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Characterization of Food-grade Microbial Xylanase and Application for the Production of Xylo-oligosaccharides (XOS): Biochemical and Regulatory Status

MASTER'S THESIS

TO OBTAIN A MASTER DEGREE AT UNIVERSITY OF NATURAL RESOURCES AND LIFE SCIENCES, VIENNA

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Abstract

Industrial enzymes are a thriving business worldwide, and food enzymes in particular are an important contributing factor. Xylanases are enzymes that are widespread in fungi, yeasts and bacteria. Suitable for a spectrum of applications, xylanases show foremost significant importance in the pulp and paper industry. A second important field of application is the baking industry. By discovering new xylanase sources deriving from Lactic acid bacteria a novel asset to the future food industry can be imparted.

For the purpose of this thesis, potential xylanase activity was investigated by screening different Lactic acid bacteria strains of several food types, mainly originating from Vietnam. Adding on to this, it is also of great importance to note that xylanases can be associated with the generation of the prebiotics, the xylo-oligosaccharides (XOS). Leading on to that fact, the current status and legislative framework of prebiotics within the European Union has been taken into consideration. The impact of the present Novel Food Regulation on prebiotics has been studied linked with a view on the progressing prebiotic market.

Table of Contents

1. Introduction

1.1. The Prebiotic Concept

To date, a number of definitions have been proposed and thus there is no full agreement on a single definition of a prebiotic (Binns, 2013).

One common definition of a prebiotic is a “non-viable food component that confers a health benefit on the host associated with modulation of the microbiota (FAO, 2007). The more recent and specific definition is given by Roberfroid et al., where a prebiotic is deemed a “selectively fermented ingredient that results in specific changes in the composition and / or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health” (Roberfroid et al., 2010). Possible health benefits of certain prebiotics include:

- improved digestive functions (e.g. resistance to gastroenteritis, pathogen inhibition, bowel movement regulation)
- positive modulation of immunity
- enhanced absorption of certain dietary minerals (e.g. calcium)
- improved functions in weight management and obesity-related disorders
- complementing probiotic functions

  (Roberfroid et al., 2010)

At present, 3 criteria are required for a prebiotic effect:

1. resistance of the prebiotic to degradation by mammalian enzymes, hydrolysis and GI (gastro intestinal) absorption
2. fermentation of the prebiotic by microbes
3. selective stimulation of the growth and/or activity of intestinal microorganisms beneficially associated with health and well-being.
   (Roberfroid et al., 2010)

Besides, qualifications for a prebiotic according to the FAO Technical Meeting on Prebiotics of 2007 were specified as followed:

- component: not an organism or drug; a substance that can be characterized chemically; in most cases this will be a food grade component
- health benefit: measurable and not due to absorption of the component into the bloodstream or due to the component acting alone; and over-riding and adverse effects
- modulation: show that the sole presence of the component and the formulation in which it is being delivered changes the composition or activities of the microbiota in the target host

In addition, a prebiotic can be a fiber but a fiber needs not to be a prebiotic (FAO, 2007).

Bearing the complete definition of a prebiotic in mind, the term fiber has to be taken into consideration as well. Fiber, more specifically dietary fiber is defined as non-digestible carbohydrates (NDC) including lignin, non-starch polysaccharides (i.e. gums, mucilages, β-glucans), resistant oligosaccharides (fructo-oligosaccharides FOS and galacto-oligosaccharides GOS and others), resistant and modified starches, some raw starch granules and retrograded amylose (EFSA, 2010a).

Summarizing, both fiber and prebiotics are typically non-digestible carbohydrates and concurrently both are fermented by gut bacteria. Nevertheless, a prebiotic differs from a fiber in that it needs to be “selectively” used in the digestive system only by beneficial bacteria of the gut microbial community (i.e. Lactic acid bacteria and Bifidobacteria) (Slavin, 2013).

1.2. Prebiotics

Currently, only carbohydrate compounds, primarily oligosaccharides have been studied with respect to prebiotic activity (Slavin, 2013). More specifically, the majority of the scientific evidence on prebiotic effects has been obtained using food ingredients or supplements belonging to the two chemical groups inulin-type fructans (ITF) and the galacto-oligosaccharides (GOS). In addition, the most extensively

tested forms of ITF in literature occur naturally in foods such as cereals, agave, leek, artichoke and chicory. However, it would take a large quantity of these foods in order to obtain their active oligosaccharides to exert a useful prebiotic effect (Roberfroid et al., 2010).

Other prebiotics include the disaccharide lactulose, polysaccharides such as polydextrose, further oligosaccharides such as gluco-oligosaccharides or xylo-oligosaccharides and resistant dextrins, arabinoxylans and resistant starches as well as some polyols such as isomalt and lactitol (Binns, 2013). These compounds have been investigated to varying degrees *in vitro*, in animal feeding studies, but rarely in human feeding studies (Roberfroid et al, 2010).

1.3. Guidelines and Assessment

Prebiotics need to be thoroughly evaluated before they can be commercialized in a product. They have to be characterized according to their source/origin, their purity, their chemical composition and structure and their vehicle, meaning the amount and concentration in which they are to be delivered to the host (FAO, 2007).

Furthermore, their functionality needs to be confirmed by substantial scientific evidence (e.g. at least one suitable sized randomized control trial; RCT). Minimum proof needs to be guaranteed by a correlation between the measurable physiological outcomes and modulation of the microbiota at a specific site (e.g. foremost the gastrointestinal tract). Examples of physiological outcomes could be the positive effects on absorption of nutrients or bowel movement and regularity (FAO, 2007).

With regard to safety, a safety assessment of a prebiotic final product needs to be established. This would mean that the product has a history of safe use in the target host, such as an acquired GRAS (generally recognized as safe) status a designation of the Food and Drug Administration (FAO, 2007).

Levels of safe consumption with minimal symptoms and side effects should be determined and the product should not contain any contaminants and impurities. Complementary to that, the prebiotic should not modify the microbiota in such a way as to have long-term harmful effects on the host. In general, guidelines for the

evaluation and substantiation of prebiotics can be illustrated in the following figure (FAO, 2007).

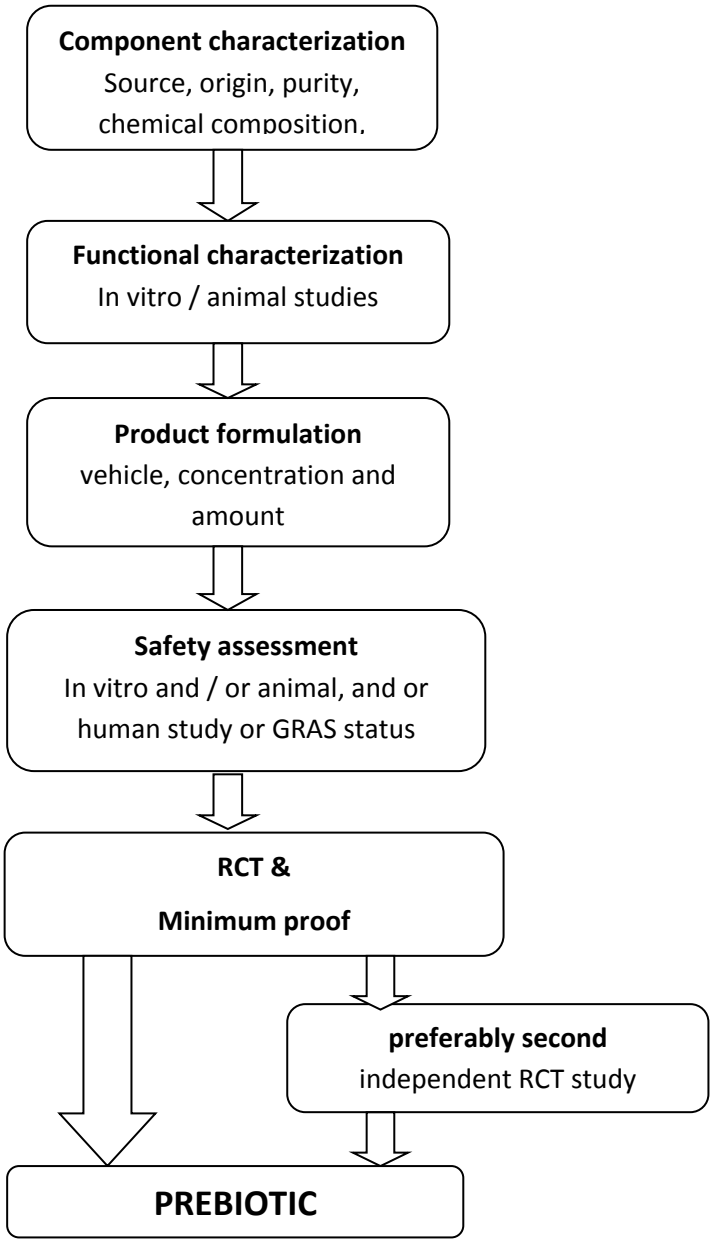

Figure 1.1.: Schematic flow of guidelines for the evaluation and substantiation of prebiotics (FAO, 2007).

1.4. Legal Obligation – the Novel Food Regulation (EC) No. 258/97

When it comes to assessing the reliability of prebiotics within the European Union, Regulation No. 258/97 (EC) has to be mentioned.

Regulation 258/97 of the European Parliament and of the Council, also known as the Novel Food Regulation (NFR) was first published on 27 January in 1997. The Regulation was established during a time when commercial interest in genetically modified (GM) crops and food ingredients arose and concurrently so did the conjoined consumer hostility. It was also at a time when BSE (Bovine Spongiform Encephalopathy also known as "mad cow disease") was still a prevailing issue and a big concern in many European Member States. The resulting uncertainties around the food safety aspects of BSE and potentially GMOs (Genetically Modified Organisms) gave rise to the development of new legislative guidelines.

The purpose for the enactment of the Novel Food Regulation was to harmonize European legislation towards the authorization of novel foods and novel food ingredients. In addition the NFR should propose a food safety assessment controlling the introduction of GM plants and their derivatives into the European market. However, the scope of the NFR was amended once on April 18th 2004 when GMOs were excluded. Hereunto, the scope is defined in article 3(1) of the NFR where the implicated "food and food ingredients" shall not:

- present a danger to the consumer
- mislead the consumer
- differ from foods or food ingredients which they are intended to replace to such an extent that their normal consumption would be nutritionally disadvantageous for the consumer.
  (European Commission, 1997a)

According to the Novel Food Regulation, a novel food is defined as a food that does not have a significant history of consumption within the European Union before the Regulation's inauguration on May 15th 1997.

In the Regulation novel foods can be classified into four categories: (European Commission, 1997a)

(c) foods and food ingredients with a new or intentionally modified primary molecular structure;

(d) foods and food ingredients consisting of or isolated from microorganisms, fungi or algae;

(e) foods and food ingredients consisting of or isolated from plants and food ingredients isolated from animals, except for foods and food ingredients obtained by traditional propagating or breeding practices and having a history of safe food use;

(f) foods and food ingredients to which has been applied a production process not currently used, where that process gives rise to significant changes in the composition or structure of the foods or food ingredients which affect their nutritional value, metabolism or level of undesirable substances;

1.6. Authorizing a Novel Food (e.g. Xylo-oligosaccharides)

If a product, like for instance a prebiotic product including xylo-oligosaccharides, falls under the NFR, it has to be previously approved as safe before being introduced to the European market as a novel food or a novel food ingredient. Thus, in order to get an approval, the cooperation of the Members States, the Standing Committee for the Food Chain and Animal Health, the European Food Safety Authority and the European Commission is required.

An approval can be pursued either through an “application” or a rather simplified procedure, the “notification”.

In case of the “application”, the applicant (i.e. the person responsible for placing the product on the Community market) submits the request to one national Member State authority and transmits a copy to the Commission. The application should be

compliant to the European Commission Recommendation 97/618/EC (European Commission, 1997b). The national authority body is thus in charge of the initial safety assessment on the basis of current scientific knowledge and decides whether additional measures are required. Accordingly, all other Member States may be included in the discussion. If no objections are raised, the applicant is instantly allowed to put the product on the European market.

The applicant may provide further data to clarify dubious presumptions, when objections may come up. If further objections are not raised, the product can be approved as a novel food or a novel food ingredient. If a Member State demands an additional assessment, the Commission will be responsible for the conduction of this additional assessment, which will be supported by scientific advice from the European Food Safety Authority (EFSA). The Standing Committee will be in charge of the formal decision-making and when no further objections are interposed the Commission will publish the decision in the Official Journal of the European Union (European Commission, 1997a).

The decision of authorization designates the scope of the authorization, which specifies as appropriate according to article 7 of the Novel Food Regulation (EC) No 258/97:

- conditions of use
- designation of the food or food ingredient
- specification and labelling requirements

Products, which are already compliant with the conditions accordant article 3 (4) of the NFR, can be put on the market through a more facilitated procedure, the so-called “notification”. These products are hence referred to as “substantially equivalent” to a product which is already on the market. This equivalence can be based on generally recognized and available scientific data or on an opinion on substantial equivalence from a national authority.

More specifically, a company's application document should show how the novel food or novel food ingredient may be substantially equivalent to an already existing food or food ingredient with respect to:

- composition (such as preparation method and the source organism)
- nutritional value
- metabolism
- intended use (such as food ingredient or supplement)
- level of undesirable substances (e.g. allergens, contaminants..)

(European Commission, 1997a)

Figure 1.2. gives a schematic overview of the application and the notification procedure.

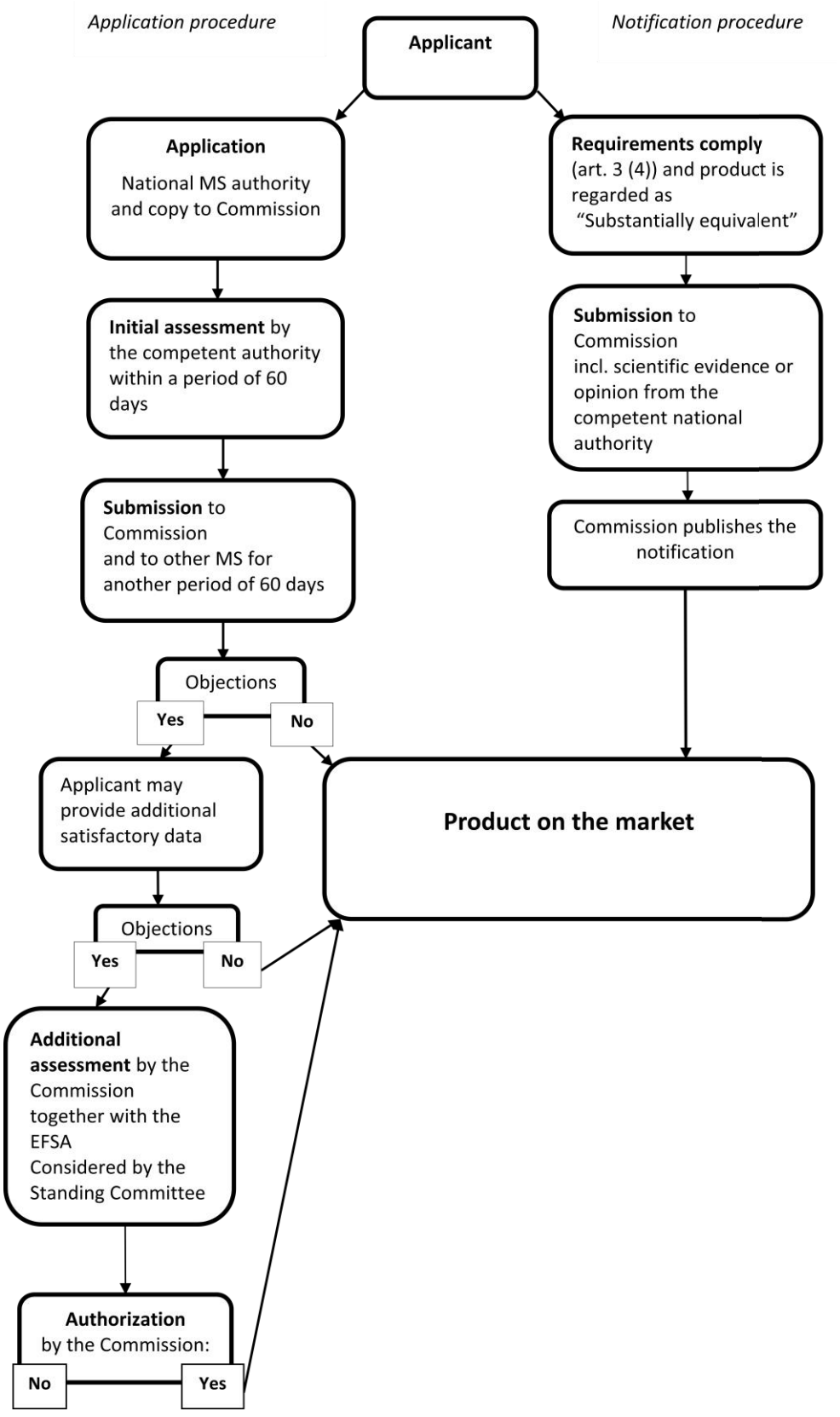

Figure 1.2.: Application and notification procedure for novel food or novel food ingredients accordinG to regulation (EC) No 258/97.

