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Xylanase activity from marine fungi

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This Bachelor's thesis was conducted at VTT Technical Research Centre of Finland. The aim was to gain information on *endo-β*-xylanase activity from marine fungi. Xylanases are industrially widely used and since the living conditions of marine fungi differ from terrestrial ones, fungi from marine habitat could be a source of novel enzymes.

Enzyme activity of ten fungi strain was first preliminarily screened with a hydrolyzing zone staining method (clearing zone) on agar medium. Five strains that were expected to possess xylanase activity were then cultivated in liquid for more detailed activity assessment. Liquid cultivations were conducted in different culture conditions in order to study their effect on fungal growth and xylanase activity.

Quantitative xylanase assays were made with samples taken from the liquid cultivations. The optimum temperature and pH, temperature stability and the effect of salinity and different xylans on *endo-β*-1,4-xylanase activity were assessed. The highest activities on birch wood xylan were observed from fungi *Calcarisporium* sp., KF525, (550 nkat/ml) and on *Lewia infectoria*, LF311, (290 nkat/ml). For both fungi, the temperature optimum for xylanase activity was found to be approximately at 50 ºC and the pH optimum at pH 5. Xylanase produced by KF525 tolerated salinity more than the enzyme from LF311. Additionally, xylan substrate from oat spelt yielded the highest xylanase activities from both fungi. The variety and ratios of xylanases produced are likely to be dependent on the source of xylan used in the cultivation.

On the basis of the promising results obtained in this thesis, it would be interesting to optimize the culture conditions even more for higher xylanase yield, for example by optimizing the incubation time, nutrients and temperature. In addition, the characterization of the xylanases produced could be assessed further (i.e. the effect of metals, or other inducing and inhibiting factors). Results obtained in this thesis can be utilized in further marine fungi studies, in xylanase production or when assessing enzyme properties in general.

Keywords	Marine fungi, enzyme activity, xylanase, clearing zone

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Työ tehtiin Teknologian tutkimuskeskus VTT Oy:lle. Tarkoituksena oli tutkia meriympäristöstä eristettyjen sienikantojen *endo-β*-ksylanaasientsyymien aktiivisuuksia ja ominaisuuksia. Elinolot meriympäristöissä eroavat maanpäällisistä, joten merten sienien entsyymeiltä voisi mahdollisesti löytyä uudenlaisia ominaisuuksia (esimerkiksi kylmänsieto). Ksylanaasientyymejä käytetään laajasti eri teollisuuden aloilla, joten uudenlaisille entsyymeille on kysyntää.

Alustava selvitys ksylanaaseja tuottavien sienikantojen tunnistamiseksi tehtiin kiinteillä agar-kasvualustoilla maljoilla kymmenelle eri sienikannalle värjäysmenetelmää käyttäen. Kaikki kymmenen kantaa osoittivat jonkinasteista entsyymiaktiivisuutta muodostuneiden hydrolyysirenkaiden perusteella, ja viisi lupaavinta kantaa valittiin jatkotutkimuksiin. Myöhemmissä tutkimuksissa ei kuitenkaan havaittu suoranaista korrelaatiota hydrolyysirenkaiden halkaisijoiden ja entsyymiaktiivisuuksien välillä.

Kvantitatiivisia entsyymimäärityksiä varten viittä kantaa kasvatettiin liuosalustoilla. Erilaisia alustoja käytettiin tutkimaan kasvatusolosuhteiden vaikutusta ksylanaasiaktiivisuuteen: mm. agaria, puskurointia sekä eri orgaanisen typen lähteitä. Tuotettujen ksylanaasien ominaisuuksista tutkittiin muun muassa lämpötila- ja pH-optimit, lämpöstabiilius sekä eri suolakonsentraatioiden ja eri ksylaanilähteiden vaikutusta entsyymiaktiivisuuteen. Korkeimmat aktiivisuudet havaittiin sienillä KF525 *Calcarisporium* sp. (550 nkat/ml) ja LF311 *Lewia infectoria* (290 nkat/ml). Molempien kantojen entsyymiaktiivisuuden optimilämpötila oli noin 50 °C ja -pH 5. *Calcarisporium*-suvun tuottama entsyymi osoittautui kestävän paremmin korkeita suolapitoisuuksia kuin *Lewia infectoria*, ja kauran ksylaani osoittautui parhaimmaksi ksylaanisubstraatin lähteeksi molemmilla kannoilla. Tuotettujen ksylanaasientsyymien määrät (nkat/ml) ja eri ksylanaasien keskinäiset suhteet ovat todennäköisesti vahvasti riippuvaisia kasvatusolosuhteista ja käytetyn ksylaanin lähteestä.

Työssä saatujen tulosten perusteella kasvatusolosuhteita voitaisiin tutkia syvällisemminkin korkeampien ksylanaasiaktiivisuuksien saavuttamiseksi (mm. eri ksylaanilähteet). Lisäksi tuotettujen ksylanaasien ominaisuuksista olisi mielenkiintoista tutkia esimerkiksi metallien ja muiden inhiboivien ja indusoivien tekijöiden vaikutusta. Tässä työssä saatuja tuloksia voidaan hyödyntää muiden merestä eristettyjen homeiden kasvatuksissa ja tutkimuksissa.

Avainsanat	Entsyymi, *endo-β*-ksylanaasi, entsyymiaktiivisuus, hydrolyysirengas

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List of Abbreviations

OD/OD_{600}	Optical density measured at a wavelength of 600 nanometers.
kat	Katal, an SI-unit (International system of Units) for enzyme activity: enzyme produced from 1 mole substrate per second.
Kr	Colony expansion rate, colony radial growth rate [µm/h].

1 Introduction

The aim of this thesis was to study xylanase activity from different marine fungi and the properties of these enzymes. This Bachelor's thesis was conducted at VTT Technical Research Centre of Finland.

The studied fungi originated from marine environments (i.e. from seaweed, sediment, mangrove, deep-sea) and they had been given to VTT as a gift from marine science institution GEOMAR Helmholtz Centre for Ocean Research Kiel, Germany.

Fungi are widely used enzyme producers and they are considered to produce higher levels of (xylanolytic) enzymes compared to bacteria or yeast. Additionally, enzymes from fungi are more often produced extracellularly than those from bacteria or yeast. [1]–[3]

The living conditions of marine fungi differ from terrestrial ones: usually more neutral pH, lower temperature, higher salinity and pressure. Therefore, marine fungi could be a source of novel enzymes or producers of known enzymes with new properties, for example an alkaline tolerant xylanase which functions in the marine habitat.

Prior to this thesis, a large survey related to marine fungi had been conducted at VTT, in which over 40 fungal strains were studied on different carbon sources, including xylan, at pH values 4.25 and 6.9 [4] [5]. Specific growth rates and optical density at 600 nm (OD_{600}) values had been determined. Additionally the growth of inoculated strains was analyzed visually from the microtiter plates, since some of the high optical density, OD_{600}, values may have resulted from for example pellets formed by the fungus being positioned directly in the beam of the OD sensor. Thus, conclusions could not be made from the reported OD without taking into account other variables as well.

On the basis of the available data for growth on birch xylan, ten strains (Table 1) that were expected to possess xylanase activity were chosen (Appendix 1, Tables 1A and 1B). Birch wood xylan was chosen as the substrate to focus on in this thesis.

2 Theory

2.1 Marine fungi

There are a large diversity of fungi living in marine environment. Marine fungi are usually responsible for decomposing organic matter in aquatic environment and therefore they play a key role in nutrient cycle. The marine fungi are typically isolated from, for example mangrove, sponges, algae, driftwood, sea floor sediment and corals. It is likely that all marine fungi have not even been discovered yet. [6], [7]

Marine fungi are generally classified as either obligate or facultative. Obligate fungi are growing exclusively in the marine environment, whereas facultative fungi originate from other habitats (e.g. terrestrial or freshwater). Sometimes the term “marine-derived fungi” is used when there is insufficient information to classify them as obligate or facultative. Terrestrial fungi can end up in water, for instance, when there is a flood or if their spores fall into the sea or river and then drift with the stream. This is why marine fungi are found to have similar characteristics as their terrestrial counterparts. [6], [8]–[10]

Although marine fungi have properties resembling terrestrial fungi, the marine habitat differs of non-water environment. In addition to high pressure, low temperature, pH extremes and salinity, the availability of light and diversity of minerals, distinguish the habitat of marine fungi from terrestrial ones. [7], [9], [11] It is expected that salinity would play a significant role in enzyme function, and since the metabolism of marine fungi may have adapted to salt conditions in the sea environment. [12] Marine fungi has been isolated from different places in the sea habitat. For example *Aspergillus* has been found in algae and sponges; and *Penicillium* in algae and sediments [13]. Due to the marine habitat, marine fungi could produce enzymes with new characteristics such as thermostability, cold-tolerance, barophilicity and salt tolerance [6].

Fungi from the marine environment have been found to produce a variety of hydrolytic enzymes: amylase, chitinase, cellulose, glucosidase, inulinase, keratinase, lignonase, lipase, phytase, protease and xylanase [6].

Some marine-derived enzymes are used in industrial applications: lipase, in cosmetics and as digestive enzymes in the medical field; protease, in production of anti-inflammatory and digestive medicines and products; and other enzymes, such as ligninase in the paper, textile and chemical industry. [6] A marine *Aspergillus* has been reported to produced enzymes with cytotoxic and antitumoral activity [13].

2.2 Xylan

Plants consist of cellulose (35-50 %), hemicellulose (20-30 %) and lignin (20-30 %). Hemicellulose in land plants consists mainly of xylan, and thereby xylan is the second most encountered polysaccharide in nature.

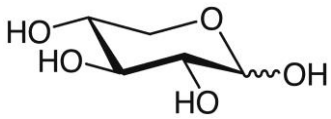

Figure 1. D-xylose monomer [14].

The backbone of xylan consists of numerous D-xylose units (Figure 1).

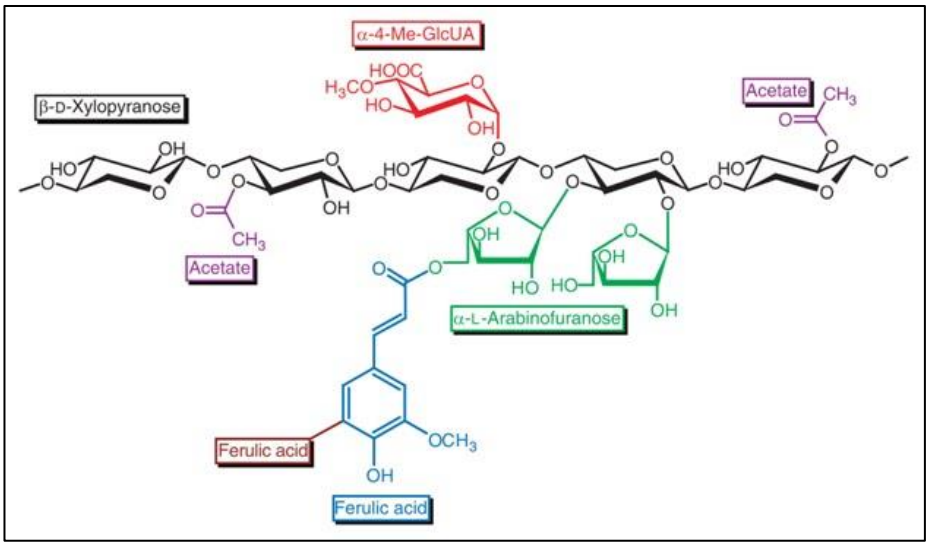

Figure 2. The structure of xylan and its sidechains [15].

Depending on the source of the xylan, the linkage between xylose may vary and different sidechains can be attached to the xylose backbone (Figure 2). The proportions of substituents depends on the origin. [16]–[19] The most common side groups in xylan are arabinosyl, acetyl and glucuronosyl. Xylans encountered in nature consist mostly of a variety of side chains, homoxylans are less common, respectively. Xylans are usually categorized based on the attached side groups, such as homoxylan, arabinoxylan and glucuronoxylan. [16], [20] The dextrorotary xylose is the one mainly encountered in nature, whereas the levorotary for, L-xylose, can be prepared in laboratory [21]. For example, in xylan from hardwood, 4-O-methyl-D-glucuronic acid and acetic acid are the principal attached side groups, whereas L-arabinofuranose acid occurs in the sidechains of softwood xylan [16], [17].

Xylan polysaccharide in hardwood (e.g. birch, teak, oak and mahogany) is O-acetyl-4-0-methylglucuronoxylan, and it consists of at least 70 β-xylanopyranose moieties with β-1,4-glycosidic linkages. Hardwood xylans are highly acetylated, for instance in birch wood xylan there is over one mole of acetic acid per two moles of D-xylose. Due to the acetic acid, xylan is partially soluble in water. [16]

Xylans in softwood (e.g. pine and fir) are arabino-4-O-methylglucuronoxylans, they are less branched and they have more 4-O-methylglucuronic acid than hardwood xylans [16], [22]. Unlike xylans from hardwood, softwood xylans are rarely acetylated. In softwood xylan the acetyl groups are replaced with α-L-arabinofuranose units with alfa-1-3-glycosidic linkages [16], [18].

Marine algae xylans have backbones consisting of β-1,3-linkages, although seaweed (like Palmaria palmate) is found to have both β-1,3- and β-1,4-linkages [23].

Xylan can be hydrolyzed with either acid or with enzymes. Acidic hydrolyzation was found first and was used industrially before xylanase enzymes were introduced. The use of acid is not as specific as enzymatic treatment, the reaction conditions are more demanding and side products occur more often than with enzymes. However, the cost of enzymes may have hindered their use in the beginning. [24]

2.3 Xylanase

Xylanases are a hydrolytic enzyme group that degrade linear polysaccharide xylan to its monomer, xylose. As illustrated earlier in Figure 2, xylan has a complex structure and therefore in order to completely degrade xylan several enzymes are required (i.e. *endo*-1,4-β-xylanase and 1,3-β-xylosidase). All substrate groups attached need their own degrading enzyme, such as acetyl xylan esterase, glucuronidase, α-arabino-furanosidase and feroryl esterase. [16], [25]

Xylanases are sometimes referred to in the singular and usually in these cases the referred xylanase type is the most common one, *endo*-1,4-β-xylanase (EC 3.2.1.8). The same has also been applied in this thesis. This *endo*-1,4-β-xylanase cleaves the 1,4-linkage in the xylan backbone yielding shorter xylooligosaccharide chains. The *endo*-xylosidase in turn breaks down small xylooligosaccharides consisting of two xylose units into xylose.

There are several already known xylanase sources, for example bacteria, fungi, yeasts, algae, seeds, snails, crustaceans, protozoans and insects, bacteria and fungi being the main commercial sources [2]. Filamentous fungi may be a promising way of producing xylanases for industrial use because of their ability to produce xylanase extracellularly and in addition, filamentous fungi are relatively easy to cultivate. Enzymes from filamentous fungi, such as xylanase and cellulose, are usually produced in submerged liquid cultures. [26]

Molecular masses of xylanases from microbial sources have reported to vary from 8 to 145 kilodalton. Bacterial and fungal *endo*-xylanases are found to have a temperature optimum at 40-60 °C, but naturally the optimum varies depending on the strain. Above this temperature range xylanases from fungi are generally found to be unstable. Optimal pH values for xylanases from fungi and bacteria are observed to be between four and seven. The higher pH optimum in bacterial xylanases is one reason why they are much used in industrial applications. [6], [27]

Xylanases from fungal sources have been reported to be low molecular mass xylanases, contrary to bacteria which produces two types of xylanases: low molecular mass, basic

xylanases and high molecular mass, acidic xylanases. [1] Two main types of fungal xylanases have been found: debranching and non-debranching. The debranching ones both hydrolyze the main chain of xylan and remove arabinose from the side chains. In contrary to non-debranching which only cuts down the main chain of xylan. [1]

Trichoderma and Aspergillus species are usually the producers of industrial fungal xylanases [28]. T. *reesei* has been reported to be one of the highest xylanase producers, 3,350 U/ml, equivalent to about 55,000 nkat/ml [1]. According to Raghukumar et al. [29], alkaline xylanases has been produced by marine-derived A*spergillus niger* and the produced alkaline xylanases should be suitable for biobleaching of paper pulp. Although the used A. *niger* was facultative marine, not obligate marine fungi. Additionally, Del-Cid et al. [30] reported from a cold-active xylanase produced by *Caldosporium* sp.

Application of xylanases

Xylanases have many application areas, including animal feed, dietary supplement and use in the paper pulp, textile, food and beverage industries. In animal feed, xylanases degrade polysaccharide, which lowers viscosity and speeds up nutrient absorption [31]. Therefore xylanases improve overall the nutritional value and digestion of fodder.

The usage of *endo*-xylanases in the paper industry for cellulose pulp bleaching decreases costs and the use of harsh chemicals, such as chlorinated agents that degrade into adsorbable organic halogens, AOX. Additionally, xylanases increase pulp brightness and reduce water discharges. [16], [32]

Xylanases are used in the food industry to clarify fruit juices, beer and wine and they are also added to wheat flour in order to improve dough quality. In human nutrition, the use of xylooligosaccarides as prebiotics has recently attracted interest [33] [34]. Xylooligosaccarides (XOS) are products of xylan degradation. The human body cannot utilize all oligosaccharides and those that cannot be digested are fermented in the colon into fatty acids. These fats improve gut microflora and contribute intestinal well-being. XOS has been reported to possess other health-promoting effects as well, such as anti-inflammatory, antiallergic and antitumoral. [35], [36]

2.4 Clearing zone method, a qualitative determination of enzyme activity

Congo red and Gram's iodine dyes are considered to work well as indicators of xylanase activity. Both have been successfully used for qualitative determination of xylanase activity on solid media. In case a strain is able to degrade xylan, a visible transparent clearing zone ("halo", hydrolyzing zone) around the fungal growth should appear. Clearance is a probable indicator that the strain is able to utilize xylan and therefore an indication of a possible xylanase activity. [37], [38]

The zone of clearance with Congo red is due to the interaction between Congo red and polysaccharides containing D-glucanosyl units with beta-(1→4)-linkages. Both Congo red and Gram's iodine stain only polysaccharides, therefore xylose as a monomer of xylan does not stain. [37] A clearing zone might in some cases be seen without staining [39] [40]. Substrate birch wood xylan and agar causes opacity to the solid media. Once the fungus starts to produce xylanases the media becomes less opaque where the xylan is broken-down into xylose, at least in theory.

These staining methods are a relatively easy way of determine possible enzyme activity when accurate quantitative data is not needed. On the other hand, Congo red is toxic and dangerous for the environment, which should be taken into account when evaluating the need to use Congo red. [41]

In this thesis, ten fungi (Table 1) were screened on agar medium for their growth on xylan and the most interesting five were then cultivated in liquid to obtain samples of their xylanases. Xylanase activity from two strains (LF311 and KF525) was characterized in term of temperature and pH optima, temperature stability and salt tolerance.

3 Methods

3.1 Strains

Ten fungal strains (Table 1) were obtained from GEOMAR and stored as spores or mycelia in 20 % (w/v) glycerol at -80 °C.

Table 1. Ten marine fungi strains selected for further xylanase activity studies.

Code of fungi strain	Name of the fungi strain
LF300	*Volucrisporia graminea*
LF311	*Lewia infectoria*
LF328	*Trichoderma* sp.
LF352	*Aspergillus sydowii*
LF371	*Penicillium granulatum*
LF458	*Penicillium pinophilum*
KF525	*Calcarisporium* sp.
KF560	*Halenospora varia*
LF580	*Scopulariopsis brevicaulis*
LF766	*Microascus trigonosporus*

3.2 Growth media

3.2.1 Solid medium for detecting xylanase activity

The solid culture media contained 6.7 g/l YNB (Yeast Nitrogen Base with ammonium sulfate, without amino acids), 5 g/l xylan from birch wood and agar as a solidifying agent (20 g/l or 15 g/l). The concentration of agar was reduced from 20 to 15 g/l to reduce the cloudiness of the media and make growth measurement easier. Medium containing agar and birch wood xylan was sterilized by autoclaving in 121 ºC for 15 minutes and 10x concentrated YNB solution was sterilized by filtering. The pH of the medium was adjusted to 5.5 with 0.25 % phosphoric acid. pH 5.5 was used, since fungal xylanase production is found to usually take place in a pH range from four to eight; pH of sea water is usually

between five and nine; and pH 5.5 is approximately average of the cultivations that was used in earlier studies at VTT [4], [5], [42].

3.2.2 Liquid media with xylan substrate

Pre-cultivation media with and without agar were used in order to assess the effect of agar on pellet formation, i.e. if smaller pellets would form with agar. The media are presented in Table 2. The pH values of the pre-cultivation media were adjusted to 5.4 or to 6.2 with ~0,025 % phosphoric acid before autoclaving.

