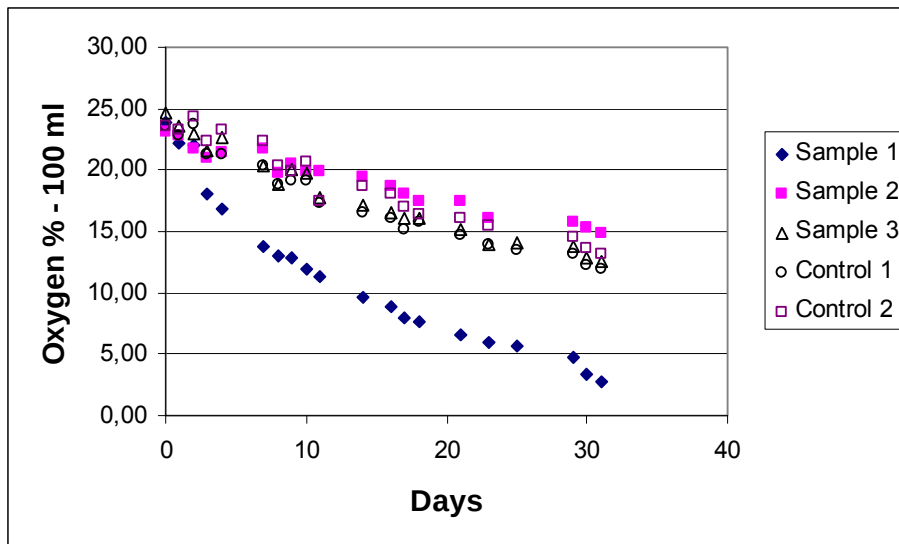


Figure 17. EnzySheet activated with liquid water at 23 °C for 100cm² of material at different start oxygen level; 8.8% oxygen and atmosphere oxygen, 23% oxygen. The glass jar has a volume of 100 ml. The oxygen level is measured with OxySense.

4.8. Rancidity test of cake and minced ham with EnzySheet

4.8.1. Cake

The hexanal concentration after storage of cakes (Figure 18 and Figure 19) is reported per g cake at the start of the incubation. Especially at the higher temperature and the and longer storage time, the cakes had become dry. However, the seemingly prooxidative effect of enzycoat in the cake experiments stands unanswered.

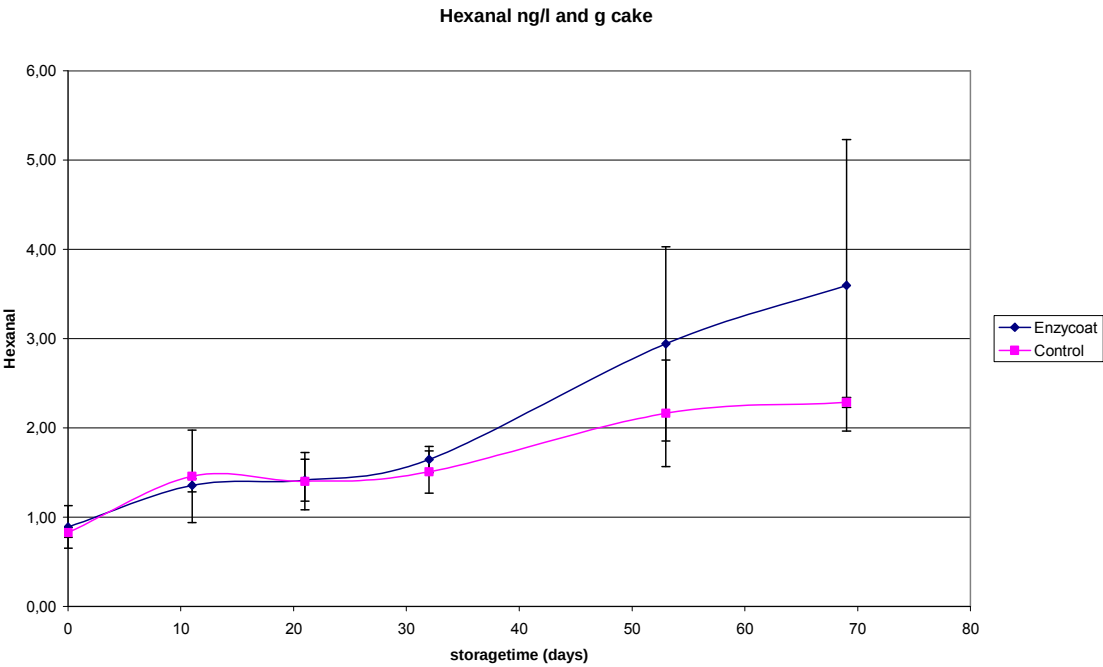


Figure 18. Hexanal concentration after storage of cakes at 23°C.