1.6. The New Proposal of the Novel Food Regulation

On 18 December 2013 the European Commission published a new “Proposal for a Regulation of the European Parliament and of the Council on novel foods (European Commission, 2013a). This draft document represents a new attempt in order to review the legal framework for novel foods.

In the past, a similar proposal was adopted by the European Commission in 2008. However, legislative discussions under Ordinary legislative procedure reached a stalemate on a limited number of issues, particularly those linked to the issue of cloning. Consequently, the proposal was withdrawn on 28 March 2011 and the Commission was asked to prepare a legislative proposal on cloning based on an impact assessment outside the NFR (European Commission, 2013b).

Similarly to the 2008 proposal, the new document includes attempts to streamline the authorization procedure, to improve its efficiency and transparency. In addition, the latest draft of the NFR straightens out the definition of a novel food, including new technologies which have an impact on food. Another aim of the proposal is the implementation of a faster and more proportionate safety assessment for traditional foods from third countries having a history of safe food use (European Commission, 2013a).

The new proposal redefines the terms “novel food” which is described in article 2 (2) as the following:

a) all food that was not used for human consumption to a significant degree within the Union before 15 May 1997, in particular

   i) food to which a new production process is applied, not used before 15 May 1997, where that production process gives rise to significant changes in the composition or structure of the food which affects its nutritional value, the way it is metabolized or the level of undesirable substances;

   ii) food containing or consisting of “engineered nanomaterials” as defined in ”Food Information to Consumers” Regulation (EU) No 1169/2011

iii) vitamins, minerals and other substances produced with a new production process or containing or consisting or engineered nanomaterials used in accordance with the" Food Supplements" Directive 2002/46/EC, regulation on the "addition of vitamins and minerals and of certain other substances to food" (EC) No 1925/2006 or Regulation on "Food for Specific Groups" (EU) No 609/2013.

iv) food used exclusively in food supplement within the Union before 15 May 1997, where it is intended to be used in foods other than food supplements […]

1.6.1. Main Changes of the New Proposal for the Novel Food Regulation

The European Commission will establish and update a "Union list of novel foods". Thus, novel foods should not be placed on the market or used as food for human consumption unless they are included in the Union list. Already authorized novel foods will be automatically included in the Union list. Moreover, a new EU authorization procedure should replace the current mutual recognition system where applications for a pre-market authorization must be submitted to an individual Member State's food assessment body which evaluates the application (European Commission, 2013a).

The initial assessment report will be transmitted to the Commission who then disseminates it to all Member States for objections and comments. In case of an objection, the Commission asks the European Food Safety Authority (EFSA) to carry out an additional assessment. This means that the current requirement to submit novel food application to national authorities will be abolished which renders a centralized EU procedure for the assessment and authorization of novel foods. With respect to that safety assessment, the Commission subsequently takes an authorization decision (European Commission, 2013a).

In general, the main changes to the current NFR can be seen in the authorization procedure, which should be harmonized and sped up. In addition, nanomaterial

intended for food use would require a novel food authorization. Moreover, novel foods will be subject to labeling requirements set out by the EU's "Food Information to Consumers" Regulation 1169/2011 and other corresponding EU food law. For every authorized novel food, additional requirements may be established in terms of description of food, its source and its condition of use (European Commission, 2013a).

Another adjustment to the current NFR would be a simplified procedure for the allowance of the marketing of traditional foods from third countries. Traditional foods are foods with a history of long-term consumption in third countries, but for which it was not possible to show a significant consumption in the EU prior to the NFR inauguration date. To that end, a traditional food may be allowed on the basis of a notification if a history of safe use can be demonstrated by the food business operator. A history of safe use implies that the food has been consumed for at least 25 years as part of a normal diet within a large part of the third country's population. More importantly, traditional food from third countries will only be considered for inclusion in the Union list if they are derived from primary production. The correspondent approval procedures are laid down in articles 13 and 17 of the proposal regulation (European Commission, 2013a).

The proposal has to be adopted under the Ordinary legislative procedure, which means that both the European Parliament and the Council conduct three readings to reach an agreement on the proposal (European Commission, 2013a).

1.7. Novel Food Applications

Since the ratification of the Novel Food Regulation in May 1997, 145 applications for authorization were submitted. Among these applications 13 were implicating genetically modified organisms and are now regulated by the Regulation (EC) No 1829/2003 (European Commission, 2003). Thus the latter were omitted in the following figure depicting the distribution of the applications divided into 5 segments.

Consequently, a total of 132 applications were considered for the statistical distribution, showing 5% were refused, 48% were authorized, 12 % were withdrawn, 34% are still in progress and 1% are not valid for the Regulation 258/97 and therefore are categorized to “other” (European Commission, 2014a).

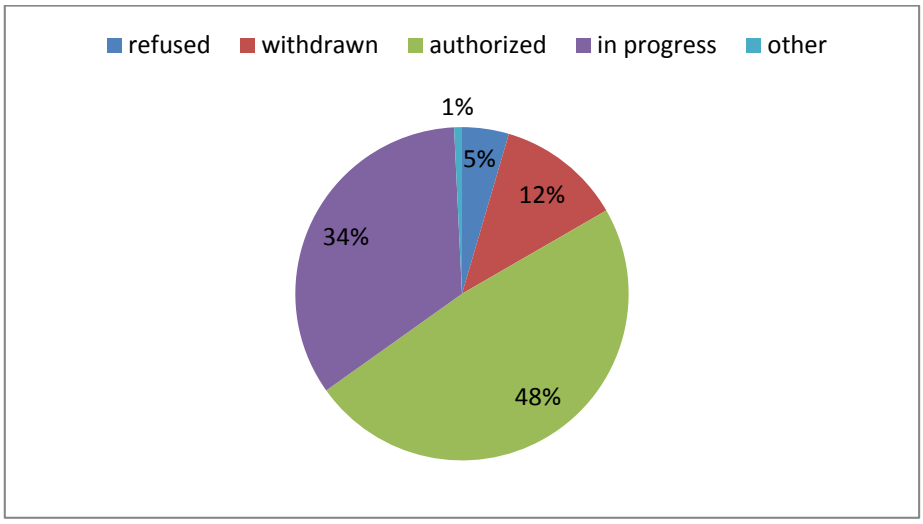

Figure 1.3.: Novel food applications from May 1997 until May 2014 and their current status

1.8. Prebiotics and the Novel Food Regulation

Since prebiotics fall under the guidelines of the Novel Food Regulation, correspondent applications have to be submitted in order to commercialize a product containing prebiotic ingredients.

Thus, these novel food compounds new to the human diet fall under the European regulatory category of “novel foods” and require legislated levels of safety and toxicological assessment before they can be included in food products (FAO, 2007).

Still, legislation covering the use of the word “prebiotic” itself on functional foods is limited. There is an increase in the collection of commercially available products which carry the label prebiotic but for which substantial scientific data is scarce or overall lacking (FAO, 2007).

When the concept of functional foods was introduced in Japan during the 1980s, they could be defined as “any food or ingredient that may trigger a health benefit beyond

traditional functions hitherto known" (Caselato de Sousa et al., 2011). This entailed that prebiotics could be considered functional food ingredients before the enactment of the Novel Food Regulation in 1997. With respect to that matter, it is known that some food ingredients that are nowadays deemed prebiotics have a long history of application within food industry (Gibson and Roberfroid, 1995).

1.8.1. Fructo-oligosaccharides and Inulin

The most prominent examples for that are fructo-oligosaccharides (FOS) and inulin. Fructo-oligosaccharides are short- and medium-length chains of β-D-fructans in with fructosyl units. These oligosaccharides have three to ten monosaccharide units joined by α-glycosidic bonds between terminal fructose and glucose. Depending on the degree of polymerization (DP), which is defined by the number of monosaccharide units, one can differentiate between FOS or oligofructose (DP<10) and inulin (DP up to 60) (Roberfroid, 2002). The linear polymer inulin derives from D-fructose and like FOS can be isolated from plants (Caselato de Sousa, 2011).

There is a wide range of common foodstuffs such as chicory, onion, garlic, artichoke, banana and asparagus that contain high levels of FOS and inulin (Caselato de Sousa, 2011). In the food industry, both FOS and inulin have been commercialized for many years (Gibson and Roberfroid, 1995). In North America FOS and inulin have been marketed as RAFTILOSE®oligo-fructose and RAFTILINE® inulin. In Europe the same company advertises oligofructose as BENEO™oligofructose and inulin BENEO Orafti® inulin.

For the generation of both products chicory is used as isolation material. They are both marketed as dietary fiber sources and claimed prebiotics which shall improve bowel regularity. Some suggested applications for Beneo™oligofructose are dairy products, fruit preparations, juices, breakfast cereals and baby food. For the inulin type product of the company some applications are bread and baked goods, dairy products, frozen desserts, chocolate, soups and sauces. In addition, Orafti®Synergy1, an oligofructose-enriched inulin is also commercially available.

Sensus is another functional food company which brands oligofructose and inulin as Frutalose® and Frutafit®. The health benefits claimed by the company include improving the digestive functions, facilitating weight management issues and enhancing bone health.

When it comes to marketing products with prebiotic character, not only FOS or their equivalents such as inulin have been placed on the market even though they do not fall under the guidelines of the Novel Food regulation.

1.8.2. Galacto-oligosaccharides

Galacto-oligosaccharides (GOS) as well share a long history as food ingredients since the introduction of the functional food concept. Companies have been focusing on the production of several GOS mixtures, produced either as powder or syrup (Playne and Crittenden, 1996). GOS consist of galactose molecules that are linked to the disaccharide lactose. Their generation is due to enzymatic conversion of lactose (transgalactosylation), which correlates positively with the amount of available lactose (Boehm et al., 2004).

Commercial products supplemented with GOS comprise Vivinal® GOS (Friesland Campina), OLIGOMATE® (Yakult), Bimuno® (Clasado), CUP Oligo (Kowa, Europe) and Purimune™ (GTC Nutrition). All these products are marketed to have prebiotic features, primarily owning a bifidogenic effect. Proposed applications are dairy products (including infant nutrition), beverages, desserts, sweets and bakery products.

1.8.3. Lactulose

SOLACTIS SAS is a biotechnology company which launched a product containing lactulose, called Solactis® Galactofructose. Lactulose is a synthetic, non-digestible disaccharide, which consists of one galactose and one fructose molecule. The commercial product is said to have a prebiotic effect and reduces transit time in

the digestive system. Some applications are found in fruit based and dairy products, supplements, beverages, baby food and cereals.

1.8.4. Polydextrose

Polydextrose is another example for a prebiotic that is available on the market. Within the food industry, polydextrose is commonly used as a thickening agent, stabilizer, humectant and carrier and is referred to as food additive with the E-number 1200 within the European Union (EFSA, 2011). The polysaccharide is synthesized by random polymerization of glucose in the presence of minor amounts of sorbitol and an acid catalyst (i.e.citric acid) under high temperature and partial vacuum (Radosta et al., 1992).

Since polydextrose is hard to digest, its ingestion can provide effects similar to those of dietary fiber (Hara et al., 2000). The joint venture of DuPont with the British agribusiness company Tate & Lyle opened the first polydextrose production line called STA-LITE® in Europe. Subsequently, the Danish company Danisco (DuPont®) placed Litesse®polydextrose on the market, “a low-calorie, low glycemic, specialty carbohydrate with prebiotic properties”. Application areas for this product include dairy, beverages, confectionery, sugar-free applications, chocolate and bakery goods. The company WINWAY also established an assortment of polydextrose products.

Furthermore, there is another prebiotic fiber which “maintains a healthy digestive system“, and is also promoted by Tate & Lyle with the PROMITOR® production line.

Table 1.1. summarizes the prebiotics available on the market that do not fall under the Novel Food Regulation (EC) No 258/97.

Table 1.1.: Prebiotics on the market without falling under the NFR

Prebiotic compound	Product name
Fructo-oligosaccharides (FOS) and oligofructose	Beneo™ Oligofructose Frutalose®
Inulin	BENEO Orafti® inulin Frutafit®
Galacto-oligosaccharides (GOS)	Vivinal® GOS OLIGOMATE® Bimuno® CUP Oligo Purimune™
Lactulose	Solactis® Galactofructose
Polydextrose	STA-LITE® Litesse®polydextrose Winway®polydextrose

1.9. Xylan

Xylan is one of the most important biopolymers in nature and is a vital structural polysaccharide in plant cell walls. Approximately one third of all renewable organic carbon earth is composed of xylan (Prade, 1995). Xylan widely prevails in hemicellulose arising from wooden fabric but it can as well be present in various other plants such as cereals, herbs and grasses (Ebringerová and Heinze, 2000). Xylan is - at least in some of its variants - a highly branched heteropolysaccharide and differs in structure and compositions among plant species (Collins et al., 2005). It comprises a backbone of residues of the monosaccharide xylose which are substituted with glucuronic acid, acetyl and arabinose residues (Rennie and Scheller, 2014).

1.10. Xylan degrading Enzymes -Xylanases

Due to the complexity of xylan, a large variety of cooperatively acting enzymes are needed in order to completely hydrolyze the polysaccharide (Puls et al.,1987; Biely,1985; Subramaniyan and Prema, 2002). Xylanolytic enzymes include endo-1,4-β-D-xylanases, β-D-xylosidases, α-D-glucuronidases, α-L-arabinofuranosidases,

acetylxylan esterases, ferulic acid esterases and *p*-coumaric acid esterases. This battery of enzymes facilitates the complete breakdown of xylan. More precisely, endo-1,4-β-D xylanases and β-D-xylosidases aim at cleaving the xylan backbone, yielding short chain xylooligomers. α-L-arabinofuranosidases, acetylxylan esterases, ferulic acid esterases and *p*-coumaric acid esterases are considered accessory enzymes and thus responsible for the removal of side substituents of xylan (Sunna and Atranikian, 1997). Generally, only few organisms contain the full complement of enzymes for total xylan hydrolysis.

Originally, xylanases were regarded pentosanases and the first reports of xylanases date back to the 1950ies (Whistler and Masek, 1955). Today, xylanases are defined as glucosidases, taking part in the hydrolysis of 1,4-β-D-glycosidic linkages in the substrate xylan. They are classified according to physic-chemical properties like for instance isoelectric point and molecular mass.

As stated by Collins et al. enzymes with xylanase activity can be confined to the so-called glycoside hydrolase (GH) families 5, 7, 8, 10, 11, 16, 26, 43, 52, and 62 (whereas generally speaking xylanases usually refer to Family 10 and Family 11) (Collins, et al., 2005).

Xylanases are widespread in nature and occur in fungi, yeasts and bacteria. More specifically, fungal xylanases have been found predominantly in *Aspergillus* spp. *Trichoderma* sp. and *Streptomyces* spp. while bacterial xylanases have been reported among *Bacillus* spp. and *Clostridium* spp. (Henrissat and Bairoch, 1993; Collins et al., 2005).

1.11. Xylanases in Industry

In the recent past, xylanases have found renewed interest due to their probable applicability in a vast range of industrial processes (Arora et al., 2009). An example of commercial applications suggested for xylanases involves the management of agroindustrial wastes to fractionally degrade xylan from biomass, presenting a renewable energy source. Another environmental friendly benefit of the application of xylanases can be seen in the paper industry where they are used for pulp bleaching

to reduce the amount of chlorine in the process (Techapun et al.; 2002 Collins et al., 2005).