The pH of the media in both un-buffered pre-cultures had lowered significantly (to under 3). Thus, potassium dihydrogen phosphate, KH_2PO_4, was added also to the pre-culture media (Table 2) to give some buffering capacity. A pH value of at least four would be desirable.

Table 2. Composition of pre-culture media.

	Un-buffered medium without agar	Un-buffered medium with agar	Medium with agar and buffer
Xylose [1]	20 g/l	20 g/l	20 g/l
Yeast Nitrogen Base without amino acids [2]	6.7 g/l	6.7 g/l	6.7 g/l
Agar [3]	-	4 g/l	4 g/l
Yeast extract [4]	1 g/l	1 g/l	1 g/l
KH_2PO_4, 200 g/l stock solution [5]	-	-	70 ml/l

[1] D-(+)-Xylose, Sigma-Aldrich ≥ 99 %, Difco™, 101956373
[2] Yeast Nitrogen Base, without amino acids, Becton, Dickinson and Company, Difco™, 291940
[3] Agar Granulated, Becton, Dickinson and Company, Difco™, 214530
[4] Yeast Extract, extract of autolyzed yeast cells, Becton, Dickinson and Company, 212750
[5] Potassium dihydrogen phosphate for analysis, Merck, Emsure® ISO, 1.04873.1000

Pre-culture (Table 2) and production media (Table 3) were made in two parts: YNB in one part and all other components in the other. YNB was used as a 10 fold concentrate

solution (i.e. 6.7 g/100 ml), which was sterilized by filtering and mixed aseptically to the rest of the media, to equal a final 6.7 g/l concentration. For all media, the YNB component was filter sterilized with a VWR Bottle Top Filtration Funnel (Polystyrene, 500 ml, 0.2 µm PES, Sterile) and other components were autoclaved (121 °C for 20 minutes).

Table 3. Composition of production media.

	Initial production medium	Production medium with yeast extract	Production medium with spent grain extract	Buffered production medium with urea
Xylan from birch wood [1]	10 g/l	10 g/l	10 g/l	10 g/l
Yeast Nitrogen Base without amino acids [2]	6.7 g/l	6.7 g/l	6.7 g/l	-
Yeast Nitrogen Base w/o amino acids and ammonium phosphate [3]	-	-	-	1.7 g/l
Yeast extract [4]	-	4 g/l	-	4 g/l
Spent grain extract [5]	-	-	200 ml/l	-
KH_2PO_4, 200 g/l stock solution [6]	-	70 ml/l	70 ml/l	-
Urea [7]	-	-	-	2.27 g/l

[1] Xylan Birch wood, Carl Roth, 7500
[2] Yeast Nitrogen Base, without amino acids, Becton, Dickinson and Company, Difco™, 291940
[3] Yeast Nitrogen Base, without amino acids and ammonium sulfate, Becton, Dickinson and Company, Difco™, 291940
[4] Yeast Extract, extract of autolyzed yeast cells, Becton, Dickinson and Company, Difco™, 212750
[5] Wheat spent grain extract 60 g/l, made by M.A., 23.7.2018
[6] Potassium dihydrogen phosphate for analysis, Merck, Emsure® ISO, 1.04873.1000
[7] Urea. Sigma-Aldrich ≥99.5 %. 10179412

3.3 Cultivation conditions

3.3.1 Cultivation on solid medium

Two microliters of each stock for strains in Table 1 were inoculated centrally per 5.5 cm Petri dish. After the inoculated spore drop had absorbed into the media, the Petri dishes were incubated upside down at four different temperatures (20, 25, 28 and 32 ºC).

Triplicate plates were inoculated, which allowed measurement of six individual replicates of the colony diameters.

The growth of the fungi on agar was measured with a ruler for three to nine days depending on how quickly each strain grew. Measurements were made approximately twice a day in two perpendicular directions on the surface of the dishes. Based on these growth measurements, colony radial growth rates, Kr, were calculated for all strains at four temperatures using equation1 (Appendix 3). Standard errors of the mean were also calculated.

$$Colony\ radial\ growth\ rate\ \left[\frac{\mu m}{h}\right] = \frac{radial\ growth\ [\mu m]}{incubation\ time\ [h]} = \frac{\frac{1}{2} diameter\ [\mu m]}{incubation\ time\ [h]} \quad (1)$$

Plates with satellite growth, where not included in the calculation of Kr, since neighboring colonies affect each other's growth. Measured vertical and horizontal growth were treated as individual measurements, since they are likely to grow independently of each other. Therefore two Kr values were calculated per dish.

3.3.2 Cultivation in liquid

Fungi were cultivated in liquid media for the enzyme activity assays. Several cultivations were made in order to optimize growth and enzyme production. Changes in cultivation media were made based on previous enzyme activity assay results. A pre-incubation in xylose preceded every production cultivation with birch wood xylan substrate in order to obtain biomass and enzyme activity more rapidly than when inoculating with spores.

Two out of the ten selected strains (Table 1) did not produce spores (LF311 and LF766), and therefore mycelia stock suspensions were used as inoculum instead of the spore suspensions. For the sporulating strains, glycerol spore stock solutions had been made in prior studies by Happonen [4] and their spore concentrations had been reported to be at least $1x10^8$ spores/ml. In this thesis, the final concentration of spores in pre-culture medium was at least $1x10^5$ spores/ml. For 20 milliliters media, this meant a total of 2 000 000 spores, i.e. minimum 20 µl of stock. A ratio of inoculum to pre-culture media of

40 µl of inoculum per 20 ml of pre-culture media was used with all strains for all pre-cultures.

For all supernatant samples taken, a Biofuge Fresco Heraeus centrifuge were used to collect the supernatant from the production culture samples. If another centrifuge has been used, it is mentioned.

Un-buffered pre-cultures (Table 2) were inoculated with 40 or 200 µl spores or mycelial fragments in 100 or 500 ml flasks containing 20 or 100 ml medium. Production cultures were inoculated with 4, 8 or 16 ml pre-culture in 40 or 80 ml medium in 250 and 500 ml flasks, respectively. Both pre-cultures and production cultures were grown with 200 rpm agitation, and 24 °C (strain KF525) or 28 °C (all other strains). KF525 was grown in lower temperature since it does not grow at temperatures above 24 ºC [5]. The size of the cultivation flasks varied (100 ml, 250 ml or 500 ml) depending on the volume of the media used in each cultivation. Only un-baffled flasks were used to keep the cultivation circumstances in that respect comparable (such as flow geometry of the media and aeration).

Two inoculum volumes 10 % (v/v) and 20 % (four and eight milliliter) were tested to assess whether a high inoculum generated more filamentous cultures than the low inoculum and produced enzyme activity sooner. Inocula were centrifuged with a Biofuge Fresco Heraeus (13,000 rpm) or a Biofuge Stratus Heraues (3,500 rpm, 15 minutes, 4 °C) centrifuge, cells were collected and re-suspended with a small volume of production media (Initial production medium in Table 3) by pipetting back and forth. These somewhat homogenized solutions were inoculated into each flask.

200 µl of spore suspension were inoculated to 100 ml of buffered pre-culture media in 500 ml Erlenmeyer flasks. The pH values of buffered pre-cultures were measured in order to assess if the pH would not decrease as much as in the agar-pre-cultures. Measurements were made on average once a day.

Haapala et al. [43] found that potassium dihydrogen phosphate had a significant effect on pH of the culture, and consequently improved fungal xylanase production [43]. Hence, the amount of KH_2PO_4 was raised to 15 g/l in order to increase the buffering capacity in

the production media. YNB contained only 1 g/l monopotassium phosphate [44]. Thus 14 g/l of KH_2PO_4 had to be added in order to yield a total concentration of 15 g/l. 14 g/l of phosphate was calculated to correspond 70 ml/l of KH_2PO_4 200 g/l stock solution. Buffered production medium also contained either spent grain extract or yeast extract as organic nitrogen source (Table 3).

From the buffered production cultivation with spent grain and yeast extract, two 2 milliliters samples from each production flask were taken on cultivation days 0, 1, 3, 5, 7 and 14. These samples (2x2 ml) were for the xylanase activity assays. Additionally, a separate pH sample was taken in order to minimize the time when assay samples were at room temperature before storing in freezer. One two ml sample was frozen without any preparations (no centrifuging etc.) and the other sample was centrifuged (13,000 rpm, 15 min., ~4 °C), and only the supernatant was frozen. The samples without centrifuging contained cells and could be used to assess whether enzyme was attached to the cell walls of the fungi (“bound enzyme”).

Alternatively, production medium contained urea (2.27 g/l) (Table 3), which replaced the $(NH_4)SO_4$ (5 g/l) in YNB with a molar equivalent of nitrogen. YNB without amino acids or ammonium sulfate was used to provide other inorganic salts. Urea was also expected to help buffer the medium, so additional phosphate was not added. Urea containing media were filter sterilized to avoid degradation of the urea. [45]–[48]

The pH of the urea containing production cultivations was measured daily and lowered aseptically if over seven with sterile 10.2 % phosphoric acid, H_3PO_4.

3.4 Enzyme activity assays

3.4.1 Qualitative assessment of enzymatic activity - Clearing zone

Clearing zones were assessed from solid media cultivations. Only a few plates had a noticeable zone of clearance without staining and therefore, a small volume (1.5-3 ml) of stain was added to most of the plates. Two dyes were used to get visible zones of clearance.

An aqueous solution of Congo red was used (0.2 g/l, 1 g/l, 3 g/l or 5 g/l). Plates were flooded with Congo red, left to stand for 15 minutes and flooded again with 1 M NaCl for 15 minutes. The other stain, Gram's iodine, contained 0.133 grams of potassium iodine and 0.067 grams of iodine, which were dissolved in 20 ml of distilled water. Plates were flooded with Gram's iodine for 10-12 minutes. After pouring off the iodine solution, plates were rinsed several times with Milli-Q water.

Teather and Wood [37] had found that hydrochloric acid enhanced the zone of clearance when used after Congo red treatment. This method was tested for one of the plates: flooded with 1 M HCl (~2 ml) after first staining with 1 g/l of Congo red. [37]

3.4.2 Quantitative xylanase activity assays

The xylanase activity assay used from M. Bailey (Appendix 4), is an international method for studying xylanase [17] [49]. This assay primarily measures the activity of *endo*-β-1,4-xylanase. The hydrolyzed sugars, xylooligosacharides, from birch xylan substrate are spectophotometrically quantified with a color reaction.

The measured color is due to the 3,5-dinitrosalicylic acid, i.e. DNS or DNSA, used in the assay. Yellow-orange DNS reduces to red-brownish 3-amino-5-nitrosalicylic acid, when xylose is oxidized (Figure 3). Boiling enhances the brownish color. The formed amino nitro salicylic acid absorbs light at wavelength of 540 nm. [50], [51]

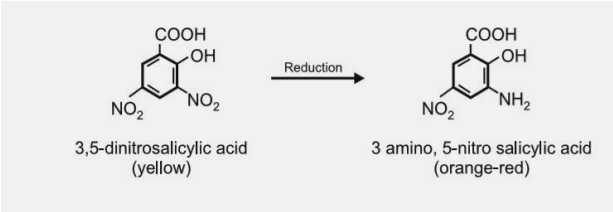

Figure 3. DNS reduces to amino-nitro salicylic acid while sugar oxidizes. [52]

Sodium citrate buffer (0.05 M) was prepared according to the Bailey activity assay (Appendix 4). Citric acid (Citric acid monohydrate, Sigma-Aldrich, 99.5-102 %) was

dissolved in distilled water. Sodium hydroxide was used to adjust the desired pH to the pH (usually pH 5). The buffer was stored at room temperature after autoclaving.

In addition, a sodium citrate buffer at pH 5 with 14 g/l and 28 g/l sea salt (Tropic Marin®, Germany) was made.

A 1 % birch wood xylan substrate was made according to the Bailey's protocol. One gram of xylan (Xylan from birch wood, Carl Roth, 7500) was mixed with a Bamix blender at 60 °C with sodium citrate buffer (at the desired pH; usually pH 5). The solution was heated to boiling point (but not boiled!), allowed to cool and was left to stir overnight. Next day, the solution was made up to a final volume of 100 milliliters and was stored in the freezer in batches of approximately 25 ml. The substrate had to be mixed well after freezing, since freezing caused sedimentation.

Substrates with alternative xylans and birch wood xylan with pH three, four, six and seven were also made. The pH of all the prepared substrates were measured after preparation.

A 0.01 M xylose stock solution was used in the assay as standard. 1.5 g xylose (D-(+)-Xylose, Sigma-Aldrich, ≥99 %, 101956373) was dissolved in one liter of sterilized water. Since the xylose was dissolved in water (not to the sodium citrate buffer), it was possible to use the same standard stock for further assays done at different pH values. The xylose standard stock solution was stored in freezer (-20 °C) in approximately 0.8 milliliters batches and was mixed well before use. The xylose stock solution was used in the assay as four different dilutions: 1:1 (un-diluted), 1:2, 1:3 and 1:5. Enzyme activities that these dilutions corresponds are presented in Table 4. With these dilutions, the absorbance was between approximately 0.08 and 0.55. The defrosted stock solution was diluted to the sodium citrate buffer (with the pH in which the assay wanted to be conduct). New standard dilutions were made for every enzyme assay.

Table 4. Xylose standard converted to enzyme activity.

Standard dilution	Xylose [µmol/ml]	Enzyme activity [nkat/ml]
1:1	10	33.3
1:2	5	16.7
1:3	3.3	11.1
1:5	2	6.7

As illustrated in equation 2, with a reaction time of 300 seconds the 1:1 xylose standard 0.01 M (=10 µmol/ml) corresponds to 33.3 nmol/ml*s of xylose, which is equal to an enzyme activity of 33.3 nkat/ml.

$$\frac{10\ \mu mol/ml}{300\ s} = 0.033\ \frac{\mu mol}{ml*s} = 33.3 \frac{nmol}{ml*s}\ (= \frac{nkat}{ml}) \quad (2)$$

The xylan degradation reaction was terminated with DNS, containing 300 g/l K-Na-tartrate ($C_4H_4KNaO_6$*$4H_2O$), 16 g/l of NaOH and 10 g/l of dinitro salicylic acid (DNS). The DNS was stored in a brown bottle in order to protect from light and carbon dioxide even though DNS has seemed to be strongly photostabile [53], [54]. DNS was filtered before use in case of precipitation. DNS absorbs light strongly at wavelength 540 nm [55], [56].

Econase (AB Enzyme Oy, Finland, lot 100068259) was used as a control enzyme to ensure that the actual enzyme assay worked. Econase is known to contain xylanase (*endo*-β-1,4-xylanase) and the amount of enzyme activity has previously been measured [57]. Econase was diluted 1:2,000 in the sodium citrate buffer (at the desired pH). With a dilution of 1:2,000 the activity observed was within the xylose standard curve (absorbance between 0 and 0.5). The Econase had been stored as a 1:10 dilution at -20 °C, thus it was diluted 1:200, first 1:10 and then 1:20, in order to achieve total dilution of 1:2,000. As with the xylose standard, a new Econase dilution was made for every assay.

3.4.2.1 Standard xylanase activity assay

The assay was done according to Bailey's (Appendix 4) xylanase activity assay, but using quarter of the volumes mentioned in the protocol to decrease chemical and sample consumption.

The xylan substrate, Econase control, enzyme samples to be measured, and the stock solution of xylan standard were allowed to defrost at room temperature after which the enzyme samples were kept on ice and returned to the freezer as soon as possible, to minimize loss of the enzyme activity at room temperature.

450 µl of defrosted and well-blended xylan solution was pipetted to ten milliliters glass test tubes and was tempered to 50 °C in a water bath for approximately five minutes. 50 µl of each (diluted) enzyme sample or Econase was added to the test tubes and incubated for exactly 300 seconds (5 min.) in the same water bath (50 °C). After exactly 300 seconds, 750 µl of filtered DNS was added to each tube in order to stop the reaction and to detect the reducing sugars released by the enzyme. Tubes were mixed with vortex, boiled for five minutes and cooled in a cold water bath for another five min. The color formed was measured with a Hitachi spectrophotometer (Hitachi U-2000, part no 1210120, Hitachi part no 121-0003) at 540 nm against the measuring zero. The absorbance values were corrected with corresponding enzyme zeros and converted into enzyme activity (equation 2).

The same number of enzyme zeros were measured, as sample, as well as concentrations, two measuring zeros and four xylose. The substrate (450 µl in each test tube) was tempered to 50 °C in a water bath, as with the enzyme samples, but 750 µl of DNS was added to the substrate *before* adding 50 µl of sample, buffer, xylose or control. The absorbance was read at 540 nm as like with the enzyme samples.

The enzyme samples, enzyme zeros, standards, Econase control and measuring zeros were all done (tempered, boiled and cooled) concurrently in order to prevent natural variation between the assay runs.

Enzyme activity was measured in katals, i.e. the amount of enzyme that catalyzes the conversion of one mole of xylan to xylose (or xylo-oligomers) per second under assay conditions.

3.4.2.2 Optimum temperature for xylanase activity

To study the effect of reaction temperature, the assay was done at four different temperature (30, 40, 55, and 60 °C) in addition to 50 °C. All other assay parameters were kept constant.

3.4.2.3 Effect of pH on xylanase activity

The effect of pH (3, 4, 5, 6 and 7) on xylanase activity was also assessed. For each pH a new Na-citrate buffer with the desired pH was made, followed by new birch wood xylan substrates with each pH. Triplicates were used.

3.4.2.4 Enzyme stability at different temperatures

In order to investigate enzyme stability at different temperatures at pH 5, the samples were pre-incubated for various times (0, 15, 30, 45 and 60 min) at different temperatures (30, 40 and 50 °C). The activity assay was conducted normally as described in section 3.4.2.1. Duplicate were used instead of triplicates in order to keep the sample consumption reasonable.

3.4.2.5 Salt tolerance of xylanase

The impact of salt on xylanase activity was studied with Tropic Marin sea salt at 14 g/l and 28 g/l. These concentrations were used since salinity of seawater is generally approximately 35 g/l [58], [59].

New sodium citrate buffers with these sea salt concentrations were made, and pH values were adjusted to 5 with NaOH, since both strain LF311 and KF525 had their highest

activities at pH 5 at approximately 50 °C (section 4.3.2). New birch wood xylan substrates were prepared from these buffers. Triplicates were used.

In the preparation of the two sea salt substrates, a smaller homogenizer (CAT, M. Zipperer X120) was used as a blender instead of the Bamix hand blender, since the prepared substrate volumes were much smaller (30 ml). It would have been unwise to use the Bamix blender, since it does not mix well suspensions with volumes under 100 ml. Homogenizers are usually more effective than ordinary blenders, and therefore mixing time was reduced from approximately one minute to about 20-30 seconds.

3.4.2.6 Effect of different xylan substrates on xylanase activity

Effect of xylans from different origin on xylanase activity were evaluated. New 1 % xylan substrates with different xylans were made in pH 5 Na-citrate buffer using the CAT homogenizator. Xylans from the seaweed *Palmaria palmate* (Elicityl OligoTech®, France), oat spelt (Fluka AG, Buchs Switzerland), low viscosity wheat arabinoxylan (Megazyme) and an insoluble wheat arabinoxylan (Megazyme) were used. Both soluble and insoluble arabinoxylans were assayed since wheat contains both forms [31]. Assays were conducted normally with triplicates at 50 °C.

4 Results

4.1 Cultivation on solid media

Media containing 20 g/l agar were turbid and thus the observation of clearing zones was challenging without staining. Decreasing the agar amount to 15 g/l was a simple and practical solution for it.

All growth measurements and calculated colony expansion rates are submitted in Appendix 2 (Table 2A). Presented Kr values are averages of the used six replicates. Standard errors of the means are shown in Figures in Appendix 3.

Ten strains (Table 1) were cultivated at temperatures 20, 25, 28 and 32 ºC. No growth was observed with LF300, *Volucrisporia* graminea, on solid media at 28 ºC and 32 ºC. Additionally, LF580 *Scopulariopsis* brevicaulis and LF560 *Halenospora* varia did not grow at 32 ºC.