Sampling was also done after 42 days but determination of hexanal concentration was mistakenly done at 30°C. These values are omitted.

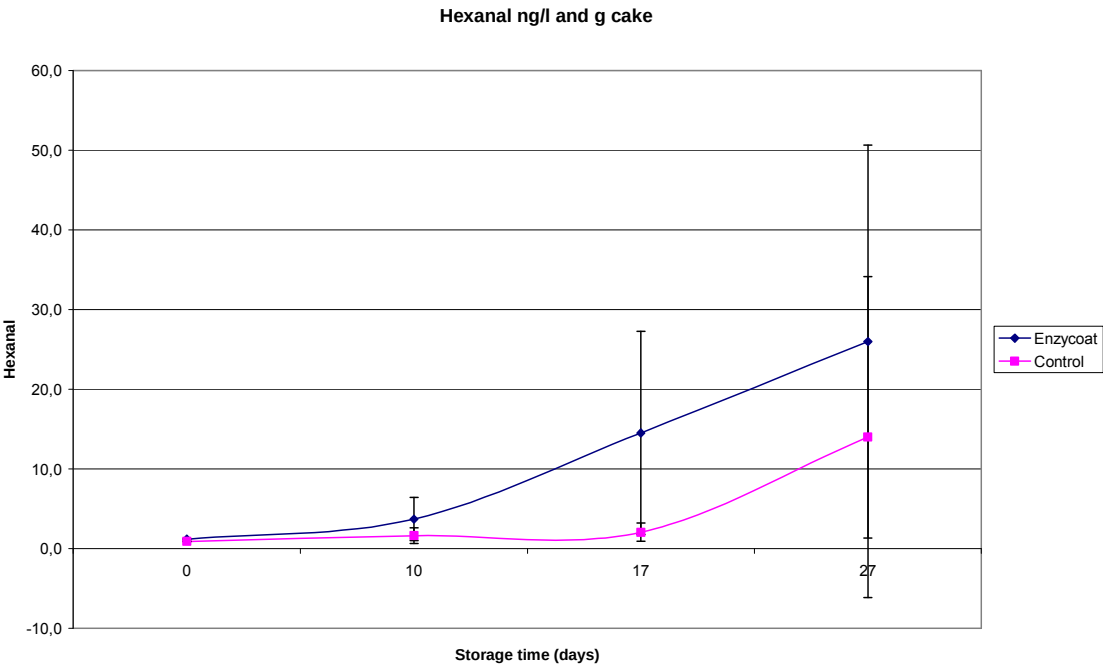


Figure 19. Hexanal concentration after storage of cakes at 37°C.

The results from the storage experiments shows that during the conditions used in the experiments the sheets coated with Enzycoat can not retard the oxidation taking place in the cakes more than control samples with sheets not coated with Enzycoat.

Given the large volume of the bags used to store the cakes in and the relatively high oxygen transmission through the bag material no retardation of lipid oxidation was achieved by the Enzycoat since the availability of oxygen was too high. However, for unknown reasons the Enzycoat actually promoted lipid oxidation. This was most obvious at the higher storage temperature but given a long enough storage time it could be seen also at the lower storage temperature. The differences were not significant in either case but the mean was repeatedly higher for the enzycoated samples.

One explanation could be that enzycoated paper actually produces additional amounts of the chosen marker hexanal from compounds present in the paper material or from other unknown sources occurring during the production of the material. However, this is a rather farfetched explanation but it has not been tested.

For cakes in bags containing enzycoated paper the differences in oxygen concentration, compared to the concentration at the start of the incubations (*Figure 20 and Figure 21*), shows that the lipid oxidation at 37°C proceeds at such a rate that oxygen concentration actually declines. For the slower rate of lipid oxidation at 23°C the influx of oxygen is higher than the consumption for both Enzycoat and control samples (*Figure 20*).