Similarly, xylanases could be used for other potential applications which include:

- the clarification of beverages such as fruit and vegetable juices
- the extraction of natural oils, starch and coffee
- improving the elasticity and dough volume of bakery goods
- enhancing the nutritional properties of agricultural feeds
  (Collins et al., 2005)

1.12. Xylo-oligosaccharides

Moreover, xylanases are receiving increasing attention because of their possible application for the production of xylo-oligosaccharides (XOS) which can be used as functional food additives or alternative sweeteners with beneficial properties (Pellerin et al., 1991).

XOS are non-digestible in the human gastrointestinal system and consist of xylose units. In general, XOS are mixtures consisting of oligosaccharides formed by xylose residues linked through β-(1→4)-linkages (Aachary and Prapulla, 2008).

Their prebiotic character can be mainly linked to their ability to stimulate the growth of beneficial bacteria (Bifidobacteria and Lactic Acid Bacteria) (Moure et al., 2006). However, this prebiotic effect of XOS has not been as profoundly studied as those of fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) (Madhukumar and Muralikrishna, 2010). Several health stimulating, biological effects of XOS are predicted such as decreasing cholesterol levels and promoting biological activity of calcium. Furthermore, XOS show an impact on skin related effects, antioxidant activity, anti-allergy, immunomodulatory action etc. (Digantkumar et al., 2012). Despite of these biological effects concerning human health, the use of XOS has been mainly implemented in animal feed and phytopharmaceutical applications (Moure et al., 2006).

For generating XOS, the xylan backbone has to be partially hydrolyzed resulting in new constitutes of lower polymerization degree. Different ways for the production of these sugar oligomers have been described. These methods include chemical, acid hydrolysis, auto hydrolysis and enzymatic treatment (Vázquez et. al., 2000). Broadly, XOS formation requires alkaline extraction of xylan and enzymatic hydrolysis (Digantkumar et al., 2012). A major benefit of the enzymatic method is that there are no undesirable by-products generated. For this XOS production method, xylanases with low exo-xylanase or β-xylosidase activity are preferred since they increase the amount of xylose causing inhibitory effects on XOS formation (Vázquez et al., 2000; Digantkumar et al., 2012,).

1.13. Lactic Acid Bacteria

At present, abundant data exists about xylanases from different microbial origin. Xylanases from fungal origin, especially from *Aspergillus* spp., are investigated and documented best, but enzymes from bacterial species such as *Bacillus* spp. and *Clostridium* spp. (Tallapragada and Venkatesh, 2011) feature also prominently. However, there is a lack of knowledge on sources arising from food and food products.

Lactic acid bacteria (LAB) have a long history of applications in food, pharmaceutical and chemical industries and abundant information is available about their growth conditions and nutrient requirements (Das and Goyal, 2012). The major and most popular known role of LAB is in the preparation of fermented dairy products including cheese, butter, yogurt, buttermilk and kefir. Moreover, they are exploited for the pickling of vegetables, wine-making, baking, curing fish, meat and meat products (Taskila and Ojamo, 2013).

As a consequence, Lactic acid bacteria could be a promising source for xylanases that have food-grade status.

LAB encompass a clade of gram positive rods or cocci, non spore forming bacteria whose main characteristic is their ability to transform sugar to lactic acid. Genera of LAB are referred to as *Lactobacillus, Leuconostoc, Lactococcus, Lactosphaera, Enterococcus, Streptococcus, Tetragenococcus, Vagococcus, Melissococcus,*

Oenococcus, Weissella and *Pediococcus*. LAB widely occur in nature and are also present in the human digestive systems (Taskila and Ojamo, 2013).

1.13.1. Xylanases and LAB

There are hardly sufficient reports existing on xylanase genes among LAB strains. Most reports available about xylanase genes are associated with fungal species. However, genes with similarity to xylanase genes could be detected among *L. brevis* and *L. lactis*, *L. plantarum* and *Lactobacillus pentosus* (Erlandson et al., 2001; Cantarel et al., 2009; Okano et al., 2009). *L. lactis* especially was shown to contain genes encoding xylanases or particularly xylanolytic enzymes (Siezen et al., 2008). For instance *xynB* encodes a β-1,4-xylosidase relevant for xylan breakdown. Additionally, *xylA* and *xylB* encode xylose isomerase and xylolukinase, respectively (Erlandson et al., 2001), indicating a certain importance of xylose utilization in *L. lactis*. Most of these genes and others in LAB, however, are merely annotated automatically as xylanase or xylosidase genes by software tools mining the ever-growing data in genomic databases, and are not experimentally confirmed as having the respective activity.

Summarizing there seems to be genetic potential among LAB regarding xylanase activity. However, a lack of reports on verifying and testing for the xylanase genes in LAB can be observed. Moreover, the data on these genes is not consistent and show a distinctive research gap.

2. Purpose of the Study

The aim of this master thesis is to seek new sources of xylanases that are considered food-grade i.e. which are qualified for human consumption. Taking this into account, Lactic acid bacteria (LAB) came up as a promising target for the pursuance of xylanase production. In order to consolidate this approach, the focus was put on Lactic acid bacteria strains all originating from food and food products.

For the practical work of this thesis, twenty two strains have been screened for possible xylanase activity using different methods divided into qualitative and quantitative assays. Most of those strains are unknown and were all collected and isolated from Asian countries, foremost Vietnam, where the practical work and research for this thesis has been performed. One key aspect of this work is to find out whether the selected Lactic acid bacteria show any sign of xylanase activity or not. Thus, this thesis tries to combine knowledge on the ever-growing importance of xylanases for future food industry and a long-term study of Lactic acid bacteria for food product applications.

Another approach of this thesis is to include European food legislation (i.e. the Novel Food Regulation (EC) No 258/97) and to discuss the fundamentals of the authorization of a novel food which in this case would be prebiotics. Since xylanases can be associated with the generation of xylo-oligosaccharides which are classified as prebiotics, it seemed plausible to further review prebiotics in detail, concerning their properties and their legal status within the European Union. This proposition will be thoroughly debated in the “Discussion” part of this thesis. In addition, a short glance on the legal framework of food enzymes in general within the EU will be given as well. This excursion part will also be dealt with in the discussion.

Overall, the following research questions can be raised:

- Is there any potential of xylanase activity among Lactic acid bacteria?
- How is the current status of prebiotics regarding the Novel Food Regulation 258/97?

Figure 2.1. highlighting the key points of this thesis.

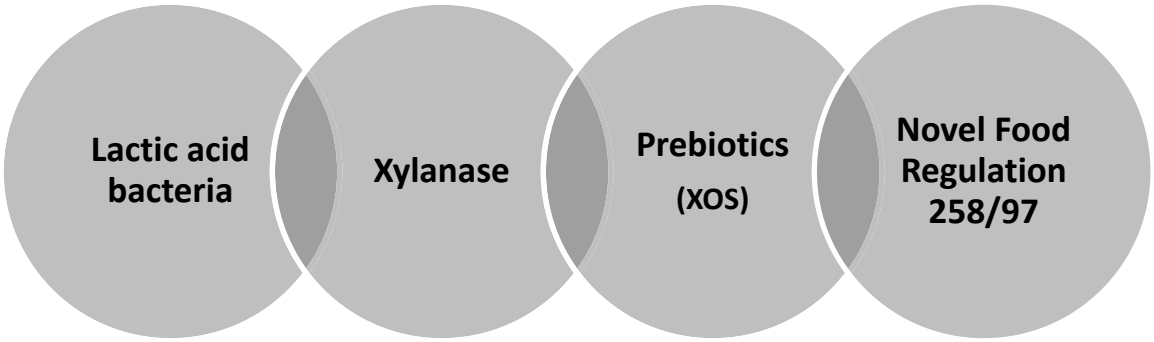

Figure 2.1.: Summarizing the key points of this thesis

3. Materials and Methods

3.1. Microorganisms

The strains that were used in this experiment are listed in table 3.1. Most of the strains were provided by the Center for Research and Development in Biotechnology of the Hanoi University of Science and Technology, Vietnam. *Lactococcus lactis* and *Lactobacillus ruminis* were provided by the Food Technology Department of the Hanoi University of Agriculture, Vietnam.

All of the strains used in this thesis, except for *L. plantarum* (from human saliva), emanate from food and food products originating from Vietnam, Korea and Japan.

Table 3.1.: Used strains and their sources

Index	Strain name	Source
SMC2	unknown	yogurt from Korea
HU5	*Lactobacillus acidophilus*	Korea
SYG1	unknown	yogurt
HL1	unknown	chili pepper
LAC7	unknown	banana peel
DSM20081	*Lactobacillus bulgaricus*	yogurt
NCTH25	unknown	nem chua* (Thanh Hóa**)
WCFSI	*Lactobacillus plantarum*	human saliva
NFRI 7306	*Lactobacillus lactis*	Japan
NCDC	unknown	nem chua*
LD12	unknown	yogurt
LCM51	unknown	egg plant in salt
NFRI 7328	unknown	Japan
CNTPH6511	unknown	nem chua*
SH1	unknown	breast milk
LM9	unknown	pickled gherkins
LD3	unknown	pickled gherkins
NFRI 7334	unknown	Japan
NFRI 7301	unknown	Japan
NFRI 7325	*Lactobacillus casei*	Japan
HUA	Lactococcus lactis	Vegetable source
HUA	Lactobacillus ruminis	Vegetable source

*fermented pork charcuterie, wrapped in banana leaves: a traditional Vietnamese snack

** mainly produced in the region of Thanh Hoá

3.2. Culturing Conditions

The bacterial strains were all grown in deMan Rogosa and Sharpe (MRS) broth medium, prepared according to the protocol of the Leibnitz Institute DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and obtained from Merck (Darmstadt, Germany). Colonies were streaked on solid MRS medium and incubated overnight at 30°C without agitation.

For further use and xylanase activity assays, the bacterial isolates were maintained in liquid MRS medium containing 0,5 % or 1% of commercial beechwood xylan from ROTH (Karlsruhe, Germany) at 30°C without shaking. The glucose content of the MRS medium was reduced from 20g/l to 2g/l. Thus, the strains would have sufficient sugar to germinate properly and consequently a growth curve could be established. For initial growth of the bacteria, a small amount of glucose had to be added to the medium. MRS broth and agar medium and required glassware were sterilized accordingly (autoclaved at 121°C for 15 min) to avoid all sources of contamination.

3.3. Collection of Natural Xylan Samples

In addition to the general investigations by means of commercial xylan, attempts for an alternative xylan source were made by collecting, preparing and using natural xylan from corn cob. Corn cobs were collected from the experimental fields of the Agronomy Department of Hanoi University of Agriculture, Vietnam. Precautionary measures were taken to minimize the contamination.

3.3. 1.Preparation of Natural Xylan

The corn stalks were de-seeded and subsequently ground to debris pieces of 0,1 – 0,5 mm in size. A solution with a 10 % concentration of sodium hydroxide was added to the resulting powder and then cooked in hot boiling water. The pH was balanced with a 10% concentrated HCl solution, then cooked again in order to

remove excess water and finally dried to a solid mass overnight. Figure 3.1. shows natural xylan isolated from corn cob

Figure 3.1.: Natural xylan from corn cob

3.4. Qualitative Assay on Xylanase Activity

Growth of the investigated strains was monitored spectrophotometrically with GeneQuant 1300 from GE Healthcare Life Sciences (Cleveland, OH) and with UV-1800 spectrophotometer from Shimadzu (Kyoto, Japan) by measuring the O.D. at 620 nm. Changes in the pH range were also observed. Samples were diluted 1:5 and collected in random order over a specific time period up to 5 days. The supernatants of the samples were then utilized as crude enzyme extracts.

3.5. Congo Red Assay

Complementary to the qualitative display of xylanase activity, the congo red staining according to Teather and Wood (1982) was performed. Isolated microorganisms were cultivated overnight on MRS agar plates containing xylan (0.5%) as sole carbon source. Therefore a volume of 30 µl of cell culture was inoculated in the agar where small cavities have been carved previously for better gathering of the liquid culture. The overnight cultures were flooded with congo red solution (BDH Chemicals Ltd., Poole, UK) in a concentration of 3 mg/ml and left for 10 minutes. Then the congo red solution was poured off and a 1 M NaCl solution was

added to the petri dishes to destain unbound regions. Positive xylanase colonies can be observed as clearing zones.

3.6. Quantitative Assay on Xylanase Activity (stopped assay)

Xylanase activity was determined by measuring the release of reducing sugars and oligosaccharides, i.e. the formation of xylose. The supernatants of 100 µl were mixed in eppendorf tubes with 800 µl of the substrate (xylan 1% concentration) and were incubated at 30°C in the incubator at 300 rpm for 30 minutes.

For the deactivated (reference) samples which were used to compare the enzyme activity, the rest of the sample (900µl) was inactivated by means of heating the samples in a 100°C water bath. Subsequently, the deactivated samples were centrifuged again at 6000 rpm at 4°C for 10 minutes. Then, the supernatants of 100 µl were mixed with 800 µl of substrate xylan 1%.

After an incubation time, i.e. reaction time of 30 minutes at 30°C with shaking at 300 rpm, all samples were inactivated by heating them in a 100 °C water bath for 5 minutes and centrifuged for 10 minutes at 4°C with 6000 rpm.

The supernatants of 800 µl were mixed with 800 µl of 3,5-dinitrosalicylic acid solution (DNS) from Merck. All samples were heated for 10 minutes at 100°C, cooled down to room temperature for 5 minutes and the absorbance was measured at 540_{nm} for xylanase activity. DNS serves as reagent and sugar reduction can be analyzed colometrically. To this end, the color of tested samples would turn from yellow to brown-red as shown in the figure 3.2. below.

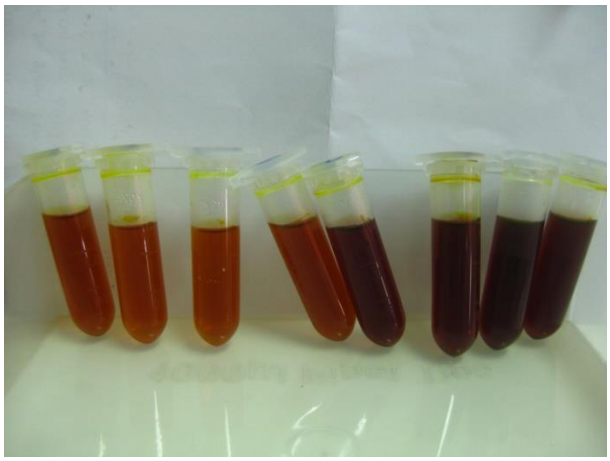

Figure 3.2.: DNS assay showing the sugar reduction via color reaction from yellow-orange to brown-red color.

3.6.1. Standard Curve

For the quantitative analysis of xylanase activity a standard curve had to be integrated. The stock solution was a 50 mM watery solution mixed with the corresponding standard xylose (Sigma, St.Louis, MO). Out of the stock solution seven different standards were made ranging from concentrations of 0.5, 0.75, 1, 1.25, 1.5, 1.75 and 2 mM. Accordingly, DNS assay was performed with the standards showing the color reaction from yellow to red brown demonstrating that xylose is present in the standards.

Table 3.2.: Composition of standards

Standard solution	1	2	3	4	5	6	7
Stock solution µl	8	12	16	20	24	28	32
H_2O µl	792	788	784	780	776	772	768
DNS µl	800						
Concentration mM	0.5	0.75	1	1.25	1.5	1,75	2

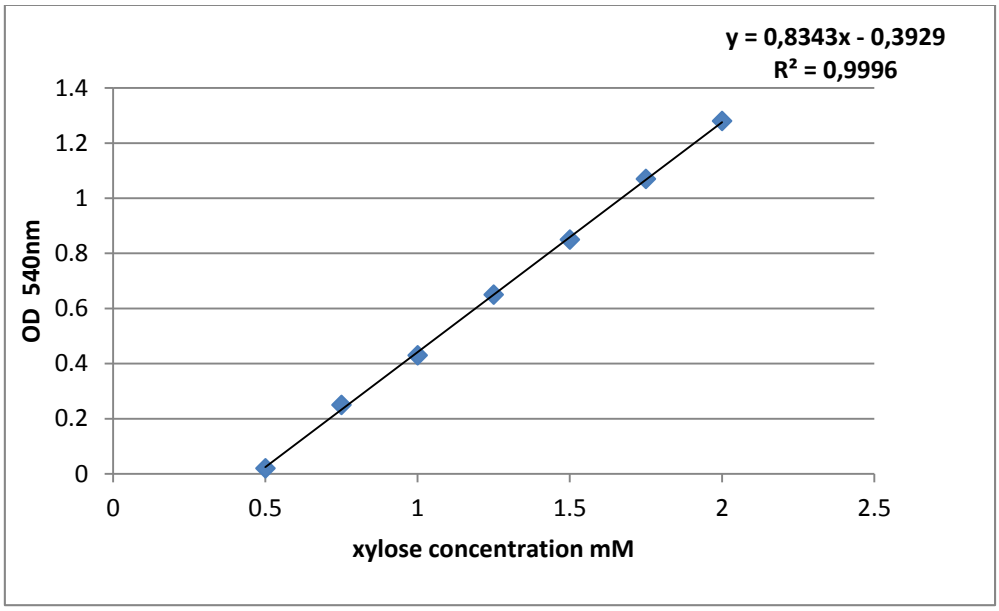

Figure 3.3.: Standard curve for quantitative xylanase activity assay

Figure 3.3. indicates the standard curve with a positive correlation of $R^2 = 0{,}9996$.

