Production, purification and characterization of a multifunctional, thermostable and acido/alkaline stable putative xylanase from the psychrotrophic bacterium, *Sphingomonas aerolata*

A thesis submitted in fulfilment of the requirements for the degree of

Master of Science (Biochemistry)

At

Rhodes University

By

Brian Nkanyiso Mathibe

Department of Biochemistry and Microbiology, Rhodes University, 6140

Grahamstown, South Africa

Dedication

I dedicate this thesis to my late father, Christopher Mathibe, who found me absconding from school in the first grade and whipped me all the way to class, and now, two decades or so later, I am submitting a thesis in fulfilment of the requirements for a Master of Science degree. Thank you Timer, for knowing what was best for me when I didn't. It breaks my heart that you never got to see me walk across the stage in a graduation gown and get my first degree, but I know I have made you proud.

Acknowledgements

I would like to thank my partner, in life and in love, and all things in between, Andile Samantha Kheswa, for forcing me to download the application forms to Rhodes University when we were in high school, then forcing me to fill in the forms and write the application essays, then going through the forms herself to make sure I had filled them out properly, then dragging me to the post office to make sure I mailed them. I can never thank her enough for her unpaid work as my advisor, and planner, and caretaker, and cheerleader for 8 years…and still counting.

I am eternally grateful to my family; my uncle and aunt, Mr and Mrs Marko Mathibe, for making sure I finished high school; my siblings, Gugu and Ndaba, Sindi and Welly, Melissa and Nceku, for their support during my years at Rhodes; and my most precious mom, Sichelesile Tshili, for always being my rock, and for always believing in me.

I would be amiss to forget to thank my friends and labmates; Sello, Matthew, Litha, Glyn, Mariska, Sinoyolo, and Mpho, for their friendship and assistance during my time in the Enzyme Science (and Technology) Programme (ESP) lab. I would also like to thank my friends and gym buddies; David, Dalitso and Phillip for keeping me entertained in gym, for the intelligent conversations and debates, and for making sure I never crushed myself with heavy weights in the gym (except that one time they got distracted admiring each other's non-existent biceps). To my dear friend and colleague, Colleen Varaidzo Manyumwa, for the long discussions on science and research, for all her brilliant ideas and suggestions, and for all her input towards my research study, I shall forever remain in her debt. To my friend (who I always take for granted) Langa "Khandakhulu" Ngwenya, thank you for your friendship that has stood the test of time, distance, and silence (a lot of silence), especially during the past few years.

And to my amazing supervisors, Professor Brett I. Pletschke and Dr Samkelo Malgas, I shall forever be grateful for the opportunity I got to work under and learn from the most brilliant scientists that I have ever known. I am truly a better researcher now after having learned from the best. Thank you!

I would also like to thank the CSIR-DST Interbursary Programme for their financial support which allowed me to conduct this research study.

Table of Contents

Dedication i

Acknowledgements ii

Abstract vii

List of abbreviations ix

List of figures xi

List of tables xiv

Chapter 1: Introduction and Literature Review 1

1.1. Industrial sustainability through biocatalysis 1

1.2. Extremophiles 2

1.3. Psychrophiles 3

1.4. Properties of psychrophiles 4

1.4.1. Sphingomonas 4

1.5. Enzymes from psychrophiles 6

1.6. Lignocellulosic biomass 7

1.7. Xylanases 11

1.9. Problem Statement 14

1.10. Hypothesis 14

1.11. Aims and objectives 14

Chapter 2: Culturing of *S. aerolata* and the production and purification of its extracellular putative xylanase 16

2.1. Introduction 16

2.1.1. Aims and objectives 17

2.2. Materials and methods 18

2.2.1. Materials 18

2.2.2. Sub-cultivation of S. aerolata cells 18

2.2.3. Determination of optimum temperature for growth.....18
2.2.4. Screening for xylanase activity.....18
2.2.5. Xylanase activity assays.....19
2.2.6. Protein determination.....19
2.2.7. Growth and activity curves for xylanase production.....19
2.2.8. Induction of xylanase production using different carbon sources.....20
2.2.9. Purification of xylanase from the S. aerolata culture.....20
2.2.10. Electrophoresis.....20
2.3. Results.....22
2.3.1. Determination of optimum growth temperature for S. aerolata.....22
2.3.2. Screening for xylanase activity.....23
2.3.3. Growth and activity curves for xylanase production.....24
2.3.4. Induction of xylanase production using different substrates as carbon sources.....25
2.3.5. Purification of S. aerolata xylanase.....26
2.4. Discussion.....29
2.4.1. Conclusions.....31
Chapter 3: Biochemical characterization of the *S. aerolata* putative xylanase.....32
3.1. Introduction.....32
3.1.1. Aims and objectives.....33
3.2. Materials and methods.....34
3.2.1. Materials.....34
3.2.2. Determination of substrate specificity.....34
3.2.3. Determination of temperature optima and stability.....34
3.2.4. Determination of pH optima.....35
3.2.5. Effect of glucose, xylose and mannose on the activity of the putative xylanase.....35

3.2.6. Effect of various metal ions and chemical reagents on the activity of the putative xylanase..35
3.2.7. Analysis of the hydrolysis of cello-oligomers and xylo-oligomers by the putative xylanase using thin layer chromatography (TLC) 36
3.3. Results..37
3.3.1. Determination of substrate specificity 37
3.3.2. Determination of temperature optima and stability 38
3.3.3. Determination of pH optima 40
3.3.4. Effect of glucose, xylose and mannose on the xylanase and "Avicel-ase" activity of the putative xylanase..41
3.3.5. Effect of various metal ions on xylanase activity of the putative xylanase 42
3.3.6. Effect of EDTA and β-mercaptoethanol on xylanase activity of the putative xylanase 43
3.4. Discussion..44
3.4.1. Conclusions..47
Chapter 4: Enzyme kinetics and substrate competition 48
4.1. Introduction...48
4.1.1. Aims and objectives...51
4.2. Materials and methods 52
4.2.1. Materials 52
4.2.2. Determination of enzyme kinetic parameters 52
4.2.3. Substrate competition assays 52
4.3. Results...54
4.3.1. Determination of kinetic parameters..54
4.3.2. Substrate competition...61
4.4. Discussion...63
4.4.1. Conclusion 66

Chapter 5: General discussion, conclusions and future recommendations..............................67
5.1. General discussion 67
5.2. Conclusions...69
5.3. Future recommendations..69
References..70
Appendices..78
Appendix I: List of reagents 78
Appendix II: Standard curves 81

Abstract

Enzymes are biological catalysts produced by living organisms in order to increase the rate of chemical reactions that are essential for life. Enzymes are now applied as catalysts in a variety of fields in response to a worldwide shift towards sustainable industrial processes, with enzymatic processes having been found to be specific, fast and to result in savings in energy and raw materials, all while generating less hazardous wastes when compared to conventional chemical processes. Microorganisms, specifically fungi and bacteria, are the most common sources for the production of enzymes because they grow rapidly meaning the production of enzymes is also rapid and can be easily manipulated using genetic engineering to obtain enzymes with desired characteristics. Due to the harsh conditions required in most industrial processes, there have been growing interests in enzymes that can withstand such harsh conditions. It has been found that extremophiles, microorganisms that can survive and grow in extreme conditions that would otherwise be detrimental to other life forms, are a source of extremozymes, enzymes that can withstand harsh conditions and therefore be utilised industrially where production processes are often harsh. Psychrophiles are examples of extremophiles, having the ability to be prolific in extremely low-temperature environments, and have been found to produce cold-active enzymes that have high specific activity at low temperatures. Cold-active enzymes from psychrophiles are of industrial and biotechnological significance as they could potentially result in energy savings when applied in industries that do not require high temperatures such as the transformation of heat-sensitive substrates at low temperatures.

In this study, a psychrophilic bacterium, *Sphingomonas aerolata*, was successfully sub-cultivated in both solid and liquid media and found to grow optimally at 28°C on media composed of (w/v): peptone, 1%; NaCl, 0.5%; yeast extract, 1%, and hydrolysed potato starch, 1%; with 1.5% agar incorporated to the solid medium formulation. This bacterium was found to produce an extracellular xylan degrading enzyme, which was purified to homogeneity using ultrafiltration with Amicon molecular weight cut-off filtration units. This enzyme was purified to a fold purification of 4.2 with percentage yield of 14.29%. A single band corresponding to approximately 36 kDa was observed using SDS-PAGE analysis of the purified putative xylanase sample. The xylanolytic activity of the purified sample was confirmed by a band of xylan hydrolysis using non-denaturing native activity-PAGE analysis, with 0.1% (w/v) beechwood xylan incorporated into the resolving gel. However, biochemical characterization of the enzyme revealed it to be also active on Avicel cellulose (22.64 U/mg), hydrolysed potato

starch (19.11 U/mg), arabinogalactan (16.82 U/mg) as well as xylan substrates, beechwood xylan (23.69 U/mg) and wheat arabinoxylan (20.15 U/mg), making it a multifunctional glycoside hydrolase enzyme. Substrate competition assays between beechwood xylan and Avicel, and between beechwood xylan and hydrolysed potato starch, revealed that the enzyme uses the same active site to hydrolyse all three substrates, confirming the cross-specificity, or substrate promiscuity, of the enzyme.

The enzyme, referred to as the putative xylanase in this study, was found to be cold-active, maintaining over 25% relative activity on beechwood xylan at 4˚C, consistent with other cold-active xylanases that have been reported in literature. The putative xylanase showed optimal xylanase activity at 40˚C and pH 6.0, and was found to be relatively thermostable, maintaining at least 78% relative activity on beechwood xylan after incubation for 24 hours at 50˚C. The enzyme was also found to be acido/alkaline stable, maintaining over 60% relative activity between pH 2.0 and 11.0, and also stable in the presence of various divalent cations, ethylenediaminetetraacetic (EDTA) and β-mercaptoethanol. The kinetic parameters of the putative xylanase on different substrates were determined using Hanes-Woolf plots. The V_{max} values of the putative xylanase on beechwood xylan, wheat arabinoxylan, Avicel and hydrolysed potato starch were determined to be 24.63, 27.40, 12.20, and 19.34 U/mg, respectively. The K_M values of the putative xylanase on beechwood xylan, wheat arabinoxylan, Avicel and hydrolysed potato starch were determined to be 3.03, 4.09, 4.49 and 2.21 mg/ml, respectively.

In conclusion, the psychrophilic bacterium, *Sphingomonas aerolata*, was successfully sub-cultivated under laboratory conditions and found to produce an extracellular putative xylanase that was successfully purified and characterized, and determined to possess a number of novel features such as the ability to hydrolyse different substrates using one active site, activity at low temperatures coupled with high thermostability, and stability at acidic and alkaline pH conditions. This novel enzyme could be very useful as a biocatalyst in different fields due to its novel features, and further study into its structure especially around the active site that has been shown to be possibly fluid enough to maintain reaction rates at low temperatures while also stable enough to maintain high reaction rates after long incubation periods at high temperatures, can bind and hydrolyse different substrates and can maintain high reaction rates across acidic, moderate and alkaline conditions, could further expand on the existing knowledge on biocatalysts and their properties.

List of abbreviations

˚C	Degree(s) Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
µmol	Micromole
BSA	Bovine serum albumin
CasMM	Casein minimal mineral medium
CAZy	Carbohydrate active enzymes
CMC	Carboxymethyl cellulose
Dha	2-keto-3-deoxy-D-lyxo-heptulosaric acid
dH_2O	distilled water
DNS	3,5-Dinitrosalicylic acid
E	Enzyme
EC	Enzyme commission number
EDTA	Ethylenediaminetetraacetic acid
ES	Enzyme-substrate complex
g	Gram
g	Gravity
GH	Glycoside hydrolase
G+C	Guanine plus cytosine
kDa	Kilo Daltons
Kdo	2-keto-3-deoxy-D-manno-octulosonic acid
LBG	Locust bean gum
M	Molar
mg	Milligram
ml	Millilitre
mM	Millimolar
MM	Molecular marker

MW	Molecular weight
nm	Nanometer
OECD	Organisation for Economic Co-operation and Development
OD	Optical density
P	Product
PAGE	Polyacrylamide gel electrophoresis
PYES	Peptone, yeast extract medium
R2A	Reasoner's 2A agar
RBB	Remazol Brilliant Blue
rpm	revolutions per minute
S	Substrate
S.D	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N, N, N', N'-tetramethylethylenediamine
TLC	Thin-layer chromatography
TSA	Trypticase soy agar
U	Units of enzyme activity
v	Volume
w	Weight

List of figures

Figure 1.1	The structures of the most common and abundant types of pectin polysaccharides	8
Figure 1.2	The primary structures of (a) homoxylan with (i) β-(1→3) or (ii) mixed β-(1→3, 1→4)-glycosidic linkages , (b) glucuronoxylan, (c) arabinoxylan and (d) (arabino)glucuronoxylan/ (glucurono)arabinoxylan	10
Figure 1.3	The two common types of catalytic mechanisms followed by xylanases in different glycoside hydrolase families (a) the inverting mechanism and (b) the retaining mechanism	12
Figure 2.1	Growth of *S.aerolata* on agar plates at different temperatures; (a) 4ºC, (b) 15ºC, (c) 22˚C, (d) 28˚C, (e) 37˚C and (f) negative control (without inoculum) using hydrolysed potato starch as a carbon source	22
Figure 2.2	Activity of the collected fractions from the *S. aerolata* culture on beechwood xylan as a substrate	23
Figure 2.3	Growth and activity curves for production of xylanase from *S. aerolata* at different temperatures	24
Figure 2.4	Effect of induction using different carbon sources on xylanase production as determined by xylanase activity of supernatants on beechwood xylan	25
Figure 2.5	SDS-PAGE analysis (a) of the BioRad unstained protein standards (MM), crude extracellular extract from an *S. aerolata* culture (Crude) and purified S. aerolata putative xylanase sample and native activity-PAGE analysis (b) of the purified putative xylanase sample	27

Figure 2.6	SDS-PAGE analysis of the BioRad unstained protein standards (MM), less than 10 kDa filtrate (<10), greater than 30 kDa but less than 100 kDa fraction (>30,<100), greater than 10 kDa but less than 100 kDa fraction (>10,<100), greater than 100 kDa fraction (>100) and crude extracellular extract from an *S. aerolata* culture (Crude)	28
Figure 3.1	Determination of optimum temperature for beechwood xylan hydrolysis by the putative *S. aerolata* xylanase	38
Figure 3.2	Thermal stability of the putative xylanase at 28˚C, 37˚C and 50˚C	39
Figure 3.3	Determination of pH optima for the *S. aerolata* putative xylanase on beechwood xylan	40
Figure 3.4	Effect of glucose, xylose and mannose on the activity of the putative xylanase on azo-wheat arabinoxylan (a) and azo-Avicel (b)	41
Figure 3.5	Effect of various divalent metal ions on the activity of the putative xylanase on beechwood xylan	42
Figure 3.6	Effect of EDTA and β-mercaptoethanol on the activity of the putative xylanase on beechwood xylan	43
Figure 4.1	The relationship between substrate concentration and the initial rate of the reaction	49
Figure 4.2	The effect of substrate concentration to reaction rate for cooperative enzymes	50
Figure 4.3	Lineweaver-Burk plot (a) and Hanes-Woolf plot (b) obtained from	

	the activity of the putative xylanase on different concentrations of beechwood xylan	54
Figure 4.4	Lineweaver-Burk plot (a) and Hanes-Woolf plot (b) obtained from the activity of the putative xylanase on different concentrations of wheat arabinoxylan	55
Figure 4.5	Lineweaver-Burk plot (a) and Hanes-Woolf plot (b) obtained from the activity of the putative xylanase on different concentrations of Avicel	56
Figure 4.6	Lineweaver-Burk plot (a) and Hanes-Woolf plot (b) obtained from the activity of the putative xylanase on different concentrations of hydrolysed potato starch	57

Appendix:

Figure II A	Bradford standard curve for protein concentration determination with BSA used as the protein standard	81
Figure II B	Xylose standard curve using the DNS assay	81
Figure II C	*p*-Nitrophenol standard curve	82
Figure II D	Remazol Brilliant Blue standard curve	82

List of tables

Table 2.1 Purification table showing the purification of *S. aerolata* xylanase using ultrafiltration with Amicon molecular weight cut-off filtration units 26

Table 3.1 Specific and relative activity of the putative *S. aerolata* xylanase on various lignocellulosic substrates 37

Table 4.1 Comparison of the kinetic parameters determined using Lineweaver-Burk plots and Hanes-Woolf plots for activity of the putative *S. aerolata* xylanase on different substrates 60

Table 4.2 Comparison of V_{max} and K_M values determined using Lineweaver-Burk plots, Hanes-Woolf plots and non-linear regression analysis using Michaelis-Menten plots on the KaleidaGraph 4.5.4 computer software 61

Table 4.3 .Comparison of the observed reaction velocities of the putative xylanase on mixtures of various concentrations of beechwood xylan and Avicel 62

Table 4.4 Comparison of the observed reaction velocities of the putative xylanase on mixtures of various concentrations of beechwood xylan and hydrolysed potato starch 63

Chapter 1: Introduction and Literature Review

1.1. Industrial sustainability through biocatalysis

Modern day society is heavily dependent on chemicals, which are essential for the production of daily life products, ranging from food processing, healthcare, personal care to household products (European Commission, 2009). Unfortunately, the production of such daily life products consumes large amounts of raw materials and energy. In addition to this, the production of such products generates large amounts of waste which has a negative impact on the environment and on human health (European Commission 2009; OECD 2009). This has resulted in a global shift towards reducing the impact of not only chemical products required to sustain human needs, but also the impact of the industrial chemical processes that are used to synthesise these chemical products on human health and the environment (Jegannathan and Nielsen, 2013). Industries around the world have, therefore, started looking into alternative technologies that can meet the increasing demand for daily life products while consuming fewer resources and having less of an impact on the environment (Jegannathan and Nielsen, 2013; OECD, 2011).

Many studies have shown that implementing enzymatic processes in place of conventional chemical processes generally results in a reduced negative impact to the environment and human health, and saves on energy and raw materials (Jegannathan and Nielsen, 2013). Enzymes are defined as proteins that are natural and specialised catalysts produced by living organisms in order to increase the rates of many diverse biochemical reactions that are essential for life (Li et al., 2012). The biotechnological applications of enzymes as catalysts in the chemical industry, referred to as biocatalysis, has increased over the years in response to an increasing world-wide focus on sustainable industrial processes (Rogers et al., 2005). Environmental concerns have also helped to drive the use of biotechnology in industry, with a focus on biocatalyst-based processes (Gavrilescu and Chisti, 2005). Enzymatic processes have been introduced to a wide range of industries in recent decades because they are specific, rapid and often save raw materials and energy compared to conventional chemical processes (Jegannathan and Nielsen, 2013). The use of enzymes has overcome limitations in the chemical industry such as the need for high temperature and pressure for catalysis, and the moderate specificity of chemical catalysts (Sundarram and Murthy, 2014). Enzymes are now applied in

numerous fields including, but not limited to, technical use, food manufacturing, animal nutrition, cosmetics, medication, and as tools for research and development (Li et al., 2012).

While enzymes can be obtained from plants, animals and microorganisms, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (de Souza and e Magalhães, 2010). The major reasons for the interest in microbial sources for enzymes are due to the rapid growth of microorganisms meaning the production of enzymes is also rapid, microorganisms are easier to handle than animals and plants as they require less space, and microorganisms can be easily manipulated using genetic engineering or other means to obtain enzymes with desired characteristics that can meet the needs of growing industries (Sundarram and Murthy, 2014). Over the past decade, the biotechnological application of enzymes that can withstand harsh conditions, and can, therefore, be utilised industrially where production processes are often harsh, has greatly increased (Demirjian et al., 2001). This has resulted in increased research into extremophiles and extremozymes (Demirjian et al., 2001).

1.2. Extremophiles

A large part of the earth's surface experiences extreme environmental conditions which are detrimental to higher life-forms (Kasana and Gulati, 2011). Such extreme environments are, however, inhabited by microorganisms that are able to grow and survive under these harsh conditions (Kasana and Gulati, 2011). Such organisms are termed extremophiles, and fall into a number of different classes such as thermophiles, acidophiles, alkaliphiles, barophiles, and psychrophiles (Demirjian et al., 2001), and are adapted to survive high temperatures, extremely low pH conditions, extremely high pH conditions, high pressure and low temperatures, respectively (Niehaus et al., 1999). All cellular components have to be stable at these extreme conditions for extremophiles to grow and survive (Elleuche et al., 2014). Their ability to adapt to such extremes in environmental conditions has also been attributed to their highly flexible metabolism (Dalmaso et al., 2015). Extremophiles, although being the most prolific of Earth's organisms, have been reported to be the least studied (Brininger et al., 2018). This could be due to the fact that these organisms inhabit harsh environments, and are therefore difficult to access and study (Brininger et al., 2018). The ability of extremophiles to survive and thrive in extremes of environmental conditions makes them desirable for applications in biotechnological processes (Dalmaso et al., 2015).

Enzymes from mesophiles are often not stable enough and therefore not well suited for the harsh reaction conditions required for industrial processes (Demirjian et al., 2001). As a result, enzymes from extremophiles have become of great biotechnological interest because they can potentially withstand the harsh reaction conditions required in industrial processes, which would otherwise denature their mesophilic counterparts (Demirjian et al., 2001). Enzymes from psychrophiles, in particular, have been found to be adapted to work at low temperatures with a high catalytic efficiency, which could potentially translate to a reduction in energy usage and costs in industrial processes (Gerday et al., 1997). Psychrophiles and their enzymes are therefore of great biotechnological interest.

1.3. Psychrophiles

Low temperatures are the most common among extreme environments (Kasana and Gulati, 2011). The marine environment covers almost three-quarters of the earth, with 90% of the ocean's waters having temperatures below 5°C (Cavicchioli et al., 2011). The permanently cold environments stretch from the deep oceans to alpine reaches and to Polar Regions (Cavicchioli et al., 2011). Cold-adapted microorganisms referred to as psychrophiles inhabit these low-temperature environments (Kasana and Gulati, 2011). Among psychrophiles, two types exist, stenopsychrophiles and eurypsychrophiles (Dalmaso et al., 2015). Stenopsychrophiles are described as incapable of growing at temperatures exceeding 20°C, while eurypsychrophiles, also referred to as psychrotolerant or psychrotrophs, have a broader range, tolerating warmer environments and capable of growing at temperatures exceeding 25°C but with upper growth limits of 40°C (Dalmaso et al., 2015). The majority of isolated psychrophilic organisms are eurypsychrophiles (Dalmaso et al., 2015). More than 100 species of psychrophiles have been identified, and these include both Gram-negative and Gram-positive bacteria from various habitats ranging from soil, sandstone, freshwater, marine lakes, sea ice and oceans (Kumar et al., 2011). Psychrophiles that have been isolated from the Antarctic and studied include the genera *Arthrobacter, Colwellia, Gelidibacter, Glaciecola, Halobacillus, Halomonas, Hyphomonas, Marinobacter, Planococcus, Pseudoalteromonas, Pseudomonas, Psychrobacter, Psychroflexus, Psychroserpens, Shewanella and Sphingomona* (Dalmaso et al., 2015). Psychrophiles are considered one of the most underutilised resources (Kumar et al., 2011), with a broad biotechnological potential that offers numerous economic and ecological advantages over mesophiles (Kasana and Gulati, 2011), chief among them being the ability to enhance yields of heat-sensitive products (Demirjian et al., 2001).

1.4. Properties of psychrophiles

Psychrophiles have developed various adaptive mechanisms to perform metabolic functions even at low temperatures (Kumar et al., 2011). While Dalmaso et al. (2015) postulated that cold shock proteins function as cold-adaptive proteins in psychrophiles, Roberts and Inniss (1992) showed that the psychrophilic bacterium, *Aquaspirillum arcticum*, produced not only cold-shock proteins as a response to sudden drops in temperature, but also cold-acclimation proteins during continuous growth at low temperatures. It has also been suggested that together with cold shock proteins, small RNA-binding proteins facilitate cold adaptation in psychrophilic bacteria by regulating RNA helicases during cold-growth (Dalmaso et al., 2015). Cold-shock or anti-freeze proteins, among their many other functions, also have the ability to retain and protect extracellular enzymes against cold degradation and also act as cryo-protectants, allowing psychrophiles to continue their activities at low temperatures (Kumar et al., 2011). The production of secondary cold-active metabolites have also been shown to be other factors involved in cold adaptation (Dalmaso et al., 2015). Proteins from psychrophiles have a higher content of α-helix sheets compared to β-sheets, which is considered an important factor in maintaining flexibility even at low temperatures (Dalmaso et al., 2015). This results in high rates of catalysis at low temperatures being achieved as a result of the flexible structure of cold-active enzymes (Dalmaso et al., 2015).

The membranes of psychrophiles contain a higher proportion of unsaturated fatty acids, and as a result can maintain their fluidity and ability to transport nutrients even under very cold conditions, which would otherwise have an adverse effect on the physical properties and functions of the membranes (Kumar et al., 2011). Other adaptations that have been suggested to increase membrane fluidity include an increased content of lipid head groups, proteins and non-polar carotenoid pigments; however, these adaptations are not widespread amongst all psychrophiles (Kumar et al., 2011).

1.4.1. Sphingomonas

Yabuuchi et al. (1990) first proposed the genus *Sphingomonas* after using cellular lipid and fatty acid analysis to separate the strains of *Sphingomonas* species from the genus *Pseudomonas*, and it accommodated strictly aerobic, chemo-heterotrophic, yellow-pigmented, Gram-negative, rod-shaped bacteria that contained glycosphingolipids as cell envelope components. The genus was loosely defined as a group of polarly flagellated species whose

guanine-plus-cytosine (G+C) content of DNA was 58-70 mol% (Yabuuchi et al., 1990). The species of the genus described were *Sphingomonas adhaesiva*, *Sphingomonas capsulata*, *Sphingomonas parapaucimobilis*, *Sphingomonas yanoikuyae* and *Sphingomonas paucimobilis* (described as the type species) (Yabuuchi et al., 1990).

Takeuchi et al. (2001) then utilised phylogenetic analysis of 16S rRNA sequences and polyamine profiles to classify the genus *Sphingomonas* into four genera, *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*. It was proposed that these four genera differ in their polyamine pattern and nucleotide signatures, forming four 16S rRNA gene sequence clusters with distance matrix analysis (Takeuchi et al., 2001). However, Yabuuchi et al. (2002) counter claimed, using numerical analysis of 27 type strains of the *Sphingomonas* species, that the members of the three new genera proposed by Takeuchi et al. (2001), *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, did not form solid clusters on the basis of phylogenetic and chemotaxonomic analyses to be classified as new genera of the genus *Sphingomonas*, and should instead be considered as later homotypic synonyms of species of the genus *Sphingomonas*. However, the nomenclature of Takeuchi et al. (2001) is still generally used. Busse et al. (2003) further emended the description of the genus *Sphingomonas* given by Yabuuchi et al. (1990) and Takeuchi et al. (2001) to include off-white- and orange-pigmented colonies. The emendation also expanded on the polar lipid profiles to contain, in addition to sphingoglycolipid, phosphatidyl glycerol as the predominant lipid, moderate to large amounts of phosphatidyl ethanolamine, diphosphatidyl glycerol, phosphatidyl dimethylethanolamine and phosphatidyl choline, and varying numbers of unidentified polar lipids (Busse et al., 2003). This emendation then allowed for the inclusion of new species, *Sphingomonas aerolata*, *Sphingomonas faeni* and *Sphingomonas aurantiaca*, which differed from the original genus description in their pigmentation and cell sizes (Busse et al., 2003). At least 45 species of the genus *Sphingomonas sensu stricto* with validly published names exist (Zhang et al., 2011).

There has been interest in the genus *Sphingomonas*, which can be attributed to reports on the broad catabolic capabilities of strains of the *Sphingomonas* species (Denner et al., 2001). The potential for biotechnological applications of these organisms include applications in the degradation, bioremediation and wastewater treatment of xenobiotic pollutants, as bacterial antagonists of phytopathogenic fungi and for the production of industrially useful exopolysaccharides (Denner et al., 2001). *Sphingomonas* sp. GY2B has previously been immobilized in polyvinyl alcohol-sodium alginate-kaolin beads and applied in the degradation of phenol (Ruan et al., 2018). The psychrotrophic *Sphingomonas* sp. FLX-7 with high

carboxylmethyl cellulase (CMCase) activity has also been isolated for the purposes of broad application to organic waste composting in the cold regions of China where difficulties in degrading cellulose from organic solid waste quickly and efficiently have been reported (Zhang et al., 2016).