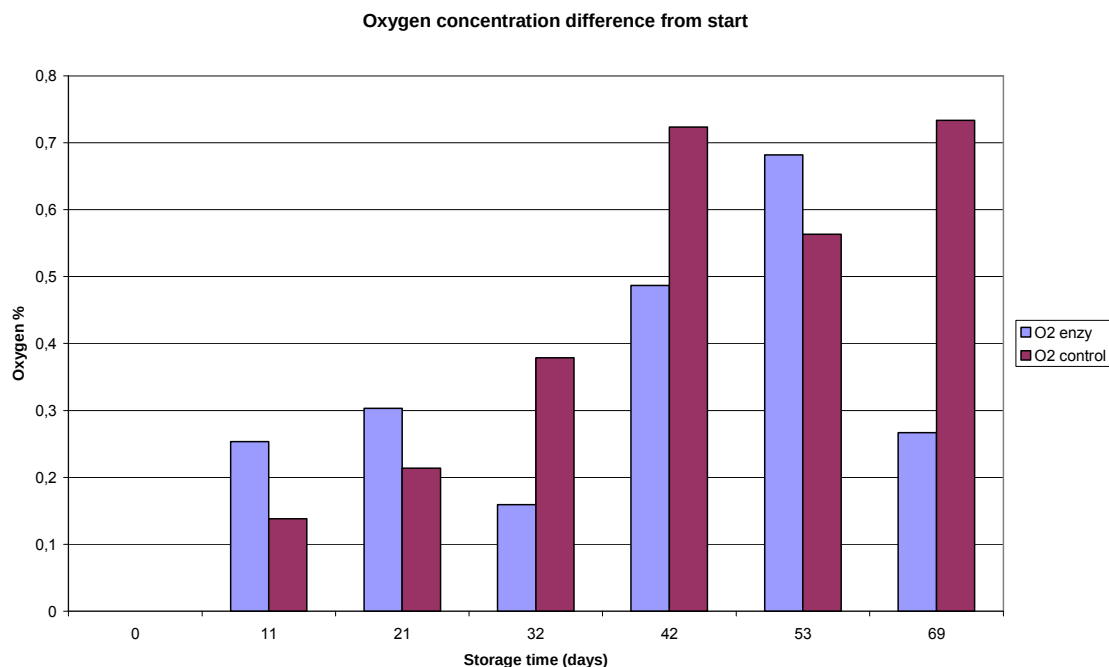


Figure 20. Differences in oxygen concentration after storage of cakes at 23°C.

The value for Enzycoat at day 69 in Figure 20 is only one samples since the other two bags had leaks giving an internal oxygen concentration similar to air.

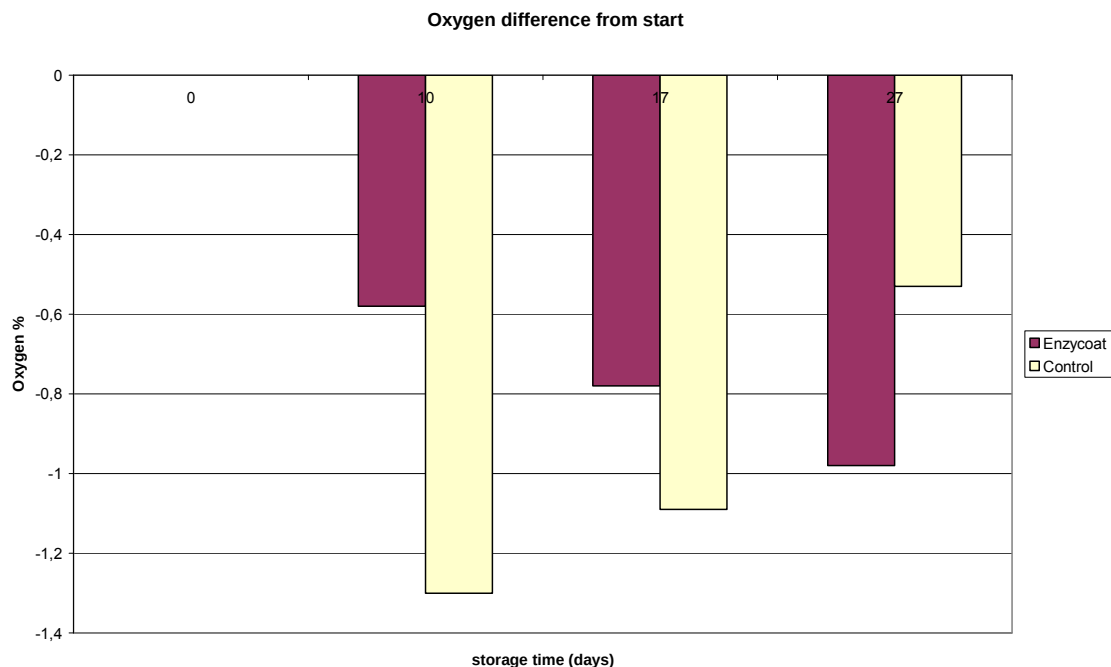


Figure 21. Differences in oxygen concentration after storage of cakes at 37°C.

