Expression, purification and characterization of proteins with unknown function homologous to a novel carbohydrate esterase

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Summary

Polysaccharides produced by plants represent a renewable resource that can be used for production of bio-derived chemicals, polymers, biofuel as well as many other high-value bioproducts. Although, utilization of carbohydrates often is associated with challenges regarding debranching and depolymerization of complex molecules. In order to address these problems Carbohydrate Active Enzymes (CAZymes) can be utilised to transform polysaccharide molecules. Nowadays, over half a million sequences are comprised in the CAZy database, however, there is still need for enzymes with novel activities that target remaining recalcitrant positions within polysaccharide structures. Recently, FjoAcXE has been characterised to have a novel acetyl esterase function, that at the same time share only 30% of sequence similarity with all known acetyl xylan esterase families. Herein, a BLASTp search identified uncharacterised proteins with sequence similarity to FjoAcXE. The objective of this project was to characterise corresponding homologues of FjoAcXE that hypothetically could comprise a novel acetyl esterase family. All together six homologues that share from 47 to 98% of sequence similarity with FjoAcXE were successfully expressed and purified. The proteins showed acetyl esterase activity on simple synthetic substrates (pNP-acetate and 4MU-acetate) and, therefore, these substrates were used to determine pH optimum and temperature stability of each studied enzyme. Further characterisation was done at optimal conditions on complex substrates, which showed that selected enzymes could deacetylate per-acetylated xylo-oligosaccharides and branched feruloylated xylo-oligosaccharides. Comparison between homologues and FjoAcXE showed slight variation within selected candidates in terms of physio-chemical characteristics, such as pH optimum and temperature stability, however, level of acetyl esterase activity was comparable on all of the tested substrates. Ongoing structural analyses of FjoAcXE and the selected homologues will help to determine whether these proteins form a novel family of carbohydrate esterase.

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4MU – 4 – Methylumbelliferone

BCA - Bicinchoninic acid assay

DTT – dithiotheitol

FPLC – Fast Protein Liquid Chromatography

FT – Flowthrough

OD – optical density

IPTG - Isopropyl β-D-1-thiogalactopyranoside

LB - Luria-Bertani medium.

MWCO – Molecular weight cut off

NTA - nitrilotriacetic acid

NMR - Nuclear magnetic resonance spectroscopy

PAHBAH – para-Hydroxybenzoic Acid Hydrazide

pNP – para-nitrophenol

RCF - relative centrifugal force

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOC - Super Optimal broth with Catabolite repression

1. Introduction

In response to growing concerns relating to renewable energy and environmental sustainability, plant polysaccharides from lignocellulose biomass attract more and more of scientific interest.

Hemicellulose is the second most abundant polysaccharide in plant cell wall after cellulose. Wide range of possible products obtained from hemicellulose vary from packaging films to food and feed ingredients (Ebringerová, 2006). Although, due to their diverse structure, nowadays hemicellulose biopolymers are still moderately utilised.
Xylan is major part of hemicellulose that is present predominantly in hardwood as glucuronoxylan and as arabinoglucuronoxylan in softwood. Xylans can be branched, for instance, hardwood xylan is usually acetylates. Side groups influence the properties of biopolymers such as solubility, interactions with other polymeric molecules and rheological properties.
Therefore, in order to control the properties of an end product, carbohydrate active enzymes with different selectivity can be applied to obtain product with desired and reproducible properties.
FjoAcXE, a newly found esterase from *Flavobacterium johnsonsae*, was characterised to be an acetyl xylan esterase and found to have a novel function - it can deacetylate Xyl-3-Ac-2MeGlcA. FjoAcXE has low susceptibility to steric hindrance, and by cleaving acetyl group from 3-O-position, it allows glucuronidases to approach previously inaccessible MeGlcpA-residues. At the same time FjoAcXE possessing a unique function shares less than 30% of sequence similarity with other carbohydrate active enzymes (CAZymes) and cannot be included in characterised families. However, in GeneBank were found uncharacterised proteins that share from 50 to 98% of sequence identity. This knowledge created a hypothesis for this thesis, that uncharacterised proteins homologous to FjoAcXE similarly to FjoAcXE are able to deacetylate Xyl-3-Ac-2MeGlcA and all together form a novel carbohydrate esterase family.

In order to confirm this hypothesis several objectives of the project were set:

- overexpress in *E.coli* and purify FjoAcXE-homologues;
- confirm that FjoAcXE-homologues possess acetyl esterase activity by performing reactions on simple synthetic substrates such as, pNP-acetate and 4MU-acetate;
- study biochemical properties such as pH optimum and temperature stability in order to understand perfect reaction conditions for FjoAcXE-homologues;
- perform secondary screening reactions studying the activity of FjoAcXE-homologues on complex synthetic and natural substrates at optimal conditions;
- study overall hydrolytic activity on variety of simple substrates;
- compare obtained characteristics between homologues and to FjoAcXE.

2. Literature review

2.1 Plant cell wall

Some of terrestrial plants reach over dozens of meters in height which apparently reveal a great need in supporting functions of a plant. Therefore, all types of cells in higher plants are surrounded by a cell wall. Cell walls are not only responsible for protective qualities of plant tissues but also take part in cell division and plant growth. Existing differentiation in literature divides cell walls into primary cell walls, usually very thin structures that continue expanding during the cell grow, and secondary walls that are formed on the inner side of primary cell wall when process of cell expansion is over (Figure 2.1.1.).

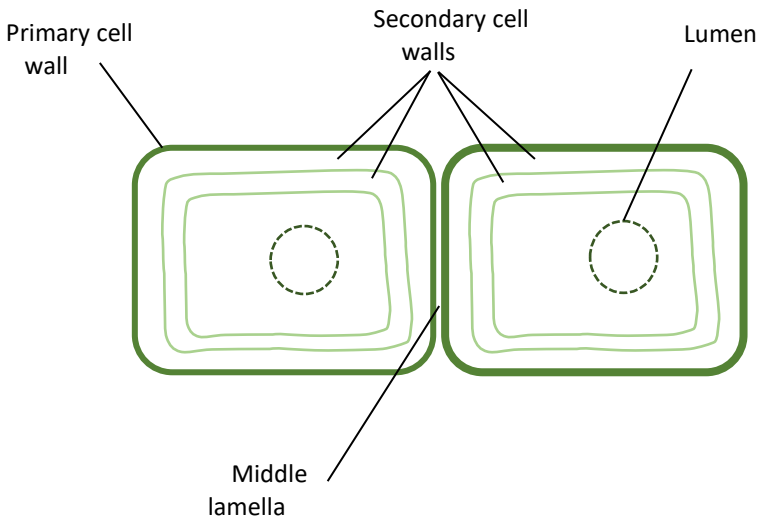

Figure 2.1.1. Schematic representation of a plant cell wall

Secondary wall is not universal and its components and size depend on the type of plant tissue but commonly it surpasses primary wall in size (Albersheim et al. 2011).

2.2. Lignocellulose material

Dry matter of plant cell walls comprises lignocellulosic biomass that consists of three major components: cellulose that accounts to 20-50% of dry weight, hemicellulose 15-35% of dry weight and lignin contributing 10-30% of dry weight (Chundawat et al. 2011). The ratio between these components varies significantly depending on the source of lignocellulose (Chen et al. 2014). Moreover, chemical content of hemicellulose and lignin differs qualitatively in hardwood, softwood and grasses (Pauly 2008). Those differences will be discussed below.

Being the most abundant component of the cell wall, cellulose is constituted by D-glucose molecules linked together by β-glycosidic bonds in positions 1→4 with varying degree of polymerisation. Those polymer units represent linear fibrils that are tightened together by hydrophobic interactions and contribute to the overall hydrophobicity of a cell wall (Rana 2017).

Hemicellulose is the second major polysaccharide of the lignocellulose that is presented by heterologous polysaccharides formed from pentoses, hexoses and uronic acid units. In this thesis hemicellulose will be discussed in more detail below.

Lignin is a phenylpropane polymer complexes that are composed of three main types of monomers: syringil, hydroxyphenyl and guaiacyl (Chen et al. 2014). Lignin in hardwood is mainly comprised by guaiacyl and syringil units, in softwood it is present by guaiacyl lignin , whereas monocots contain guaiacyl-syringil-hydroxyphenyl ligin (Chundawat et al. 2011; Chen et al. 2014). Lignin is thought to create lignin carbohydrate complexes (LCC) due to covalent bond formation with side chains of branched hemicellulose. It is speculated that LCCs could limit enzymatic or chemical hydrolysis of the cell walls, although this mechanism has not yet been explained in literature (Chundawat et al. 2011). Due to its physical properties, lignin contributes to stiffness of a cell wall and its supportive functions (Chen et al. 2014).

Amongst other components of a cells walls are pectins, polysaccharides that mainly can be found in primary cell wall and middle lamella as they are deposited in a cell during its initial growth stages (Voragen et al. 2009). Mostly, pectins are represented by hetero-polysaccharides that mainly contain linked galacturonic acid residues that can be substituted

with side chains and esters. Grasses contains from 2 to 10% of pectin, whereas in hardwood this number is around 5% (Voragen et al. 2009).

2.3. Hemicellulose

After cellulose molecules, hemicelluloses comprise the second most abundant group of polysaccharides present in plant cell wall. In comparison with cellulose which is chemically very well-defined and differs only in degree of polymerisation (DP) and crystallinity, hemicellulose are structurally complex molecules, consisting of various sugar residues, that furthermore vary in ratio of those sugar units and substituents (Gatenholm et al. 2003).

During formation of cellulose molecules, hemicelluloses can incorporate between cellulose microfibrils and readily build hydrogen bonding with adjacent molecules, contributing to the strength and flexibility of the cell wall. Furthemore, hemicelluloses are speculated to build covalent interactions with lignin and between each other (Gatenholm et al. 2003, Pauly et al. 2013, Kuhad and Singh 2007). Those interactions hinder enzyme degradation of the plant polysaccharide representing protective mechanisms of a cell wall.

Some literature sources refer to hemicelluloses as to the group of polysaccharides that can be extracted in alkali conditions with prior removal of pectic substances (Gatenholm et al. 2003, Scheller and Ulvskov 2010). This classification may not be applicable in cases when pectins also require alkali conditions for their extraction (Pauly et al. 2013). That is why it is suggested to classify hemicelluloses as polysaccharides that share equatorial β(1-4)-linkage in their backbone. They are comprised of pentoses and hexoses building polymers with general formula $(C_5H_8O_4)_n$ and $(C_6H_{10}O_5)_n$ and DP from 80 to 200 on average (Gatenholm et al. 2003). Therefore, hemicelluloses include xylans, mannans, β-(1→3,1→4)- linked glucans, as well as xyloglucans and glucomannans. The ratio of main sugar components vary depending on type of a plant species, thereby mannan-containing hemicelluloses are more common for softwood, whereas hardwood is mainly based on xylan-containg components.

2.4. Xylan

Xylan can be found in almost all plant cell walls. It comprises up to 35% of dry weight in hardwood and 15% in sofwoods. The backbone of xylan consists of β(1-4)- linked D-xylose units. The structure of xylan polymers may differ from a linear structure to diversely branched. Glucuronic acid substitutions can occur as α(1→2)- glycosidic linkages to backbone xylopyranose unit in glucuronoxylan. Generally, one methyl-glucuronic acid substitution occurs per 8-20 xylopyranoside units (Sjöström 1993). Xylose units that have glucuronic acid moieties in 2-O-position can at the same time be acetylated at position C-3 (Figure 2.4.1). Intrinsic degree of polymerisation for hardwood is about 100-220 (Ek et al. 2009).

Softwood comprise 5-10% w/w of arabinoglucuronoxylan. Arabinofuranosyl substitutions occur in C-3 position (Figure 2.4.1). Methyl-glucuronic acid ratio is one per 5-6 xylose units, whereas arabinofuranose substitutions occur once per 8-9 xylose units. Degree of polymerisation of those molecules reaches 90-120 (Ek et al. 2009).

Acetylation naturally prevents cell wall from degradation or invasion of pathogens (Hill 2006). Hardwood xylan is usually *O*-acetylated, with average of 70% of xylose units substituted with acetyl groups (Tenkanen 1992). Xylose can be esterified with acetyl group at 2-*O*- and/or 3-*O*-positions On the contrary, softwood xylans are not acetylated.

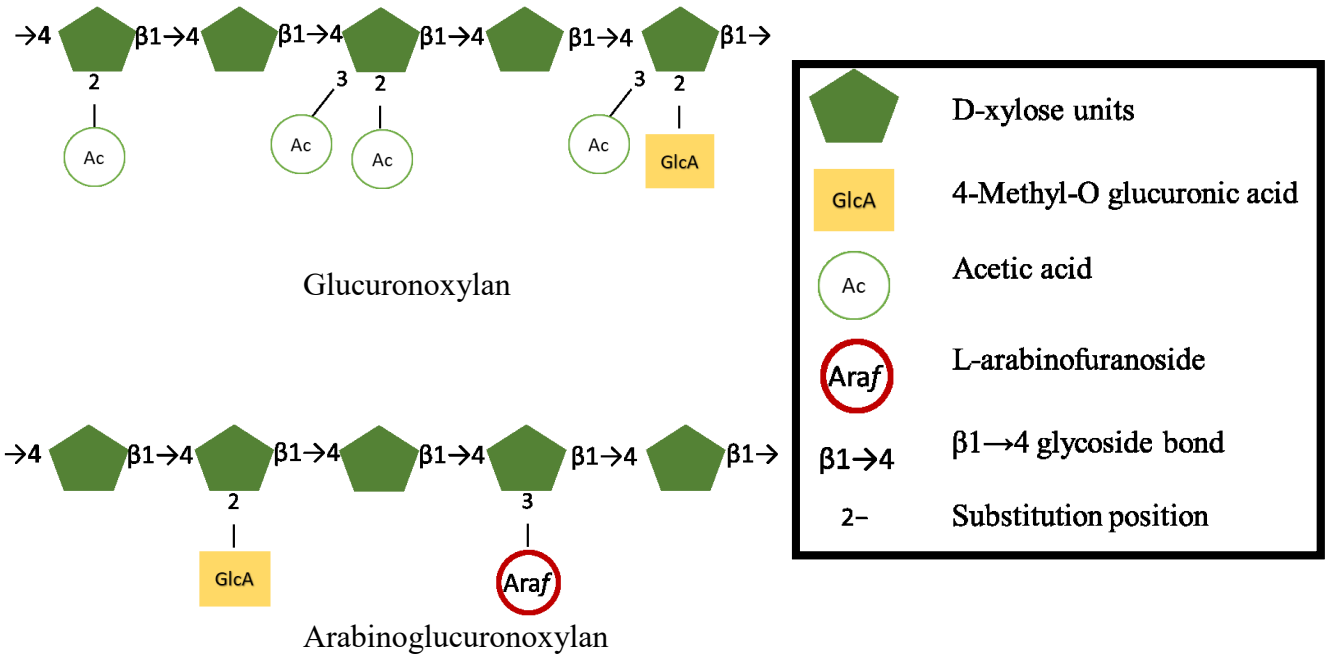

Figure 2.4.1 Schematic representation hardwood xylan (glucuronoxylan) and softwood xylan (arabinoglucuronoxylan)

2.5. Carbohydrate-active enzymes

Carbohydrate-active enzymes (CAZy) are a large group of enzymes that show a wide range of activities on carbohydrates: catalysing assembling, degradation or modification. In order to systemize the large number of CAZymes in 1998 there was introduced a CAZymes database (Lombard et al. 2013). In this database all enzymes are differentiated to the following classes: glycoside transferases (GT), glycoside hydrolases (GH), polysaccharide lyases (PT), auxiliary activities (AA) and carbohydrate esterases (CE). Apart from these classes, the classification of enzymes includes also enzyme associated non-catalytic carbohydrate-binding modules (CBM). Moreover, experimentally based classification allows to divide characterised enzymes to families (Cantarel et al. 2008). Enzymes catalysing hydrolytic removal of esters or amides in O- and N- positions from polysaccharides are called carbohydrate esterases. Currently 16 CE families are well-characterised in Carbohydrate Active Enzyme database (CAZy). In this work we will concentrate on describing CE removing acetic acid from

hemicelluloses of soft- and hardwood. Currently, studied acetyl xylan esterases have been included in families CE 1-7, 12 and 16 (Biely et al. 2014).