S. aerolata sp. was proposed by Busse et al. (2003) as a novel airborne species of the genus *Sphingomonas* isolated from the Antarctic. Cells of *S. aerolata* were described as small rods, 0.6-0.8 by 1.5-2.6 µm, with growth observed on Czapek-Dox, R2A, CasMM, PYES and TSA agar (Busse et al., 2003). Cells were found to grow at 4-28˚C, but not at 37˚C, and Gram-negative as determined by Gram staining, KOH and aminopeptidase tests (Busse et al., 2003). Cells were motile, with no endospores observed, and the colonies were circular, slightly convex, opaque and orange-pigmented (Busse et al., 2003). The G+C content of the genomic DNA of the type strain was found to be 65.4 mol% (Busse et al., 2003).

S. aerolata is of special interest as it is airborne, and has the potential to survive in unfavourable environments, potentially withstanding increased radiation and desiccation (Busse et al., 2003).

1.5. Enzymes from psychrophiles

Microorganisms that inhabit cold environments are diverse and as such have the potential to be suitable sources of enzymes that can function effectively in the cold (Cavicchioli et al., 2011). Psychrophiles, in order to survive extremely low temperatures, require adequate chemical reaction rates at such temperatures to enable metabolic rates comparable to homologous organisms living at moderate temperatures (Gerday et al., 1997). As most chemical reactions are catalysed by enzymes, these organisms contain enzymes that exhibit high specific activities at low temperatures and so can maintain metabolic rates even at such low temperatures (Kumar et al., 2011). These enzymes, referred to as cold-active enzymes, are generally thermo-labile (Kumar et al., 2011).

Due to the cold temperature environments inhabited by psychrophiles, psychrophilic proteins face a thermodynamic challenge, which is reduced molecular motion as a result of decreased entropy and enthalpy (Brininger et al., 2018). As a result, psychrophilic enzymes are adapted to increase kinetic energy and catalytic efficiency despite the low energy environment (Brininger et al., 2018). Cold-active enzymes are characterized by an increase in flexibility of the polypeptide chain which enables for easier accommodation of substrates at low temperatures (Gerday et al., 1997). The compositional and structural features that are believed

to be responsible for this increase in flexibility include decreased core hydrophobicity, lower arginine/lysine ratio, weaker inter-domain and inter-subunit interactions, decreased secondary structure content, increased glycine residues, decreased proline residues in loops, increased proline residues in α-helices, less and weaker metal-binding sites, a reduced number of disulphide bridges, fewer electrostatic interactions, reduced oligomerisation and an increase in conformational entropy of the unfolded state (Cavicchioli et al., 2011). While some cold-active enzymes have been found to have a whole structure that is uniformly flexible, some have been shown to have flexibility that is localised in the structures surrounding or comprising the active site (Cavicchioli et al., 2011).

Cold-active enzymes have been reported to have a reaction rate that is largely independent of temperature (Dalmaso et al., 2015). As a result of their high specific activity at low temperatures and inactivation at higher temperatures, enzymes from psychrophiles offer great potential as biocatalysts in biotechnological applications requiring low temperatures (Kumar et al., 2011). These enzymes have the potential to provide economic benefits by being more productive than their mesophilic or thermophilic counterparts at low temperatures, which translates to energy savings during the processes that the enzymes are used in (Cavicchioli et al., 2011). Other potential benefits from the use of cold-adapted enzymes include minimising undesirable chemical reactions that could occur at higher temperatures, the use of the enzymes to transform heat-sensitive substrates at low temperatures and the ability to inactivate these enzymes rapidly by heating (Cavicchioli et al., 2011). Enzymes from psychrophiles that have previously been isolated and studied include proteases, amylases, cellulases, peptidases, lipases, xylanases and other classes of enzymes (Dalmaso et al., 2015).

1.6. Lignocellulosic biomass

Lignocellulosic biomass has been identified as a rapidly renewable bio-resource comprised of cellulose microfibrils embedded in a matrix of hemicellulose and pectin, all encapsulated by lignin, which gives the cell walls rigidity and strength (Morrison et al., 2011). Pectin has been described as the most complex of the biomass polysaccharides, consisting of esterified α-D-1,4-linked galacturonic acid residues (Lagaert et al., 2009). Pectic polysaccharides have been classified into homogalacturonan, xylogalacturonan, apiogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II (Harholt et al., 2010). Homogalacturonan is a linear unbranched homopolymer of α-D-1,4-linked galacturonic acid residues, with some of the carboxyl groups

methylesterified (Harholt et al., 2010; Lagaert et al., 2009). Substitution of the linear homopolymer of galacturonic acid residues at various positions with xylose yields xylogalacturonan, while substitution with apiofuranose yields apiogalacturonan (Harholt et al., 2010). Rhamnogalacturonan I has a backbone composed of L-rhamnosyl and α-D-1,4-linked galactosyluronic acid residues, with side chains of predominantly linear and branched chains of arabinosyl and galactosyl residues attached to some the rhamnosyl residues (Lagaert et al., 2009). The backbone of rhamnogalacturonan II is composed of α-D-1,4-linked galactosyluronic acid residues substituted with 4 different side chains, with the side chains, in turn, containing up to 12 different glycosyl residues, among them rare monosaccharides such as 2-*O*-methyl-L-fucose, 2-*O*-methyl-D-xylose, apiose, 2-keto-3-deoxy-D-lyxo-heptulosaric acid (Dha) and 2-keto-3-deoxy-D-manno-octulosonic acid (Kdo) (Lagaert et al., 2009). Of these pectic polysaccharides, the most studied and most common pectic polysaccharides are homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II (Lagaert et al., 2009). The structures of different pectic polysaccharides are shown in Figure 1.1.

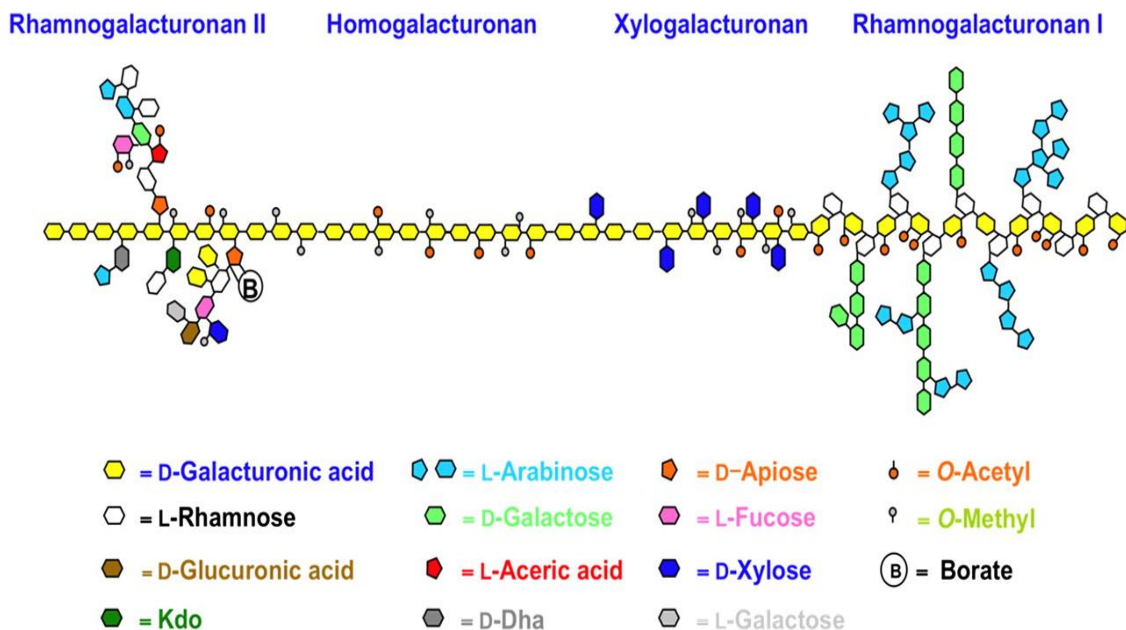


Figure 1.1: The structures of the most common and abundant types of pectin polysaccharides (obtained from Harholt et al., 2010)

Cellulose is the major component of plant biomass and is the most abundant polysaccharide in nature (Cheng et al., 2014). It is a homo-polysaccharide composed of chains of β-1,4-linked glucose units, which are in turn comprised of cellobiose as the basic coupling unit (Cheng et

al., 2014). The linkages between the chains are strong hydrogen bonds that render cellulose crystalline in structure and recalcitrant to degradation (Van Dyk and Pletschke, 2012). The saccharification of cellulose is of great industrial significance and is achieved by the synergistic action of three types of cellulose degrading enzymes; endo-1,4-β-glucanases (carboxymethyl-cellulases) (EC 3.2.1.4), cellobiohydrolases (exoglucanases or Avicelases) (EC 3.2.1.91) and β-glucosidases (cellobiases) (EC 3.2.1.21) (Ray, 2015). Endo-glucanases hydrolyse internal β-1,4-glucosidic bonds randomly along the cellulose chain, while cellobiohydrolases move along the cellulose chain cleaving off cellobiose units from the ends and β-glucosidases hydrolyse cellobiose to glucose and cleave off glucose units from cellooligosaccharides (Jørgensen et al., 2007). A new paradigm for cellulose saccharification has been presented, in which the hydrolytic activities of classical cellulases such as endo-glucanases, cellobiohydrolases and β-glucosidases on mainly crystalline cellulose, are facilitated by the oxidative cleavage of cellulose at C1, and in some cases C4 and C6 carbons, by lytic polysaccharide monooxygenases (LPMOs) such as CBM33 (now known as AA10) and GH61 (now known as AA9) hydrolases (Horn et al., 2012). LPMOs therefore play a crucial role in the degradation of cellulose by contributing to the synergistic action of cellulose degrading enzymes (Horn et al., 2012).

Lignin on the other hand is a complex polyphenolic compound that forms the epidermal tissue of plants (Himmel et al., 2007). The basic units of lignin are phenylpropanoid derivatives that combine into high molecular substances by ether bonds and carbon-carbon bonds (Himmel et al., 2007). Lignin is linked to hemicellulose by chemical bonding, mainly via galactose residues and arabinose residues on the side chains of hemicellulose polymers, respectively (Himmel et al., 2007).

Hemicellulose is more varied in composition when compared to cellulose, comprising of mannan, galactan, xylan and arabinan polymers (Van Dyk and Pletschke, 2012). It is a heteropolymer of pentoses (xylose and arabinose) and hexoses (glucose, galactose and mannose) (Himmel et al., 2007). The major component of hemicellulose is xylan, accounting for approximately a third of all renewable carbon on earth (Shi et al., 2015). Xylan is a complex, highly branched heteropolysaccharide that varies in structure from one plant species to another (Collins et al., 2005). It consists of a homopolymeric backbone chain of β-D-1,4-linked xylopyranosyl units substituted to varying degrees with glucuronopyranosyl, 4-*O*-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl and/or *p*-coumaroyl side chains depending on the biomass source (Collins et al., 2005).

The side chain substitutions on the xylan backbone differentiate different types of xylan structures from each other (Dahlman et al., 2003). The main types of xylan are homoxylans, glucuronoxylans, arabinoxylans, (arabino)glucuronoxylans and (glucurono)arabinoxylans (Ebringerová, 2006). Homoxylans are unsubstituted xylan polymers commonly found in seaweeds of Palmariales and Nemaliales, and have either β-(1→3) or mixed β-(1→3, 1→4)-glycosidic linkages within their D-xylopyranose backbones (Ebringerová, 2006). Glucuronoxylans, also referred to as 4-*O*-methyl-α-D-glucurono-D-xylan, are commonly found in hardwoods and have single 4-*O*-methyl-α-D-glucopyranosyl uronic acid residues attached to position 2 of the main D-xylopyranose backbone chain (Ebringerová, 2006). Arabinoxylans are substituted with α-L-arabinofuranose groups either on O-2 or O-3 position and/or on both positions of the D-xylopyranose backbone chain (Ebringerová, 2006). (Arabino)glucuronoxylans have a backbone chain that is heavily substituted with 4-*O*-methyl-D-glucuronic acid, while the backbone chain of the (glucurono)arabinoxylan structure is heavily substituted with α-L-arabinofuranose groups (Ebringerová, 2006). The structures of the different types of xylan are illustrated in Figure 1.2 below.

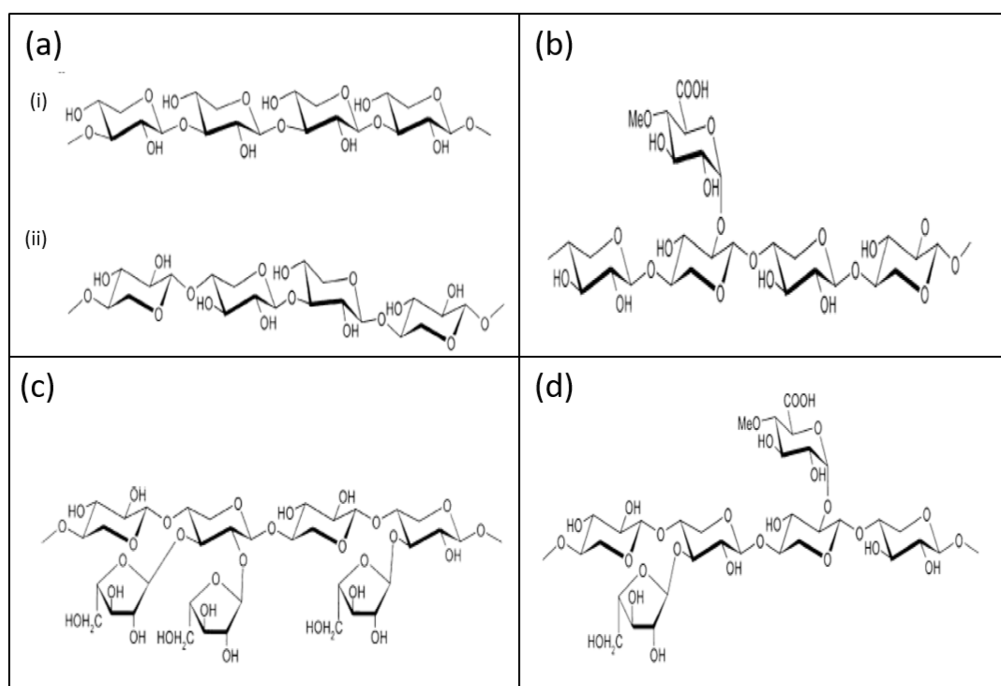


Figure 1.2: The primary structures of (a) homoxylan with (i) β-(1→3) or (ii) mixed β-(1→3, 1→4)-glycosidic linkages , (b) glucuronoxylan, (c) arabinoxylan and (d) (arabino)glucuronoxylan/ (glucurono)arabinoxylan (obtained from Ebringerová (2006))

1.7. Xylanases

Due to its heterogeneous and complex structure, the complete hydrolysis of xylan requires the combined and synergistic action of a variety of enzymes (Shi et al., 2015). Endo-1,4-β-xylanases (EC 3.2.1.8) randomly cleave the xylan backbone releasing xylo-oligosacharides, while β-D-xylosidases (EC 3.2.1.37) cleave xylose monomers from the non-reducing end of xylo-oligosaccharides and xylobiose (Collins et al., 2005). Reducing-end xylose-releasing exo-oligoxylanases (EC 3.2.1.156) cleave xylan substrates in an exo-acting manner, liberating xylopyranosyl units from the reducing end of the xylan chain (Valenzuela et al., 2016). The removal of branched substituents of xylan is catalysed by α-glucuronidases (EC 3.2.1.139), acetylxylan esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73), *p*-coumaric acid esterases (EC 3.1.1-) and α-L-arabinofuranosidases (EC 3.2.1.55) (Collins et al., 2005). Endo-1,4-β-xylanases are the most crucial enzymes required during the hydrolysis of xylan (Singh et al., 2003).

Endo-1,4-β-xylanases, commonly referred to as xylanases, are *O*-glycoside hydrolases that catalyse the hydrolysis of β-D-1,4-xylosidic linkages in xylan to yield short oligosaccharides or xylose (Collins et al., 2005; Meng et al., 2015). They are a widespread group of enzymes produced by various organisms including bacteria, algae, fungi, protozoa, gastropods and anthropods (Collins et al., 2005). The heterogeneity and complexity of xylan have given rise to a number of diverse xylanases with varying specificities, primary sequences and folds (Collins et al., 2005). Xylanases have since been classified into glycosyl hydrolase (GH) families 5, 7, 8, 10, 11, 30 and 43, based on the similarities of their amino acid sequences (Shi et al., 2015). However, most characterised xylanases belong to GH10 and GH11 (Meng et al., 2015). Xylanases from the different families differ in their physico-chemical properties, structure, mode of action and substrate specificity (Collins et al., 2005). Families 5, 7, 10 and 11 have been found to contain xylanases that follow an overall retention catalytic mechanism, while those of GH families 8 and 43 follow an inverting catalytic mechanism (http://www.cazy.org/Glycoside-Hydrolases.html).

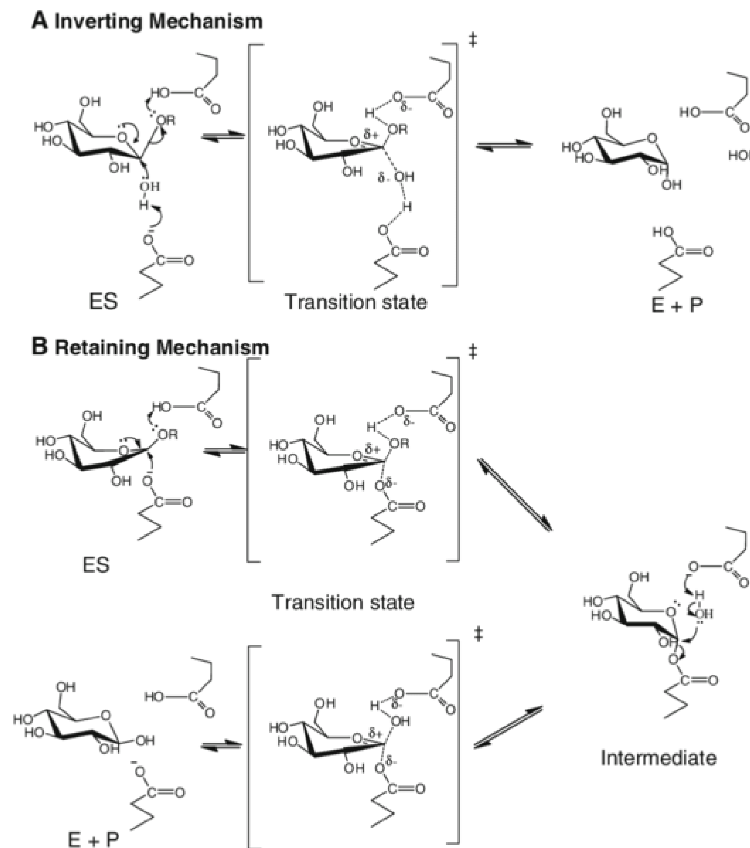


Figure 1.3: The two common types of catalytic mechanisms followed by xylanases in different glycoside hydrolase families (a) the inverting mechanism and (b) the retaining mechanism (adapted from Davies and Henrissat, 1995)

The majority of studied xylanases have been from fungal or bacterial origin and most have been found to be optimally active at or close to mesophilic temperatures (around 40-60°C) and neutral or slightly acidic pHs (Collins et al., 2005). However, xylanases that are stable and active at extremes of pH and temperature have been reported (Collins et al., 2005). Of these, xylanases from thermophiles, alkaliphiles and acidophiles have been the most extensively studied, while cold-adapted xylanases have been much less investigated (Collins et al., 2005). Despite cold-temperature environments being the most abundant on earth, only a few psychrophilic xylanase producers have been identified (Collins et al., 2005).

1.8. Application of xylanases

Xylanases have potential applications in a wide range of industrial processes, including, but not limited to animal feed, where they are applied to decrease the content of non-starch polysaccharides and improve the nutritional value of feeds; brewing, to reduce haze in the final brewed product; baking, to improve the elasticity and strength of the dough resulting in larger

loaf volumes and improved bread texture; bio-bleaching in the paper and pulp industry resulting in reduced chlorine consumption and toxic wastes; waste treatment; the production of biofuels from lignocellulosic biomass and the production of pharmacologically active polysaccharides for use as antimicrobial agents (Collins et al., 2005; Singh et al., 2003).

Xylanases from extremophiles are of special interest for their potential utility in many biotechnological processes (Collins et al., 2005). For instance, thermostable xylanases could be utilised in processes where high temperatures are needed to increase the solubility of substrates and increase yields and reduce the risk of contamination, while cold-active xylanases can be utilised in processes where heating could be economically counterproductive or where low temperatures are required to prevent the alteration of ingredients and/or product quality and product denaturation (Collins et al., 2005).

The major current use of xylanases is in bio-bleaching where they are utilised to selectively degrade the accessible hemicellulose fraction of woods and to enhance the extractability of lignin (Singh et al., 2003). Increased brightness and viscosity has been achieved when xylanases from *T. lanuginosus* were applied as bleaching agents in kraft and sulphite pulp production from sugar cane bagasse, *Eucalyptus* and *Fagus* (Singh et al., 2003). However, due to the high temperatures of the paper and pulp processes (55-70°C) and the alkaline pH of the pulp substrate, thermo-alkaliphilic xylanases are most suitable for application to achieve efficient bio-bleaching (Collins et al., 2005).

Cold-active enzymes could potentially offer economic benefits through energy savings in processes where heat is not a requirement (Cavicchioli et al., 2002). These enzymes function effectively at low temperatures, increasing reaction yields, accommodating a high level of stereospecificity, minimising undesirable chemical reactions that can occur at higher temperatures and exhibiting thermal lability for quick and easy inactivation when required (Cavicchioli et al., 2002). The most promising potential application for cold-adapted xylanases, currently, is in the food industry (Collins et al., 2005). These enzymes would be especially suited for use in the baking industry as dough preparation and proofing is generally carried out at temperatures not exceeding 35°C (Collins et al., 2005).

The isolation and characterization of a cold-active xylanase from the psychrotrophic bacterium, *Sphingomonas aerolata*, is therefore of great interest, so as to increase the knowledge base on cold-active xylanases, which so far have not been as well studied as their mesophilic and

thermophilic counterparts. In order to achieve this, the airborne psychrotrophic bacterium isolated from the Antarctica had to be first cultured and grown under laboratory conditions.

1.9. Problem Statement

Enzymes from psychrophiles have a vast potential for biotechnological applications as a result of their high catalytic efficiency even at low temperatures and their high levels of stereospecificity (Cavicchioli et al., 2011, 2002; Dalmaso et al., 2015; Kumar et al., 2011). However, psychrophiles are still considered one of the most underutilised resources (Kumar et al., 2011), presenting a need to explore psychrophiles and their enzymes further. In addition, literature on the purification and characterization of cold-active xylanases is currently very scant, and there is currently no available literature on the isolation and biochemical characterization of any enzymes from the novel psychrophilic bacterium *S. aerolata*. Therefore, the current project on the isolation and biochemical characterization of a xylanase from this bacterium should present novel findings.

1.10. Hypothesis

The psychrotrophic bacterium, *S. aerolata*, produces a novel cold-active xylanase that has the potential for application in industries that do not require high temperatures, resulting in energy savings and therefore economical savings.

1.11. Aims and objectives

- *Aims*

The aims of this project were to produce, isolate and biochemically characterize a cold-active xylanase from the psychrotrophic bacterium, *S. aerolata*.

- *Objectives*

The objectives of this project were to:

  - subcultivate *S. aerolata* cells;
  - isolate a putative xylanase enzyme from the culture;

- develop a suitable enzyme assay for xylanase activity;
- biochemically characterize the putative xylanase (molecular weight, temperature and pH studies, substrate specificity, inhibition by simple sugars, effect of metal ions);
- determine kinetic parameters (apparent V_{max}, K_M, and k_{cat} values);
- determine the mode of action of the putative xylanase and identify the products of xylan degradation by the putative xylanase using thin layer chromatography (TLC).

Chapter 2: Culturing of *S. aerolata* and the production and purification of its extracellular putative xylanase

2.1. Introduction

Psychrophiles have been defined as organisms that cannot grow at temperatures above 20°C, and those organisms that can grow at low temperatures and also above 20°C are referred to as psychrotolerant or psychrotrophs (Russell, 1997). Dalmaso et al. (2015) classified the two types of psychrophiles as stenopyschrophiles to denote true psychrophiles that cannot grow above 20°C and eurypsychrophiles to denote psychrotrophs that can grow at temperatures exceeding 20°C. Morita (1975) defined psychrophiles as organisms that can grow at 0°C and have an optimum growth temperature below 15°C and a maximum temperature below 20°C. Psychrotolerants, on the other hand, are less likely to grow at 0°C, but will grow between 3 and 5°C, and have optimum and maximum growth temperatures above 20°C (Russell, 1997). Cavicchioli et al. (2002), consistent with the definitions put forward by Russell (1997), distinguished psychrotrophs as organisms that have the fastest growth rates above 20°C, while psychrophiles grow fastest at temperatures lower than 15°C.

It is therefore common practice to cultivate Antarctic strains, which are considered psychrophilic, at approximately 5°C in laboratories (Gerday et al., 2000). This temperature is considered to be the optimum temperature which does not induce cellular stress for Antarctic strains (Gerday et al., 2000). It has also been found that higher temperatures, above 20°C, may shorten the growth period, but will induce cellular stress leading to low densities of cells and poor extracellular enzyme production (Gerday et al., 2000). Organisms believed to be psychrotrophic, however, can be cultivated at temperatures above 20°C, but not exceeding 30°C (Cavicchioli et al. 2002).

While cells can contain as many as 20 000 different proteins, the isolation of only one protein from these other proteins is of great scientific and biotechnological significance as it allows for the detailed study of the properties of only that one protein (Campbell and Farrell, 2012; Wilson and Walker, 2010). Generally, the techniques used to isolate proteins focus on size, charge and polarity (Campbell and Farrell 2012) and the final degree of purity required depends on the purposes for which the isolated protein will be used (Wilson and Walker 2010). For the detailed study of enzymes, a homogenous sample comprising of only one protein molecule is required

(Campbell and Farrell, 2012; Ratledge and Kristiansen, 2001). Even though numerous techniques are utilised to eliminate contaminants and purify the protein of interest to homogeneity, in practice, only four different fractionation steps are usually required to purify a given protein (Campbell and Farrell, 2012; Wilson and Walker, 2010). The first step in purifying a soluble protein, as the xylanase from *S. aerolata* is expected to be, from a liquid culture is centrifugation, which will separate the cells and nuclei from the soluble protein of interest (Campbell and Farrell, 2012). A combination of salting out, using a salt such as ammonium sulphate to precipitate the protein of interest from solution, and chromatography techniques such as size-exclusion chromatography, affinity chromatography and ion-exchange chromatography, can then be utilized to arrive at a pure sample of the protein of interest (Campbell and Farrell, 2012). A purification table allows for the tracking of the recovery and purification process so as to gauge the success of the purification protocol (Campbell and Farrell, 2012). SDS-polyacrylamide-gel electrophoresis (SDS-PAGE) analysis of the sample of interest can be used to determine the purity of the sample and to estimate the molecular weight of the protein sample (Campbell and Farrell, 2012).

2.1.1. Aims and objectives

- *Aims*

The aims of this chapter were to determine the optimum conditions for the cultivation of the *S. aerolata* bacterium isolated from Antarctica and for the production and purification of a cold-adapted xylanase derived from this organism.

- *Objectives*

The objectives of this chapter were to:

  - subcultivate *S. aerolata* cells;
  - determine optimal growth conditions for *S. aerolata*;
  - determine optimal conditions for the production of a xylanase from *S. aerolata* cultures;
  - develop a suitable enzyme assay for xylanase activity;
  - purify the xylanase enzyme from the *S. aerolata* culture.

2.2. Materials and methods

2.2.1. Materials

An *S. aerolata* bacterium stock sample in glycerol was kindly donated by Dr G. Matcher (Department of Biochemistry and Microbiology, Rhodes University) and stored at -20°C. All other chemicals and reagents used were of analytical grade and purchased from various chemical companies (Appendix 1).

