Characterization of ulvan polysaccharide degrading enzymes from *Wenyingzhuangia fucanilytica*

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Popular science summary

Climate change and eutrophication of seas and oceans, due to the overfertilization of farmlands, has given rise to an increase in seasonal algal blooms know as green tides. One proposed way to combat this problem is to cultivate fast-growing macro algae, or seaweed as it is also called, in affected areas and/or close to estuaries emerging from agricultural lands. The cultivated algae can then be used in several different applications from human food and animal feed to industrial applications such as cosmetics. The algae also contain rare sugar molecules that are not commonly found in plants grown on land, sugar molecules which are believed to possess several health benefits such as reducing inflammation and promoting good intestinal bacteria and gut health. However, they are locked in long chains of repeating sugar molecules, called polysaccharides, and need to be degraded. The best way to perform this degradation is with the use of enzymes. Enzymes are proteins that help chemical reactions, such as the ones that break down polysaccharides, and they are found in every living organism. Every enzyme catalyzes a specific reaction so to get a certain result several enzymes need to be investigated.

Two of these enzymes that degrade polysaccharides from algae was investigated in this report. They were isolated from the chromosome of the bacteria *Wenyingzhuangia fucanilytica* which was found in fermenting algae in shallow coastal waters. To be able to analyze these enzymes they first needed to be produced. This is done by modifying the genes of another bacteria, *Escherichia coli*, which is a bacteria that is easy to cultivate and fast growing in laboratory conditions. The gene modified cells are then grown and harvested. From the cells, the enzymes are then purified and characterized confirming activity.

One of the studied enzymes, which catalyze the first reaction in breaking down the polysaccharides, was active on ulvan, which is a polysaccharide derived from green seaweed, and could be introduced to raw polysaccharide treatment obtaining products of high additional value. The other enzyme, a glucoside hydrolase which catalyze the last reaction in breaking down the polysaccharides, was producible in a soluble form and catalyzes the reaction into the rare sugar molecules that are also of biotechnological interest. Both characterized enzymes were active at temperatures close to room temperature and were most active at mildly acidic conditions. Further investigation will allow to test both enzymes in polysaccharide ulvan disruption processes.

Abstract

The aim of this thesis was to fully characterize the enzyme WFp-105, including bioinformatic analysis, structural modeling, full activity and stability characterization and product determination. As well as for the enzyme WFp-37; optimize protein production in regard to expression strains and cultivation temperature, analyzing the genomic context as well as performing an initial activity detection.

From the genome of *Wenyingzhuangia fucanilytica* two enzymes were cloned, one glucoside hydrolase termed WFp-105 and one polysaccharide lyase termed WFp-37 attributed to polysaccharide lyase family 37. Both were heterologously produced in *Escherichia coli* and purified using affinity chromatography. WFp-37 showed activity on raw ulvan in the crude cell lysate. However, it was prone to aggregation as pure enzyme. The glucoside hydrolase attributed to glycoside hydrolase family 105 by gene phylogenetic analysis and structural modeling. Recombinant WFp-105 was purified to high concentrations and remained soluble. Enzyme demonstrated activity on unsaturated oligosaccharides from ulvan in the temperature range 10-40°C and pH 5-7.5. WFp-105 activity optima corresponded to algae containing ulvan growth conditions. Hydrolysis product detection with thin layer chromatography as well as product profiling with high-performance anion exchange chromatography - pulsed amperometric detection confirmed generation of monosaccharides from unsaturated oligosaccharides, in particular xylose, as well as three other products that was not successfully attributed to any mono- or oligosaccharide. Further investigation is needed in order to fully investigate potential of studied enzymes to be implemented for biotechnological applications.

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Introduction

Green algae are found all over the world and grows in several different types of waters *i.e.*, fresh-, saline and brackish water. Macroalgae have for a long time been cultivated for human consumption all over south-east Asia and several other geographic regions (Kidgell *et al.*, 2019). The possibility to grow in saline and brackish waters poses as an opportunity for largescale marine agriculture, or mariculture, both in Sweden and worldwide (Salinas & French, 2017).

Algal blooms and green tides in eutrophicated waters are problems globally (Watson *et al.*, 2015). A solution for this could be the cultivation of fast-growing macro algal species in eutrophicated waters as well as in proximity to estuaries that dispense water from agricultural lands. This has the potential to keep the nutrients from fertilized lands out of seas and oceans (Tiwari *et al.*, 2017).

In recent years the use of algae has found new areas of research and technological innovation such as biofuel production and health and medicine. Green algae, such as algae from the genus *Ulva,* have cell walls that contain different types of branched polysaccharides. These are mainly cellulose, glucuronan, xyloglucan and ulvan. Ulvan, an irregularly branched polysaccharide is composed of disaccharides constructed of sulfated monosaccharides. These monosaccharides are believed to possess several health benefits such as anti-inflammatory, anti-cancer as well as prebiotic and other potential effects. To utilize ulvan it has to be degraded by enzymes to obtain the desired products. Microorganisms naturally fermenting seaweeds are valuable resources of ulvan-active enzymes. Organisms such as *Wenyingzhuangia fucanilytica* (Chen *et al.*, 2016). From the genome of *W. fucanilytica* the sequences of two potential ulvan degrading enzymes, AXE80_05965 and AXE80_06090, were isolated which will be analyzed in this thesis.

The aim of this thesis was to fully characterize the enzyme WFp-105 attributed to gene-sequence AXE80_05965, including bioinformatic analysis, structural modeling, full activity and stability characterization and product determination. As well as for the enzyme WFp-37 attributed to gene-sequence AXE80_06090; optimize protein production in regard to expression strains and cultivation temperature, analyzing the genomic context as well as performing an initial activity detection.

Background

Ulvan

Ulvan is a water-soluble polysaccharide that can be extracted from the cell wall of green algae such as *Ulva lactuca*. It is shown to possess several beneficial biological activities including, but not exclusive to, inflammation regulation, bacterial inhibitory effects (Chen *et al.*, 2019) and anti-cancerogenic potential (Kidgell *et al.*, 2019). Ulvan consists mainly of four monosaccharides, *i.e.*, xylose, sulfated rhamnose and two uronic acids, iduronic and glucuronic acid. These monosaccharides are mainly present in the form of repeating disaccharides (*figure 1*) (Kidgell *et al.*, 2019). What makes these unique is that the sugars are rare in terrestrial plants. Rhamnose and iduronic acid are examples of these rare sugars. They are commonly used in several industrial and medical applications (Onda *et al.*, 2017). Rhamnose can be used in wound dressing and healing applications (Chen *et al.*, 2019) and uronic acids have been used in several different applications such as the cosmetic and food industries (Li *et al.*, 2020). The enzymatic disruption of ulvan is still a relatively novel area in carbohydrate active enzyme research with only few enzymes acting on ulvan described (Kidgell *et al.*, 2019). Ulvan extraction from raw algal biomass as well as subsequent polysaccharide pretreatment is also only sporadically investigated, while purification strategies for ulvan disaccharide is yet to be developed (Podolean *et al.*, 2022).

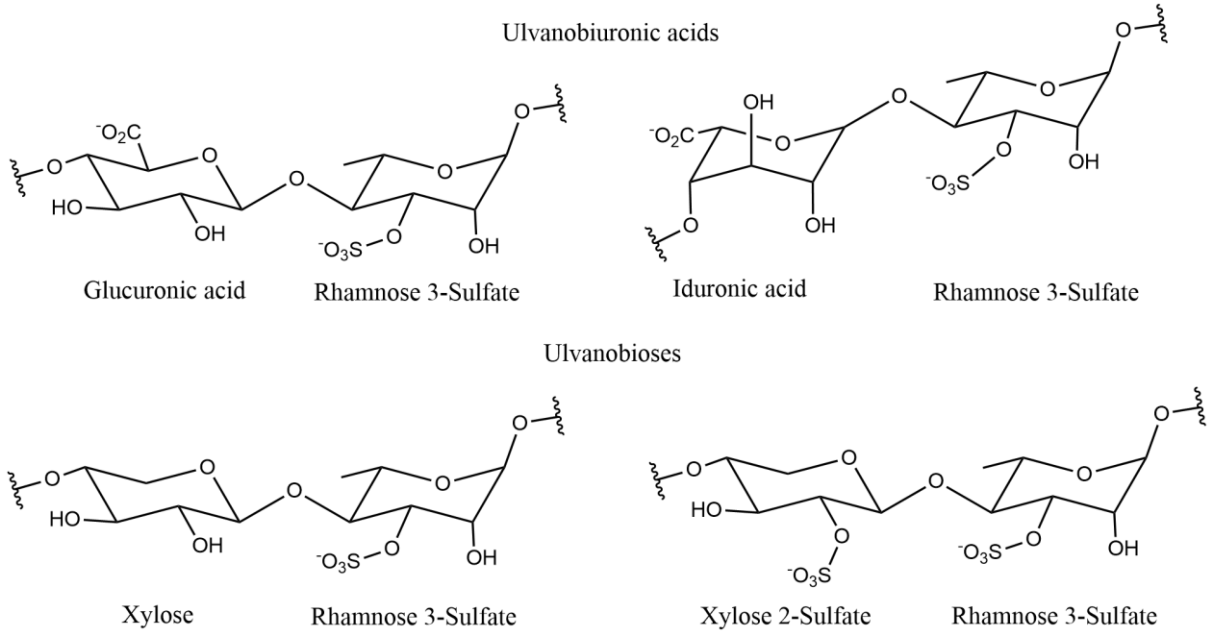

Figure 1. Structures of the four main constituents making up ulvan polysaccharide from the green algae Ulva (Kidgell et al., 2019).