The standard curve enables the calculation of enzyme activity measured in Units per ml (U/ml).

Calculation:

$$\frac{\mathrm{U}}{\mathrm{ml}} = \frac{\mathrm{ODsample} - \mathrm{ODreference}}{x} \,\mathrm{x}\, \frac{1}{\mathrm{t}} \,\mathrm{x}\, \frac{\mathrm{V1} + \mathrm{V2}}{1000\mu\mathrm{l}} \,\mathrm{x\ dil}$$

$\frac{\mathrm{U}}{\mathrm{ml}}$	activity of xylanase
OD	absorption measured at 540 nm
Sample	samples activated
reference	samples inactivated
x	slope of standard curve
t	reaction time (30 min)
V1	volume of sample (100 µl)
V2	volume of xylan (800 µl)

dil dilution factor

The absorbance of the activated test samples was deduced from the values of the inactivated (reference) samples.

One Unit releases 1.0 milligram of reducing sugar equivalents from the substrate xylan per minute.

3.7. HPLC

High performance Liquid Chromatography (Agilent Technologies, USA) was performed for checking the formation of xylose. The method implemented for this analysis was isocratic elution which means the concentration of mobile phase is kept constant during the whole chromatographic analysis.

The column which was used for HPLC was the Aminex HPX-87P column from BioRad (Hercules, CA) applicable for pentoses such as xylose. The conditions for the HPLC run were set at a maximum flow rate of 0.6ml/min ($V_{max)}$ at a maximum temperature of 60°C (T_{max}).

A 10mM sulfuric acid solution served as eluent and the detector for measuring the analyte was the Refractive Index Detector (RID) from BioRad set at 35°C. Five standards in a concentration range of (0.07815 – 2.5 mg/ml) were used for the standard curve. The expected peak for xylose is after a retention time of 15 minutes.

4. Results

4.1. Qualitative Data of Xylanase Activity

For the prescreening of all the strains used in this study the congo red assay was performed and subsequent results should help aiming for the strains of interest to be further investigated for xylanase activity. 17 different strains were stained on 5 different pertri dishes with congo red. No specific halo zones could be observed but five different colonies grew on the surface of the xylan containing agar. Since possible reasons for the growth of the colonies were not clear they were further cultivated and analyzed.

All LAB strains were checked pH-metrically and spectrophotometrically so that it could be observed immediately if the strains were growing appropriately.

In theory, it was expected that the LAB strains would show a lag-, log-, and stationary phase using up all the glucose at first and eventually trying to start using the xylan as new carbon source in order to prolong lifetime. This would then mean that after the stationary phase, a new growth curve could begin again and this could be observed by measuring the pH and the OD_{620}.

On the first attempt, five strains were screened, measuring the two above mentioned parameters. These five strains were: *L. plantarum*, *L. acidophilus*, CNTP6511, LAC7 and one colony (colony 5) from the prescreening with congo red assay. The medium containing no bacteria culture was used as a control. The following figures show their results regarding changes in OD_{620} and pH.

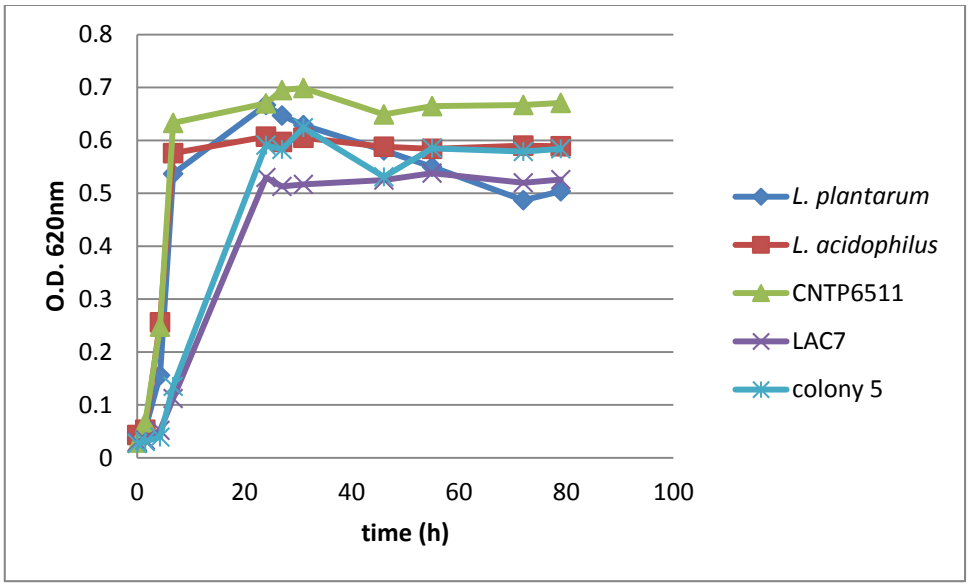

Figure 4.1.: Growth curve of *L. plantarum*, *L. acidophilus*, CNTP6511, LAC7 and colony 5 in MRS medium without xylan.

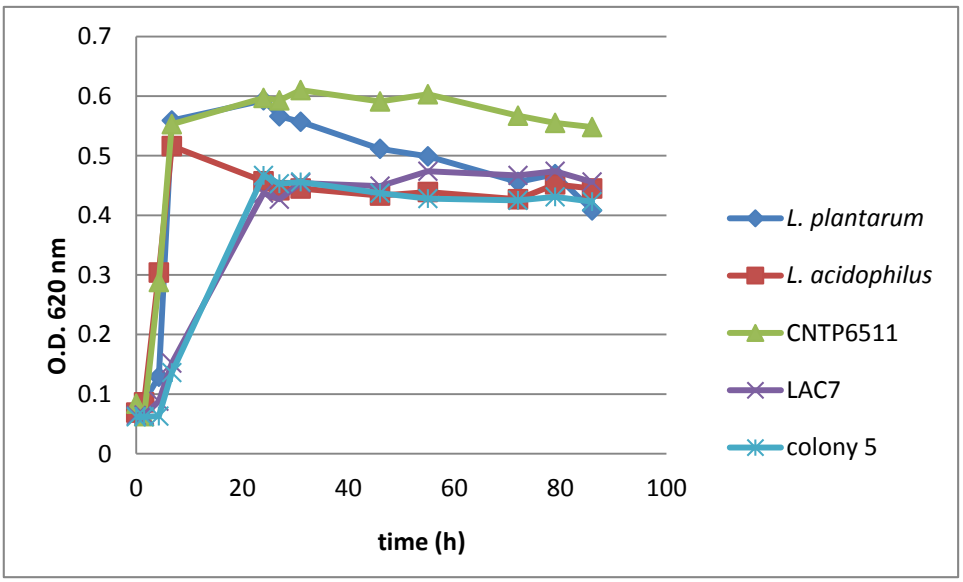

Figure 4.2.: Growth curve of *L. plantarum, L. acidophilus*, CNTP6511, LAC7 and colony 5 in MRS medium with 1% xylan

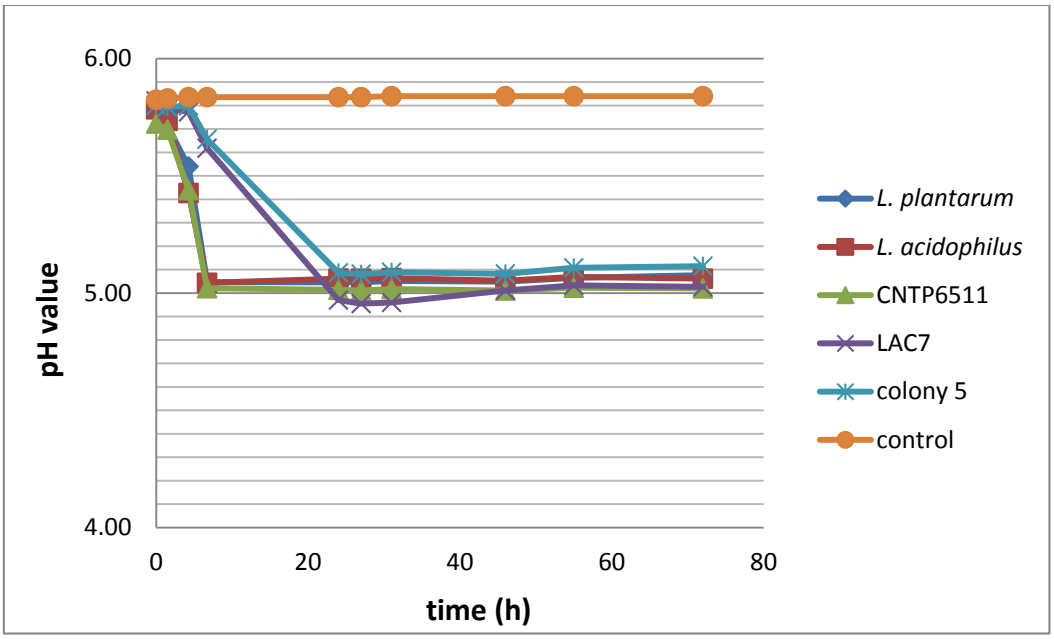

Figure 4.3.: Developing of pH value of *L. plantarum*, *L. acidophilus*, CNTP6511, LAC7, colony 5 and control (medium only) in MRS medium without xylan.

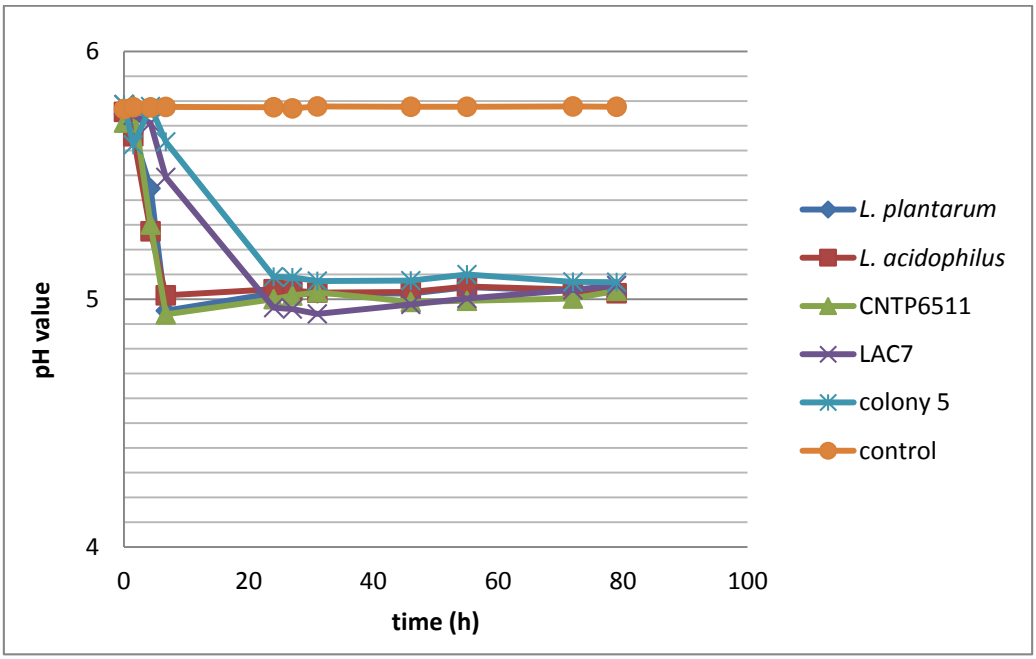

Figure 4.4.: Developing of pH value of *L. plantarum*, *L. acidophilus*, CNTP6511, LAC7, colony 5 and control (medium only) in MRS medium with 1% xylan.

Additionally, each medium was observed to check whether there is any difference between the MRS medium with xylan compared to the MRS medium without xylan. MRS medium with xylan tends to have a slightly higher OD (approximately +

0.035nm) and a smaller pH than MRS medium without xylan. Figure 4.5. and 4.6. show the comparison of the two media in OD and pH.

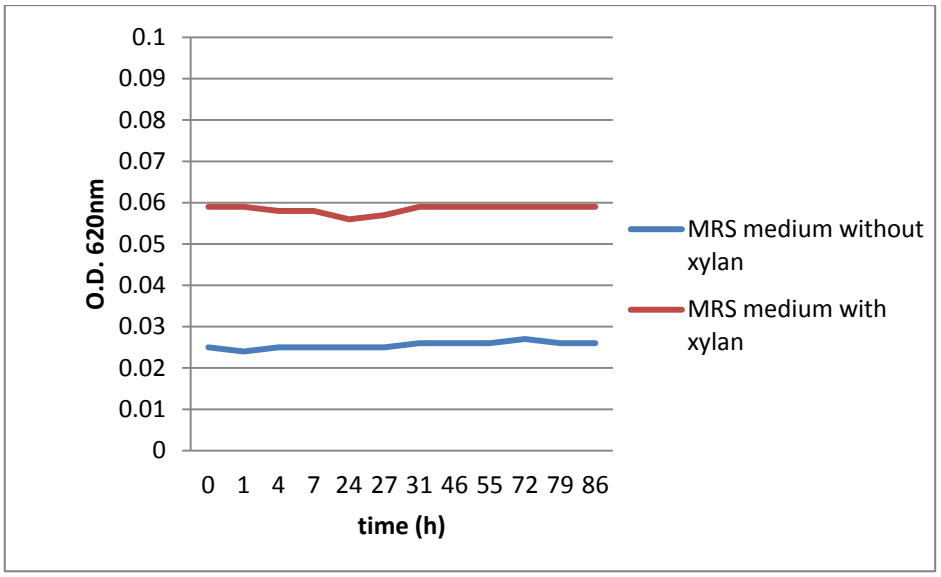

Figure 4.5.: Comparison in O.D. of MRS medium with and without xylan

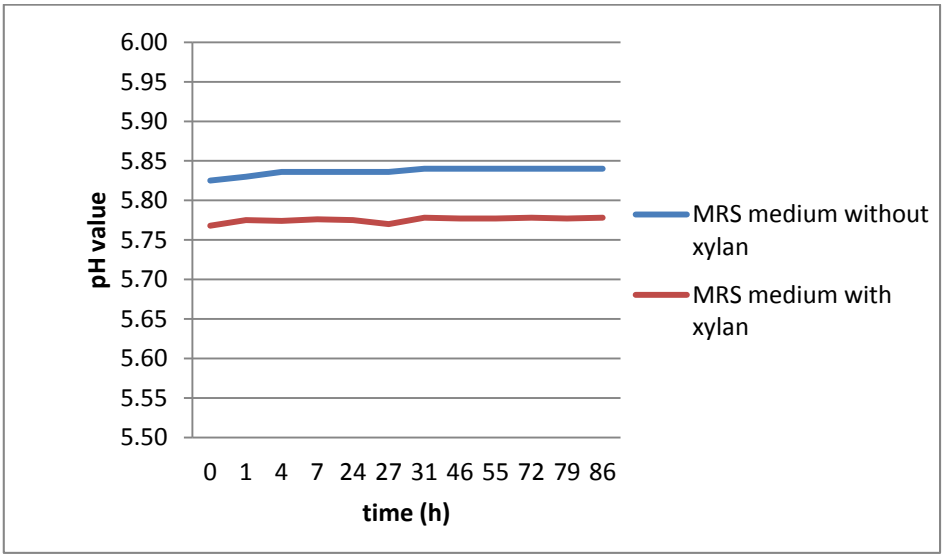

Figure 4.6.: Comparison in pH value of MRS medium with and without xylan

Then, in another trial, 10 different strains including *L. plantarum*, *L. acidophilus*, CNTP6511, LAC7, LCM51 and the five colonies from prescreening with congo red assay were further analyzed by means of the same parametric measures than before over a time period of 96 hours. Figure 4.7. and 4.8. show their results in O.D. and pH.