2.2.2. Sub-cultivation of S. aerolata cells

S. aerolata was subcultivated in a modified liquid medium containing (w/v): peptone, 1%; NaCl, 0.5%; yeast extract, 0.5%, and hydrolysed potato starch, 1%, as the carbon source. For culturing in solid medium, 1.5% agar was added to the above formulation.

2.2.3. Determination of optimum temperature for growth

S. aerolata was sub-cultivated on agar plates (agar, 1.5% (w/v); peptone, 1% (w/v); NaCl, 0.5% (w/v); yeast extract, 0.5% (w/v), and hydrolysed potato starch, 1% (w/v), as the carbon source) at 4, 15, 22, 28 and 37°C for 96 hours.

2.2.4. Screening for xylanase activity

S. aerolata was grown in liquid broth containing (w/v): peptone, 1%; NaCl, 0.5%; yeast extract, 0.5%, and hydrolysed potato starch, 1%, for 48 hours at 28°C. After 48 hours, the culture was centrifuged (15 000 $\times g$ for 15 minutes at 4°C) to obtain the supernatant (extracellular component) and pellet. The pellet was re-suspended in lysis buffer (pH 7.0) and sonicated (10 seconds sonication with 30 second rest period, repeated 5 times). The sonicated sample was centrifuged to obtain the supernatant (intracellular component). The pellet was then re-suspended in sufficient volume of phosphate buffer (pH 7.0) to make a 1 mg/ml suspension (membrane-bound fraction). These fractions were assayed for xylanase activity. In the initial screening stages, activity was expressed as U/ml, to allow for the equitable comparison of xylanase activity produced by each unit volume of fraction harvested.

2.2.5. Xylanase activity assays

Reactions on beechwood xylan were set up by mixing 50 µl of each sample (extracellular, intracellular and membrane-bound fractions) with 150 µl of 1.33% (w/v) beechwood xylan substrate. The reactions, including substrate controls which contained 150 µl of 1.33% (w/v) beechwood xylan and 50 µl of phosphate buffer (pH 7.0), and enzyme controls which contained 50 µl of each sample (extracellular, intracellular and membrane-bound fractions) and 150 µl of phosphate buffer (pH 7.0), were incubated at 37°C for 1 hour, after which they were heat-treated at 100˚C for 5 minutes and then assayed for reducing sugars using a modified dinitrosalicylic acid (DNS) assay method (Miller, 1959), with xylose used as the standard. The DNS reagent was composed of sodium hydroxide (2 g), 3,5-dinitrosalicylic acid (2 g), potassium sodium tartrate (40 g), phenol (0.4 g) and sodium metabisulfite (0.1 g) in 200 ml dH_2O. The heat-treated reactions were centrifuged at 16 060 $\times g$ to remove insoluble substrate and the supernatants from each reaction (150 µl) were mixed with DNS reagent (300 µl). A blank comprised of 150 µl of phosphate buffer (pH 7.0) and 300 µl of DNS reagent was also prepared. These mixtures were heated at 100˚C for 7 minutes, cooled on ice for another 7 minutes, transferred to a 96-well plate and readings were measured at 540 nm using a PowerWaveX Microplate Reader.

2.2.6. Protein determination

The Bradford method (Bradford, 1976) was modified to allow for the accurate determination of low protein concentrations. Each sample (120 µl) was incubated with Bradford's reagent (135 µl) in a 96-well plate for 15 minutes at room temperature before readings were taken at 595 nm using a PowerWaveX Microplate Reader. A standard curve using Bovine Serum Albumin (BSA) as standard was used to determine the protein concentration in each sample.

2.2.7. Growth and activity curves for xylanase production

S. aerolata cells were cultivated in production media (peptone, 1% (w/v); NaCl, 0.5% (w/v); yeast extract, 0.5% (w/v), and hydrolysed potato starch, 1% (w/v)) at 24, 28 and 37°C with shaking at 150 rpm. Samples of the extracellular fractions of the cultures were collected every 6 hours over a total period of 72 hours.

2.2.8. Induction of xylanase production using different carbon sources

S. aerolata was grown on three different modified media at 28°C with shaking at 150 rpm. The first contained hydrolysed potato starch as the carbon source (peptone, 1% (w/v); NaCl, 0.5% (w/v); yeast extract, 1% (w/v), and hydrolysed potato starch, 1% (w/v)), the second contained beechwood xylan as the carbon source (peptone, 1% (w/v); NaCl, 0.5% (w/v); yeast extract, 1% (w/v), and beechwood xylan, 1% (w/v)), and the third contained a combination of hydrolysed potato starch and beechwood xylan as the carbon sources (peptone, 1% (w/v); NaCl, 0.5% (w/v); yeast extract, 1% (w/v); hydrolysed potato starch, 0.5% (w/v) and beechwood xylan, 0.5% (w/v)). After 24 hours, the supernatants were collected by centrifugation at 15 000 ×*g* for 15 minutes at 4˚C and assayed for xylanase activity.

2.2.9. Purification of xylanase from the S. aerolata culture

S. aerolata was grown in liquid broth (peptone, 1% (w/v); NaCl, 0.5% (w/v); yeast extract, 0.5% (w/v), and hydrolysed potato starch, 1% (w/v)) for 24 hours at 28°C in 2 separate flasks. The cell-free supernatant from the first flask was harvested by centrifugation at 15 000 ×*g* for 15 minutes at 4˚C. This supernatant was purified using ultrafiltration with Amicon molecular weight cut-off filtration units (10 kDa and 100 kDa molecular weight cut-off filtration units) at 4 000 ×*g* for 20 minutes at 4˚C.

2.2.10. Electrophoresis

SDS-PAGE was performed using 12% (w/v) acrylamide resolving gels and 4% (w/v) acrylamide stacking gels as described by Laemmli (1970) for purity analysis and molecular weight determination. The SDS-PAGE gel was resolved at 120 V for 90 minutes and the protein bands were stained using Pierce Silver Stain Kit as per the manufacturers' guidelines. Native activity-PAGE was performed by incorporating 0.1% (w/v) beechwood xylan in the 12% (w/v) SDS-free acrylamide resolving gels. The samples were not heat-treated and were mixed with SDS-free and β-mercaptoethanol-free sample buffer prior to loading onto the 4% (w/v) SDS-free stacking gel. The electrode buffer for native activity-PAGE was also SDS-free. After electrophoresis, the gels were incubated in phosphate buffer (pH 7.0) at 37˚C for 6-12 hours. After incubation, the gels were stained with 0.3% (w/v) Congo Red for 15 minutes, then destained using 0.1M NaCl until bands appeared. As soon as the bands appeared, the gels were

counterstained with 0.5% (v/v) acetic acid. The SDS-PAGE and native activity-PAGE gels were visualised and photographed using an UVIprochemi geldoc system (Whitehead Scientific).

2.3. Results

2.3.1. Determination of optimum growth temperature for S. aerolata

An *S. aerolata* stock culture was sub-cultivated on agar plates at 4°C, 15°C, 22°C, 28°C and 37°C for 96 hours after which the plates were photographed. Figure 2.1 shows the growth results of the bacteria at various temperatures.

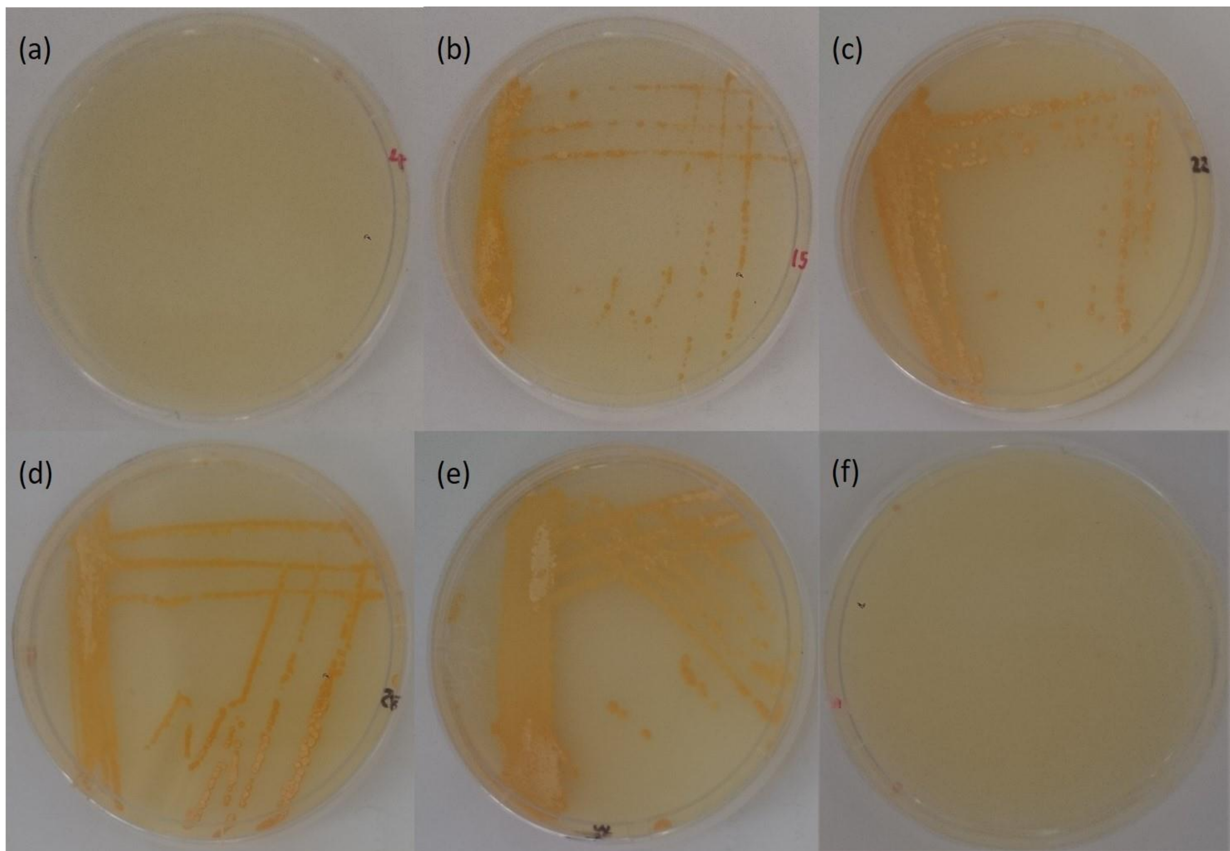


Figure 2.1: Growth of S. aerolata on agar plates at different temperatures; (a) $4^{o}C$, (b) $15^{o}C$, (c) 22˚C, (d) 28˚C, (e) 37˚C and (f) negative control (without inoculum) using hydrolysed potato starch as a carbon source.

The growth of *S. aerolata* was not observed at 4°C after 96 hours with growth only observed between 22˚C and 37˚C (Fig. 2.1b to 2.1e). Optimal growth was observed at 28°C (Fig. 2.1d).

2.3.2. Screening for xylanase activity

The extracellular, intracellular and membrane-bound fractions from a culture of *S. aerolata* cultured in liquid broth were collected and assayed for xylanase activity using beechwood xylan as a substrate and the DNS assay was used to detect reducing sugars. The results are presented in Figure 2.2:

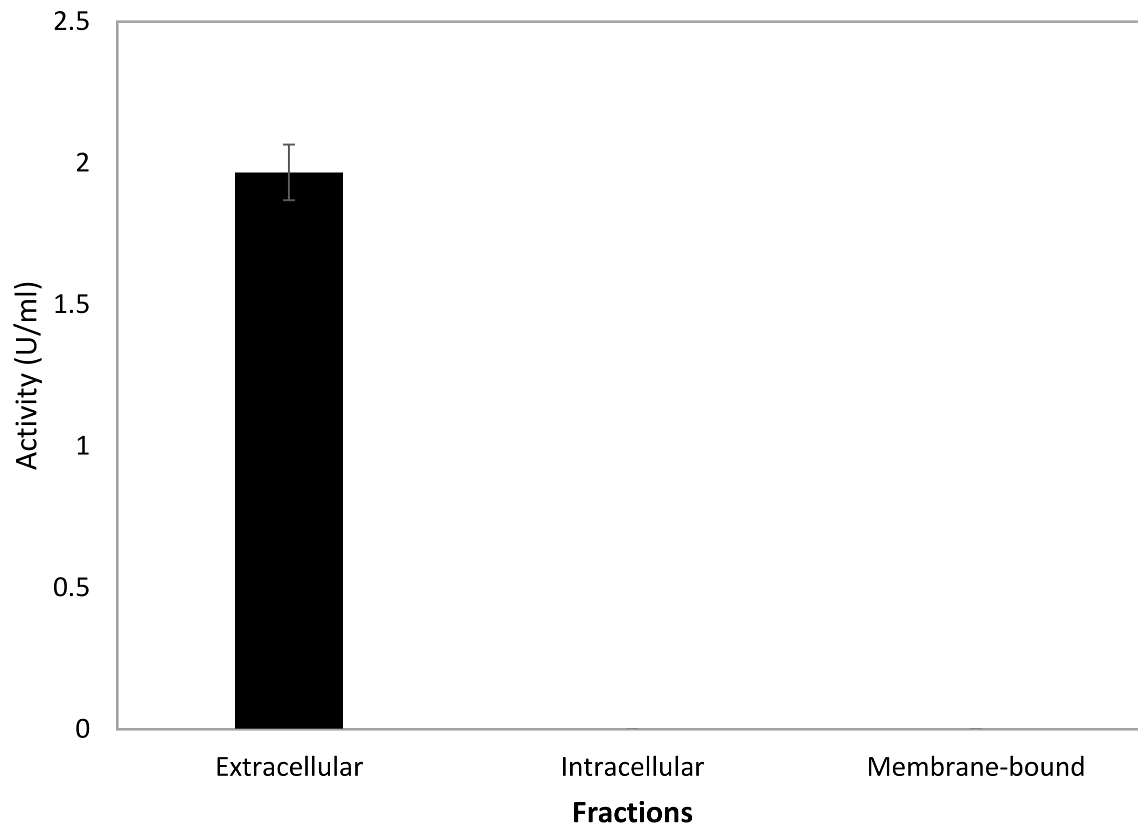


Figure 2.2: Activity of the collected fractions from the S. aerolata culture on beechwood xylan as a substrate (n=3, S.D within 5%)

Xylanase activity was only detected in the extracellular fraction (2 U/mL), while no activity was detected in the intracellular and membrane-bound fractions of *S. aerolata*.

2.3.3. Growth and activity curves for xylanase production

Samples were collected from *S. aerolata* cultures grown at 24, 28 and 37°C every 6 hours for OD_{600} readings and activity assays on beechwood xylan. The results are illustrated in Figure 2.3 below.

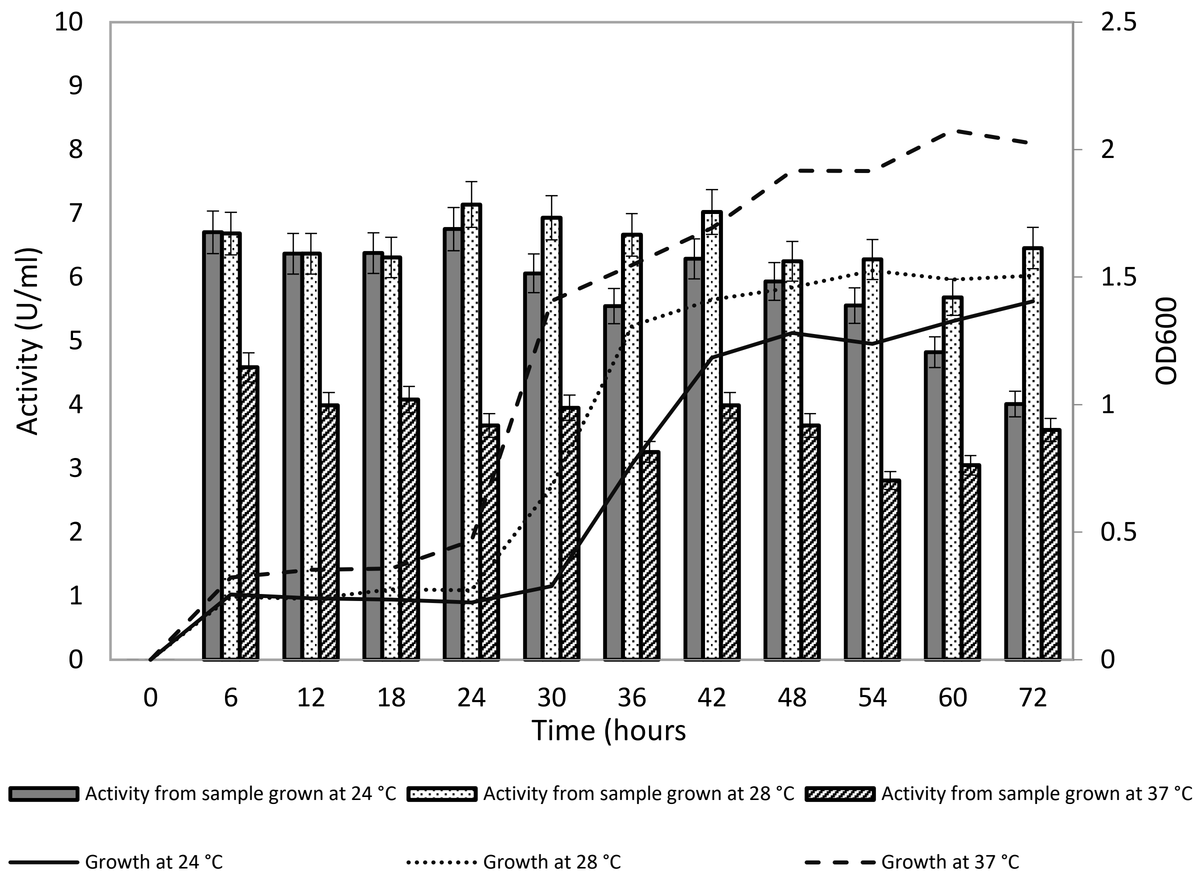


Figure 2.3: Growth and activity curves for production of xylanase from S. aerolata at different temperatures (n=3, S.D within 5%)

Growth curves extrapolated from OD_{600} readings taken at 6 hour intervals from cultures of *S. aerlata* grown at three different temperatures indicated that the bacterium grew the fastest at 37°C. However, activity assays using the same samples showed that the highest activity was obtained when *S. aerolata* was grown at 28°C.

2.3.4. Induction of xylanase production using different substrates as carbon sources

The extracellular fractions from *S. aerolata* cultures induced with hydrolysed potato starch or beechwood xylan or a combination of the two, were assayed for xylanase activity using the DNS method and the results obtained are illustrated in Figure 2.4:

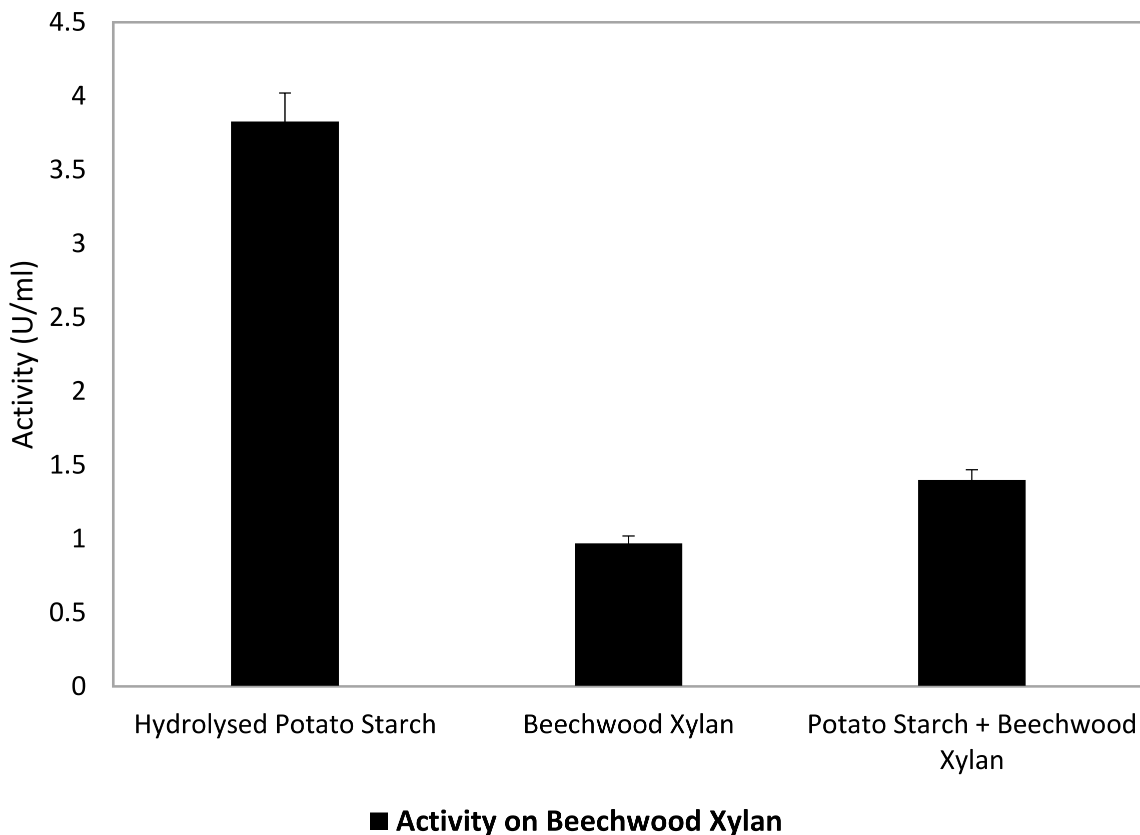


Figure 2.4: Effect of induction using different carbon sources on xylanase production as determined by xylanase activity of supernatants on beechwood xylan (n=3, S.D within 5%)

Xylanase production was found to be most induced by hydrolysed potato starch as compared to beechwood xylan. A combination of the two substrates as inducers for xylanase production resulted in higher xylanase activity than when beechwood xylan was used as the sole inducer. However, this improvement was still significantly less than when hydrolysed potato starch was used as the sole carbon source.

2.3.5. Purification of S. aerolata xylanase

The cell-free supernatant from the *S. aerolata* culture containing the xylanase was harvested by centrifugation at 15 000 × g for 15 minutes at 4˚C. This supernatant was then purified using ultrafiltration with Amicon molecular weight cut-off filtration units (10 kDa and 100 kDa molecular weight cut-off filtration units) at 4 000 ×*g* for 20 minutes at 4˚C. The purification table (Table 2.1) for the purification of the putative xylanase from *S. aerolata* using ultrafiltration is presented below.

Table 2.1: Purification table showing the purification of S. aerolata xylanase using ultrafiltration with Amicon molecular weight cut-off filtration units

Fraction	Volume (mL)	Total protein (mg)	Activity (U/mL)	Total activity (U)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude	50	8.07	0.96	47.80	5.93	1	100
Ultrafiltration	8	0.27	0.85	6.83	24.88	4.20	14.29

The xylanase was purified using 10 kDa and 100 kDa molecular weight cut-off filtration units and was found to be in the greater than 10 kDa but less than 100 kDa fraction. The putative xylanase was purified to a fold purification of 4.2 with a percentage yield of 14%.

SDS-PAGE analysis was performed on the crude and purified samples to check for purity and to estimate the molecular weight of the putative xylanase. Native activity-PAGE analysis was also performed to confirm that the purified protein was a xylanase. The results are presented in Figure 2.5 below.

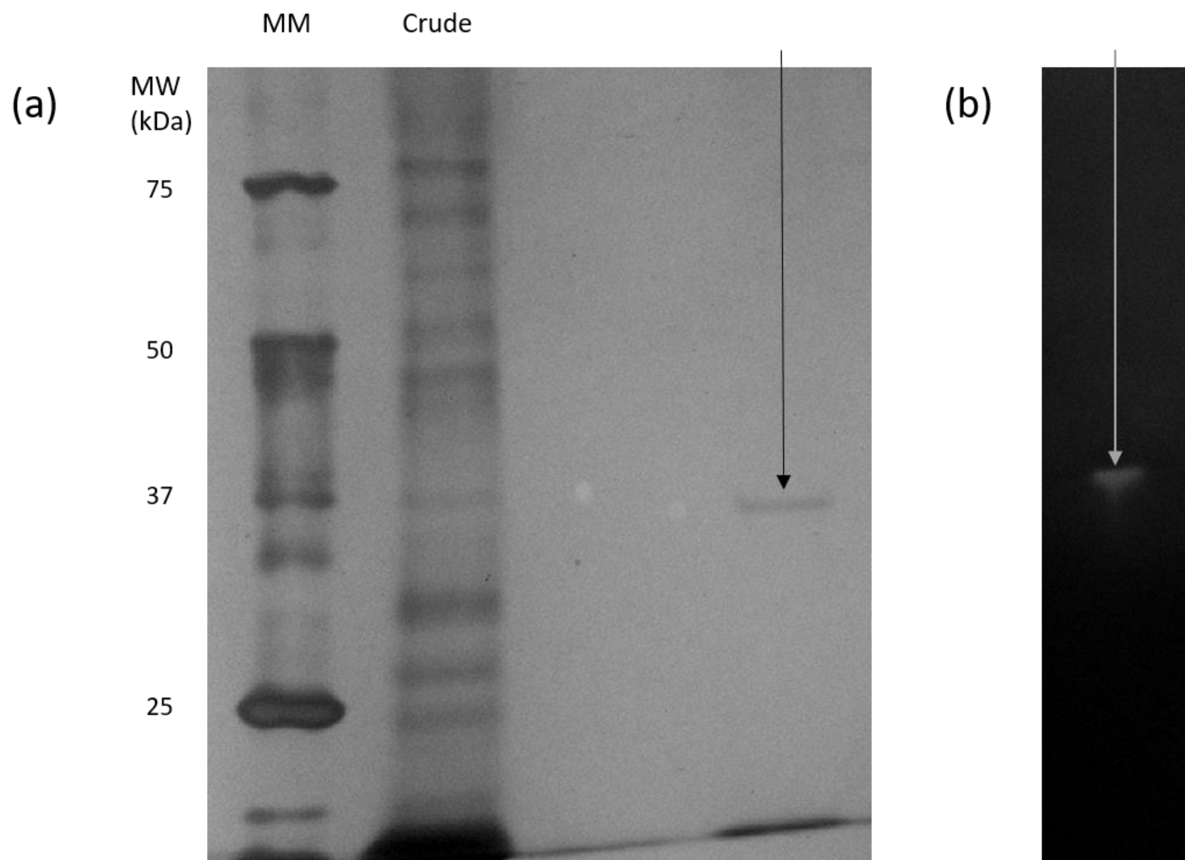


Figure 2.5: SDS-PAGE analysis (a) of the BioRad unstained protein standards (MM), crude extracellular extract from an S. aerolata culture (Crude) and purified S. aerolata putative xylanase sample and native activity-PAGE analysis (b) of the purified putative xylanase sample.

The purified sample from the *S. aerolata* culture appeared to have only one band on SDS-PAGE (shown by the black arrow) (Fig. 2.5a), indicating that purification using only ultrafiltration with 10 kDa and 100 kDa Amicon molecular weight cut-off filtration units was successful. The band on the purified sample was estimated to have a molecular weight of approximately 36 kDa. This sample was analysed using non-denaturing native activity-PAGE (Fig. 2.5b) by incorporating 0.1% (w/v) beechwood xylan in the resolving gel, and xylanase activity of this purified sample was confirmed by a band showing activity on the beechwood xylan resolving gel (indicated by the grey arrow).

All the purification fractions collected during the purification protocol were also analysed using SDS-PAGE and the results are shown in Figure 2.6.