Enzymes disrupting ulvan

Several enzymes with different activity are needed for the full degradation of polysaccharides into their respective constituents, as a result in genomes, genes encoding carbohydrate active enzymes are accumulated in polysaccharide utilization loci (PUL). Typically, regulatory elements are also present in PUL ensuring enzyme correct order of gene expression. Each enzyme in a PUL catalyze a specific reaction in the cascade of the degradation of polysaccharides, starting with the depolymerization of the polysaccharides into smaller polysaccharides and finishing with oligosaccharide hydrolysis to monosaccharides performed typically after transport into cell. The Carbohydrate Active Enzyme database (CAZy) is an extensive catalogue of characterized and hypothetical enzymes responsible for the breakdown of poly- and oligosaccharides (Terrapon *et al.*, 2017). The database was used as reference standard for novel putative enzyme sequence characterization.

The CAZy family PL37 is a novel family with only a single enzyme biochemically characterized (Drula *et al.*, 2021). *W. fucanilytica* encodes putative ulvan lyase attributed to CAZy PL37 family selected for characterization here termed as WFp-37. Target enzyme WFp-37, catalyzes the β-elimination reaction of the 4,1-glycosidic bond within the ulvan polysaccharide chain (figure 2), producing a new reducing end on one product and an unsaturated oligosaccharide on the other product (Garron & Cygler, 2010).

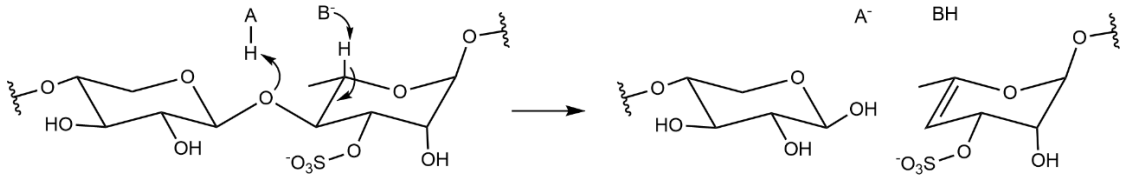

Figure 2. β-elimination reaction performed on an ulvan polysaccharide by a CAZy family PL27 enzyme.

The CAZy family GH105 is well studied, and several enzymes have previously been characterized (Drula *et al.*, 2021). GH105 share high sequence and activity identity with enzymes in the CAZy family GH88 with differences, among others, in the catalytic amino acid motif of the active site (Jongkees & Withers, 2011). GH105 family proteins use an aspartic acid as the catalytic residue and utilizes a combination of tryptophan and arginine for the binding of ulvan (Collén *et al.*, 2014). The putative unsaturated oligosaccharide hydrolase attributed to GH105 family from *W. fucanilytica* here termed as WFp-105 was selected as a target for characterization aiming investigate enzymes activity on ulvan oligosaccharides. WFp-105 was predicted to be acting as unsaturated rhamnogalacturonidase acting upon the 4-5 unsaturated bond at the non-reducing end of ulvan oligosaccharides, which are the products of ulvan degrading lyases performing β-elimination. The reaction results in a linear monosaccharide (figure3). WFp-105 was predicted to be a part of the putative PUL 7 (figure 4).

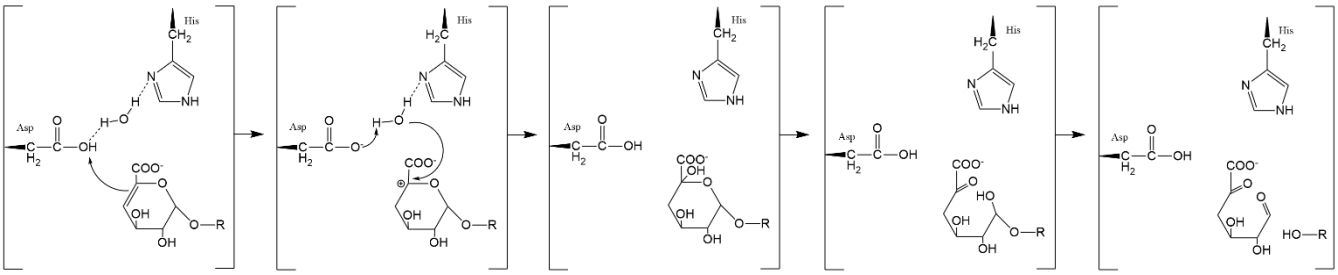

Figure 3. Reaction mechanism of degrading an unsaturated ulvan oligosaccharide hydrolysis by GH105 family oligosaccharide hydrolases

Figure 4. Structure of a predicted PUL from Wenyingzhuangia fucanilytica PUL7 responsible for ulvan degradation containing target protein GH105 gene from the CAZy database of PULs. (Terrapon et. al, 2015)

Recombinant protein production

When producing protein for research purposes batch cultivation in shake flasks is a common method as a convenient method suitable for lab-scale protein expression purposes. *Escherichia coli* is the most common expression host as it is easy to cultivate and readily grows to high OD values (Ganjave *et al.,* 2022). To be able to produce protein recombinantly the cells first need to be transformed via, for example, thermal shock where an expression construct containing the target protein, an ori native to the organism used, a promoter for the recombinant gene, a selection method to distinguish transformed cells such as a resistance gene for antibiotics. A commonly used method for selection is antibiotics which applies a selective pressure to the cells thus isolating the transformed cells. To initiate protein expression an inducer is used, an example of which is isopropyl β-D-1-thiogalactopyranoside (IPTG). Research indicates that lower concentrations of IPTG (Einsfeldt *et al.,* 2011) and sub optimal cultivation temperatures (Mahnič *et al.*, 2012) leads to higher production yields of soluble recombinant protein as a result of increased folding. To increase the expression and solubility of proteins in *E. coli* expression hosts it is possible to utilize solubility tags. Tags such as GST, small ubiquitin-related modifier (Steinmetz & Auldridge, 2017) and the Fh8 fusion tag (Costa *et al.,* 2014) have been shown to increase solubility in an *E. coli* expression host.

Enzyme activity analysis methods

3,5- dinitrosalicylic acid (DNS) assay is and has been the go-to analysis method when working with polysaccharide degrading enzymes such as hydrolases and lyases. It works by reacting with the reducing end produced by hydrolysis of poly- and oligosaccharides producing a colored product capable of absorbing light at $A_{540\text{-}550\ nm}$. This developed color change is directly proportional to the number of reducing ends present in the sample, thus ensuring a linear detection of reaction product (Miller, 1959).

When working with unsaturated oligosaccharides there is the possibility of direct detection of the carbon/carbon- double bond (C=C) present in the sugar molecule produced by the β-elimination of ulvan degrading lyases as unsaturated bond absorbs in the UV-spectra. This method has been for example used to monitor the enzymatic degradation of biomass and continuously detect the formation of unsaturated oligosaccharide products (Limtiaco *et al.,* 2011). Direct detection of double bond by absorption measurement was implemented for ulvan lyase as well as unsaturated oligosaccharide hydrolase activity characterization as ensuring higher reliability of activity measurements that initially applied DNS assay.

Materials and methods

Protein sequence analysis

Amino acid sequences were aligned analysis sequence identity and conservation using NCBI BlastP against non-redundant (nr) and Protein Data Bank (pdb) databases. Phylogenetic three of WFp-105 sequence was constructed using alignment containing all family GH88 and family GH105 sequences indexed in CAZy database as characterized. Manually extracted sequences were re-aligned with ClustalW implemented in the MEGA 11 suite under default parameter values for WFp-105 phylogenetic analysis. MEGA 11 suite was also used for calculation of a maximum-likelihood three under default parameter values with 500 bootstrap replications. Attribution to operon as well as gene transcription regulatory elements were analyzed with using Softberry's prediction tool BPROM under default parameter values including the upstream to WFp-37 gene genome region performing final prediction consideration manually.