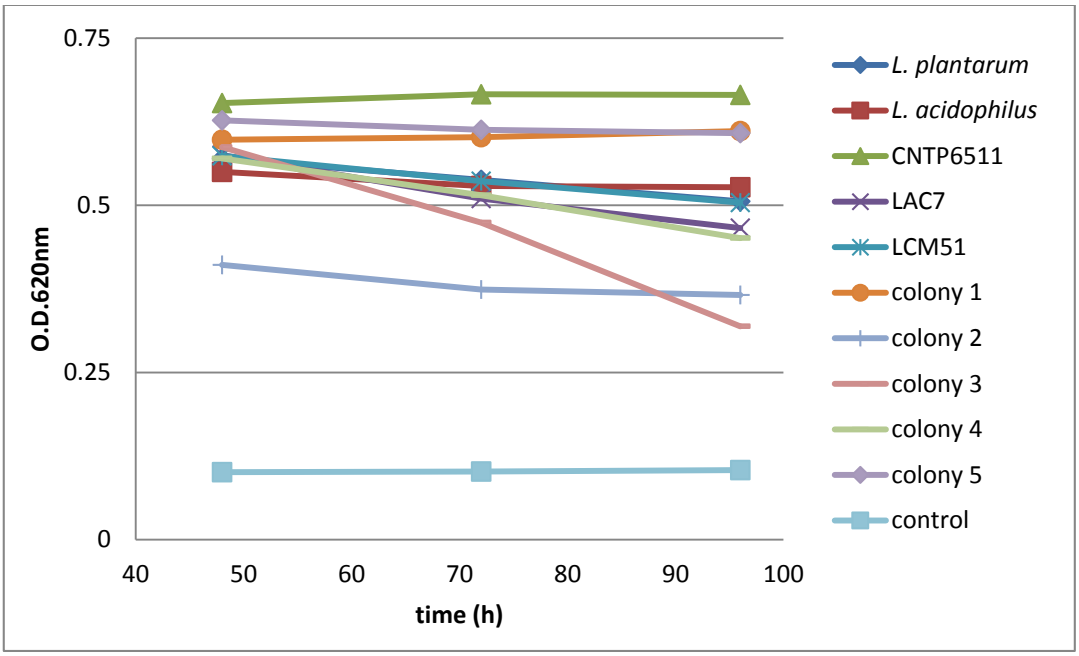

Figure 4.7.: O.D.$_{620nm}$ of 10 strains and control (medium with 0.5% xylan)

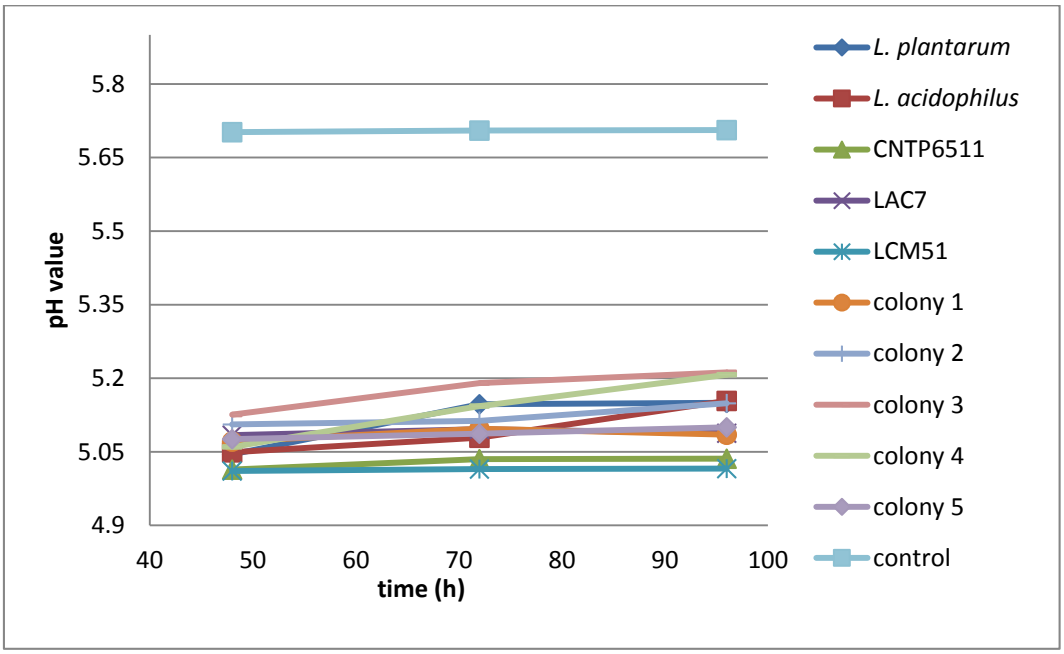

Figure 4.8.: pH value of 10 strains and control (medium with 0.5% xylan)

Furthermore, two more LAB strains such as *L. ruminis* and *L. lactis* were screened accordingly as indicated in figure 4.9. and 4.10..

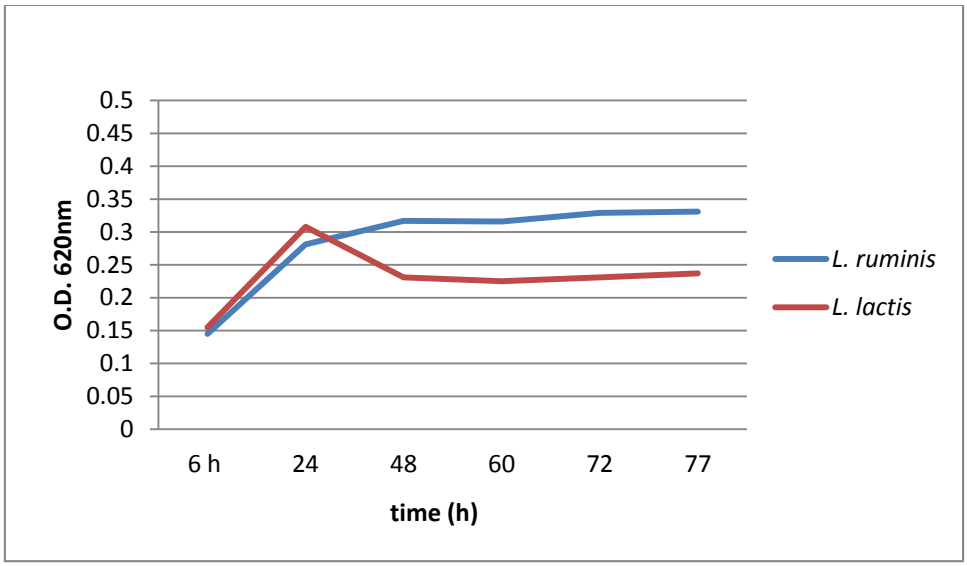

Figure 4.9.: O.D.$_{620nm}$ of *L. ruminis* and *L. lactis*

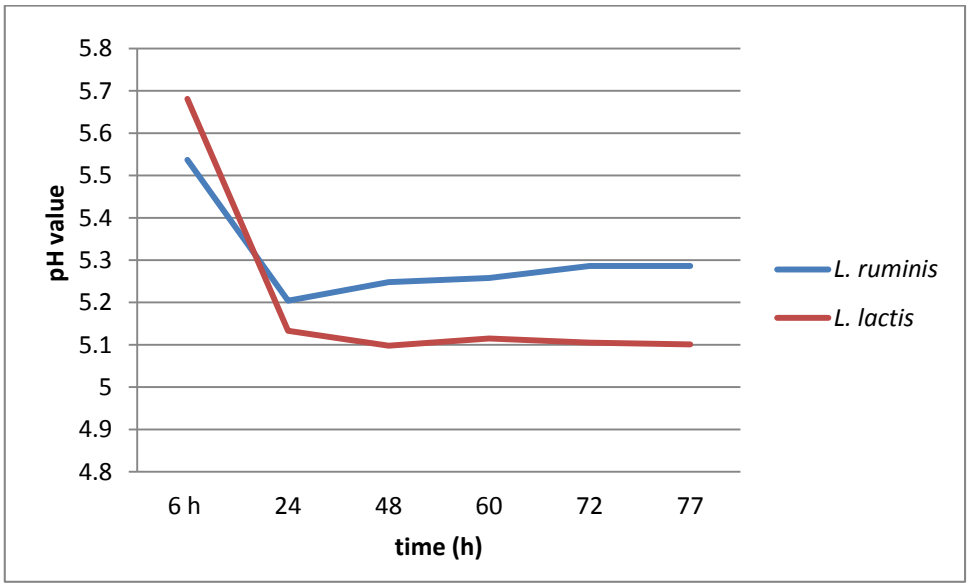

Figure 4.10.: pH value of *L. ruminis* and *L. lactis*

In order to complement these results the congo red assay was performed as well for those 10 strains from figure 4.7. and 4.8.. No halo zones could be seen; therefore no sign of xylan degrading activity could be noted. Results can be seen in the following figures.

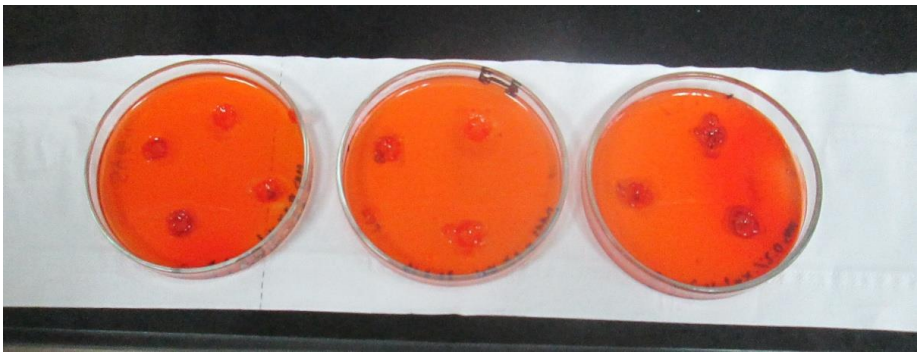

Figure 4.11.: Congo red assay of 10 LAB strains

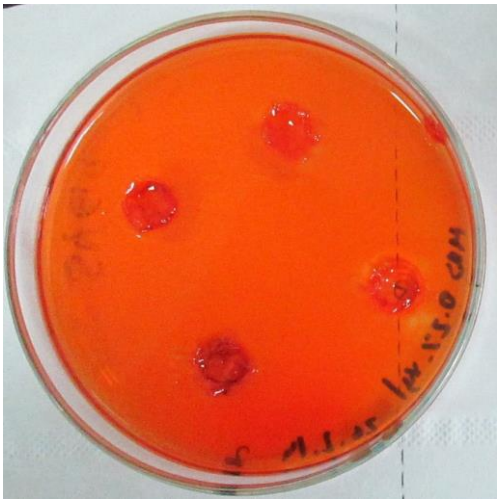

Figure 4.12.: Congo red assay of LCM51, *L. plantarum*, *L. acidophilus* and CNTP6511

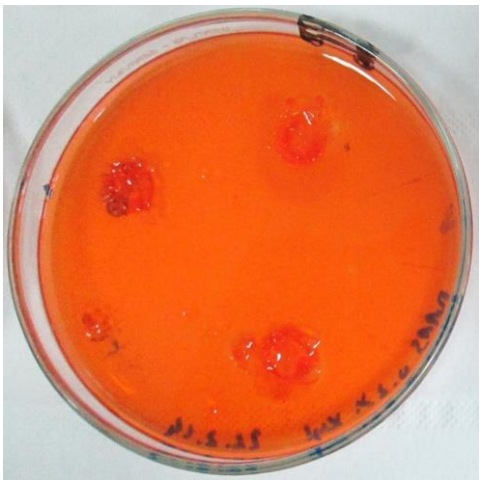

Figure 4.13.: Congo red assay of LAC7,colony 1 and colony 2

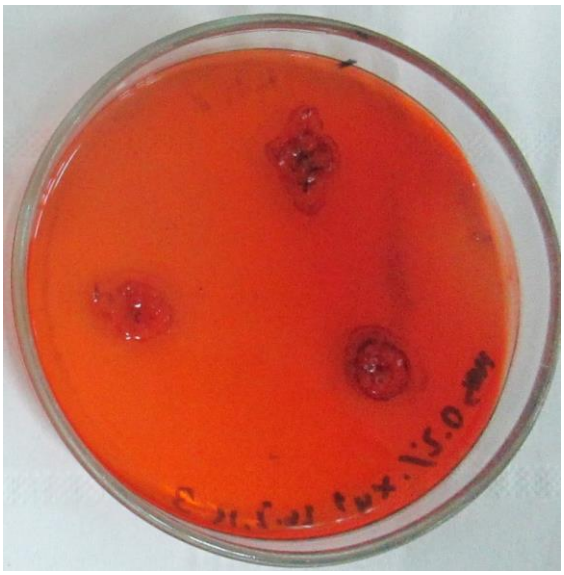

Figure 4.14.: Congo red assay of colony 3, 4, and 5

Further congo red assay results of 12 LAB strains can be seen in the following figures.