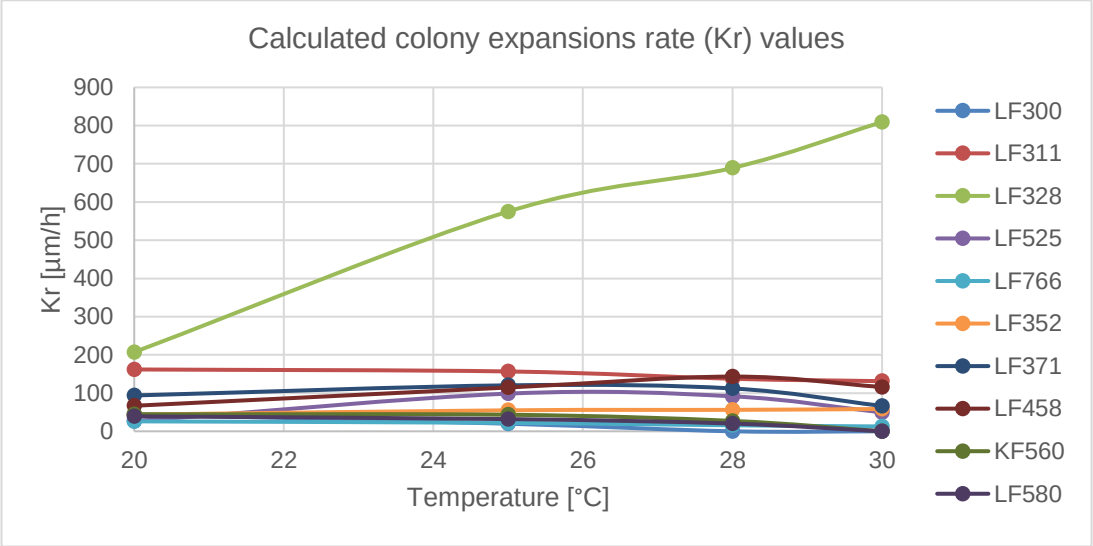

Figure 4. Calculated colony expansion rates [µm/h] for ten fungi.

Strains LF311 *Lewia infectoria* and LF328 *Trichoderma* sp. had the highest colony expansion rates at all studied temperatures (Figure 4). The Kr values for LF311 varied between 131 and 161 µm/h and for LF328 between 205 and 804 µm/h. LF311 had its highest expansion rate at the lowest temperature (20 ºC) and the smallest Kr at the 32 ºC. In contrast, LF328 expanded fastest at high temperatures. Although LF328 preferred temperatures 28-32 ºC, it had the highest Kr values at all temperatures.

4.2 Cultivation in liquid medium

4.2.1 Pre-cultivation media with and without agar

Five strains (LF311, LF352, LF371, KF525 and LF766) were included in liquid cultivations with and without agar in the pre-culture media.

In the first pre-cultivation, media with and without agar were used in order to detect possible pellet formation (Figures 5 and 6). Large pellets were formed without agar. Only strain KF525 seemed to have relatively good-looking growth on both pre-cultures (not shown). KF525 was the only strain from which inocula from pre-cultures with and without agar were both used. For all other strains inocula were taken from only the agar containing culture. All subsequent pre-cultivations were conducted with agar in order to reduce pellet formation.

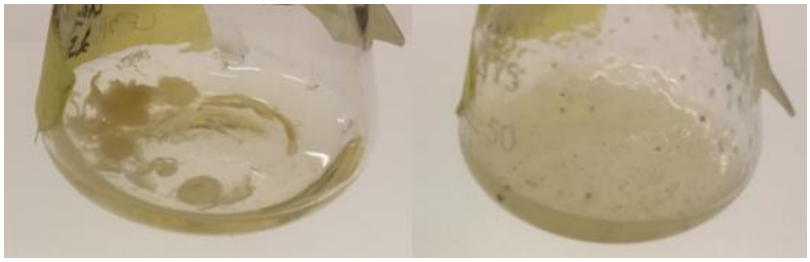

Figure 5. Pre-culture of strain LF311 without (on left) and with 4 g/l agar (on right) at incubation time 164 h. Big pellets were formed without agar.

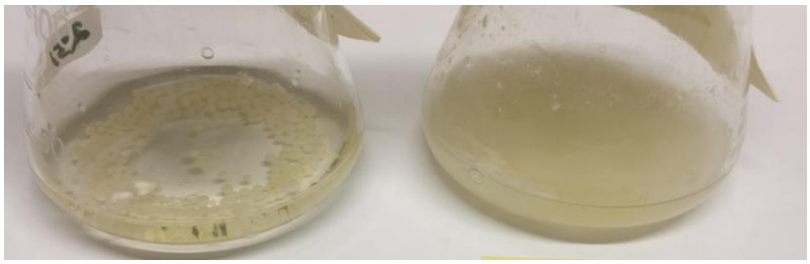

Figure 6. Strain LF371's pre-cultivation without (on left) and with 4 g/l agar (on right) after 72 hours of incubation. Pellets were formed without agar.

Strains LF311 and KF525 were inoculated to production media after seven days and LF352 and LF371 already after three. All four strains were cultivated in production for seven days. Strain LF766 grew very slowly and it did not seem to have enough growth within ten days to be transferred to production. LF766 was not included in further cultivations.

Although two inoculum volumes, 10 and 20 % (v/v), from the agar containing pre-culture were used, no significant differences in growth were observed.

The pH of the pre-cultures with and without agar were not measured, but the pH in the production cultures was approximately 0.3 pH units higher in cultures with 8 ml of inoculum compared to those with 4 ml inoculum. For all strains, the differences between four and eight milliliter in pH values reduced toward the end of each cultivation.

4.2.2 Buffered production media with different nitrogen sources

Xylanase activities from the un-buffered cultivations were low, almost zero, and therefore re-cultivations were needed. Higher pre-incubation pH and a buffered production media were thought to have positive effects on the enzyme activities. Four strains were included: LF311, LF352, LF371 and KF525. LF766 was left out as a result of growing too slowly and forming pellets in the previous pre-culture.

Both buffered production media contained KH_2PO_4 and either yeast extract or spent grain extract as an organic nitrogen source. Agar containing pre-cultures were used.

The pH of the pre-cultures were measured but only pH of cultures with KF525 was raised. The pH of KF525's pre-culture decreased under 3.3 after the third incubation day and after this the pH was raised to over six every time it dropped under 3.5 (i.e. on cultivation days three, five and seven).

The pH of the buffered production media stayed relatively stable throughout the cultivations (between 5.7 and 6.5). No pH adjustments were needed. Only small variations in the pH were measured within the first 100 hours for all strains.

No visible difference in growth was detected between media with yeast extract and media with spent grain. However, differences would be difficult to detect since spent gran stained the media darker and the hyphae were less visible than in yeast extract.

The yeast extract and potassium phosphate culture of strain LF352 was found to be contaminated (microscopic examination) and was not included in subsequent analyses.

4.2.3 Buffered pre-culture

Despite the slightly lower starting pH in the buffered pre-culture, the pH stayed higher (4.5) throughout the pre-cultivation than in the previous pre-cultivations (~2.4), presumably due to the added KH_2PO_4 buffer. Since the culture pH values did not drop under four, the pH values in the buffered pre-cultures were not adjusted at all, contrary to previous pre-cultivations.

For both strains, LF311 and KF525, the pH of the production culture with urea had to be adjusted, since production pH values rose above 7. The pH of KF525's culture increased above 7 within 48 hours and with LF311 the pH value of 7 was exceeded already within 24 hours. Once the pH values rose over 7 the media had to be adjusted daily for both of these strains. In contrast, yeast extract media did not need any pH adjustments.

KF525 appeared to grow similarly in both production media - urea and yeast extract, with the exception of LF311. LF311 mycelium in yeast extract medium appeared to be soft, whereas mycelia in the urea medium looked more solid with sharp edges, like small pellets.

Enzyme activity assays from yeast extract production culture showed a peek in endo-β-1,4-xylanase activity within 45 hours with strain LF311. Therefore, samples (2 ml + 2 ml) from the production cultivation were taken every five hours for the first 48 hours, in order to detect the activity peak as accurately as possible. After 48 hours, samples were taken ones a day for five days.

Contrary to LF311, samples from KF525 were taken only once a day for total of nine days. It was found that only once a day should be enough and the highest activity might be at cultivation hours between 140-200 h. In other word, the xylanase production with KF525 should not be as rapid as with LF311.

Pre-cultivation of LF311 and KF525 lasted for seven days. Strain LF311 was grown in production for five days and KF525 for 15 days.

4.3 Enzyme activity assays

4.3.1 Clearing zones

With strains LF352, LF371, LF458 and LF766 a light hazy clearing zone around the colony in xylan plates was observed without using any dye. Since these zones were very pale, almost unnoticeable, and clearing zones were not detected on most of the plates, two dyes were used to visualize the zones.

The first stain tried was Congo red at concentrations of 0.2 g/l, 1 g/l, 3 g/l and 5 g/l (Figure 7). Some of the clearing zones were visible with 5 g/l Congo red and only on a couple of the plates with 3 g/l. No clearing zones were detected with 0.2 g/l or 1 g/l. Addition of 2 ml of 1 M HCl solution to 1 g/l Congo red plate did not result in a more prominent zone. The HCl changed the medium color to blue as reported in earlier studies, but no effect on the visibility of the zone was detected. [37]

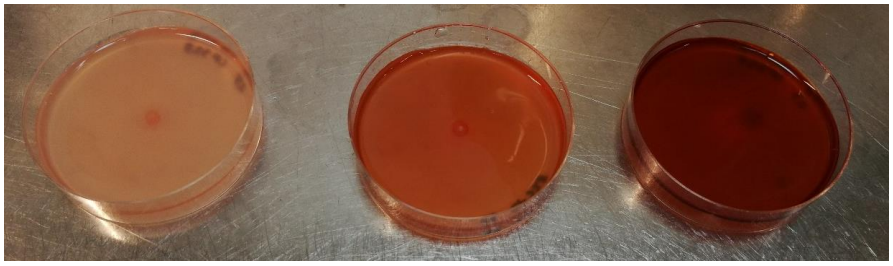

Figure 7. Congo red at concentrations 0.2 g/l, 1 g/l and 5 g/l.

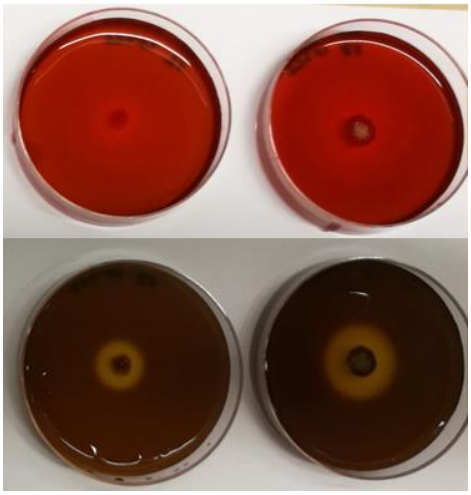

Figure 8. Strain LF766 after 5 days of cultivation at 25 ºC (dishes on left) and 32 ºC (dishes on right). Clearing zones were easier to detect when stained with Gram's iodine (dishes below) than Congo red 5 g/l (dishes on top). Zone diameters: 14 mm and 20 mm.

Prominent clearing zones (Figure 8) developed for all ten strains after staining with grams iodine. Strains LF311, LF352, LF371, KF525 and LF766 gave the clearest and most sharp-edged zones.

Based on the formed zones of clearance, five strains (Table 5) were chosen for liquid cultivations and for the enzyme activity assays. The criteria assessed were the sharpness and the color of the zone.

Table 5. Selected 5 fungal strains to cultivate in liquid.

Code of fungi strain	Name of the fungi strain
LF311	*Lewia infectoria*
LF352	*Aspergillus sydowii*
LF371	*Penicillium granulatum*
KF525	*Calcarisporium* sp.
LF766	*Microascus trigonosporus*

4.3.2 Quantitative xylanase activity assay

Triplicates or duplicates were used when practical in all assays and the error bars as standard errors of the means are shown in the presented figures. The original data of the xylanase assays are in Appendix 5.

Substrates were prepared several time and the pH of all of them were measured to ensure that the pH values were approximately those wanted (Appendix 6). Birch wood xylan substrate at pH 3 differed the most from that planned, with a measured pH of 3.24. The added salts; buffers at different pH values; and different xylan substrates had hardly any effect on the pH values of the final substrates.

Econase, with a known activity, was used as a control enzyme for xylanase activity assays. Econase gave a xylanase activity within the documented range (39,000 - 41,000 nkat/ml), and thus the enzyme activity protocol itself was okay. In case the assay would have given too low xylanase activity for Econase, there could have been a problem with either the assay or the activity (e.g. too long storage or some error while making the dilutions).

No xylanase activity was observed in supernatants from any strain, when they were grown in un-buffered medium.

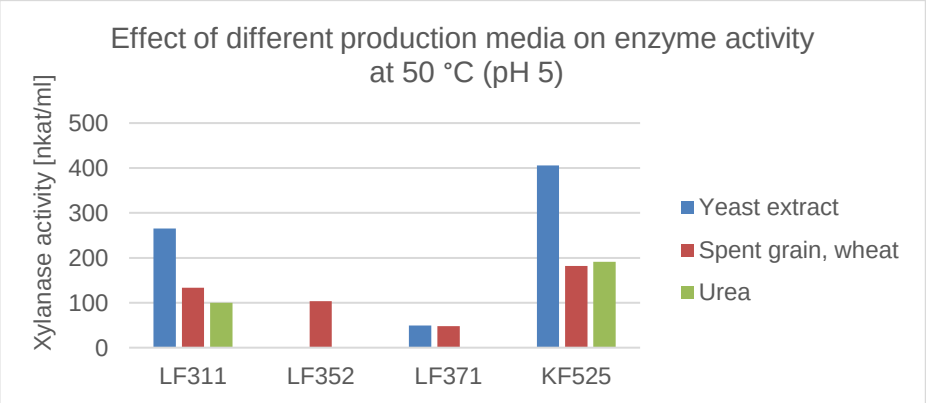

Figure 9. Xylanase activity from different fungi. Un-buffered pre-culture and buffered production media with different nitrogen sourced were used.

As seen from Figure 9, strains LF352 and LF371 produced less xylanase activity than enzyme produced by LF311 (270 nkat/ml) and LF525 (400 nkat/ml). Based on activity results, only strains LF311 and KF525 were included in further studies. Both LF311 and KF525 produced more activity when yeast extract was included in the media than with spent grain or urea (Figure 9). Activities in the supernatant samples were approximately 50 nkat/ml higher than samples with mycelium present (data not shown). Therefore, the enzyme assays focused on supernatant samples from media containing yeast extract.

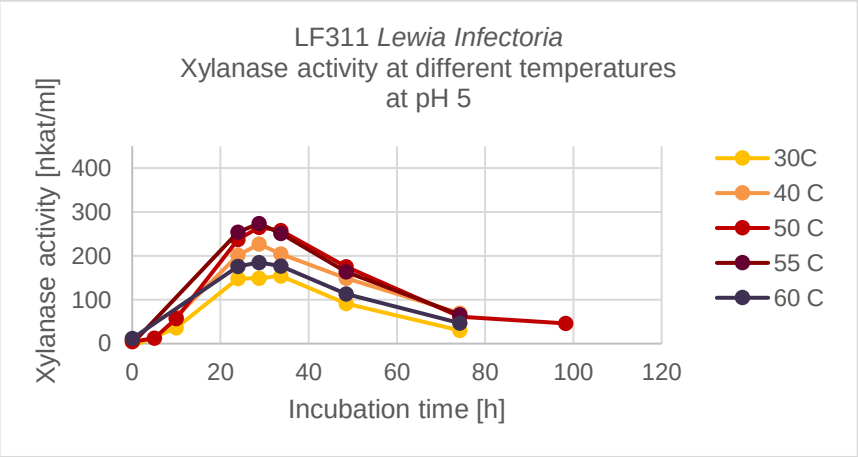

Figure 10. Xylanase activity on strain LF311 at different temperatures.

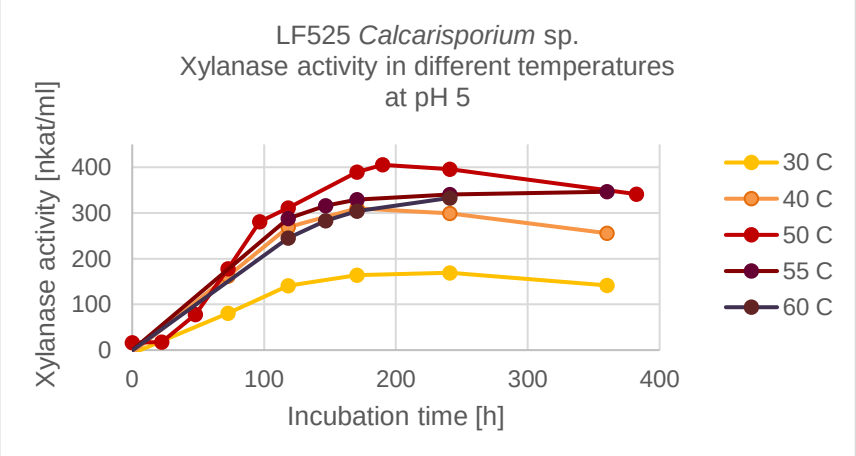

Figure 11. Xylanase activity on strain LF311 at different temperatures.

For strain KF525 maximum xylanase activity was found to be around 190 hours after inoculation (Figure 10) and for LF311 at 29 hours (Figure 11).

4.3.2.1 Temperature optimum

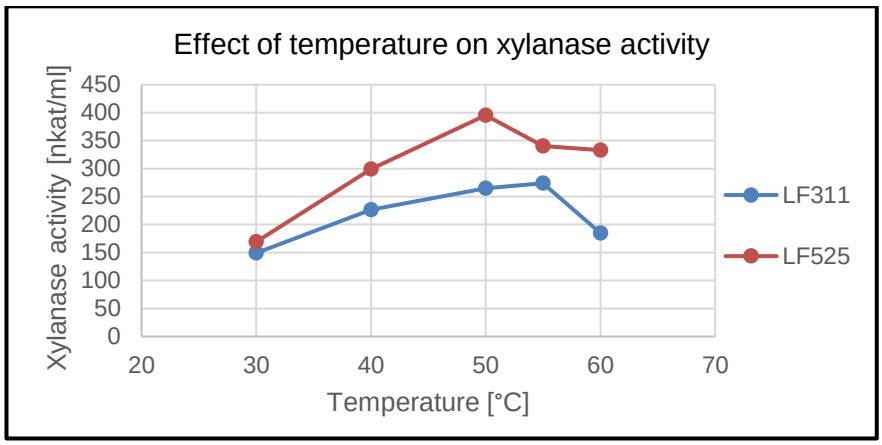

Figure 12. Effect of temperature on xylanase activity from LF311 and KF525.

As seen in Figure 12, activity for xylanase produced by LF311 was highest at 55 °C (270 nkat/ml), whereas xylanase from KF525 was measured at 50 °C (400 nkat/ml). At 60 °C the xylanase activity of LF311 had decreased 30 %. The decrease in activity at temperatures above 50 °C for KF525 was less, about 15 % of the maximum.

4.3.2.2 Temperature stability

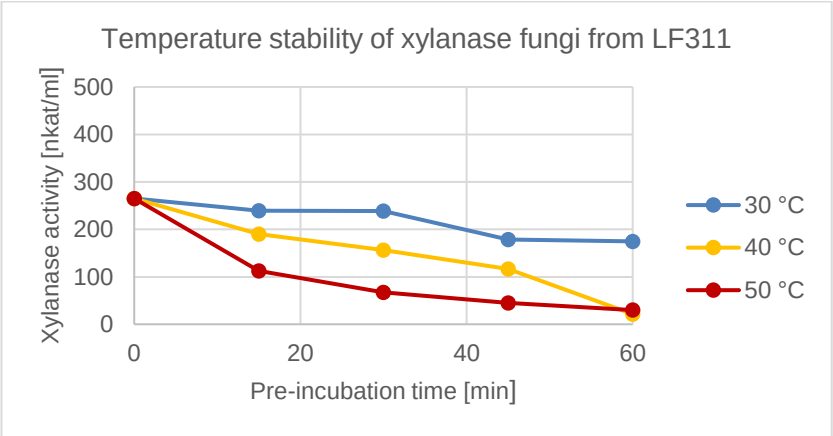

Figure 13. Effect of incubation temperature and time on xylanase activity from LF311.

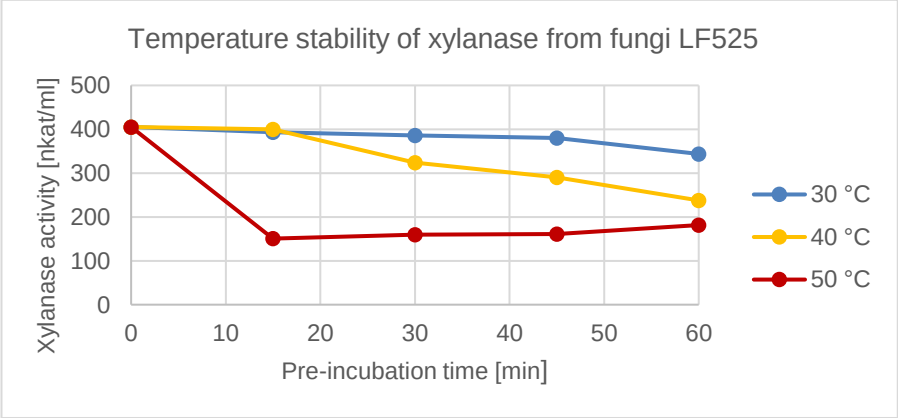

Figure 14. Effect of incubation temperature and time on xylanase activity from KF525.