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2.6. Carbohydrate esterases

Acetylation in xylans predominantly occurs at xylose positions 2 and 3 (Biely et al. 2014). It is also possible that at the same time both of the positions can be acetylated. Although, other positions are also possible for O-Ac substitutions, in case when those positions are typical to some plant species, for instance, xyloglucans of annual plants are acetylated 6-O-positions (Biely et al. 2012).

Presence of acetyl groups decreases accessibility of a substrate for glycosyl hydrolases, which is important, for example, in perspective of further polysaccharide degradation. Moreover, acetylation of a substrate can create hindrance in solubility, thereby substrates with completely removed acetyl groups as well as show lower solubility in comparison to partly acetylated ones. It was shown in literature that acetic acid residues create hindrances in forming hydrogen bonds and thus, xylan has lower binding ability to cellulose when acetylated (Biely et al. 2012).

Taking into account all the above listed reasons, lower degree of acetylation facilitates access of polysaccharide degrading enzymes. In order to deacetylate substrate, alkali pre-treatment can be used. Although, in some cases where it is preferred not to use high pH, deacetylation can be made with help of esterases. Acetyl xylan esterases belong to a class of carbohydrate esterases. They are removing acetic acid attached to the xylopyranosyl units that creates accessible positions for glycosyl hydrolases. Acetyl xylan esterases produced by fungal species are well-characterised, on the contrary, bacterial AcXE are not so well described in literature(Biely at al. 2012).

2.7 Discovery of new enzymes

Animals that feed on plants use a diverse mixture of plant cell-wall polysaccharides as their main energy source (Flint et al. 2008). Therefore, gut microbiota of those animals developed a diverse variety of CAZymes that is used in order to cooperatively degrade polysaccharides (Martens et al. 2008). Bacterial phyla Bacteroidetes are the dominant representatives of mammalian gut microbiota that produce a multienzyme complex for glycan degradation that is called polysaccharide utilisation loci or PULs. (Martens et al. 2009). PULs imply to physically linked genes that produce ensemble of proteins that work on degrading of polysaccharides.

Discovery of new PULs is done by performing metagenomic studies and afterwards PULs are located in a database. In return, unknown parts of PULs can be a source for finding new CAZymes.

PUL description started with starch utilization system (Sus) from *Bacteroides thetaiomicron.* It was the first PUL to be analysed that contained eight genes: susRABCDEFG (Martens et al., 2008). Proteins from that PUL coordinately together, where SusD, and supposedly with help of SusE, SusF, attach starch molecule to bacterial surface where then SusG encoded α-amylase degrade starch molecule. SusC serve as transporter delivering oligosaccharides inside the cell. Further degradation is done with the help of neopullunase from SusA and α-glucosidase that is encoded by SusB (Martens et al. 2008). Regulation of PUL expression is done by SusR that activates the transcription. Usually, regulators in PULs are located on the inner membrane and are activated or suppressed by products od polysaccharide degradation (Martens et al, 2008). Nowadays, all known PULs conserve transporter SusC and SusD that coordinates the attachment to the polysaccharide (Terrapon et al. 2015). Presence of those genes can be a predictor of a new PUL.

Recently, it has been shown in literature that complete characterisation of all genes that are assembled in one PUL could reveal novel enzyme complexes for degradation of specific polysaccharides (Terrapon et al. 2015). For example, Larsbrink et al, 2014 described *B. ovatus* PUL that can brake xyloglucan molecules to monosaccharides.

2.7. FjoAcXE acetyl xylan esterase

FjoAcXE also known as FjoAcXE is an unknown protein was recently studied in work of F.M. Razeq (2017). Protein was chosen from polysaccharide utilisation loci database (PULDB) PUL 20 and proven to be an acetyl xylan esterase. It was shown that FjoAcXE can target various types of residues, having low influence from steric hindrance of the molecule. Moreover, a novel esterase activity that has not been previously described amongst known CE families was detected. It was shown that FjoAcXE has synergism with xylan α-glucuronidase, since FjoAcXE removes acetic acid from 3-O-acetylated xylopyranosyl that are at the same time substituted with (4-O methyl) glucuronic acid at 2-O position allowing glucuronidases to access to previously inaccessible xylopyranosyl residues.

Alignment of sequences of FjoAcXE and all carbohydrate esterases listed CAZy database has shown that FjoAcXE shares less than 30% sequence similarity. Although, at the same time studies of predicted 3D structure of FjoAcXE showed that it is structurally similar to one of carbohydrate esterase from families CE2 and CE12. BLASTp search revealed that proteins that share 44% and more sequence similarity are uncharacterised lipases from GDSL-families or SGNH hydrolases.

3. Materials and methods

3.1 Materials.

3.1.1 Studied enzymes

In this work, all together eight homologues of the enzyme FjoAcXE (Accession number ABQ06890.1, EC 3.1.1.72) were examined. Candidates were chosen according to the BLASTp search of FjoAcXE-homologues executed in the Master's thesis study completed by F.M. Razeq (University of Toronto). Chosen candidates share from 47 to 98% of sequence identity with FjoAcXE and come from various bacterial organisms (Table 3.1.1.). Protein sequences of all studied in this thesis FjoAcXE-homologues are listed in Appendix 1, summary information about FjoAcXE is given in Appendix 2.

Table 3.1.1. Overall information of FjoAcXE-homologues.

Full name	Accession number (GeneBank ID)	Species	Identity with FjoAcXE, %	Signal sequence	Size, kDa
K392DRAFT_2214	WP_073411463.1	*Flavobacterium sp. 40S8*	98.1	+	43.500
FF52_18088	WP_008467835.1.	*Flavobacterium sp. F52*	69.4	+	44.043
EW79DRAFT_2436	WP_047423182.1	*Chryseobacterium sp. YR480*	53.3	-	44.006
T426DRAFT_00687	WP_027586161.1	*Prolixibacter bellariivorans*	51.6	+	43.141
WP_035683315.1	WP_035683315.1	*Flavobacterium reichenbachii*	73.9	+	43.229
ZPR_3026	WP_013072442.1	*Zunongwangia profunda SM-A87*	52.5	-	46.481
CHSO_3300	WP_045502788.1	*Chryseobacterium sp. StRB126*	51.3	-	46.224

AlkimDRAFT_0871	WP_044117739.1	*Alkaliflexus imshenetskii DSM 15055 AlkimDRAFT_0871*	47	+	42.238

3.1.2 Buffers

Table 3.1.2. Overall information of buffers used in this study.

Buffer	pH	Content	Utilisation step
Cell lysis			
Cell Lysis buffer	7.4	20 mM HEPES 500 mM NaCl 88266 Pierce™ Protease Inhibitor Tablets, EDTA-Free, Thermo Fisher Scientific Inc.	Ultrasonication (small scale); Emulsiflex lysis (large scale).
Purification			
Binding buffer	7.4	20 mM HEPES 500 mM NaCl	Purification with Ni-NTA agarose beads (small scale); FPLC (large scale)
Washing buffer	7.4	20 mM HEPES 500 mM NaCl 20 mM imidazole	Purification with Ni-NTA agarose beads (small scale); Purification with Ni-NTA agarose on Preppy™ 12-port vacuum system (large scale)
Elution buffer 100mM	7.4	20 mM HEPES 500 mM NaCl 100 mM imidazole	Purification with Ni-NTA agarose beads (small scale);

			Purification with Ni-NTA agarose on Preppy™ 12-port vacuum system (large scale)
Elution buffer 500mM	7.4	20 mM HEPES 500 mM NaCl 500 mM imidazole	Purification with Ni-NTA agarose beads (small scale); FPLC (large scale); Purification with Ni-NTA agarose on Preppy™ 12-port vacuum system (large scale)
Desalting (buffer exchange)			
Desalting buffer	7.4	10 mM HEPES 10%glycerol	
Enzyme assays			
NaAcO buffer	5.5	50 mM NaAcO	PAHBAH, pNP, 4MUA assays, Minimal enzyme concentration assay
HEPES	7.0	50 mM HEPES	
HEPES	8.5	50 mM HEPES	
Universal buffer (50mM)	3.5-9.0 pH adjusted with NaOH	Stock solution 200 mM: 67 mM $NaCH_3COO$ 67 mM H_3BO_3 67 mM H_3PO_4 Milli-Q water to 1L	pH optimum Temperature stability

3.1.3 Substrates

Table 3.1.3. Substrates used in this study

Substrate name	IUPAC name	Producer	Activity detection method
Wheat arabinoxylan (medium viscosity)	Arabinoxylan (1,2;1,3-α-Araf)	Megazyme	PAHBAH assay
Arabinoglucuronoxylan (oat spelt water soluble part separated after boiling)	Arabinoglucuronoxylan	Molekula	PAHBAH assay
CM-Cellulose 4M (medium viscosity)	Cellulose	Megazyme	PAHBAH assay
Galacto-xyloglucan (Tamarind, high viscosity)	Galacto-xyloglucan	Megazyme	PAHBAH assay
Lichenan (Icelandic moss)	Glucan (1,3; 1,4-β-D-)	Megazyme	PAHBAH assay
Pullulan	Glucan (1,4; 1,6-α-D-)	Megazyme	PAHBAH assay
Dextran	Glucan (1,6-α-D-)	Megazyme	PAHBAH assay
Glucomannan Konjac (low viscosity)	Glucomannan, acetylated	Megazyme	PAHBAH assay
Mannan	Mannan (1,4-β-D-)	Megazyme	PAHBAH assay
Arabinan (sugar beet)	Pectin (1,5-α-Araf)	Megazyme	PAHBAH assay
Arabinogalactan (Larch wood)	Pectin (1,3;1,4;1,6-β-Gal; 1,4;1,6-α-Araf)	Megazyme	PAHBAH assay
Pectic galactan (Lupin)	Pectin (Galactan (1,4-β-D))	Megazyme	PAHBAH assay
Galactan (Lupin)	Pectin (Galactan (1,4-β-D-)); Arabinofuranosidase treated lupin pectic galactan)	Megazyme	PAHBAH assay

Rhamnogalacturonan (soy bean)	Rhamnogalacturon	Megazyme	PAHBAH assay
Rhamnogalacturonan I (potato)	Rhamnogalacturonan I	Megazyme	PAHBAH assay
Glucuronoxylan	Glucuronoxylan	Megazyme	PAHBAH assay
pNP-arabinofuranoside	4-NP-α-L- arabinofuranoside	Sigma-Aldrich Inc., USA	pNP-assay
pNP-glucopyranoside	4- Nitrophenyl β-D-glucopyranoside	Sigma-Aldrich Inc., USA	pNP-assay
pNP-mannopyranoside	4-Nitrophenyl β-D-mannopyranoside	Sigma-Aldrich Inc., USA	pNP-assay
pNP-xylopyranoside	4-Nitrophenyl-xylopyranoside	Sigma-Aldrich Inc., USA	pNP-assay
pNP-chitobioside	4-Nitrophenyl-N,N,'-diacetyl-β-chitobioside	Sigma-Aldrich Inc., USA	pNP-assay
pNP-α-galactopyranoside	4-Nitrophenyl-α-D-galactopyranoside	Sigma-Aldrich Inc., USA	pNP-assay
pNP-fucopyranoside	4-Nitrophenyl- α-L-fucopyranoside	Sigma-Aldrich Inc., USA	pNP-assay
pNP-β-galactopyranoside	4-Nitrophenyl- β-D-galactopyranoside	Sigma-Aldrich Inc., USA	pNP-assay
pNp-acetate	4-Nitrophenyl-acetate	Sigma-Aldrich Inc., USA	pNP-assay, Minimal enzyme concentration assay;

			pH optimum assay
4MU-acetate	4-Methylumbelliferyl acetate	Sigma-Aldrich Inc., USA	4MU-acetate-assay, Minimal enzyme concentration assay; pH optimum assay
Xylo-oligosaccharide mixture from corn fibre			Secondary screen
Per-acetylated xylo-oligosaccharide mixture	(2,3-di-O-acetyl-β-D-Xylp-(1,4)-1,2,3-tri-O-acetyl-α-D-Xylp) (2,3-di-O-acetyl-β-D-Xylp-(1,4)-2,3-di-O-acetyl-α-D-Xylp)	LISBP, France	Secondary screen

3.2. Enzyme expression

3.2.1 Preparation of competent cells

Targeted proteins were expressed in *E.coli* BL21(DE3) strain (New England BioLabs Inc.), whereas *E.coli* Top10 strain served as the cloning strain. Accordingly, chemically competent cells were made from both strains prior to transformation. In each case, an overnight was made in 3 ml LB medium with addition of 50 µg/ml of kanamycin, and incubated at 37˚C. A 1ml of the preculture was transferred into 100 ml of LB medium with 50 µg/ml kanamycin and incubated at 37 ˚C with shaking at 300 rpm until the optical density (OD) at 600 nm reached between 0.25-0.3. The culture was then cooled on ice and centrifuged for 10 min at 3200 rcf at 4˚C. The supernatant was discarded and the pellet was suspended in 0.1 M $CaCl_2$ and kept on ice for 30 min. Following centrifugation, the supernatant was again discarded and the cell pellet was suspended in 0.1 M $CaCl_2$ containing 15% glycerol, before being aliquoted as 1 ml volumes in Eppendorf tubes, flash-frozen in liquid N_2, and then stored at -80˚C.

3.2.2. Transformation

DNA plasmids encoding the proteins listed in Table 1 were purchased from GenScript Laboratories Inc. At first, lyophilised plasmid was diluted in deionised water and incubated at 50˚C for 15 min. Transformation was performed with both strains at the same time the *E.coli* Top10 to create -80˚C glycerol stocks and *E.coli* BL21(DE3) to generate the expression strains. After adding 10 µl of plasmid DNA, competent cells were incubated firstly on ice for 30 min with subsequent incubation at 42˚C for 45 sec, after which they were again transferred to ice for 5 min. Then, 250 µl of SOC was added to the tube containing transformed cell prior to incubation at 37˚C for 1 h, shaking 220 rpm. A 25 µl aliquot of transformed cells was then transferred to an LB agar plate containing 50 µl/ml kanamycin, and then incubated overnight at 37˚C. Stock samples were prepared from a single colony from each plate, where the colony was transferred to 4 ml LB containing 50 µl/ ml kanamycin and incubated overnight at 37˚C. Culture suspension was mixed with 50% glycerol in ratio 1:1, flash-frozen in liquid N_2 and stored as glycerol stocks at -80˚C.

3.2.3. Small scale expression in 6 ml of Magic Media

In order to estimate the expression potential of the recombinant proteins, each transformant was initally cultivated in small scale, using 6 ml Magic medium containing 50 µl/ml kanamycin. Flow chart representation of small scale expression is shown in Figure 3.2.3. Incubations were performed for 24 h at 30˚C in 50 ml falcon tubes sealed with breathable tape to insure good aeration. Cultivation was done in duplicates and after measuring the final OD, the best growing replicate was chosen for lysis to assess the level of recombinant protein expression. Before lysis, 50 µl of sample was collected for SDS/PAGE analysis, centrifuged (4000 rcf, 4 ˚C, phases were separated into two Eppendorf tubes: supernatant and cell debris), cell debris was dissolved in MilliQ water and then both samples further prepared for the SDS-PAGE as described below (Table 3.3.3.). The rest of the culture was also centrifuged at 4000 rcf for 5 min and the pellet was suspended in lysis buffer (Table 3.1.3) before lysis by ultrasonication. The intensity of the ultrasonification was adjusted to 20% of amplitude, using pulse-mode (1 sec ON followed by 1 sec OFF) for in total of 40 sec. To avoid protein degradation due to an increase in temperature during sonication, Eppendorf tubes containing cell suspension were kept on ice at all times during sonication. A 50 µl sample was collected and then centrifuged (12000 rcf, 4˚C, 5 min), and both supernatant and the insoluble fraction were analysed by SDS-PAGE analysis (Table 3.3.3.). The remaining lysate was centrifuged at 12000 rcf at 4˚C for 5 min, and the supernatant was recovered and then stored in the fridge if the purification process was done the following day.