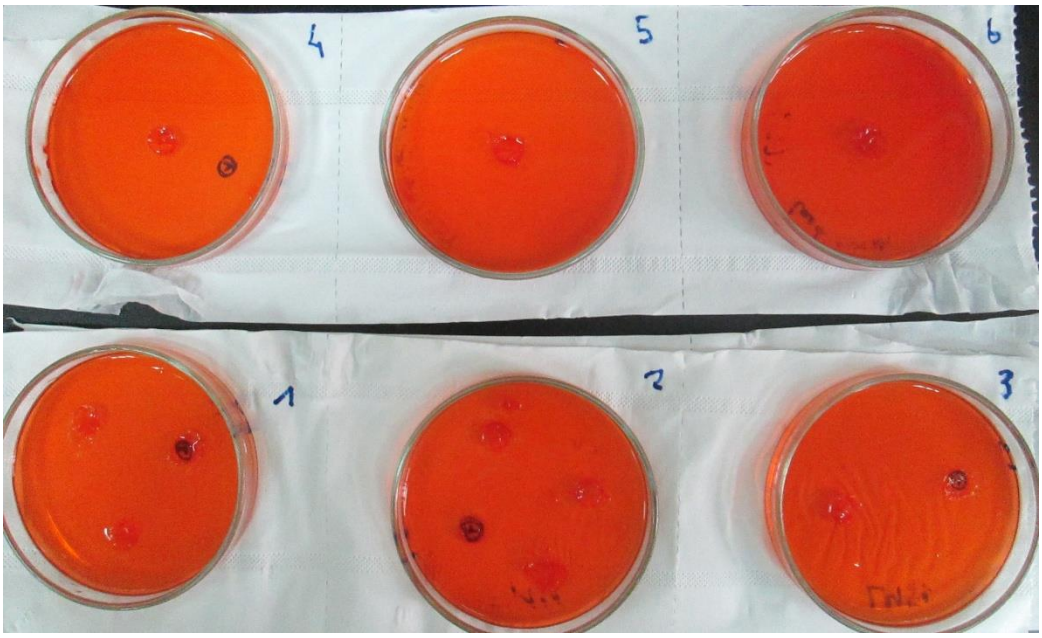

Figure 4.15.: Congo red assay of 12 LAB strains

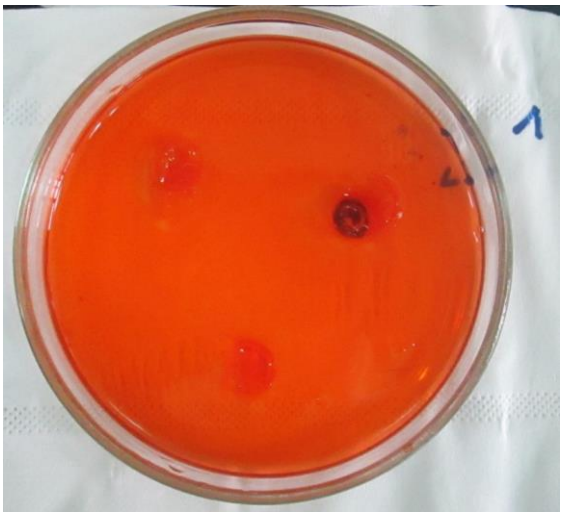

Figure 4.16.: Congo red assay of *L. plantarum*, *L. acidophilus* and CNTP6511

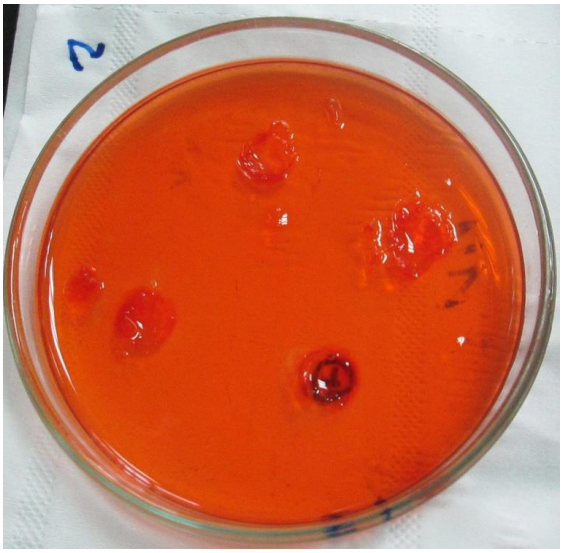

Figure 4.17.: Congo red assay of colony 3, 4, 5 and LCM51

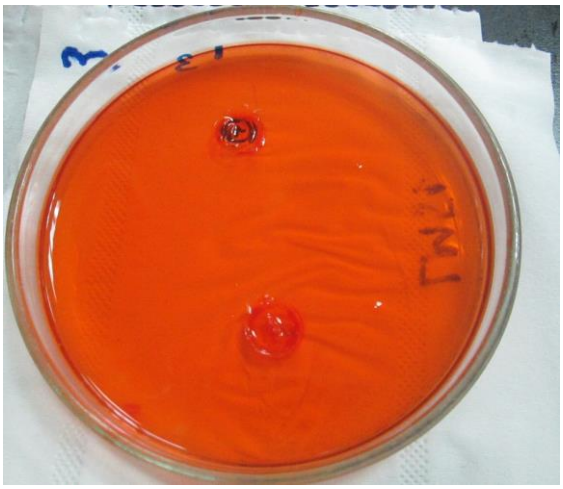

Figure 4.18.: Congo red assay of *L. ruminis* and Lactococcus *lactis*

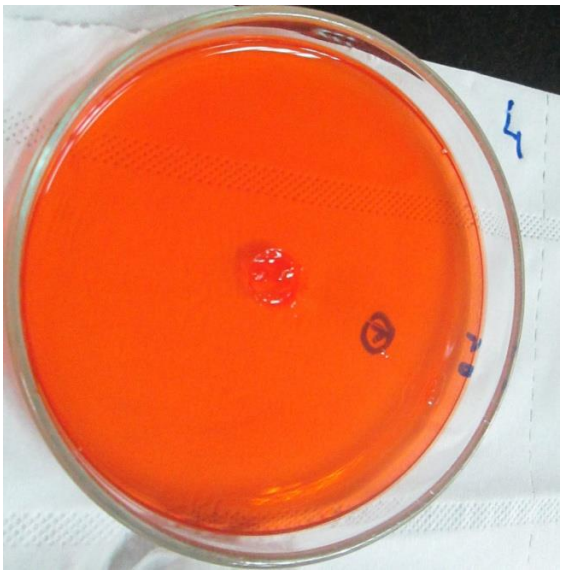

Figure 4.19.: Congo red assay of LAC7

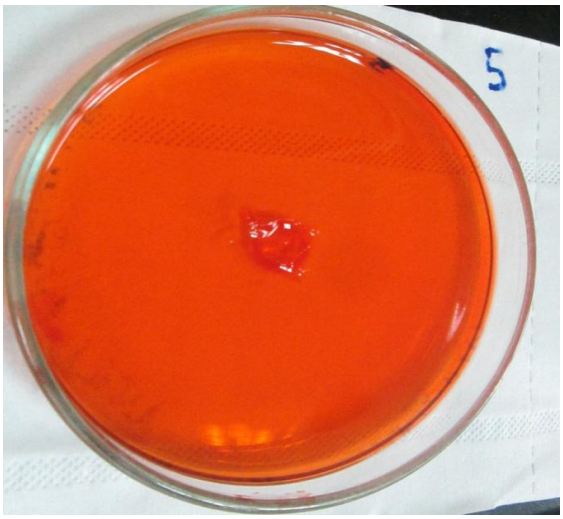

Figure 4.20.: Congo red assay of colony 1

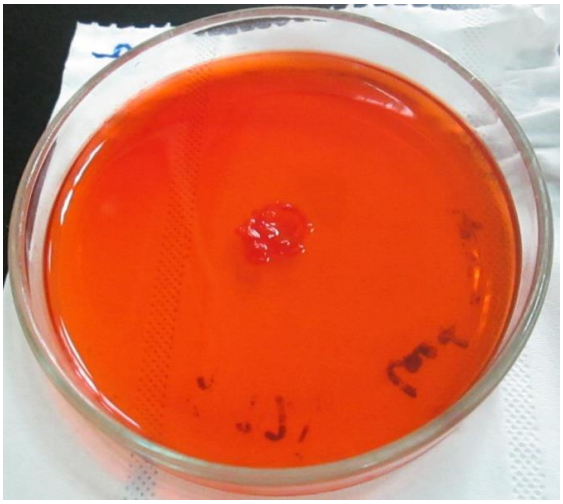

Figure 4.21.: Congo red assay of colony 2

Figure 4.22.. shows the comparison in OD_{620nm} of other 12 strains grown in MRS medium without xylan and MRS medium with 0.5% of xylan after 48 hours.

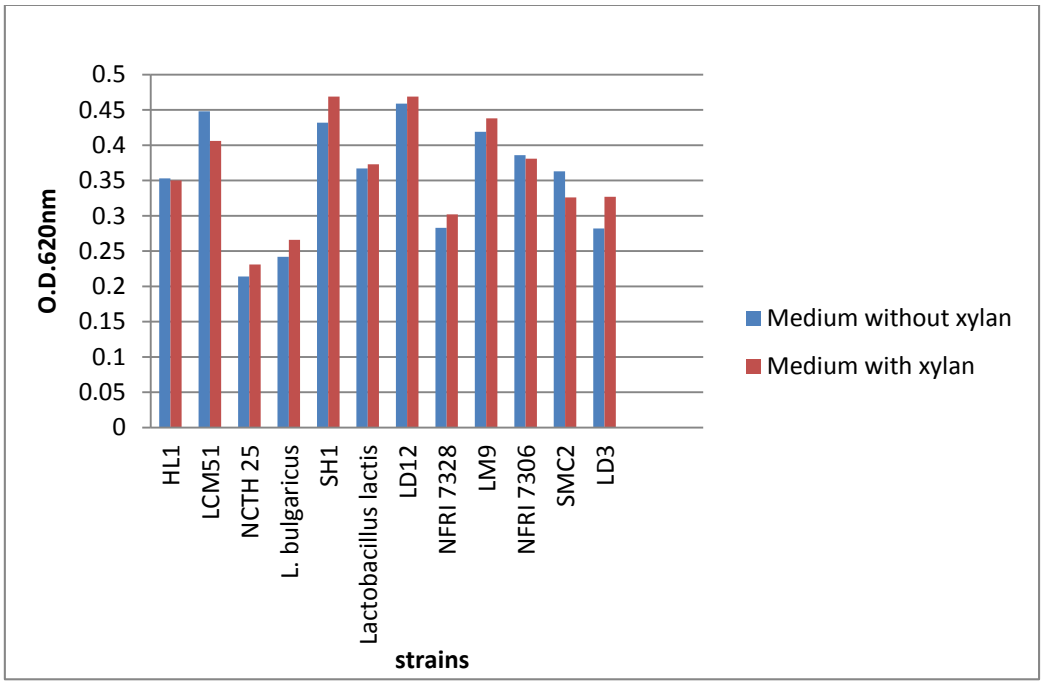

Figure 4.22.: Comparison in O.D. at 620 nm of 12 LAB strains grown in medium without and with xylan

Correspondingly, figure 4.23. depicts the comparison of the two different media in pH value in all fifteen strains together with a control (no cultures added).

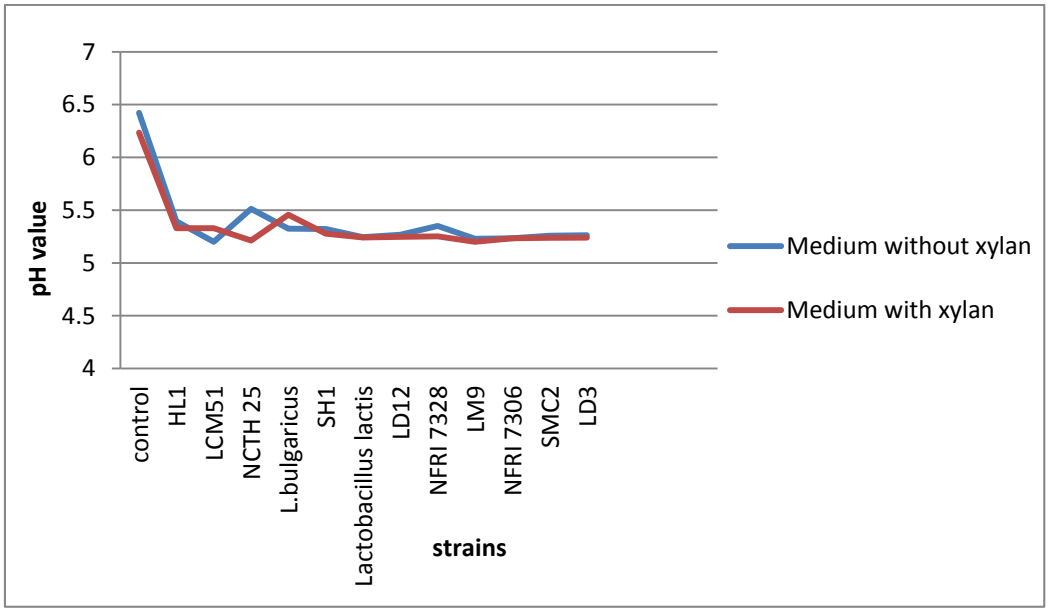

Figure 4.23.: Comparison in pH value of 12 LAB strains and control, grown in medium without and with xylan

To this end, congo red staining was made again in order to supplement the data of those 12 strains.

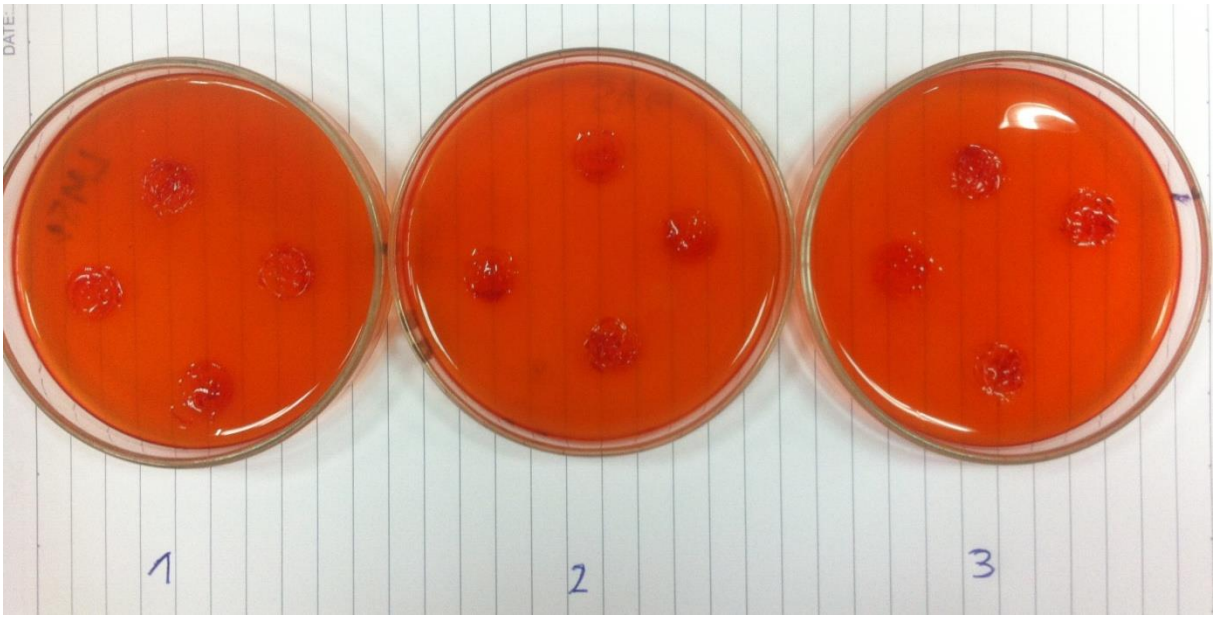

Figure 4.24.: Congo red assay of the 12 strains

Additionally, it was also intended to check if there is any sign of living in MRS broth solely containing natural xylan from corn cob in a concentration of 6 %.

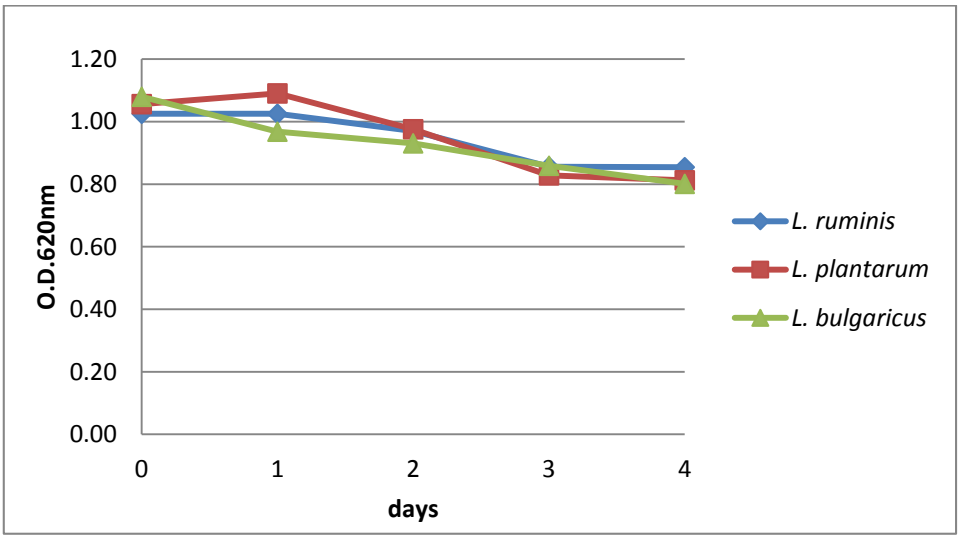

Figure 4.25.: Growth curve of *L. ruminis*, *L. plantarum* and *L. bulgaricus* in MRS medium with 6% of natural xylan from corn cob.

HPLC analysis was made in order to check qualitatively whether some of the strains show any xylose formation. See appendix.

4.2. Quantitative Data of Xylanase Activity

Enzyme activity was measured colorimetrically by means of DNS solution. Since results of congo red assay, pH and O.D. showed no explicit sign of any xylan utilization by the bacteria, only a few bacteria strains such as *L. plantarum, L. ruminis, L. lactis, L. bulgaricus*, were selected for further analysis with DNS. Since these four strains are known and have been sequenced already they were chosen for the quantitative proof of xylanase activity.

In one trial, *L. ruminis*, *L. plantarum* and *L. bulgaricus* were observed during a time period of 40 hours starting after 40 hours of cultivation in MRS medium broth supplemented with xylan 0.5%. Figure. 4.26. shows the results and the U/ml of enzyme product.