Xylanase from both stains, LF311 and KF525, retained activity better at lower pre-incubation temperatures (30 to 40 °C) than at higher (Figures 13 and 14). The KF525's enzyme retained 88 % of its activity after one hour at 30 °C, while xylanase from LF311 had only 63 % of its activity left.

The activity of xylanase from LF311 decreased more the longer it was incubated at all temperatures (Figure 13). LF311's activity dropped almost to zero, after one hour pre-

incubation at 40 or 50 °C. KF525's xylanase lost 73 % of its activity within 15 minutes incubated at 50 °C, but was then stable. The first 15 minutes the enzyme of KF525 seemed to be totally stable at both 30 °C and 40 °C, but after 15 minutes the activity decreased almost linearly.

4.3.2.3 Effect of pH

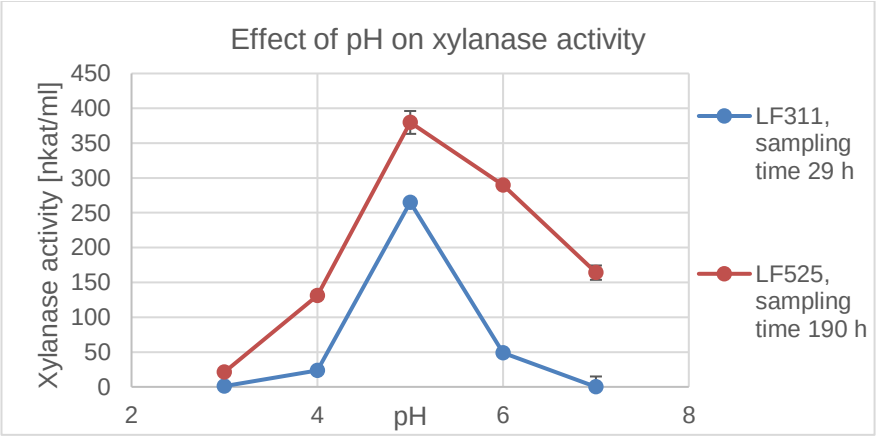

Figure 15. The effect of pH on xylanase activity produced by LF311 and KF525.

Both strains seem had highest enzyme activity at pH 5 (Figure 15). The activities seemed to decrease significantly above and below that pH. For the KF525 enzyme, the decrease was more notable when changing the pH from five to four than change from five to six. The LF311 enzyme had almost no activity at pH values above or below pH 5.

4.3.2.4 Salt tolerance

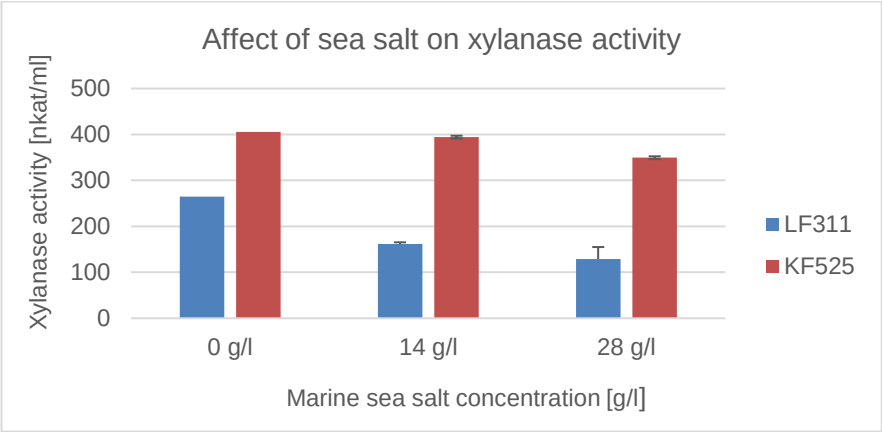

Figure 16. Effect of salinity on xylanase activity for strains LF311 and KF525.

As shown in Figure 16, addition of 14 g/l marine salts affected enzyme activity more on xylanase produced by LF311 than by KF525. Xylanase activity from fungus KF525 decreased only slightly (3 %) with addition of 14 g/l salt, whereas LF311's enzyme activity declined 39 %. Increasing the salinity from 14 g/l to 28 g/l resulted in loss of 14 % KF525's xylanase activity and 51 % of LF311's, compared to the maximum without salt addition (Figure 16).

4.3.2.5 Alternative xylan substrates

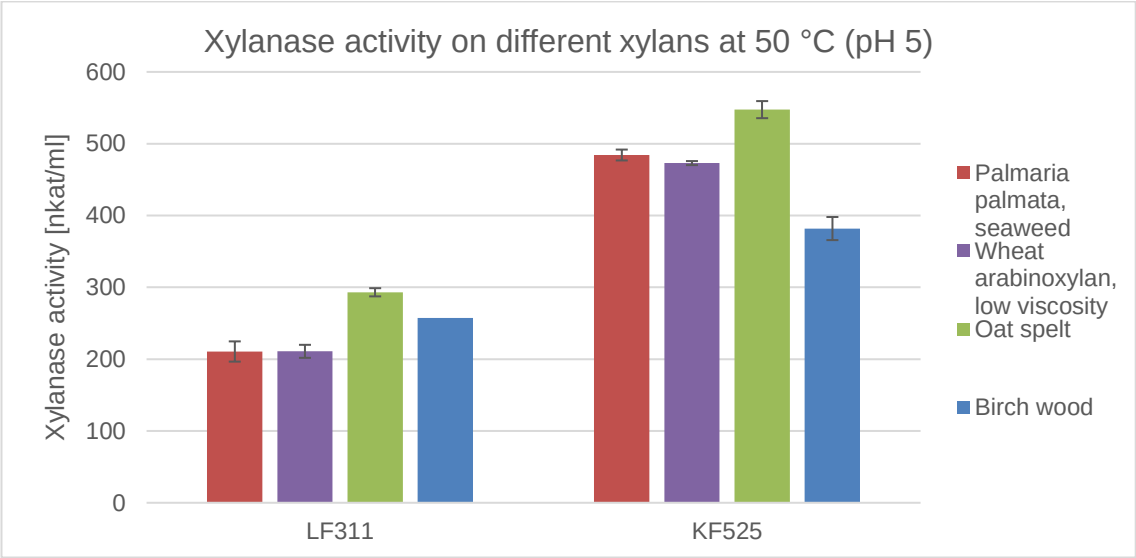

Figure 17. Enzyme activity on different xylan substrates on strains LF311 and KF525.

As can be seen from Figure 17, the highest xylanase activity from both fungi was found to be on xylan from oat spelt: L. *infectoria* 300 nkat/ml and *Calcarisporium* sp. 550 nkat/ml.

Enzyme activity on insoluble wheat arabinoxylan was also studied (data not shown), but the results were not reproducible; even with multiple repetitions. The measured activity varied between -117 and 50 nkat/ml and even Econase did not give reproducible results.

5 Discussion

This thesis focused on characterization of xylanase activity in marine fungi. Although ten marine fungi were observed to degrade xylan in a clearing zone assay, only two, *Lewia infectoria* LF311 and *Calcarisporium* sp. KF525, produced considerable amounts in liquid. Two other strains produced detectable, but low levels of activity in liquid (Figure 9).

After selecting ten fungi with good potential for xylanase production from previous data, the fungi were initially screened on solid medium. This provided information on their secrete enzyme production (clearing zones) and on their growth at different temperatures. Although colony radial growth rate (Kr) cannot be used to compare growth or specific growth rates of different strains, which may differ considerably in their branching behavior, it was expected that it would provide a good estimate of the temperature optimum for growth. Most of the ten strains considered here had temperature optima between 25 and 28 °C. LF328 grew best at 32 °C and KF560 and LF580 had optima between 20 and 25 °C. KF525, which is reported to not grow above 24 °C [5], grew at both 28 and 32 °C, suggesting the stock had become contaminated. Fresh stock was used for further studies.

Two fungi, LF311 and KF525, were found to produce good amounts of xylanase activity. The activity of LF311 was optimal at 55 °C and at pH 5, but the enzyme activity was not stable at 50 °C. Also KF525 had its xylanase activity optimum at pH 5, but at 50 °C (although it does not grow above 24 °C [5]). The temperature optimum at 50-55 °C for marine fungi correlates to reported averages in other studies [2], [16], [18]. Xylanase activity decreased significantly on both LF311 and KF525 after 15 minutes at 50 °C. Therefore a high hydrolysis temperature (around 50 °C) may lead to enzyme degradation. The xylanase from KF525 seemed to tolerate marine salt addition better than enzyme produced by LF311. The enzyme activities obtained in this thesis were not as high as reported in some studies of other marine fungi from Raghukumar et al. [29] and dos Santos et al. [60]. However, it should be taken into account that the xylanase activity has been determined with different methods and thus the yielded activity results might not be unequivocally comparable.

Although *endo*-1,4-*β*-xylanase is the most common xylanase, other xylanase activities (such as *endo*-xylosidase) could have been tested as well, especially since changes in the cultivation conditions may also affect the amounts and ratios of the xylanases produced [61], [62]. Different growth conditions may promote different xylanases, as Xiong et al (2004) observed: the fungus modified the production of different xylanases when pH of the culture was altered [63].

Yeast extract as nitrogen source was found to be a better inducer for xylanase production than wheat spent grain, since higher xylanase activities were observed on yeast extract for both LF311 and KF525. Other nitrogen sources such as KNO3, NH4Cl or tryptone could be tested as well, since they have been reported to induce xylanase production [36]. Especially the use of refuses from other processes would be interesting to test from a recycling point of view.

The poor activity at low pH for both LF311's and KF525's xylanase suggests that poor buffering was the reason no activity was observed in the initial test. Additionally, the optimum temperature for growth (as interpreted from Kr) may not be optimal for enzyme production.

Highest xylanase activities from LF311 and KF525 were observed on xylan substrate from oat spelt. Also seaweed substrate gave high activity for strain KF525, but LF311's xylanase yielded considerably lower activity than on oat spelt or birch. This might suggest that xylanase from KF525 is able to degrade more effectively both *β*-1,4- and β-1,3-linkages than xylanase from LF311, since xylan from oat has predominantly *β*-1,4-linkages whereas seaweed xylan has both 1,3- and 1,4-linkages [23].

Xylanases are used in the paper industry and the effect of different metal ions could be tested. Pulp contains usually a variety of different metal ions and some of them have been found to enhance xylanase activity (Mn^{2+}, Zn^{2+}, Ca^{2+}), some to inhibit (K^{2+}) and some to have no effect on enzyme activity (Cu^{2+}, Mg^{2+}) [64]. Other inhibiting or inducting factors could be tested as well. [36] Additionally, the xylanase activity at lower temperatures than 30 °C would be interesting to assess. since xylanases active at low temperatures could have applications in bread making [65].

The glucuronoxylan from birch wood (Carl Roth) which was used is no longer commercially available. Some of the Roth's xylan that Happonen [4] had used in his studies was still left from those prior studies. The Carl Roth xylan was also used in cultivations in this thesis in order to make the results somewhat comparable with the earlier work. However, beechwood glucuronoxylan could be an alternative for birch wood's xylan, since beechwood is found to be quite well comparable with that from birch [33].

Although considerable xylanase activity was observed with two strains, LF311 and KF525, some activity was detected also from LF352 and LF371. Possibly with more culture condition optimizing the activity levels would have been high enough to characterize the enzymes.

Cultivations and enzyme assays made in this thesis should be reproducible, as observed in successful repetitions within this study. The promising results obtained could be utilized in further marine fungi studies, in xylanase productions or assessing enzyme properties in general.

6 Conclusions

The objective of this thesis was to grow marine fungi and study their xylanase activity and the properties of these enzymes.

Preliminary assessment on xylanase activity were made using two dye. Gram's iodine was found to be more effective than Congo red, since Gram's iodine generated more easily detected zones. There seemed to be no clear correlation between the diameter of the clearing zone and the spectrophotometrically measured enzyme activity in this thesis.

Cultivations in liquid media in this thesis highlighted the importance of cultivation conditions, especially pH and buffering capacity. The pH should be kept high enough (for filamentous fungi usually over pH value 4) for instance by buffering and/or adjusting the pH while cultivating.

Production media with yeast extract as an organic nitrogen source and KH_2PO_2 as buffer resulted in higher xylanase activities than enzyme produced on media containing buffer and urea or buffer and wheat spent grain.

Temperature optima for xylanase activity for strains LF311 and KF525 were found to be around 50 ºC. Similar results with fungal xylanases have been reported in several studies [11], [66]–[68]. The highest xylanase activities determined were from *Calcarisporium* sp.

(550 nkat/ml) and from *Lewia infectoria* (290 nkat/ml) on oat spelt xylan at 50 °C at pH 5.

The enzymes studied in this thesis could be industrially more interesting if the enzymes would have optimum activity at lower temperatures in order to reduce heating costs or facilitate simultaneous saccharification and fermentation in industrial processes.

Studies with LF311 should be easy to conduct and repeat, since it grew relatively fast, though its xylanase activity was not as high as with xylanase produced by KF525. The optimum pH was found to be approximately at pH 5 for both fungi. Xylanase produced by KF525 tolerated salinity more than enzyme from LF311. L. *infectoria* (LF311) retained 51 % of its activity at a sea salt concentration of 28 g/l compared to no salt addition. Xylanase from KF525 had even 86 % of activity left at 28 g/l sea salt.

Information on marine fungi were obtained, but naturally much more could have learned if the study would have been continued even further. Five fungal strains were cultivated in liquid media and some characteristics of xylanases from two strains were studied. *Lewia infectoria* (LF311) and *Calcarisporium* sp. (KF525) were the most promising strains based on their xylanase activity.

The study could be continued by determine more precisely the optimum cultivation conditions for maximum xylanase yield: optimum incubation time (with help of HPLC) and culturing in a richer medium (with for example trace elements). A high performance liquid chromatograph, HPLC, could be used to monitor sugar consumption in the liquid culture and thus evaluate the optimum incubation time. Additionally, the characterization of the produced xylanases would be interesting to assess further, for example the effect of metals, or other inducing and inhibiting factors.

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Specific growth rates and OD_{600} values for marine fungi from Happonen [4] **and Wang et al.** [5]**.** Selected strains are marked with *.

Table 1A. Specific growth rates for marine fungi from prior studies.

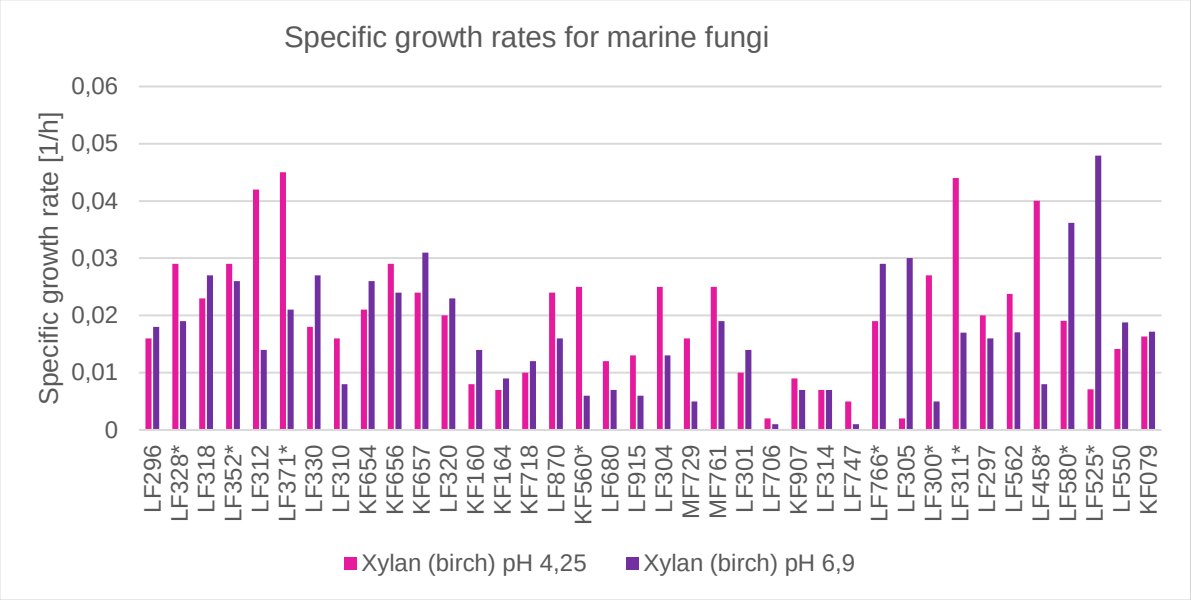
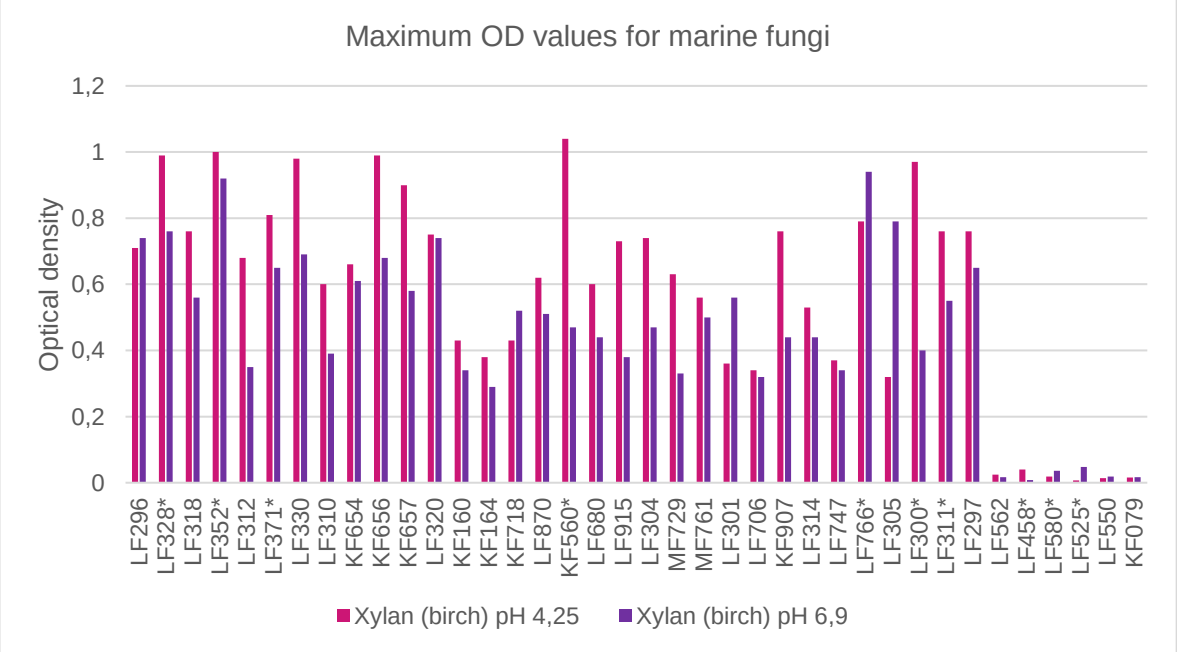


Table 1B. Optical density (OD_{600}) values for marine fungi from prior studies.



Measurements of fungal growth on solid media and calculated colony expansion rates (Kr).

Table 2A. Colonies with satellite growth are marked with grey (not included in the calculation of Kr).

