CONSTRUCTION AND CHARACTERIZATION OF A XYLANASE CELL SURFACE DISPLAY SYSTEM USING ICE NUCLEATION PROTEIN IN *Escherichia coli* FOR DEGRADATION OF PINEAPPLE HEMICELLULOSE

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ABSTRACT

Cell surface display is a method of anchoring enzymes on the surface of cells. It can be used as a whole-cell biocatalyst to catalyse the breakdown of substrates extracellularly. An anchoring motif is an essential tool for the construction of a surface display system in cells. By far, ice nucleation protein (INP) has been among the most successful anchoring motifs studied. However, some problems still arise in relation to its limited expression on the cell surface and also its stability during display. Therefore, exploring a wider selection of INP anchors could be useful in providing one with better surface display efficiency. An INP from *Erwinia ananas*, InaA, is the anchor employed in this study for the functional display of xylanase enzyme (EC 3.2.1.8). The surface display using InaA fused to xylanase (InaAxyl) was compared with two other established INPs and gave the highest enzyme activity of 92.2 U/g dry cell weight which was up to three times higher than the other two INPs used. The proper expression of InaAxyl on the surface of the cell was confirmed by SDS-PAGE, Western blot, immunofluorescence microscopy and flow cytometry analysis. Quantitative data from flow cytometry showed that surface anchoring using InaA was up to four times more effective than the other two constructs used. Conditions for expression of InaAxyl were optimized using one-factor-at-a-time (OFAT) method. The conditions are post-induction harvest time of 8 h using LB medium with 0.3 mM inducer concentration and agitation rate of 200 rpm at 25 ˚C. The cell surface display system was then used for the hydrolysis of hemicellulose from pineapple waste. The degradation of lignocellulosic biomass has not been done using a bacterial surface display system before. After subjecting the raw pineapple waste to pretreatment for the breakdown of lignin, hemicellulose extraction was carried out. The hemicellulose extracted pineapple was analysed using Fourier-transform infrared spectroscopy which confirmed the successful extraction of hemicellulose. The morphology of the pineapple waste before and after hemicellulose extraction was also studied using field emission scanning electron microscope. The rough surfaces of the recalcitrant lignin structure before the pretreatment changed to smooth after the extraction of hemicellulose. Then, screening of reaction conditions for InaAxyl with pineapple waste was studied using OFAT. Optimization for pH, cell loading and temperature of reaction was investigated using response surface methodology Box-Behnken design in the DESIGN EXPERT software. A total of 2.129 mg/ml of reducing sugar was produced under the optimized conditions of pH 7.5 using 100 g/L wet cell weight of cells at 30 ˚C. High performance liquid chromatography (HPLC) was used for qualitative and quantitative analysis to determine the type and amount of xylooligosaccharides (XOS) produced from the reaction. The XOS detected were xylobiose and xylotriose with a total yield of 5.4 mg/g of pineapple substrate. Based on the results of this study, it can be concluded that InaAxyl was well expressed on the cell surface in its active and stable form. The cell surface display system successfully degraded pineapple waste into XOS.