Recombinant WFp-105 and WFp-37 production

Expression constructs

The expression constructs were prepared by cloning of *de novo* synthesized sequences (BioCat) of interest into the expression vector pET-21b(+) (Novagen) in order to obtain expression construct for WFp-105 or into the expression vector pET-41a(+) (Novagen) in order to obtain expression construct for WFp-37.

Transformation

The expression constructs were transformed to selected *E. coli* strains by heat shock transformation cultivating on LB-Lennox agar plates with appropriate antibiotics no more than 14 h at 37°C. Obtained discrete colonies were replated to fresh antibiotic containing plates for each protein expression. The *E. coli* strain used for WFp-105 expression construct was BL21, with plates containing 100μg/ml ampicillin. The *E. coli* strains used for WFp-37 expression construct was BL21 and Tuner(DE3), with plates containing 50μg/ml kanamycin.

Heterologous overexpression

Expression cultures were inoculated with overnight inoculum up to 1% (v/v). Heterologous overexpression was performed in 1 L baffled shake-flasks using LB-Lennox supplemented with appropriated antibiotic. All cells were transferred into 1l shake flasks and cultivated on 200 ml LB media with shaking at 180 rpm. WFp-105 transformed cells were induced with 0.2 M IPTG at OD_{600} = 1 and grown for three hours at 30°C. WFp-37 cells were induced with 1mM IPTG at OD_{600} = 0.8. The cells were then cultivated at three different temperatures for 16 h. BL21 cells cultivated at 30°C and the Tuner cells were cultivated 28°C and 16°C respectively. Expression cultures were harvested by centrifugation at 8000G for 20 min.

Affinity purification

Affinity purification was performed using indicated buffers. For the purification different buffers were prepared in accordance with the recommendations in context of selected chromatography resin, with the exception that Tris-HCl was used instead of phosphate buffered saline.

GH105 Binding Buffer	GH105 Elution Buffer	GH105 Desalting Buffer	PL37 Binding Buffer	PL37 Elution Buffer
- 50 mM Tris-HCl, pH 7.4/RT - 50 mM imidazole - 500 mM NaCl - 5% glycerol	- 50 mM Tris-HCl, pH 7.4/RT - 500 mM imidazole - 500 mM NaCl - 10% glycerol	- 50 mM HEPES-NaOH, pH 7.4/RT - 10% glycerol	- 50 mM Tris-HCl, pH 7.4/RT - 5% glycerol	- 50 mM Tris-HCl, pH 8.0/RT - 10 mM reduced glutathione - 5% glycerol

The cell pellets containing WFp-105 were resuspended in GH105 binding buffer and disrupted by sonication for 15 min with an amplitude of 60% and a 0.5 cycle using a dr.hielscher UP 400s Ultraschallprozessor. Cell debris was separated by centrifugation 12 000G for 20 min The supernatant was saved, and the pellet discarded except for a small amount saved for analysis.

Target proteins were purified by affinity chromatography applying nickel affinity chromatography for WFp-105 and glutathione affinity chromatography for WFp-37 using HisTrap HP 1 mL column (Cytiva) or, respectively, GSTrap 1 mL column (Cytiva), with ÄKTA start FPLC purification system (GE Healthcare Life Sciences). The samples were filtered using a 0.2 µm regenerated cellulose filter (GE Healthcare Life Sciences) prior to loading onto column. Target proteins were eluted by single step elution. Purified WFp-105 was desalted using HiTrap desalting column (Cytiva). Protein purity and integrity was by 4–15% glycine-SDS-PAGE (Laemmli, 1970). The protein concentration was determined by measuring A_{280nm} using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

Enzyme activity characterization

As there were no commercially available substrates for WFp-105 this needed to be produced. Ulvan polysaccharide from *Ulva amoricana* super fine grade (Elicityl) was used as initial raw ulvan for pretreatment. The raw ulvan polysaccharides at 0.5% (w/v) was lyase-pretreated with recombinant family PL25 ulvanase (Sivakumar, 2022) performing enzymatic pretreatment under optimal conditions for lyase activity. Lyase was irreversibly inactivated by heating at 95°C for 2 min. Enzyme assay contained 95 µL of the substrate in PCR tubes to which 5 µL either blank buffer, as control, or enzyme solution was added to a final enzyme concentration of 5 µg/ml. The samples were then incubated for 22 h at varying temperatures to determine temperature optimum. To determine the pH optimum substrate was lyase-pretreated in buffers with varying pH after which the samples were incubated for 22 h at 25°C. Temperature and pH stability was determined by incubating the enzyme solution at temperatures 10, 20, 40 and 60°C and pH 5, 6, 7.4 and 8 for 30 min. After which the activity was analyzed after incubation for 22 h at 25°C. All samples were measured spectrophotometrically at $A_{235\ nm}$. The initial activity detection of WFp-37 was analyzed by adding enzyme to raw ulvan polysaccharides at 0.5% (w/v) to a final concentration of 5 µg/ml parallel to control samples where equal volume of blank buffer was added as protein. The samples were then incubated at 25°C for 24h and then analyzed spectrophotometrically at $A_{235\ nm}$ and compared with control samples.

Product determination

WFp-105 reaction products were visualized with thin layer chromatography (TLC) and profiled with high-performance anion exchange chromatography – pulsed amperometric detection (IC) was performed with xylose, rhamnose and glucuronic acid as standards. For IC, each sample was diluted x50 and filtered using 0.2 µm polytetrafluoroethylene membrane syringe filter (Pall). The samples were run on an IC using a PA-200 column (Thermo Fisher Scientific) with the mobile phase of 400 mM sodium acetate and 200 mM sodium hydroxide to separate mono- and oligosaccharides. For TLC, 3 µl of each sample was loaded onto a TLC silica gel 60 F_{254} sheet. The mono- and oligosaccharides were then separated using 2:1:1 mixture of butanol, acetic acid and water as a mobile phase and visualized using a spray consisting of 95 mL methanol, 5 mL H_2SO_4 and 100 mg oricinol. Plate development was performed by heating at 130°C until visible.

Structure modeling

A modeling of WFp-105 was performed to predict structural organization. Swiss Institute of Bioinformatics' online tool SWISS-MODEL (Waterhouse *et al.*, 2018) was selected for modeling using the structure of a GH105 protein from *Nonlabens ulvanivorans* (Collén *et al.*, 2014) as a model template due to the 70% identity between sequences of WFp-105 and GH105 protein from *N. ulvanivorans*.

Results

WFp-105

The phylogenetic and sequence alignment analyses demonstrated that the enzyme originating from *W. fucanilytica* should be attributed to to the CAZy family GH105 as enzyme sequence demonstrates homology confirmed by high sequence identity with putative as well as characterized proteins from family GH105 (figure 5). The phylogenetic tree confirmed a clear divergence (figure 6) between the sequences from two families GH105 and GH88 analogous in substrate specificity with WFp-105 positioned in GH105 clade. The alignment (figure 5) also alowed to discriminate conserved regions, in particular, the catalytic and binding sites, complementingWFp-105 characterisation as GH105 protein. The highest identity among characterized and among structurally characterized enzymes, was with the unsaturated glucuronyl hyrdolase from *N. ulvanivorans* showing a 70% likeness in amino acid residues.

Figure 5. Alignment of amino acid sequence of structurally characterized proteins in the family GH105. Performed using UniProt online alignment tool and ClustalW. Marked are the conserved catalytic residues and residues ensuring substrate binding of GH105 enzymes.