				Incubation time [h]											Kr [µm/h]	r2
	T [C]	Dish number	Measurement	0	21	45	69	117	141	165	169	190	195	217		
LF300	20	1	Vertical	4	4	4	4	6	7	7	7	9	9	11	39	0,99
			Horizontal	4	4	4	4	6	7	7	7	9	9	11	39	0,99
		2	V	4	4	4	4	7	7	7	7				0	#DIV/0!
			H	4	4	4	4	7	7	7	8				125	1,00
		3	V	4	4	4	4	6	6	7	8	8	9	10	25	0,87
			H	4	4	4	4	6	7	7	7	8	9	11	39	0,95
	25	4	V	4	4	4	4	4	4	4	5	5	5	6	20	0,97
			H	4	4	4	4	4	4	4	5	5	5	6	20	0,97
		5	V	4	4	4	4	4	4	4	4	5	5	6	20	0,97
			H	4	4	4	4	4	4	4	4	5	5	6	20	0,97
		6	V	4	4	4	4	4	4	4	5	5	5	6	20	0,97
			H	4	4	4	4	4	4	4	5	5	5	6	20	0,97
	28	7	V	4	4	4	4	4	4	4	4	4	4	4	0	#DIV/0!
			H	4	4	4	4	4	4	4	4	4	4	4	0	#DIV/0!
		8	V	4	4	4	4	4	4	4	4	4	4	4	0	#DIV/0!
			H	4	4	4	4	4	4	4	4	4	4	4	0	#DIV/0!
		9	V	4	4	4	4	4	4	4	4	4	4	4	0	#DIV/0!
			H	4	4	4	4	4	4	4	4	4	4	4	0	#DIV/0!
	32	10	V	4	4	4	4	4	4	4	4	4	4	4	0	#DIV/0!
			H	4	4	4	4	4	4	4	4	4	4	4	0	#DIV/0!
		11	V	4	4	4	4	4	4	4	4	4	4	4	0	#DIV/0!
			H	4	4	4	4	4	4	4	4	4	4	4	0	#DIV/0!
		12	V	4	4	4	4	4	4	4	4	4	4	4	0	#DIV/0!
			H	4	4	4	4	4	4	4	4	4	4	4	0	#DIV/0!
LF311	20	13	V	4	4	4	13	32	33						160	0,96
			H	4	4	4	10	29	34						165	0,99
		14	V	4	4	4	12	29	35	42	42	46	50	50	165	1,00
			H	4	4	4	13	30	38	42	45	48	50	50	177	1,00
		15	V	4	4	4	15	26	35	43	44	44	50	50	152	0,98
			H	4	4	4	8	27	31	41	41	46	50	50	152	0,98

	25	16	V	4	4	8	18	35	37	42	42	45	43	51	156	0,97
			H	4	4	9	19	34	40	41	44	45	47	51	160	0,99
		17	V	4	4	9	17	35	40						167	0,99
			H	4	4	8	17	34	40						169	1,00
		18	V	4	4	8	18	32	35	37	40	42	42	50	142	0,97
			H	4	4	8	17	31	36	37	40	40	42	50	146	0,99
	28	19	V	4	4	8	16	29	28	29	30	28	29	30	144	1,00
			H	4	4	9	16	29	28	29	30	29	28	30	138	1,00
		20	V	4	4	9	18	30	33	37	37	40	43	55	143	0,99
			H	4	4	9	17	30	34	35	35	40	43	55	144	1,00
		21	V	4	4	9	16	28	31	36	36	40	442	55	131	1,00
			H	4	4	9	16	27	30	35	35	40	42	55	124	1,00
	32	22	V	4	4	6	9	21	25	30	30	36	38	42	117	0,99
			H	4	4	6	10	23	25	29	29	36	37	43	123	0,98
		23	V	4	4	5	11	21	25	30	31	37	38	44	127	1,00
			H	4	4	6	10	21	25	30	31	37	38	45	132	0,99
		24	V	4	4	6	11	20	22	29	30	37	38	44	147	1,00
			H	4	4	6	11	21	24	29	30	37	38	45	141	0,99
				0	**3**	**24**	**28**	**32**	**49**	**50**	**55**	**73**	**76**			
LF328.1	20	25.1	V	4	4	4	4	5	12	13	13	28	31		204	0,99
			H	4	4	4	4	5	12	13	13	28	31		204	0,99
		26.1	V	4	4	4	4	5	12	13	13	29	32		204	0,99
			H	4	4	4	4	5	12	13	13	30	32		204	0,99
		27.1	V	4	4	4	4	4	12	13	13	29	31		214	0,98
			H	4	4	4	4	4	12	13	13	28	32		214	0,98
	25	28.1	V	4	4	6	9	12	33	35	35	55	55		571	0,99
			H	4	4	6	9	12	34	35	35	55	55		581	0,99
		29.1	V	4	4	6	9	12	33	35	35	55	55		571	0,99
			H	4	4	6	9	12	34	36	36	55	55		592	0,99
		30.1	V	4	4	7	9	13	34	35	35	55	55		566	0,99
			H	4	4	7	10	13	35	35	35	55	55		570	0,99
	28	31.1	V	4	4	10	15	19	43	50	50	55	55		665	1,00
			H	4	4	10	15	20	44	50	50	55	55		685	1,00
		32.1	V	4	4	10	15	20	44	50	50	55	55		685	1,00
			H	4	4	10	15	20	45	50	50	55	55		706	1,00
		33.1	V	4	4	10	15	19	44	50	50	55	55		686	1,00
			H	4	4	10	15	19	45	50	50	55	55		708	1,00
	32	34.1	V	4	4	11	15	22	49	53	53	55	55		808	1,00
			H	4	4	11	16	21	50	53	53	55	55		815	1,00

		35.1	V	4	4	12	17	21	50	53	53	55	55		797	0,99
			H	4	4	12	17	21	50	53	53	55	55		797	0,99
		36.1	V	4	4	11	16	20	50	53	53	55	55		819	0,99
			H	4	4	11	16	20	50	53	53	55	55		819	0,99
				0	**21**	**45**	**69**	**117**	**141**	**165**	**169**	**190**	**195**	**217**		
KF525	20	37	V	4	4	4	6	8							27	0,96
			H	4	4	4	6	8							27	0,96
		38	V	4	4	4	6	8							27	0,96
			H	4	4	4	7	8							25	0,79
		39	V	4	4	4	6	8	8	9	9	10	10	11	27	0,96
			H	4	4	4	6	8	8	8	9	10	10	11	27	0,96
	25	40	V	4	4	7	7	17	20	25	25	27	25	28	92	0,99
			H	4	4	6	7	18	21	23	23	26	27	43314	85	0,96
		41	V	4	4	6	7	19	23	25	25	29	27	31	96	0,96
			H	4	4	7	7	18	20	25	25	2/	29	30	92	0,98
		42	V	4	4	5	6	18	22						113	0,99
			H	4	4	5	6	18	22						113	0,99
	28	43	V	4	4	8	13	21	25	27	27	30	29	31	88	1,00
			H	4	4	7	12	22	26	27	28	30	30	32	95	1,00
		44	V	4	4	9	13	22	26	28	29	31	30	32	91	1,00
			H	4	4	8	13	23	27	28	30	32	30	33	98	1,00
		45	V	4	4	9	13	22	25	28	29	30	30	31	88	1,00
			H	4	4	9	13	22	25	27	27	30	30	30	88	1,00
	32	46	V	4	4	5	8	14	11	18	19	20	19	21	54	0,97
			H	4	4	5	9	14	11	18	19	20	21	21	55	0,98
		47	V	4	4	5	9	14	13	14	20	13	17	20	55	0,98
			H	4	4	6	8	13	13	13	19	13	16	20	47	1,00
		48	V	4	4	6	10	10							32	0,77
			H	4	4	6	10	14							54	0,98
LF766	20	49	V	4	4	4	4	4	4	4	4	5	5		19	0,97
			H	4	4	4	4	4	4	4	4	5	5		19	0,97
		50	V	4	4	4	4	4	4	4	4	5	5	5	19	0,97
			H	4	4	4	4	4	4	4	4	5	5	5	19	0,97
		51	V	4	4	4	4	4	4	4	4	4	4	5	0	#DIV/0!
			H	4	4	4	4	4	4	4	4	4	4	5	0	#DIV/0!
	25	52	V	4	4	4	4	4	6						12	0,57
			H	4	4	4	4	4	6						12	0,57
		53	V	4	4	4	4	4	7						18	0,57
			H	4	4	4	4	4	6						12	0,57

		54	V	4	4	4	4	4	6	8	10	11	11	12	20	0,75
			H	4	4	4	4	4	6	8	9	11	12	13	20	0,75
	28	55	V	4	4	4	4	4	8	10	11	13	13	14	24	0,57
			H	4	4	4	4	5	8	10	11	13	13	14	25	0,79
		56	V	4	4	4	4	4	8	10	11	13	13	15	24	0,57
			H	4	4	4	4	4	7	10	11	13	13	15	18	0,57
		57	V	4	4	4	5	5	8	10	12	13	13	15	18	0,57
			H	4	4	4	4	4	8	10	12	13	13	15	24	0,57
	32	58	V	4	4	4	4	6	9						25	0,86
			H	4	4	4	4	5	9						23	0,71
		59	V	4	4	4	4	6	8						21	0,91
			H	4	4	4	4	5	9						23	0,71
		60	V	4	4	4	4	7	11	12	13	15		16	35	0,88
			H	4	4	4	4	6	10	12	13	15		16	29	0,82
				0	**19**	**27**	**44**	**49**	**67**	**73**	**91**	**163**	**170**			
LF352	20	61	V	4	4	4	4	4	6	6	7	9,5	9,5		35	0,93
			H	4	4	4	4	4	6	6	7	8,5	9		35	0,93
		62	V	4	4	4	4	4	6	6	8	8,5	9		47	0,98
			H	4	4	4	4	4	6	7	8	9	9		48	0,96
		63	V	4	4	4	4	4	6	7	8	10	10		48	0,96
			H	4	4	4	4	4	6	7	8	9	9,5		48	0,96
	25	64	V	4	4	5	7	7	10	10	11				51	0,97
			H	4	4	5	7	7	10	10	11				51	0,97
		65	V	4	4	5	7	7,5	10	10	12	19	19		56	0,99
			H	4	4	5	8	8	10	11	12,5	19	19		60	0,98
		66	V	4	4	5	7	8	9	10	12	18	18		54	0,99
			H	4	4	5	8	8,5	10	11	12	19	19		57	0,97
	28	67	V	4	4	5	7	8	10	10	13,5	20	20		56	0,99
			H	4	4	5	9	8	11	11	13	20	20		54	0,97
		68	V	4	4	5	7	8	11	11	13	20	20		56	0,99
			H	4	4	5	7	8	11	11	13	20	20		56	0,99
		69	V	4	4	5	7	8	11	12	13	21	21		59	0,99
			H	4	4	5	7	8	11	11	12	20	20		55	0,99
	32	70	V	4	4	5	7	8	10						63	1,00
			H	4	4	5	7	8	10						63	1,00
		71	V	4	4	5	7	7	10	10,5	12	20	20		56	1,00
			H	4	4	5	7	7	10	10,5	12	19	19		52	0,99
		72	V	4	4	5	7	7,5	9	10	11	20	20		54	0,99
			H	4	4	5	7	7,5	10	11	12	22	20		62	1,00

	20	73	V	4	4	4	6	8	10	11	14	29	31		95	0,99
			H	4	4	4	7	8	11	11	15	29	31		94	1,00
		74	V	4	4	4	6	8	10	11	15	29	31		96	1,00
			H	4	4	4	7	7	10	11	15	29	31		95	1,00
		75	V	4	4	4	8	8	11	12	15	29	29		89	1,00
			H	4	4	4	7	7	10	11	14	28	31		93	0,99
	25	76	V	4	4	5	9	10,5	15	16	21	38	39		120	1,00
			H	4	4	6	9	10,5	15	17	21	38	39		118	1,00
		77	V	4	4	5	9	11	15						126	0,99
			H	4	4	5	9	10	15						125	1,00
		78	V	4	4	6	9	11	15	16	21	37	38		114	1,00
LF371			H	4	4	5	9	11	15	16	20	38	39		119	1,00
	28	79	V	4	4	6	10	11	16	17	21	35	36		104	1,00
			H	4	4	6	9	11	17	18	21	38	40		118	1,00
		80	V	4	4	6	9	11	15	17	20	36	37		109	1,00
			H	4	4	7	9	11	15	17	20	36	37		108	1,00
		81	V	4	4	7	10	11	16	16	21				110	0,99
			H	4	4	6	9	11	16	17	21				122	0,99
	32	82	V	4	4	4	6	7	10	10	13	22	23		66	1,00
			H	4	4	5	6	7	10	10	13	22	23		65	1,00
		83	V	4	4	4	5	6	10	10	12,5	21	22		64	0,99
			H	4	4	4	6	6	8	8	12,5	24	25		77	0,99
		84	V	4	4	5	6	7	10	10	12	22	23		65	1,00
			H	4	4	4	6	7	10	11	12	21	22		62	0,99
				0	**19**	**27**	**44**	**49**	**67**	**73**	**91**	**163**	**170**			
	20	85	V	4	4	4	4	4	7	8	9	19	21		63	0,99
			H	4	4	4	4	5	7	7	8	20	21		75	0,99
		86	V	4	4	4	4	5	7	7	12	19	23		62	0,94
			H	4	4	4	4	5	8	8	9	19	21		63	0,99
		87	V	4	4	4	5	5	8	8	10	20	20		67	1,00
			H	4	4	4	5	5	8	8	9	20	21		69	0,99
LF458	25	88	V	4	4	4	8	8	14						140	0,96
			H	4	4	5	8	9	14						133	1,00
		89	V	4	4	5	7	7	12	12	17	35	38		101	0,95
			H	4	4	4	7	7	11	13	17	37	39		106	0,97
		90	V	4	4	4	8	9	14	15	21	37	39		126	1,00
			H	4	4	4	8	9	14	15	21	38	40		126	1,00
	28	91	V	4	4	6	9	9	12	15	17	38	39		133	0,99
			H	4	4	6	9	9	13	13	18	40	40		151	1,00

		92	V	4	4	6	9	13	21	23	30	50	53		147	1,00
			H	4	4	6	10	13	18	22	27	49	52		151	1,00
		93	V	4	4	6	10	11	17	21	26				139	1,00
			H	4	4	6	11	11	19	21	26				139	1,00
	32	94	V	4	4	6	13	15	22						191	1,00
			H	4	4	7	13	15	22						186	1,00
		95	V	4	4	7	8	10	12	13	17	32	32		78	0,94
			H	4	4	6	7	10	12	12	17	32	33		82	0,93
		96	V	4	4	6	7	10	12	12	16	31	32		82	0,93
			H	4	4	7	7	10	12	12	16	32	32		77	0,88
				0	**19**	**27**	**44**	**49**	**67**	**73**	**91**	**163**	**170**			
KF560	20	85	V	4	4	4	5	5	6	7	9	15	15		41	0,99
			H	4	4	4	5	5	6	8	9	16	16		45	0,99
		86	V	4	4	4	5	5	8						50	0,89
			H	4	4	4	6	6	8						49	0,98
		87	V	4	4	4	6	6	8	8	10	15	15		38	0,99
			H	4	4	4	6	6	8	8	10	16	17		44	1,00
	25	88	V	4	4	4	5	5	7	8	9	14	15		38	0,99
			H	4	4	4	5	5	7	8	9	14	15		38	0,99
		89	V	4	4	4	6	7	8	9	11				53	0,98
			H	4	4	4	6	6	8	8	10				45	0,99
		90	V	4	4	4	6	6,5	8	9	10	16	16		41	0,99
			H	4	4	4	6	6,5	8	9	10	16	17		43	1,00
	28	91	V	4	4	4	5	5	5	6	7	11	11		26	0,98
			H	4	4	4	5	5	5	6	7	11	11		26	0,98
		92	V	4	4	5	5	5	7	7	8	12	12		27	0,98
			H	4	4	4	5	5	7	7	8	12	13		31	0,99
		93	V	4	4	4	5	5	7	7	8	12	13		31	0,99
			H	4	4	4	5	5	7	7	8	12	13		31	0,99
	32	94	V	4	4	4	4	4	4	4	4	4	4		0	#DIV/0!
			H	4	4	4	4	4	4	4	4	4	4		0	#DIV/0!
		95	V	4	4	4	4	4	4	4	4	4	4		0	#DIV/0!
			H	4	4	4	4	4	4	4	4	4	4		0	#DIV/0!
		96	V	4	4	4	4	4	4	4	4	4	4		0	#DIV/0!
			H	4	4	4	4	4	4	4	4	4	4		0	#DIV/0!
LF580	20	109	V	4	4	4	4	6	6	8	9	10,5	17		36	0,84
			H	4	4	4	4	6	6	8	9	10,5	17		36	0,84
		110	V	4	4	4	4	6	7	9					53	0,84
			H	4	4	4	4	7	7	9					52	0,78

		111	V	4	4	4	4	6	6	7	8	9	15		30	0,80
			H	4	4	4	4	6	6	7	8	8	15		28	0,72
	25	112	V	4	4	4	5	5	6	8	8	9	16		31	0,77
			H	4	4	4	4	5	6	8	8	10	15		32	0,86
		113	V	4	4	4	4	6	7	8	9	10	17		35	0,81
			H	4	4	4	4	6	7	8	9	10	17		35	0,81
		114	V	4	4	4	4	5	6	8	8	8	15		28	0,72
			H	4	4	4	4	5	6	8	8	9	15		30	0,80
	28	115	V	4	4	4	4	5	5	6	7	8			16	0,87
			H	4	4	4	4	4	5	6	7	8			17	0,86
		116	V	4	4	4	4	5	5	5	6	7	11		19	0,79
			H	4	4	4	4	4	5	5	6	7	11		20	0,81
		117	V	4	4	4	4	5	5	6	7	8	13		25	0,81
			H	4	4	4	4	5	5	7	7	8	13		25	0,79
	32	118	V	4	4	4	4	4	4	4	4	4	4		0	#DIV/0!
			H	4	4	4	4	4	4	4	4	4	4		0	#DIV/0!
		119	V	4	4	4	4	4	4	4	4	4	4		0	#DIV/0!
			H	4	4	4	4	4	4	4	4	4	4		0	#DIV/0!
		120	V	4	4	4	4	4	4	4	4	4	4		0	#DIV/0!
			H	4	4	4	4	4	4	4	4	4	4		0	#DIV/0!

Calculated colony expansion rates, Kr, for all ten fungi at four different temperatures.

Table 3A. Colony expansion rates at 20 °C.

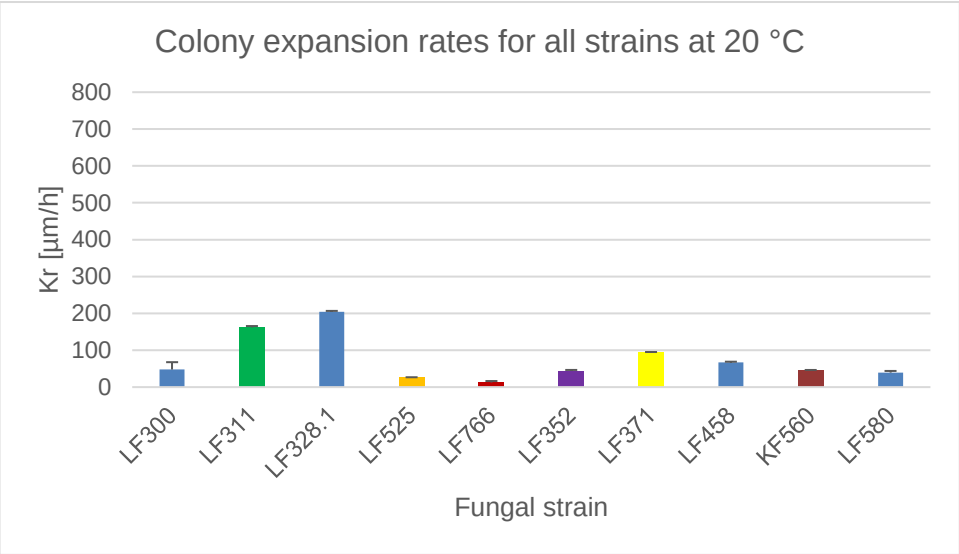


Table 3B. Colony expansion rates at 25 °C.

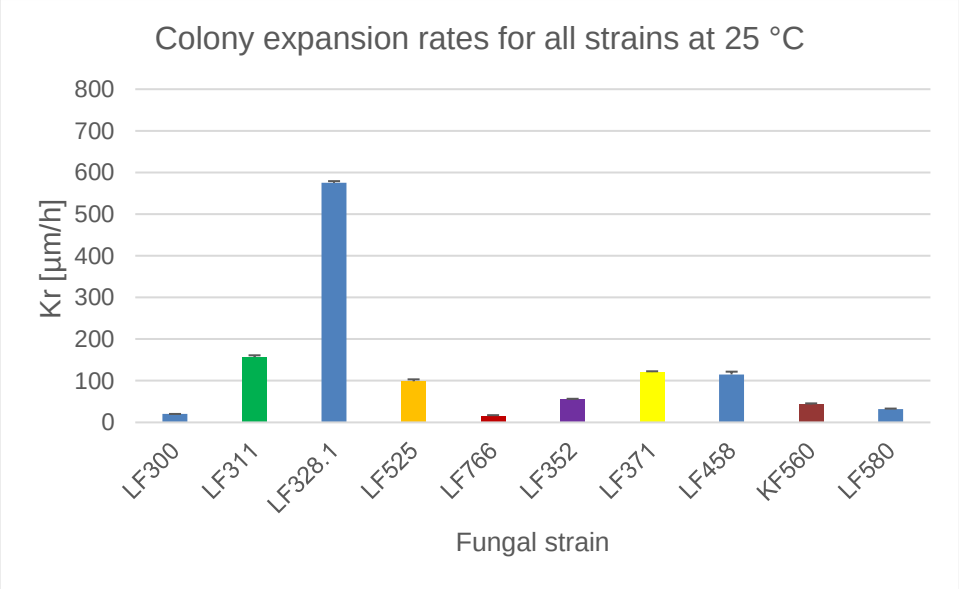


Table 3C. Colony expansion rates at 28 °C.