ABSTRAK

Paparan permukaan sel merupakan kaedah untuk melekatkan enzim pada permukaan sel. Kaedah ini boleh digunakan sebagai mangkinbio keseluruhan sel bagi memangkinkan pemotongan substrat di luar sel. Motif pelekatan adalah amat penting dalam pembinaan sistem paparan permukaan sel. Sehingga kini, protein penukleusan ais (INP) merupakan antara motif pelekatan yang paling berkesan yang telah dikaji. Namun, masih terdapat masalah yang timbul berhubung dengan pengekspresian terhad di permukaan sel dan kestabilan ketika dipaparkan. Oleh itu, penerokaan motif pelekatan INP dari sumber yang lebih luas mungkin dapat menemukan INP yang lebih efisien dari segi paparan permukaan pada sel. INP daripada *Erwinia ananas*, InaA, ialah motif pelekatan yang digunakan dalam kajian ini untuk paparan berfungsi enzim xilanase (EC 3.2.1.8). Paparan permukaan menggunakan InaA yang digabungkan dengan xilanase (InaAxyl), memberikan aktiviti enzim yang hampir tiga kali ganda lebih tinggi iaitu 92.2 U/g berat sel kering berbanding dengan dua INP lain yang digunakan. Pengekspresian InaAxyl pada permukaan sel telah disahkan dengan analisis SDS-PAGE, Western blot, mikroskopi imunopendarfluor, dan aliran sitometri. Data kuantitatif aliran sitometri menunjukkan motif pelekatan InaA adalah hampir empat kali ganda lebih efektif berbanding konstruk INP yang lain. Keadaan pengekspresian InaAxyl telah dioptimumkan menggunakan kaedah satu faktor pada satu masa (OFAT). Keadaan yang dioptimumkan ialah masa selepas aruhan iaitu 8 jam menggunakan medium LB dengan kepekatan pengaruh 0.3 mM dan kadar pengadukan 200 rpm pada suhu 25 ˚C. Sistem paparan permukaan sel ini kemudiannya digunakan untuk menghidrolisis hemiselulosa daripada sisa nanas. Sehingga kini, masih tiada kajian yang dilakukan terhadap degradasi biojisim lignoselulosik menggunakan sistem paparan permukaan pada bakteria. Setelah sisa mentah nanas melalui proses prarawatan untuk membuang lignin, pengekstrakan hemiselulosa telah dijalankan. Bahagian hemiselulosa nanas yang telah diekstrak kemudiannya dianalisa menggunakan spektroskopi inframerah jelmaan Fourier yang mengesahkan kejayaan proses pengekstrakan tersebut. Morfologi sisa nanas sebelum dan selepas pengekstrakan hemiselulosa dikaji menggunakan mikroskop pengimbasan elektron pancaran medan. Permukaan lignin yang kasar sebelum prarawatan berubah menjadi licin selepas pengekstrakan hemiselulosa. Kemudian, penyaringan parameter tindak balas InaAxyl dengan sisa nanas dikaji dengan kaedah OFAT. Pengoptimuman bagi pH, kepekatan sel dan suhu dikaji menggunakan kaedah sambutan permukaan dengan reka bentuk Box-Behnken daripada perisian DESIGN EXPERT. Jumlah gula ringkas yang terhasil ialah 2.129 mg/ml pada keadaan optimum iaitu pH 7.5 menggunakan 100 g/L berat sel basah pada suhu 30 ˚C. Kromatografi cecair prestasi tinggi (HPLC) digunakan untuk analisis kualitatif dan kuantitatif bagi menentukan jenis dan kuantiti xilooligosakarida (XOS) yang terhasil daripada tindak balas ini. XOS yang dikesan ialah xilobiosa dan xilotriosa dengan hasil 5.4 mg/g substrat nanas. Berdasarkan kajian ini, dapat disimpulkan bahawa InaAxyl telah diekspres dengan baik pada permukaan sel dalam keadaan enzim yang aktif dan stabil. Sistem paparan permukaan sel yang digunakan telah berjaya menukarkan sisa nanas kepada XOS.

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LIST OF ABBREVIATIONS

BBD	-	Box-Behnken Design
BG	-	Bacterial ghost
bp	-	base pair
BPP	-	Beta-propellar phytase
BSA	-	Bovine serum albumin
CRD	-	Central repeating domain
DCW	-	Dry cell weight
DF	-	Dilution factor
DMSO	-	dimethlysulfoxide
DNA	-	deoxyribonucleic acid
DNS	-	3,5-dinitrosalicylic acid
FESEM	-	Field emission scanning electron microscope
FOSHU	-	Food for Specified Health Use
FTIR	-	Fourier Transform infrared spectroscopy
GFP	-	Green fluorescent protein
GPI	-	Glycosylphosphatidylinositol
GRAS	-	Generally regarded as safe
HPLC	-	High performance liquid chromatography
INP	-	Ice nucleation protein
IPTG	-	isopropyl β-D-1-thiogalactopyranoside
kb	-	kilo base
LB	-	Luria-Bertani
MW	-	Molecular weight
OFAT	-	One-factor-at-a-time
OMP	-	Outer membrane protein
PAA	-	Peracetic acid
PBS	-	Phosphate buffered saliine
PCR	-	Polymerase chain reaction
PEG	-	Polyethylene glycol
RMS	-	Response Surface Methodology

SDS-PAGE	-	Sodium-dodecyl sulphate polyacrylamide gel electrophoresis
SOB	-	Super optimal broth
TB	-	Terrific broth
M9	-	Minimal medium
TSS	-	Transformation storage solution
t	-	time
v/v	-	volume per volume
w/v	-	weight per volume
X1	-	xylose
X2	-	xylobiose
X3	-	xylotriose
X4	-	xylotetraose
X5	-	xylopentaose
X6	-	xylohexaose
XOS	-	xylooligosaccharide

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CHAPTER 1

INTRODUCTION

1.1 Background of Research

The use of microorganisms in industrial processes is rapidly growing. In 2010, biotechnological processes products amount to 91.9 billion Euros while the projected amount of sales for these products are about 432 billion Euros in the year 2020. About 5% of the volume of biotechnological products is from the usage of biocatalyst (Schüürmann *et al.*, 2014). These figures highlight the growing potential of biocatalyst in industrial biotechnology and the room for its growth and improvement. Since 1990, many enzymes were commercialized mostly for the production of fine chemicals for use in pharmaceutical industry, plant protecting agents, and fragrances (Reetz, 2013). Currently, various strategies are being applied for extracellular enzyme production. Extracellular protein production is especially important if the substrates are large polymers resulting in difficult passage through the cell surface and its minimal uptake (Ni and Chen, 2009). Thus, if intracellular enzymes are produced, contact with substrates becomes an issue. Besides that, complex bioprocess is avoided through extracellular enzyme production and the quality of enzyme is preserved (Ni and Chen, 2009).