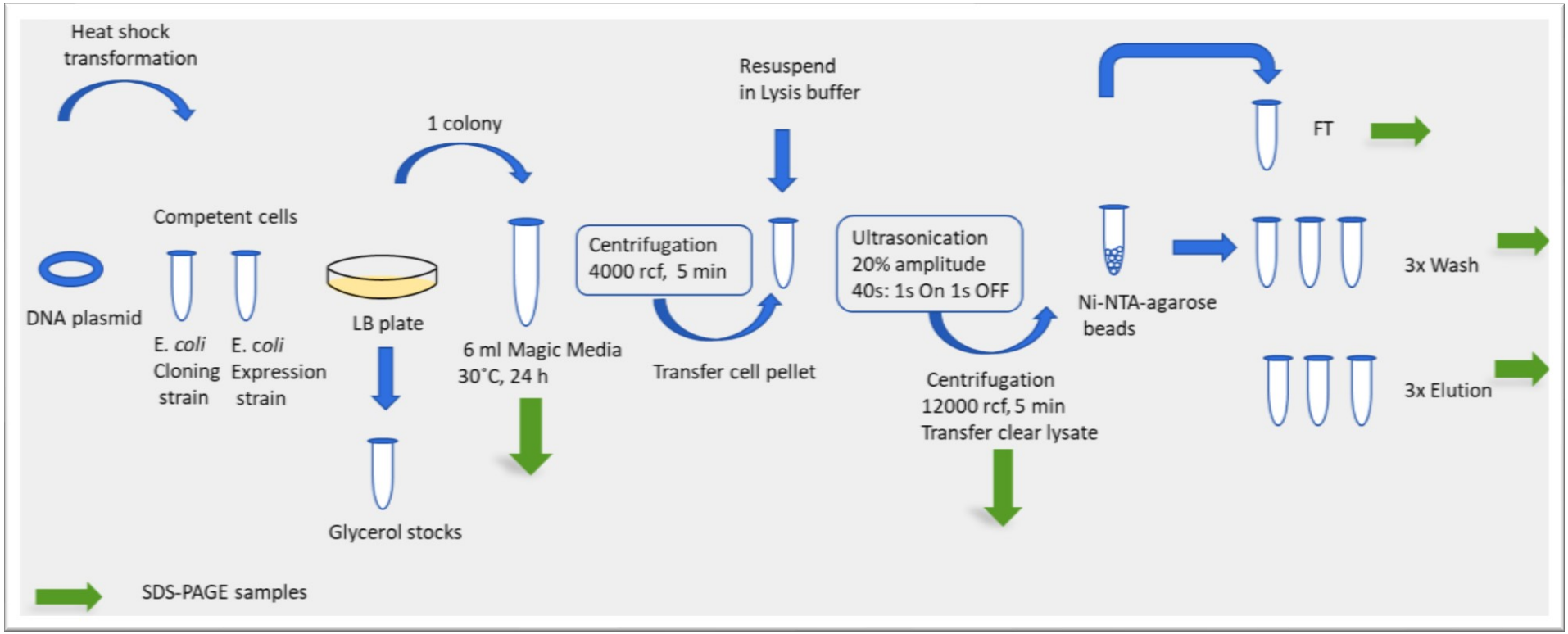


Figure 3.2.3. Flow-chart representation of small scale expression and purification.

3.2.4. Large scale expression in 500 ml LB Medium

Flow chart of large scale expression presented in Figure 3.2.4. Large scale expression was performed in 2 l flasks. First precultures were prepared by transferring a small sample of frozen stock for the selected transformant to 4 ml LB with 50 µl/ml kanamycin after 4 h of incubation at 37˚C 2 ml were transferred to 25 ml LB with 50 µl/ml to make second preculture and then incubated at 30˚C overnight. A 10 ml sample of the second preculture was transferred to 500 ml LB with 50 µl/ml kanamycin, containing 0.5 M sorbitol and 0.025 M glycine betaine with a final cultivation volume of 550 ml. Incubation was done at 30˚C until the OD600 reached the range between 0.5-0.6. After induction with 0.5 mM IPTG, the temperature was decreased to 16˚C to facilitate protein folding. After 16 h of incubation the final OD600 was measured. A 50 µl sample was collected and centrifuged (4000 rcf, 4 ˚C, 5 min). Supernatant was transferred to the Eppendorf tube and cell debris was suspended in 50 µl of milliQ water, both were then prepared for SDS-PAGE analyses (Table 3.3.3.). The remaining cultures were then centrifuged at 4000 rcf for 30 min, the supernatant was separated, and the pellet was transferred to a 50 ml flacon tube. In case the purification was not done the same week, the pellet was stored at -20˚C. For purification, the pellet was suspended in lysis buffer (Table 3.1.2.), and cell disruption was performed using the Emulsiflex (Avestin Inc, Canada). Disruption was performed using pulsation mode with pressure set between 10000-15000 psi for 10 min. In order to ensure efficient lysis, the volume of cell suspension was around 40 ml. A 50 µl sample was collected and centrifuged at 12000 rcf and 4 ˚ for 5 min (Table 3.3.3.). The cell suspension was then transferred to Sorvall tubes and centrifuged at 12000 rcf and 4˚C for 45 min to obtain the clear lysate.

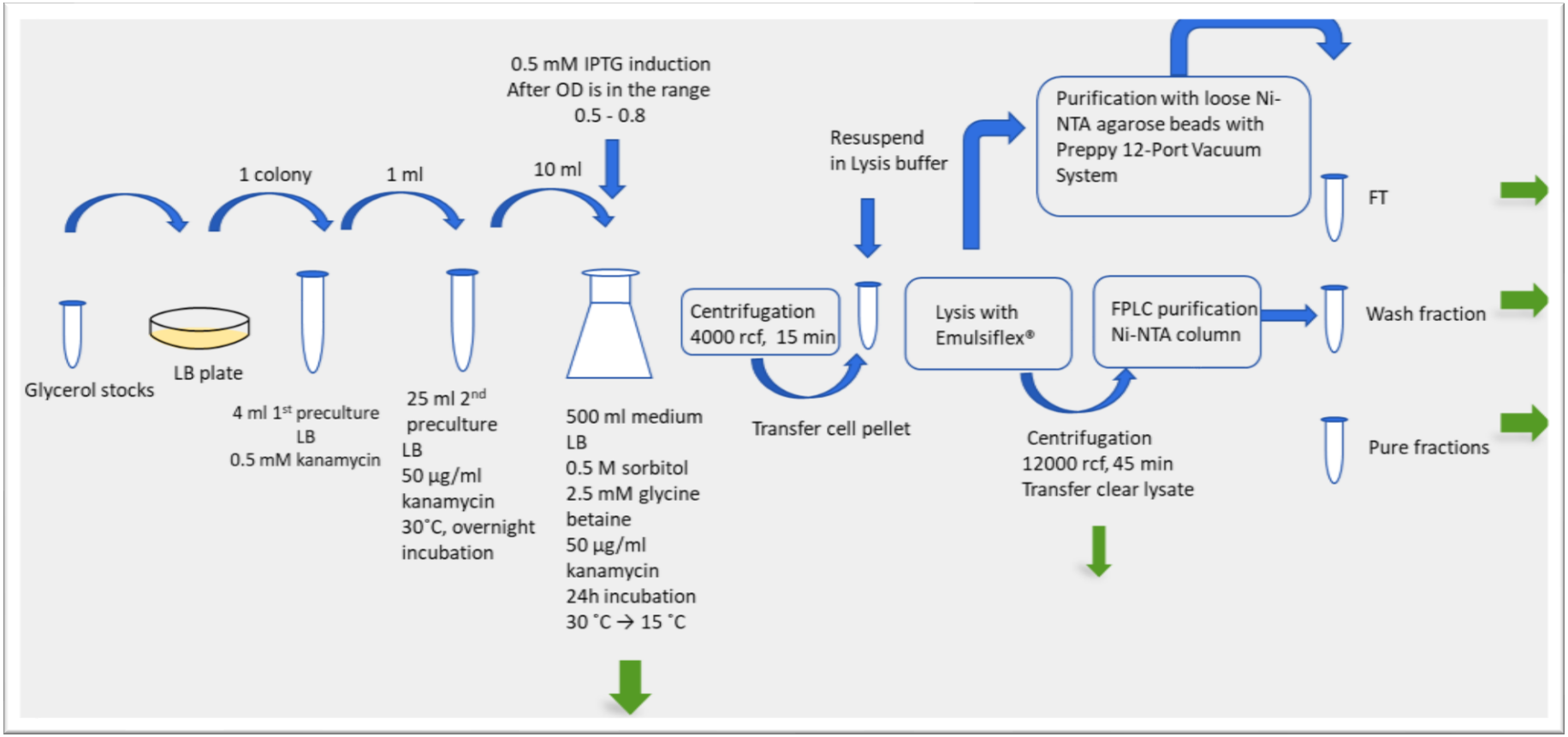


Figure 3.2.4. Flow-chart representation of large scale expression and purification

3.3. Enzyme purification

3.3.1 Small scale purification with 250 µl of Ni-NTA agarose resin

Purification of the studied enzymes in small scale was performed with Ni-NTA agarose beads in 2 ml Eppendorf tubes. At first, 250 µl of Ni-NTA agarose resin (Ni-NTA Agarose, Qiagen Inc.) was washed three times with three resin-bed volume of binding buffer (Table 3.1.2.), every time followed by spinning (4˚C, 5 min), centrifuging at 1500 rcf and discarding the wash. Clear lysate was loaded for overnight spinning on head-over-tail mixer at 4˚C and after centrifuging at 1500 rcf, the supernatant was collected as flow-through. Then, beads were washed three times with three resin-bed volume of washing buffer (Table 3.1.2.) as described above and supernatants were collected. The first protein elution was performed with two resin-bed volumes of 100 mM imidazole elution buffer (Table 3.1.2.). The second protein elution was done with two resin-bed volume of 500 mM imidazole elution buffer (Table 3.1.2.), and the third protein elution used three resin-bed volume of the same buffer, supernatants were collected in separate Eppendorf tubes. At each purification step, a 50 µl sample was collected to track protein recovery (Table 3.3.3.).

3.3.2. Large scale purification

One of FjoAcXE-homologues, T426DRAFT_00687, was purified at large scale using Ni-NTA agarose and the Preppy™ 12-port vacuum system. Liquid flow and sample collection within the system is performed by the application of vacuum (max. pressure 2.8 psi). The clear lysate recovered from the cell pellet was diluted with binding buffer up to approximately 70 ml (~ $1/8^{th}$ of the original culture volume – 550 ml) and divided into two 50 ml falcon tubes before adding 50% resin (to 1/3 of the lysate volume) and transferring the suspension to 4˚C for overnight mixing on head-over-tail mixer. Following the overnight incubation, the resin was allowed to settle for 1h at room temperature. The liquid was then decanted and saved as “flow through”. The resin was washed three times with 15 ml of washing buffer (Table 2) and wash fractions were collected. The first protein elution was made with 15 ml of elution buffer 100 mM (Table 3.1.2.), and the second and third elutions were performed with 15 ml of elution buffer 500 mM (Table 3.1.2.). A 50 µl sample was

collected from each step of the purification to track protein recovery (Table 3.3.3.). Following the protein purification, the NiNTA resin was washed with Milli-Q water and stored in 20% ethanol at 4˚C.

Large scale purifications of all other targeted enzymes were performed using an FPLC (ÄKTA purifier, Amersham Bioscience Inc.) using a Ni-NTA column. Buffers used for purification are listed in Table 3.1.2. Protein elution was performed using a gradient reaching 4% of elution buffer in 1 min and keeping it constant until the first peak appears on the chromatogram or, otherwise, for 10 min. The gradient was then increased to 100% of elution buffer in 30 min. The volume of the eluted fractions was 5 ml, which were collected in 15 ml falcon tubes. A 50 µl sample was collected from the flow-through and all of the fractions that gave a peak on chromatogram detected by sensor at 280 nm (Table 3.3.3.).

Desalting of the purified fractions was done by centrifugation at 4900 rcf at 4˚C in spin columns (Vivaspin 20 10,000- Da MWCO, Vivascience Inc.). Desalting was generally done according to producer's protocol. Each column was filled with 5 ml of the purified fraction and 15 ml of buffer, and then two washes with 15 ml buffer. The protein solution that remained in the spin column was collected in a falcon tube and the membrane of the spin column was washed with 0.5-0.75 ml of buffer and added to protein solution. The concentration of recovered protein samples was assessed using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol.

Aliquoting of desalted protein for the future experiments was made using a 10 ml syringe to create drops of the protein solution that were directly frozen in liquid N_2 and then collected to 2 ml Eppendorf tubes and stored at -80˚C. All of the pure enzymes were run on SDS-PAGE. For that, beads of the protein were defrosted and diluted in milliQ water with the final protein concentration 0.5 mg/ml (A Guide to Polyacrilamide Gel and Detection, Bio-Rad Laboratories Inc.).

3.3.3. SDS-PAGE run

In order to analyse protein expression and purification, samples were run on SDS-PAGE. For this thesis, hand-casted gels were made using the Multi-Casting Chamber Model 49 (Bio-Rad). 10% resolving gel and 4% stacking gel were prepared and run according to manufacturer's protocol (A Guide to Polyacrilamide Gel and Detection, Bio-Rad Laboratories Inc.). Staining of the gels was made with PageBlue™ Protein Staining Solution (Thermo Fisher Scientific Inc.). Samples from expression and purification steps were collected at each step of the process and prepared in Eppendorf tubes by adding 4x Laemmli sample buffer with 50 mM DTT and boiled at 95˚C for 10 min. Molecular weight of the protein was determined using the PageRuler™ Unstained protein Ladder (Thermo Fisher Scientific Inc.) and Precision Plus Protein™ Dual Color Standards (Bio-Rad Laboratories Inc.). Cell pellet and cell debris material (about 0.5 µl) was transferred to 30 µl of deionised water before addition of sample buffer in order to avoid excess protein loading. A list of samples analysed by SDS-PAGE is presented in Table 3.3.3.

Table 3.3.3. SDS-PAGE samples list, collected throughout the enzyme expression and purification process.

Part of the process where sample is collected	Small scale expression and purification on Ni-NTA agarose beads	Large scale expression and purification on FPLC	Large scale T426DRAFT_00687 expression and purification with Ni-NTA agarose on Preppy™ 12-port vacuum system (large scale)
Cultivation (collected after centrifugation)	Cell Medium Cell Pellet	Cell Medium Cell Pellet	Cell Medium Cell Pellet
Cell Lysis	Cell Debris Clear lysate	Cell Debris Clear lysate	Cell Debris Clear lysate
Enzyme purification	FT Wash samples (3)	FT	FT Wash samples (3)

	Elution samples (3)	Fractions that give peaks on chromatogram	Elution samples (3)

3.4. Enzyme activity assays

3.4.1. Primary screen: determination of exo-activity

Exo-activity was measured using colorimetric pNP-linked substrates and 4MU-linked acetate assays. Substrates used for this experiment are presented in Table 3. The activity was measured at three pH values: 5.5, 7.0 and 8.5 (buffers are listed in Table 3.1.2.). Assays were performed in 96-well plates (200 µl) and measurements were done after 30 min incubations at 40˚C and shaking at 300 rpm.

For the 4MU-acetate assays plates were made separately for different time points due to substrate precipitation that can affect OD measurements. Standard curves consisted of eight standards in range from 0.5 mM to 2.5 mM of 4MU and was prepared in the same buffer. Reaction was initiated by adding 1 mM 4MU-acetate to the enzyme diluted in buffer in concentration 10% per gram dry matter substrate.

After 30-min incubation, the 96-well plates were centrifuged at 3900 rpm for 2 min, then liquid was transferred with the multipipet to a new 96-well plate and OD was measured at 354 nm. In case with pNP-linked substrates, it was possible to perform continuous reaction on one plate, with OD measurements at 2 h, 4 h, and 24 h incubation time. Plates at first were centrifuged to avoid condensation and then measured using the plate reader. Enzyme activity was assessed by amount of released product, using standard curve. For the 4MU-acetate assay, the standard curve consisted of eight standards in range from 0.5 mM to 2.5 mM of 4MU, diluted in HEPES buffer (pH 7.0).