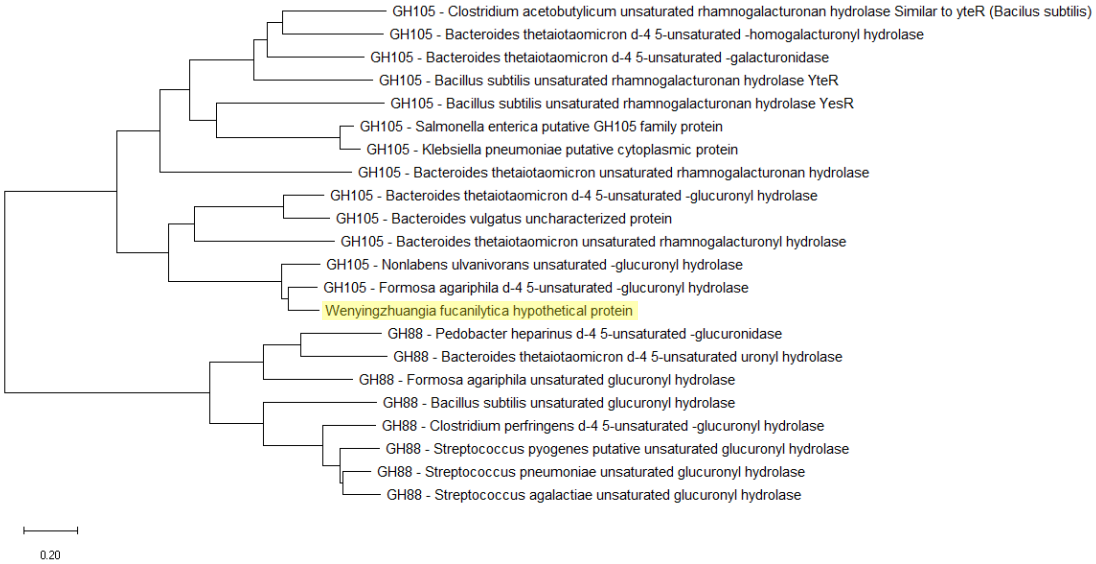

Figure 6. Unrooted phylogenetic tree of characterized family GH105 and GH88 enzymes from different bacteria. Highlighted in yellow is target protein WFp-105.

WFp-105 expression and purification

The enzyme was successfully overexpressed in *E. coli* BL21 cells. Target enzyme was expressed obtaining high yield. Spectroscopic analysis at A_{280}, yielded the total soluble concentration 19.2 mg/ml. Analysis of the band intensity of protein electrophoresis gel gave the yield in the soluble fraction 13.7 mg/ml WFp-GH105 which corresponds to 37.9% of the total amount of WFp-105 (table 1). The enzyme was successfully purified using nickel affinity chromatography to obtaining high protein recovery yield of intact protein (figure 7). Recombinant enzyme demonstrated comparatively high affinity to chromatography resin even when binding was performed at elevated concentration of imidazole, what allowed to achieve purity near to homogeneity by single step affinity chromatography. Pure WFp-105 remained stably soluble after affinity purification as well as after enzyme sample desalting. Aggregation of WFp-105 was not observed after prolonged incubation at 4°C reaching one or even two months. The first purification was performed in three separate instances giving the 4°C incubation times one and two months. As visualized by protein electrophoresis WFp-105 detecting bands at around 80 kDa and around 120 kDa WFp-105 demonstrates a high trend of oligomerization resulting in homodimer that probably further interacts to dimer of homodimers.

Table 1. WFp-105 yields in the soluble and insoluble fraction after sonication and centrifugation.

	Relative band intensity (%)	Total conc. (mg/ml)	Conc. WFp-105 (mg/ml)
Soluble fraction	71.6	19.2	13.7
Insoluble fraction	55.9	40.1	22.4

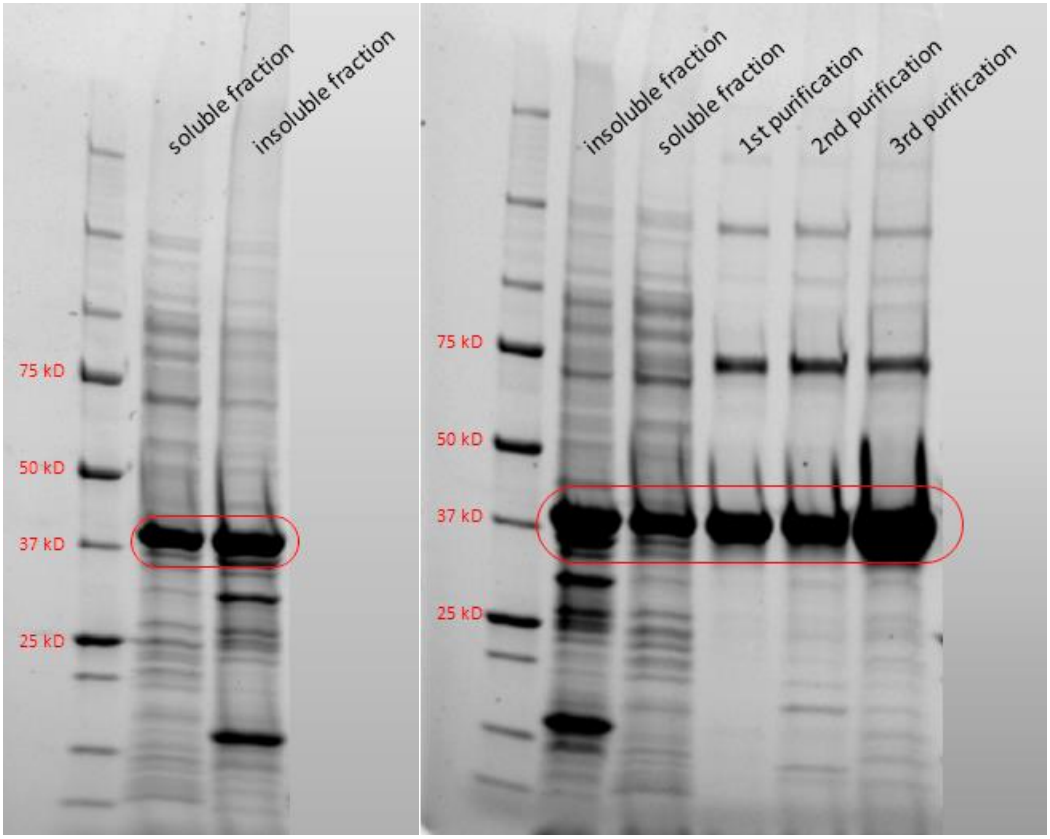

Figure 7. (Left) Results of WFp-105 expression visualized via protein electrophoresis. (Right) Results of WFp-105 purification using affinity chromatography visualized via protein electrophoresis. Last purification was used for subsequent enzyme characterization. Highlighted is 42kD target protein WFp-105. Visible in the purified sample are also possible di- and trimers of target protein. Protein molecular mass standard used was Precision Plus Protein™ from Bio-Rad.

Enzyme activity characterization

As DNS method was not ensuring reliable detection for WFp-105 activity due to the initial complexity of lyase-pretreated ulvan mixture, preliminary activity detection results obtained using DNS omitted from the results (included in appendix). Initially, the activity of WFp-105 was assayed after performing reaction incubation at 25°C for 24 h. Assay was performed with freshly produced enzyme, enzyme after prolonged three months incubation as well as enzyme that had been frozen and enzyme that had been desalted in a size exclusion chromatography. Remarkably, the assay showed no decrease in the activity regardless of the treatment (table 1).

Table 2. Activity confirmation of WFp-105 performed at 25°C for 24 h, measured using A_{235}.

	Control	Old, purified protein	Fresh, purified protein	Frozen protein	Desalted protein
Absorbance (A_{235nm})	1,135	0,215	0,194	0,192	0,185
	1,098	0,199	0,193	0,202	0,173

The results of WFp-105 reaction product profiling performed using high-performance anion exchange chromatography – pulsed amperometric detection (IC) (figure 8), enabled to detect in a number of products. Glucuronic acid eluted roughly 10 min after the last reaction sample peak and is therefore not depicted in the chromatograms. Analysis of chromatograms allowed to identify two obvious peaks (figure 8, peak 5 and 6) that did not correspond to any of the standards used. Apart from the peaks that are not present in activity reaction control profiles, the peaks 3 and 4 to increased. Peak 3 directly corresponds to the xylose standard, which indicates that the reaction is resulting in monosaccharide production one of which is xylose.

WFp-105 reaction at optimal conditions were also visualized via TLC (figure 9). Comparison with standard migration patterns two main differences are noticeable. The right most band in the reaction sample, which is not present in the reaction migration pattern, is likely a monosaccharide that does not correspond with the xylose nor the rhamnose bands. The left indication shows another band present in the reaction that is not present in the control, likely to be a smaller oligosaccharide produced after hydrolysis.