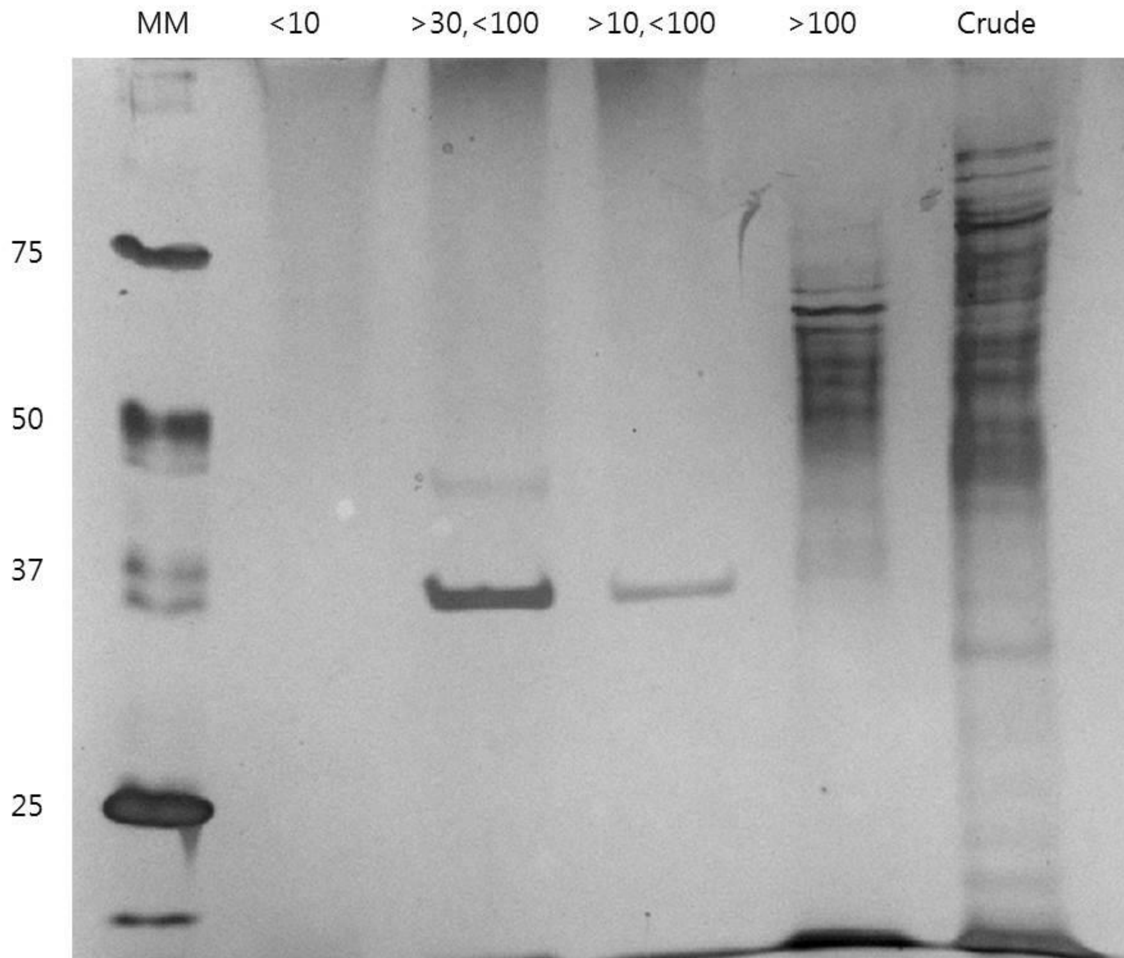


Figure 2.6: SDS-PAGE analysis of the BioRad unstained protein standards (MM), less than 10 kDa filtrate (<10), greater than 30 kDa but less than 100 kDa fraction (>30,<100), greater than 10 kDa but less than 100 kDa fraction (>10,<100), greater than 100 kDa fraction (>100) and crude extracellular extract from an S. aerolata culture (Crude)

There were no protein bands observed in the less than 10 kDa fraction. There were two protein bands observed in the greater than 30 kDa but less than 100 kDa fraction, with one of the bands corresponding to the putative xylanase. Only one band, corresponding to the putative xylanase, was observed in the greater than 10 kDa but less than 100 kDa fraction, corroborating the results from Figure 2.5, that the 10 kDa and 100 kDa Amicon molecular weight cut-off filtration units can be used to purify the putative xylanase to homogeneity. Multiple protein bands between 37 kDa and 75 kDa were observed in the greater than 100 kDa fraction, suggesting that most proteins were retained in the 100 kDa Amicon molecular weight cut-off filtration unit, with the exception of the two proteins observed in greater than 30 kDa but less than 100 kDa fraction.

2.4. Discussion

The psychrotrophic bacterium, *S. aerolata*, was successfully sub-cultivated in both solid and liquid media and found to grow optimally at 28°C on media composed of (w/v): peptone, 1%; NaCl, 0.5%; yeast extract, 1%, and hydrolysed potato starch, 1%; with or without 1.5% agar. While the growth of *S.aerolata* was observed at 15, 22, 28 and 37°C after 96 hours, no growth was observed at 4°C after the same time period. It is likely that at such a low temperature, an extended period of time exceeding 96 hours is required before the growth of *S. aerolata* can be observed. The ability of the bacterium to grow at 15°C and also at temperatures exceeding 20°C is consistent with the definitions of europsychrophiles, or psychrotrophs, as presented by Cavicchioli et al. (2002), Dalmaso et al. (2015), Morita (1975) and Russell (1997). The optimal temperature for growth, determined after comparing the growth patterns on agar plates incubated at different temperatures for 96 hours, was found to be 28°C. Cavicchioli et al. (2002) distinguished psychrotrophs as organisms with the fastest growth rates at temperatures exceeding 20°C, which is consistent with the findings from our study. Zhang et al. (2011) and Busse et al. (2003) also cultured different species of bacteria from the *Sphingomons* genus, including *Sphingomonas aerolata* by the latter, at 25°C and room temperature, respectively, confirming as we did that bacterial species from the *Sphingomonas* genus are psychrotrophic and have optimal growth temperatures exceeding 20°C.

Different fractions (intracellular, extracellular and membrane-bound fractions) isolated from a liquid culture of *S. aerolata* grown at 28°C with shaking at 150 rpm for 24 hours, were screened for xylanase activity and it was found that only the extracellular fraction was active on beechwood xylan. These findings were expected as xylanases are endo-acting enzymes and are usually produced and released into the extracellular fraction by xylanase producing micro-organisms (Alvarez-Cervantes et al., 2016). Growth and activity curves indicated that the optimal conditions for xylanase production was growth of *S. aerolata* at 28°C, with shaking at 150 rpm for 24 hours. Xylanases are not constitutively produced by xylanolytic microorganisms and require induction by an appropriate substrate, usually the substrate that the respective xylanase will be active on, for their synthesis by the microorganism (Alvarez-Cervantes et al., 2016). Interestingly, it was found in this study that hydrolysed potato starch was a more effective inducer of xylanase production than beechwood xylan, a natural substrate for xylanase. It is also believed that xylanase production is actually induced by the products of hydrolysis of the substrates in the medium, rather by than the actual substrates, as they are

usually too large to enter the cell matrix and induce expression of the xylanases (Alvarez-Cervantes et al., 2016). It is therefore quite possible that there are other glycoside hydrolase enzymes that the *S. aerolata* bacterium constitutively expresses into the medium in the initial stages of its growth, that act on hydrolysed potato starch to release sugars of low molecular weights which can easily enter the cells and induce expression of the putative xylanase.

Proteins are usually separated and purified by using their two most prominent physical properties; size and charge (Gajera et al., 2008). However, affinity purification strategies that utilise the biological function or specific recognition properties of a target protein can also be used (Gajera et al., 2008). In this study, two methods were used to purify the xylanase from the crude extracellular extract of the *S. aerolata* liquid culture. The first was ultrafiltration using Amicon molecular weight cut-off filtration units, and the second was a combination of acetone precipitation and ultrafiltration using the molecular weight cut-off filtration units. It was found that the first method using only ultrafiltration and molecular weight cut-off filtration units was the best strategy as it resulted in a higher fold purification (4.2 compared to 0.2) and percentage yield (14.2% compared to 4.2%). The sample purified using ultrafiltration alone also had a higher specific activity (24.88 U/mg compared to 1.09 U/mg). These results show that purification of the *S. aerolata* putative xylanase could be performed more successfully using ultrafiltration using 10 kDa and 100 kDa Amicon molecular weight cut-off filtration units than using a combined protocol of acetone precipitation and ultrafiltration.

The *S. aerolata* xylanase purified using ultrafiltration and molecular weight cut-off units was analysed using SDS-PAGE to check for purity and to determine the molecular weight of the xylanase. Only one band was visualised in the lane where the purified putative xylanase was loaded, indicating that the xylanase was purified successfully to homogeneity. The molecular weight of the putative xylanase was determined to be approximately 36 kDa. All the purification fractions collected during the purification protocol were also analysed using SDS-PAGE (Figure 2.6). It was found that most of the bands observed in the crude sample, even bands corresponding to molecular weights as small as 37 kDa, were retained by the 100 kDa Amicon molecular weight cut-off filtration unit (Figure 2.6). This suggests that the proteins observed in the SDS-PAGE analysis of the crude sample might be produced by the bacteria as a multi-protein complex. This would explain how proteins seemingly smaller than 100 kDa, as observed by SDS-PAGE analysis, were retained by the 100 kDa Amicon molecular weight filter. Subsequent treatment of the sample retained by the 100 kDa filter using SDS and β-mercaptoethanol prior to SDS-PAGE analysis, unwound the multi-protein complex resulting

in the visualisation of proteins as single bands corresponding to molecular weights smaller than 100 kDa. Therefore, the working hypothesis for the observed successful purification to homogeneity of the putative xylanase, from a crude sample containing numerous protein species using only ultrafiltration and molecular weight cut-off filtration units, is that the observed proteins in the crude sample occur as a multi-protein aggregate or multi-protein complex that is retained by the 100 kDa filter, allowing only the target protein to pass through the filter. The xylanase activity of the purified sample was successfully confirmed using non-denaturing native activity-PAGE with 0.1% (w/v) beechwood xylan incorporated in the resolving gel, with a band of xylan hydrolysis visualised by staining the gel with 0.3% (w/v) Congo Red, de-staining it with 1 M NaCl and counterstaining with 5% (v/v) acetic acid.

2.4.1. Conclusions

S. aerolata was found to grow optimally at 28˚C on media composed of (w/v): peptone, 1%; NaCl, 0.5%; yeast extract, 1%, and hydrolysed potato starch, 1%. It was found to produce a xylanase into the extracellular fraction of the liquid culture. This xylanase was successfully purified to homogeneity by ultrafiltration using 10 kDa and 100 kDa Amicon molecular weight cut-off filtration units and was determined to have a molecular weight of approximately 36 kDa. The xylanase activity was confirmed using native activity-PAGE with 0.1% (w/v) beechwood xylan incorporated into the resolving gel.

The aims of this chapter, to determine the optimum conditions for the cultivation of *S. aerolata* and the production and purification of a xylanase from this extremophile, were achieved. The putative xylanase was purified to homogeneity, making it suitable for further detailed studies of its biochemical characteristics. The next step in this study was to biochemically characterize the *S. aerolata* putative xylanase.

Chapter 3: Biochemical characterization of the *S. aerolata* putative xylanase

3.1. Introduction

Xylanases (EC 3.2.1.8) have been defined as glycoside hydrolase enzymes that have specific activity on the substrate xylan, a complex plant cell wall polysaccharide (Collins et al., 2005; Lagaert et al., 2009). Xylanases exist in diverse forms that all display varying structures, mechanisms of action, substrate specificities, hydrolytic activities and physicochemical characteristics (Collins et al., 2005). The existence of such diversity of xylanases has been attributed to be due to the complexity and heterogeneity of the xylan substrate (Collins et al., 2005). Glycoside hydrolases, such as xylanases, are however classified and organised into different glycoside hydrolase (GH) families based on amino acid sequence homologies, not substrate specificity (St John et al., 2016). This organisation of GH families is maintained by the active efforts of the curators of the Carbohydrate Active Enzymes (CAZy) database (St John et al., 2016).

Xylanases have been classified into GH families 5, 8, 10, 11, 30, 43, 62 and 98 (www.cazy.org). Most xylanases that have been studied have been found to have evolved from two main scaffolds, the TIM-barrel $(\alpha/\beta)_8$ and the β-jelly roll (Karlsson et al., 2018). GH 5, GH10 and GH30 xylanases are known to have TIM-barrel $(\alpha/\beta)_8$ structures while GH 11 xylanases are known to have β-jelly roll structures (Karlsson et al., 2018). GH 5 xylanases have been found to accept arabinose substituents in certain sites on the xylan backbone and to require an arabinose substituent in the -1 subsite for binding and hydrolysis of xylan (Karlsson et al., 2018). GH 30 xylanases, in a similar manner to GH 5 xylanases, also have a preference for substituted xylan, in this case uronic acid substituted xylan, requiring a uronic acid subsituent in the -2 subsite for binding and hydrolysis of xylan (Karlsson et al., 2018). Most xylanases, however, have generally been classified into only GH families 10 and 11 (Meng et al., 2015). GH 10 xylanases are characterised by $(\alpha/\beta)_8$ barrel fold structures and molecular masses typically greater than 30 kDa, while GH 11 xylanases have β-jelly roll structures and molecular masses typically lower than 30 kDa (Ko et al., 2016). Due to their typically smaller molecular sizes, GH 11 xylanases have been found to be more active on insoluble substrates compared to GH 10 xylanases (Karlsson et al., 2018). While most xylanases, with the exception of GH 5 and GH 30 xylanases, generally hydrolyse unsubstituted xylan more efficiently, compared to

GH 10 xylanases, GH 11 xylanases are even more restricted by substituents on the xylan backbone (Karlsson et al., 2018).

The majority of xylanases that have been studied are from microbial sources, with optimum operating temperatures at, or near the mesophilic range of approximately 40-60˚C (Collins et al., 2005). These xylanases have also been found to be optimally active at neutral pHs (bacterial xylanases) or slightly acidic pHs (fungal xylanases) (Collins et al., 2005). However, xylanases have also been reported that are active at the extremes of pH (pH 2.0 to pH 11.0) and temperature (5˚C to 105˚C) (Collins et al., 2005). Such xylanases are produced by extremophilic organisms, and of the extremophilic xylanases, thermophiles, acidiphiles and alkaliphiles have been the most studied (Collins et al., 2005).

Industrial demands for enzymes that can operate at process conditions have resulted in numerous xylanases from extremophilic organisms being isolated and studied (Collins et al., 2005). Xylanases from thermophiles, alkaliphiles and acidiphiles have been isolated and studied; however, little attention has been paid to xylanases from psychrophiles (Collins et al., 2005). The use of psychrophilic xylanases in industrial processes can be advantageous as colder reaction conditions would preserve the properties of heat-labile products and also result in energy savings (Lee et al., 2006). This study focused on the characterization of a putative xylanase isolated from a psychrophilic bacterium, *S. aerolata*.

3.1.1. Aims and objectives

- *Aims*

The aims of this chapter were to biochemically characterize the purified *S. aerolata* putative xylanase.

- *Objectives*

The objectives of this chapter were to:

  - determine the substrate specificity of the putative xylanase;
  - determine the optimum temperature for the putative xylanase;
  - determine the thermostability of the putative xylanase;
  - determine the optimum pH for the putative xylanase;
  - determine inhibition profiles of the putative xylanase by simple sugars;

- determine the effect of various metal ions on the putative xylanase.

3.2. Materials and methods

3.2.1. Materials

All chemicals and reagents used were of analytical grade and purchased from various chemical companies (Appendix I).

3.2.2. Determination of substrate specificity

Hydrolytic activities of the purified putative xylanase sample were assayed on a wide range of substrates, including cellulose substrates (Avicel and carboxymethyl cellulose (CMC)), starch substrates (maize amylopectin, soluble starch and hydrolysed potato starch), mannan substrates (konjac glucomannan, guar galactomannan and locust bean gum (LBG)) and xylan substrates (beechwood xylan and wheat arabinoxylan). Reactions were set up and assayed as per the protocols detailed in section 2.2.5. Glucose was used as the standard for cellulose and starch substrates, mannose as the standard for mannan substrates and xylose as the standard for xylose substrates. Reactions on *p*-nitrophenyl-based substrates (*p*-nitrophenyl β-D-xylopyranoside, *p*-nitrophenyl α-L-arabinopyranoside, *p*-nitrophenyl β-D-glucopyranoside and *p*-nitrophenyl α-D-galactopyranoside) were also set up by incubating 25 µl of the purified putative xylanase with 5 µl of 100 mM *p*-nitrophenyl substrates. Enzyme controls containing 25 µl of the putative xylanase sample and 5 µl of phosphate buffer (pH 7.0) and substrate controls containing 25 µl of phosphate buffer (pH 7.0) and 5 µl of 100 mM *p*-nitrophenyl substrates were also included. The reactions were made up to 250 µl using phosphate buffer (pH 7.0) and incubated at 37˚C for 15 minutes. The reactions were then stopped by the addition of 250 µl of 2 M Na_2CO_3, and 250 µl of each reaction was transferred to a 96-well plate with absorbance readings taken at 405 nm.

3.2.3. Determination of temperature optima and stability

Activity of the putative *S. aerolata* xylanase on beechwood xylan was assayed at different temperatures (4-90˚C) using the standard activity assay protocol outlined in section 2.2.5. The thermal stability of the putative xylanase was determined by incubating the enzyme at 28˚C,

37˚C and 50˚C for 1, 3, 6 and 24 hours, respectively, before enzyme activity determination was performed using the protocol outlined in section 2.2.5.

3.2.4. Determination of pH optima

A universal buffer (50 mM Tris, 50 mM boric acid, 33 mM citric acid, and 50 mM Na_2PO_4) (Britton and Robinson, 1931) was prepared and adjusted using either NaOH or HCl to obtain a range of pH values (pH 2.0 - 11.0). Activity of the putative *S. aerolata* xylanase on beechwood xylan (1.33% (w/v)) was estimated over this range of pH values (pH 2.0 - 11.0) using the standard activity assay protocol outlined in section 2.2.5.

3.2.5. Effect of glucose, xylose and mannose on the activity of the putative xylanase

The effect of glucose, xylose and mannose on the xylanase and "Avicel-ase" activity of the putative *S. aerolata* xylanase was investigated by mixing 50 µl of the enzyme sample with 50 µl of the azo-based substrates (azo-wheat arabinoxylan and azo-Avicel) (4% w/v), and adjusting the volumes of the glucose, xylose and mannose solutions (100 mM) along with the volume of the citrate buffer (pH 6.0) to achieve final concentrations of the sugars at 5, 20, 50 and 100 µM and a total reaction volume of 200 µl. Enzyme controls (50 µl of enzyme sample plus 150 µl of citrate buffer (pH 6.0)), and substrate controls (50 µl of azo substrates plus 150 µl of citrate buffer (pH 6,0) were also prepared. The reactions were incubated at 37˚C for an hour and then stopped by addition of 800 µl of 95% ethanol. The reactions were then allowed to stand for 5 minutes before they were centrifuged at 16 060 ×*g* for 5 minutes in a Heraeus Biofuge Pico Benchtop Centrifuge. The supernatants of each reaction (250 µl) were transferred to a 96-well plate and absorbance readings were taken at 590 nm with Remazol Brilliant Blue (RBB) used as a suitable standard.

3.2.6. Effect of various metal ions and chemical reagents on the activity of the putative xylanase

The effect of Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} ions, as well as EDTA and β-mercaptoethanol, on the activity of the putative xylanase during beechwood xylan degradation was investigated by preparing 100 mM stock solutions of $CaCl_2$, $CuSO_4$, $FeSO_4$, $MgSO_4$, $MnSO_4$, $ZnSO_4$, EDTA and β-mercaptoethanol. These solutions were incorporated into the

standard xylanase activity determination reactions to produce final concentrations of 5 mM and 10 mM of each chemical. In addition, enzyme controls and substrate controls were prepared according to the protocol outlined in section 2.2.5. Metal ion controls containing each metal ion plus substrate and no enzyme were also prepared. These reactions were assayed according to the standard xylanase activity assay protocol described in section 2.2.5.

3.2.7. Analysis of the hydrolysis of cello-oligomers and xylo-oligomers by the putative xylanase using thin layer chromatography (TLC)

Reactions with the putative xylanase (100 µl) on 1% (w/v) wheat arabinoxylan, beechwood xylan, xylobiose, xylotriose, xylotetraose, xylopentaose, Avicel, cellobiose, cellotriose, cellotetraose, and cellopentaose substrates (300 µl) were incubated at 37°C for 24 hours. Control reactions consisting of 100 µl citrate buffer (pH 6.0) and 300 µl of each substrate were also set up and incubated at 37°C for 24 hours. After 24 hours, the reactions were terminated by heating at 100°C for five minutes and 5 µl of each sample was applied on HPTLC plates. The plates were developed twice with butanol: acetic acid: water (2: 1: 1, v/v/v). The plates were subsequently dried at 37°C until dry before they were developed the second time using the butanol, acetic acid and water mixture. The plates were dried again before they were soaked in a solution of 0.3% (w/v) α-naphthol in 5% (v/v) sulphuric acid in methanol for approximately 7 minutes, and then dried again at 37°C until completely dry. The sugars developed on the plates were visualised by heating the plates at 120°C for 10 minutes.

3.3. Results

3.3.1. Determination of substrate specificity

Activity of the putative xylanase was tested on a wide range of lignocellulosic substrates, and the results are presented in Table 3.1 below.

Table 3.1: Specific and relative activity of the putative S. aerolata xylanase on various lignocellulosic substrates (n=3).

Substrate	Specific Activity (U/mg)	Relative Activity (%)
Beechwood xylan	23.69 ± 0.81	100
Wheat arabinoxylan	20.15 ± 0.48	85.02
Avicel	22.64 ± 2.24	95.53
Carboxymethyl cellulose (CMC)	5.50 ± 0.01	23.20
Maize amylopectin	0	0
Soluble starch (Maize)	0	0
Hydrolysed potato starch	19.11 ± 0.93	80.63
Konjac glucomannan	0	0
Guar galactomannan	9.48 ± 0.02	40.01
Locust bean gum (LBG)	7.07 ± 0.01	29.82
Arabinogalactan	16.82 ± 0.16	70.98
4-Nitrophenyl β-D-xylopyranoside	0.31 ± 0.004	1.32
4-Nitrophenyl α-L-arabinopyranoside	0.76 ± 0.003	3.20
4-Nitrophenyl β-D-glucopyranoside	1.59 ± 0.01	6.71
4-Nitrophenyl α-D-galactopyranoside	1.47 ± 0.01	6.21

The putative xylanase was found to have high activity towards xylan substrates (Table 3.1), but it also displayed relatively high activity towards Avicel cellulose (96%) and hydrolysed potato starch (81%) with beechwood xylan specified as 100% relative activity, indicating a possible multifunctional nature of the putative xylanase.

3.3.2. Determination of temperature optima and stability

Activity of the putative xylanase on beechwood xylan (n=3) was determined after incubation of the reactions at different temperatures (4-90˚C) for 30 minutes, before assaying for xylanase activity using the DNS protocol described in section 2.2.5. The results are presented in Figure 3.1 below.

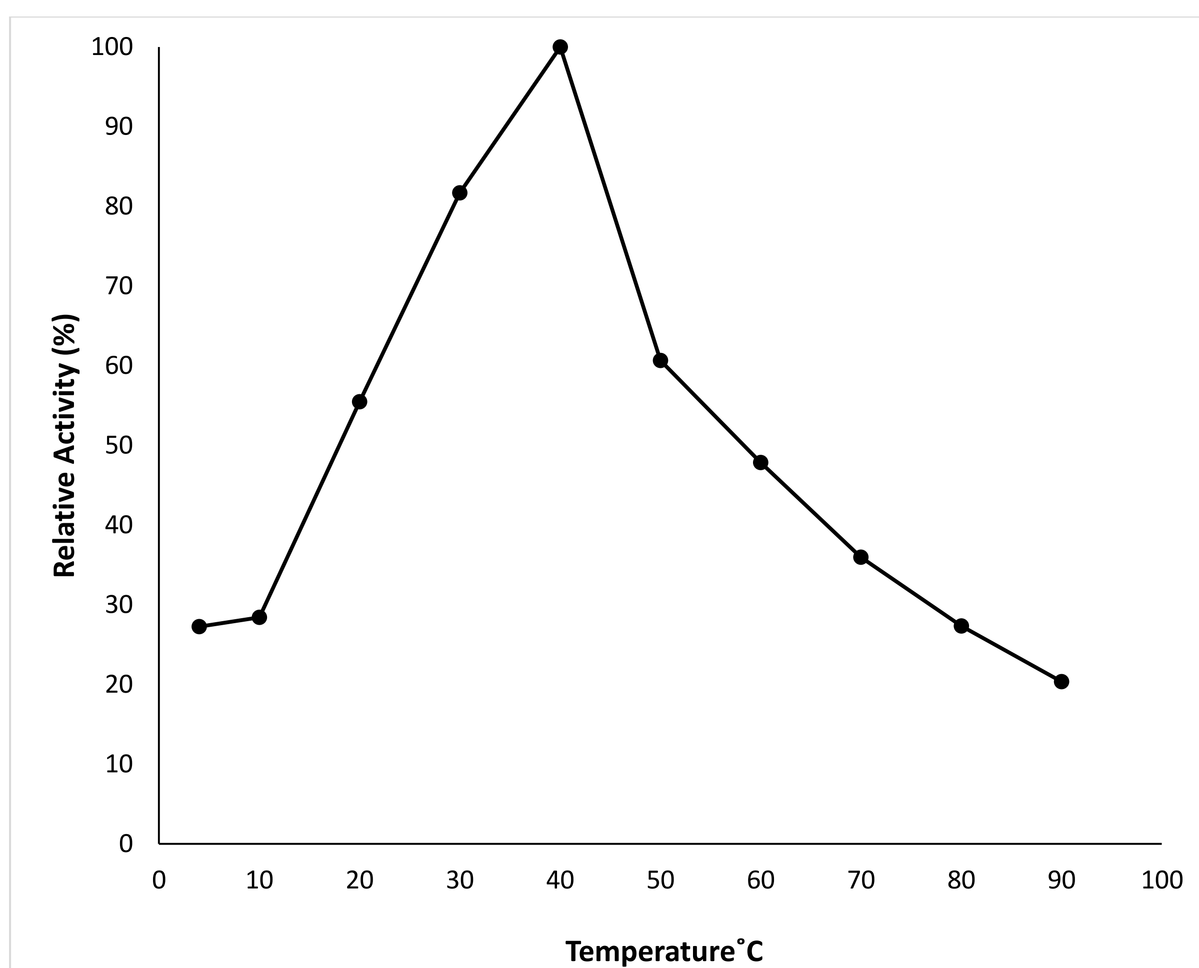


Figure 3.1: Determination of optimum temperature for beechwood xylan hydrolysis by the putative S. aerolata xylanase.

The putative xylanase was found to have an optimum temperature of 40˚C (Fig. 3.1). Subsequent xylanase activity determination for the putative xylanase was continued at 37˚C for the remainder of the study as this was close to its optimum temperature.

Stability of the putative xylanase was determined by incubating the xylanase at 28˚C, 37˚C and 50˚C for 1, 3, 6 and 24 hours prior to enzyme activity estimation (n=3). The results are presented in Figure 3.2.

