Cloning, expression, partial characterisation and application of a recombinant GH10 xylanase, XT6, from *Geobacillus stearothermophilus* T6 as an additive to chicken feeds

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

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Abstract

Monogastric animal farming has largely been sustained by feeding animals with grain feedstocks containing non-starch polysaccharides (NSPs) and anti-nutritive factors, which cause adverse effects, such as increased digesta viscosity and entrapment of nutrients, which leads to the inaccessibility of nutrients. These effects have been linked to a reduction in nutrient digestion and absorption, which results in a decreased feed conversion ratio, energy metabolism and animal growth. Monogastric animals do not produce enzymes that can hydrolyse these NSPs. The application of exogenous enzymes as supplements to animal feeds has been implemented to reduce viscosity and increase nutrient absorption in poultry and pigs over the past few decades. The aim of this study was to clone, express, partially characterise and apply a glycoside hydrolase (GH) family 10 xylanase (XT6), derived from *Geobacillus stearothermophilus* T6, as an additive to locally produced chicken feeds.

The *xt6* gene (1,236 bp) was subcloned and expressed in *Escherichia coli* DH5α and BL21(DE3) cells, respectively. Upon expression, XT6 had a molecular weight of 42 kDa and was partially purified by Ni-NTA chromatography and ultrafiltration. The purification step resulted in a yield of 66.7% with a 16.8-fold increase in purification. XT6 exhibited maximal activity when incubated at a pH and temperature of pH 6.0 and 70°C, respectively, with a high thermostability over a broad range of pH (2–9) and temperature (30–90 °C). The specific activities of XT6 on extracted soluble and insoluble wheat flour arabinoxylans were 110.9 U/mg and 63.98 U/mg, respectively. Kinetic data showed that XT6 displayed a higher catalytic activity and affinity (V_{max} = 231.60 µmol/min/mg and K_M = 2.759 mg/ml) for soluble wheat arabinoxylan, compared to insoluble wheat arabinoxylan (V_{max} = 99.02 µmol/min/mg and K_M = 5.058 mg/ml).

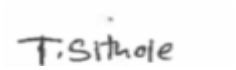
High-performance liquid chromatography (HPLC) analysis showed that the enzyme hydrolysed wheat flour, arabinoxylan and chicken feeds, producing a range of xylooligosaccharides (XOS), with xylotetraose and xylopentaose being the predominant XOS species. Hydrolysis of both soluble and insoluble wheat flour arabinoxylans by XT6 led to a significant reduction in substrate viscosity. The effects of simulated gastrointestinal fluid contents, such as proteases, bile salts and mucins, on XT6 stability were also studied. Exposure of XT6 to pepsin did not significantly reduce its activity; however, the inhibitory effect of trypsin and mucin on XT6 was much greater. The presence of gut-derived bile salts had no

significant effect on XT6 activity. Finally, it was shown that the XOS produced from the hydrolysis of chicken feeds (starter and grower feeds) by XT6 significantly enhanced the growth of the probiotic bacteria *B. subtilis*, while there was no significant improvement in the growth of *S. thermophilus* and *L. bulgaricus*.

In conclusion, the recombinantly produced XT6 demonstrated efficient hydrolysis of starter and grower feeds, and produced XOS that showed prebiotic activity on selected probiotic bacteria. In addition, the pH, temperature and simulated gastric juice content stability of XT6 renders it an attractive candidate as an additive for chicken feeds.

Plagiarism declaration

I, Tariro Sithole, declare that this thesis is my own, original and unaided work. It is being submitted for the Master of Science at Rhodes University. It has not been submitted before, for any degree or examination, at any other university.