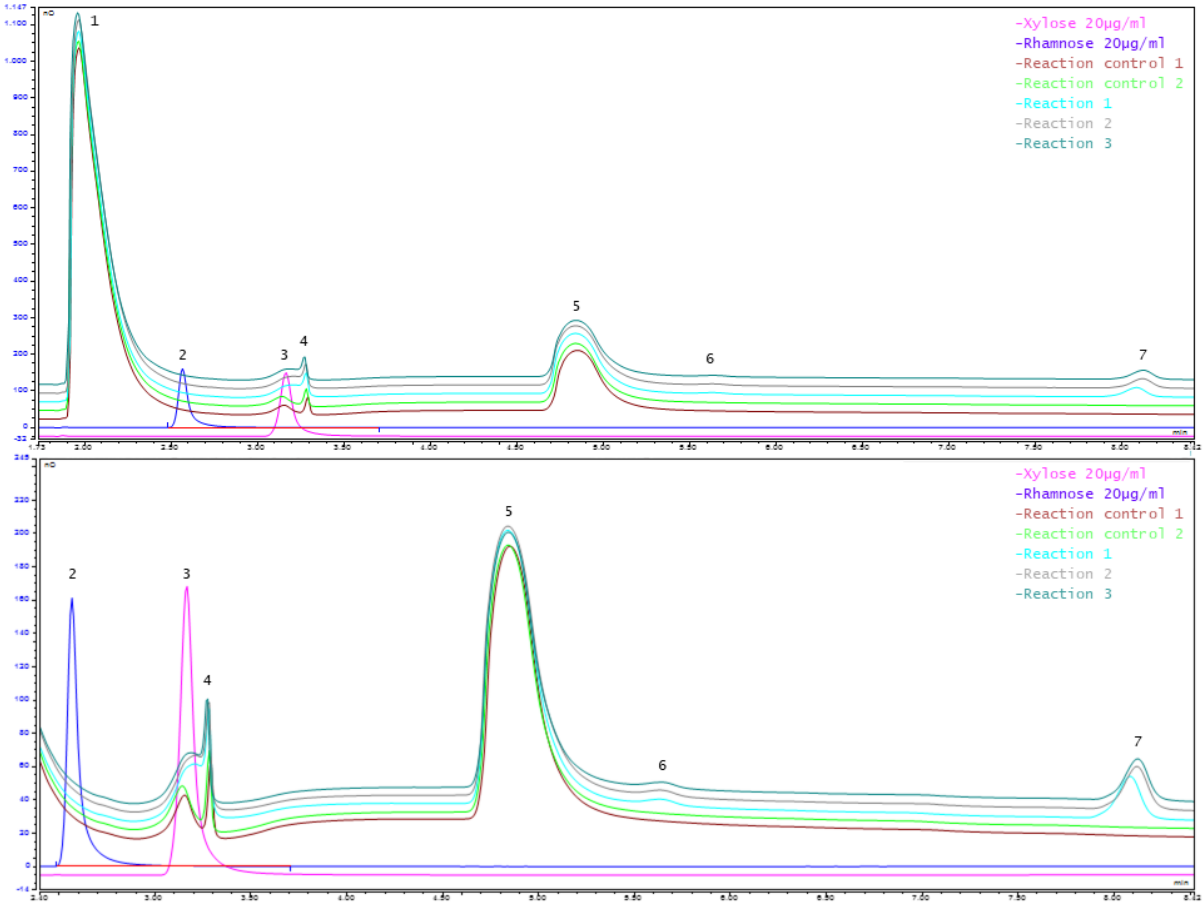

Figure 8. (Top) Chromatogram of IC run on a PA-200 column for WFp-105 reaction at optimal conditions with xylose and rhamnose as standards. (Bottom) Closer image of the chromatogram to show differences in peak height. Reaction 1,2 and 3 corresponds to triplicate reactions and control 1 and 2 to dublicates.

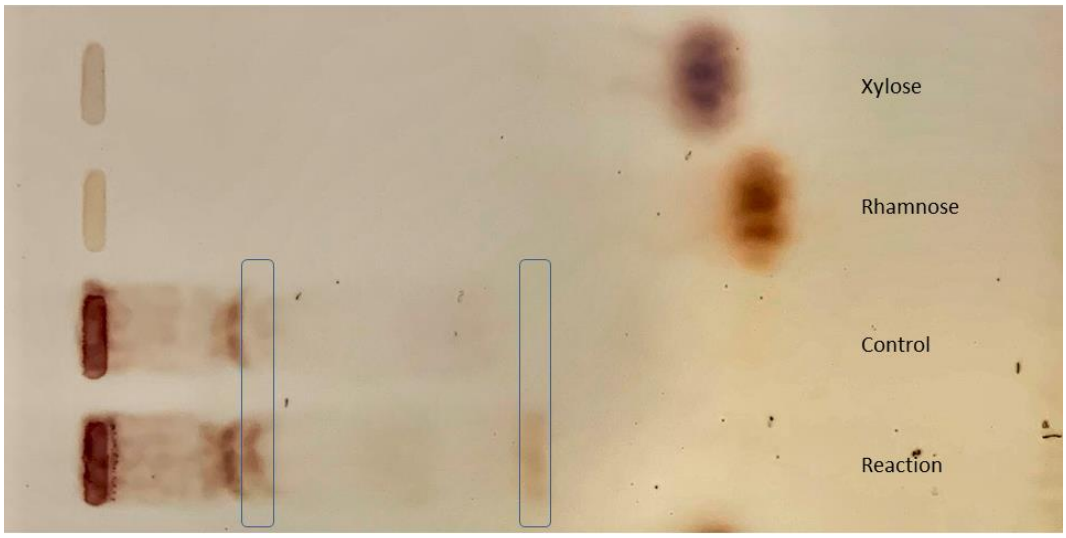

Figure 9. TLC plate of WFp-105 reaction and control with xylose and rhamnose as standards.

Characterization of WFp-105 activity optima and activity stability spectrophotometric detection was successfully applied measuring reduction of absorbance at wavelength absorbed by double bond in unsaturated oligosaccharides. The enzyme demonstrated almost identical catalytic activity level at temperature range 10-40°C. Mild activity loss was observed after 35°C, with subsequent sharp activity loss after 40°C. At 60°C recombinant WFp-105 almost completely loss activity No clear peak was detected (figure 10) indicating a sharp temperature optimum. This was confirmed by direct comparison of activity level values in outlined temperature range noting activity level difference within 10-30°C only about 3%.

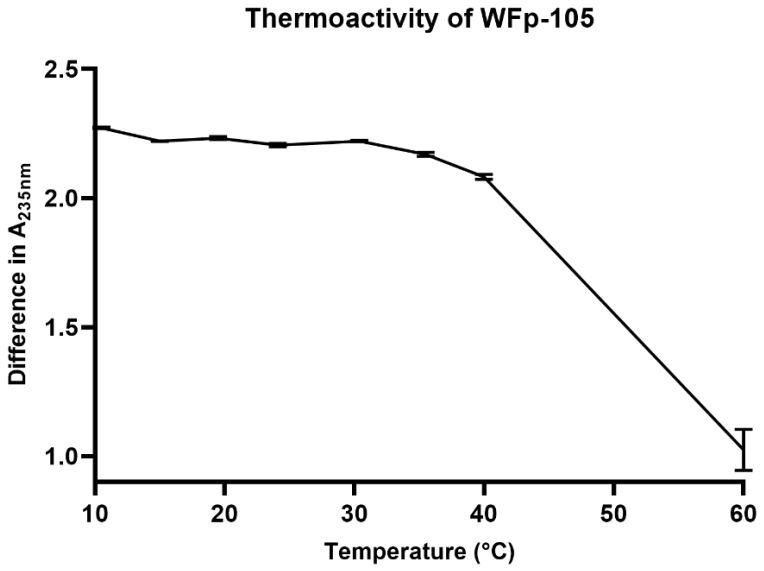

Figure 10. Thermoactivity of WFp-105. The graph shows the difference in absorbance after 22h reaction at varying temperatures. Samples run in triplicates, error bars showing 99% confidence interval.

The influence of pH on WFp-105 was successfully characterized (figure 11). The enzyme was most active at slightly acidic conditions, with activity optima at pH 5. WFp-105 was losing activity in alkaline pH range, however relevant activity loss was not shown as enzyme remain active at pH 8 as well. No significant difference of WFp-105 activity between pH 7.4 and 8.

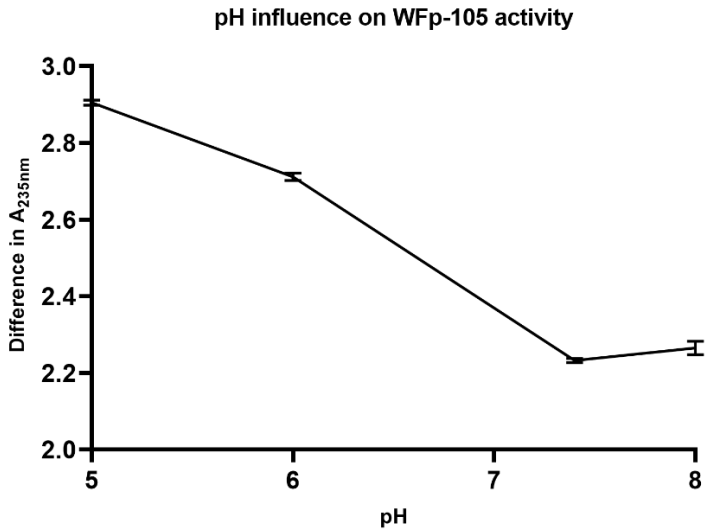

Figure 11. pH influence on the activity of WFp-105 activity. The graph shows the difference in absorbance after 22h reaction at varying pH. Samples run in triplicates error bars showing 99% confidence interval.