4.8.2. Minced ham

The results are given in mg malon dialdehyde/kg sample. The results shown in *Table 9* and *Figure 22* are average numbers for each day and in the figure the Standard deviation is shown for each measurement.

Table 9. Average absorbance and malon dialdehyde (mg/kg) for pork meat with (A) or without Enzycoat (B).

	A		B	
days	abs	mg/kg	abs	mg/kg
0	0.01	0.0780	0.01	0.078
3	0.011	0.09	0.021	0.1638
10	0.015	0.12	0.024	0.1872
17	0.016	0.12	0.025	0.195
24	0.023	0.1794	0.0310	0.2418
31	0.027	0.2106	0.0353	0.2750

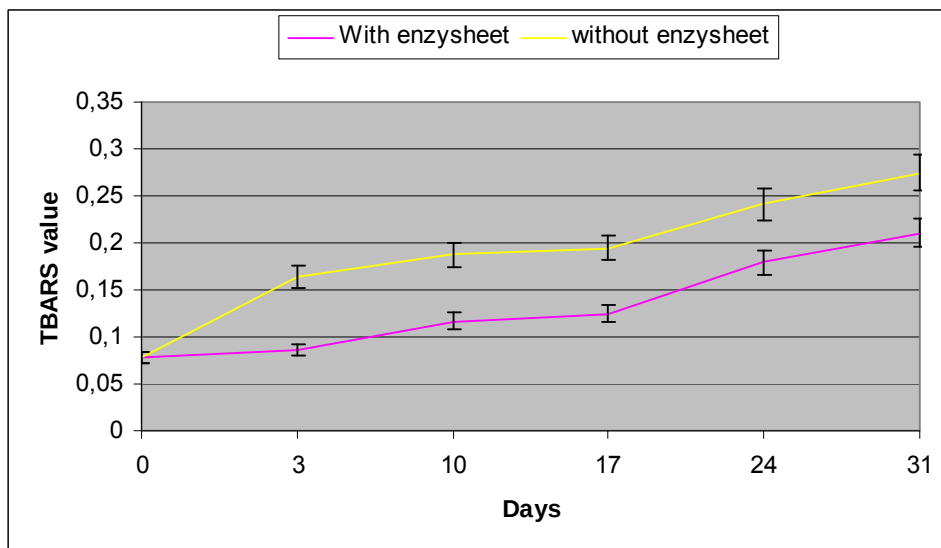


Figure 22. TBARS value after storage of minced ham at room temperature..

For the minced meat the OTR characteristics was not known and the oxygen concentration in the vacuumised bags was not measured. Typical OTR values for the type of material used would be between 10 and 25 cc/m² day atm. However, one reason for the ability of the enzyme-coated sheets to significantly retard the lipid oxidation could be a lower availability of oxygen than for the cakes. Direct contact between the enzyme-sheets and water from the minced meat could also play a role since it has been shown that the enzymatic preparations used in the production of Enzycoat need liquid water to be effective.

The enzyme-coated paper sheets could retard lipid oxidation in minced ham in relation to minced ham stored with sheets not containing Enzycoat.

5. Conclusions

It was possible to coat paperboard, with retained enzymatic activity, by dispersion coating methods including air drying. The enzyme was active for at least three months after coating despite storage conditions. Even high temperature for a short time did not substantially affect the activity.

For the removal of oxygen from an ambient aqueous solution, it is of importance for high enzymatic activity that multi-layer structures of enzyme-containing coatings are designed to keep the enzyme in the uppermost surface.

APG gave the same result as glucose when used as substrate for glucose oxidase. APG showed to be a good candidate as substrate for glucose oxidase.

Oxygen transmission rate for the mineral-coated paperboard (Natura Performa) used decreased substantially when enzymes were included in the coating formulation. When Natura Barr was used, the OTR values were as low as about 10 cm³/m² day for all dispersion-coated samples irrespective of whether enzymes were present or not. At least the oxygen transmission rate of the mineral-coated paperboard (Natura Performa) indicated a positive effect of the enzyme coating (reduction of OTR) when measured at 50 % RH without any additional pre-activation step.