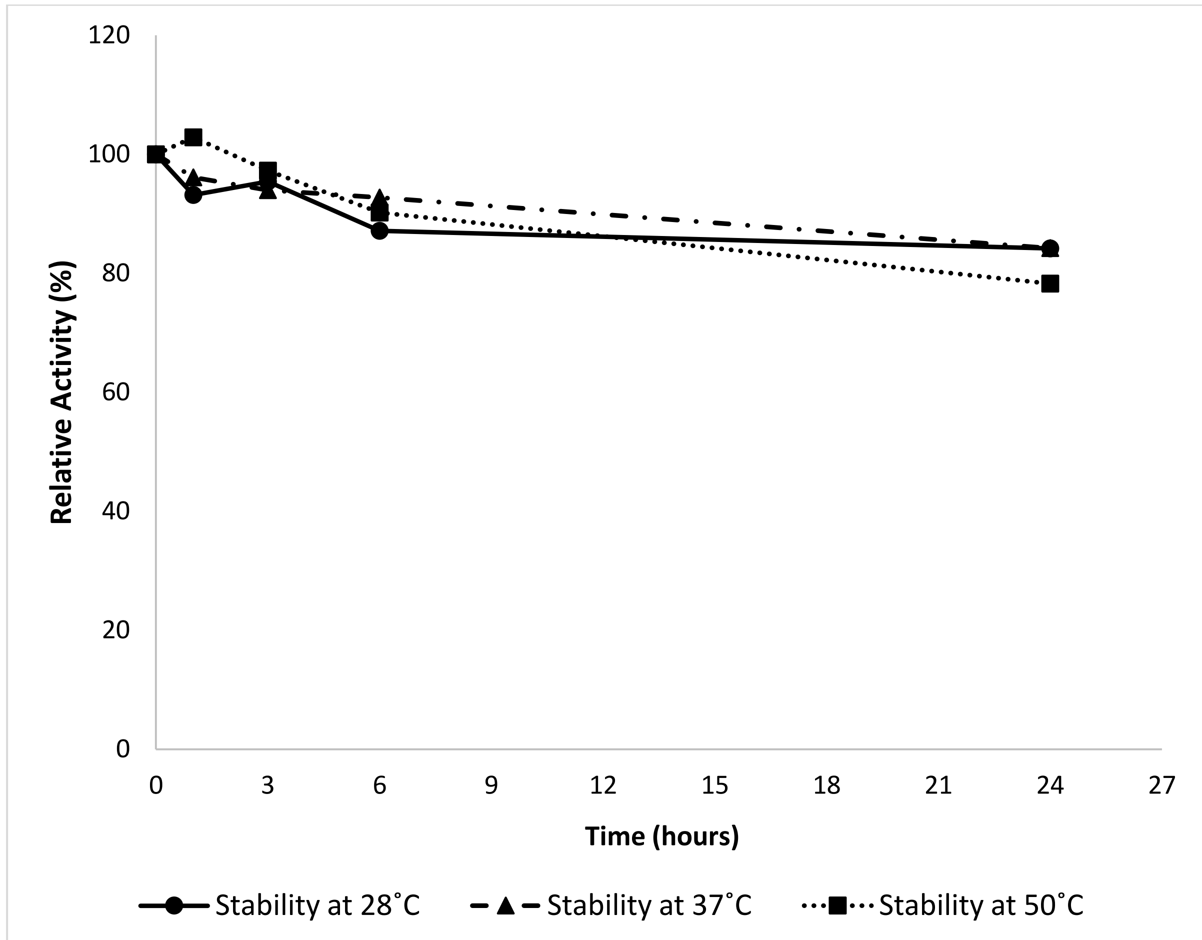


Figure 3.2: Thermal stability of the putative xylanase at 28˚C, 37˚C and 50˚C (n=3)

The putative xylanase was found to maintain approximately 80% of its relative activity on beechwood xylan even after incubation at 50˚C for 24 hours (Fig. 3.2). These results indicate that the putative xylanase is relatively thermostable, which is unexpected for an enzyme from a psychrophilic source.

3.3.3. Determination of pH optima

Relative activity of the putative *S. aerolata* xylanase was determined over a range of pH values and the results are presented in Figure 3.3.

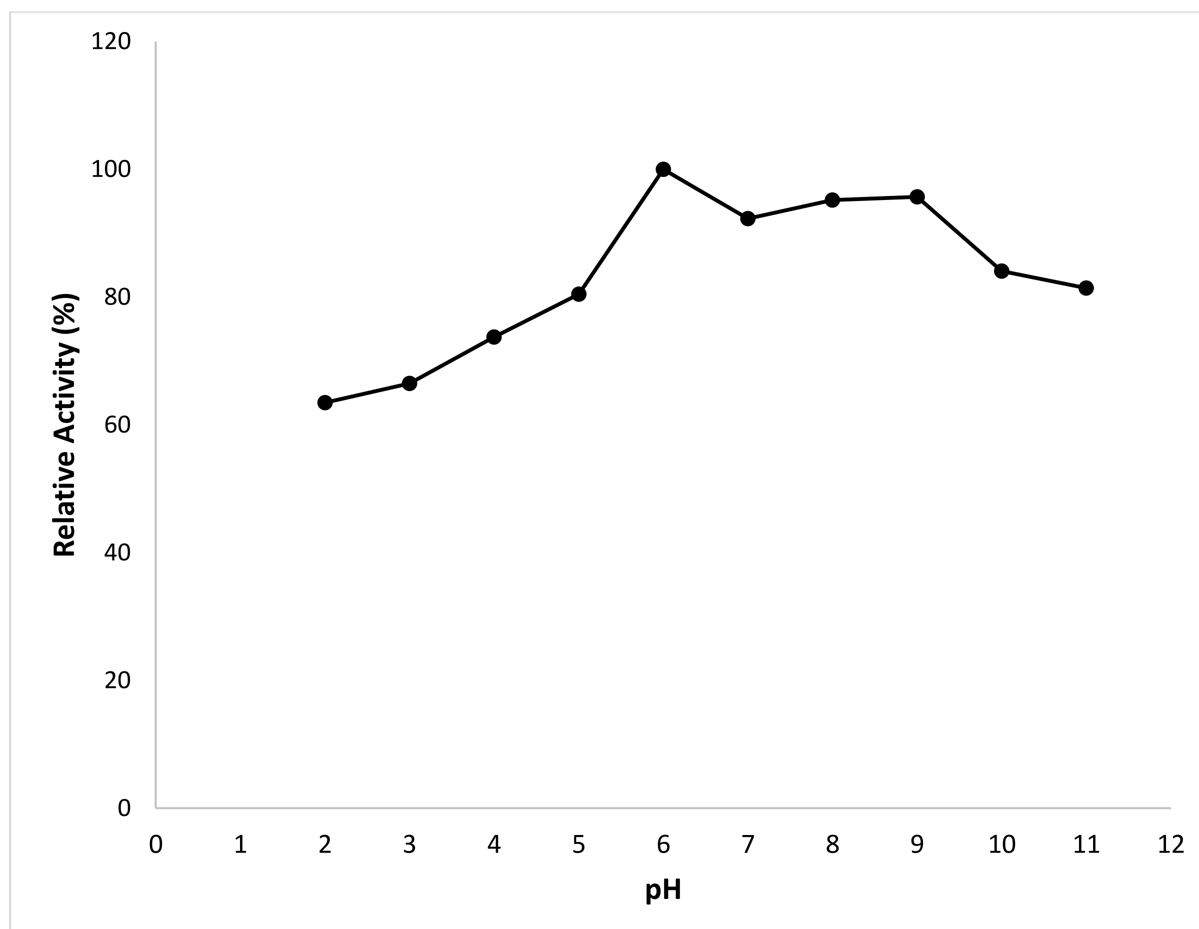


Figure 3.3: Determination of pH optima for the S. aerolata putative xylanase on beechwood xylan

The putative xylanase from *S. aerolata* was found to have an optimum pH of 6.0 (Fig. 3.3). The putative *S. aerolata* xylanase also appeared to be relatively stable at acidic pH, maintaining over 60% of its relative activity at pH 2.0. The putative xylanase also maintained over 80% relative activity at basic pH (pH 8.0-11.0). Subsequent reactions were carried out using citrate buffer (0.05 M, pH 6.0), which is the optimum pH for the putative xylanase activity.

3.3.4. Effect of glucose, xylose and mannose on the xylanase and "Avicel-ase" activity of the putative xylanase

The effect of glucose, xylose and mannose on the xylanase and "Avicel-ase" activity of the putative *S. aerolata* xylanase was determined using azo-wheat arabinoxylan and azo-Avicel as substrates and the results are presented in Figure 3.4.

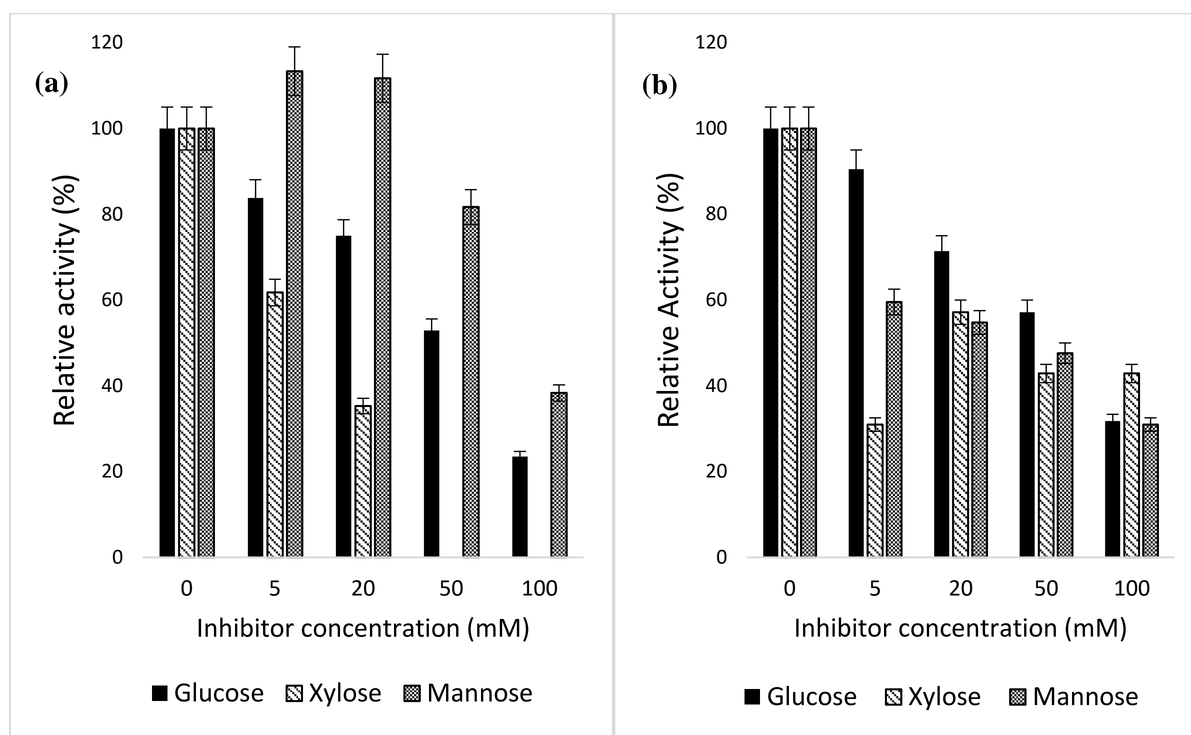


Figure 3.4: Effect of glucose, xylose and mannose on the activity of the putative xylanase on azo-wheat arabinoxylan (a) and azo-Avicel (b) (n=4, SD within 5%)

The activity of the *S. aerolata* putative xylanase on azo-wheat arabinoxylan was found to be inhibited by glucose (to approximately 80% relative activity) and xylose (to approximately 60% relative activity) even at a low concentration of 5 mM (Fig. 3.4a). Inhibition of xylanase activity by glucose and xylose appeared to be directly proportional to the concentration of the sugars. At xylose concentrations of 50 mM and 100 mM, activity of the putative xylanase on azo-wheat arabinoxylan was completely abolished. At mannose concentrations of 5 mM and 20 mM, xylanase activity increased to over 110% relative to xylanase activity in the absence of any sugars. Inhibition of xylanase activity by mannose only occurred at high mannose concentrations of 50 mM and 100 mM. Avicelase activity of the putative *S. aerolata* xylanase was inhibited by all three sugars; glucose, xylose and mannose, with inhibition appearing to be directly proportional to the concentration of the sugars (Fig. 3.4b).

3.3.5. Effect of various metal ions on xylanase activity of the putative xylanase

Activity of the putative xylanase on beechwood xylan was assayed in the presence of various metal ions at different concentrations and the results are presented in Figure 3.5.

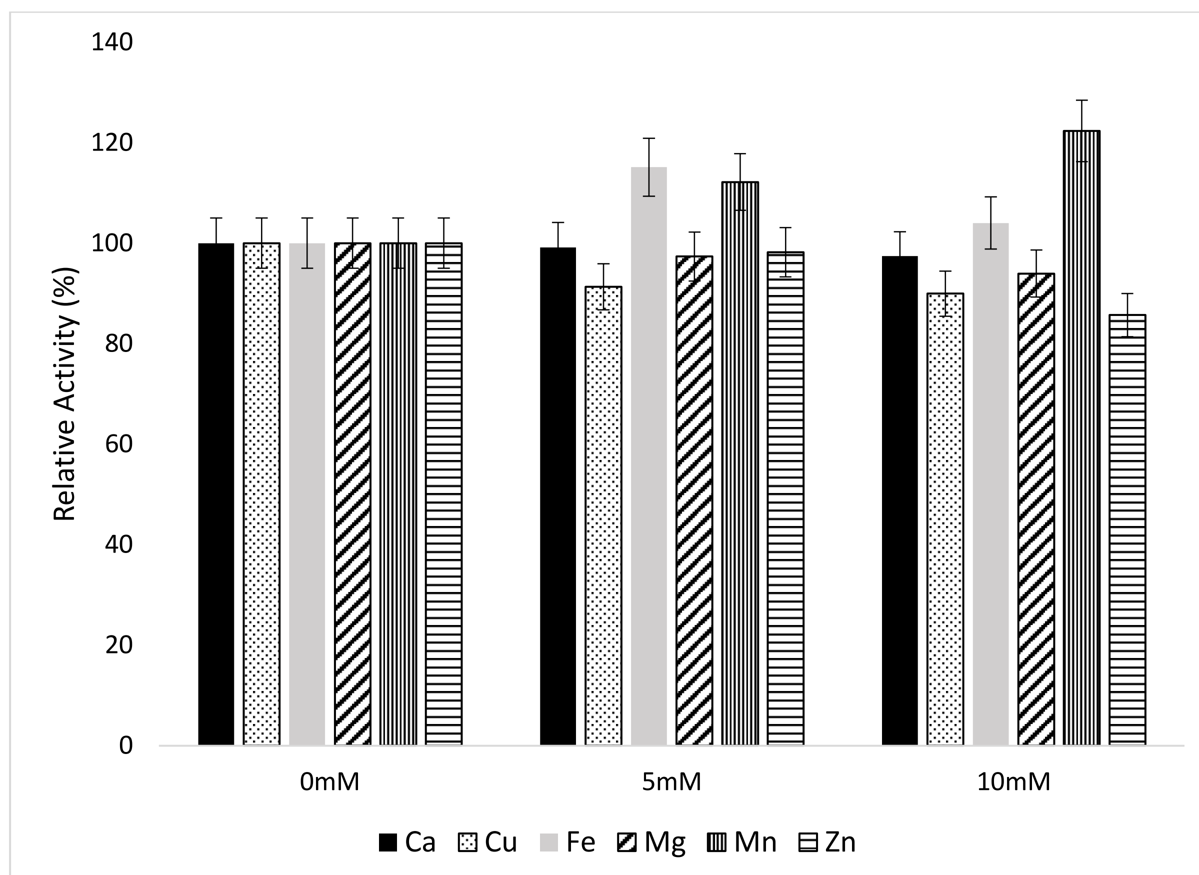


Figure 3.5: Effect of various divalent metal ions on the activity of the putative xylanase on beechwood xylan (n=3, SD within 5%)

The putative *S. aerolata* xylanase was found to be relatively stable in the presence of various divalent metal ions (Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}), maintaining at least 80% of its relative activity (with activity in the absence of any metal ions regarded as 100%) in the presence of 10 mM concentrations of any of the metal ions (Fig. 3.5). The presence of 5 mM Fe^{2+} and Mn^{2+} was found to result in an increase in relative activity, while the presence of 5 mM Ca^{2+}, Mg^{2+} and Zn^{2+} had a negligible effect on the activity of the putative xylanase. Relative activity increased with an increase in concentration of Mn^{2+}, while it decreased with increase in concentration of Cu^{2+}, Mg^{2+} and Zn^{2+}.

3.3.6. Effect of EDTA and β-mercaptoethanol on xylanase activity of the putative xylanase

The effect of chemical reagents, EDTA and β-mercaptoethanol, on xylanase activity of the putative *S. aerolata* xylanase was investigated and the results are presented in Figure 3.6.

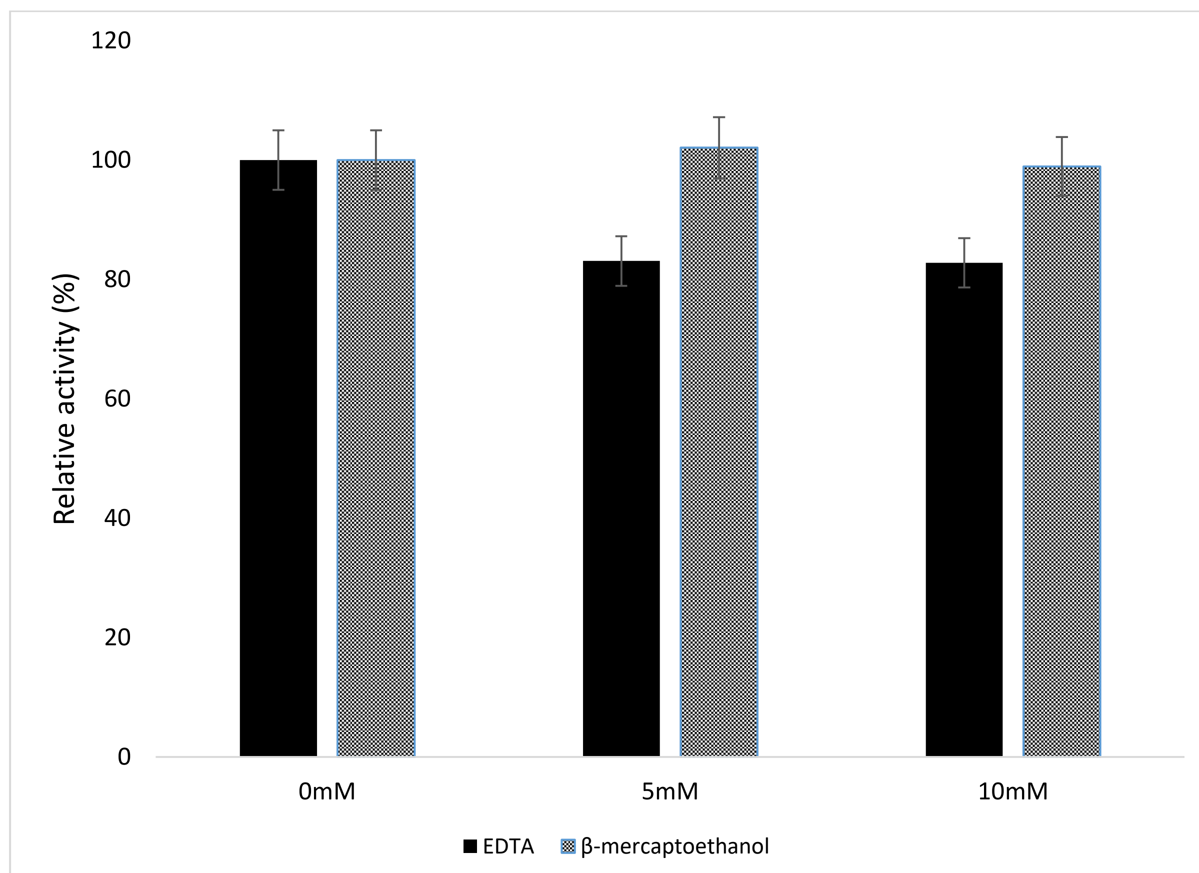


Figure 3.6: Effect of EDTA and β-mercaptoethanol on the activity of the putative xylanase on beechwood xylan (n=3, SD within 5%)

The putative *S. aerolata* xylanase was found to be relatively stable in the presence of both EDTA and β-mercaptoethanol (Fig. 3.6). EDTA slightly inhibited the xylanase activity. However, the putative xylanase was still able to maintain over 80% of its relative activity in the presence of 10 mM EDTA. β-mercaptoethanol appeared to have no effect on the putative xylanase activity, increasing the relative activity slightly to 102% at 5 mM, and slightly decreasing the relative activity to 99% at 10 mM.

3.4. Discussion

Enzymes are well known for their specificity when it comes to the substrates that they recognise and bind to and the reactions that they catalyse. For instance, amylases are known to specifically bind and hydrolyse the substrate starch, while cellulases specifically recognise and hydrolyse cellulose substrates. Similarly, xylanases are glycoside hydrolase enzymes that catalyse the hydrolysis of 1,4-β-D-xylosidic linkages in xylan (Collins et al., 2005). Due to the specificity of enzymes, a xylan degrading enzyme purified to homogeneity, such as the putative xylanase in this study, is expected to only show activity towards xylan substrates. However, the purified putative *S. aerolata* xylanase was found to be highly active towards Avicel (microcrystalline cellulose) (22.64 µmol/min/mg) and hydrolysed potato starch (19.11 µmol/min/mg), as well as xylan substrates, beechwood xylan (23.69 µmol/min/mg) and wheat arabinoxylan (20.15 µmol/min/mg) (Table 3.1). These results show that the putative xylanase retained at least 80% relative activity towards non-xylan substrates (Avicel and hydrolysed potato starch), which was unexpected and indicates that the putative xylanase produced by *S. aerolata* is a novel enzyme. In contrast to these findings, a cold-active xylanase, XynA, isolated from the marine bacterium, *Glaciecola mesophila*, was found to be only active on xylan substrates and had no activity on cellulose or starch substrates (Guo et al., 2009). Similarly, another cold-active xylanase from an environmental DNA library (Xyn8) which was cloned and characterised, was also found to have negligible activity on cellulose substrates, carboxymethyl cellulose and Avicel, or any other glucose-based substrates (Lee et al., 2006). A thermophilic xylanase from *Thermomyces lanuginosus* CBS 288.54 was also found to be only active on xylan substrates, retaining no activity on Avicel and starch (Li et al., 2005). These findings in literature contradict the findings from our study, and highlight the cross-specificity of our putative xylanase. The term cross-specificity is used to describe the ability of some enzymes to be active on more than one substrate (Van Dyk and Pletschke, 2012).

While bi-functional xylanases (active on xylan and one other non-xylan substrate) have been reported in literature (Aygan and Arikan, 2009; Bhattacharya et al., 2016; Chen et al., 2006; Fernandes et al., 1999; Meryandini et al., 2009; Rouau and Odier, 1986; Shi et al., 2010), there are currently no reports on multi-functional xylanases that are capable of hydrolysing cellulose, starch as well as xylan substrates. Interestingly, while the putative xylanase was active on hydrolysed potato starch, it was found to have no activity on other starch substrates such as maize amylopectin and soluble starch from maize. This would suggest that the structure of hydrolysed potato starch is different from the structures of the starches from maize, and this

difference allows the putative xylanase to recognise and bind to hydrolysed potato starch but not the other starch substrates. Overall, these results indicate that the putative xylanase is possibly a multifunctional glycoside hydrolase enzyme exhibiting activities towards xylan substrates, Avicel cellulose as well as hydrolysed potato starch.

Using the definitions from the characterisation of multifunctional enzymes, the putative *S. aerolata* xylanase can be classified as a substrate promiscuous multifunctional enzyme because of its broad substrate specificity (Cheng et al., 2012). It can be postulated that the increased flexibility in the polypeptide chain (that has been reported for enzymes from psychrophilic sources) allows for the ease in accommodation of substrates at the active sites of cold-adapted enzymes (Gerday et al., 1997), which could be a contributing factor to the substrate promiscuity of this putative xylanase, which was isolated from a psychrophilic bacterium, *S. aerolata*. It has been hypothesised that multifunctional enzymes provide competitive survival edges to the organism by utilising alternative approaches to coordinate multiple activities and regulate their own expression (Cheng et al., 2012). The multifunctional nature of the putative xylanase could also be beneficial to the survival of the psychrophilic *S. aerolata* bacterium by using the ability to hydrolyse a variety of substrates to maintain metabolic rates necessary for survival, even in environments where one substrate might be lacking.

Xylanases are applied in different industries that usually require harsh reaction conditions which could be intolerable for most xylanase enzymes (Collins et al., 2005). It is, therefore, crucial that xylanases which can operate at industrial conditions be identified for the purposes of application in these industries. This putative xylanase was found to maintain over 25% relative activity at 4˚C using beechwood xylan as a substrate (Figure 3.1), consistent with Xyn8 which was found to maintain 29% relative activity on beechwood xylan at 5˚C (Lee et al., 2006). However, the cold-active xylanase (Xyn8) maintained close to 60% relative activity at 10˚C (Lee et al., 2006), while the *S. aerolata* putative xylanase only maintained 30% relative activity at that temperature, consistent with another cold-active xylanase, the recombinant Xyn10C, from the marine bacterium, *Saccharophagus degradans*, which maintained just over 40% relative activity at 10˚C (Ko et al., 2016). The optimum temperature of the putative xylanase was found to be 40˚C, similar to two cold-active xylanases from the Antarctic krill, *Euphausia superba* Dana (Turkiewicz et al., 2000), while Xyn8 was found to be optimally active at 20˚C (Lee et al., 2006) and Xyn10C at 30˚C (Ko et al., 2016). At high temperatures, the relative activities of Xyn8 and Xyn10C steadily decreased until there was no activity detected beyond 60˚C (Ko et al., 2016; Lee et al., 2006), while the putative xylanase in our

study maintained 47% relative activity at 60˚C, and 20% relative activity at 90˚C. While both cold-active *E. superba* xylanases were found to have weak thermostability (rapid inactivation at temperatures above 25˚C) (Turkiewicz et al., 2000), the putative xylanase in this study was found to be relatively thermostable, maintaining at least 78% relative activity on beechwood xylan after incubation at 28˚C, 37˚C and even 50˚C for 24 hours (Figure 3.2), while an incubation period of only 1 hour at 60˚C resulted in no Xyn8 activity being detected. These results indicate that the putative xylanase might be relatively cold-active as it maintains comparable relative activity at 4˚C as the cold-active Xyn8 did at 5˚C, and comparable activity at 10˚C as the cold active Xyn10C did at this temperature. However, unlike Xyn8 and Xyn10C, the putative xylanase was also found to be relatively thermostable, retaining up to 78% relative activity after incubation for a period as long as 24 hours at temperatures as high as 50˚C, which was unexpected from an enzyme from a psychrophilic source. Xyn10C only retained 20% of its relative activity after an incubation period of only 20 minutes at 50˚C (Ko et al., 2016). These results indicate that the putative multifunctional xylanase is both a cold-active and thermostable enzyme.

While the putative xylanase was found to have an optimum pH for beechwood xylan hydrolysis of pH 6.0, it was also found to be acido- and alkaline-stable, maintaining over 60% relative activity at pH 2.0 and 80% relative activity at pH 11.0 (Figure 3.3). The optimal pH of the putative xylanase was the same as that of the two cold-active krill xylanases (Turkiewicz et al., 2000). Xyn10C, also from a bacterial source, was found to be optimally active at pH 7.0, comparable to the optimal pH of the putative xylanase in this study, and also consistent with findings of Collins et al. (2005). However, unlike the putative *S. aerolata* xylanase, Xyn8 and Xyn10C were not active at pH 4.0 or lower, and retained less than 10% relative activity at pH 10.0 (Ko et al., 2016). The acido/alkaliphile nature of the putative xylanase is novel, and could allow for its applications in a wide range of industries that might require acidic pH or alkaline pH without the enzyme being denatured or inactivated.

While it has been found that the products of polysaccharide hydrolysis such as monooligosaccharides can inhibit the activity of glycoside hydrolases (Alvarez-Cervantes et al., 2016), the activity of the putative xylanase on azo-wheat arabinoxylan was found to be only slightly inhibited by glucose and xylose at a concentration of 5 mM, while mannose (at concentrations of 5 mM and 20 mM) increased xylanase activity to over 110% relative to xylanase activity in the absence of any sugars (Figure 3.4). The presence of various divalent cations also had a negligible effect on the activity of the putative xylanase on beechwood xylan

(Figure 3.5). Similarly, the presence of 5 mM Ca^{2+}, Mg^{2+} and Zn^{2+} also had a negligible effect on the activity of a *Geobacillus thermoleovorans* xylanase (Verma and Satyanarayana, 2012). EDTA and β-mercaptoethanol were also found to have a negligible effect on the activity of the putative xylanase (Figure 3.6). Similarly, EDTA (5 mM) only inhibited the activity of the xylanase from *G. thermoleovorans* by just over 20% to 78.67% relative activity (Verma and Satyanarayana, 2012). However, unlike with the putative xylanase in this study, β-mercaptoethanol (5 mM) had an effect on the activity of the *G. thermoleovorans* xylanase, inhibiting it to 74.76% relative activity (Verma and Satyanarayana, 2012).

There were no hydrolysis products observed, using TLC, for the hydrolysis of cellotriose, cellotetraose, cellopentaose, Avicel, xylotriose, xylotetraose, xylopentaose, beechwood xylan and wheat arabinoxylan. These results are inconsistent with other characterized xylanases that have been shown, using TLC, to hydrolyse xylan substrates and xylo-oligomers of high degrees of polymerisation to even shorter xylo-oligomers such as xylotriose, xylobiose, and in some cases, xylose (Guo et al., 2009; Ko et al., 2016; Lee et al., 2006; Monclaro et al., 2016; Shi et al., 2015; Verma and Satyanarayana, 2012). These results could indicate that an oligomer sequence longer than five residues is required for the active binding site of the putative xylanase on cellulose and xylan substrates. However, due to the lack of products observed for the hydrolysis of substrates with degrees of polymerisation greater than five, such as Avicel, beechwood xylan and wheat arabinoxylan, it is more likely that the products of the reaction samples required concentration prior to TLC analysis for any hydrolysis products to be observed.