Enzyme stability

The thermostability and pH stability characterization demonstrated that WFp-105 being quite resilient. No significant loss of activity was detected after WFp-105 30 min incubation at 40°C, however when enzyme was incubated at 60°C no residual activity was detected. WFp-105 seem to be more resilient toward acidic conditions showing no statistically significant difference between pH 5-7, and only having a mild decrease of activity after 30 min incubation at pH 8. Although, the difference in activity is minor with only being about 3% decrease between pH 7.4 and 8.

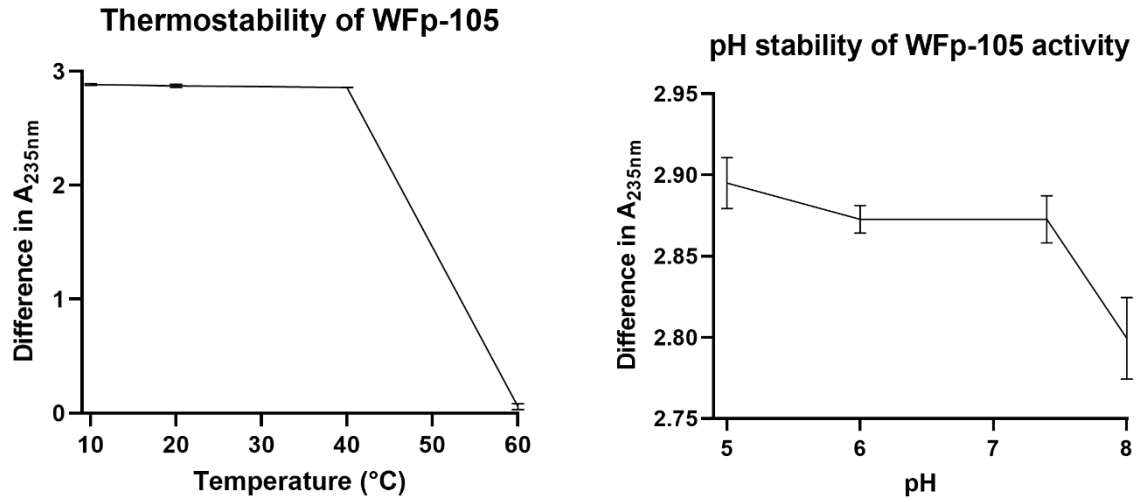

Figure 12. (Left) residual activity of WFp-105 after enzyme incubation for 30 min at indicated temperatures. (Right) residual activity of WFp-105 after enzyme incubation for 30 min at indicated pH values. Activity was measured under optimal condition after preincubations. Error bars showing 99% confidence interval.

Structure modeling

High sequence identity to GH105 protein allowed apply structural modeling of WFp-105 structure (figure 13). Modeled structure represents globular tube-like shaped protein, mainly composed of α-helixes. Structural assessment of the model gives the overall quality estimate QMEAN= 0.87±0.05 with the highest scores in the internal structure composed of five α-helixes one of which contain the catalytic residue Asp162 (figure 14). Substarte binding domain was also predicted for WFp-105 . Taken together, the good distribution ramchandran plot (figure 14) indicate high reliability of creted model.

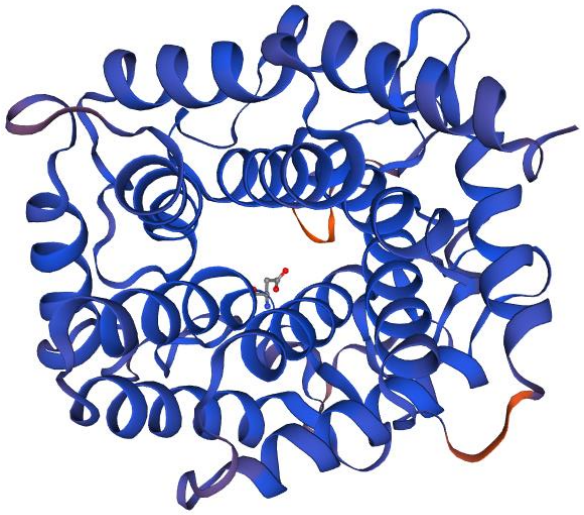

Figure 13. Model of WFp-105 structure. Modeled using SWISS-MODEL with the structure of a GH105 protein from Nonlabens ulvanivorans 4CE7_1 as a model template. The outlined amino acid residue is the catalytic residue Asp162. Blue color indicates good model score and red indicates a lower reliability score.

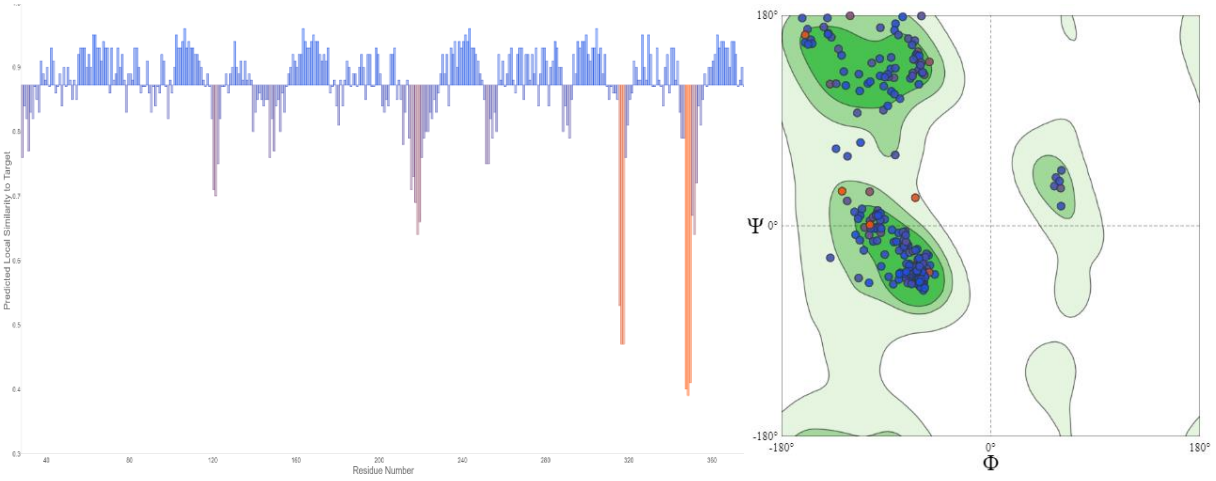

Figure 14. (Left) WFp-105 model quality score for each amino acid compared to the homologous protein from Nonlabens ulvanivorans. (Right) Ramchandran plot shoing the torion angels of the bonds in the peptide back bone of the WFp-105 model.

WFp-37

Genome context analysis of WFp-37 gene

WFp-105 gene was originally annotated as an open reading frame demonstrating homology to putative ulvan lyases attributed to family PL37. Deliberate analysis of WFp-37 gene genome context with the BPROM search resulted in no clear indication for a promotor for the WFp-37 gene sequence. The predicted promoters, located several hundred nucleotides upstream of the protein gene sequence, indirectly indicate that WFp-37 expression is likely part of an operon containing several other proteins thus dependent on upstream protein transcription. The most probable promotor sequence was predicted to be located position 35 and 17 downstream of first codon (figure 15). No conclusive results were found regarding the terminator for the operon responsible for transcription of WFp-37 nor for any proteins in proximity.

```
>W. fucanilytica strain CZ1127, complete genome 1464653-1466198
 Length of sequence-      1546
 Threshold for promoters -  0.20
 Number of predicted promoters -      4
 Promoter Pos:     98 LDF- 11.18
 -10 box at pos.     83 TTTTAAAAT Score    79
 -35 box at pos.     65 TAGATA    Score    21
```

Figure 15. Results of BPROM analysis including the 100 bp upstream region of the fifth protein upstream of WFp-37, gene annotation AXE80_06065 showing the promotor responsible for regulation of the operon containing AXE80_06065 and possibly subsequent proteins AXE80_06070, AXE80_06075, AXE80_06080 and WFp-37.