One of the methods for extracellular protein production is to fuse the target protein with an anchoring motif to create a cell surface display system (Lee *et al.*, 2003). Cell surface display is the presentation of the target protein on the surface of cells (van Bloois *et al.*, 2011). This method is particularly useful as enzyme fused to the cell can be easily separated from the medium of reaction through simple methods (Schüürmann *et al.*, 2014). Surface display of enzymes reduces the complexity of bioprocess as it eliminates the time and cost needed for enzyme purification (Dong *et al.*, 2020). Complex substrates which cannot pass through the complex membrane barriers of Gram-negative bacteria in order for reaction to take place can also be

overcome. Among the other applications of cell surface display are the development of live vaccines (Lee *et al.*, 2000), bioremediation (Shimazu *et al.*, 2001a) and high-throughput screening of peptide libraries (Boder and Wittrup, 1997).

A potential use of cell surface display is in the hydrolysis of lignocellulosic biomass. Lignocellulosic biomass is plant material made up mainly of cellulose, hemicellulose and lignin. It includes forestry residues, waste from agriculture, and grasses which are produced in abundance from agro-industries (Anwar *et al.*, 2014). Many industries underutilize lignocellulose as only a portion of the biomass is used for industrial processes while the unused lignocellulose streams are gone to waste (Mathews *et al.*, 2015). These streams have the potential to be used for bioconversion into useful products. Hemicellulose is the second most abundant polymer in lignocellulose and is made up of sugars such as xylan, arabinan, galactan and mannan with xylan being the most abundant (Beg *et al.*, 2001; Mathews *et al.*, 2015). Xylan, when hydrolysed by xylanolytic enzymes yields xylooligosaccharides (XOS) which is a useful product for various industries. The main enzyme used in the hydrolysis of xylan is xylanase (E.C. 3.2.1.8). Therefore, for the hydrolysis of xylan using cell surface display, xylanase has to be anchored to the surface of the cell to catalyse the reaction.

Xylanases can be obtained from many sources such as algae, yeast, bacteria and fungi. Xylanases from fungi has drawn the attention of researchers because it is secreted into the medium for reaction and is produced in high amounts (Polizeli *et al.*, 2005). Enzymatic hydrolysis of xylan has several benefits such as milder reaction conditions and side reactions which produce unwanted byproducts can be avoided (Motta *et al.*, 2013).

In this study, a cell surface display system using ice nucleation protein (INP) as an anchor to display xylanase for the hydrolysis of xylan in pineapple was constructed. INP is an outer membrane protein that can be found in ice nucleation active bacteria such as Pseudomonas, Xanthomonas and Erwinia. This protein catalyzes the formation of ice in supercooled water (Vali *et al.*, 1976). The first study on the surface display ability of INP in *E. coli* was done in 1997 where levansucrase

was successfully displayed on the surface of the cell (Jung *et al.*, 1998a). After this discovery, the display of other enzymes using INP followed such as carboxymethylcellulase (Jung *et al.*, 1998b), organophosphorus (Shimazu *et al.*, 2001a) and transglucosidase (Wu *et al.*, 2006b). To the best of our knowledge, cell surface display using INP for xylanase enzyme has yet been done. Besides that, INP from *Erwinia ananas*, InaA, which has not been reported for cell surface display was used in this study.

1.2 Problem Statement

The use of enzymes as catalysts in chemical reactions bears advantages such as its high specificity for substrates and mild reaction conditions. In many bio-based production processes, purified enzymes are used as catalyst. Unfortunately, enzyme purification is an expensive process and it also takes up time (Schüürmann *et al.*, 2014). The use of microbes as whole-cell biocatalyst is able to solve this problem as enzymes need not undergo further purification procedures.

On the other hand, whole-cell biocatalyst with enzymes produced intracellularly requires the substrate to penetrate the membrane of the cells, especially Gram-negative bacteria. This is particularly difficult if not impossible for large substrates such as polysaccharides to pass through the membrane barrier (Muñoz-Gutiérrez and Martinez, 2013). Besides that, a cell produces many enzymes with reactions that might interfere with the desired reaction (Schüürmann *et al.*, 2014).