Signature: 

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List of Abbreviations

ANFs	Anti-Nutritive Factors
AX	Arabinoxylan
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
DNS	3, 5-Dinitrosalicylic acid
DP	Degree of polymerization
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FACE	Fluorophore-Assisted Carbohydrate Electrophoresis
g	Grams
g	Relative centrifugal force
GAXs	Glucuronoarabinoxylans
GH	Glycoside Hydrolase
HPLC	High-Performance Liquid Chromatography
IMAC	Immobilized metal-affinity chromatography
IPTG	Isopropyl β-D-Thiogalactopyranoside
M	molar
mg	milligram
ml	millilitre
mM	millimolar
NSPs	Non-Starch Polysaccharides
OD	Optical density

RS	Reducing sugars
SCFAs	short-chain fatty acids
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SOC	Shorthand for super optimum broth with catabolite repression
TAE	Tris-acetate
TLC	Thin-Layer Chromatography
U/mg	µmol/mim/mg of protein
V	Volts
v/v	Volume per volume
w/v	Weight per volume
XOS	Xylo-oligosaccharide(s)
XT6	Xylanase T6
µl	Microlitre
YT	Yeast extract tryptone
℃	Degrees Celsius

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I also wish to acknowledge the Department of Science (now Innovation) of South Africa and the Technology Innovation Agency (DSI-TIA) for funding this study and for providing me with a bursary. Any opinion, findings and conclusions or recommendations expressed in this material are those of the author(s) and therefore the DSI-TIA does not accept liability in this regard.

Research outputs

Manuscript in preparation: Sithole T., Mzimkulu N.H., Lebogang R., Malgas S. and Pletschke B.I. (2021) The effect of enzyme additives on agri-industrial feeds – their effect on the feed intake, digestion, and growth performance of monogastric animals.

Manuscript in preparation: Sithole T., Malgas S. and Pletschke B.I. (2021) Cloning, expression, partial characterisation and application of a recombinant GH10 xylanase, XT6, from *Geobacillus stearothermophilus* T6 as an additive to chicken feeds.

Chapter 1: Literature review

1.1.BACKGROUND ON ANIMAL FEEDS

Monogastric animal (e.g., pigs and poultry) farming has been sustained by feeding animals with grain feedstocks. Commonly used grains include wheat, barley and maize (Nørgaard et al., 2019; Pedersen et al., 2015). These have been used as commercial animal feeds for the past few decades (Cowieson et al., 2006; Schramm et al., 2017). The primary staple cereals produced and utilised in South Africa are maize and wheat (Nhemachena and Kirsten, 2017). The South African Department of Agriculture, Forestry and Fisheries (DAAF) has reported that the annual wheat production in South Africa ranges from 1.5 to 3 million tonnes (Nhemachena and Kirsten, 2017). On the other hand, about 10.5 million tons of maize are produced annually in South Africa. According to the Animal Feed Manufacturer Association (AFMA), about 4.5 million tons of maize are used in animal feeds (Nhemachena and Kirsten, 2017). Most of these cereals are used for human consumption as a source of starch. Cereal by-products produced after separating the starch portion in grains contain the highest values of anti-nutritive factors (ANFs) and non-starch polysaccharides (NSPs) (Sethy et al., 2015). These by-products are subsequently used in various bioprocessing industries and animal feeds (Choct, 2015; Sethy et al., 2015; Yuan et al., 2017).

NSPs have been reported to be either soluble (viscous) or insoluble (non-viscous) in aqueous solutions (Choct, 2006; Pluske, et al., 2001). Wheat, oats, barley, rye and triticale are considered to be viscous, while corn, sorghum, millet and rice are considered non-viscous (Choct, 2006). Soluble NSPs have been associated with a reduction in nutrient digestion efficiency in the animal feed industry, as the nutrients are embedded into the cell wall's insoluble parts, making the nutrients inaccessible for absorption by the animal (Cowan, 1995). The soluble NSPs have been reported to have adverse effects, including increased intestinal viscosity and lowered net energy utilisation due to indigestibility (Sethy et al., 2015; Zhang et al., 2018). High viscosity has been associated with impaired nutrient digestion and absorption, increasing digesta viscosity, and interference with the gut microflora and digestive tract physiology (Choct, 2006). The application of exogenous enzymes as supplements in animal feeds has been implemented for the past few decades to overcome this problem (Bedford and Schulze, 1998). These enzymes include xylanases, proteases and β-glucanases (Bedford, 2018;

Ponte et al., 2004). These exogenous enzymes have been reported to improve feed intake and digestion in animals, leading to increased growth performance, as illustrated in Figure 1.1.