3.4.2. Overall hydrolytic activity (reducing end assay)

The overall activity of the studied enzymes was determined using the PAHBAH assay. Substrates used for this assay are listed in the Table 3. Preliminary, primary screen 96-well plates were prepared at three pH values: 5.5, 7.0 and 8.5 (Table 3.1.2.) by performing 24 h

incubation of enzyme with substrate in buffer (40˚C, shaking 300 rpm). The enzyme dose used for this assay was 10% per gram dry matter substrate. After the incubation, the 96-well plates were stored at -20˚C and were defrosted right before performing PAHBAH assay. For the reaction, 10ul from the primary screen plate was transferred and the reaction was initiated by adding 200 µl of PAHBAH reagent and following 30-min incubation at 40˚C and shaking. Optical density was measured at 405 nm. Activity of enzyme was estimated by substracting substrate blank absorbance from absorbance of enzyme reaction with substrate. This value was then compared to enzyme blank absorbance.

3.4.3. Biochemical characterisation of enzymes

Minimal concentration of the studied enzymes was determined on 0.3 mM pNP-acetate and 0.5 mM 4MU-acetate in HEPES buffer on pH 7.0 (Table 2) as described above. In order to determine the linear relation between enzyme concentration and absorbance values for the pH optimum assay, tested enzyme concentrations were taken in range of 0.05-7.5% per gram dry matter substrate.

The pH optimum assay was then performed with determined minimal enxyme concentration incubated 30 min at 40˚C with 0.5 mM 4MU-acetate and 50 mM universal buffer (Table 3.1.2) at pH values: from 3.5 to 5.5 and from 8.0 to 9.0 with 1 pH unit step, from 6.0 to 8.0 with 0.5 pH unit step. Activity was determined upon amount of released product, detected at 354 nm.

Temperature stability was determined with 0.5 mM 4MU-acetate with 50 mM Universal buffer (Table 3.1.2.) optimal pH, 30 min incubations at four different temperature points (20, 40, 55 and 70˚C) and seven time points (0 min, 30 min, 1, 2, 4, 6 and 24 h). Zero min time point was made as comparison point when incubation was made immediately after unfreezing enzyme bead. Activity was determined upon amount of released product, detected at 354 nm.

3.4.4. Assay on natural and synthetic complex substrates

Assay was performed on corn fibre fraction and xylo-oligosaccharide mixture substrates (Table 3.1.2).

Corn fibre fraction implies to highly branched mixture of feruloylated xylooligosaccharides previously described by Appeldoorn et al, 2013.

Per-acetylated mixture of xylo-oligosaccharides was a kind gift from Dr. R. Faure, made by LISBP, France. Expected degree of polymerisation was from 4 to 7 (Figure 3.4.2)

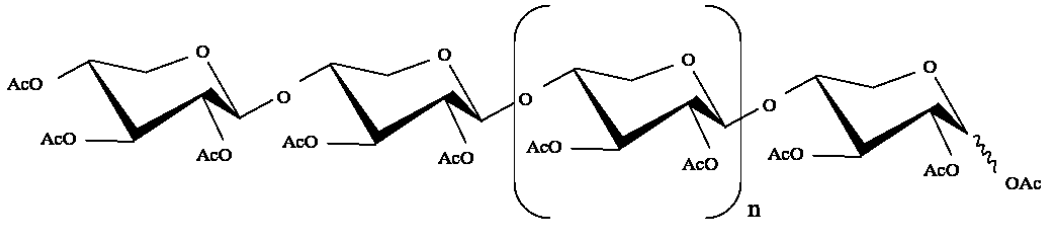


Figure 3.4.2. Expected structure of per-acetylated xylo-oligosaccharide mixture

For incubations with corn fibre fraction substrate two time points were used: 20 min (two pHs 7.0 and 8.0) and 4 h (pH 7.0). Enzyme dose used for these incubations was 1% w/v. Incubation with xylo-oligosaccharide mixture were done on three time points 20 min, 4 h and 20 h. Enzyme dose, chosen was 1 and 2% for 20 min incubations and 2% for 4 and 20 h. Afterwards, release of acetic acid was determined with acetic acid kit (K-ACETRM, Megazyme) according to enclosed protocol. In order to estimate maximal theoretical amount of acetyl group substitutions chemical saponification with 0.5 M NaOH was performed with both substrates (30 min, 70˚C, shaking 450 rpm). Release of acetic acid was measured with acetic acid kit (K-ACETRM, Megazyme).

4. Results and discussion

4.1 Comparison of preselected candidates

BLASTp search for FjoAcXE homologues against a GeneBank showed high similarity with eight homologues that contain Lipase_GDSL_2 (PF13472), SGNH_hydro domain. Sequence alignment of eight preselected candidates in reference to FjoAcXE was performed with full protein sequences containing signal sequence where present. As expected, results showed that all eight homologues contain four conserved SGNH-residues: Ser-Gly-Asn-His (conserved positions in FjoAcXE full protein sequence 217, 258, 294 and 395) and putative GDSL-catalytic trial Ser-His-Asp (conserved positions in FjoAcXE full protein sequence 217, 392 and 395).

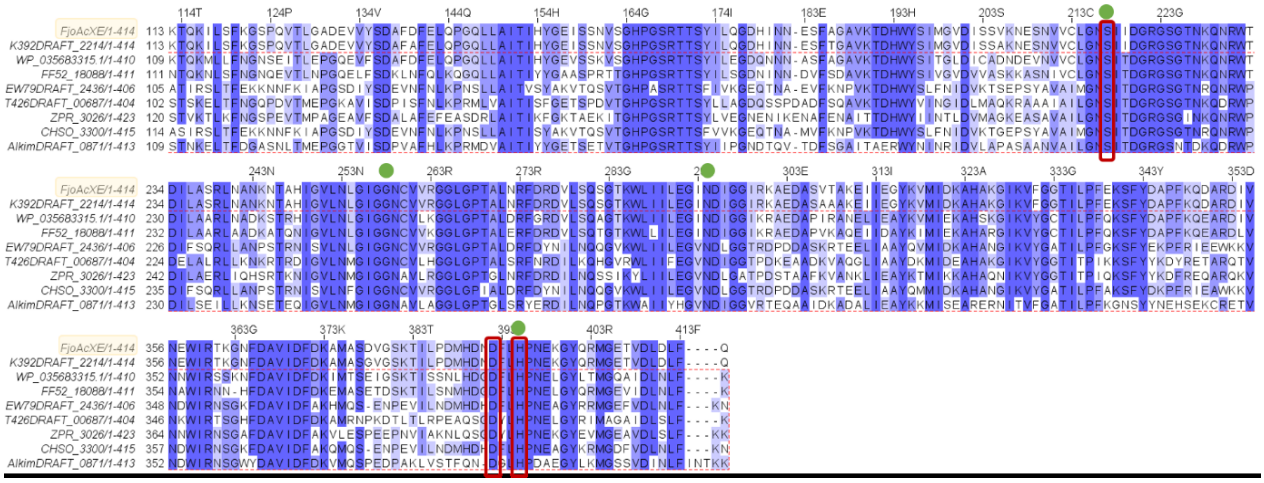


Figure 4.1.1. Multiple sequence alignment of FjoAcXE and homologues

Enzymes containing GDSL-catalytic triad present a wide range of lipases and esterases that have a flexible active cite and therefore show low susceptibility to steric hindrance of substrates (Akoh et al. 2004).

4.2. Purification

4.2.1. Small scale expression and purification

Transformations of DNA plasmids containing sequences of all chosen FjoAcXE-homologues were performed using heat shock method. In order to separate successful transformants vector plasmids contained a selective marker – resistance to kanamycin. As a results all plasmids were successfully introduced in *E.coli* expression and cloning strains as clones showed growth on LB plates containing kanamycin.

After transformation, six of the FjoAcXE-homologues (K392DRAFT_2214, FF52_18088, EW79DRAFT_2436, T426DRAFT_00687, WP_035683315.1, AlkimDRAFT_0871) were expressed in small scale in self-inductive Magic Media. Following purification on Ni-NTA agarose beads. Performing small scale expression and purification helped to estimate the feasibility of purification using Ni-NTA agarose.

Several attempts to express ZPR_3026 and CHSO_3300 in *E.coli* using Magic Media or LB Media did not give any positive results and no dominant bands of corresponding weights were seen in cell pellet and medium samples on SDS-PAGE (Figure 4.2.1).

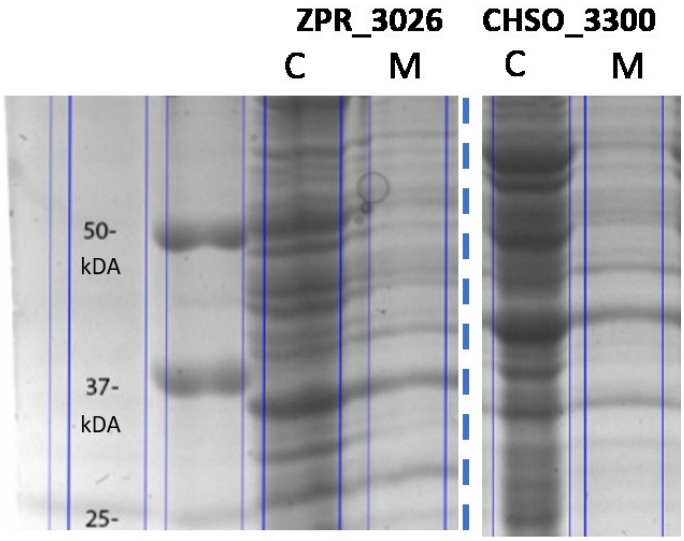


Figure 4.2.1. SDS-PAGE image of ZPR_3026 and CHSO_3300 attempt of small scale expression. C – cell pellet sample, M – culture medium sample. Molecular weights of ZPR_3026 and CHSO_3300 is 46.48 and 46.22 kDa respectively

Notably, only ZPR_3026, CHSO_3300 and EW79DRAFT_2436 did not have a signal sequence, although, only first two were not express. Moreover, CHSO_3300 and EW79DRAFT_2436 derive from the same genus of gram negative bacteria – *Cryseobacterium sp.* Therefore, it does not seem possible that genus of microorganism could affect the expression of the corresponding enzyme.

4.2.2 Large scale expression and purification

As expected from small scale expression results, FjoAcXE- homologues were sufficiently expressed in 500 ml of LB Media with addition of 0.5 M sorbitol and 2.5 mM glycine betaine. Overall six proteins (K392DRAFT_2214, FF52_18088, EW79DRAFT_2436, T426DRAFT_00687, WP_035683315.1, AlkimDRAFT_0871) were expressed in *E.coli*. Following purification of expressed proteins was performed on NI-NTA, as all proteins contain 6His-tag attached to C-terminus of amino acid sequence. Five of the proteins – K392DRAFT_2214, FF52_18088, EW79DRAFT_2436, WP_035683315.1 and AlkimDRAFT_0871were purified using FPLC Äkta purifier with Ni-NTA column. T426DRAFT_00687 was purified with on-the-bench purification method applying Preppy 12-Port Vacuum System with loose Ni-NTA agarose beads. There was no significant difference between these two methods in purity of obtained protein fractions, therefore, after first try out with T426DRAFT_00687 it was decided to continue with automated FPLC purification method for other proteins. Considering that imidazole can significantly interfere with the absorbance (Molina et al. 1996), there was a need to carry out buffer exchange of pure protein fractions that were eluted with imidazole-containing buffer. Size of expressed protein was confirmed with SDS-PAGE.

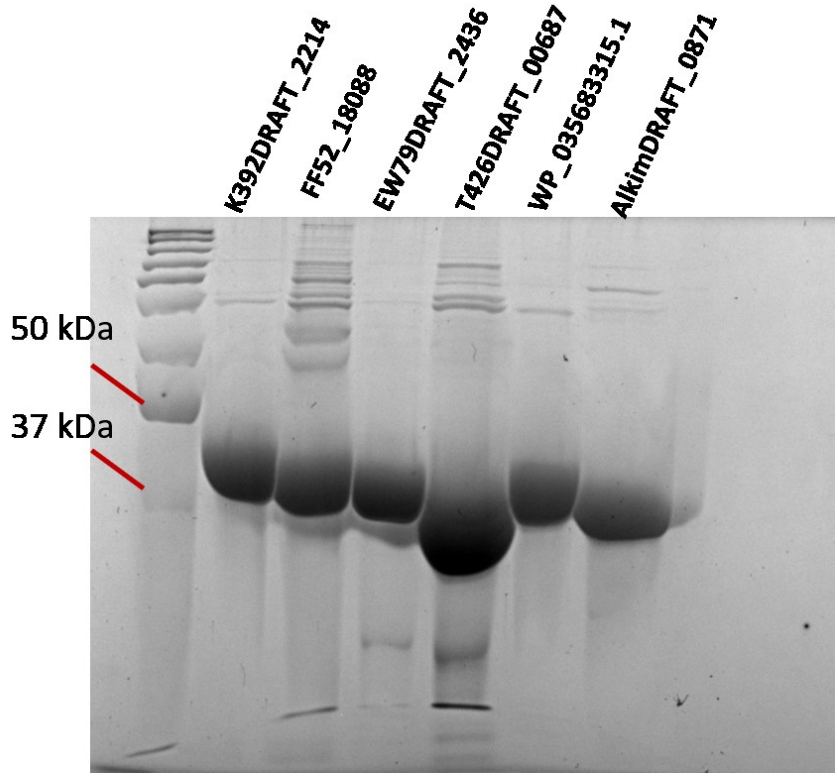


Figure 4.2.2. Pure fractions of FjoAcXE-homologues, SDS-PAGE run.

Molecular mass and theoretical pI was calculated with ProtParam Tool (http://web.expasy.org/protparam/).Yields of the purified FjoAcXE-homologues are listed in Table 4.2.1.

Table 4.2.1. Yields of purified FjoAcXE-homologues

Enzyme code	Sequence identity, %	Signal sequence	Molecular mass, kDa	Theoretical pI	Purification	Yield, mg
K392DRAFT_2214	98.1	+	43.5	6.71	+	40
FF52_18088	69.4	+	42.93	6.67	+	9.2
EW79DRAFT_2436	53.3	+	44.01	8.88	+	12
T426DRAFT_00687	51.6	+	43.14	9.35	+	8
WP_035683315.1	73.9	+	43.23	6.43	+	8.3
AlkimDRAFT_0871	47.0	+	42.24	5.32	+	14
ZPR_3026	52.5	-	46.48	9.39	not expressed	
CHSO_3300	51.3	-	46.22	9.45	not expressed	

FjoAcXE-homologues have similar molecular masses varying from 42.93 to 46.48 kDa. Molecular weight of FjoAcXE was determined to be in the same range and was found to be 45.2 kDa. Relatively small molecular weight is typical for esterases containing GDSL-domain (Akoh et al. 2004).

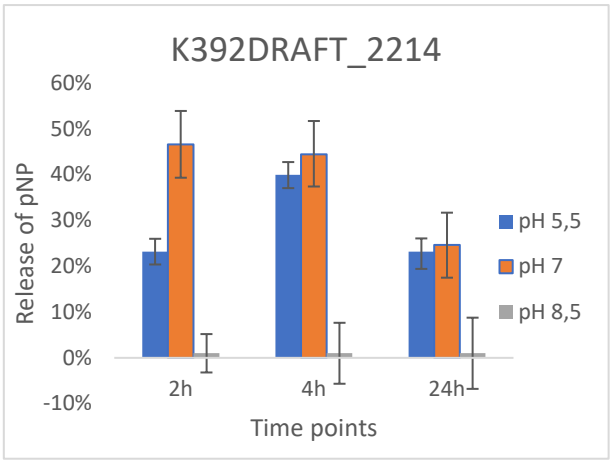
Interestingly, three of eight homologues (K392DRAFT_2214, WP_035683315.1 and FF52_18088) derive from same bacterial genus as FjoAcXE and show the highest similarity with FjoAcXE – 98.1, 73.9 and 69.4% of sequence identity respectively. Moreover, those homologues also have theoretical pI values 6.71, 6.43 and 6.67 respectively that is close to FjoAcXE pI of 6.4. Theoretical pI of homologues that derive from other genera varies from 5.32 to 9.45.