For reduction of oxygen in ambient atmosphere an activation process with liquid water was needed. The samples without activation with liquid water did not absorb oxygen. Activation in 100% water vapour was not effective enough. Therefore the current compositions are not useful for oxygen absorption for dry products. The need for activation for oxygen absorption but not for oxygen transmission indicated different equilibrium conditions between absorption and transmission. The capacity of the enzyme could be sufficient for reduction of oxygen penetrating through the material also without activation, while this apparently not was the case for oxygen absorption.

Improvement of the Enzysheet for low temperature is needed. The oxygen absorption speed at 23°C is much faster. The oxygen was decreased to 0.4% within 2 days at a start oxygen level of 1.2 %.

The Enzysheet is proved to be able to decrease the oxygen level dramatically for a very high start concentration of oxygen (8.8% and atmosphere oxygen).

A problem appears regard different responses between samples of equal treatment. It might be related to differences of the Enzysheet but stronger evidence point to the activation method. In practice it is not suitable to use liquid water for the activation, thus, the Enzysheet need improvement. However, the Enzysheet showed great potential for fast absorption of oxygen in conditions of room temperature and appropriate activation.

The sample without activation with liquid water does not absorb oxygen. The samples with activation showed response within 2-3 days. Most samples showed moderate absorption speed.

Enzysheets did reduce the oxidation (rancidity) of packed minced pork meat. However, during the conditions used in the experiments of packed cakes, the Enzysheets could not retard the oxidation taking place in the cakes more than control samples without enzymes.

The coating without TiO₂ is heatsealable however even at high sealing temperatures, times and pressure, it produce what could be a hermetic seal, but too weak to be of practical packaging use. When TiO₂ was included no seal was obtainable.

6. Literature

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System Manual Hansatech, (2000), "Oxygen Measurements in the Liquid-Phase, version 1.00", Hansatech Instrument Ltd., Norfolk, England, www.hansatech-instruments.co.uk.

Zohrehvand, S. and te Nijenhuis, K., (2006), "Film formation from monodispersed acrylic lattices Part 3: Drying and ageing of acrylic latex films", Progress in Organic Coatings, 55, 11-19.

Appendix 1

Table A1. EnzySheet activated with **100% water vapor** at 23°C for 70cm² and for 100cm² of material. The glass jar had a volume of 100 ml. The oxygen level was measured with OxySense. The measurement was carried out 24/9-27/9 2007.

Day	0	1	3	4
Area 70				
1	1.2	1.1	1.1	1.1
2	1.2	1.2	1.2	1.1
3	1.1	1.1	1.1	1.1
mean 70	1.2	1.1	1.1	1.1

Day	0	1	3	4
Area 100				
1	1.1	1.1	1.0	1.0
2	1.1	1.1	1.0	1.1
mean 70	1.1	1.1	1.0	1.1

Table A2. EnzySheet activated with **liquid water** at 23°C for 70cm² and for 100cm² of material. The glass jar had a volume of 100 ml. The oxygen level was measured with OxySense. The measurement was carried out 27/9-3/10 2007.

Day	0	1	3	4	5	6
Area 70						
1	1.2	1.2	0.7	0.4	0.1	0.1
2	1.2	1.4	0.6	0.9	0.5	0.4
3	1.2	1.3	1.1	0.9	0.8	0.6
mean 70	0.4	0.4	0.2	0.1	0.1	0.0

Day	0	1	3	4	5	6
Area 100						
1	1.1	1.1	0.1	0.0	0.1	0.1
2	1.0	1.2	0.6	0.2	0.1	0.1
3	1.2	1.2	1.2	0.7	0.5	0.1
mean 100	0.0	0.3	1.0	1.3	1.7	2.0

Appendix 2

Table A3. Enzysheet activated with liquid water at 5°C for 100cm² of material. The glass jar had a volume of 100 ml. The oxygen level was measured with OxySense.

	08-okt	09-okt	10-okt	11-okt	12-okt	15-okt
Sample\Day	0	1	2	3	4	7
1	1.2	1.3	1.2	1.1	1.1	1
2	1.2	1.1	1	0.9	0.9	0.8
3	1.2	1.3	1.2	1.1	1.1	0.9
4	1.4	1.4	1.4	1.2	1.3	1.1
5	1.2	1.2	1.1	1.1	1.1	1.2
mean	1.2	1.3	1.2	1.1	1.1	1.0

Table A4. Enzysheet activated with liquid water at 23°C for 100cm² of material. The glass jar had a volume of 100 ml. The oxygen level was measured with OxySense.