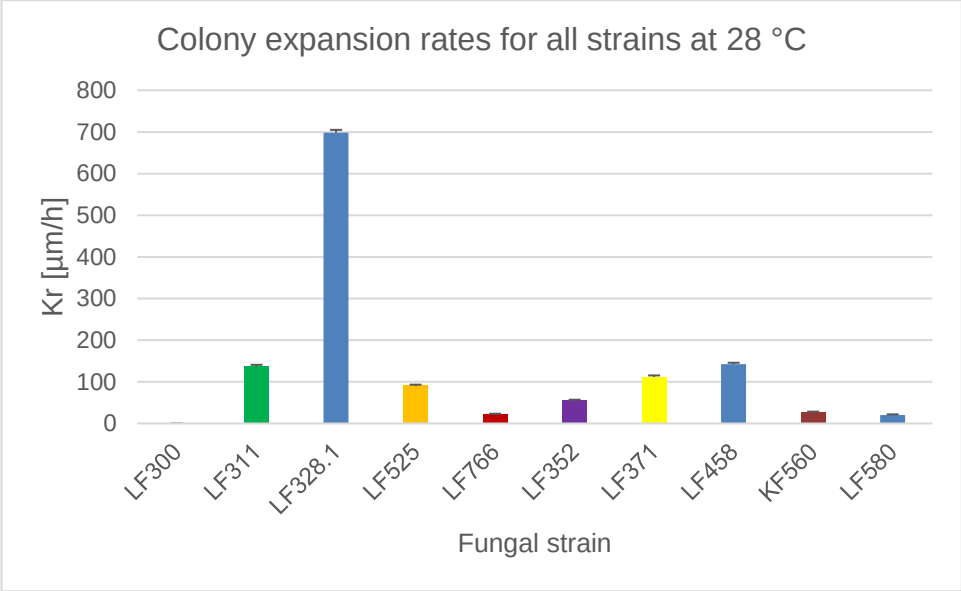
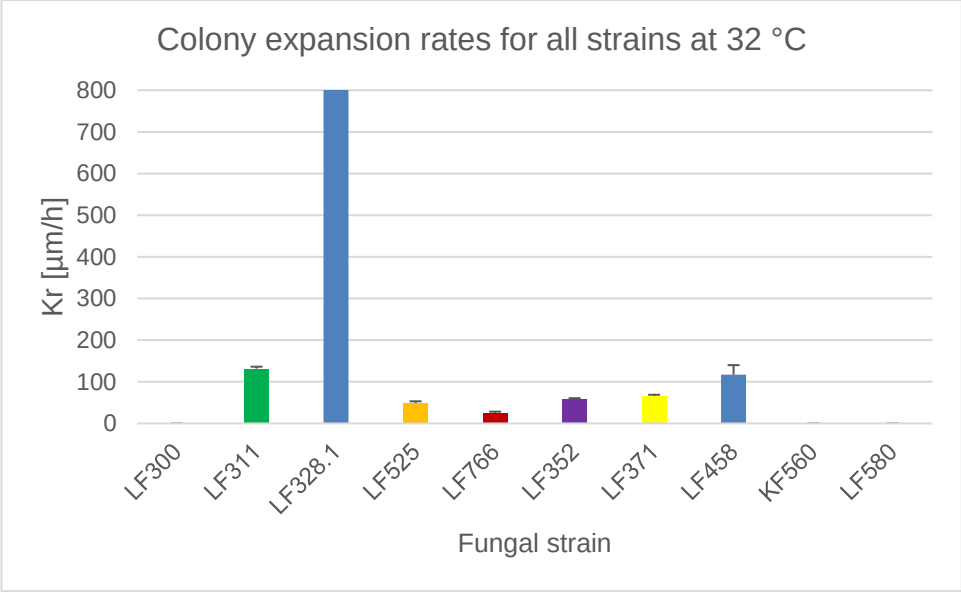


Table 3D. Colony expansion rates at 32 °C.



Xylanase activity assay.

KSYLANAASIN MÄÄRITTÄMINEN

1. Tarkoitus

Endo-β-1,4-ksylanaasiaktiivisuuden (EC 3.2.1.8) määrittäminen.

2. Soveltamisalue

Menetelmä soveltuu erityyppisille liuosnäytteille, tyypillisesti mikrobien kasvuliuoksille ja niiden puhdisteille. Menetelmä ei sovellu ilman esikäsittelyä sellaisille näytteille, joissa matala aktiivisuus ja samalla korkea sokerikonsentraatio.

3. Periaate

Ksylaanisubstraatista entsyymin vaikutuksesta pilkkoutuvat sokerit (ksylo-oligosakkaridit) kvantifioidaan värireaktiossa.

4. Työturvallisuus

Dinitrosalisylihappoliuos (DNS, ks erillinen ohje) on nimestään huolimatta vahvasti **emäksinen**. Katso käyttöturvallisuustiedote (2-hydroxy-3,5-dinitrobenzoic acid).

5. Reagenssit

5.1. Substraatti: 1,0% koivun glukuronoksylaani (Roth 7500) 0,05 M Na-sitraattipuskurissa, pH 5,0 (home-entsyymeille***) tai 6,5 (useimmille bakteereille). 1,0 g ksylaania sekoitetaan tehosekoittimella n. 80 ml:aan puskuria 60°C:ssa ja sen jälkeen kuumennetaan magneettisekoituslevyllä kiehumispisteeseen (**ei keitetä!**). Annetaan jäähtyä ja jatketaan magneettisekoitusta yön yli. Täytetään puskurilla 100 ml:ksi. Pakastetaan esim. 25 ml:n erissä. Sekoitettava hyvin sulatuksen jälkeen!
*** *Huom: ennen 1.1.1999 käytettiin pH 5,3 home-entsyymeille!*

5.2 Puskuri: Standardimenetelmässä käytetään 0,05M Na-sitraattipuskuria, pH 5,0 (tai 6,5). Punnitaan sitruunahappo, lisätään tislattua vettä 75% kokonaistilavuudesta, säädetään pH NaOH:lla, täytetään merkkiin.

5.3 Stopping-reagenssi: Dinitrosalisylihapporeagenssi (DNS, katso erillinen ohje VTT-3783-93)

6. Laitteet

Normaalien laboratoriolaitteiden lisäksi (koeputkia, pipettejä, vortex-sekoitin, vesihaude, jne) tarvitaan spektrofotometri, joka mittaa aallonpitoisuudella 540 nm.

7. Suoritus

7.1 1,8 ml substraattiliuosta temperoidaan 50°C:een.

7.2 Lisätään 200 µl Na-sitraattipuskuriin laimennettua entsyymiä, sekoitetaan.

7.3 Inkuboidaan 300 s (5 min), 50°C.

7.4 Lisätään 3,0 ml DNS, sekoitetaan, keitetään 5 min, jäähdytetään kylmävesihauteessa.

7.5 Mitataan muodostunut väri spektrofotometrillä (540 nm) mittausnollaa vastaan.

7.6 Näyteputken absorbanssiarvo (ks. 7.5) korjataan tarpeen vaatiessa vähentämällä entsyyminollan absorbanssiarvo (ks. alla).

7.7 Korjattu absorbanssiarvo muutetaan standardisuoran avulla entsyymiaktiivisuus-yksiköiksi (nkat ml^{-1}).

7.8 Alkuperäisen (laimentamattoman) näytteen aktiivisuus saadaan kertomalla aktiivisuusyksiköt (ks. 7.7) laimennuskertoimella.

Mittausnolla	**Entsyyminolla**
1,8 ml substraattiliuosta 5 min, 50°C 3,0 ml DNS 0,2 ml puskuria Keitto, jäähdytys. Tällä liuoksella nollataan spektrofotometri.	1,8 ml substraattiliuosta 5 min, 50°C 3,0 ml DNS 0,2 ml entsyymilaimennusta (***Huomaa pipetointijärjestys!***) Keitto, jäähdytys. Entsyyminolla tarvitaan vain, jos näytteen laimennuskerroin on melko pieni ja/tai jos näyte sisältää huomattavasti sokeria.

8. Standardisuora ja tuloksen laskeminen

Standardina käytetään puhdasta ksyloosia (esim. Merck 8692, MW = 150), kantaliuoksen vahvuus 0,01 M (= 0,15 g/100 ml puskuria). Kantaliuosta voidaan säilyttää pakastettuna pienissä erissä -20°C:ssä. Sulatuksen jälkeen putket on sekoitettava erityisen huolellisesti, koska pakastuminen aiheuttaa liuoksen kerrostumista. Huonosti sekoitetusta liuoksesta tulee kelvoton standardisuora!

Kantaliuosta laimennetaan seuraavasti (puskuriin):

1:1 (= laimentamaton)	= 10,0 µmol ml^{-1} --->	33,3 nkat ml^{-1}*
1:2	= 5,0	16,7
1:3	= 3,3	11,1
1:5	= 2,0	6,7

*Esimerkki:

Jos reaktion aikana on tuotettu väriä, joka vastaa vahvinta standardipistettä (10 mmol ml^{-1}), on tuotettu ksyloosia seuraavasti:

$$\frac{10\ \mu mol\ ml^{-1}}{300\ s} = 0{,}033\ \mu mol\ ml^{-1}\ s^{-1} = 33{,}3\ nmol\ ml^{-1}\ s^{-1}\ (= nkat\ ml^{-1})$$

Standardiliuokset käsitellään kuten esim. entsyyminollat:
1,8 ml substraattiliuosta
5 min, 50°C
3,0 ml DNS
0,2 ml standardiliuosta
Keitto, jne, mitataan mittausnollaa vastaan. Standardisuora rakennetaan neljän standardipisteen avulla (Absorbanssit Y-akselilla, nkat ml^{-1} X-akselilla).

9. Huomioitavaa

1. On suositeltavaa, että ylläkuvattu standardisuora valmistetaan joka määrityskerralla, jolloin mahdolliset erot esim. DNS-erien, keitto-olosuhteiden, substraattiliuosten ja pipettien kalibroinnin välillä tulevat tasoitetuiksi.
2. Standardisuora perustuu pelkästään kemialliseen reaktioon ja on lineaarinen hyvinkin pitkälle. **Entsymaattisen** reaktion lineaarisuus on tutkittava tekemällä ko. mikrobin tuottamalla entsyymillä laimennussarja. Esimerkiksi *Trichoderma reesei*n ja *Aspergillus niger*in tuottamat ksylanaasiaktiivisuudet ovat näissä olosuhteissa lineaarisia lukemaan n. 0,550.
3. Tällä menetelmällä mitattu aktiivisuus on aina huomattavasti korkeampi kuin samasta näytteestä mitattu ksylanaasiaktiivisuus Somogyi-Nelson-lopetuksella (ks. viite).
4. Tällä menetelmällä mitataan kaikkien ksylaania hydrolysoivien entsyymien kokonaisaktiivisuutta. Entsyymiseos, joka sisältää esim. paljon ß-ksylosidaasia mutta vain vähän endoksylanaasia, saattaa antaa lupaavan tuloksen määrityksessä mutta toimia huonosti ksylaanin hydrolyysissä.

10. Aiemmat versiot

Määritys kehitetty 22.8.1985/MJB.
Modifioitu (uusi substraatti) 16.3-2.4.90/MJB. Edellinen substraatti (pyökkiksylaani, J. Puls/BFH/Hamburg) ei ollut kaupallisesti saatavissa.
1.1.1999 alkaen pH 5,0 (home-entsyymit) aikaisemman pH 5,3 sijasta.

11. Kirjallisuus

Bailey MJ, Biely P and Poutanen K (1992) Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 23:257-270.

Huom! Tämä on kansainvälinen standardimenetelmä (ks. viite), jota ei saisi ilman painavaa syytä muuttaa!

Determined xylanase activities from all cultivations.

First cultivation
Second cultivation
Third cultivation

B = Birch wood xylan substrate
S = S sample (without cells)
C = sample with cells, non-centrifuged

			Assay conditions			Pre-incubation										Enzyme activity	
Strain	Xylan substrate	Media	pH	Time [min]	Temp [°C]	Temp [°C]	Time [min]	Sample	R2 of standard	number of assayed sample	Dilution	Abs.	$E_{0\,abs}$	Abs.-E_0	nkat/ml diluted	nkat/ml
T.reesei	B		5	5	50	-	0	S	0.9998	0 , 0 h	1	0,091	0,087	0,004	2,6	2,6
T.reesei	B		5	5	50	-	0	S	0.9998	1 , 18 h	1	0,195	0,058	0,137	11,0	11,0
T.reesei	B		5	5	50	-	0	S	0.9998	2 , 24 h	1	0,28	0,034	0,246	18,0	18,0
T.reesei	B		5	5	50	-	0	S	0.9998	3 , 42 h	1	0,486	0,02	0,466	32,0	32,0
T.reesei	B		5	5	50	-	0	S	0.9998	4 , 47 h	1	0,577	0,015	0,562	38,1	38,1
Econase	B		6	5	50	-	0	S	0.9998	control	2000	0,187	-0,032	0,219	16,3	40665
LF352	B		5	5	50	-	0	S	0.9997	0 , 0 h	1	0,109	0,115	-0,006	-0,9	-0,9
LF352	B		5	5	50	-	0	S	0.9997	1 , 17 h	1	0,166	0,149	0,017	0,8	0,8
LF352	B		5	5	50	-	0	S	0.9997	2 , 22 h	1	0,364	0,342	0,022	1,2	1,2
LF352	B		5	5	50	-	0	S	0.9997	3 , 41 h	1	0,428	0,355	0,073	5,0	5,0
LF352	B		5	5	50	-	0	S	0.9997	4 , 47 h	1	0,141	0,141	0	-0,4	-0,4
LF352	B		5	5	50	-	0	S	0.9997	5 , 89 h	1	0,154	0,137	0,017	0,8	0,8
LF352	B		5	5	50	-	0	S	0.9997	6 , 96 h	1	0,146	0,133	0,013	0,5	0,5
LF352	B		5	5	50	-	0	S	0.9997	7 , 161 h	1	0,112	0,137	-0,025	-2,3	-2,3
LF352	B		5	5	50	-	0	S	0.9991	0 , 0 h	1	0,212	0,201	0,011	-0,3	-0,3
LF352	B		5	5	50	-	0	S	0.9991	1 , 17 h	1	0,292	0,264	0,028	1,0	1,0
LF352	B		5	5	50	-	0	S	0.9991	2 , 22 h	1	0,454	0,424	0,03	1,1	1,1
LF352	B		5	5	50	-	0	S	0.9991	3 , 41 h	1	0,234	0,158	0,076	4,5	4,5
LF352	B		5	5	50	-	0	S	0.9991	4 , 47 h	1	0,154	0,154	0	-1,1	-1,1
LF352	B		5	5	50	-	0	S	0.9991	5 , 89 h	1	0,147	0,131	0,016	0,1	0,1
LF352	B		5	5	50	-	0	S	0.9991	6 , 96 h	1	0,148	0,151	-0,003	-1,3	-1,3
LF352	B		5	5	50	-	0	S	0.9991	7 , 161 h	1	0,15	0,153	-0,003	-1,3	-1,3
LF371	B		5	5	50	-	0	S	0.9988	0 , 0 h	1	0,147	0,137	0,01	1,7	1,7

LF371	B		5	5	50	-	0	S	0.9988	1 , 17 h	1	0,289	0,267	0,022	2,5	2,5
LF371	B		5	5	50	-	0	S	0.9988	2 , 22 h	1	0,427	0,398	0,029	3,0	3,0
LF371	B		5	5	50	-	0	S	0.9988	3 , 41 h	1	0,317	0,279	0,038	3,5	3,5
LF371	B		5	5	50	-	0	S	0.9988	4 , 47 h	1	0,25	0,252	-0,002	0,9	0,9
LF371	B		5	5	50	-	0	S	0.9988	5 , 89 h	1	0,234	0,247	-0,013	0,2	0,2
LF371	B		5	5	50	-	0	S	0.9988	6 , 96 h	1	0,225	0,204	0,021	2,4	2,4
LF371	B		5	5	50	-	0	S	0.9988	7 , 161 h	1	0,208	0,207	0,001	1,1	1,1
LF311	B		5	5	50	-	0	S	0.9996	0 , 0 h	1	0,096	0,11	-0,014	-1,1	-1,1
LF311	B		5	5	50	-	0	S	0.9996	1 , 68 h	1	0,12	0,132	-0,012	-0,9	-0,9
LF311	B		5	5	50	-	0	S	0.9996	2 , 93 h	1	0,125	0,138	-0,013	-1,0	-1,0
LF311	B		5	5	50	-	0	S	0.9996	3 , 116 h	1	0,125	0,14	-0,015	-1,1	-1,1
LF311	B		5	5	50	-	0	S	0.9996	4 , 140 h	1	0,125	0,132	-0,007	-0,6	-0,6
LF311	B		5	5	50	-	0	S	0.9996	5 , 164 h	1	0,121	0,151	-0,03	-2,2	-2,2
LF311	B		5	5	50	-	0	S	0.9991	0 , 0 h	1	0,087	0,098	-0,011	-0,9	-0,9
LF311	B		5	5	50	-	0	S	0.9991	1 , 68 h	1	0,108	0,118	-0,01	-0,8	-0,8
LF311	B		5	5	50	-	0	S	0.9991	2 , 93 h	1	0,107	0,116	-0,009	-0,7	-0,7
LF311	B		5	5	50	-	0	S	0.9991	3 , 116 h	1	0,116	0,12	-0,004	-0,4	-0,4
LF311	B		5	5	50	-	0	S	0.9991	4 , 140 h	1	0,115	0,14	-0,025	-1,8	-1,8
LF311	B		5	5	50	-	0	S	0.9991	5 , 164 h	1	0,111	0,128	-0,017	-1,3	-1,3
KF525	B		5	5	50	-	0	S	0.9991	0 , 0 h	1	0,055	0,054	0,001	1,8	1,8
KF525	B		5	5	50	-	0	S	0.9991	1 , 68 h	1	0,463	0,429	0,034	4,0	4,0
KF525	B		5	5	50	-	0	S	0.9991	2 , 93 h	1	0,654	0,593	0,061	5,9	5,9
KF525	B		5	5	50	-	0	S	0.9991	3 , 116 h	1	0,642	0,599	0,043	4,6	4,6
KF525	B		5	5	50	-	0	S	0.9991	4 , 140 h	1	0,615	0,577	0,038	4,3	4,3
KF525	B		5	5	50	-	0	S	0.9991	5 , 164 h	1	0,565	0,537	0,028	3,6	3,6
KF525	B		5	20	25	-	0	S	0.9993	0 , 0 h	1	0,032	0,055	-0,023	0,1	0,1
KF525	B		5	20	25	-	0	S	0.9993	1 , 68 h	1	0,439	0,457	-0,018	0,4	0,4
KF525	B		5	20	25	-	0	S	0.9993	2 , 93 h	1	0,627	0,605	0,022	3,2	3,2
KF525	B		5	20	25	-	0	S	0.9993	3 , 116 h	1	0,619	0,576	0,043	4,6	4,6
KF525	B		5	20	25	-	0	S	0.9993	4 , 140 h	1	0,557	0,558	-0,001	1,6	1,6
KF525	B		5	20	25	-	0	S	0.9993	5 , 164 h	1	0,524	0,486	0,038	4,3	4,3
KF525	B		5	5	50	-	0	S	0.9922	0 , 0 h	1	0,146	0,149	-0,003	1,4	1,4
KF525	B		5	5	50	-	0	S	0.9922	1 , 68 h	1	0,047	0,06	-0,013	0,7	0,7
KF525	B		5	5	50	-	0	S	0.9922	2 , 93 h	1	0,047	0,05	-0,003	1,4	1,4
KF525	B		5	5	50	-	0	S	0.9922	3 , 116 h	1	0,052	0,056	-0,004	1,3	1,3
KF525	B		5	5	50	-	0	S	0.9922	4 , 140 h	1	0,052	0,081	-0,029	-0,3	-0,3
KF525	B		5	5	50	-	0	S	0.9922	5 , 164 h	1	0,055	0,085	-0,03	-0,4	-0,4
LF352	B	1b	5	5	50	-	0	S	0.9994	0 , 0 h	1	0,252	0,283	-0,031	-1,8	-1,8
LF352	B	1b	5	5	50	-	0	S	0.9994	1 , 17 h	1	1,315	0,3	1,236	84,1	84,1
LF352	B	1b	5	5	50	-	0	S	0.9994	2 , 22 h	1	1,34	0,079	1,265	86,0	86,0
LF352	B	1b	5	5	50	-	0	S	0.9994	3 , 41 h	1	1,386	0,075	1,318	89,6	89,6