In order to address this problem, a cell surface display system where the targeted protein is presented on the surface of cells for the reaction to occur extracellularly was designed. Surface display of enzymes allows contact with the substrate without the need of the substrate to pass the membrane barrier and can also be easily separated from the reaction mixture. Hitherto, INP has been used for the display of many enzymes and it has been found that in some cases, transportation of intracellularly expressed INP-enzyme to the surface of the host cell was limited (Li *et*

al., 2012; Li *et al.*, 2004). Besides that, the instability of the anchoring motif caused by proteolytic degradation also affected the efficiency of the biocatalyst (Li *et al.*, 2009). Therefore, INP variants from other sources should be explored to address this problem.

1.3 Objectives

The objectives of this research are:

(a) To construct and analyse the performance of INP from *Erwinia ananas*, InaA, as a surface display system for enzymes in *E. coli*.

(b) To evaluate the effect of expression parameters on performance of the constructed system.

(c) To study the reaction parameters for conversion of pineapple hemicellulose to XOS using cell surface display of xylanase in *E. coli*.

1.4 Scope

The scope of study listed below will be used to achieve the objectives stated.

(a) The first objective was defined with the following scope; Construction of plasmid containing INP and xylanase enzyme through molecular cloning. Analysing and confirming surface display on the outer membrane of *E. coli* and its stability using fluorescence microscopy and flow cytometry.

(b) The scope for the second objective was optimising conditions for expression by studying the effects of (1) post-induction time harvest time, (2) type of medium, (3) inducer concentration, (4) temperature of expression, and (5) agitation rate on surface display of xylanase.

(c) The final objective has the following scope; Screening for reducing sugar from reaction of surface displayed xylanase using one-factor-at-a-time on a few parameters: (1) cell loading, (2) pH, (3) agitation rate, (4) substrate loading, and (5) temperature. Optimisation of reaction using cell surface displayed xylanase on the hydrolysis of pineapple pomace by Box-Behnken Design (BBD) for maximum amount of reducing sugar produced. Analysing the quantity of XOS production from pineapple pomace after reaction at optimum conditions.

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LIST OF PUBLICATIONS

Journal with Impact Factor

1. **Wee, M. Y. J.**, Murad, A. M. A., Bakar, D. D. A., Low, K, O., and Illias, R. M. (2019). Expression of xylanase on *Escherichia coli* using truncated ice nucleation protein of Erwinia ananas (InaA). *Process biochemistry*. 78, 25-32. https://doi.org/10.1016/j.procbio.2019.01.005 **(Q2, IF:2.952)**

Conference Proceedings with ISBN

1. **Wee, M. Y. J.,** Illias, R. M. (2015). Functional Cell Surface Display using Ice Nucleation Protein from *Erwinia ananas* on *Escherichia coli*. In *17th International Conference on Bioinformatics and Molecular Biology (ICBMB)* 3-4 December 2015. Bayview Hotel, Georgetown, Penang, Malaysia.

2. **Wee**, **M. Y. J.**, Illias, R. M., (2016). Reaction Optimization of Cell Surface Display of Xylanase on *Escherichia coli*. In *3rd International Conference on Chemical, Biological, and Environmental Sciences, ICCBES'15,* 31 Decermber 2015-1 January 2016. Hotel Mercure Bangkok Siam, Bangkok, Thailand.