3.4.1. Conclusions

The purified putative xylanase was found to be a possible multifunctional glycoside hydrolase, as it was active towards xylan substrates, Avicel cellulose and hydrolysed potato starch. The enzyme was found to have a temperature optimum of 40˚C and a pH optimum of 6.0 during beechwood xylan hydrolysis. The enzyme was also found to be thermostable, acido/alkali stable, as well as stable in the presence of various divalent cations, EDTA and β-mercaptoethanol. The next objectives of our study were to determine the kinetic parameters of the putative multifunctional xylanase and to explore the multifunctional nature of the enzyme using substrate competition assays.

Chapter 4: Enzyme kinetics and substrate competition

4.1. Introduction

Living organisms undergo thousands of biochemical reactions in a minute, and these reactions are essential to maintain and sustain life (Biaglow et al., 2010). A major determining factor in the survival of organisms is, therefore, the rates at which these biochemical reactions occur (Biaglow et al., 2010). Enzymes are biological catalysts which are responsible for the moderation of biological chemical processes by increasing the rate at which biochemical reactions occur (Biaglow et al., 2010; Li et al., 2012). In the absence of enzymes, biochemical reactions would occur at rates too slow to sustain life, and as a result, organisms are dependent on enzymes to speed up necessary reactions (Biaglow et al., 2010). Enzymes usually function by converting one chemical, referred to as the substrate, into another chemical, referred to as the product (Biaglow et al., 2010). Enzymes are usually larger than their substrates and interact with them by binding to them using active sites on the enzyme; these active sites are created by the specific three-dimensional folding of the enzyme (Wilson and Walker, 2010). The binding of the enzyme to the substrate results in the formation of a transition state, which has a significantly reduced activation energy barrier compared to reactions which are not catalysed by enzymes (Wilson and Walker, 2010). As a result, reactions catalysed by enzymes can be up to millions of times faster than non-catalysed reactions (Wilson and Walker, 2010).

The study of the rate of the reactions catalysed by enzymes is termed enzyme kinetics, and these studies can help shed light on the catalytic processes of enzymes (Biaglow et al., 2010). Studying the effect that substrate concentration has on the initial rate of the enzyme-catalysed reaction is a major focus of enzyme kinetics (Biaglow et al., 2010). The effect of substrate concentration on the initial rate of an enzyme catalysed reaction generally follows the pattern illustrated in Figure 4.1.

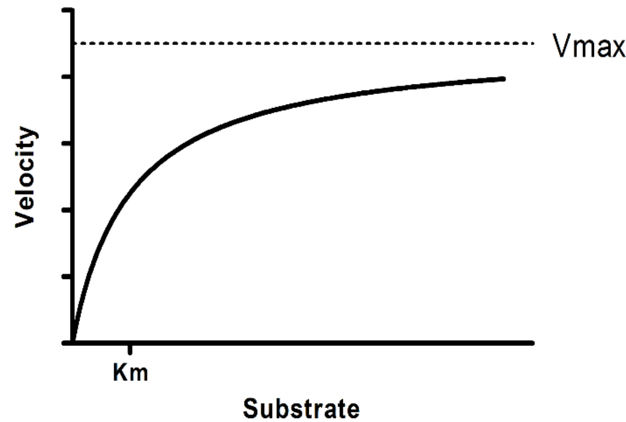
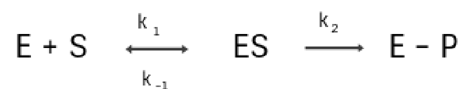


Figure 4.1: The relationship between substrate concentration and the initial rate of the reaction (adapted from Biaglow et al., 2010)

The most basic enzyme kinetic model deduced by Leonor Michaelis and Maud Leonora Menten over a 100 years ago elucidated that the conversion of the substrate to a product was via an intermediate state, termed the enzyme substrate complex (Biaglow et al., 2010; Johnson and Goody, 2011; Qian, 2008). This model can be represented using the equation below:



where E refers to the enzyme, S to the substrate, ES to the enzyme substrate complex, P to the product, k_1 and k_{-1} to the forward and reverse rate constants for substrate binding, respectively, and k_2 is the catalytic rate constant (Torres and Santos, 2017).

The main aim of enzyme kinetics is to estimate the values of K_M and V_{max} from the Michaelis-Menten equation;

$$v_o = (V_{max} \cdot [S])/(K_M + [S])$$

where v_o is the initial rate at substrate concentration S, V_{max} is the maximum, limiting rate at saturating substrate concentration and K_M is the Michaelis constant, which is also an indication of the affinity of the enzyme to the substrate (Torres and Santos, 2017).

To transform the Michaelis-Menten equation into a more useful way to plot the experimental data, Hans Lineweaver and Dean Burk took the reciprocal of both the substrate concentration and initial rate of reaction to give the double reciprocal plot, now commonly known as the Lineweaver-Burk Plot (Gajera et al., 2008). The Hanes-Woolf Plot is another way that

rearranges the Michaelis-Menten equation by plotting the substrate concentration divided by the rate of the reaction at that substrate concentration ([S]/v_o) versus the substrate concentration ([S]) to linearize the data and allow for the rapid calculation of K_M and V_{max} (Gilbert, 2000). The Lineweaver-Burk Plot has been widely used for the representation of kinetic data, however, for the accurate calculation of kinetic parameters such as K_M and v_{max}, the Hanes-Woolf Plot and non-linear regression computer software are now preferred.

In some cases, two substrate molecules can bind to the same enzyme protein molecule, a phenomenon known as cooperativity (Torres and Santos, 2017). In these cases, the rate versus substrate concentration plots resemble S-shaped curves (Figure 4.2) (Torres and Santos, 2017), different from the curve illustrated in Figure 4.1. Cooperativity is only possible when an enzyme has multiple active sites and it has been observed that cooperative enzymes are usually dimers, trimers, tetramers and so forth (Gajera et al., 2008). The Michaelis-Menten equation for cooperative enzymes can then be adapted and represented as the equation below;

$$v_o = [S]^n/(K + [S]^n)$$

Where n>1.

The effect of substrate concentration on the initial rate of a cooperative enzyme catalysed reaction generally follows the pattern illustrated in Figure 4.2.

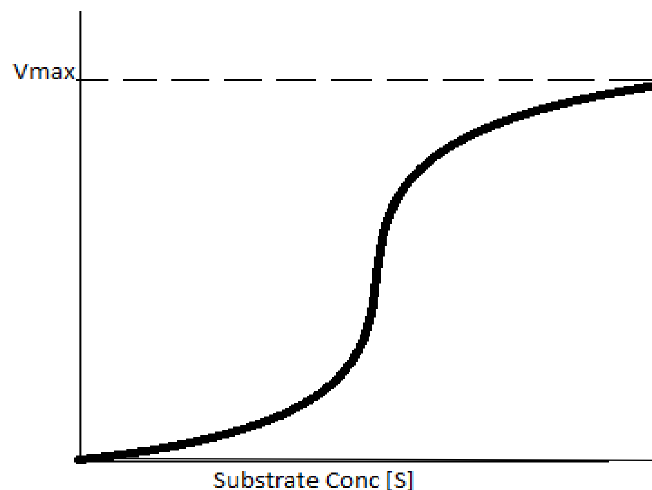


Figure 4.2: The effect of substrate concentration on reaction rate for cooperative enzymes (adapted from Gajera et al., 2008)

While enzymes are generally very specific in terms of the reactions they catalyse and in their choice of substrates (Gajera et al., 2008; Gilbert, 2000), enzymes that catalyse more than one

reaction and/or recognise more than one substrate are known to exist (Cheng et al., 2012). Enzymes with relaxed or broad substrate specificity, such as the putative xylanase from *S. aerolata* which has displayed activity towards xylan substrates as well as Avicel and hydrolysed potato starch, are referred to as substrate promiscuous enzymes (Cheng et al., 2012). Substrate promiscuous xylanases have been previously studied (Bhattacharya et al., 2016; Chen et al., 2006; Fernandes et al., 1999; Meryandini et al., 2009; Shi et al., 2015, 2010). Substrate competition assays have been useful in elucidating whether the substrate promiscuous xylanases use the same active site or different active sites for recognition and hydrolysis of the different substrates (Bhattacharya et al., 2016; Chen et al., 2006; Shi et al., 2010).

4.1.1. Aims and objectives

- *Aims*

One of the aims of this chapter was to determine the enzyme kinetic parameters for the putative *S. aerolata* xylanase on xylan substrates as well as Avicel and hydrolysed potato starch.

Another aim of this chapter was to conduct substrate competition assays so as to determine whether the putative xylanase utilises the same or multiple catalytic/active centres to hydrolyse its different substrates.

- *Objectives*

The objectives of this chapter were to:

  - determine the kinetic parameters of the putative xylanase on beechwood xylan, wheat arabinoxylan, Avicel and hydrolysed potato starch;
  - determine the substrate that the putative xylanase has the highest affinity for by comparing the kinetic parameters of the putative xylanase on different substrates;
  - determine whether the xylanase utilises the same or multiple catalytic centers for hydrolysis of beechwood xylan, Avicel and hydrolysed potato starch using substrate competition assays.

4.2. Materials and methods

4.2.1. Materials

All chemicals and reagents used were of analytical grade and purchased from various chemical companies (Appendix I).

4.2.2. Determination of enzyme kinetic parameters

Activity of the putative xylanase was assayed on beechwood xylan, wheat arabinoxylan, Avicel and hydrolysed potato starch at different concentrations of each substrate ranging from 0.125% to 2% (w/v) using the activity assay protocol outlined in section 2.2.5. The kinetic parameters, V_{max}, K_M and k_{cat}, were calculated graphically using Lineweaver-Burk and Hanes-Woolf plots.

4.2.3. Substrate competition assays

Activity of the putative xylanase was assayed on combinations of beechwood xylan and Avicel, and combinations of beechwood xylan and hydrolysed potato starch at final concentrations of 20 mg/ml of combined substrates in the reactions and the activity assay protocol described in section 2.2.5 was followed. To determine whether the putative xylanase uses the same active site or different active sites for the hydrolysis of the different substrates, the methods of Chen et al. (2006) were used. If the putative xylanase uses the same active site to hydrolyse beechwood xylan, Avicel and hydrolysed potato starch, the substrates would competitively inhibit each other during the process of enzyme substrate binding (Chen et al., 2006) and the overall rate of hydrolysis of the substrates (V_s) would be calculated using the equation:

$$V_s = \frac{\left(\frac{V_{mx} \times X}{K_{mx}}\right) + \left(\frac{V_{ma} \times A}{K_{ma}}\right)}{1 + \left(\frac{X}{K_{mx}}\right) + \left(\frac{A}{K_{ma}}\right)} \quad \text{or} \quad V_s = \frac{\left(\frac{V_{mx} \times X}{K_{mx}}\right) + \left(\frac{V_{mh} \times H}{K_{mh}}\right)}{1 + \left(\frac{X}{K_{mx}}\right) + \left(\frac{H}{K_{mh}}\right)}$$

where V_{mx}, V_{ma} and V_{mh} are the maximum velocities for beechwood xylan, Avicel and hydrolysed potato starch, respectively; X, A and H are the concentrations of beechwood xylan, Avicel and hydrolysed potato starch, respectively; K_{mx}, K_{ma} and K_{mh} are the Michaelis-Menten constants for beechwood xylan, Avicel and hydrolysed potato starch, respectively.

If the putative xylanase uses different active sites to hydrolyse the substrates, or different enzymes are responsible for the hydrolysis of the substrates, then the substrates would not competitively inhibit each other (Chen et al., 2006), and the overall rate of hydrolysis (V_d) would be calculated using the equation:

$$V_d = \frac{(V_{mx} \times X / K_{mx})}{1 + (X / K_{mx})} + \frac{(V_{ma} \times A / K_{ma})}{1 + (A / K_{ma})} \qquad \text{or} \qquad V_d = \frac{(V_{mx} \times X / K_{mx})}{1 + (X / K_{mx})} + \frac{(V_{mh} \times H / K_{mh})}{1 + (H / K_{mh})}$$

where V_{mx}, V_{ma} and V_{mh} are the maximum velocities for beechwood xylan, Avicel and hydrolysed potato starch, respectively; X, A and H are the concentrations of beechwood xylan, Avicel and hydrolysed potato starch, respectively; K_{mx}, K_{ma} and K_{mh} are the Michaelis-Menten constants for beechwood xylan, Avicel and hydrolysed potato starch, respectively.

4.3. Results

4.3.1. Determination of kinetic parameters

The V_{max} and K_M values of the putative xylanase on beechwood xylan calculated using the Lineweaver-Burk plot (Fig. 4.3a) were found to be 25.00 U/mg and 3.25 mg/ml, respectively. Using the Hanes-Woolf plot (Fig. 4.3b), the V_{max} and K_M values were determined to be 24.63 U/mg and 3.03 mg/ml, respectively.

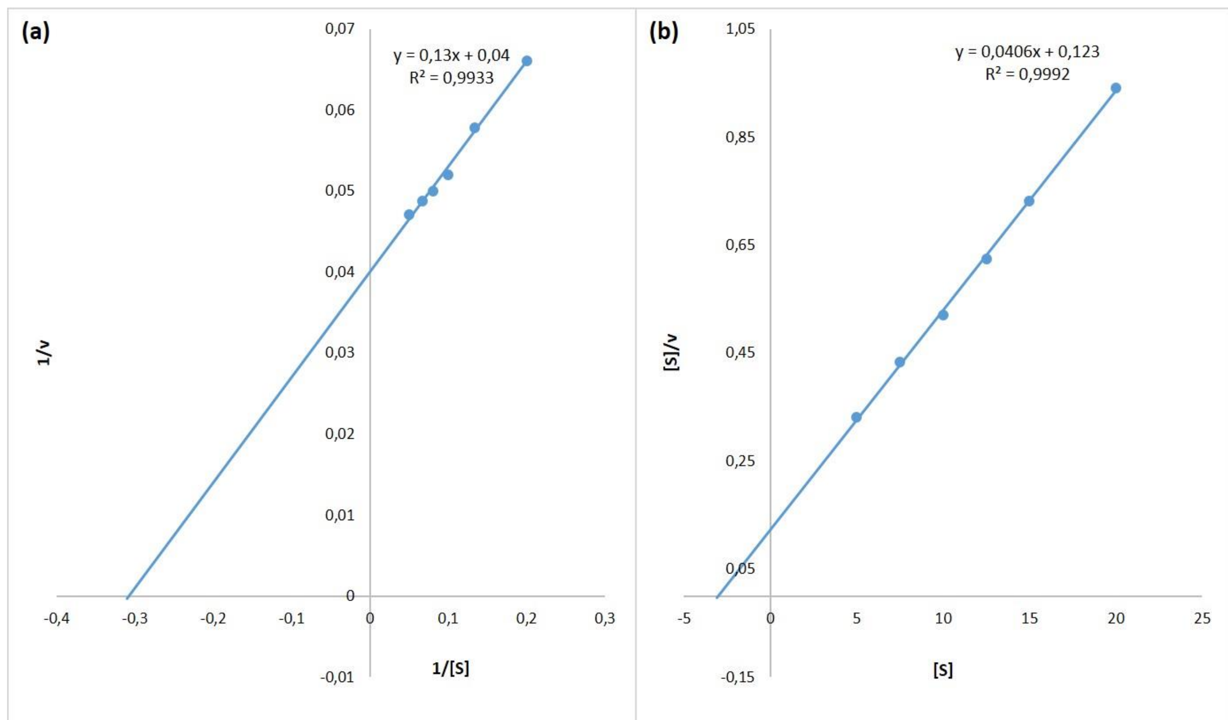


Figure 4.3: Lineweaver-Burk plot (a) and Hanes-Woolf plot (b) obtained from the activity of the putative xylanase on different concentrations of beechwood xylan (n=3)

The V_{max} and K_M values of the putative xylanase on wheat arabinoxylan calculated using the Lineweaver-Burk plot (Fig. 4.4a) were found to be 26.48 U/mg and 3.59 mg/ml, respectively, while using the Hanes-Woolf plot (Fig. 4.4b), the V_{max} and K_M values were determined to be 27.40 U/mg and 4.09 mg/ml, respectively.

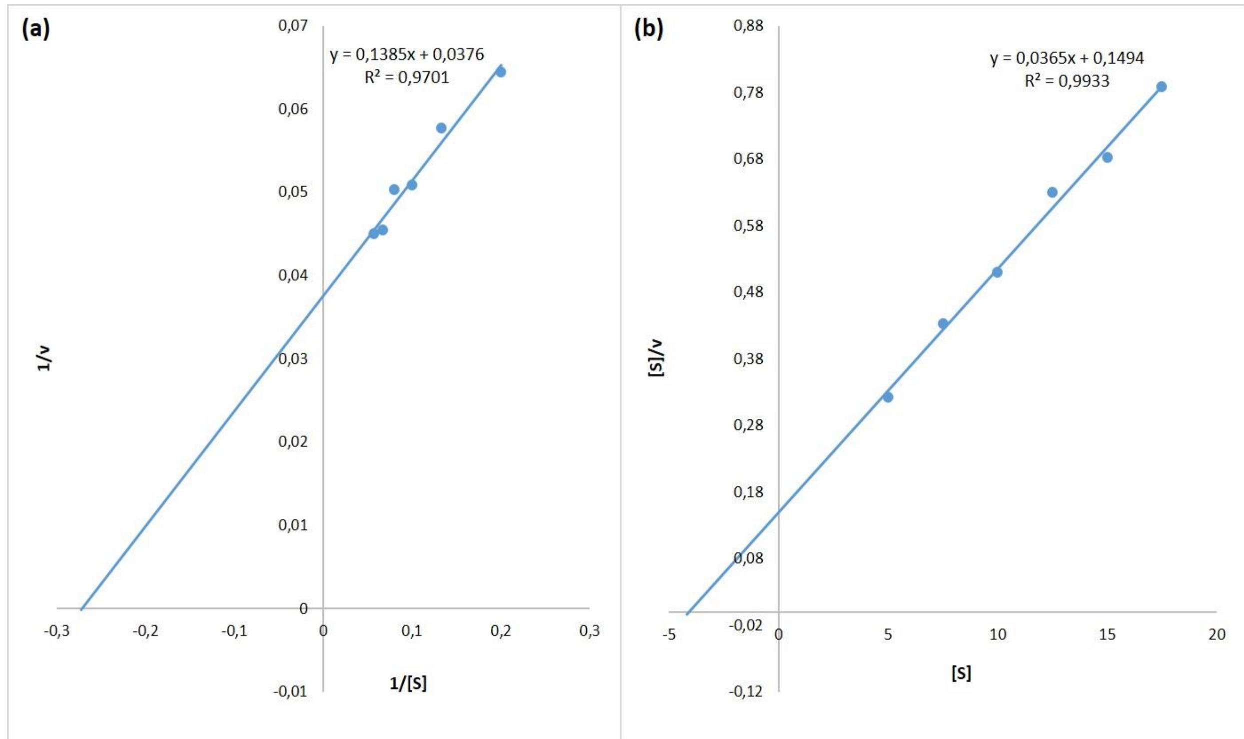


Figure 4.4: Lineweaver-Burk plot (a) and Hanes-Woolf plot (b) obtained from the activity of the putative xylanase on different concentrations of wheat arabinoxylan (n=3)

The V_{max} and K_M values of the putative xylanase on Avicel calculated using the Lineweaver-Burk plot (217.39 U/mg and 290 mg/ml, respectively) (Fig. 4.5a) were very different from the

V_{max} and K_M values calculated using the Hanes-Woolf plot (12.20 U/mg and 4.49 mg/ml, respectively) (Fig. 4.5b).

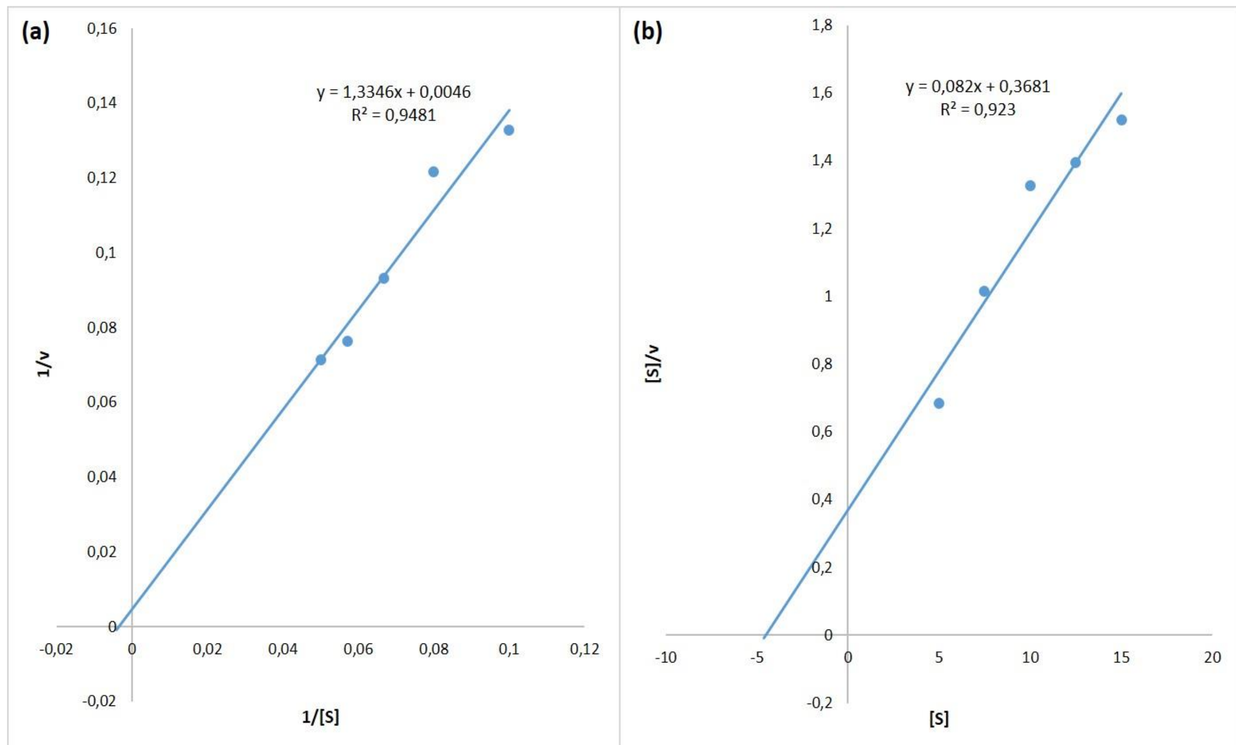


Figure 4.5: Lineweaver-Burk plot (a) and Hanes-Woolf plot (b) obtained from the activity of the putative xylanase on different concentrations of Avicel (n=3)

The V_{max} and K_M values of the putative xylanase on hydrolysed potato starch were calculated using the Lineweaver-Burk plot (24.88 U/mg and 5.6 mg/ml, respectively) (Fig. 4.6a) and using the Hanes-Woolf plot (19.34 U/mg and 2.21 mg/ml) (Fig. 4.6b).

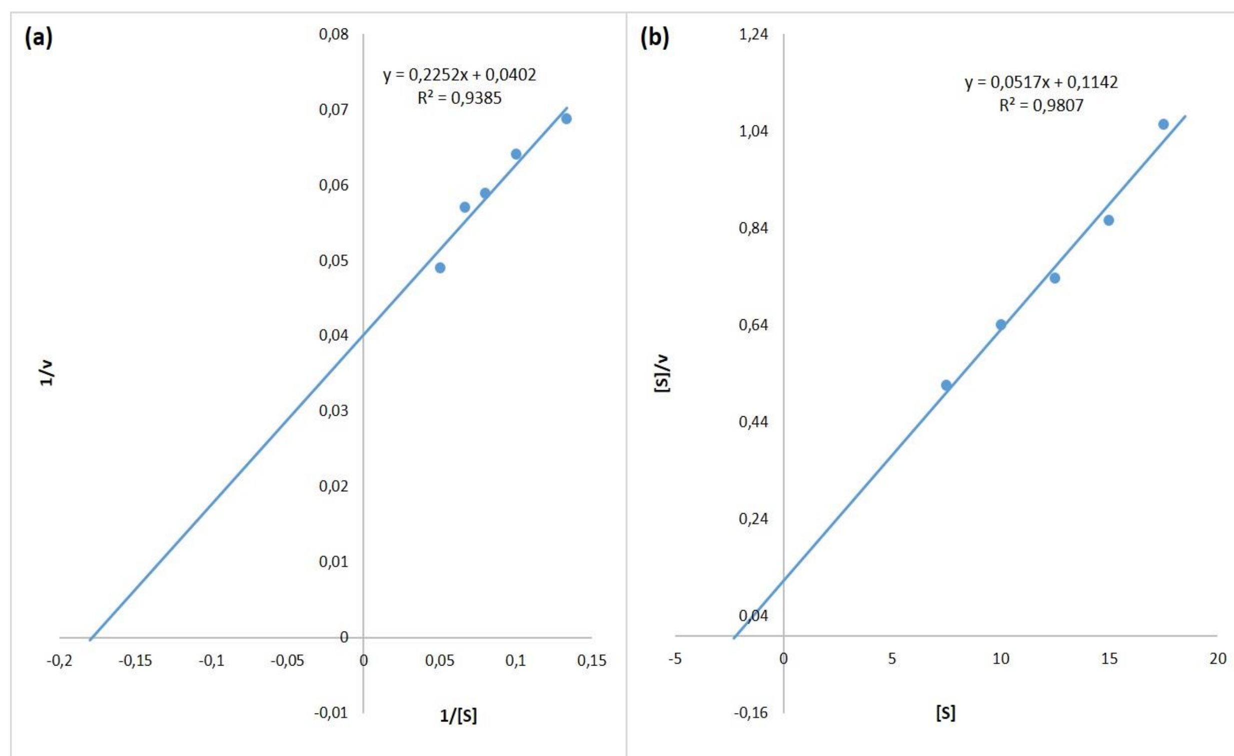


Figure 4.6: Lineweaver-Burk plot (a) and Hanes-Woolf plot (b) obtained from the activity of the putative xylanase on different concentrations of hydrolysed potato starch (n=3)

Comparison of the kinetic parameters obtained from the activity of the putative xylanase on beechwood xylan, wheat arabinoxylan, Avicel and hydrolysed potato starch showed that the Lineweaver-Burk and Hanes-Woolf plots gave similar results for the hydrolysis of xylan substrates, but highly contradictory results for the hydrolysis of Avicel (Table 4.1). The putative xylanase exhibited the highest V_{max} (217.39 µmol. min^{-1}. mg^{-1}) and K_M (290.13 mg/ml) on Avicel, as determined using Lineweaver-Burk plots, which were very different from the V_{max} (12.20 µmol. min^{-1}. mg^{-1}) and K_M (4.49 mg/ml) calculated using Hanes-Woolf plots (Table 4.1). The catalytic efficiency (k_{cat}/K_M) of the putative xylanase on Avicel was found to be extremely low in comparison to its catalytic efficiency on the other substrates as determined by Lineweaver-Burk plots. The kinetic parameters determined on xylan substrates, beechwood xylan and wheat arabinoxylan, using Lineweaver-Burk plots, were consistent with the kinetic parameters determined using Hanes-Woolf plots. The lowest K_M determined using Hanes-

Woolf plots was on hydrolysed potato starch (2.21 mg/ml), indicating that the putative xylanase had the highest binding affinity towards that substrate. However, using Lineweaver-Burk plots, the lowest K_M was found to be on beechwood xylan (3.25 mg/ml).

Due to the variation in enzyme kinetic parameters calculated using the Lineweaver-Burk and Hanes-Woolf plots for the hydrolysis of Avicel, non-linear regression using Michaelis-Menten plots on KaleidaGraph 4.5.4 software was then used to calculate V_{max} and K_M values. The results were compared to the kinetic parameters determined from the Lineweaver-Burk and Hanes-Woolf plots in Table 4.2.