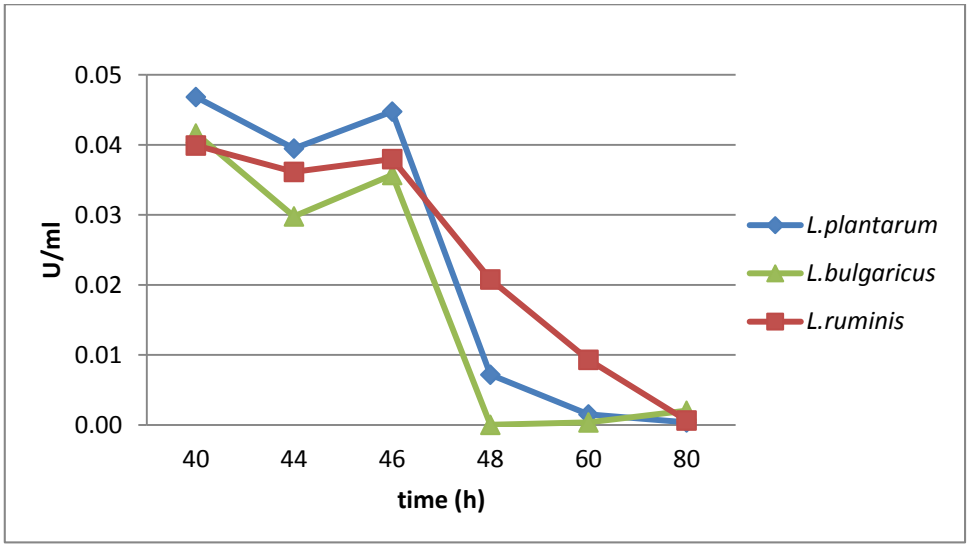

Figure 4.26.: Quantitative xylanase activity results from *L. ruminis, L. plantarum* and *L. bulgaricus*

Additionally, *L. lactis* was investigated after 48 hours of cultivation in MRS medium broth with 0.5% xylan. Samples were taken randomly lasting until 120 hours of cultivation. The result is indicated in figure 4.27.

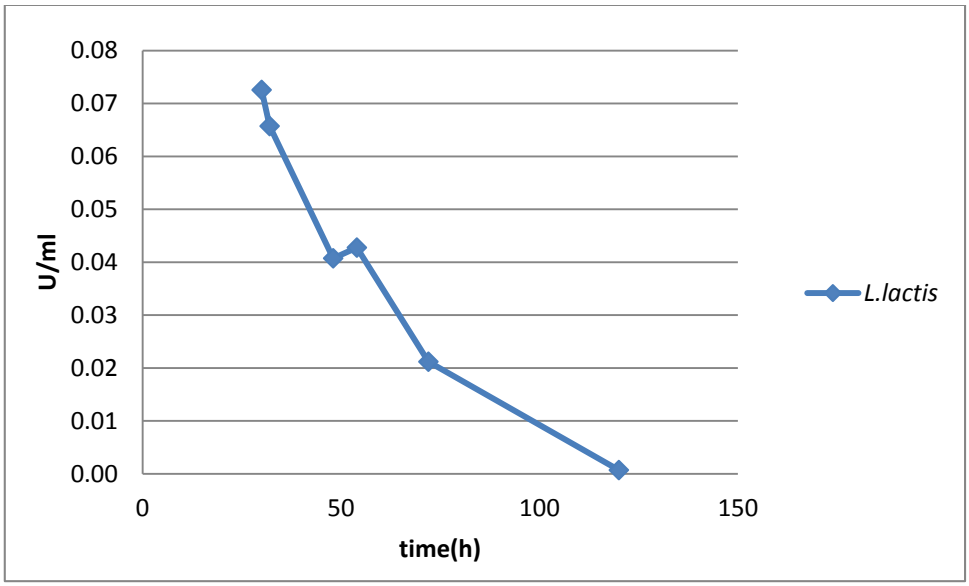

Figure 4.27.: Quantitative xylanase activity result from *L. lactis*

No evidence of xylanase activity was found regarding quantitative assay with DNS.

5. Discussion

5.1. Xylanase from Lactic Acid Bacteria

Since enzyme technology has become more and more prevalent in the food industry a wide spectrum of research has been performed on enzymes implemented in food production processes. Xylanase is one of these enzymes, which has drawn increasing attention in the past few years. Currently the most widely used application of xylanase can be seen in the bleaching of Kraft pulp. By means of xylanase the number of bleaching steps can be reduced as well as the chemical consumption (Techapun et al., 2002). The second major application of xylanases is in the baking industry. Xylanases are of great importance in baking as they have been found to improve the elasticity and the strength of the dough, thereby allowing easier handling, a bigger bread volume and improved crumb structure (Collins et al., 2005; Butt et al., 2008). In addition, stickiness of the dough and staleness of the bread can be reduced by means of xylanase application. So far, there is strong evidence of literature dealing with the application of xylanases in breadmaking, where, as a consequence, they potentially may substitute different emulsifiers and chemical additives (Butt et al., 2008).

Lactic acid bacteria strains were chosen for this experiment since they are used in a wide range of food biotechnology applications and can thus be a food grade source for xylanase production.

Twenty two strains were screened for xylanase production and no particular sign of present xylanase evidence could be confirmed for any of them. LAB strains were isolated from food and food produces mainly originating in Vietnam. Prescreening with congo red assay showed that no clear halo zones could be perceived on any petri dishes, indicating a lack of xylanase production.

More than that, with regard to qualitative xylanase survey, no xylanase activity could be observed neither via spectrophotometric methods nor via measuring pH values. In all attempts the growth curve of the used bacteria strains showed a steady state after an average time of 48 hours. This concludes that all of the supplemented glucose

has been used up while in the meantime no xylan has been utilized at all by the bacteria. Simultaneously, MRS medium containing natural xylan (isolated from corn cob) as sole carbon source, showed no sign of proper growth of the bacteria.

Commercial xylan which was added to the cultural medium caused a slight decrease in pH value and O.D. values were scarcely higher than in those samples grown in medium without any xylan. However, these marginal differences are not of decisive importance regarding xylanase activity.

Quantitative analysis seemed insignificant given the previous results from qualitative analysis. However, the subsequent results should point out the argument of xylanase absence among all of those LAB strains. The units per ml of enzyme activity all were around 0.04 in average.

In addition, HPLC results show no sign in xylose peaks confirming that no xylose could be formed resulting from xylanase activity.

5.2. Reasoning and Improvement

Overall, and as equally stated by Zendo, T. (from the Lactic Acid Fermentation research group of the Kyushu University, Japan) xylanase production among Lactic acid bacteria is very hard to estimate and to harvest. In particular, cultural conditions of xylan have turned out to be difficult to optimize in order to maximize xylanase activity in Lactic acid bacteria (Zendo, T. 2014, personal communication). To that end, focus should be put on the impact of the parameters ph values, temperature and time on xylan. In our present study, xylan degradation could not be observed by the used methodology since there seemed to be a shortage of previous investigation on the different properties and structures of xylanases and the complete xylanolytic enzyme system regarding their efficiency and extent of hydrolysis. As a result, no clear evidence of xylan degradation by Lactic acid bacteria could be confirmed.

At first, the impression was that cultural conditions were (medium optimization, definition of incubation time and temperature and estimation of xylan properties and concentration) were unsuitable for the formation of xylanase activity. This does not appear reasonable, since in all other known biological systems the presence of a

suitable polysaccharide substrate is conducive to the formation of the respective hydrolytic enzyme (Stricker et al, 2008). It should, however, be investigated whether the standard media for LAB (which are nutritionally demanding and require a number of supplements) do not contain sufficient amounts of easily metabolizable carbon sources that are able to repress the formation of polysaccharide-utilizing enzymes such as xylanases. This mechanism is well known in fungal systems and may be at work here as well (Hasper, A. et al 2000; Stricker et al., 2008).

Another plausible explanation for the observed lack of xylanase activity is that, accessory enzymes such as, for instance, α-L-arabinofuranosidase, are lacking in the LAB complement of xylanolytic enzymes, and that the used substrates (beechwood xylan) would have required such activities. LAB are not known for being able to entirely hydrolyze complex plant cell wall derived polysaccharides with a complete enzymatic complex, but would probably rather utilize hemicellulose fractions that are the product of pre-hydrolysis by the enzyme systems of other commensal organisms. The xylanolytic enzymes that are produced by LAB may be very sensitive to, e.g., steric hindrances imposed by xlyan backbone substitutions, and the commercially available substrate beechwood xylan may be inaccessible for initial enzymatic attack by the enzymes that the LAB in the screening program are able to produce.

Finally it has to be taken into consideration that the enzymes encoded by the genes annotated as (potential) xylanase genes based on sequence similarities do not, in fact, encode true xylanases. Sequence similarities have been shown to be often misleading, and due to the vast amount of genomic information that is accumulating in the databases, only a fraction of the annotated genes is experimentally confirmed to constitute, in fact, an active gene (i.e. a gene that is transcribed under certain conditions resulting in an mRNA that is translated into a functional polypeptide), and not an evolutionarily degenerated pseudogene or an artefact of electronic annotation. In addition, even among correctly annotated active genes the substrate specificity or catalytic specificity very often is incorrectly predicted. Recent experimental approaches in heterologously expressing genes annotated as xylanases from LAB in *E. coli* and *L. plantarum* resulted in minor yields of soluble protein, but the predicted endoxylanase activity could not be detected, and it is quite possible that the electronic annotation of the genes is not correct (Nguyen Hoang Anh and C. Peterbauer, personal communication).

5.3. Xylo-oligosaccharides (XOS): Market and NFR

Since XOS represent one key point within this thesis, their status regarding commercialization and Novel Food Regulation shall be briefly scrutinized. As previously mentioned, xylo-oligosaccharides (XOS) are classified as prebiotics (Cummings et al., 2001). XOS do not only possess prebiotic properties, but they show additional technological features, including resistance to heat, stability in acidic media and ability for providing lower available energy (Oku and Sadako, 2002). When it comes to current and potential market demand, XOS' most promising applications relate to ingredients for functional foods such as soft drinks, tea or cocoa drinks, nutritive preparations, cakes, biscuits, pastries, puddings, dairy products, and special preparations for health food for elder people and children (Moure et al., 2006).

The functional food market has boomed rapidly over the past given consumer's awareness of the interest between health, nutrition and diet. Simultaneously, the interest of food manufacturers has grown due to the increase value that the added ingredients deliver to food. XOS have been marketed mainly in Japan and also the United States (Moure et al., 2006). Companies that have been producing XOS are for instance, Suntory Ltd., Marushige Ueda Co., Enzamin Laboratory Inc. and Lotte Co. (Vázquez et al., 2000). Within the European Union XOS have not been commercialized as such however there is an example where they have been placed on the market according to the Novel Food Regulation (European Commission, 2014).

On 8 February 2011, the Commission declared "no objections" on an application for the authorization of wheat bran extract as a novel food ingredient that has been submitted by the Belgian company Fugeia NV (European Commission, 2014). The application was for the placing in the market of Wheat bran extract (WBE) as a novel food ingredient on various products as source of prebiotic dietary fiber. WBE is an off-white powder, which is obtained by extraction from wheat bran and shows a high amount of arabinoxylan-oligosaccharides. Arabinoxylan-oligosaccharides comprise a mixture of xylo and arabinoxylo-oligosaccharides. Thus WBE is intended to be added to various food products as a prebiotic ingredient, which shall additionally contribute to an increased fiber intake (EFSA, 2010b). WBE falls under the category as

described under article 1(2) of the NFR, which is a complex novel food from non-GM sources which shows a history of safe use in the EU. Concomitantly, this corresponds to class 2.1.under the Commission Recommendation 97/618/EC where in the case of WBE all the requirements for a submission were met (European Commission, 1997b).

5.4. Guidelines for the outline of a request dossier according to the Bundesministerium für Gesundheit

In order to better understand the heavy work load of required documentation for the authorization of novel foods, the following information was provided. It shall give a brief overview on the guidelines for the establishment of a request dossier, drawn up by (e.g. in Austria) the Federal Ministry of Health (Bundesministerium für Gesundheit, 2014). At the same time, the applicant will forward a copy of the request to the Commission.

The Bundesministerium für Gesundheit is the competent authority to which the application dossier shall be submitted. Thus, it bears the responsibility of the initial assessment on the basis of current scientific knowledge and decides if additional measures are required.

5.4.1. Administrative Information

Full name of the applicant/manufacturer of the NF/responsible person of the dossier

Address of the applicant /manufacturer of the NF/responsible person of the dossier

5.4.2. General description

General information of the novel food / novel food ingredient shall be given with consideration of article 1 and 2 of the Novel Food Regulation.

This means that the applicant should make sure that the scope of the Novel Food Regulation applies to the novel food or novel food ingredient which he/she wishes to place on the market.

5.4.3. Identification of essential information requirements

The applicant should use table II of Part I of the Commission's Recommendation No. 97/618 (EC) in order to determine which of the schemes I-XIII are essential to provide data permitting a safety and nutritional evaluation of the NF.

Table 5.1.: Structured schemes according to Commission Recommendation No. 97/618 (EC).

	Structured scheme
I.	Specification of the NF
II.	Effect of the production process applied to the NF
III.	History of the organism used as the source of the NF
IV.	Effect of the genetic modification on the properties of the host organism
V.	Genetic stability of the GMO
VI.	Specificity of expression of novel genetic material
VII.	Transfer of genetic material from GM microorganisms
VIII.	Ability to survive in and colonize the human gut
IX.	Anticipated intake/extent of use of the NF
X.	Information from previous human exposure to the NF or its source
XI.	Nutritional information on the NF
XII.	Microbiological information on the NF
XIII.	Toxicological information on the NF

In this case, scheme number IV.-VIII. can be omitted.

I. Specification of the NF

Parameters that should characterize the product from a safety and nutritional point of view should be considered. This includes:

- Species and taxonomic identity:i.e. *Lactobacillus* spp.
- Chemical composition relating to particularly nutritional properties and possible antinutritional/toxicological concerns: i.e. mixture of oligosaccharides formed by xylose residues;
  prebiotic properties (e.g. stimulating the growth of beneficial bacteria such as Bifidobacteria and Lactic acid bacteria)

II. Effect of the production process applied to the NF

Description of the technical details has to be sufficiently detailed in order to permit a distinction between novel and existing processes and to predict whether the potential of the process to introduce physical, chemical and/or biological changes in the food might have impact on the nutritional, toxicological and microbiological parameters of the final product.

In this case, abundant documentation, describing for instance the. enzymatic hydrolysis conditions and specifications for the generation of XOS by means of xylanase, shall be submitted.

III. History of the organism used as the source of the NF

The novelty of the food microorganisms in relation to these guidelines is defined by their novelty in the European food supply.

With respect to this case, a full description can be omitted since LAB strains will be the source of the NF, and they show generally recognized use in the diet (GRAS status).

Scheme IV. – VIII. were omitted for this case.

IX. Anticipated intake/extent of use of the NF

Projections of anticipated intakes are required in order to evaluate the dietary and nutritional significance of the NF. This assessment focuses

on the nature of the NF and its anticipated use based upon properties i.e. as a prebiotic. This is an aspect that shall be further investigated thoroughly since there is lack of population studies with XOS treatment in order to determine e.g. intake levels of XOS.

X. Information from previous human exposure to the NF or its source

Relevant documentation is needed on previous use of the NF source in the community and/or the NF in other parts of the world.

In this case, this means history of safe use of LAB strains is required in detail in order to establish a baseline for assessment. The information should deal with aspects, where traditional handling and preparation of the microorganism prevent misuse or adverse short and long term health effects.

XI. Nutritional information on the NF

This shall include a systematic review of the NF's composition, preparation and role which it is expected to have in the diet. Normal and maximum levels of consumption and the nutritional compositional data should be taken into account. Additionally, the effects of storage, further processing, cooking and the antinutritional effects (e.g. inhibiting mineral absorption) have to be considered as well.

The numbers involved in study groups should support the evidence and displays statistical power. All studies and tests should comply with ethical principles of guidelines of good clinical practice (GCP) and good laboratory practice (GLP).

Overall, it is important to note that more specific population based studies are needed in order to fulfill the provisions of this scheme.

XII. Microbiological information on the NF

In general, the intentionally used source organism for the NF has to be recognised as:

- non-pathogenic,
- non-toxigenic microorganism,
- with a known genetic stability, that does not affect the desirable properties of the normal intestinal flora

The examination should include a characterization of the microorganisms present and the analysis of their metabolites.

For this case, LAB strains do comply with all the required data.

XIII. Toxicological information on the NF

The set of toxicological information needed for this scheme depends on the range of scenarios foods for which substantial equivalence can be established.

In this case, a nutritional-toxicological testing program may not be necessary since the source organism shows abundant evidence of non toxicological origin.

5.4.4. Consultation of structured schemes (decision trees)

All the relevant information according to the schemes I to XIII (via a decision tree questionnaire) shall be included. The schemes lead through a decision-tree-like set of questions and thus shall enable to decide whether the data available to the applicant are sufficient or if further information is required.