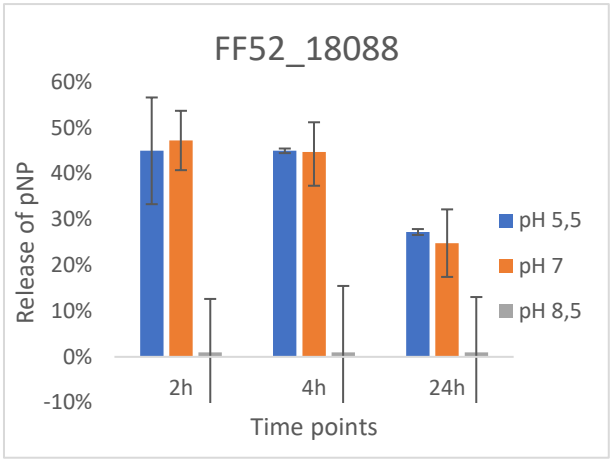
4.3 Primary screening

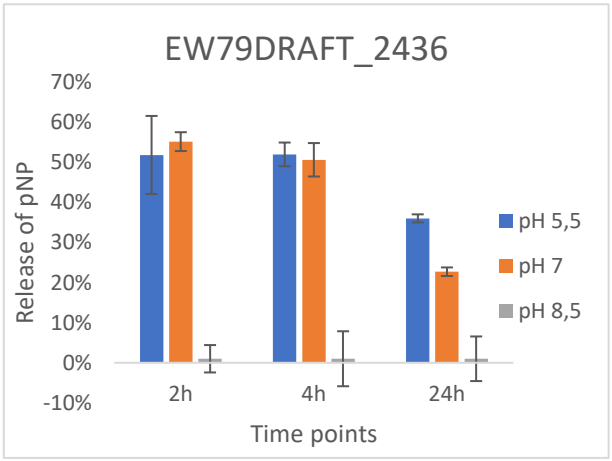
4.3.1 Esterase activity

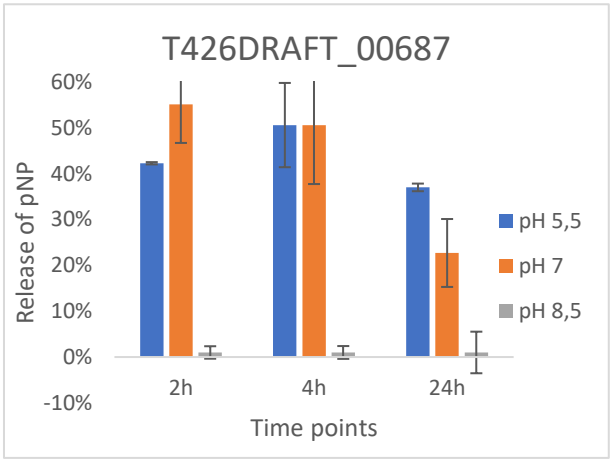
In order to estimate acetyl esterase activity of FjoAcXE-homologues they were tested on pNP-acetate substrate on three pHs: 5.5, 7.0 and 8.5 Results are present as release of pNP from pNP-acetate(Figure 4.3.1).

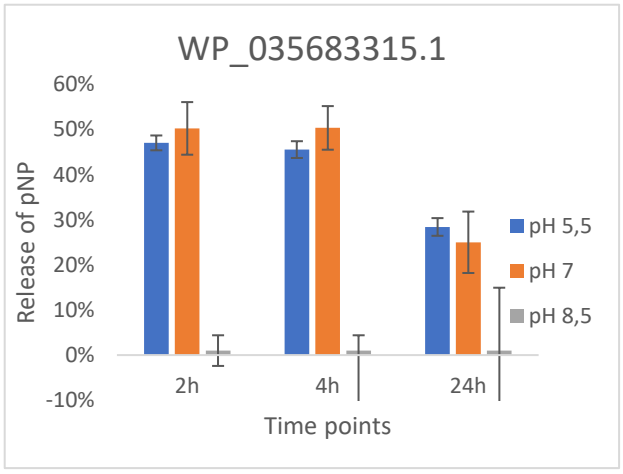
All six FjoAcXE-homologues showed release of pNP from pNP-acetate on pH 5.5 and 7.0 (Figure 4.3.1). No release of pNP related to enzymatic degradation was detected on pH 8.5, due to a complete degradation of the substrate already after 2 h incubation. General pattern that can be seen from this experiment that all homologues show highest activity on pH 7.0 after 2 and 4 h incubation. Release of pNP after 24 h incubation decreases significantly, which can be evidence of low enzyme stability decreasing already within 24 h.











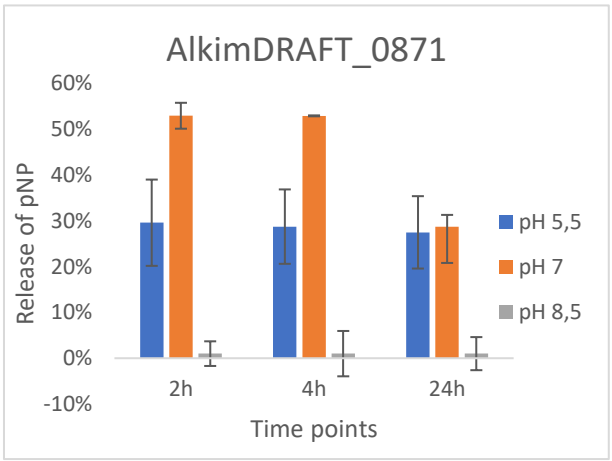


Figure 4.3.1. pNP release detected on pNP-acetate. Reactions were done in 200µl volume with concentration of pNP-acetate 1mM for pH 5.5 and 7.0 and 0.3mM for pH 8.5 and enzyme dose of 10% per gram dry matter substrate (n=3, error bars represent standard deviation). Release was calculated in % to theoretical maximum.

In order to confirm acetyl-esterase activity, assay on 4MU-acetate was performed. Due to the higher stability of 4MU-acetate in comparison to pNP-linked acetate, activity can be also seen after 2 h incubations on pH 8.5 (Figure 4.3.2.), however, after 24 h incubation 4MU-acetate was also completely degraded and, thus, it was not possible to detect enzymal degradation of a substrate at high pH. (Figure 4.3.3.).

Surprisingly, despite that concentration of the enzyme was equal when tested on pNP-acetate and 4MU-acetate, release of product after incubation with 4MU-acetate is generally higher than on pNP-acetate. For instance, T426DRAFT_00687 and WP_035683315.1 reach 100% of product release on pH 7.0.

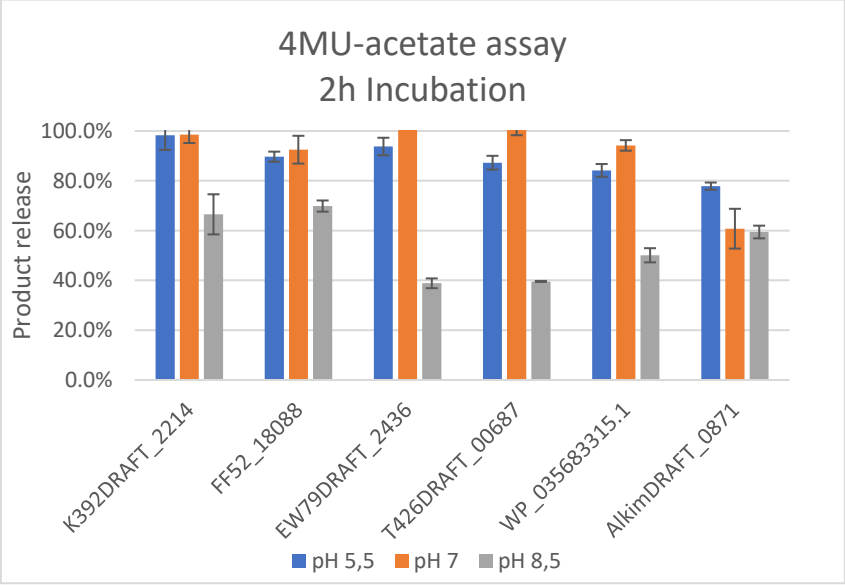


Figure 4.3.2. 4-MU release after 2 h incubations of FjoAcXE-homologues with 4MU-acetate. In 200µl reaction volume 1mM 4MU-acetate in 50 mM buffer (pH 5.5, 7.0 and 8.5) was incubated with 10% of enzyme per gram dry matter substrate (n=3, error bars represent standard deviation).

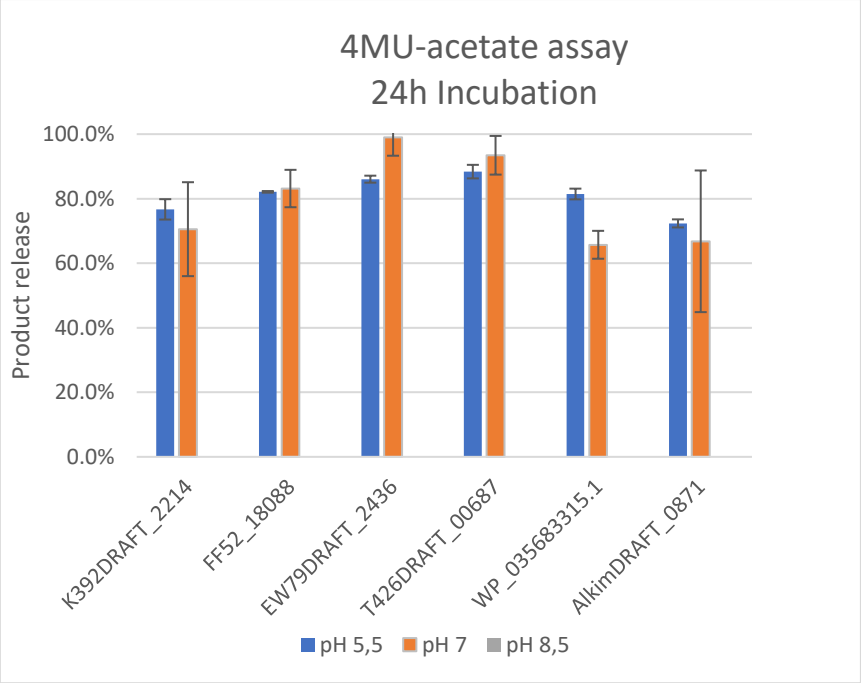


Figure 4.3.3. 4-MU release after 24 h incubations of UNK4-homologues with 4MU-acetate. In 200µl reaction volume 1mM 4MU-acetate in 50 mM buffer (pH 5.5, 7.0 and 8.5) was incubated with 10% of enzyme per gram dry matter substrate (n=3, error bars represent standard deviation).

As it was determined with pNP-assay product release on 4MU-acetate decreases respectively with time of an incubation.

Due to conserved GDSL-residues, studied FjoAcXE-homologues were excpected to show an esterase activity. Results in this work obtained after primary screen on both pNP-linked and 4MU-acetate show that homologues are able to cleave acetic acid from simple synthetic acetylated substrates. This knowledge is also consistent to the results demonstrated in Razeq work. FjoAcXE activity was tested after incubation with pNP-linked substrates for 2 h at 30˚C and pH 8 and the activity was detected only on pNP-acetate.

4.3.2. Hydrolytic activity

In this thesis, together with pNP-acetate, FjoAcXE-homologues were tested on nine other pNP-linked substrates (Table 3.1.3.) in order to discover other possible hydrolytic activities. Interestingly, two enzymes preparations (FF52_18088 and AlkimDRAFT_0871) showed release of pNP on other substrate than pNP-acetate and activity was detected from pNP-β-galactopyranoside on pH 7.0 and pH 8.5 (Figure 4.3.4). Moreover, release of the product increases respectively with increase of pH and time of the reaction, reaching the maximum on pH 8.5 after 24 h incubation. It has been reviewed in literature (Tryland,1998; Lederberg, 1950) that *E.coli* produces β-D-galactosidase and one could suspect that glycoside hydrolase activity comes from impurities in prepared protein fraction. According to the SDS-PAGE image (Figure 4.2.2.), enzyme preparation of FF52_18088 contains significantly more extrinsic bands than that of AlkimDRAFT_0871. That could explain the fact that FF52_18088 has higher release of pNP from pNP-β-galactopyranoside on tested pHs. It is preferable to test the unbound fraction from purification step to determine if the same activity can be detected on *E.coli*- derived enzymes.

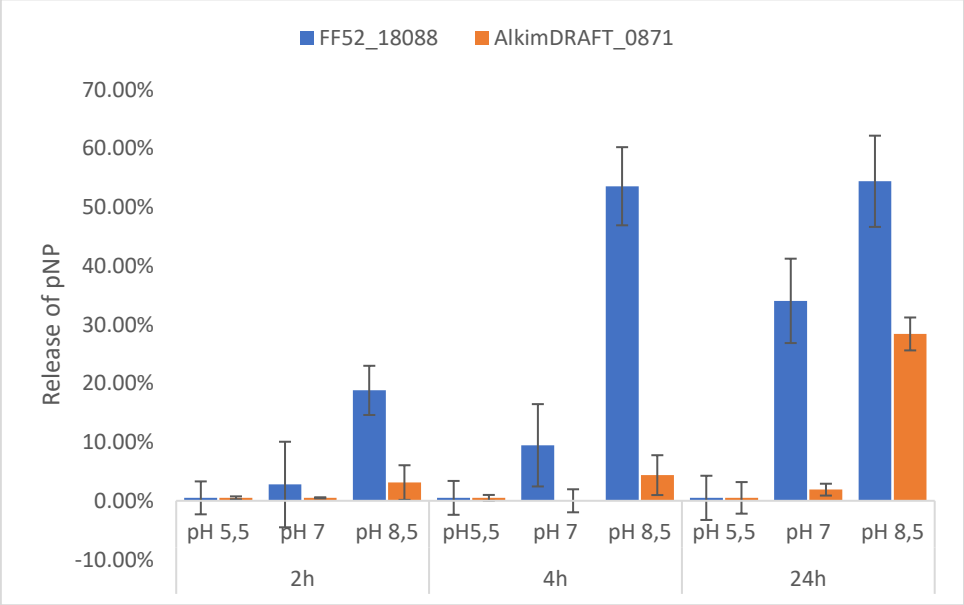


Figure 4.3.4. pNP release from pNP-β-galactopyranoside in incubations with FF52_18088 and AlkimDRAFT_0871. Reactions were done in 200µl volume with concentration of pNP-acetate 1mM for pH 5.5 and 7.0 and 0.3mM for pH 8.5 and enzyme dose of 10% per gram dry matter substrate. Release was calculated in % to theoretical maximum (n=3, error bars represent standard deviation).

In order to obtain a general knowledge of an overall hydrolytic activity of FjoAcXE-homologues they were additionally tested by performing PAHBAH reducing end assay on a broad variety of substrates and three pHs – 5.5, 7.0 and 8.5. All in all 17 substrates were tested (Table 3.1.3). Reducing ends were measured with 4-Hydrobenzoic acid Hydrazide that creates bis-hydrazones with fragments of sugars from hydrolysed polysaccharides implementing a complex releasing colour (Sinnott 2013). The amount of reducing ends was estimated upon the difference between absorbances of enzyme incubation with substrate blank, which then was compared to absorbance of enzyme blank. This experiment revealed that only minor levels of released xylose and hexose (reducing ends) were detected on some substrates on whole range of tested pH. General pattern that can be observed is that reducing ends have been determined on pullulan incubations with all of the enzymes. It is reported in literature that acetyl xylan esterases facilitate hydrolysis of acetylated substrate by increasing the accessibility of the substrate to hydrolases, but it has not been proven that AcXE have ability to hydrolyse polysaccharides themselves. It is important to mention that standard deviation in this method is rising with the pH; moreover, sensitivity of the assay starts from 0.010 absorbance difference (https://secure.megazyme.com/files/Booklet/K-FRUC_DATA.pdf).