Appendix A

Gene sequences and amino acid sequences

Appendix A1: InaA from *Erwinia ananas* IN-10

A1.1: Gene sequence

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>gi|296095|emb|X17316.1| E.ananas inaA gene
CCCGGGTTTTTGCCGAATCGGATACCCAGCCGCAGCAAAGTCATGTTTGCCGATCATCTGCTGCGCTA
TGTGCCGCTGGCCGCCTTGATTAAAACCGTGCTGGATGAACGTACCCGGTCATCGTGCGCCAACGCAT
GCGTTTCGAGCAGGAGCTTGGAAGACTCACTGGCTGACAGGCGCCAAAGAGACGCTGGACAGCGATCA
ACTGGGGCCGCTATGCAGAGATCTTCTCTTATAACGATCAGACCGAATATTTTAGTCTGGAAGACGTT
GAGTTTTAATTCTGCTCGCTCAGCAAAACGTTGCGCTATCCCTTACACCATCCCGTTACTCTGCACGG
GATGGTGCCTGAAAGATTCTCAAGGCAATAATCCTTTCTGAAAGATAAATAAACGCGGTTTACACTGT
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TGAATATTTAATTCTGTTTTATTTATTAATTTTATGGTTTCTGAAAATTAAGTGGCGGTAATTTATTA
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TAAGCGGTATCGTAGAGTGTAAATACTGGAAGCCGGTTAAAGGCTTTGAGAACGGACTAACGGGGCTA
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GGCTGCCGATGAGTGTATCGCTATTGAAACTCATGGCTGGATTAAATTTCCCCGTGCTGAGGTTCTTC
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GAAATGCAGGCCGGGCCTGGAGGGCCTGATGTCACTTCAGAGGCAAAGGCAGGCAATCGATCGTTGCC
TGTAACAGATGATATTGATGCGACCATCGAATCAGGCAGTACGCAGCCGACACAAACGATTGAAATCG
CAACCTATGGCAGTACGCTCAGCGGCACGCATCAGAGTCAGCTGATTGCCGGATATGGCAGTACTGAG
ACGGCGGGTGATAGCAGCACATTAATTGCCGGTTATGGTAGCACCGGTACAGCGGGATCAGACAGCAC
ATTAGTCGCGGGCTACGGTAGTACCCAAACCGCAGGTGAAGAGAGCAGCCAGATGGCGGGTTACGGCA
GCACGCAGACCGGCATGAAAGGCAGCGATCTCACCGCCGGTTACGGCAGTACCGGCACGGCGGGCGCC
GACAGCTCCCTGATCGCCGGTTACGGCAGTACCCAGACGGCGGGCGAAGACAACTCGCTGACAGCCGG
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CGCAGAAGGGCAGCGATCTTACGGCCGGTTATGGCAGTACCGGCACGGCGGGTGCCGACAGTTCATTA
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TGCCGACAGTTCATTAATTGCGGGCTACGGCAGCACCCAGACGGCCGGGGAAGAGAGCACCCAGACAG
CCGGTTATGGCAGCACCCAGACCGCGCAGAAGGGCAGCGACCTTACGGCCGGTTACGGCAGTACCGGT
ACGGCGGGTGACGACAGCTCCCTGATCGCCGGTTACGGCAGTACCCAGACCGCGCAGAAGGGCAGCGA
TCTTACGGCCGGTTATGGCAGTACCTCTACGGCAGGCTATGAAAGTTCATTGATCGCGGGCTATGGCA
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GAAAGCGATCTCATCACCGGCTATGGCAGTACGTCTACCGCCGGGGCAAATAGTTCCCTGATCGCAGG
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CGATCGCAGCAAACTCACGGCAGGCAATGACTGCATCCTCATGGCGGGAGACCGAAGCAAGCTGACGG
CGGGCATCAACAGCATTCTCACCGCCGGATGCCGTAGCAAGCTGATAGGAAGCAATGGTTCAACCCTG
ACCGCCGGGGAAAACTCAGTTCTGATTTTTCGCTGCTGGGATGGAAAGCGCTACACTAATGTGGTCGC
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ATCCCTAACCCCGGCTTGTCATTGACGTAAGCATAGCCCTGATCCACTTCAGGACAGCCCGGGAAGAC
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A1.2: Amino acid sequence

>sp|P20469|1-1322

MKEDKVLILRTCANNMADHGGIIWPLSGIVECKYWKPVKGFENGLTGLIWGKGSDSPLSLHADARRVV
AEVAADECIAIETHGWIKFPRAEVLHVGTQNSAMQFILHHRADYVACTEMQAGPGGPDVTSEAKAGNR
SLPVTDDIDATIESGSTQPTQTIEIFRCWDGKRYTNVVAKTGKGGIEADMPYQMDEDNNIVNKPEE

Underlined : sequence for N-terminal domain
Dashed undeline : sequence for C-terminal domain
Bold : stop codon (not included in synthetic gene)

(Markings/legends are applied to all sequences in Appendix A)

Appendix A2: InaK from *Pseudomonas syringae* KCTC 1832

A2.1: Gene sequence

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>gi|2331278|gb|AF013159.1| Pseudomonas syringae ice nucleation
protein (inaK) gene, complete cds
AGATCTGTCGCGCGCGACGGATCGATCAGCGTCTGGTGCTGTATGTCGAGCACTACCTGAGACCGGAG
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GGTGGAATCCAGATACTGGCAGTCAACCAGGCGGCATGAGAATGGTCTGGTCGGTTTACTGTGGGGCG
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CAGGATCAGCGCGTCACACTTCATTTCGGCACGTCAGGCCGACCCTGCGTCAACGTCAACGTCAACGT
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CGGGAAGTGACAGCGCCCTGACCGCAGGCTATGGCAGCACGCAAACGGCCCAGGAAGGCAGCAATCTC
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GCAATCTGACGGCGGGGTACGGCAGCACGGGTACAGCAGGTGTCGACAGTTCGTTGATCGCCGGATAT
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A2.1: Amino acid sequence

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CEVAVADIISLEEPGMVKFPRAEVVHVGDRISASHFISARQADPASTSTSTSTSTLTPMPTAIPTPMP
AVASVTLPVAEQARHEVFDVASVSAAAAPVNTLPVTTPQNLQTFRLWDGKRYRQLVARTGENGVEADI
PYYVNEDDDIVDKPDEDDDWIEVK
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Appendix A3: InaZ from *Pseudomonas syringae* S203