Table 4.1: Comparison of the kinetic parameters determined using Lineweaver-Burk plots and Hanes-Woolf plots for activity of the putative S. aerolata xylanase on different substrates

Substrate	Lineweaver-Burk				Hanes-Woolf			
	V_{max} (U/mg)	K_M (mg/ml)	k_{cat} (min^{-1})	k_{cat}/K_M	V_{max} (U/mg)	K_M (mg/ml)	k_{cat} (min^{-1})	k_{cat}/K_M
Beechwood xylan	25.00 ± 1.27	3.25 ± 0.28	900.00 ± 1.91	276.92 ± 1.69	24.63 ± 0.98	3.03 ± 0.86	886.70 ± 1.37	292.68 ± 2.35
Wheat arabinoxylan	26.48 ± 0.77	3.59 ± 0.62	953.39 ± 1.19	265.54 ± 2.10	27.40 ± 1.31	4.09 ± 0.53	986.30 ± 2.82	240.96 ± 1.96
Avicel	217.39 ± 5.66	290.13 ± 2.78	7826.09 ± 1.63	26.97 ± 1.75	12.20 ± 1.44	4.49 ± 0.64	439.02 ± 1.90	97.80 ± 1.55
Hydrolysed potato starch	24.88 ± 1.29	5.60 ± 1.12	895.52 ± 1.07	159.86 ± 1.32	19.34 ± 0.53	2.21 ± 0.14	696.32 ± 2.13	315.24 ± 2.97

Table 4.2: Comparison of V_{max} and K_M values determined using Lineweaver-Burk plots, Hanes-Woolf plots and non-linear regression analysis using Michaelis-Menten plots on the KaleidaGraph 4.5.4 computer software

Substrate	**Lineweaver-Burk**			**Hanes-Woolf**			**Non-linear regression analysis using KaleidaGraph 4.5.4 software**		
	V_{max} (U/mg)	**K_M (mg/ml)**	**k_{cat}/ K_M**	**V_{max} (U/mg)**	**K_M (mg/ml)**	**k_{cat}/ K_M**	**V_{max} (U/mg)**	**K_M (mg/ml)**	**k_{cat}/ K_M**
Beechwood xylan	25.00 ± 1.27	3.25 ± 0.28	276.92 ± 1.69	24.63 ± 0.98	3.03 ± 0.86	292.68 ± 2.35	25.29 ± 0.59	3.35 ± 0.11	264.69 ± 1.99
Wheat arabinoxylan	26.48 ± 0.77	3.59 ± 0.62	265.54 ± 2.10	27.40 ± 1.31	4.09 ± 0.53	240.96 ± 1.96	26.91 ± 0.71	3.86 ± 0.22	255.44 ± 1.36
Avicel	217.39 ± 5.66	290.13 ± 2.78	26.97 ± 1.75	12.20 ± 1.44	4.49 ± 0.64	97.80 ± 1.55	12.08 ± 0.92	4.25 ± 0.53	103.36 ± 1.65
Hydrolysed Potato Starch	24.88 ± 1.29	5.60 ± 1.12	159.86 ± 1.32	19.34 ± 0.53	2.21 ± 0.14	315.24 ± 2.97	20.00 ± 0.90	2.68 ± 0.59	259.70 ± 1.94

4.3.2. Substrate competition

Activity of the putative xylanase was assayed on combinations of beechwood xylan and Avicel and combinations of beechwood xylan and hydrolysed potato starch at final concentrations of 20 mg/ml in the reactions. The observed overall velocities were compared to theoretical values calculated using the methods of Chen et al. (2006) to determine whether the putative xylanase uses the same active site or different active sites to hydrolyse the multiple substrates, or alternatively, that different enzymes are responsible for the hydrolysis of the multiple substrates. The results are presented in Table 4.2 and Table 4.3 below.

Table 4.3: Comparison of the observed reaction velocities of the putative xylanase on mixtures of various concentrations of beechwood xylan and Avicel

Reaction number	**Concentration of substrates in reaction mixture (mg/ml)**		**Observed values (U/mg)**	**Theoretical values (U/mg) for:**	
	Beechwood xylan	**Avicel**		**Same active center**	**Different active center**
1	17.5	2.5	20.59 ± 0.89	20.33	25.36
2	15.0	5.0	17.36 ± 0.97	19.18	26.92
3	5.0	15.0	11.20 ± 0.53	13.59	24.72
4	2.5	17.5	8.97 ± 0.65	11.86	20.84

The observed reaction velocities of the putative xylanase on combinations of beechwood xylan and Avicel were consistent with the calculated theoretical values for the hydrolysis of the two substrates using the same active center on the putative xylanase. These results indicate that the putative xylanase binds to and hydrolyses beechwood xylan and Avicel using the same active site.

Table 4.4: Comparison of the observed reaction velocities of the putative xylanase on mixtures of various concentrations of beechwood xylan and hydrolysed potato starch

Reaction number	Concentration of substrates in reaction mixture (mg/ml)		Observed values (U/mg)	Theoretical values (U/mg) for:	
	Beechwood xylan	Hydrolysed potato starch		Same active center	Different active center
1	17.5	2.5	20.76 ± 0.67	20.76	31.26
2	15.0	5.0	19.36 ± 1.20	20.18	33.91
3	5.0	15.0	17.20 ± 0.71	18.22	32.20
4	2.5	17.5	16.85 ± 0.55	17.81	28.31

The observed values for the reaction velocities of the putative xylanase on combinations of beechwood xylan and hydrolysed potato starch were consistent with the calculated theoretical reaction velocities for the hydrolysis of the two substrates at the same active site of the putative xylanase. These results indicate that the putative xylanase uses the same active site to bind to and hydrolyse beechwood xylan and hydrolysed potato starch.

In summary, the results from the substrate competition assays show that the putative xylanase uses the same active site to hydrolyse the three different substrates; beechwood xylan, Avicel and hydrolysed potato starch. This confirms that the putative xylanase is a multifunctional glycoside hydrolase enzyme.

4.4. Discussion

The original Michaelis-Menten plot presented difficulties in determining the maximum velocity (V_{max}) of the enzyme-catalysed reaction experimentally due to the curved nature of the plot (Silverman, 2000). This difficulty resulted in the transformation of the Michaelis-Menten equation into linear forms which would allow for more accurate determination of K_M and V_{max} values from experimental data (Silverman, 2000). The most common linear plots derived from the Michaelis-Menten equation are the Lineweaver-Burk plot and the Hanes-Woolf plot (Silverman, 2000). In this study, both plots were used to determine the kinetic parameters (V_{max}, K_M, k_{cat} and k_{cat}/K_M) of the putative xylanase on beechwood xylan, wheat arabinoxylan, Avicel and hydrolysed potato starch.

While it was found that the kinetic parameters obtained for the putative xylanase activity on xylan substrates, beechwood xylan and wheat arabinoxylan, were consistent using both the Lineweaver-Burk and Hanes-Woolf plots, there was significant disparity in the values of the kinetic parameters obtained for the activity of the putative xylanase on Avicel and hydrolysed potato starch between the two plots (Table 4.1). The Lineweaver-Burk plot is a reciprocal of the Michaelis-Menten plot, and while it is a popular means to obtain kinetic parameters from experimental data, it is not accurate and reliable due to the fact that small errors in the determination of v are magnified when the reciprocals are taken (Silverman, 2000). The Lineweaver-Burk plot also has the added disadvantage of condensing together data points at high concentrations into a small region and emphasizing the data points at low concentrations (Silverman, 2000). The Hanes-Woolf plot, on the other hand, is a plot of $[S]/v$ versus [S], and over a wide range of [S] values, the errors in $[S]/v$ reflect a fair reflection of the errors in v, making it a more accurate means to determine V_{max} and K_M (Silverman, 2000). Non-linear regression analysis using KaleidaGraph 4.5.4 computer software, a much more accurate method of calculating enzyme kinetic parameters, was found to yield V_{max} and K_M values, for the activity of the putative xylanase on all four of its substrates, that were more consistent with those determined using Hanes-Woolf plots in this study, further confirming the accuracy of the Hanes-Woolf plot in comparison to the Lineweaver-Burk plot. The kinetic parameters determined using Hanes-Woolf plots and confirmed by non-linear regression analysis using Michaelis-Menten plots on KaleidaGraph 4.5.4 were therefore used in subsequent substrate competition studies.

Using the results obtained from the Hanes-Woolf plots, it was found that the putative xylanase had the lowest K_M, and therefore the highest affinity for hydrolysed potato starch (2.21 mg/ml),

followed by beechwood xylan (3.03 mg/ml), wheat arabinoxylan (4.09 mg/ml) and Avicel (4.49 mg/ml), (Table 4.1). These results were consistent and comparable to the results obtained using KaleidaGraph 4.5.4 non-linear regression analysis software (Table 4.2). The highest V_{max} value was found to be on wheat arabinoxylan (27.40 U/mg) followed by beechwood xylan (24.63 U/mg) and hydrolysed potato starch (19.34 U/mg), while the lowest V_{max} value was found to be on Avicel (12.20 U/mg). An interesting aspect to note is that, while the putative xylanase had higher affinity for hydrolysed potato starch, it displayed the highest reaction rates on xylan substrates. This suggests that while the putative xylanase recognises and binds to hydrolysed potato starch more strongly, it still hydrolyses xylan substrates at faster rates than it does hydrolysed potato starch. Of the xylan substrates, the lowest K_M was observed on beechwood xylan, suggesting that the putative xylanase has a higher binding affinity for glucuronoxylan than for arabinoxylan, such as is observed for typical GH 30 xylanases (St John et al., 2014). However, while GH 30 xylanases depend on the presence of an uronic acid subsituent in the -2 subsite for binding and hydrolysis of xylan (Karlsson et al., 2018), resulting in the GH 30 xylanases not being active on arabinoxylan or neutral xylo-oligosaccharides (St John et al., 2014), the putative xylanase in this study was also active on wheat arabinoxylan, ruling it out as a GH 30 xylanase.

The putative xylanase was found to have the highest catalytic efficiency (k_{cat}/K_M) on hydrolysed potato starch (315.24 ml mg^{-1} min^{-1}) followed by beechwood xylan (292.68 ml mg^{-1} min^{-1}), wheat arabinoxylan (240.96 ml mg^{-1} min^{-1}) and Avicel (97 ml mg^{-1} min^{-1}), using kinetic parameters measured using Hanes-Woolf plots. These results follow the same pattern that was observed when comparing the K_M values measured for the activity of the putative xylanase on the four different substrates. These results suggest that the putative xylanase not only has a higher affinity towards hydrolysed potato starch than the other substrates, but also hydrolyses hydrolysed potato starch more efficiently than it hydrolyses xylan substrates. However, using non-linear regression analysis using KaleidaGraph 4.5.4 software, the highest catalytic efficiency for the putative xylanase was found to be on beechwood xylan (264 ml mg^{-1} min^{-1}) followed by hydrolysed potato starch (259.70 ml mg^{-1} min^{-1}), wheat arabinoxylan (255.44 ml mg^{-1} min^{-1}) and Avicel (103.36 ml mg^{-1} min^{-1}). While there is inconsistency in the catalytic efficiency results obtained using the two methods (Hanes-Woolf plots and KaleidaGraph 4.5.4 non-linear regression software), the general conclusion from these results is that the putative xylanase hydrolyses xylan substrates and hydrolysed potato starch more efficiently than it hydrolyses Avicel cellulose.

Cold-active enzymes are characterised by their higher catalytic efficiencies when compared to their mesophilic and thermophilic counterparts (Brininger et al., 2018). However, a comparison of the kinetic parameters determined in this study for the activity of the cold-active putative xylanase on beechwood xylan, with the kinetic parameters determined for the activity of a thermophilic *Geobacillus stearothermophilus* wild-type xylanase on beechwood xylan (Wang et al., 2013) exhibited the contrary. The thermophilic *G. stearothermophilus* xylanase was found to have a catalytic efficiency of 17 880 ml mg^{-1} min^{-1} (Wang et al., 2013), compared to only 292.68 ml mg^{-1} min^{-1} observed for the activity of the putative xylanase on beechwood xylan. These results indicate that the thermophilic xylanase is over 60 times more catalytically efficient than the cold-active putative xylanase in this study. Similarly, the cold-active xylanase, Xyn8, was also found to have a lower catalytic efficiency towards beechwood xylan (6 656 ml mg^{-1} min^{-1}) (Lee et al., 2006) than the thermophilic *G. stearothermophilus* xylanase. An important aspect to bear in mind, however, is that the experiments to determine the kinetic parameters were conducted at 20˚C for the cold-active Xyn8, 37˚C for the putative xylanase in this study, and 55˚C for the thermophilic *G. stearothermophilus* xylanase (Lee et al., 2006; Wang et al., 2013). The effect of temperature on the catalytic efficiency of the different xylanases cannot be overlooked and it is possible that at low temperatures (10˚C and lower), the cold-active xylanases may be more catalytically efficient than the thermophilic xylanase.

Multifunctional enzymes, such as the putative *S. aerolata* xylanase, are known to exist and to play multiple physiological roles (Cheng et al., 2012). They are classified as moonlighting enzymes or promiscuous enzymes, with moonlighting enzymes shown to have at least a single catalytic domain as well as an additional non-catalytic domain, while promiscuous enzymes have been characterised as enzymes with catalytic domains that can execute several functions (Cheng et al., 2012). Promiscuous enzymes are further classified as condition, substrate and catalytic promiscuous enzymes (Cheng et al., 2012). The putative xylanase from this study can be categorised as a substrate promiscuous multifunctional enzyme as it recognizes and hydrolyses more than one substrate.

Substrate competition assays were performed so as to determine whether the putative xylanase uses the same active site to hydrolyse the different substrates or whether it uses different active sites, or alternatively that more than one enzyme is involved in the hydrolysis of the different substrates. Using calculations obtained from Chen at al. (2006), the theoretical values for the overall rate of hydrolysis of combinations of beechwood xylan and Avicel, assuming the putative xylanase was using the same active site or different active sites to hydrolyse the

substrates, were determined (Table 4.3). The actual values for the overall rate of hydrolysis obtained using experimental data were found to be similar to the theoretical values for the overall rate of hydrolysis of both beechwood xylan and Avicel using the same active site. The theoretical values for the overall rate of hydrolysis of combinations of beechwood xylan and hydrolysed potato starch, assuming the putative xylanase was using the same active site to hydrolyse both substrates, were consistent with the actual values for the overall rate of hydrolysis of the two substrates obtained from experimental data. These results indicate that the putative xylanase uses the same active site to hydrolyse three different substrates, beechwood xylan, Avicel cellulose and hydrolysed potato starch, and confirmed that the putative xylanase is a substrate promiscuous multifunctional glycoside hydrolase enzyme.

A complete *Sphingomonas aerolata* NW12 genomic sequence (Accession number NZ_PZZN00000000) is available on the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov). An endo-1,4-β-xylanase gene (Accession number WP_107933884) was identified in the genomic sequence; however, a search for identical proteins retrieved a GH family 5 cellulase (Accession number PTM44987) from the same genomic sequence. These findings suggest that the algorithms used by the NCBI database to identify genes within a genomic sequence determined that the same gene sequence from the *S. aerolata* NW12 genome was both a xylanase and a cellulase, consistent with findings in this study that the same enzyme from an *S. aerolata* bacterium hydrolysed both xylan and Avicel cellulose, using the same active site.

4.4.1. Conclusion

The *S. aerolata* putative xylanase was confirmed to be a substrate promiscuous multifunctional glycoside hydrolase enzyme, which hydrolyses three different substrates - beechwood xylan, Avicel and hydrolysed potato starch - using the same active site. These findings are novel, as there are currently no enzymes reported in literature that use the same active site to hydrolyse three different substrate classes, specifically xylan, crystalline cellulose and starch.

Chapter 5: General discussion, conclusions and future recommendations

5.1. General discussion

In this study, a psychrotrophic bacterium, *Sphingomonas aerolata*, was successfully sub-cultivated under laboratory conditions in both solid and liquid modified PYEs media (peptone, 1%; yeast extract, 1%; NaCl, 0.5%, and hydrolysed potato starch, 1%; with 1.5% agar incorporated for solid media). Psychrophiles are cold-adapted microorganisms known to be capable of surviving and growing in extremely low-temperature environments, ranging from below 5˚C and not exceeding 20˚C (Cavicchioli et al., 2002; Kumar et al., 2011). Psychrotrophs, also referred to as psychrotolerant or eurypsychrophiles, are psychrophiles with a broader temperature range, capable of growing at temperatures higher than 25˚C but not exceeding 40˚C (Dalmaso et al., 2015). Psychrophiles are considered an underutilised resource (Kumar et al., 2011), with a broad biotechnological potential that offers numerous economic and ecological advantages over mesophiles (Kasana and Gulati, 2011), thus warranting further research studies into their properties and potential applications. The optimal temperature for *S. aerolata* growth in this study was found to be 28˚C, consistent with definitions of psychrotrophic microorganisms (Cavicchioli et al., 2002; Dalmaso et al., 2015; Russell, 1997) and consistent with the temperatures used in previous studies when culturing different species of bacteria from the *Sphingomonas* genus (Busse et al., 2003; Zhang et al., 2011).

S. aerolata was found to produce an extracellular, xylan degrading enzyme at optimal culturing conditions of 28˚C with shaking at 150 rpm for 24 hours in modified PYES liquid media with hydrolysed potato starch (1%). It was found that hydrolysed potato starch was a more effective inducer for the production of the putative xylanase than beechwood xylan, a natural substrate for xylanases. It is possible that the induction of xylanase production is actually by the products of the hydrolysis of the substrates in the culture medium, rather than the actual substrates, as these are usually too large to enter the cell matrix and induce expression of xylanases (Alvarez-Cervantes et al., 2016). Therefore, it is quite probable that the products of the hydrolysis of hydrolysed potato starch produces smaller sugars that can enter the cell and induce xylanase production, while the hydrolysis of beechwood xylan does not produce such products. This could explain how the production of the putative xylanase by *S. aerolata* was induced by hydrolysed potato starch more efficiently than by beechwood xylan.

The putative xylanase produced by *S. aerolata* was purified to homogeneity using ultrafiltration with the aid of Amicon molecular weight cut-off filtration units and determined to have a molecular weight of approximately 36 kDa. This molecular weight is consistent with the molecular weight of a hypothetical GH5 enzyme (accession number PT47425.1) from the *S. aerolata* NW12 genome, which was calculated to be approximately 38.8 kDa by running the amino acid sequence of the GH5 enzyme on the ProtParam tool of the ExPASy resource portal (web.expasy.org/protparam). The xylanase activity of the purified sample was successfully confirmed using non-denaturing native activity-PAGE with 0.1% (w/v) beechwood xylan incorporated in the resolving gel, with a band of xylan hydrolysis visualised by staining the gel with 0.3% (w/v) Congo Red, de-staining it with 1 M NaCl and counterstaining with 5% (v/v) acetic acid. However, this putative xylanase was also determined to be active on other substrates, making it a substrate promiscuous multifunctional glycoside hydrolase. The multifunctional GH enzyme was found to hydrolyse beechwood xylan, Avicel and hydrolysed potato starch using the same active site. These findings are novel, as there are currently no enzymes reported in literature that can hydrolyse three different substrates using the same active site.

The multifunctional putative xylanase was also found to be active on beechwood xylan at low temperatures, consistent with other studies of cold-active xylanases isolated from psychrophiles (Ko et al., 2016; Lee et al., 2006; Turkiewicz et al., 2000). It was also found to have an optimum temperature for xylan hydrolysis of 40˚C, similar to two cold-active xylanases from the Antarctic krill, *Euphausia superba* Dana (Turkiewicz et al., 2000). However, unlike any of the cold-active xylanases reported in literature, the multifunctional putative *S. aerolata* xylanase was found to be thermostable, maintaining over 78% relative activity on beechwood xylan after an incubation period of 24 hours at 50˚C. These findings indicate that the multifunctional putative xylanase is a cold-active thermostable enzyme, another novel finding, as it has been shown that cold-active enzymes from psychrophilic microorganisms are usually thermo-labile (Cavicchioli et al., 2011; Dalmaso et al., 2015). In addition to being thermostable, the multifunctional putative xylanase was also found to be highly alkali- and acido-stable, maintaining over 60% relative activity between pH 2.0 and 5.0 and over 80% relative activity between pH 8.0 and 11.0.

5.2. Conclusions

A novel cold-active, thermostable, acido/alkaliphile, and substrate-promiscuous multifunctional putative xylanase was successfully purified from a liquid nutrient culture medium of the psychrotrophic bacterium *Sphingomonas aerolata*. The multifunctional enzyme was found to hydrolyse beechwood xylan, Avicel and hydrolysed potato starch using the same active site, making it a truly novel glycoside hydrolase enzyme.

5.3. Future recommendations

While the *S. aerolata* multifunctional glycoside hydrolase enzyme purified and characterised in this study was classified as a putative xylanase, because it exhibited the highest activity on beechwood xylan, its activities on the other substrates such as Avicel cellulose, hydrolysed potato starch and even arabinogalactan, were significant enough to cast doubt on the classification of this enzyme using only biochemical characterisation. A recommendation for future studies would be to use in-gel tryptic digestion of the purified putative xylanase band on SDS-PAGE to obtain the sequence of the protein. This sequence could be used to identify and accurately classify the enzyme using bioinformatic studies, which would also shed more light on the structure of the active site, which is capable of accommodating different substrates. Another recommendation would be to concentrate the products from the hydrolysis of the multifunctional GH enzyme on beechwood xylan, Avicel, hydrolysed potato starch and arabinogalactan, and use HPLC to identify the hydrolysis products, which would also further shed light on the mode of action of this multifunctional glycoside hydrolase.

References

Alvarez-Cervantes, J., Dominguez-Hernandez, E., Mercado-Flores, Y., O'Donovan, A., Diaz-Godinez, G., 2016. Mycosphere Essay 10 : Properties and characteristics of microbial xylanases. Mycosphere 7, 1600–1619. https://doi.org/10.5943/mycosphere/si/3b/12

Aygan, A., Arikan, B., 2009. Production and characterization of multifunctional endoxylanase by *Bacillus* sp. X13. Turkish J. Biol. 33, 231–237. https://doi.org/10.3906/biy-0806-18

Bhattacharya, A., Mafa, M., Rashamuse, K., Pletschke, B.I., 2016. A bi-functional xylanase/ xyloglucanase, Gh5H, from termite metagenome improves the hydrolysis of pretreated biomass. Int. J. Adv. Sci. Eng. Technol. 4, 213–216.

Biaglow, A., Erickson, K., McMurran, S., 2010. Enzyme kinetics and the Michaelis-Menten equation. Primus 20, 148–168. https://doi.org/10.1080/10511970903486491

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248–254.

Brininger, C., Spradlin, S., Cobani, L., Evilia, C., 2018. The more adaptive to change, the more likely you are to survive: Protein adaptation in extremophiles. Semin. Cell Dev. Biol. 1–12. https://doi.org/10.1016/j.semcdb.2017.12.016

Britton, H.T.S., Robinson, R.A., 1931. CXCVIII.—Universal buffer solutions and the dissociation constant of veronal. J. Chem. Soc. 1456–1462. https://doi.org/10.1039/JR9310001456

Busse, H.J., Denner, E.B.M., Buczolits, S., Salkinoja-Salonen, M., Bennasar, A., Kämpfer, P., 2003. *Sphingomonas aurantiaca* sp. nov., *Sphingomonas aerolata* sp. nov. and *Sphingomonas faeni* sp. nov., air- and dustborne and Antarctic, orange-pigmented, psychrotolerant bacteria, and emended description of the genus *Sphingomonas*. Int. J. Syst. Evol. Microbiol. 53, 1253–1260. https://doi.org/10.1099/ijs.0.02461-0

Campbell, M.K., Farrell, S.O., 2012. Biochemistry, 7th ed. 117–165. Brooks/Cole Cengange Learning.

Cavicchioli, R., Charlton, T., Ertan, H., Omar, S.M., Siddiqui, K.S., Williams, T.J., 2011. Biotechnological uses of enzymes from psychrophiles. Microb. Biotechnol. 4, 449–460. https://doi.org/10.1111/j.1751-7915.2011.00258.x

Cavicchioli, R., Siddiqui, K.S., Andrews, D., Sowers, K.R., 2002. Low-temperature extremophiles and their applications. Curr. Opin. Biotechnol. 13, 253–261. https://doi.org/10.1016/S0958-1669(02)00317-8

Chen, H., Wang, M., Huang, H., Yan, X.I.N., Jia, X., Wang, J., 2006. Purification and characterization of novel bifunctional xylanase, XynIII, isolated from *Aspergillus niger* A-25. J. Microbiol. Biotechnol. 16, 1132–1138.

Cheng, X.Y., Huang, W.J., Hu, S.C., Zhang, H.L., Wang, H., Zhang, J.X., Lin, H.H., Chen, Y.Z., Zou, Q., Ji, Z.L., 2012. A global characterization and identification of multifunctional enzymes. PLoS One 7, 1–8. https://doi.org/10.1371/journal.pone.0038979

Cheng, Y.S., Chen, C.C., Huang, C.H., Ko, T.P., Luo, W., Huang, J.W., Liu, J.R., Guo, R.T., 2014. Structural analysis of a glycoside hydrolase family 11 xylanase from *Neocallimastix patriciarum*: Insights into the molecular basis of a thermophilic enzyme. J. Biol. Chem. 289, 11020–11028. https://doi.org/10.1074/jbc.M114.550905

Collins, T., Gerday, C., Feller, G., 2005. Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol. Rev. 29, 3–23. https://doi.org/10.1016/j.femsre.2004.06.005

Dahlman, O., Jacobs, A., Sjöberg, J., 2003. Molecular properties of hemicelluloses located in the surface and inner layers of hardwood and softwood pulps. Cellulose 10, 325–334. https://doi.org/10.1023/A:1027316926308

Dalmaso, G.Z.L., Ferreira, D., Vermelho, A.B., 2015. Marine extremophiles a source of hydrolases for biotechnological applications. Mar. Drugs 13, 1925–1965. https://doi.org/10.3390/md13041925

Davies, G., Henrissat, B., 1995. Structures and mechanisms of glycosyl hydrolases. Structure 3, 853–859. https://doi.org/10.1016/S0969-2126(01)00220-9

de Souza, P.M., e Magalhães, P. de O., 2010. Application of microbial α-amylase in industry - a review. Brazilian J. Microbiol. 41, 850–861. https://doi.org/10.1590/S1517-83822010000400004

Demirjian, D.C., Morís-Varas, F., Cassidy, C.S., 2001. Enzymes from extremophiles. Curr. Opin. Chem. Biol. 5, 144–151. https://doi.org/10.1016/S1367-5931(00)00183-6

Denner, E.B.M., Paukner, S., Kämpfer, P., Moore, E.R.B., Abraham, W.R., Busse, H.J., Wanner, G., Lubitz, W., 2001. *Sphingomonas pituitosa* sp. nov., an exopolysaccharide-

producing bacterium that secretes an unusual type of sphingan. Int. J. Syst. Evol. Microbiol. 51, 827–841. https://doi.org/10.1099/00207713-51-3-827

Ebringerová, A., 2006. Structural diversity and application potential of hemicelluloses. Macromol. Symp. 232, 1–12. https://doi.org/10.1002/masy.200551401

Elleuche, S., Schröder, C., Sahm, K., Antranikian, G., 2014. Extremozymes-biocatalysts with unique properties from extremophilic microorganisms. Curr. Opin. Biotechnol. 29, 116–123. https://doi.org/10.1016/j.copbio.2014.04.003

European Commission, 2009. Towards 2020 : Making Chemicals Safer. The EU's Contribution to the Strategic Approach to International Chemicals Management. Eur. Comm.

Fernandes, A.C., Fontes, C.M., Gilbert, H.J., Hazlewood, G.P., Fernandes, T.H., Ferreira, L.M., 1999. Homologous xylanases from *Clostridium thermocellum*: evidence for bi-functional activity, synergism between xylanase catalytic modules and the presence of xylan-binding domains in enzyme complexes. Biochem. J. 342, 105–110. https://doi.org/Doi 10.1042/0264-6021:3420105

Gajera, H.P., Patel, S. V, Golakiya, B.A., 2008. Enzymes and Kinetics. In *Fundamentals of Biochemistry*. 181–196. International Book Distributing Co.