Taking into account above mentioned facts following interpretation can be suggested. On the one hand, presence of other bands than corresponding recombinant protein bands in pure protein fractions on SDS-PAGE reveals remained impurities from the expression host. On the other hand, it is very likely that a very small absorbance differences that were detected can be caused by standard deviation, especially on pH 7.0 and pH 8.5. Therefore, at first, it is suggested to test the unbound fraction containing proteins coming from host organism obtained at purification step. Secondly, to repeat the experiment with increased enzyme dose on the substrates where reducing ends were detected.

4.4. Effect of pH and temperature

4.4.1. Minimal enzyme concentration

To ensure reliable calculations when determining pH optimum and temperature stability, these assays were done in linear range of enzyme activity. Therefore, minimal enzyme concentration was determined by plotting a curve showing an increase in enzyme activity on 4MU-acetate depending of enzyme concentration (Figure 4.4.1.).

Based on the results of assays on pNP- and 4MU-linked substrates, minimal enzyme concentration assay was done in HEPES buffer pH 7.0, as the enzyme activity in both of these assays was the highest at those conditions. Generally, enzyme activity curve looks similar with all homologues, giving a saturation of the enzyme activity at enzyme doze of 0.5% per gram dry matter substrate in case of K392DRAFT_2214, EW79DRAFT_2436, T426DRAFT_00687 and WP_035683315.1 and 0.2% in case of FF52_18088 and AlkimDRAFT_0871. Based on the activity curve, 0.1% per gram dry matter substrate was chosen as a minimal enzyme concentration for proteins FF52_18088, EW79DRAFT_2436, T426DRAFT_00687, WP_035683315.1 and AlkimDRAFT_0871 and 0.15% per gram dry matter substrate was chosen for K392DRAFT_2214 due to a very low product release at 0.1% (Figure 4.5.1).

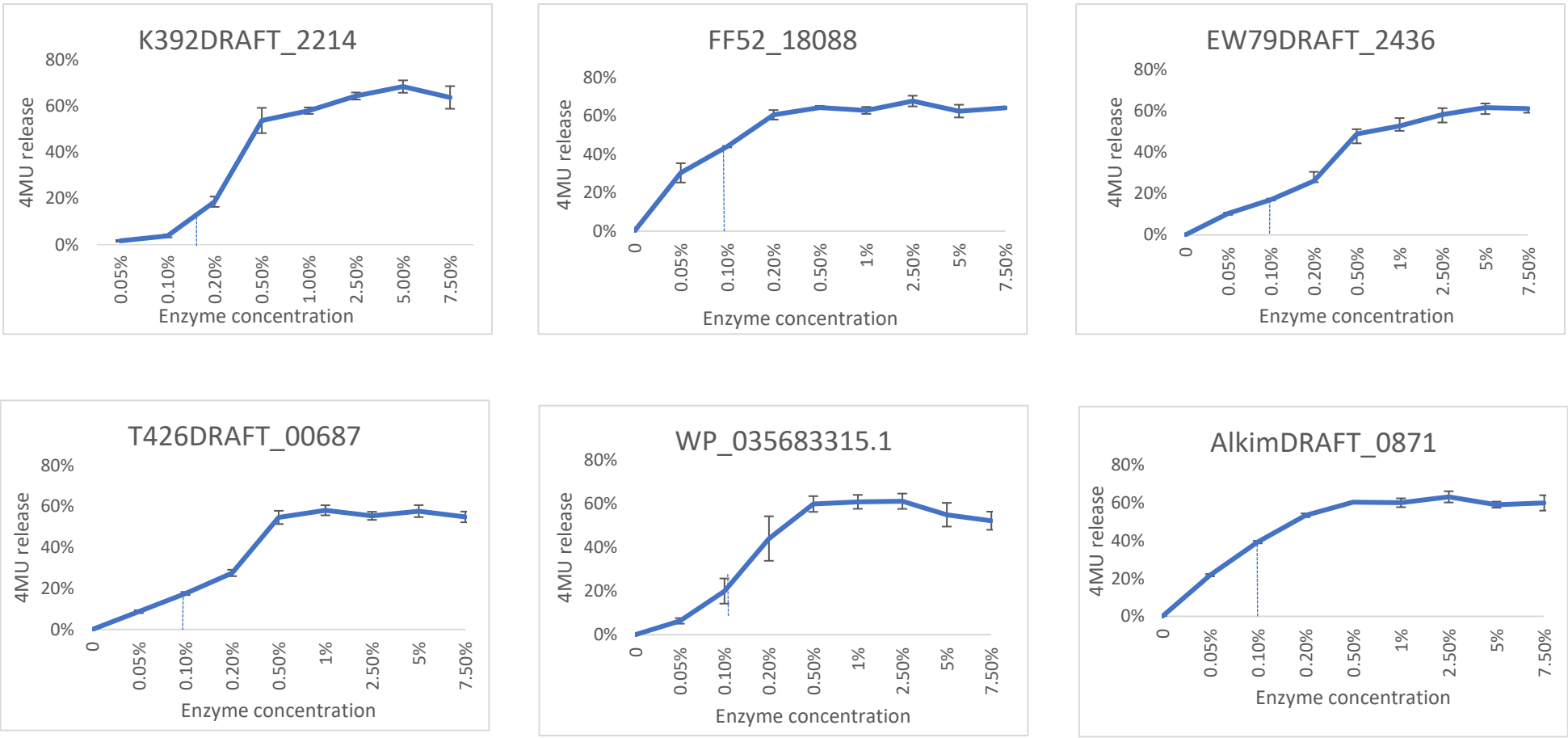


Figure 4.4.1. Saturation curve of enzyme activity in relation to increase of enzyme doze determined for FjoAcXE-homologues. In 200 µl reaction volume 0.5 mM 4MU-acetate was incubated for 30 min with enzyme in concentration range 0.05-7.5% per gram dry matter substrate. Intermittent line shows chosen minimal enzyme concentration for pH optimum and temperature stability assays (n=3, error bars represent standard deviation).

4.4.2. pH optimum

Optimal pH conditions were determined on 4MU-acetate and minimal enzyme concentration described above. A broad range of pH was tested with 1,0 pH unit step from 3.5 to 5.5 and from 8.0 to 9.0 and smaller steps in the range of predicted pH – 0.5 pH unit step from 5.5 to 8.0. All FjoAcXE-homologues show activity on the whole tested pH range (Figure 4.4.2). K392DRAFT_2214, FF52_18088, T426DRAFT_00687 show highest activity from 6.0 to 7.5; WP_035683315.1 and AlkimDRAFT_0871 have their optimum from pH 5.5 to 7.5, whereas EW79DRAFT_2436 has more narrow pH optimum between 6.5 and 7.0.

It was expected that homologues would share same range of pH that is optimal with FjoAcXE, especially K392DRAFT_2214, FF52_18088 and WP_035683315.1 that derive from the same genus of microorganism - *Flavobacterium sp.* However, obtained results slightly differ from the FjoAcXE pH optimum determined in F.M. Razeq work. There FjoAcXE showed more distinct pH optimum at pH 8.0. In contrast, K392DRAFT_2214, FF52_18088 and WP_035683315.1 show a broader pH optimum. It is also interesting, that pH optimum of K392DRAFT_2214 that shares maximal sequence similarity with FjoAcXE also significantly differs from that of FjoAcXE. According to obtained results, no correlation between sequence similarity to FjoAcXE and optimum of pH was found.

In literature, bacterial acetyl xylan esterases are not characterised as well as fungal. Although, it was reviewed in literature that, for example, AcXE from *Fibrobacter succinogenes S85* also has wide optimal pH range, retaining about 70% of relative activity in range of pH between 6.0 and 8.0 (McDermid et al, 1990). Moreover, wide optimal pH range that was shown by FjoAcXE-homologues is also similar to characterised in literature GDSL-lipases, SGNH-hydrolases, for instance, Alr1529 from *Anabaena sp*. (Bakshy et al. 2008) and AlipB from Anaerovibrio lipolyticus 5ST (Prive et al. 2013).

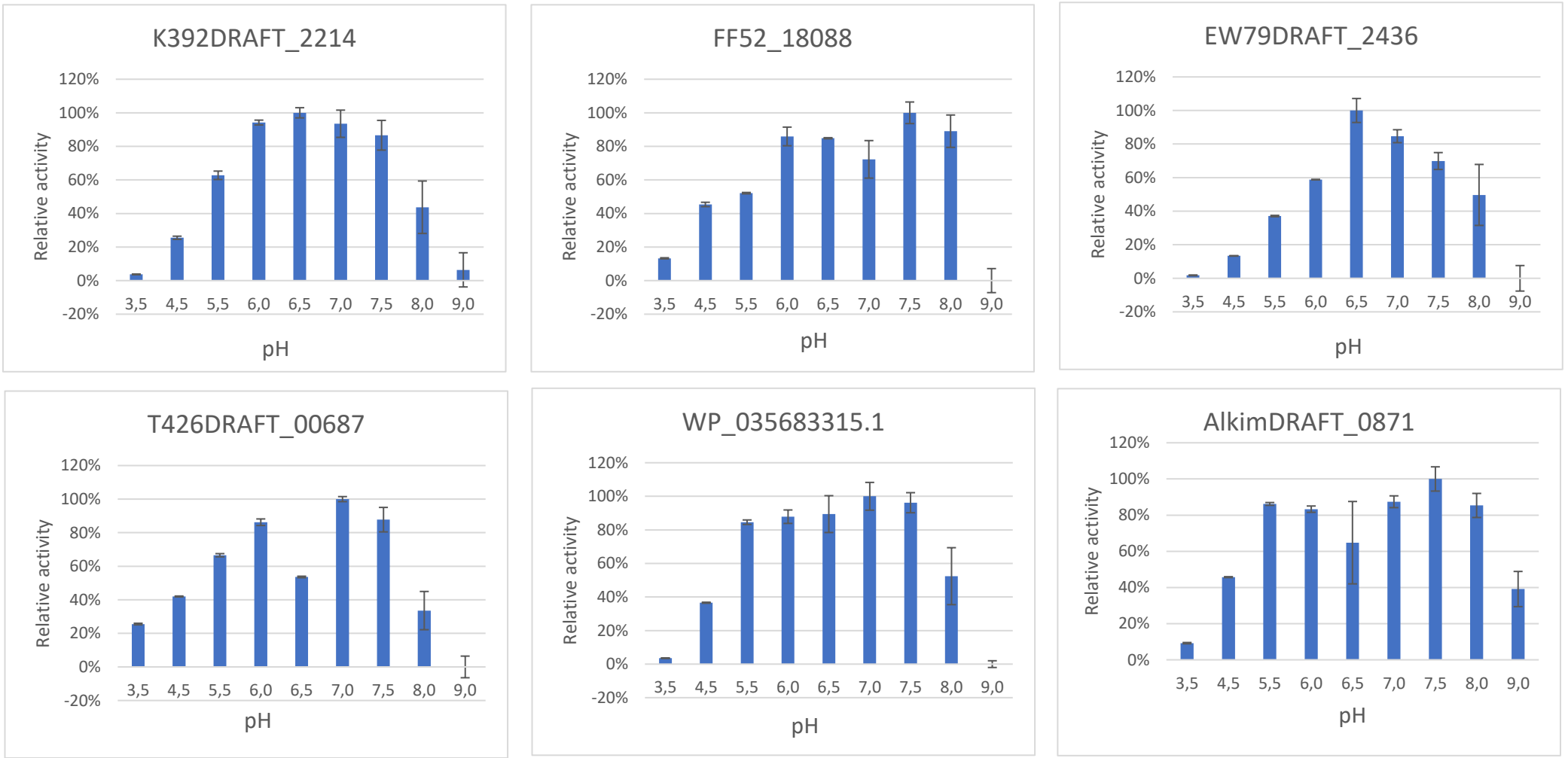


Figure 4.4.2. Change of relative enzyme activity of FjoAcXE-homologues according to pH change. In 200 µl reaction volume 0.5 mM 4MU-acetate was incubated for 30 min with enzyme concentration of 0.1% per gram dry matter substrate (0.15% for K392DRAFT_2214) and 50 mM universal buffer on pH range from 3.5 to 9.0 (n=3, error bars represent standard deviation).

4.4.3. Temperature stability

Results of temperature stability test showed FjoAcXE-homologues are most stable at between 20˚C and 40˚C (Figure 4.4.3.). At 20˚C K392DRAFT_2214, FF52_18088 and WP_035683315.1 lose 50% of their activity between 6 and 24 h, whereas AlkimDRAFT_0871 loses 50% already between 2 and 4 h incubation time. On the other hand, two of the homologues, T426DRAFT_00687 and EW79DRAFT_2436, show higher stability in comparison to other proteins: remaining 98 and 63% of relative activity respectively. That corresponds with results of deacetylating 4MU-acetate after 24 h incubation (Figure 4.3.4), where T426DRAFT_00687 and EW79DRAFT_2436 showed highest release compared to other homologues. It is interesting that K392DRAFT_2214, FF52_18088 and WP_035683315.1, that derive from *Flavobacterium sp.,* give close to each other temperature stability curves at 20˚C with an increase in relative activity after 30 min incubation.

Relative activity curves at 40˚C mimic those of 20˚C, where K392DRAFT_2214, FF52_18088 lose 50% relative activity already after 6 h. Lowest stability at 40˚C was detected with WP_035683315.1 (remaining 50% of relative activity between 4 and 6 h) and AlkimDRAFT_0871(loosing 50% of relative activity already between 1 and 2 h). Highest stability at 40˚C was detected with T426DRAFT_00687 and EW79DRAFT_2436, similarly as at 20˚C, retain more than 50% of relative activity after 24 h.

Raising the temperature to 55˚C significantly decreases enzyme stability. Relative activity of AlkimDRAFT_0871 plunges to 30% already at first time point – 30 min, proteins K392DRAFT_2214 and WP_035683315.1 show decrease to 50% of relative activity between 30 min and 1h incubation at 55 ˚C, similarly, EW79DRAFT_2436 retain 50% activity between 1 and 2 h incubation, whereas T426DRAFT_00687 and FF52_18088 lose 50% activity between 4 and 24 h.

As expected incubation of proteins at 70˚C gave no detected activity already after 30 min incubation, which provides evidence to protein degradation.

FjoAcXE showed significantly higher temperature at 20 and 40˚C stability than homologues retaining 100% of its activity after 16h of incubation, K392DRAFT_2214 despite very high similarity does not give a corresponding with FjoAcXE temperature stability.

Obtained results can be related to previously discussed enzymes belonging to GDSL/SGNH subfamily Alr1529 from *Anabaena sp*. (Bakshy et al, 2008) and AlipB from *Anaerovibrio lipolyticus* 5ST (Prive et al, 2013) showing relatively low temperature stability. AlipB losing more than 50% of activity after 1h incubation at 50˚C and Alr1529 retaining about 10% of activity after 2,5h incubation at 50 ˚C.