A3.1: Gene sequence

>gi|45828|emb|X03035.1| Pseudomonas syringae S203 ice nucleation gene

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TATTGTCGATAAACCCGACGAGGACGATGACTGGATAGAGGTAAAG**TAG**CCCGCGTTATTCAAGCACT
CCACGACTGAATCATCGACAGGGCGCGTCGATGGAATTC

A3.2: Amino acid sequence

>sp|P06620|ICEN_PSESY Ice nucleation protein OS=Pseudomonas syringae pv. syringae GN=inaZ PE=1 SV=1
MNLDKALVLRTCANNMADHCGLIWPASGTVESRYWQSTRRHENGLVGLLWGAGTSAFLSVHADARWIV
CEVAVADIISLEEPGMVKFPRAEVVHVGDRISASHFISARQADPASTSTSTLTPMPTAIPTPMPAVAS
VTLPVAEQARHEVFDVASVSAAAAPVNTLPVTTPQNVQTATYGSTLSGDNHSRLIAGYGSNETAGNHS
DLIAGYGSTGTAGSDSWLVAGYGSTQTAGGDSALTAGYGSTQTAREGSNLTAGYGSTGTAGSDSSLIA
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Appendix A4: Xylanase from *Aspergillus fumigatus* RT-1

A4.1: Gene sequence

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>gi|305377695|gb|GQ458016.1| Aspergillus fumigatus strain RT-1
endoxylanase (xynG1) gene
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GAGAAGTATGCGTCCTCTCCCTGCTTGTTAGGTTCAAGCTAATGGATTCAGAGCGGTCACCTACAGCG
GCTCCTGGCAGACCAGCGGAAACGGCTACCTCTCCGTGTACGGCTGGACGACCAGTCCGCTGGTCGAA
TTCTACATCGTGGAGAGTTACGGCTCCTATGACCCCTCCACGGGAGCCACCCATCTCGGCACCGTCGA
GAGCGACGGGGCCACGTACAACCTCTACAAGACGACGCGGACGAATGCGCCGTCCATCCAGGGCACGG
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GGGGTACCAGAGCAGCGGCTCTGCTACTATCACTGTTTCT
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Appendix B

Mediums and buffers for experimental work

Appendix B1: Buffers

1L PBS buffer

8 g NaCl

0.2 g KCl

1.78 g $Na_2HPO_4.2H_2O$

0.24 g KH_2PO_4

Autoclave

Buffers for screening of cultural conditions for hydrolysis of pineapple pomace

	0.1 M acetic acid	0.1 M sodium acetate
pH 4	84.7	15.3
pH 5	35.7	64.3

	0.2 M NaH_2PO_4	0.2 M Na_2HPO_4
pH 6	87.7	12.3

	0.2 M glycine	0.2 M NaOH	dH_2O
pH 9	25	4.4	Top up to 100
pH 10	25	19.3	Top up to 100

Appendix B2: Medium

1 L SOB medium

20 g tryptone

5 g yeast extract

0.5 g NaCl

10 ml of 250 mM KCl

5 ml of sterilized 2 M $MgCl_2$ (added after autoclaving the above chemicals in dH_2O)

1 L TB medium

12 g tryptone

24 g yeast extract

4 ml glycerol

Autoclave the above chemicals in about 900 ml of dH_2O and allow to cool. Then add 100 ml of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 which have been autoclaved separately.

1 L M9 medium

200 ml 5× M9 salts

2 ml 1 M $MgSO_4$

20 ml 20 % glucose solution

0.1 ml 1 M $CaCl_2$

The 1 M $MgSO_4$ and 1 M $CaCl_2$ were prepared and autoclaved separately while the glucose solution was filter sterilized. The mixture was top up to 1 L with sterile dH_2O.

<u>1 L of 5× M9 salts</u>

64 g $Na_2HPO_4.7H_2O$

15 g anhydrous KH_2PO_4

2.5 g NaCl

5 g NH_4Cl

LB medium/agar

10 g tryptone

5 g NaCl

5 g yeast extract

1 % (w/v) agar

Appendix B3: Working solution for SDS-PAGE

2 X SDS-PAGE sample buffer

Tris-HCl pH 6.8	125 mM
Glycerol	20 %
SDS	4 %
Bromophenol blue	0.1 %

5% β-merchaptoethanol was added to the sample buffer before use.