Gavrilescu, M., Chisti, Y., 2005. Biotechnology - A sustainable alternative for chemical industry. Biotechnol. Adv. https://doi.org/10.1016/j.biotechadv.2005.03.004

Gerday, C., Aittaleb, M., Arpigny, J.L., Baise, E., Chessa, J.P., Garsoux, G., Petrescu, I., Feller, G., 1997. Psychrophilic enzymes: A thermodynamic challenge. Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol. 1342, 119–131. https://doi.org/10.1016/S0167-4838(97)00093-9

Gerday, C., Aittaleb, M., Bentahir, M., Chessa, J.P., Claverie, P., Collins, T., D'Amico, S., Dumont, J., Garsoux, G., Georlette, D., Hoyoux, A., Lonhienne, T., Meuwis, M.A., Feller, G., 2000. Cold-adapted enzymes: From fundamentals to biotechnology. Trends Biotechnol. 18, 103–107. https://doi.org/10.1016/S0167-7799(99)01413-4

Gilbert, H.F., 2000. Basic Concepts in Biochemistry: A Student's Survival Guide, Second Edi. ed. 80–119. McGraw-Hill.

Guo, B., Chen, X., Sun, C., Zhou, B.-C., Zhang, Y.-Z., 2009. Gene cloning , expression and characterization of a new from marine *Glaciecola mesophila* KMM 241. Appl. Microbiol.

Biotechnol. 84, 1107–1115. https://doi.org/10.1007/s00253-009-2056-y

Harholt, J., Suttangkakul, A., Scheller, H.V., 2010. Biosynthesis of pectin. Plant Physiol. https://doi.org/10.1104/pp.110.156588

Himmel, M.E., Ding, S.-Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D., 2007. Biomass recalcitrance: Engineering plants and enzymes for biofuels production. Nature 454, 804–807. https://doi.org/10.1126/science.1137016

Horn, S.J., Vaaje-Kolstad, G., Westereng, B., Eijsink, V.G.H., 2012. Novel enzymes for the degradation of cellulose. Biotechnol. Biofuels 5, 2–12.

Jegannathan, K.R., Nielsen, P.H., 2013. Environmental assessment of enzyme use in industrial production e a literature review. J. Clean. Prod. 42, 228–240. https://doi.org/10.1016/j.jclepro.2012.11.005

Johnson, K.A., Goody, R.S., 2011. The original Michaelis constant: Translation of the 1913 Michaelis-Menten paper. Biochemistry 50, 8264–8269. https://doi.org/10.1021/bi201284u

Jørgensen, H., Kristensen, J.B., Felby, C., 2007. Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. Biofuels Bioprod. Biorefining 1, 119–134. https://doi.org/10.1002/bbb

Karlsson, E.N., Schmitz, E., Linares-pastén, J.A., Adlercreutz, P., 2018. Endo-xylanases as tools for production of substituted xylooligosaccharides with prebiotic properties. Appl. Microbiol. Biotechnol. 102, 9081–9088.

Kasana, R.C., Gulati, A., 2011. Cellulases from psychrophilic microorganisms: A review. J. Basic Microbiol. 51, 572–579. https://doi.org/10.1002/jobm.201000385

Ko, J.K., Ko, H., Kim, K.H., Choi, I., 2016. Characterization of the biochemical properties of recombinant Xyn10C from a marine bacterium, *Saccharophagus degradans*. Bioprocess Biosyst. Eng. 39, 677–684. https://doi.org/10.1007/s00449-016-1548-2

Kumar, P.S., Ghosh, M., Pulicherla, K.K., Rao, K.R.S.S., 2011. Cold Active Enzymes from the Marine Psychrophiles: Biotechnological Perspective. Adv. Biotechnol. 10, 16–20.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature 227, 680–685.

Lagaert, S., Beliën, T., Volckaert, G., 2009. Plant cell walls: Protecting the barrier from

degradation by microbial enzymes. Semin. Cell Dev. Biol. 20, 1064–1073. https://doi.org/10.1016/j.semcdb.2009.05.008

Lee, C.C., Kibblewhite-Accinelli, R.E., Wagschal, K., Robertson, G.H., Wong, D.W.S., 2006. Cloning and characterization of a cold-active xylanase enzyme from an environmental DNA library. Extremophiles 10, 295–300. https://doi.org/10.1007/s00792-005-0499-3

Li, S., Yang, X., Yang, S., Zhu, M., Wang, X., 2012. Technology Prospecting on Enzymes: Application, Marketing and Engineering. Comput. Struct. Biotechnol. J. 2, e201209017. https://doi.org/10.5936/csbj.201209017

Li, X., Jiang, Z., Li, L., Yang, S., Feng, W., Fan, J., Kusakabe, I., 2005. Characterization of a cellulase-free, neutral xylanase from *Thermomyces lanuginosus* CBS 288.54 and its biobleaching effect on wheat straw pulp. Bioresour. Technol. 96, 1370–1379. https://doi.org/10.1016/j.biortech.2004.11.006

Meng, D.D., Ying, Y., Chen, X.H., Lu, M., Ning, K., Wang, L.S., Li, F.L., 2015. Distinct roles for carbohydrate-binding modules of glycoside hydrolase 10 (GH10) and GH11 xylanases from *Caldicellulosiruptor* sp. strain F32 in thermostability and catalytic efficiency. Appl. Environ. Microbiol. 81, 2006–2014. https://doi.org/10.1128/AEM.03677-14

Meryandini, A., Hendarwin, T., Fahrruzozi, Akhdiya, A., Saprudin, D., Lestari, Y., 2009. Characterization and Purification a Specific Xylanase Showing Arabinofuranosidase Activity from *Streptomyces* spp. 234P-16. Biodiversitas, J. Biol. Divers. 10, 115–119. https://doi.org/10.13057/biodiv/d100302

Miller, G.L., 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal. Chem. 31, 426–428.

Monclaro, A., Aquino, E., Faria, R., Ricart, C., Freitas, S., Midorikawa, G., Miller, R., Michelin, M., Polizeli, M., Filho, E., 2016. Characterization of multiple xylanase forms from *Aspergillus tamarii* resistant to phenolic compounds. Mycosphere 7, 1554–1567. https://doi.org/10.5943/mycosphere/si/3b/7

Morita, R.Y., 1975. Psychrophilic bacteria. Bacteriol. Rev. 39, 144–167. https://doi.org/10.1016/S0140-6736(59)91597-1

Morrison, D., van Dyk, J.S., Pletschke, B.I., 2011. The effect of alcohols, lignin and phenolic compounds on the enzyme activity of *Clostridium cellulovorans* XynA. BioResources 6, 3132–3141.

Niehaus, F., Niehaus, F., Bertoldo, C., Bertoldo, C., Kähler, M., Kähler, M., Antranikian, G., Antranikian, G., 1999. Extremophiles as a source of novel enzymes for industrial application. Appl. Microbiol. Biotechnol. 51, 711–729. https://doi.org/10.1007/s002530051456

OECD, 2011. Industrial Biotechnology and Climate Change: Opportunities and Challenges. Organ. Econ. Co-operation Dev. Publ.

OECD, 2009. Metrics to support informed decision-making for consumers of biobased products. Organ. Econ. Co-operation Dev. Publ.

Qian, H., 2008. Cooperativity and specificity in enzyme kinetics: A single-molecule time-based perspective. Biophys. J. 95, 10–17. https://doi.org/10.1529/biophysj.108.131771

Ratledge, C., Kristiansen, B., 2001. Basic Biotechnology. Cambridge University Press.

Ray, R.R., 2015. Microbial avicelase: An overview. Bull. Environ. Pharmacol. Life Sci. 4, 3–13.

Roberts, M.E., Inniss, W.E., 1992. The synthesis of cold shock proteins and cold acclimation proteins in the psychrophilic bacterium *Aquaspirillum arcticum*. Curr. Microbiol. 25, 275–278. https://doi.org/10.1007/BF01575861

Rogers, P.L., Jeon, Y.J., Svenson, C.J., 2005. Application of Biotechnology to Industrial Sustainability. Process Saf. Environ. Prot. 83, 499–503. https://doi.org/10.1205/psep.05005

Rouau, X., Odier, E., 1986. Purification and properties of two enzymes from *Dichomitus squalens* which exhibit both cellobiohydrolase and xylanase activity. Carbohydr. Res. 145, 279–292.

Ruan, B., Wu, P., Chen, M., Lai, X., Chen, L., Yu, L., Gong, B., Kang, C., Dang, Z., Shi, Z., Liu, Z., 2018. Immobilization of *Sphingomonas* sp. GY2B in polyvinyl alcohol–alginate–kaolin beads for efficient degradation of phenol against unfavorable environmental factors. Ecotoxicol. Environ. Saf. 162, 103–111. https://doi.org/10.1016/j.ecoenv.2018.06.058

Russell, N.J., 1997. Psychrophilic bacteria - molecular adaptations of membrane lipids. Comp. Biochem. Physiol. Part A Physiol. 118, 489–493. https://doi.org/10.1016/S0300-9629(97)87354-9

Shi, P., Du, Y., Yang, H., Huang, H., Zhang, X., Wang, Y., Yao, B., 2015. Molecular

characterization of a new alkaline-tolerant xylanase from *humicola insolens* y1. Biomed Res. Int. 2015. https://doi.org/10.1155/2015/149504

Shi, P., Tian, J., Yuan, T., Liu, X., Huang, H., Bai, Y., Yang, P., Chen, X., Wu, N., Yao, B., 2010. *Paenibacillus* sp. Strain E18 Bifunctional Xylanase-Glucanase with a Single Catalytic Domain. Appl. Environ. Microbiol. 76, 3620–3624. https://doi.org/10.1128/AEM.00345-10

Silverman, R.B., 2000. The Organic Chemistry of Enzyme-Catalyzed Reactions. Academic Press.

Singh, S., Madlala, A.M., Prior, B.A., 2003. *Thermomyces lanuginosus*: Properties of strains and their hemicellulases. FEMS Microbiol. Rev. 27, 3–16. https://doi.org/10.1016/S0168-6445(03)00018-4

St John, F.J., Crooks, C., Dietrich, D., Hurlbert, J., 2016. Xylanase 30 A from *Clostridium thermocellum* functions as a glucuronoxylan xylanohydrolase. Journal Mol. Catal. B, Enzym. 133, S445–S451. https://doi.org/10.1016/j.molcatb.2017.03.008

St John, F.J., Dietrich, D., Crooks, C., Porzharski, E., Gonzalez, J.M., Bales, E., Smith, K., Hurlbert, J.C., 2014. A novel member of glycoside hydrolase family 30 subfamily 8 with altered substrate specificity. Acta Crystallogr. Sect. D 70, 2950–2958. https://doi.org/10.1107/S1399004714019531

Sundarram, A., Murthy, T.P.K., 2014. α-Amylase production and applications : A review. J. Appl. Environ. Microbiol. 2, 166–175. https://doi.org/10.12691/jaem-2-4-10

Takeuchi, M., Hamana, K., Hiraishi, A., 2001. Proposal of the genus *Sphingomonas sensu stricto* and three new genera. Int. J. Syst. Evol. Microbiol. 51, 1405–1417. https://doi.org/10.1099/00207713-51-4-1405

Torres, N., Santos, G., 2017. Simple simulator to teach enzyme kinetics dynamics. Application in a Problem-Solving exercise. High. Educ. Pedagog. 2, 14–27. https://doi.org/10.1080/23752696.2017.1307693

Turkiewicz, M., Kalinowska, H., Zielinska, M., Bielecki, S., 2000. Purification and characterization of two endo-1, 4-β-xylanases from Antarctic krill, *Euphausia superba* Dana. Comp. Biochem. Physiol. Part B 127, 325–335.

Valenzuela, S. V, Lopez, S., Biely, P., Sanz-Aparicio, J., Pastor, J.F.I., 2016. The Glycoside Hydrolase Family 8 Reducing-End Xylose-Releasing Exo-oligoxylanase Rex8A from

Paenibacillus barcinonensis BP-23 Is Active on Branched Xylooligosaccharides. Appl. Environ. Microbiol. 82, 5116–5124. https://doi.org/10.1128/AEM.01329-16.Editor

Van Dyk, J.S., Pletschke, B.I., 2012. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes - Factors affecting enzymes, conversion and synergy. Biotechnol. Adv. 30, 1458–1480. https://doi.org/10.1016/j.biotechadv.2012.03.002

Verma, D., Satyanarayana, T., 2012. Cloning , expression and applicability of thermo-alkali-stable xylanase of *Geobacillus thermoleovorans* in generating xylooligosaccharides from agro-residues. Bioresour. Technol. 107, 333–338. https://doi.org/10.1016/j.biortech.2011.12.055

Wang, Y., Feng, S., Zhan, T., Huang, Z., Wu, G., Liu, Z., 2013. Improving catalytic efficiency of endo-β-1, 4-xylanase from *Geobacillus stearothermophilus* by directed evolution and H179 saturation mutagenesis. J. Biotechnol. 1–7. https://doi.org/10.1016/j.jbiotec.2013.09.014

Wilson, K., Walker, J., 2010. Principles and Techniques of Biochemistry and Molecular Biology, Seventh Ed. ed. Cambridge University Press. https://doi.org/10.1016/0014-5793(94)01289-X

Yabuuchi, E., Yano, I., Oyaizu, H., Hashimoto, Y., Ezaki, T., Yamamoto, H., 1990. Proposals of *Sphingomonas paucimobilis* gen . nov . and comb . nov ., *Sphingomonas parapaucimobilis* sp . nov ., *Sphingomonas yanoikuyae* sp . nov ., *Sphingomonas adhaesiva* sp . nov ., *Sphingomonas capsulata* comb . nov ., and Two Genospecies of the Genus *Sphingomonas*. Microbiol. immunol. 34, 99–119.

Zhang, D.C., Busse, H.J., Liu, H.C., Zhou, Y.G., Schinner, F., Margesin, R., 2011. *Sphingomonas glacialis* sp. nov., a psychrophilic bacterium isolated from alpine glacier cryoconite. Int. J. Syst. Evol. Microbiol. 61, 587–591. https://doi.org/10.1099/ijs.0.023135-0

Zhang, L., Wang, Y., Liang, J., Song, Q., Zhang, X.H., 2016. Degradation properties of various macromolecules of cultivable psychrophilic bacteria from the deep-sea water of the South Pacific Gyre. Extremophiles 20, 663–671. https://doi.org/10.1007/s00792-016-0856-4

Appendices

Appendix I: List of reagents

Acetone	Merck (Cat. No. 822251)
Acrylamide	Sigma (Cat. No. A8887)
Agarose	Sigma (Cat. No. A9539)
Ammonium persulfate	Sigma-Aldrich (Cat. No. A3678)
Amylopectin from maize	Sigma-Aldrich (Cat. No. 10120)
Arabinogalactan from larch	Megazyme (CAS No. 9036-66-2)
Avicel	Fluka (Cat. No. 11365)
Azo-Avicel	Megazyme (Cat. No. I-AAVIC)
Azo-wheat arabinoxylan	Megazyme (Cat. No. S-AWAXP)
Beechwood xylan	Megazyme (CAS No. 9014-63-5)
Bovine Serum Albumin	Sigma (Cat. No. A7906)
Bradford's Reagent	Sigma (Cat. No. B6916)
Bromophenol blue	Sigma (Cat. No. 114391)
Calcium chloride dihydrate	Merck (Cat. No. 208290)
Calcium chloride	Sigma (Cat. No. C1016)
Carboxymethyl cellulose	Calbiochem (Cat.No. 217277)
Citric acid	Merck (Cat.No. 1.00244)
Coomassie Brilliant Blue R250	Merck (Cat. No. 1.12553)
Copper (II) sulphate pentahydrate	Merck (Cat No. 102790)
3,5-Dinitrosalicylic acid	Sigma (Cat. No. D0550)
Di-potassium hydrogen phosphate	Merck (Cat No. 137010)
Di-sodium hydrogen orthophosphate	Saar Chem (Cat. No. 5822860)

Ethanol	Merck (Cat. No. 8.18700)
Ethylenediaminetetraacetic acid (EDTA)	Sigma (Cat. No. E9884)
Folin Ciolcateau’s reagent	Sigma (Cat. No. F9252)
Glacial acetic acid	Merck (Cat. No. 1.00063)
D-Glucose	Merck (Cat. No. 346351)
Glycerol	Merck (Cat. No. 818709)
Glycine	Merck (Cat. No. 104169)
Hydrolysed potato starch	Sigma (Cat. No. S5651)
Hydrochloric acid	Merck (Cat. No. 100319)
Iron (II) sulfate heptahydrate	Sigma (Cat. No. F7002)
Locust bean gum	Sigma-Aldrich (Cat No. G0753)
Magnesium sulphate anhydrous	Merck (Cat No. 106067)
Manganese (II) sulphate monohydrate	Sigma (Cat. No. M7364)
D-Mannose	Sigma (Cat. No. M2069)
β-Mercaptoethanol	Sigma (Cat. No. M6250)
Methanol	Merck (Cat. No. 822283)
N,N-Methylenebisacrylamide	Sigma (Cat. No. M7279)
p-Nitrophenol	Sigma (Cat. No. 42,575-3)
4-Nitrophenyl α-L-arabinopyranoside	Sigma-Aldrich (Cat. No. N3512)
4-Nitrophenyl α-D-galactopyranoside	Sigma-Aldrich (Cat. No. N0877)
4-Nitrophenyl β-D-glucopyranoside	Sigma-Aldrich (Cat. No. N7006)
4-Nitrophenyl β-D-xylopyranoside	Sigma-Aldrich (Cat. No N2132)
Phenol	Sigma (Cat. No. P3653)
Protein Unstained Molecular Marker	BioRad (Cat. No. 1610363)
Sodium azide	Merck (Cat. No. 8.22335)
Sodium carbonate	Merck (Cat. No. 106392)

Sodium chloride	Merck (Cat. No. 137017)
Sodium dodecyl sulphate	Merck (Cat. No. 817034)
Sodium hydroxide	Sigma (Cat. No. 795429)
Sodium metabisulfite	Sigma-Aldrich (Cat. No. 255556)
Sodium potassium tartrate	Merck (Cat. No. 1.08087)
Soluble starch	Sigma-Aldrich (Cat. No. S9765)
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Sigma (Cat. No. T9281)
Trichloroacetic acid	Sigma (Cat. No. T6399)
Tris (hydroxymethyl) aminomethane	Merck (Cat. No. 108382)
Tryptone	Merck (Cat. No. 107213)
Wheat arabinoxylan	Megazyme (CAS No.9040-27-1)
Yeast Extract	Merck (Cat. No. 111962)
Zinc sulphate heptahydrate	Sigma (Cat. No. Z0251)

Appendix II: Standard curves

Bradford standard curve

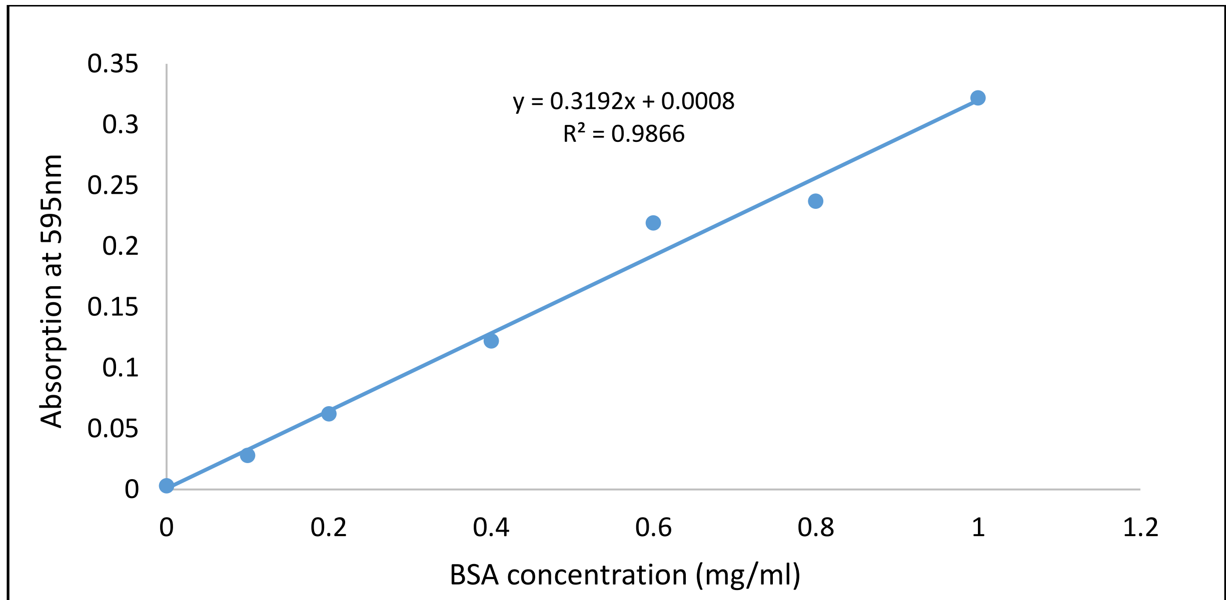


Figure II A: Bradford standard curve for protein concentration determination with BSA used as the protein standard (n=3)

Xylose standard curve

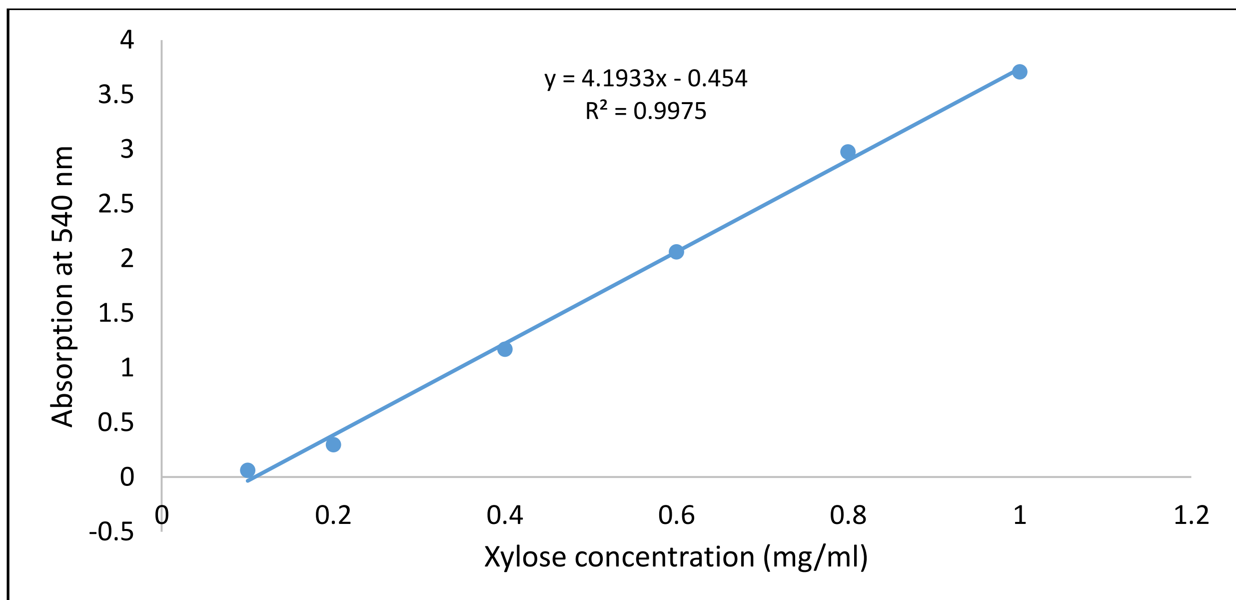


Figure II B: Xylose standard curve using the DNS assay (n=3)

p-Nitrophenol standard curve

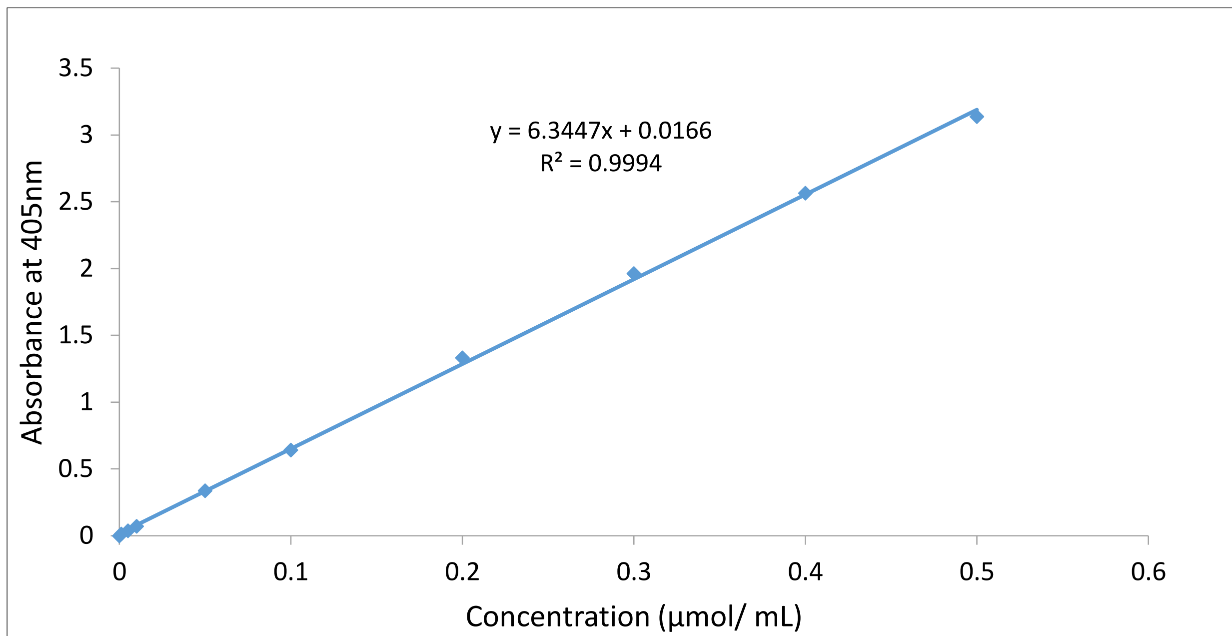


Figure II C: p-Nitrophenol standard curve (n=3)

Remazol Brilliant Blue standard curve

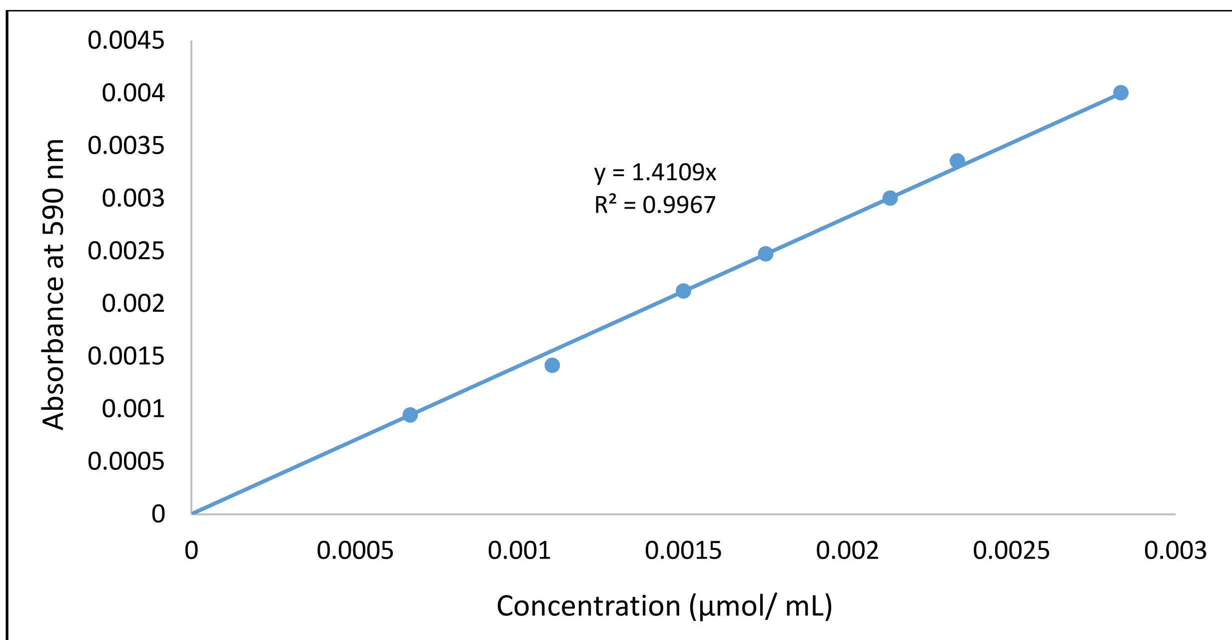


Figure II D: Remazol Brilliant Blue standard curve (n=4)