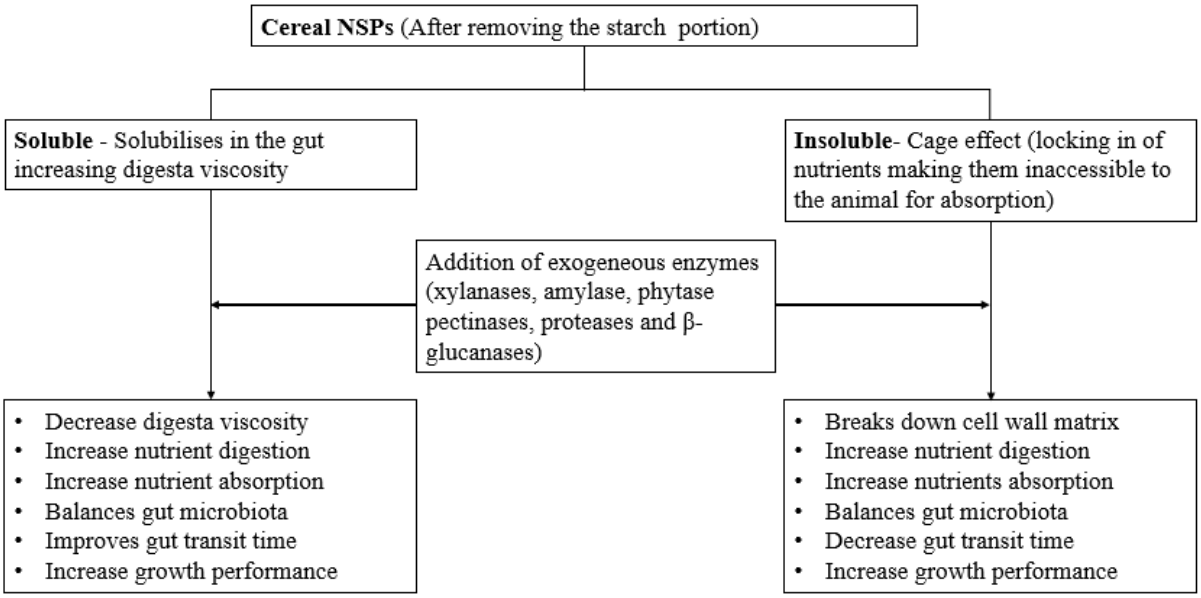

Figure 1.1: Types of cereal NSPs, their effects on monogastric animals and the effect of supplementing exogeneous enzymes on monogastric animals. Adapted from Chesson (2001) and Smits and Annison (1996).

NSPs form part of the cell wall polysaccharides of cereal grains (Baker et al., 2021; Izydorczyk., 2021, Navarro, et al., 2019). The main components of the cereal grain cell walls include the bran (aleurone layer and the pericarp layer), the germ layer, and the endosperm (Figure 1.2; Fardet, 2010). In cereal grains, the primary cell wall consists of approximately 70% to 80% of non-cellulosic polysaccharides (NCP) and 20% to 30% cellulose (linear β-glucan chains) (Navarro et al., 2019). In comparison, the secondary cell walls contain about 50% of cellulose and 50% non-cellulosic polysaccharides (Navarro et al., 2019). Non-cellulosic polysaccharides include pentoses (such as arabinose and xylose), hexoses (such as glucose, galactose, and mannose), 6-deoxyhexoses (such as rhamnose and fucose), uronic acids (which include glucuronic and galacturonic acids) and pectic polysaccharides (polygalacturonic acids) (Pluske et al., 2001; Navarro et al., 2019).