LF352	B	1b	5	5	50	-	0	S	0.9994	4 , 47 h	1	1,558	0,068	1,49	101,3	101,3
LF352	B	1b	5	5	50	-	0	S	0.9994	5 , 89 h	1	1,685	0,068	1,685	114,5	114,5
LF352	B	1b	5	5	50	-	0	S	0.9978	0 , 0 h	10	0,001	0,009	-0,008	1,0	9,5
LF352	B	1b	5	5	50	-	0	S	0.9978	1 , 24 h	10	0,123	0,035	0,102	8,0	79,9
LF352	B	1b	5	5	50	-	0	S	0.9978	2 , 69 h	10	0,11	0,021	0,089	7,2	71,6
LF352	B	1b	5	5	50	-	0	S	0.9978	3 , 97 h	10	0,102	0,021	0,086	7,0	69,7
LF352	B	1b	5	5	50	-	0	S	0.9978	4 , 145 h	10	0,135	0,016	0,109	8,4	84,4
LF352	B	1b	5	5	50	-	0	S	0.9978	5 , 220 h	10	0,139	0,026	0,139	10,4	103,6
LF371	B	1a	5	5	50	-	0	S	0.9978	0 , 0 h	1	0,177	0,237	-0,06	-2,0	-2,0
LF371	B	1a	5	5	50	-	0	S	0.9978	2 , 69 h	1	0,807	0,076	0,717	49,1	49,1
LF371	B	1a	5	5	50	-	0	S	0.9978	4 , 145 h	1	0,441	0,09	0,335	24,0	24,0
LF371	B	1a	5	5	50	-	0	S	0.9978	6 , 297 h	1	0,377	0,106	0,377	26,7	26,7
LF371	B	1b	5	5	50	-	0	S	0.9978	0 , 0 h	1	0,22	0,242	-0,022	0,5	0,5
LF371	B	1b	5	5	50	-	0	S	0.9978	2 , 69 h	1	0,769	0,104	0,695	47,6	47,6
LF371	B	1b	5	5	50	-	0	S	0.9978	4 , 145 h	1	0,526	0,074	0,428	30,1	30,1
LF371	B	1b	5	5	50	-	0	S	0.9978	6 , 297 h	1	0,373	0,098	0,373	26,5	26,5
LF311	B	1a	5	5	50	-	0	S	0.9948	0 , 0 h	1	0,489	0,444	0,045	3,2	3,2
LF311	B	1a	5	5	50	-	0	S	0.9948	2 , 45 h	1	2,906	0,075	2,817	192,3	192,3
LF311	B	1a	5	5	50	-	0	S	0.9948	4 , 143 h	1	2,04	0,089	1,961	133,9	133,9
LF311	B	1a	5	5	50	-	0	S	0.9948	5 , 188 h	1	1,44	0,079	1,44	98,4	98,4
LF311	B	1a	5	5	50	-	0	S	0.9973	0 , 0 h	10	0,04	0,049	-0,009	-0,5	-5,2
LF311	B	1a	5	5	50	-	0	S	0.9973	2 , 45 h	10	0,377	0,027	0,323	22,1	221,4
LF311	B	1a	5	5	50	-	0	S	0.9973	4 , 143 h	10	0,152	0,054	0,121	8,4	83,5
LF311	B	1a	5	5	50	-	0	S	0.9973	5 , 188 h	10	0,109	0,031	0,109	7,5	75,3
LF311	B	1b	5	5	50	-	0	S	0.9973	0 , 0 h	10	0,05	0,065	-0,015	-0,9	-9,3
LF311	B	1b	5	5	50	-	0	S	0.9973	2 , 45 h	10	0,238	0,036	0,194	13,3	133,3
LF311	B	1b	5	5	50	-	0	S	0.9973	4 , 143 h	10	0,108	0,044	0,089	6,2	61,7
LF311	B	1b	5	5	50	-	0	S	0.9973	5 , 188 h	10	0,102	0,019	0,102	7,1	70,6
KF525	B	1a	5	5	50	-	0	S	0.9972	0 , 0 h	10	0,007	0,015	-0,008	-3,6	-36,0
KF525	B	1a	5	5	50	-	0	S	0.9972	2 , 46 h	10	0,379	0,046	0,333	24,5	245,4
KF525	B	1a	5	5	50	-	0	S	0.9972	3 , 70 h	10	0,458	0,017	0,441	33,5	334,5
KF525	B	1a	5	5	50	-	0	S	0.9972	4 , 167 h	10	0,502	0,01	0,492	37,7	376,6
KF525	B	1a	5	5	50	-	0	S	0.9901	0 , 0 h	10	0,016	0,045	-0,029	-5,3	-53,3
KF525	B	1a	5	5	50	-	0	S	0.9901	1 , 24 h h	10	0,173	0,073	0,1	5,3	53,1
KF525	B	1a	5	5	50	-	0	S	0.9901	2 , 46 h	10	0,397	0,046	0,351	26,0	260,2
KF525	B	1a	5	5	50	-	0	S	0.9901	3 , 70 h	10	0,484	0,055	0,429	32,5	324,6
KF525	B	1a	5	5	50	-	0	S	0.9901	4 , 167 h	10	0,561	0,032	0,529	40,7	407,1
KF525	B	1a	5	5	50	-	0	S	0.9901	5 , 196 h	10	0,499	0,047	0,452	34,4	343,6
KF525	B	1a	5	5	50	-	0	S	0.9901	6 , 238 h	10	0,441	0,061	0,38	28,4	284,2
KF525	B	1a	5	5	50	-	0	S	0.9901	7 , 336 h	10	0,375	0,05	0,325	23,9	238,8
KF525	B	1b	5	5	50	-	0	S	0.9972	0 , 0 h	10	0,019	0,034	-0,015	-4,2	-41,8

KF525	B	1b	5	5	50	-	0	S	0.9972	2 , 46 h	10	0,333	0,045	0,288	20,8	208,3
KF525	B	1b	5	5	50	-	0	S	0.9972	3 , 70 h	10	0,317	0,021	0,296	21,5	214,9
KF525	B	1b	5	5	50	-	0	S	0.9972	4 , 167 h	10	0,276	0,02	0,256	18,2	181,8
LF311, A	B	1a	5	5	50	-	0	S	0.993	0 , 0 h	10	0,031	0,035	-0,004	0,4	4,2
LF311, A	B	1a	5	5	50	-	0	S	0.993	1 , 5 h	10	0,095	0,086	0,009	1,2	12,4
LF311, A	B	1a	5	5	50	-	0	S	0.993	1 , 10 h	10	0,167	0,088	0,079	5,7	56,6
LF311, A	B	1a	5	5	50	-	0	S	0.993	3 , 24 h	10	0,419	0,054	0,365	23,7	237,2
LF311, A	B	1a	5	5	50	-	0	S	0.993	4 , 29 h	10	0,447	0,038	0,409	26,5	265,0
LF311, A	B	1a	5	5	50	-	0	S	0.993	5 , 34 h	10	0,434	0,037	0,397	25,7	257,5
LF311, A	B	1a	5	5	50	-	0	S	0.993	6 , 49 h	10	0,307	0,04	0,267	17,5	175,3
LF311, A	B	1a	5	5	50	-	0	S	0.993	7 , 74 h	10	0,127	0,04	0,087	6,2	61,6
LF311, A	B	1a	5	5	50	-	0	S	0.993	8 , 98 h	10	0,092	0,03	0,062	4,6	45,8
LF311, B	B	1a	5	5	50	-	0	S	0.9993	0 , 0 h	10	0	0,021	-0,021	-2,3	-23,1
LF311, B	B	1a	5	5	50	-	0	S	0.9993	1 , 14 h	10	0,183	0,052	0,131	7,7	76,9
LF311, B	B	1a	5	5	50	-	0	S	0.9993	2 , 19 h	10	0,181	0,046	0,135	8,0	79,5
LF311, B	B	1a	5	5	50	-	0	S	0.9993	3 , 24 h	10	0,292	0,019	0,273	17,0	170,3
LF311, B	B	1a	5	5	50	-	0	S	0.9993	4 , 40 h	10	0,237	0,029	0,208	12,8	127,6
LF311, B	B	1a	5	5	50	-	0	S	0.9993	5 , 64 h	10	0,112	0,037	0,075	4,0	40,0
LF311, B	B	1a	5	5	50	-	0	S	0.9993	6 , 88 h	10	0,07	0,036	0,034	1,3	13,1
LF311, B	B	1a	5	5	50	-	0	S	0.9993	7 , 110 h	10	0,052	0,045	0,007	-0,5	-4,7
LF311, A	B	1a	5	5	30	-	0	S	0.9999	0 , 0 h	10	0,027	0,048	-0,021	-0,6	-6,2
LF311, A	B	1a	5	5	30	-	0	S	0.9999	2 , 10 h	10	0,124	0,078	0,046	3,6	35,6
LF311, A	B	1a	5	5	30	-	0	S	0.9999	3 , 24 h	10	0,251	0,025	0,226	14,8	148,0
LF311, A	B	1a	5	5	30	-	0	S	0.9999	4 , 29 h	10	0,247	0,019	0,228	14,9	149,2
LF311, A	B	1a	5	5	30	-	0	S	0.9999	5 , 34 h	10	0,263	0,027	0,236	15,4	154,2
LF311, A	B	1a	5	5	30	-	0	S	0.9999	6 , 49 h	10	0,159	0,024	0,135	9,1	91,2
LF311, A	B	1a	5	5	30	-	0	S	0.9999	7 , 74 h	10	0,055	0,018	0,037	3,0	30,0
LF311, A	B	1a	5	5	40	-	0	S	0.9976	0 , 0 h	10	0,05	0,045	0,005	1,3	13,3
LF311, A	B	1a	5	5	40	-	0	S	0.9976	2 , 10 h	10	0,163	0,076	0,087	6,4	63,6
LF311, A	B	1a	5	5	40	-	0	S	0.9976	3 , 24 h	10	0,344	0,032	0,312	20,2	201,5
LF311, A	B	1a	5	5	40	-	0	S	0.9976	4 , 29 h	10	0,383	0,031	0,352	22,6	226,0
LF311, A	B	1a	5	5	40	-	0	S	0.9976	5 , 34 h	10	0,393	0,077	0,316	20,4	204,0
LF311, A	B	1a	5	5	40	-	0	S	0.9976	6 , 49 h	10	0,243	0,015	0,228	15,0	150,0
LF311, A	B	1a	5	5	40	-	0	S	0.9976	7 , 74 h	10	0,1	0	0,1	7,2	71,6
LF311, A	B	1a	5	5	55	-	0	S	0.9927	0 , 0 h	10	0,036	0,053	-0,017	0,0	-0,1
LF311, A	B	1a	5	5	55	-	0	S	0.997	2 , 10 h	10	0,412	0,014	0,398	25,4	254,2
LF311, A	B	1a	5	5	55	-	0	S	0.7933	3 , 24 h	10	0,461	0,031	0,43	27,4	273,9
LF311, A	B	1a	5	5	55	-	0	S	0.16863	4 , 29 h	10	0,43	0,037	0,393	25,1	251,2
LF311, A	B	1a	5	5	55	-	0	S	0.25793	5 , 34 h	10	0,291	0,041	0,25	16,4	163,5
LF311, A	B	1a	5	5	55	-	0	S	0.34723	6 , 49 h	10	0,127	0,037	0,09	6,5	65,4
LF311, A	B	1a	5	5	60	-	0	S	0.999	0 , 0 h	10	0,044	0,042	0,002	1,2	11,5

LF311, A	B	1a	5	5	60	-	0	S	0.999	2 , 10 h	10	0,3	0,03	0,27	17,6	175,8
LF311, A	B	1a	5	5	60	-	0	S	0.999	3 , 24 h	10	0,316	0,031	0,285	18,5	185,0
LF311, A	B	1a	5	5	60	-	0	S	0.999	4 , 29 h	10	0,294	0,023	0,271	17,6	176,4
LF311, A	B	1a	5	5	60	-	0	S	0.999	5 , 34 h	10	0,192	0,024	0,168	11,3	113,3
LF311, A	B	1a	5	5	60	-	0	S	0.999	6 , 49 h	10	0,073	0,013	0,06	4,7	47,1
LF311, A	B	3	5	5	50	-	0	S	0.9998	0 , 0 h	10	0,024	0,03	-0,006	-1,9	-18,8
LF311, A	B	3	5	5	50	-	0	S	0.9998	1 , 5 h	10	0,074	0,082	-0,008	-2,0	-20,1
LF311, A	B	3	5	5	50	-	0	S	0.9998	2 , 10 h	10	0,051	0,083	-0,032	-3,7	-36,6
LF311, A	B	3	5	5	50	-	0	S	0.9998	3 , 24 h	10	0,188	0,038	0,15	8,8	87,9
LF311, A	B	3	5	5	50	-	0	S	0.9998	4 , 29 h	10	0,213	0,045	0,168	10,0	100,2
LF311, A	B	3	5	5	50	-	0	S	0.9998	5 , 34 h	10	0,167	0,037	0,13	7,4	74,3
LF311, A	B	3	5	5	50	-	0	S	0.9998	6 , 49 h	10	0,028	0,031	-0,003	-1,7	-16,7
LF311, A	B	3	5	5	50	-	0	S	0.9998	7 , 74 h	10	0,02	0,043	-0,023	-3,0	-30,4
LF311, A	B	3	5	5	50	-	0	S	0.9998	8 , 98 h	10	0,016	0,037	-0,021	-2,9	-29,0
LF311, B	B	3	5	5	50	-	0	S	0.9981	0 , 0 h	10	0,028	0,015	0,013	2,8	28,1
LF311, B	B	3	5	5	50	-	0	S	0.9981	1 , 14 h	10	0,171	0,055	0,116	9,4	94,3
LF311, B	B	3	5	5	50	-	0	S	0.9981	2 , 19 h	10	0,162	0,037	0,125	10,0	100,1
LF311, B	B	3	5	5	50	-	0	S	0.9981	3 , 24 h	10	0,21	0,031	0,179	13,5	134,8
LF311, B	B	3	5	5	50	-	0	S	0.9981	4 , 40 h	10	0,094	0,014	0,08	7,1	71,1
LF311, B	B	3	5	5	50	-	0	S	0.9981	5 , 64 h	10	0,029	0,01	0,019	3,2	31,9
LF311, B	B	3	5	5	50	-	0	S	0.9981	6 , 88 h	10	0,029	0,011	0,018	3,1	31,3
LF311, B	B	3	5	5	50	-	0	S	0.9981	7 , 110 h	10	0,023	0,008	0,015	2,9	29,4
KF525	B	1a	5	5	50	-	0	S	0.999	0 , 0 h	10	0,045	0,055	-0,01	-0,2	-2,0
KF525	B	1a	5	5	50	-	0	S	0.999	1 , 22 h	10	0,09	0,097	-0,007	0,0	-0,2
KF525	B	1a	5	5	50	-	0	S	0.999	2 , 48 h	10	0,136	0,049	0,087	5,8	57,9
KF525	B	1a	5	5	50	-	0	S	0.999	3 , 73 h	10	0,359	0,048	0,311	19,6	196,4
KF525	B	1a	5	5	50	-	0	S	0.999	4 , 97 h	10	0,485	0,057	0,428	26,9	268,7
KF525	B	1a	5	5	50	-	0	S	0.999	5 , 118 h	10	0,581	0,045	0,536	33,5	335,5
KF525	B	1a	5	5	50	-	0	S	0.999	6 , 147 h	10	0,702	0,043	0,659	41,2	411,5
KF525	B	1a	5	5	50	-	0	S	0.999	7 , 171 h	10	0,606	0,046	0,56	35,0	350,3
KF525	B	1a	5	5	50	-	0	S	0.999	8 , 190 h	10	0,642	0,049	0,593	37,1	370,7
KF525	B	1a	5	5	50	-	0	S	0.999	9 , 214 h	10	0,617	0,032	0,585	36,6	365,8
KF525	B	1a	5	5	50	-	0	S	0.999	10 , 360 h	10	0,578	0,031	0,547	34,2	342,3
KF525	B	1a	5	5	50	-	0	S	0.999	11 , 383 h	10	0,538	0,03	0,508	31,8	318,2
KF525	B	1a	5	5	50	-	0	C	0.999	0 , 0 h	10	0,025	0,031	-0,006	0,0	0,4
KF525	B	1a	5	5	50	-	0	C	0.999	1 , 22 h	10	0,075	0,067	0,008	0,9	9,1
KF525	B	1a	5	5	50	-	0	C	0.999	2 , 48 h	10	0,149	0,025	0,124	8,1	80,8
KF525	B	1a	5	5	50	-	0	C	0.999	3 , 73 h	10	0,314	0,027	0,287	18,2	181,6
KF525	B	1a	5	5	50	-	0	C	0.999	4 , 97 h	10	0,54	0,032	0,508	31,8	318,2
KF525	B	1a	5	5	50	-	0	C	0.999	5 , 118 h	10	0,385	0,021	0,364	22,9	229,2
KF525	B	1a	5	5	50	-	0	C	0.999	6 , 147 h	10	0,43	0,015	0,415	26,1	260,7

KF525	B	1a	5	5	50	-	0	C	0.999	7 , 171 h	10	0,522	0,019	0,503	31,5	315,1
KF525	B	1a	5	5	50	-	0	C	0.999	8 , 190 h	10	0,516	0,017	0,499	31,3	312,6
KF525	B	1a	5	5	50	-	0	C	0.999	9 , 214 h	10	0,534	0,011	0,523	32,7	327,4
KF525	B	1a	5	5	50	-	0	C	0.999	10 , 360 h	10	0,45	0,005	0,445	27,9	279,2
KF525	B	1a	5	5	50	-	0	C	0.999	11 , 383 h	10	0,438	-0,001	0,439	27,6	275,5
KF525	B	1a	5	5	30	-	0	S	0.9999	0 , 0 h	10	0,046	0,069	-0,023	-0,7	-7,4
KF525	B	1a	5	5	30	-	0	S	0.9999	3 , 73 h	10	0,167	0,049	0,118	8,1	80,6
KF525	B	1a	5	5	30	-	0	S	0.9999	5 , 118 h	10	0,272	0,057	0,215	14,1	141,1
KF525	B	1a	5	5	30	-	0	S	0.9999	7 , 171 h	10	0,307	0,055	0,252	16,4	164,2
KF525	B	1a	5	5	30	-	0	S	0.9999	9 , 214 h	10	0,312	0,052	0,26	16,9	169,2
KF525	B	1a	5	5	30	-	0	S	0.9999	10 , 360 h	10	0,267	0,051	0,216	14,2	141,7
KF525	B	1a	5	5	30	-	0	S	0.9999	11 , 383 h	10	0,259	0,045	0,214	14,0	140,5
KF525	B	1a	5	5	40	-	0	S	0.9999	0 , 0 h	10	0,054	0,075	-0,021	-0,6	-6,2
KF525	B	1a	5	5	40	-	0	S	0.9999	3 , 73 h	10	0,302	0,053	0,249	16,2	162,3
KF525	B	1a	5	5	40	-	0	S	0.9999	5 , 118 h	10	0,459	0,039	0,42	26,9	269,1
KF525	B	1a	5	5	40	-	0	S	0.9999	7 , 171 h	10	0,527	0,042	0,485	31,0	309,6
KF525	B	1a	5	5	40	-	0	S	0.9999	9 , 214 h	10	0,51	0,042	0,468	29,9	299,0
KF525	B	1a	5	5	40	-	0	S	0.9999	10 , 360 h	10	0,448	0,049	0,399	25,6	255,9
KF525	B	1a	5	5	40	-	0	S	0.9999	11 , 383 h	10	0,437	0,026	0,411	26,3	263,4
KF525	B	1a	5	5	55	-	0	S	0.9927	0 , 0 h	10	0,035	0,065	-0,03	0,0	-0,2
KF525	B	1a	5	5	55	-	0	S	0.9927	5 , 118 h	10	0,487	0,046	0,441	28,8	288,1
KF525	B	1a	5	5	55	-	0	S	0.9927	6 , 147 h	10	0,533	0,046	0,487	31,6	316,2
KF525	B	1a	5	5	55	-	0	S	0.9927	7 , 171 h	10	0,568	0,06	0,508	32,9	329,1
KF525	B	1a	5	5	55	-	0	S	0.9927	9 , 214 h	10	0,586	0,059	0,527	34,1	340,7
KF525	B	1a	5	5	55	-	0	S	0.9927	10 , 360 h	10	0,608	0,045	0,563	36,3	362,7
KF525	B	1a	5	5	60	-	0	S	0.999	0 , 0 h	10	0,048	0,066	-0,018	-0,1	-0,9
KF525	B	1a	5	5	60	-	0	S	0.999	5 , 118 h	10	0,428	0,056	0,372	24,5	245,2
KF525	B	1a	5	5	60	-	0	S	0.999	6 , 147 h	10	0,477	0,045	0,432	28,3	283,1
KF525	B	1a	5	5	60	-	0	S	0.999	7 , 171 h	10	0,52	0,055	0,465	30,4	303,9
KF525	B	1a	5	5	60	-	0	S	0.999	9 , 214 h	10	0,566	0,055	0,511	33,3	332,9
KF525	B	1a	5	5	60	-	0	S	0.999	10 , 360 h	10	0,611	0,037	0,574	37,3	372,7
KF525	B	1a	5	5	55	-	0	S	0.9992	10 , 360 h	10	0,592	0,029	0,563	35,8	358,3
KF525	B	1a	5	5	55	-	0	S	0.9992	10 , 360 h	10	0,582	0,038	0,544	34,6	346,4
KF525	B	1a	5	5	55	-	0	S	0.9992	11 , 383 h	10	0,604	0,034	0,57	36,3	362,7
KF525	B	1a	5	5	50	-	0	S	0.9903	0 , 0 h	10	0,028	0,036	-0,008	1,6	16,3
KF525	B	1a	5	5	50	-	0	S	0.9903	1 , 22 h	10	0,072	0,078	-0,006	1,8	17,5
KF525	B	1a	5	5	50	-	0	S	0.9903	2 , 48 h	10	0,132	0,04	0,092	7,8	77,6
KF525	B	1a	5	5	50	-	0	S	0.9903	3 , 73 h	10	0,291	0,035	0,256	17,8	178,2
KF525	B	1a	5	5	50	-	0	S	0.9903	4 , 97 h	10	0,461	0,038	0,423	28,1	280,7
KF525	B	1a	5	5	50	-	0	S	0.9903	5 , 118 h	10	0,508	0,036	0,472	31,1	310,7
KF525	B	1a	5	5	50	-	0	S	0.9903	6 , 147 h	10	0,513	0,027	0,486	31,9	319,3