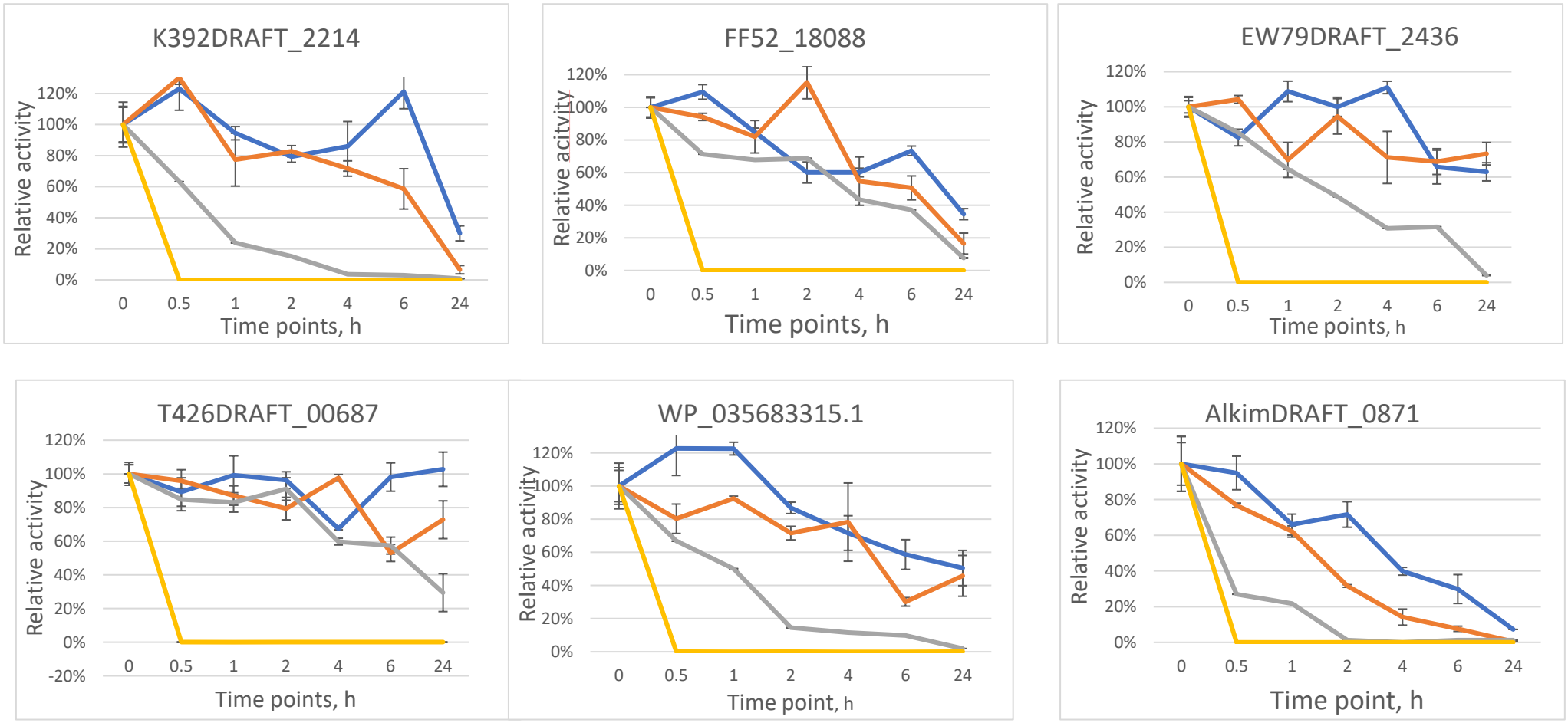


Figure 4.4.3. Temperature stability results. Prior to determination of activity enzyme was incubated in 50 mM Universal buffer, at pH 6, at 4 temperature points (20, 40, 55 and 70 ˚C) and seven time points (0, 30 min, 1, 2, 4, 6 and 24 h); In 200 µl reaction volume with 0.5 mM 4MU-acetate was incubated for 30 min with final enzyme concentration of 0.1% per gram dry matter substrate (0.15% for K392DRAFT_2214); n=3, error bars represent standard deviation.

4.5. Secondary screening

4.5.1. Activity on complex natural and synthetic substrates

GDSL-family esterases are characterised in literature as enzymes with wide substrate specificity and broad regioselectivity. Thus, it was of great interest to test activity of the FjoAcXE-homologues on more branched and complex substrates.

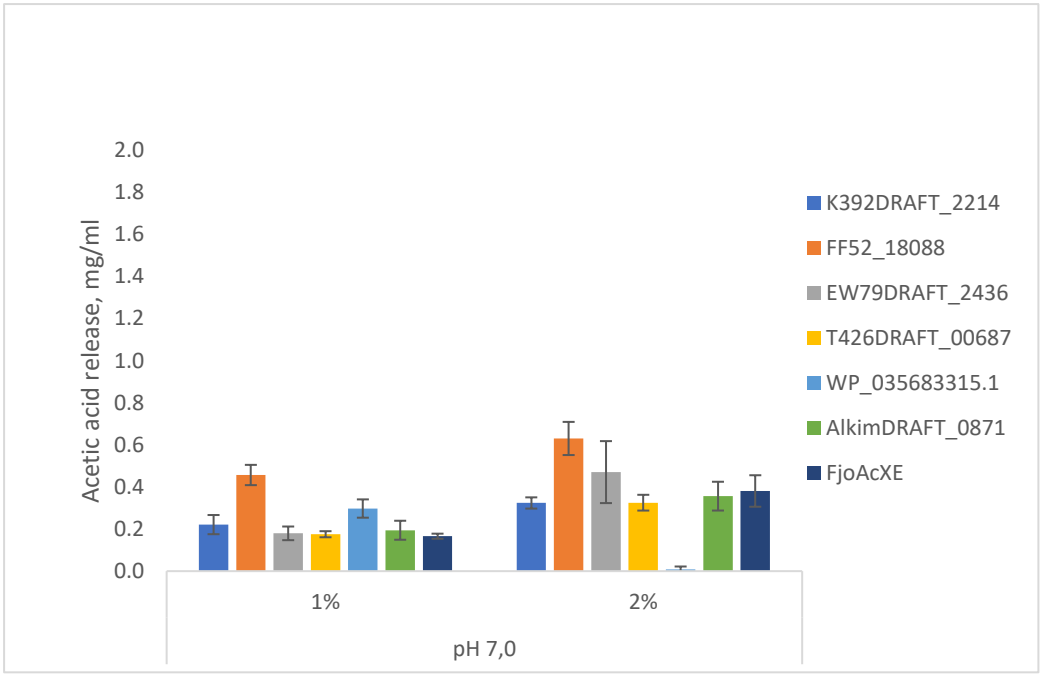
Thus, in order to estimate how steric hindrance affects the activity of enzymes, FjoAcXE and FjoAcXE-homologues were tested on mixture of per-acetylated xylo-oligosaccharides (acetic acid substitutes in positions 1-,2- and 3-O) with a different degree of polymerisation from 4 to 7 (Figure 4.5.1 and Figure 4.5.2) and on corn fibre (Figure 4.5.3 and Figure 4.5.4). Release of acetic acid was tested with acetic acid kit, incubation times for xylo-oligosaccharide mixture were 20 min, 4 h and 20 h incubation time and 20 and 4 h for corn fibre fraction.

Reactions with per-acetylated xylo-oligosaccharides were performed for 20 min at pH 7.0 and pH 8.0 (Figure 4.5.1.). Two pH points were used due to the fact that previous experiments with FjoAcXE-homologues has shown highest activity at pH 7.0, but at the same time, in Razeq work FjoAcXE had shown optimum at pH 8.0.

Release of acetic acid was detected for all proteins at both tested pHs. There can be seen an increase in the amount of released acetic acid with the increase of pH and, similarly, with the increase of the enzyme dose. Release of acetic acid varied within tested proteins, with highest release of 0.630 mg/ml detected at pH 7 with 2% w/v of FF52_18088. A closer look at the data indicates that K392DRAFT_2214 that shares 98.1% of sequence similarity with FjoAcXE gives approximately the same release of acetic acid as FjoAcXE in comparison to other homologues.

Data obtained after 20 min incubations at pH 8 goes in correspondence with pH optimum described above. T426DRAFT_00687 retained only 20% of its relative activity at pH 8 and showed lowest release of acetic acid among all tested proteins (Figure 4.5.1. B).

A



B

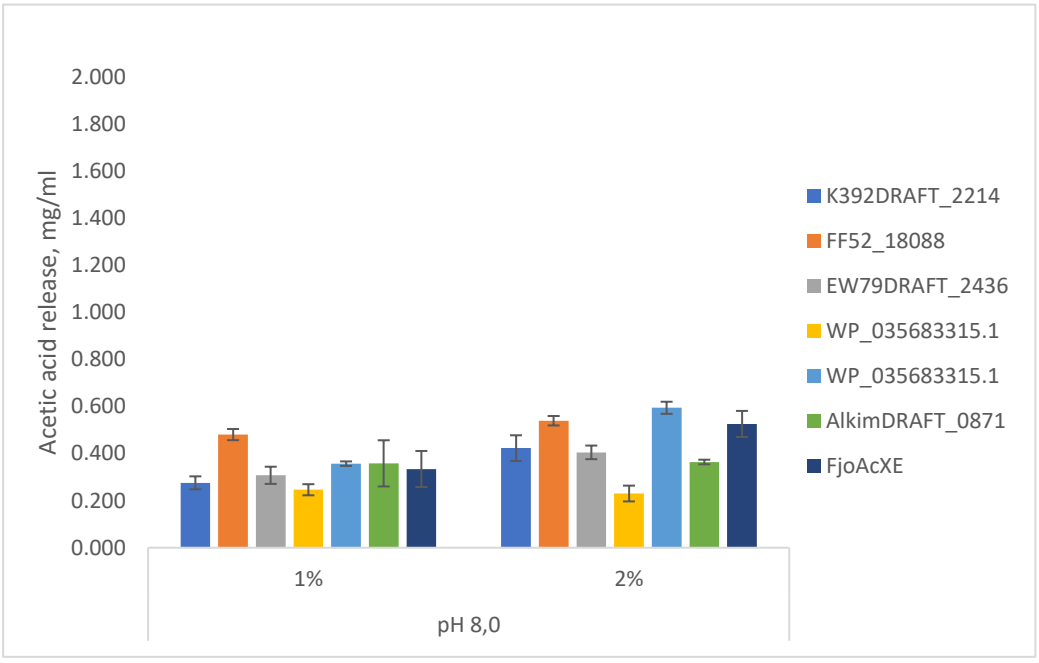


Figure 4.5.1 Acetic acid release detected with acetic acid kit after 20 min incubation with per-acetylated xylo-oligosaccharide mixture. A. Acetic acid release measured on 1 and 2 % w/v enzyme dose incubated with substrate in HEPES buffer at pH 7. B. Acetic acid release measured on 1 and 2% w/v enzyme doze incubate with substrate in HEPES buffer, pH 8 (n=3, error bars represent standard deviation)

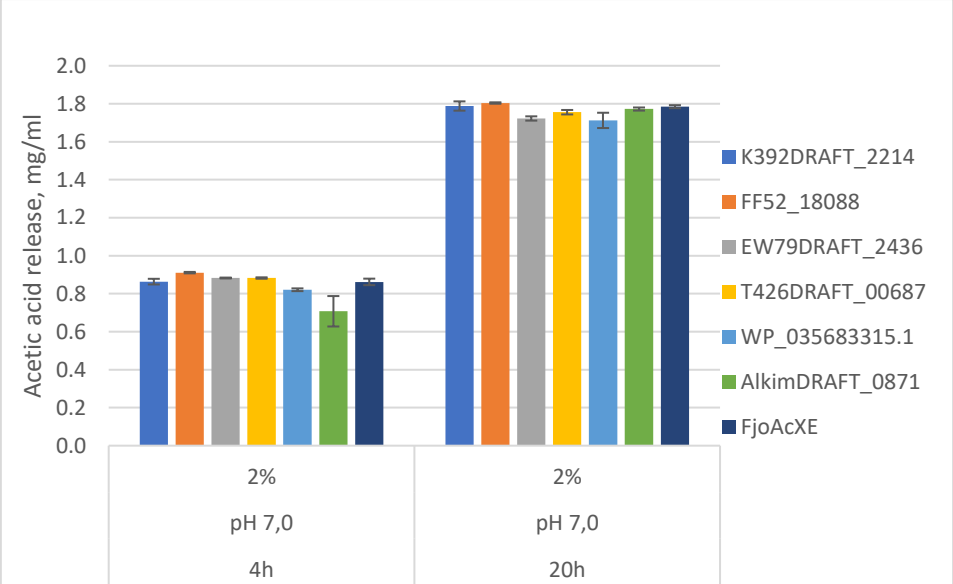


Figure 4.5.2. Acetic acid release detected with acetic acid kit after 4 and 20 h incubations with per-acetylated xylo-oligosaccharide mixture. Acetic released was measured on 2% w/v enzyme incubated in HEPES buffer pH 7(n=3, error bars represent standard deviation).

Incubations with per-acetylated xylo-oligosaccharides were also done for 4 and 20h (Figure 4.5.2.). Incubations were done with 2% w/v enzyme concentration and pH 7.0. Maximum of released acetic acid is detected after 20 h incubation giving almost three times higher values and 4 h incubation giving two times higher values compared to 20 min incubation with the same buffer and enzyme concentration. Given that theoretical maximal yield of acetic acid was calculated to be 3 mg/ml, after 20 h incubation FjoAcXE-homologues release on average 60% of expected theoretical maximum of acetic acid and after 4 h this number is 20%. Interestingly, while after 20 min incubation the release slightly differs, after 20 h incubations FjoAcXE and homologues give approximately the same acetic release from the substrate. These experiments show that FjoAcXE-homologues have activity that closely resembles activity of FjoAcXE.

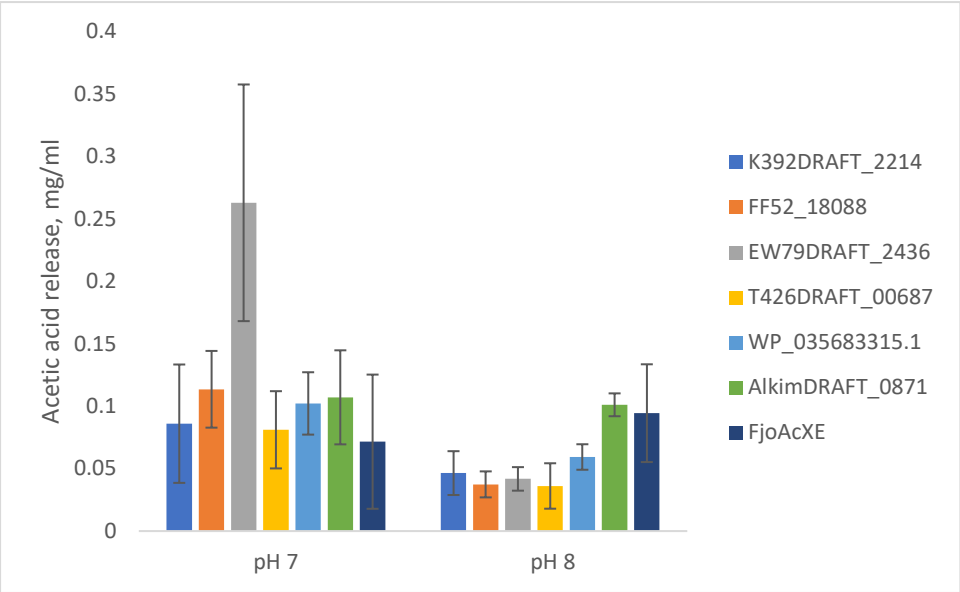


Figure 4.5.3. Acetic acid release detected with acetic acid kit after 20 min incubations with xylo-oligosaccharides from corn fibre. Acetic released was measured on 1% w/v enzyme incubated in 50mM HEPES buffer pH 7 and pH 8 (n=5, error bars represent standard deviation).

FjoAcXE together with homologues also showed a release of acetic acid after incubation with xylo-oligosaccharides from corn fibre (Figure 4.5.3 and Figure 4.5.4). Generally, detected release of acetic acid from corn fibre is poorer than from xylo-oligosaccharide mixture. Interestingly, with increase of pH from 7.0 to 8.0 acetic acid release from homologues K392DRAFT_2214, FF52_18088, T426DRAFT_00687 and WP_035683315.1 slightly decreases. Surprisingly, it significantly decreases for EW79DRAFT_2436. Even though relative activity of EW79DRAFT_2436 decreases from 80% to 50% with increase in pH from 7.0 to 8.0 (Figure 4.4.2.), this pattern was not reproduced in incubations with per-acetylated xylo-oligosaccharides (Figure, 4.5.1). Therefore, it is possible that such substantial decrease in acetic acid release is related to large standard deviation. Acetic acid release of AlkimDRAFT_0871 remains on the same level. It is likely related to AlkimDRAFT_0871 pH optimum curve that is shifted to higher pH in comparison with other homologues (retained about 40% of its activity at pH 9.0). As was expected FjoAcXE releases more acetic acid on optimal pH 8.0 than on pH 7.0.