WFp-37 expression and purification

WFp-37 was successfully expressed obtaining moderate yield of recombinant protein in *E. coli* BL21(DE3) and Tuner(DE3) cells (figure 16). Spectroscopic analysis at A_{280}, showed the highest concentration of WFp-37 was achieved in the Tuner cells cultivated at 16°C with a concentration 3.08 mg/ml WFp-37 (table 3). While the lower incubation temperature provided a higher total yield, the higher temperature provided more enzyme remaining in the soluble phase. Glutathione affinity chromatography was successfully implemented for WFp-37 (figure 16); however, pure GST-tagged WFp-37 was highly prone to aggregation resulting in catalytically inactive, amorphous aggregates.

Table 3. WFp-37 yields in the soluble and insoluble fraction after sonication and centrifugation.

	Relative band intensity (%)	Total conc. (mg/ml)	Conc. WFp-37 (mg/ml)
Tuner 16 sol. frac.	19.4	15.9	3.08
Tuner 16 insol. frac.	16.7	13.3	2.22
Tuner 28 sol. frac.	19.0	15.7	2.98
Tuner 28 insol. frac.	6.3	4.9	0.31
BL21 sol. frac.	15.7	9.28	1.46
BL21 insol. frac.	19.1	7.23	1.38

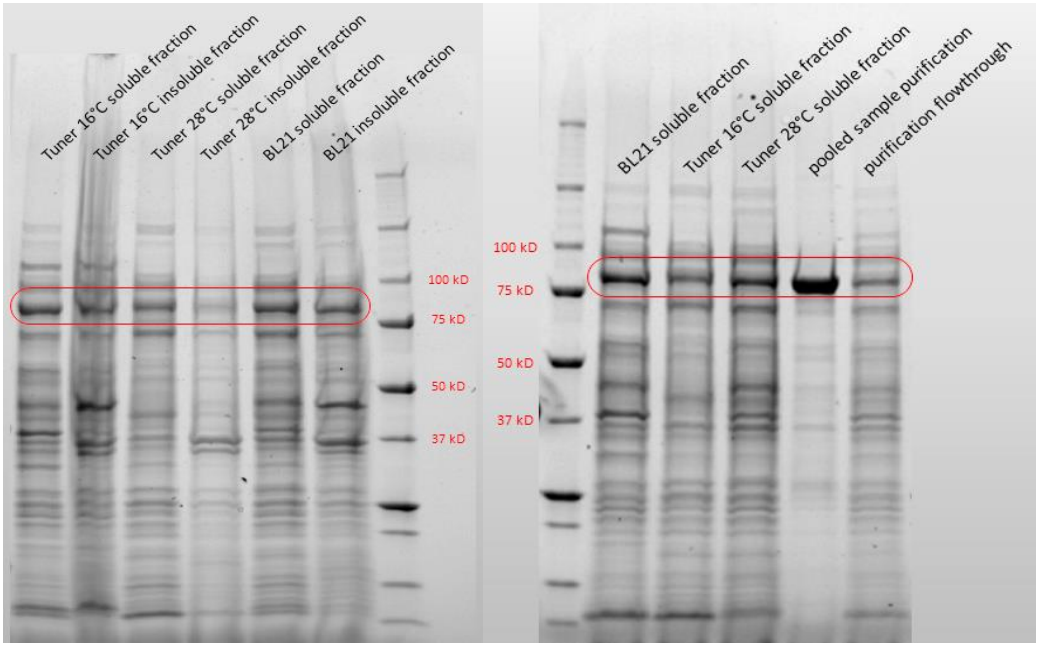

Figure 16. (Left) Results of WFp-37 heterologous overexpression in indicated expression strains performing induction under indicated temperatures visualized via protein electrophoresis. (Right) Results of WFp-37 purification using glutathione affinity chromatography, visualized via protein electrophoresis. Highlighted is GST-tagged target protein WFp-37 of total 98 kDa. Protein molecular mass standard used was Precision Plus Protein™ from Bio-Rad.

WFp-37 activity

Recombinant WFp-37 activity was assessed in crude clarified extract of soluble protein fraction as well as in eluate obtained after glutathione affinity chromatography. Activity of non-purified recombinant WFp-37 was successfully confirmed (table 4). However, amorphous aggregates of WFp-37 had no remaining activity on raw ulvan. Activity assessment data (table 4) shows that WFp-37 has ulvan degrading activity in the crude cell lysate. The highest activity was detected when target enzyme was heterologously overexpressed in in *E. coli* Tuner(DE3) cells and cultivated for 4h at 28°C with protein expression induction ad OD_{600} = 0.8 with 0.2 mM IPTG.

Table 4. WFp-37 initial catalytic activity determination results on raw ulvan analyzed at A235nm of the pooled purified sample containing enzyme from all cultivation, lysate of BL21 cells cultivated at 30°C and lysate from two cultivation of Tuner(DE3) cells cultivated at 16°C and 28°C respectively.

			Crude cell lysates		
	Control	Purified	BL21	Tuner(DE3) 16°C	Tuner(DE3) 28°C
A_{235nm}	0.312	0.316	0.627	0.695	0.851
	0.317	0.323	0.637	0.791	0.782

Discussion

WFp-105

WFp-105 was expressed obtaining high yield of stably soluble enzyme exploiting standard recombinant protein expression strategy in *E. coli* performing induction at 28°C. Characterized glycoside hydrolase recovery yield after affinity purification was also high indirectly confirming that WFp-105 was not aggregating during purification. Desalting was not causing recombinant enzyme aggregation either, which shows the high potential of WFp-105 implementation in biotechnological processes from production perspective. The WFp-105 sequence is homologous to characterized glycoside hydrolases, which allowed the successful modeling of the structural organization and prediction of catalytic residues. Further investigation is needed for in-depth description of enzyme native oligomeric organization. Activity characteristics outlines WFp-105 as actively hydrolyzing ulvan unsaturated oligosaccharides in wide temperature as well as pH range. It is highly expected that native WFp-105 in *W. fucanilytica* participates in conjunction with secrete ulvan lyase, which gene was also annotated in PUL.

Substrate shortage and substrate preparation

The shortage of commercial substrates available for WFp-105 activity assessment caused difficulties in the enzymatic assay. This, in combination with the absence of standards for spectrophotometric measurements and catalytic reaction product identification by chromatography, meant complete novel enzyme characterization including kinetic parameter determination and product determination was not possible. Performing preparative size-exclusion chromatography is needed for pure ulvan oligosaccharide separation after raw ulvan lyase-pretreatment.

Characterization was performed after efficient inactivation of non-thermostable pretreatment ulvan lyase by short incubation at high temperature, since initial attempts to separate oligosaccharides by filtration were not successful. Direct estimation of WFp-105 activity without consideration of protein concentration – as lyase was not separated after raw ulvan pretreatment represents initial results of temperature and pH influence on glycoside hydrolase activity and stability.

Activity and stability

The classic method of measuring enzymes active on sugars activity, the DNS method (Miller, 1959), was not granting reliable results when used for WFp-105 activity determination most probably by comparative high background of alpha reducing ends in complex mixtures of lyase-pretreated raw ulvan. Thus, causing the shift towards analysis of A_{235nm}. Glycoside hydrolase activity measurements exploiting unsaturated bondage at A_{235nm} has previously been successfully applied for ulvan disrupting enzyme characterization (R. Mondal, 2020) providing confidence in the analysis.

The fact that no commercial substrate was available on the market introduced variables into the assays that were hard to consider. One example being the fact that there was another enzyme present throughout the reactions an enzyme that could've been active, even though the data suggest otherwise, and thus led to the indication that WFp-105 had lower activity than there actually was. It's also possible that the enzyme had stabilizing properties leading to the temperature and pH optimum being skewed. To further understand the specific activities of WFp-105 a pure, specified substrate is needed. When previous research has been performed into unsaturated rhamnogalacturonidases and D-4,5-unsaturated β-glucuronyl hydrolases, breaking down ulvan oligosaccharides, a purification of lyase reaction products have been made thus providing a specified substrate. As this was not in the scope of this thesis it was not looked into whether this would be possible in the case of WFp-105 however, would be interesting looking into in future research.

The results of the WFp-105 activity determination characterize enzyme as non-thermoactive favoring lower temperatures and slightly acidic conditions which is to be expected as enzyme originates from *W. fucanilytica* which is a costal organism present in fermenting seaweeds which likely is a slightly acidic environment since fermentation commonly lowers the pH and the ocean temperatures being low (Chen *et al*., 2016). Comparing temperature optimum to the temperature stability results clearly indicate the substrate having stabilizing properties, which is not unexpected for glycoside hydrolases. The activity measurement at 60°C allowed the detection of activity present in the enzyme albeit low, whereas the enzyme kept for 30 min at 60°C had no residual activity after incubation.