15 % resolving gel	5 ml
H_2O	1.1 ml
30 % acrylamide	2.5 ml
1.5 M Tris (pH 8.8)	1.3 ml
10 % SDS	0.05 ml
10 % ammonium persulphate	0.05 ml
TEMED	0.002 ml

5 % stacking gel	2ml
H_2O	1.38 ml
30 % acrylamide	0.33 ml
1 M Tris (pH 6.8)	0.25 ml
10 % SDS	0.02 ml
10 % ammonium persulphate	0.02 ml
TEMED	0.002 ml

Staining solution A

50 % ethanol

10 % acetic acid

Staining solution B 50 ml

5 % ethanol

7.5 % acetic acid

200 µl of 0.25 % solution of Coomassie brilliant blue in 95 % ethanol

Appendix B4: Working solution for Western blot

Transfer buffer

25 mM Tris base

192 mM glycine

10 % methanol or isopropanol

10 × TBS 250 ml

6.05 g Tris base pH 7.6

21 g NaCl

1 × TBST 500 ml

1 × TBS + 500 µL of 0.1 % Tween 20

Blocking buffer 30 ml

5 % non-fat dry milk to 30 ml 1 × TBST

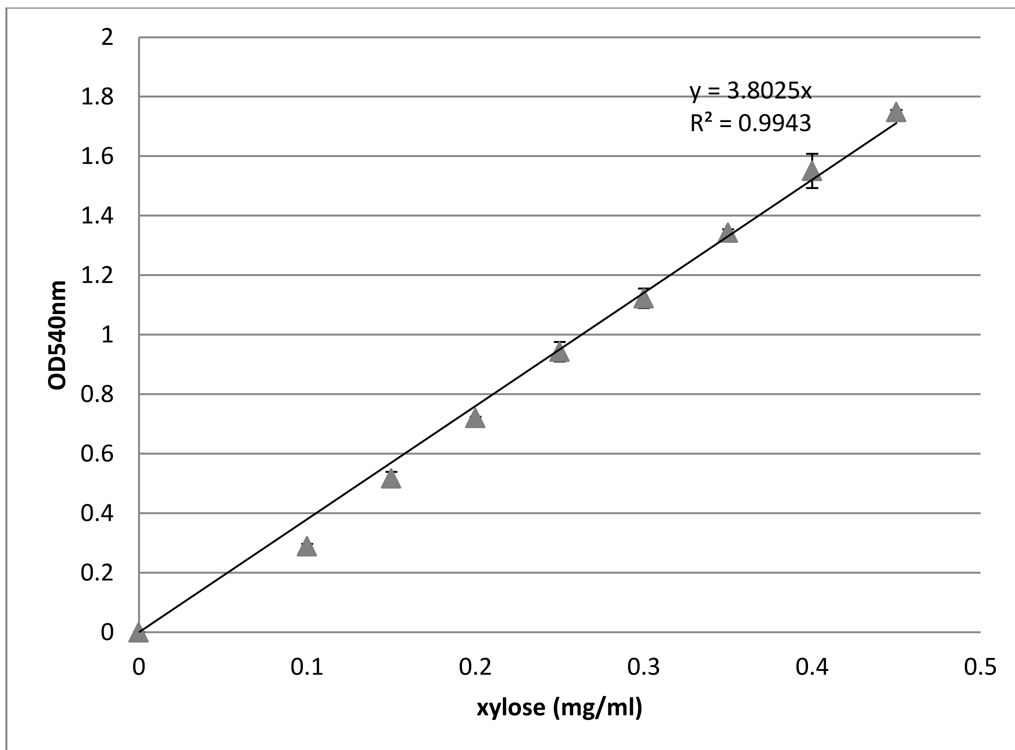
OR