KF525	B	1a	5	5	50	-	0	S	0.9903	7 , 171 h	10	0,631	0,031	0,6	38,9	389,3
KF525	B	1a	5	5	50	-	0	S	0.9903	8 , 190 h	10	0,655	0,029	0,626	40,5	405,2
KF525	B	1a	5	5	50	-	0	S	0.9903	9 , 214 h	10	0,634	0,024	0,61	39,5	395,4
KF525	B	1a	5	5	50	-	0	S	0.9903	10 , 360 h	10	0,493	0,007	0,486	31,9	319,3
KF525	B	1a	5	5	50	-	0	S	0.9903	11 , 383 h	10	0,528	0,007	0,521	34,1	340,8
KF525	B	3	5	5	50	-	0	S	0.9993	0 , 0 h	10	0,028	0,054	-0,026	-0,8	-8,3
KF525	B	3	5	5	50	-	0	S	0.9993	1 , 22 h	11	0,061	0,081	-0,02	-0,4	-4,9
KF525	B	3	5	5	50	-	0	S	0.9993	2 , 48 h	10	0,269	0,041	0,228	15,4	154,1
KF525	B	3	5	5	50	-	0	S	0.9993	3 , 73 h	10	0,485	0,042	0,443	29,2	291,7
KF525	B	3	5	5	50	-	0	S	0.9993	4 , 97 h	10	0,522	0,023	0,499	32,7	327,5
KF525	B	3	5	5	50	-	0	S	0.9993	5 , 118 h	10	0,529	0,026	0,503	33,0	330,0
KF525	B	3	5	5	50	-	0	S	0.9993	6 , 147 h	10	0,469	0,012	0,457	30,1	300,6
KF525	B	3	5	5	50	-	0	S	0.9993	7 , 171 h	10	0,351	0,002	0,349	23,2	231,5
KF525	B	3	5	5	50	-	0	S	0.9993	8 , 190 h	10	0,28	-0,006	0,286	19,1	191,2
LF311, A	B	1a	3	5	50	-	0	S	0.9908	4 , 29 h	10	-0,012	0,003	-0,015	0,6	6,4
LF311, A	B	1a	3	5	50	-	0	S	0.9908	4 , 29 h	10	-0,016	0,019	-0,035	-0,7	-7,0
LF311, A	B	1a	3	5	50	-	0	S	0.9908	4 , 29 h	10	-0,03	-0,012	-0,018	0,4	4,4
KF525	B	1a	3	5	50	-	0	S	0.9908	8 , 190 h	10	0,019	-0,008	0,027	2,0	20,1
KF525	B	1a	3	5	50	-	0	S	0.9908	8 , 190 h	10	0	-0,038	0,038	2,7	27,1
KF525	B	1a	3	5	50	-	0	S	0.9909	8 , 190 h	10	-0,015	-0,036	0,021	1,6	16,3
LF311, A	B	1a	4	5	50	-	0	S	0.9968	4 , 29 h	10	0,043	0,01	0,033	1,9	18,7
LF311, A	B	1a	4	5	50	-	0	S	0.9968	4 , 29 h	10	0,032	-0,014	0,046	2,8	28,3
LF311, A	B	1a	4	5	50	-	0	S	0.9968	4 , 29 h	10	0,011	-0,029	0,04	2,4	23,9
KF525	B	1a	4	5	50	-	0	S	0.9968	8 , 190 h	10	0,185	0,013	0,172	12,1	121,2
KF525	B	1a	4	5	50	-	0	S	0.9968	8 , 190 h	10	0,183	-0,006	0,189	13,4	133,7
KF525	B	1a	4	5	50	-	0	S	0.9968	8 , 190 h	10	0,195	-0,001	0,1964	13,9	139,2
LF311, A	B	1a	6	5	50	-	0	S	0.9998	4 , 29 h	10	0,106	0,032	0,074	4,8	48,2
LF311, A	B	1a	6	5	50	-	0	S	0.9998	4 , 29 h	10	0,09	0,025	0,065	4,3	42,7
LF311, A	B	1a	6	5	50	-	0	S	0.9998	4 , 29 h	10	0,104	0,017	0,087	5,6	56,2
KF525	B	1a	6	5	50	-	0	S	0.9998	8 , 190 h	10	0,5	0,029	0,471	29,2	292,4
KF525	B	1a	6	5	50	-	0	S	0.9998	8 , 190 h	10	0,48	0,022	0,458	28,4	284,4
KF525	B	1a	6	5	50	-	0	S	0.9998	8 , 190 h	10	0,486	0,015	0,471	29,2	292,4
LF311, A	B	1a	7	5	50	-	0	S	0.9989	4 , 29 h	10	0,032	0,096	-0,064	-4,8	-47,9
LF311, A	B	1a	7	5	50	-	0	S	0.9989	4 , 29 h	10	0,028	0,013	0,015	0,3	3,4
LF311, A	B	1a	7	5	50	-	0	S	0.9989	4 , 29 h	10	0,02	0,054	-0,034	-2,8	-28,5
KF525	B	1a	7	5	50	-	0	S	0.9989	8 , 190 h	10	0,282	0,034	0,248	15,5	154,7
KF525	B	1a	7	5	50	-	0	S	0.9989	8 , 190 h	10	0,285	0,04	0,245	15,3	152,8
KF525	B	1a	7	5	50	-	0	S	0.9989	8 , 190 h	10	0,333	0,039	0,294	18,5	184,6
LF311, A	B	1a	5	5	50	30	60	S	0.9954	4 , 29 h	10	0,252	-0,026	0,278	18,4	183,9
LF311, A	B	1a	5	5	50	30	60	S	0.9954	4 , 29 h	10	0,229	-0,032	0,261	17,3	172,8
LF311, A	B	1a	5	5	50	30	45	S	0.9954	4 , 29 h	10	0,249	-0,015	0,264	17,5	174,7

LF311, A	B	1a	5	5	50	30	45	S	0.9954	4 , 29 h	10	0,247	-0,017	0,264	17,5	174,7
LF311, A	B	1a	5	5	50	30	30	S	0.9954	4 , 29 h	10	0,349	-0,024	0,373	24,6	245,8
LF311, A	B	1a	5	5	50	30	30	S	0.9954	4 , 29 h	10	0,333	-0,018	0,351	23,1	231,5
LF311, A	B	1a	5	5	50	30	15	S	0.9954	4 , 29 h	10	0,349	-0,018	0,367	24,2	241,9
LF311, A	B	1a	5	5	50	30	15	S	0.9954	4 , 29 h	10	0,348	-0,012	0,36	23,7	237,3
KF525	B	1a	5	5	50	30	60	S	0.9954	8 , 190 h	10	0,52	0,004	0,516	33,9	339,1
KF525	B	1a	5	5	50	30	60	S	0.9954	8 , 190 h	10	0,533	0,003	0,53	34,8	348,2
KF525	B	1a	5	5	50	30	45	S	0.9954	8 , 190 h	10	0,584	0,011	0,573	37,6	376,3
KF525	B	1a	5	5	50	30	45	S	0.9954	8 , 190 h	10	0,59	0,006	0,584	38,3	383,5
KF525	B	1a	5	5	50	30	30	S	0.9954	8 , 190 h	10	0,603	0,004	0,599	39,3	393,3
KF525	B	1a	5	5	50	30	30	S	0.9954	8 , 190 h	10	0,593	0,017	0,576	37,8	378,3
KF525	B	1a	5	5	50	30	15	S	0.9954	8 , 190 h	10	0,603	0,018	0,585	38,4	384,1
KF525	B	1a	5	5	50	30	15	S	0.9954	8 , 190 h	10	0,617	0,004	0,613	40,2	402,4
LF311, A	B	1a	5	5	50	40	60	S	0.9954	4 , 29 h	10	-0,01	-0,01	0	1,5	14,8
LF311, A	B	1a	5	5	50	40	60	S	0.9954	4 , 29 h	10	-0,017	-0,038	0,021	2,8	28,0
LF311, A	B	1a	5	5	50	40	45	S	0.9954	4 , 29 h	10	0,149	-0,006	0,155	11,2	112,3
LF311, A	B	1a	5	5	50	40	45	S	0.9954	4 , 29 h	10	0,144	-0,024	0,168	12,0	120,5
LF311, A	B	1a	5	5	50	40	30	S	0.9954	4 , 29 h	10	0,191	-0,034	0,225	15,6	156,3
LF311, A	B	1a	5	5	50	40	30	S	0.9954	4 , 29 h	10	0,199	-0,027	0,226	15,7	156,9
LF311, A	B	1a	5	5	50	40	15	S	0.9954	4 , 29 h	10	0,253	-0,037	0,29	19,7	197,2
LF311, A	B	1a	5	5	50	40	15	S	0.9954	4 , 29 h	10	0,23	-0,037	0,267	18,3	182,7
KF525	B	1a	5	5	50	40	60	S	0.9954	8 , 190 h	10	0,418	-0,019	0,437	24,4	243,7
KF525	B	1a	5	5	50	40	60	S	0.9954	8 , 190 h	10	0,425	-0,014	0,439	23,2	231,7
KF525	B	1a	5	5	50	40	45	S	0.9954	8 , 190 h	10	0,345	-0,019	0,364	29,0	289,6
KF525	B	1a	5	5	50	40	45	S	0.9954	8 , 190 h	10	0,34	-0,005	0,345	29,1	290,8
KF525	B	1a	5	5	50	40	30	S	0.9954	8 , 190 h	10	0,622	0,02	0,602	32,5	324,8
KF525	B	1a	5	5	50	40	30	S	0.9954	8 , 190 h	10	0,626	0,004	0,622	32,3	322,9
KF525	B	1a	5	5	50	40	15	S	0.9954	8 , 190 h	10	0,492	-0,001	0,493	39,3	393,3
KF525	B	1a	5	5	50	40	15	S	0.9954	8 , 190 h	10	0,475	-0,015	0,49	40,6	405,9
LF311, A	B	1a	5	5	50	50	60	S	0.9954	4 , 29 h	10	0,018	-0,014	0,032	2,2	22,2
LF311, A	B	1a	5	5	50	50	60	S	0.9954	4 , 29 h	10	0,033	-0,022	0,055	3,8	37,7
LF311, A	B	1a	5	5	50	50	45	S	0.9986	4 , 29 h	10	0,058	-0,01	0,068	4,6	46,4
LF311, A	B	1a	5	5	50	50	45	S	0.9986	4 , 29 h	10	0,046	-0,017	0,063	4,3	43,0
LF311, A	B	1a	5	5	50	50	30	S	0.9986	4 , 29 h	10	0,096	-0,014	0,11	7,5	74,7
LF311, A	B	1a	5	5	50	50	30	S	0.9986	4 , 29 h	10	0,089	0,001	0,088	6,0	59,9
LF311, A	B	1a	5	5	50	50	15	S	0.9986	4 , 29 h	10	0,168	0,01	0,158	10,7	106,9
LF311, A	B	1a	5	5	50	50	15	S	0.9986	4 , 29 h	10	0,183	0,008	0,175	11,8	118,4
KF525	B	1a	5	5	50	50	60	S	0.9954	8 , 190 h	10	0,271	-0,021	0,292	19,7	197,1
KF525	B	1a	5	5	50	50	60	S	0.9954	8 , 190 h	10	0,261	0,016	0,245	16,5	165,5
KF525	B	1a	5	5	50	50	45	S	0.9986	8 , 190 h	10	0,243	-0,016	0,259	17,5	174,9
KF525	B	1a	5	5	50	50	45	S	0.9986	8 , 190 h	10	0,242	0,023	0,219	14,8	148,0

KF525	B	1a	5	5	50	50	30	S	0.9986	8 , 190 h	10	0,243	-0,012	0,255	17,2	172,2
KF525	B	1a	5	5	50	50	30	S	0.9986	8 , 190 h	10	0,235	0,018	0,217	14,7	146,6
KF525	B	1a	5	5	50	50	15	S	0.9986	8 , 190 h	10	0,223	0,005	0,218	14,7	147,3
KF525	B	1a	5	5	50	50	15	S	0.9986	8 , 190 h	10	0,232	0,004	0,228	15,4	154,0
LF311, A	14 g/l salt birch	1a	5	5	50	-	-	S	0.9994	4 , 29 h	10	0,26	0,021	0,239	15,6	156,3
LF311, A	14 g/l salt birch	1a	5	5	50	-	-	S	0.9994	4 , 29 h	10	0,271	0,029	0,242	15,8	158,5
LF311, A	14 g/l salt birch	1a	5	5	50	-	-	S	0.9994	4 , 29 h	10	0,269	0,012	0,257	16,9	169,3
KF525	14 g/l salt birch	1a	5	5	50	-	-	S	0.9994	8 , 190 h	10	0,601	0,034	0,567	39,3	393,5
KF525	14 g/l salt birch	1a	5	5	50	-	-	S	0.9994	8 , 190 h	10	0,581	0,019	0,562	39,0	389,9
KF525	14 g/l salt birch	1a	5	5	50	-	-	S	0.9994	8 , 190 h	10	0,58	0,004	0,576	40,0	400,0
LF311, A	28 g/l salt birch	1a	5	5	50	-	-	S	0.993	4 , 29 h	10	0,232	0,143	0,089	8,5	85,0
LF311, A	28 g/l salt birch	1a	5	5	50	-	-	S	0.993	4 , 29 h	10	0,302	0,14	0,162	12,6	126,5
LF311, A	28 g/l salt birch	1a	5	5	50	-	-	S	0.993	4 , 29 h	10	0,301	0,053	0,248	17,5	175,4
KF525	28 g/l salt birch	1a	5	5	50	-	-	S	0.993	8 , 190 h	10	0,448	-0,117	0,565	35,6	355,6
KF525	28 g/l salt birch	1a	5	5	50	-	-	S	0.993	8 , 190 h	10	0,488	-0,062	0,55	34,7	347,0
KF525	28 g/l salt birch	1a	5	5	50	-	-	S	0.993	8 , 190 h	10	0,484	-0,064	0,548	34,6	345,9
LF311, A	Palmaria palmata	1a	5	5	50	-	-	S	0.9998	5 , 34 h	10	0,416	0,063	0,353	20,0	200,2
LF311, A	Palmaria palmata	1a	5	5	50	-	-	S	0.9998	5 , 34 h	10	0,497	0,084	0,413	23,9	238,6
LF311, A	Palmaria palmata	1a	5	5	50	-	-	S	0.9998	5 , 34 h	10	0,479	0,137	0,342	19,3	193,2
KF525	Palmaria palmata	1a	5	5	50	-	-	S	0.9998	9 , 190 h	10	0,996	0,017	0,979	56,5	565,0
KF525	Palmaria palmata	1a	5	5	50	-	-	S	0.9998	9 , 190 h	10	0,952	0,044	0,908	52,5	524,8
KF525	Palmaria palmata	1a	5	5	50	-	-	S	0.9998	9 , 190 h	10	0,941	-0,016	0,957	55,3	552,5
LF311, A	Oat spelt	1a	5	5	50	-	-	S	0.996	5 , 34 h	10	0,517	0,006	0,511	30,0	299,9
LF311, A	Oat spelt	1a	5	5	50	-	-	S	0.996	5 , 34 h	10	0,493	0,014	0,479	28,2	281,8
LF311, A	Oat spelt	1a	5	5	50	-	-	S	0.996	5 , 34 h	10	0,528	0,021	0,507	29,8	297,7

KF525	Oat spelt	1a	5	5	50	-	-	S	0.996	9 , 190 h	10	0,996	0,017	0,979	56,5	565,0
KF525	Oat spelt	1a	5	5	50	-	-	S	0.996	9 , 190 h	10	0,952	0,044	0,908	52,5	524,8
KF525	Oat spelt	1a	5	5	50	-	-	S	0.996	9 , 190 h	10	0,941	-0,016	0,957	55,3	552,5
LF311, A	Wheat arabino, insoluble	1a	5	5	50	-	-	S	0.9632	5 , 34 h	10	0,292	0,014	0,278	14,3	143,0
LF311, A	Wheat arabino, insoluble	1a	5	5	50	-	-	S	0.9632	5 , 34 h	10	0,273	0,002	0,271	13,9	139,5
LF311, A	Wheat arabino, insoluble	1a	5	5	50	-	-	S	0.9632	5 , 34 h	10	0,407	-0,024	0,431	22,0	220,2
KF525	Wheat arabino, insoluble	1a	5	5	50	-	-	S	0.9632	9 , 190 h	10	0,241	0,4005	-0,1595	-7,8	-77,8
KF525	Wheat arabino, insoluble	1a	5	5	50	-	-	S	0.9632	9 , 190 h	10	0,358	0,672	-0,314	-15,6	-155,7
KF525	Wheat arabino, insoluble	1a	5	5	50	-	-	S	0.9632	9 , 190 h	10	-0,037	-0,1	0,063	3,5	34,5
LF311, A	Wheat arabino, low viscosity	1a	5	5	50	-	-	S	0.9987	5 , 34 h	10	0,576	0,188	0,388	22,7	226,6
LF311, A	Wheat arabino, low viscosity	1a	5	5	50	-	-	S	0.9987	5 , 34 h	10	0,548	0,218	0,33	19,5	195,2
LF311, A	Wheat arabino, low viscosity	1a	5	5	50	-	-	S	0.9987	5 , 34 h	10	0,526	0,167	0,359	21,1	210,9
KF525	Wheat arabino, low viscosity	1a	5	5	50	-	-	S	0.9987	9 , 190 h	10	1,055	0,211	0,844	47,3	473,4
KF525	Wheat arabino, low viscosity	1a	5	5	50	-	-	S	0.9987	9 , 190 h	10	1,051	0,199	0,852	47,8	477,7
KF525	Wheat arabino, low viscosity	1a	5	5	50	-	-	S	0.9987	9 , 190 h	10	1,046	0,212	0,834	46,8	468,0
LF311, A	Wheat arabino, insoluble	1a	5	5	50	-	-	S	0.9787	5 , 34 h	10	0,138	0,046	0,092	-0,2	-1,7
LF311, A	Wheat arabino, insoluble	1a	5	5	50	-	-	S	0.9787	5 , 34 h	10	0,228	0,056	0,172	5,3	53,2
LF311, A	Wheat arabino, insoluble	1a	5	5	50	-	-	S	0.9787	5 , 34 h	10	0,140	0,194	-0,054	-10,2	-101,9
KF525	Wheat arabino, insoluble	1a	5	5	50	-	-	S	0.9787	9 , 190 h	10	0,249	0,074	0,175	5,5	55,2
KF525	Wheat arabino, insoluble	1a	5	5	50	-	-	S	0.9787	9 , 190 h	10	0,140	0,083	0,057	-2,6	-25,7
KF525	Wheat arabino, insoluble	1a	5	5	50	-	-	S	0.9787	9 , 190 h	10	-0,013	0,001	-0,014	-7,4	-74,5

Measured pH values for prepared xylan substrates.

Table 6A.

1 % xylan substrate	Desired pH	Measured pH
Basic birch wood xylan	5	5.04
Birch wood xylan, pH 3	3	3.24
Birch wood xylan, pH 4	4	4.02
Birch wood xylan, pH 6	6	5.95
Birch wood xylan, pH 7	7	7.02
Birch wood xylan with 14 g/l sea salt	5	5.09
Birch wood xylan with 28 g/l sea salt	5	5.09
Seaweed Palmaria palmata	5	5.00
Oat spelt xylan	5	5.05
Wheat arabinoxylan, low viscosity	5	5.08
Wheat arabinoxylan, insoluble/gel	5	5.05