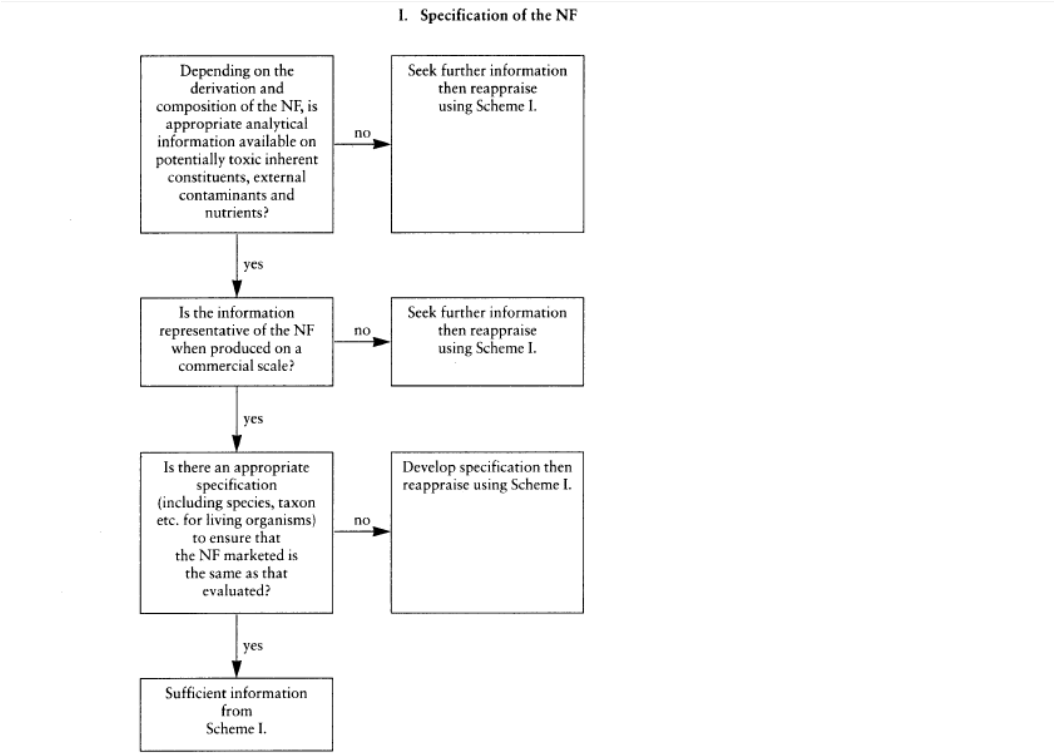

Figure 5.1.: Example of a decision-tree-like Scheme

5.4.5. Evaluation and conclusion of the applicant

After having evaluated the total information assembled should be presented covering the key issues relevant to the NF.

5.4.6. Summary of the dossier

The applicant should provide a summary which shall be forwarded to other Member States as foreseen in article 6 (2) of the Novel Food Regulation.

5.5. *Excursion* - Food Enzymes and their Legal Framework

Industrial enzymes are a flourishing business worldwide with food enzymes making up for most of the market value (Spök, 2006). The Association of Manufacturers and Formulators of Enzyme Products lists almost 250 enzymes manufactured for food industry purposes as of April 2014 (AMFEP, 2014). Most food

enzymes are used within the beverage and bakery industry, in the production of dairy products and in the processing of starch (Spök, 2006).

Bearing the rising interest for enzymes within the food industry in mind, appropriate legislation has to be put into consideration. The basis of the legal framework of food enzymes within the European Union is Regulation (EC) No 1332/2008, the so-called "Food Enzyme Regulation". According to article 3 of this regulation, food enzymes are defined as products obtained from plants, animals or micro-organisms or products thereof, including a product obtained by a fermentation process using micro-organisms:

- containing one or more enzymes capable of catalyzing a biochemical reaction; and
- added to food for technological reasons during the manufacturing, processing, preparation, treatment, packaging, transport or storage of foods.
  (European Commission, 2008a).

5.5.1. The Food Enzyme Regulation (EC) No 1332/2008

Regulation (EC) No 1332/2008 aims to harmonize rules on food enzymes within the EU and ordains a deadline of 24 months for the submission of applications concerning authorization (European Commission, 2008). Furthermore, this regulation was adopted on 16 December 2008 together with Regulation (EC) No 1333/2008, Regulation (EC) No 1334/2008 and Regulation (EC) No 1331/2008. All these regulations entered into force on 20 January 2009 and form the so-called "Food Improvement Agent package" (FIAP). The FIAP deals with common EU authorization procedure for all food additives, food flavorings and food enzymes (European Commission, 2011a).

Regulation (EC) No 1332/2008 applies to enzymes that are added during food processing or to novel food and novel food ingredients performing a technological function, and additionally includes enzymes used as processing aids.

It does not address food enzymes when they are used in the production of food additives since there is another regulation aiming on that matter, Regulation (EC) No

1333/2008 (“Food Additives Regulation”) applies. Moreover, enzymes which are intended for nutritional or digestive functions are also not covered by the Food enzyme Regulation (European Commission, 2008a). Thus, article 6 of the Food Enzyme Regulation states the general conditions where a food enzyme is included in the Union list if:

- it does not pose a health concern to the consumer;
- there is a technological need;
- its use does not mislead the consumer.
  (European Commission, 2008a)

5.5.2. Authorization – the Union List of Food Enzymes

A common procedure for the assessment and authorization of food enzymes is laid down in Regulation (EC) No 1331/2008 (European Commission, 2008b). Food enzymes that have been permitted to be placed on the Community market shall be included in the Community list (“positive list”) which today may also be referred to as Union list of food enzymes (European Commission 2008b; European Commission 2011b). The Union list should describe the enzymes and specify any conditions governing their use, including, when necessary, information on their function in the final food. The list should be supplemented with criteria on purity and the origin of the food enzyme (European Commission, 2008a).

Until a full EU positive list is established, the current national rules on the use and marketing of food enzymes will apply. This means that food enzymes producers may continue putting existing products on the markets while assessment and authorization is still in process according to article 24 of the Food enzyme Regulation (EFSA, 2014).

The common procedure for updating the Community list may be started either from the Commission’s side or following an application. Applications shall be sent to the Commission, which subsequently seeks scientific opinion from the EFSA. The common procedure will end with the adoption by the Commission of a regulation implementing the update (European Commission, 2008b).

5.5.3. Safety Assessment of Food Enzymes

In order to enable EFSA to carry out the safety assessment, the following critical issues have to be taken into account:

- the source (animals, plants, microorganisms)
- the food enzyme (enzyme proteins)
- intended and unintended reaction products (from enzymatic or chemical reactions)
- the dietary exposure of the consumer (residue concentration of food enzyme) (EFSA, 2009)

Furthermore, information on the food enzyme shall be given more precisely in a dossier covering inter alia administrative, technical and toxicological data among others. After that the food enzymes can be considered for inclusion on the list of approved food enzymes by EU decision makers *(*EFSA, 2009).

Regarding the source of food enzymes, given the facts of this thesis, the production from microbial sources should be considered.

This means that adequate information about the strain used for food enzyme production should be provided, as below:

- the taxonomic identity of the strain
- details of any documented history of use with absence of human health adverse effects including Qualified Presumption of Safety (QPS) status. (EFSA, 2009)

In addition, information on the monitoring, pathogenicity, toxigenicity and antimicrobial resistance of the production strain should be included (EFSA, 2009).

With respect to the topic of this thesis, i.e. xylanases from wild type LAB strains, toxicological testing (as required in the dossier) may be reduced or completely waived. According to EFSA toxicological data may be excluded for instance when the food enzymes produced by microorganisms that have been given a status of QPS.

Many *Lactobacillus* spp. show a long history of safe use within food processing and they have received a QPS status given by the EFSA (Gharaei-Fathabad and Eslamifar (2011); EFSA (2012)). Due to this fact authorization procedure for xylanases from LAB sources might be smoothed and simplified regarding future perspectives.

5.5.4. Amended Legislation with Regard to Xylanases

On November 2012, an amendment to the Food enzyme Regulation was published in Regulation (EU) No 1056/2012 (European Commission, 2012). This amendment aimed at the modification of the time period of applications for assessing enzymes. To that end, past experience has shown to be insufficient for the parties involved to present all the necessary data (European Commission, 2011a). Therefore the period was extended from 24 months to 42 months and the deadline for applications remains 11 March 2015 (European Commission, 2011a; EFSA, 2014).

In addition, the timeframe and the data requirements for submission of applications were set down in a proper regulation, regulation (EU) No 234/2011. Thus the new regulation shall simplify common approval procedure for food enzymes, implementing the provisions of regulation (EC) No 1331/2008. This regulation enacts a submission period for existing and designated food enzymes starting from 11 September 2011 (European Commission, 2011b).

To date, xylanases from *Aspergillus niger*, *Humicola insolens*, *Trichoderma reesei* and *longibrachiatum* have been evaluated according to SCF (Scientific Committee for Food) guidelines and fall under Regulation (EU) No 234/2011 (European Commission, 2014b).

6. Conclusion

To sum up, this thesis endorses the challenging and ambiguous research on xylanase enzyme characterization and highlights that the current knowledge about xylanase associated with Lactic acid bacteria is still lacking, especially when it comes to distinguishing the characteristics of certain enzyme groups. Moreover, this thesis accentuates the demand for optimizing culturing conditions with respect to medium composition, temperature, pH value and time.

Still, it is for sure known that some LAB strains have genes encoding xylanases, however these findings should be verified and proven more extensively in order to facilitate which specific strains harbor xylanase genes and subsequently tend to be more susceptible for further xylanase analysis like screening. Since it is hard to pin down the exact potential of a LAB strain regarding xylanase, it seems even more difficult to track down the exact coordination of xylanases which shall degrade the substrate xylan as shown in this study. This means that not only research on the genomic level should be emphasized but also research on the interaction of different kinds of xylanases, which are said to be present, has to be broadened.

This consciousness opens up new directions of significant magnitude for future food biotechnology research. Simultaneously, it underlines the importance of the continuous unremitting thirst for seeking the perfect food grade LAB strain with the highest xylanase activity facilitating the future food industry.

These resultant xylanases could be used for example for the production of xylo-oligosaccharides (XOS), which are classified as prebiotics. XOS share a long history of human exposure as hemicelluloses from grain fiber and possess GRAS (*generally recognized as safe*) status.
The Novel Food Regulation involves the assessment of the reliability of prebiotics within the European Union. Surprisingly, a range of prebiotics are already available on the market that do not fall under the Novel Food Regulation such as fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), lactulose and polydextrose. It seems that these prebiotics can be concomitantly regarded amongst others as fiber sources or food additives, which might have helped them to be placed on the market more easily.

Xylo-oligosaccharides have been commercialized foremost in Japan and the United States. However, there is one product including XOS sources, which has been authorized by the EU in 2011, complying with the provisions of the Novel Food Regulation.

In general, XOS offer an array of dietary benefits to consumers such as immunostimulating effects, positive effects on skin and allergy and cholesterol levels. However, human experimental data on XOS is scarce and simultaneously evidence of prebiotic efficacy of XOS is limited due to the fact that this is a relatively new area of nutritional sciences. Most of the human studies have been dedicated to the effects of XOS on the colonic microflora. Moreover, XOS do not only possess prebiotic properties but also additional technological features, including resistance to heat, stability in acidic media and ability for providing lower available energy. This enhances their value and status within food industry.

Overall, XOS show a great potential as agents to improve and maintain a well balanced intestinal microflora which eventually results in enhanced health and well-being. Their spectrum of their incorporation into food products is versatile. Future research is needed to provide exact explanations on the effects of XOS for instance in gastrointestinal disorders or skin related diseases. Also the opportunities of XOS combined with probiotics may be encouraged as well.

It is important to note that population based studies are costly. One potential way of funding this initiative may be to forge industry-government collaborations. It is a big concern to find the right strategy that is most likely to succeed with regulatory agencies like EFSA. This thesis demonstrates how much documentation is needed in order to submit a proper application and how difficult it is to eventually get an authorization to be placed on the market. However, the legal framework is constantly being updated and altered in order to facilitate, harmonize and above all speed up authorization procedures within the EU as indicated in the latest proposal of the Novel Food Regulation of last year. The establishment of a centralized EU procedure for the assessment and authorization of a novel food and the generic Union list of authorized novel foods, are both good examples of the Commission’s work for alleviating the future pathway of a successful authorization process.

7. Summary

7.1. Summary (English)

Food enzymes have spurred great interest over the last decades thereby improving food production processes among many sectors in food industry for instance in baking industry.

Xylanases are enzymes that are widespread in nature and can be found in fungi, yeasts and bacteria. Since most reports are about xylanases deriving from fungal species, the aim of this thesis was to focus on the presence of xylanases of bacteria species which in this study are Lactic acid bacteria.

Lactic acid bacteria (LAB) have a long history of applications in food and abundant information is available about their nutrient requirements and growth conditions. Bearing these understandings in mind, lactic acid bacteria came up as a promising target for the pursuance of food-grade xylanase production. For the practical work of this thesis different LAB strains (mainly originating from Vietnam) have been screened for possible xylanase activity using different methods divided into qualitative and quantitative assays.

In addition, xylanases can be applied for the generation of xylo-oligosaccharides which are classified as prebiotics. The Novel Food Regulation focuses on the assessment of the reliability of prebiotics within the European Union. There is a range of prebiotics such as for instance galacto-oligosaccharides that are available on the market and do not fall under the Novel Food Regulation. Xylo-oligosaccharides have been commercialized foremost in Japan and the United states. Since xylo-oligosaccharides show great potential for various food application sectors such as dairy, beverages, bakery and baby food, future prospects of xylo-oligsaccharides are proficient concerning the authorization and commercialization of them within the European Union.

7.2. Summary (German)

Der Einsatz von Lebensmittelenzymen hat in den vergangenen Jahrzehnten stark an Bedeutung gewonnen wie beispielsweise in der Backindustrie. Hier könnten vor allem Enzyme wie Xylanasen von großem Interesse sein, da ihre Zugabe die Qualität des Brotes im Hinblick auf Teigelastizität, Haltbarkeit und Volumen verbessern könnte.

Xylanasen findet man zahlreich in Pilzen, Hefen und auch Bakterien. Die meiste Literatur gibt es über Xylanasen aus Pilzquellen. Deshalb wurden für diese Masterarbeit Xylanasen von Bakterien gezielt untersucht. Zu diesem Zweck wurde der Fokus auf Milchsäurebakterien gelegt, da diese schon lange in der Lebensmittelindustrie eingesetzt werden und es reichlich Literatur über deren geeignete Kultivierung, Wachstums,- und Ernährungskonditionen gibt. Für diese Arbeit wurden mehrere Milchsäurebakterienstämme aus unterschiedlichen Quellen gescreened, um deren Potential, Xylanasen zu produzieren, auszuschöpfen.

Ein weiteres potentielles Einsatzziel von den daraus resultierenden Xylanasen stellt die Herstellung von Xylo-oligosacchariden dar, welche als Präbiotika klassifiziert werden. Xylo-oligosaccharide können wie andere Präbiotika auch in der Produktion von Backwaren, Getränken und Milchprodukten zugesetzt werden. Bislang werden sie hauptsächlich in Japan und den USA vermarktet. Die Zukunft verspricht den Einsatz der Xylo-oligosaccharide vermehrt wissenschaftlich zu durchleuchten um damit auch die Zulassung und Vermarktung innerhalb der EU zu erleichtern.

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9. Appendix

Abbreviations

AMFEP	Association of Manufacturers and Formulators of Enzyme Products
BSE	Bovine Spongiform Encephalopathy
DNS	3,5-dinitrosalicylic acid solution
DP	Degree of Polymerization
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FIAP	Food Improvement Agent Package
FOS	Fructo-oligosaccharides
GH	Glycoside Hydrolase (family)
GMO	Genetically Modified Organism
GOS	Galacto-oligosaccharides
GRAS	Generally Recognized as Safe
HPLC	High Performance Liquid Chromatography
ISAPP	International Scientific Association for Probiotics and Prebiotics
ITF	Inulin-type Fructans
LAB	Lactic Acid Bacteria
MRS	deMan Rogosa and Sharpe (medium)
MS	Member State
NDC	Non-digestible carbohydrates
NFR	Novel Food Regulation (EC) No 258/97
OD	Optical Density
QPS	Quality Presumption of Safety
RID	Refractive Index Detector

RTC Randomized Controlled trial

SCF Scientific Committee for Food (European Commission)

XOS Xylo-oligosaccharides

Table of Figures

Table of Tables