3 % BSA in 1 × TBST

Appendix C

Graphs of standard curves

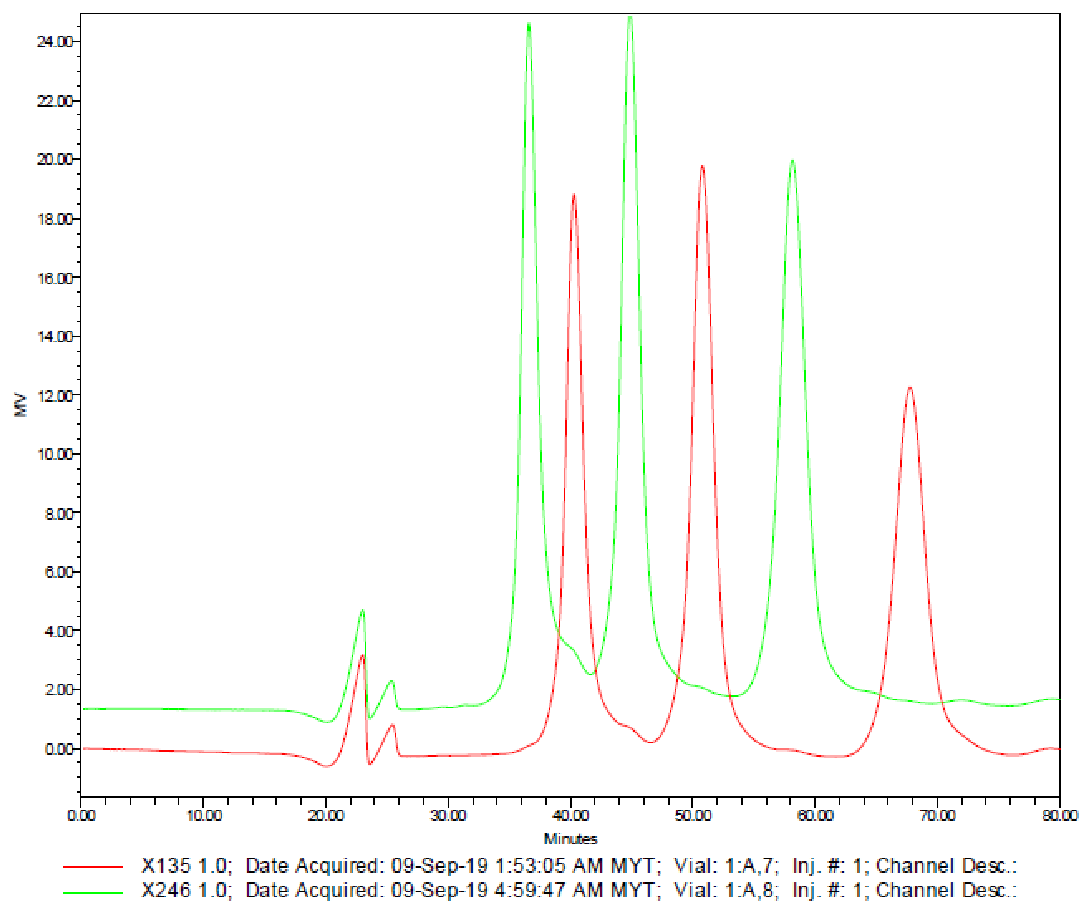
Appendix C1: Standard curve for reducing sugar assay (using xylose)



One of the standard curves used for DNS assay.

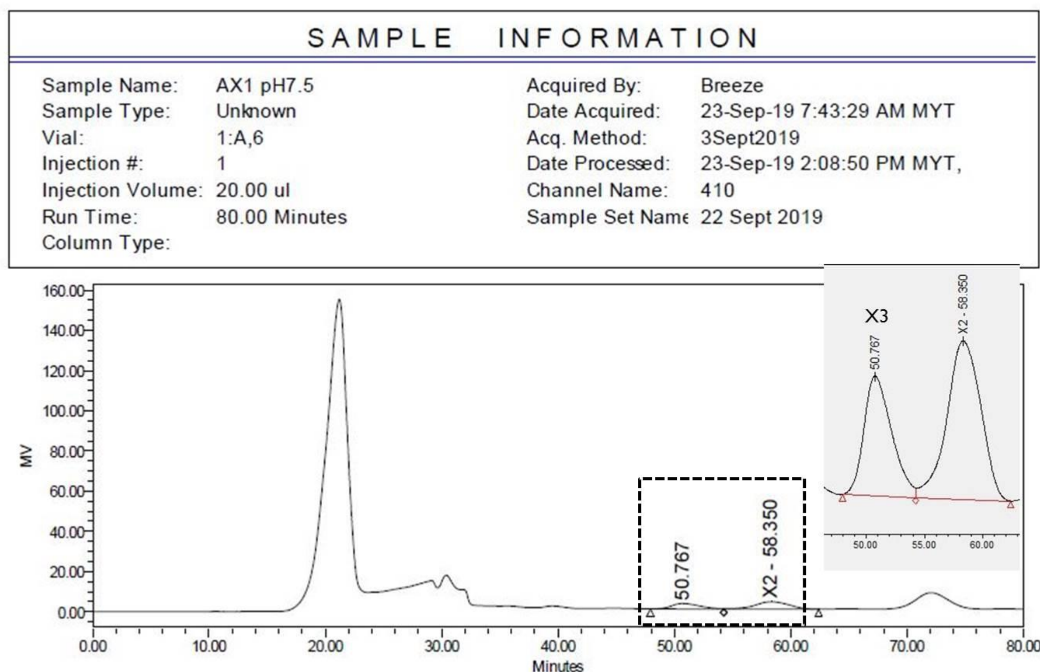
Appendix C2: XOS standard peaks using HPLC

UTM
Project Name Joanne
Reported by User: Breeze user (Breeze)



X135 refers to the combined XOS of xylose, xylotriose and xylopentaose in a single vial with equal concentration of 1 mg/ml while X246 refers to the combined XOS of xylobiose, xylotetraose and xylohexaose with equal concentration of 1 mg/ml.

Appendix C3: Example of chromatogram for HPLC result



Product of reaction using InaAxyl and pineapple pomace. X2 refers to xylobiose while X3 refers to xylotriose. The Figure embedded is the zoomed peak of X2 and X3.