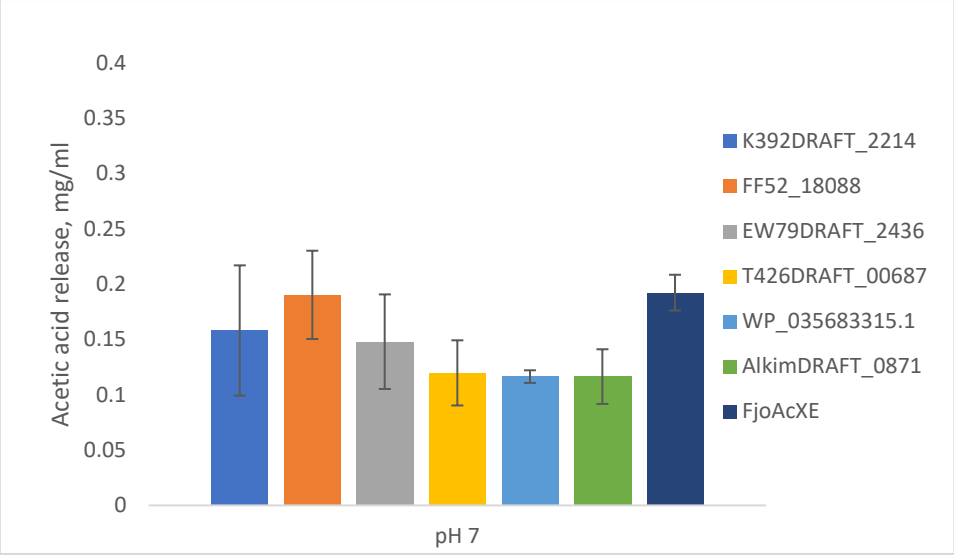


Figure 4.5.4. Acetic acid release detected with acetic acid kit after 4 h incubations with xylo-oligosaccharides from corn fibre. Acetic released was measured on 1% w/v enzyme incubated in 50mM HEPES buffer pH 7.0 (n=3, error bars represent standard deviation).

Longer incubation time gives increase in release: two times higher values of the acetic acid release after 4 h incubation in comparison to 20 min incubation at the same pH. Theoretical maximum of acetic acid release was calculated to be 1,2 mg/ml. Therefore, FjoAcXE and FF52_18088 both release only around 16% of acetic acid from theoretical maximum after 4 h incubation. Minimal obtained release was 10% for proteins WP_035683315.1 and AlkimDRAFT_0871.

Performed experiments give an understanding that even complex substrates are degraded by FjoAcXE and homologous to it proteins. Obtained results prove that FjoAcXE-homologues also show acetyl xylan esterase activity. However, it is notable, that FjoAcXE-homologues give a substantially lower acetic acid release from substrate that contains adjacent ferulic acid in comparison to complex per-acetylated substrate. Steric hindrance of a substrate is likely to decrease the efficiency of deacetylation performed by studied proteins, therefore, it is advisable to study longer incubation time with corn fibre fraction in order to estimate if acetic acid release increases further with reaction time.

5. Conclusions

All in all, eight FjoAcXE-homologues that shared from 47 to 98% sequence similarity with FjoAcXE were chosen for examination in this work. All of the homologues contained GDSL-lipase and SGNH-hydrolase conserved residues and were uncharacterised. Three of eight homologues K392DRAFT_2214, WP_035683315.1 and FF52_18088 derived from *Flavobactrium sp.,* same genus as FjoAcXE.

In total of six homologues were expressed in *E.coli* (K392DRAFT_2214, FF52_18088, EW79DRAFT_2436, T426DRAFT_00687, WP_035683315.1, AlkimDRAFT_0871). Two of preselected candidates, ZPR_3026 and CHSO_3300, were not expressed after several attempts of cultivation in Magic Media or LB Media, likely, due to defected protein encoding sequence.

In this work, proteins were purified only using one step of purification, therefore, pure fractions contained extrinsic bands, detected on SDS-PAGE gel. Those bands could affect the results in primary screening (activity detected pNP-β-galactopyranoside and presence of minor level of reducing ends). Therefore, for further characterisation it is recommended to use second-step purification (for example, size exclusion chromatography, that will help to separate proteins in HPLC fraction according to molecular weight and prevent presence of *E.coli* derived proteins in pure fraction).

In this work pH optimum and temperature stability were determined for the studied FjoAcXE homologues. Results has shown that there was no correlation between sequence similarity to FjoAcXE and optimum of pH; FjoAcXE-homologues possess broader optimal pH range than that of FjoAcXE. At the same time pH optima results resemble that of proteins that belong to GDSL/SGNH subfamily. Generally, temperature stability of FjoAcXE-homologues was highest at 20 and 40˚C, decreasing significantly at 55˚. No activity was detected after incubation at 70˚C.

According to the results presented in this thesis, it can be concluded that FjoAcXE - homologues showed their ability to deacetylate complex per-acetylated substrates giving a release of acetic around 60% of theoretical maximum after incubation for 20h. Furthermore, all homologues showed release of acetic acid from corn fibre fraction containing complex of highly branched feruloylated xylo-oligosaccharides. Release of acetic acid from complex substrates was similar to that of FjoAcXE and, therefore, homologues were proven to act as

acetyl xylan esterases. However, it is not yet possible to conclude that FjoAcXE and its homologues present a novel carbohydrate esterase family, as in this thesis, secondary screen was performed using only colorimetric methods of detection, it was not possible to determine at what positions deacetylation takes place.

Summarizing results listed above, it can be advised to include following considerations in further research:

1) Results in this work show that studies enzymes partly deacetylate complex substrates, but do not give a thorough understanding about the positions from which acetic acid is predominantly cleaved. In that perspective, it is recommended to perform NMR spectroscopy of reactions used in secondary screen. In addition, in order to prove the unique activity, FjoAcXE-homologues also has to be tested on glucuronoxylan. It is likely that FjoAcXE-homologues have broad substrate specificity also towards other branched xylans, therefore, it could be also tested on other branched polysaccharides, for instance, acetylated glucuronoarabinoxylan from sugarcane fraction.

2) In this work biochemical characterisation was stopped after determination of pH optima and temperature stability. Further steps can include determination of enzyme kinetics and determination of 3D- and crystal structures to understand their mechanism of catalysis and to detect the structural similarities to other carbohydrate esterase families.

3) FF52_18088 and AlkimDRAFT_0871 should be purified using second step of purification and then tested on pNP-β-galactopyranoside and lactose in order to confirm side-activity.

4) Results of this thesis showed that FjoAcXE-homologues are able to deacetylate highly substituted fractions corn fibre. Next step could be to see synergistic interactions of FjoAcXE-homologues with other enzymes working on following degradation of this substrate, for example, incubations together with members of GH families.

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Table A.1. FjoAcXE-homologues summarised information

Name	K392DRAFT_2214	FF52_18088	EW79DRAFT_2436	T426DRAFT_0067	WP_035683315.1	AlkimDRAFT_0871	CHSO_3300	ZPR_3026
Accession number	WP_073411463.1	WP_008467835.1.	WP_047423182.1	WP_027586161.1	WP_035683315.1	WP_044117739.1	WP_045502788.1	WP_013072442.1
Microorganism	Flavobacterium sp. 40S8	Flavobacterium sp. F52	Chryseobacterium sp. YR480	Prolixibacter bellariivorans	Flavobacterium reichenbachii	Alkaliflexus imshenetskii DSM 15055 AlkimDRAFT_0871	Chryseobacterium sp. StRB126	Zunongwangia profunda SM-A87
Amino acid number	414	411	406	404	410	413	415	423
Signal sequence	+	+	-	+	+	+	-	-
Pfam domain	PF13472							
Original amino acid sequence	MNVKIKSVKWIVLLNLLFVMAGCTSNKALIKTGEKKETQNWTGTWATAQMLVEPKNMPPEPGLAQNTLRQIIKVSLGGKQIRLRFSNLFSDQPAVLKSVSVANVTEAPAVDIKTQKILSFKGSPQVTLGADEVVYSDAFAFELQP	MIKRYLKYILLLQLFTLILGCKTNQPLANSSEKTKAENWVGSWACAPMLVEPNNMPPAPGLAENTLRQIIKVSIGGEHIRLRFSNLFSDQPTVLKKVSIANVLEAPAIDKNTQAIDKNTQKNLSFNGNQEVTLN	MRFKSLYIFIISGFLVSCSSSQSLEKQSSKGQWLTTWATAPQLVEPKNLPPEPGLSGNTLRQIVRVSVGGKKLRLRFSNKYSMDSLAVKAVSIAVPSDSSNVDAATIRSLTFEKKNNFKIAPGSDIYSDEVNFNLKPNSLLAITVSYAKVTQSVTGHPASRTTSFIVKGEQTNAEVFKNPVKTDHWYSLFNIDVKTSEPSYAVAIMGNSIT	MKRLILIMAVLLTTGTTAISQNKTTASKSWVGTWATAPQLVEPRNMPPAPGLTNSTLRQVVCVSIGGKQLQFRFSNRFSKSPVTMKTVHIAVSKGGSEIEPSTSKELTFNGQPDVTMEPGKAVISDPISFNLKPRMLVAITISFGETSPDVTGHPGSRTTSYLLAGDQSSPDADFSQAVKTDHWYVINGIDLMAQKRAAAIAILGNSI	MLRRFFFKSLLLLKTLLFIILTGACNKSLRQVVRVSIGGSLRQVVRVSIGGGTWATAPQLVEPHNMPPAPGLTNNGTVISDPVAFHLKPRMDVAITIYYGETSETVTGHPYGETSETVTGHPRWYNINRIDVLAPASAANVAILGN	MNLKRRYIKSIVLLSFLFLIAGCKSSSSVSKKDHQNWTGSWASAQMLVEPQNMPPSPGLAQHTLRQIIKVSLGGKRIKLRFSNLFSDVSVGGKKLRLRLSNKYSVDSLAVKAVSIAVPSDSSNVVIDAPVIDPKTQKMLLFNGNSEITLETGGHTIDISTNKEVIDAPVIDPKTQKMLLFNGNSEITLETFDGASNLTMEPGQEVFSDAFDFELQPGQLLAITIHELQPGQLLAITIHYGEVSSKVSGHPLAITISYAKVTQSYGEVSSKVSGHPVTGHPGSRTTSFVVKGEQTNAMVNNNASFAGAVKTADNDEVNVVCL	MRKNEKTEKMRFKNLYIFIISGFLVSCSSSQSLAKQSSKDRWLTTWATAPQLVEPKNLPPEPGLSGNTLRQIVRVSVGGKKLRLRLSNKYSVDSLAVKAVSIAVPSDSSNVDAASIRSLTFEKKNNFKIAPGSDIYSDEVNFNLKPNSLLAITIHDEVSSKVSGHPLAITISYAKVTQSVVKGEQTNAMVFKNPVKTDHWYSLFNIDVKTGEPSYTDHWYIINTLDV	MSRHLKYRFVKGFIGKRNVIFVLFLMLLASCGTSSSIVQNDSKNQQKWVGSWSTAPQLVEPRNVPPAPGLTGNTLRQIIRPSLGGKSIRLKFSNAFSDLPVEMISVEIAIVTEQPLVKTSTVKTLKFNGSPEVTMPAGEAVFSDALAFEFEASDRLAITIKFGKTAEKITGHPGSRTTSYLVEGNENIKENAFENAITTDHWYIINTLDV

	GQLLAITIH YGEISSNVS GHPGSRTTS YILQGDHIN NESFTGAV KTDHWYSI MGVDISSA KNESNVVC LGNSIIDGR GSGTNKQN RWTDILASR LNANKNTA HIGVLNLGI GGNCVVRG GLGPTALN RFDRDVLS QSGTKWLII LEGINDIGGI RKAEDASA AAKEIIEGY KVMIDKAH AKGIKVFG GTILPFEKSF YDAPFKQD ARDIVNEWI RTKGNFDA VIDFDKAM ASGVGSKTI LPDMHDND FLHPNEKG YQRMGETV DLDLFQ	PGQELFSD KLNFQLK QGQLLAIT IYYGAASP RTTGHPGS RTTSYILS GDNINND VFSDAVK TDHWYSI VGVDVVA SKKASNIV CLGNSITD GRGSGTN KQNRWTD ILAARLAA DKATQNI GVLNLGIG GNCVVKG GLGPTALE RFDRDVL SQTGTKW LLILEGIN DIGGIKRA EDAPVKA QEIIDAYK IMIEKAHA RGIKVYG CTILPFQK SFYDAPFK QEARDLV NAWIRNN HFDAVIDF DKEMASE TDSKTILS NMHDGDF LHPNELG YQRMGEV IDLNLFK	DGRGSGTNRQNR WPDIFSQRLLANP STRNISVLNLGIG GNCVVRGGLGPT ALDRFDYNILNQ QGVKWLIILEGV NDLGGTRDPDDA SKRTEELIAAYQ VMIDKAHANGIK VYGATILPFGKSF YEKPFRIEEWKK VNDWIRNSGKFD AVIDFAKHMQSE NPEVILNDMHDH DFLHPNEAGYRR MGEFVDLNLFKN	TDGRGSGTNKQD RWPDELALRLLK NKRTRDIGVLNM GIGGNCVLHGGL GPTALSRFNRDIL KQHGVRWLIIFE GVNDIGGTPDKE AADKVAQGLIAA YDKMIDEAHAK GIKVYGGTITPIK KSFYYKDYRETA RQTVNKWIRTSG HFDAVIDFDKAM RNPKDTLTLRPE AQSGDYLHPNEL GYRIMAGAIDLS LFK	SITDGRGSNTDK QDRWPDILSEILL KNSETEQIGVLN MGIGGNAVLAG GLGPTGLSRYER DILNQPGTKWAII YHGVNDIGGVRT EQAAIDKADALIE AYKKMISEARER NITVFGATILPFK GNSYYNEHSEKC RETVNDWIRNSG WYDAVIDFDKV MQSPEDPAKLVS TFQNDGLHPDAE GYLKMGSSVDIN LFINTKK	GNSITDGRGSGT NKQNRWTDILAA RLNADKSTRHIG VLNLGIGGNCVL KGGLGPTALDRF GRDVLSQAGTK WLIILEGINDIGGI KRAEDAPIRANE LIEAYKVMIEKA HSKGIKVYGCTIL PFQKSFYDAPFK QEARDIVNNWIR SSKNFDAVIDFD KIMTSEIGSKTISS NLHDGDFLHPNE LGYLTMGQAIDL NLFK	AVAIMGNSITDG RGSGTNRQNRWP DIFSQRLLANPST RNISVLNFGIGGN CVVRGGLGPIAL DRFDYNILNQQG VKWLIILEGVND LGGTRDPDDASK RTEELIAAYQMM IDKAHANGIKVY GATILPFAKSFYD KPFRIEAWKKVN DWIRNSGKFDAV IDFAKQMQSENP EVILNDMHDHDF LHPNEAGYKRM GDFVDLNLFKN	MAGKEASAVAIL GNSITDGRGSGIN KQNRWPDILAER LIQHSRTKNIGVL NMGIGGNAVLRG GLGPTGLNRFDR DILNQSSIKYLIIL EGVNDLGATPDS TAAFKVANKLIE AYKTMIKKAHA QNIKVYGGTITPI QKSFYYKDFREQ ARQKVNNWIRNS GAFDAVIDFAKV LESPEEPNVIAKN LQSGDYLHPNEK GYEVMGEAVDL SLFKK

Table A.2. FjoAcXE information

Name	FjoAcXE
Accession number	ABQ06890.1
Microorganism	Flavobacterium johnsoniae UW101
Amino acid number	414
Signal sequence	+
Pfam domain	PF13472
Original amino acid sequence	MNVKIKSVKWIVLLNLLFVMAGCTSNKALIKTGDKKEDQNWTGT WATAQMLVEPKNMPPEPGLAQNTLRQIIKVSLGGKQIRLRFSNLFS DQPAVLKSVSVANVTEAPAVDIKTQKILSFKGSPQVTLGADEVVY SDAFDFELQPGQLLAITIHYGEISSNVSGHPGSRTTSYILQGDHINNE SFAGAVKTDHWYSIMGVDISSVKNESNVVCLGNSIIDGRGSGTNK QNRWTDILASRLNANKNTAHIGVLNLGIGGNCVVRGGLGPTALNR FDRDVLSQSGTKWLIILEGINDIGGIRKAEDASVTAKEIIEGYKVMI DKAHAKGIKVFGGTILPFEKSFYDAPFKQDARDIVNEWIRTKGNFD AVIDFDKAMASDVGSKTILPDMHDNDFLHPNEKGYQRMGETVDL DLFQ