Analysis of WFp-105 activity reaction measurement results also indirectly indicates that even though A measurement at 235 nm is very specific for double bond overall comparatively low concentration of unsaturated oligosaccharide in pretreated ulvan that was used as a substrate which decreased the precision of activity measurement. This was noticeable for example in enzyme temperature optima determination as at the temperatures 10 through 30°C. This could indicate that the assays suffer from limitations due to substrate depletion of unsaturated oligosaccharides. Shorter incubation times and activity assay optimization with pure unsaturated ulvan oligosaccharides is needed seeking increase WFp-105 activity quantification assay precision. The pH optima determination could be further investigated measuring WFp-105 catalytic activity between pH 4.5-5.

Product determination

The reaction products of WFp-105 were difficult to specify. The TLC show oligosaccharides being consumed resulting in monosaccharide production what were assumed by decrease of oligosaccharide band intensity and simultaneous observation of a faint band in the range of monosaccharides.

General model of ulvan composition allows predict the repertoire of produced monosaccharides, however without objective ulvan oligosaccharide standards estimation of WFp-105 preference in context of ulvan disaccharide type (ulvanobiuronic acids or ulvanbioses). Characterized glycoside hydrolase rection product profiling allowed confirm production xylose from ulvan oligosaccharides confirming enzyme catalytic activity. Without available standards other detected WFp-105 reaction products could be identified by NMR.

Theoretically, the majority of ulvan consist of five monosaccharides allowing the expectation of five peaks present on the IC chromatogram of the reaction products. As outlined only one peak from detected product peak was successfully identified as xylose and none as glucuronic acid. Unfortunately, there were no iduronic acid standard available at the time of analysis and could therefore not be included, one of the unidentified peaks could therefore correspond to iduronic acid. What remains unknown is if the sulfate groups present on the xylose and rhamnose affect the elution times or if the influence is minor. The remaining peaks could thus be attributed to sulfated monosaccharides which there is no standards available on the market to date. Furthermore, as the glucuronic acid standard peak did not correspond to any of the products this seem to indicate that WFp-105 has some substrate specificity in that it doesn't catalyze the hydrolysis of one of the ulvanobiuronic acids. The reason no glucuronic acid was present among the products could also be attributed to the substrate. It could be that there were no oligosaccharides with unsaturated glucuronic acid at the non-reducing end present. To analyze substrate specificity there is again need of pure unsaturated di- or oligosaccharides from ulvan. Regardless, accumulated results allow the suggestion that recombinant WFp-105 produces xylose, xylose 3-sulfate and rhamnose 3-sulfate when hydrolyzing a mixture of unsaturated oligosaccharides from ulvan.

WFp-37

Recombinant protein expression and optimization

Initial expression and affinity purification trials of his-tagged WFp-37 resulted in determination of low solubility of this ulvan lyase attributed of only sporadically characterized family with no clear comprehension neither on structural organization neither catalytic site architecture. In context of this study an attempt of WFp-37 solubility increment by protein sequence fusion with GST-tag was performed. Affinity tag was beneficial for WFp-37 folding as was indirectly confirmed by determined activity of recombinant protein in crude clarified soluble protein fraction. However, WFp-37 remain aggregation prone if purified. Buffer optimizations provide a solution for the aggregation problem as there is no problem with solubility in the cell lysate. Other solubility tags that enhance solubility of proteins in *E. coli* could also be an alternative.

The different cell lines showed a clear difference in protein yield, both cultivations of Tuner cells provided a higher amount of soluble protein after disruption of the cells. The cultivation temperature of the Tuner cells also seemed to have an influence on the amount of protein in the soluble phase compared to the insoluble phase where the higher temperature showed a clear increase. When working with PL37 in the future Tuner cells seem to give the best results in terms of yield and also the amount of protein left in the soluble phase. Those are also the samples that show the highest activity in the rough activity trials.

Genomic context analysis

The genomic organization analysis of WFp-37 gene indicated that the transcription of WFp-37 happens in conjunction with the five upstream proteins present in the genome suggesting gene attribution to an operon. Having in mind that several PUL were identified in *W. fucanilytica* genome it would be likely that the genome of this microorganism also contains additional PUL for ulvan utilization with WFp-37 as ulvan lyase.

Future product determination

Since activity of recombinant WFp-37 was successfully confirmed in the cell lysate a preliminary characterization of the reaction products could be performed by separating the products on TLC and then analyzing the bands of the WFp-37 rough trials using for example NMR to see which oligosaccharides are produced this analysis would be allowing accumulate valuable characterization data on novel lyase family PL37.

Conclusion

W. fucanilytica has several enzymes contributing to the disruption of ulvan polysaccharides, two of which are WFp-105 and WFp-37. The glucoside hydrolase enzyme WFp-105 most likely belong to the CAZy family GH105 due to the high homology as well as the conserved regions within both the amino acid sequence as well as the potential modeled 3D-structure. In terms of WFp-105 it is possible to recombinantly express, cultivate and purify soluble and active protein with a preference for lower temperatures and acidic conditions. The potential to utilize WFp-105 to produce rare sugars such as rhamnose and iduronic acid need to be further researched but the results seem to indicate that there is a possibility of success with the use of WFp-105 in combination with an ulvan lyase that provides short, unsaturated oligosaccharides to produce rare monosaccharides. The polysaccharide lyase WFp-37 was not possible to produce as a soluble protein after purification using GST as a solubility tag. There is activity present pre purification showing the future potential of the protein. With optimization of binding and elution buffers or the inclusion of other solubility tags it is likely that it is possible to achieve pure, active and soluble enzyme.

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Appendix

DNS assay results

Table A, B and C represents omitted results of initial activity measurement of recombinant glycoside hydrolase WFp-105 using DNS method. Initial activity measurement results were omitted due to statistically low reliability.

Table A. Calibration curve for DNS reagent with xylose used as a standard

Concentration	0mM	1mM	3mM	5mM	7mM	10mM	15mM	20mM
Absorbance (A)	0,118	0,236	0,820	1,42	2,03	2,75	3,80	>6
	0,120	0,223	0,832	1,43	2,10	2,72	3,67	>6
	0,119	0,226	0,749	1,38	2,00	2,79	3,93	>6

Table B. Initial activity measurement results of WFp-105 performing reaction at 40°C for 24 h.

Sample	Blank			Reaction		
Absorbance	0,769	0,784	0,771	0,864	0,839	0,865

Table C. Initial activity measurement results of trials for WFp-105 performing reaction incubated at 25°C for 24 hours.

Sample	Blank				Reaction						
Absorbance	0,740	0,750	0,769	0,772	0,810	0,833	0,818	0,792	0,801	0,803	0,821

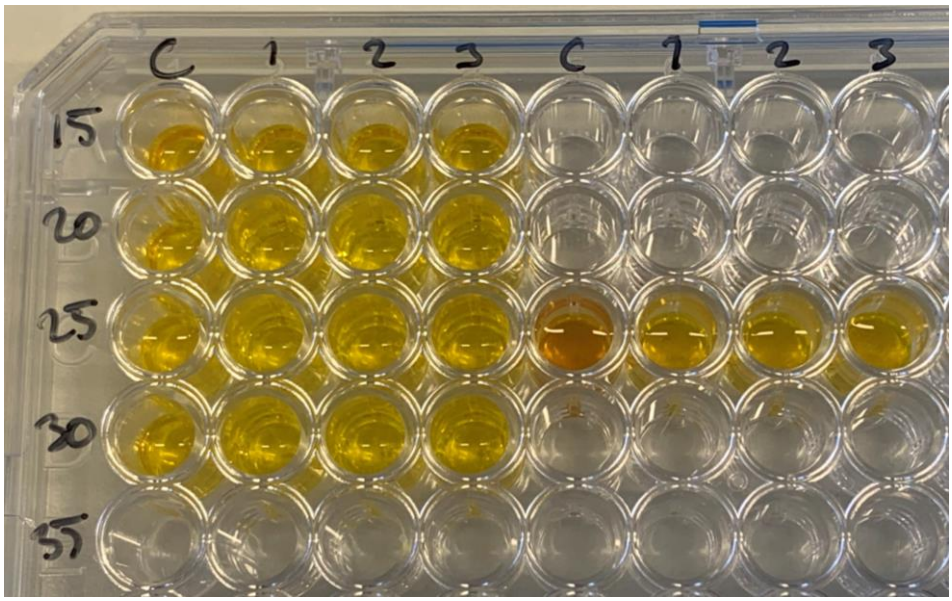

Figure A. Image of 96-well plate with WFp-105 reaction mixtures as well as controls after DNS reagent development. Fifth column, third row represents the blank control samples indicating of a higher amount of reducing ends in comparison with reactions.