	08-okt	09-okt	10-okt	11-okt	12-okt	15-okt
Sample\Day	0	1	2	3	4	7
1	1.2	1.2	0.4	0.2	0.1	0.1
2	1.3	1.1	0.4	0.2	0.2	0.2
3	1.3	1.2	1	0.5	0.2	0.2
4	1.2	1	0.3	0.1	0.1	0.1
mean	1.3	1.1	0.5	0.3	0.2	0.2

Appendix 3

Table A5. EnzySheet activated with liquid water at 23°C for 100cm² of material with start oxygen level at **8.8% oxygen**. The glass jar had a volume of 100 ml. The oxygen level was measured with OxySense. The reference samples produced without enzymes are denoted as control.

		5.11.07	6.11.07	7.11.07	8.11.07	9.11.09	12.11.07	13.11.07
days		0	1	2	3	4	7	8
8.80% 100 ml	1	8.60	8.30	7.70	7.20	6.60	5.50	5.10
	2	8.80	8.60	6.90	5.30	3.90	1.00	0.40
	3	9.30	8.30	7.40	6.20	5.50	4.20	4.00
	control	9.90	9.80	9.30	8.90	8.50	7.70	7.20
	control	8.80	8.20	8.10	8.00	7.70	8.10	8.10
		14.11.07	15.11.07	16.11.07	19.11.07	21.11.07	22.11.07	23.11.07
days		9	10	11	14	16	17	18
8.80% 100 ml	1	4.90	4.50	4.20	3.50	3.10	2.80	2.60
	2	0.10	0.10	0.10	0.00	0.10	0.10	0.00
	3	4.00	3.90	3.90	3.90	4.00	4.00	4.00
	control	7.10	6.80	6.50	5.70	5.40	5.00	4.90
	control	8.10	8.30	8.20	7.80	7.70	7.40	7.70
		26.11.07	28.11.07	30.11.07	3.12.07	4.12.07	5.12.07	
days		21	23	25	29	30	31	
8.80% 100 ml	1	2.10	1.90	1.80	1.40	0.80	0.60	
	2	0.10	0.10	0.10	0.10	0.10	0.10	
	3	4.40	4.50	4.60	5.00			
	control	4.40	4.30	4.10	3.80	3.20	3.00	
	control	7.60	7.50	7.60	7.20	7.30	7.60	

Table A6 Enzysheet activated with liquid water at 23°C for 100cm² of material with start oxygen level at atmosphere oxygen level, **23% oxygen**. The glass jar had a volume of 100 ml. The oxygen level was measured with OxySense. The reference samples produced without enzymes are denoted as control.

		5.11.07	6.11.07	7.11.07	8.11.07	9.11.09	12.11.07	13.11.07
days		0	1	2	3	4	7	8
23% 100 ml	1	23.90	22.20	22.00	18.00	16.90	13.70	13.00
	2	23.10	22.80	21.80	21.00	21.40	21.80	19.80
	3	24.60	23.60	23.00	21.60	22.60	20.40	18.80
	control	23.60	22.80	23.80	21.20	21.20	20.30	18.90
	control	23.60	23.20	24.30	22.30	23.20	22.40	20.40
		14.11.07	15.11.07	16.11.07	19.11.07	21.11.07	22.11.07	23.11.07
days		9	10	11	14	16	17	18
23% 100 ml	1	12.80	12.00	11.30	9.60	8.90	8.00	7.60
	2	20.50	19.90	19.90	19.40	18.70	18.00	17.50
	3	20.10	19.70	17.80	17.20	16.60	16.10	16.10
	control	19.10	19.10	17.30	16.50	16.00	15.20	15.70
	control	19.90	20.70	17.40	18.60	18.10	17.00	16.40
		26.11.07	28.11.07	30.11.07	3.12.07	4.12.07	5.12.07	
days		21	23	25	29	30	31	
23% 100 ml	1	6.60	6.00	5.70	4.80	3.30	2.70	
	2	17.50	16.10		15.70	15.30	14.80	
	3	15.20	14.00	14.10	13.70	12.80	12.60	
	control	14.70	13.90	13.50	13.10	12.30	11.90	
	control	16.10	15.40		14.50	13.60	13.10	



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