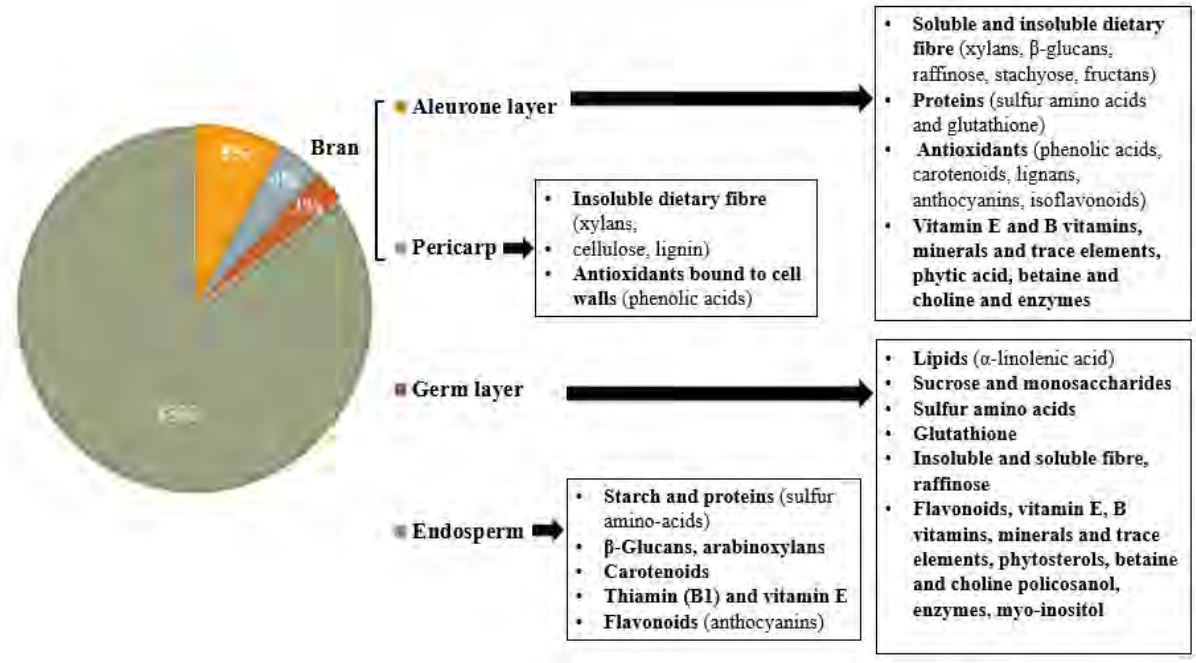

Figure 1.2: A pie chart showing the composition of whole grain major components (bran, germ and endosperm) and their main bioactive compounds (including NSP components). Modified from Fardet (2010).

The NSP content in cereal grains differs between species and the parts of the grain they are located in (Navarro et al., 2019). Wheat, barley, oats, rye and triticale have been reported to have the highest NSP content, while corn (maize), sorghum and rice have the lowest (Izydorczyk., 2021; Navarro et al., 2019). Rye, wheat barley, oats, rice, sorghum, maize and millet are cereals that contain arabinoxylans (AX) in the endosperm and aleurone of their cell walls, their bran tissues and the husk. Glucuronoarabinoxylans (GAXs) are acidic xylans found in the husk and bran of cereal grains (Izydorczyk and Biliaderis, 2007). The ratios of arabinose to xylose in grains differ in the substituents' arrangement within side-chains and linkages in the structure (Izydorczyk and Biliaderis, 1995).

The arabinose substituents contained in the AX found in the aleurone layer are more esterified than those of AX from the endosperm, forming a covalent link with other cell wall polysaccharides (such as ferulic acid dihydrodiferulic acid) (Mathew et al., 2017; Izydorczyk and Dexter 2008). The alkali-labile cross-linkages between arabinoxylan and polysaccharides on the cell wall affect cereal solubility (Sinha et al., 2011). The ability of the AX to bind to other cell wall polysaccharides cause them to absorb water, resulting in increased solubility in water, forming viscous solutions (Mathew et al., 2017; Sinha et al., 2011). The arabinose to xylose ratio influences the solubility of AX in water. The greater the arabinose residue

substitution, the more water soluble the AX. Grains, such as wheat and rye, have a large proportion of arabinose to xylose, which make them more soluble in water than barley and oats with a large proportion of xylose to arabinose (Mathew et al., 2017).

Grain legumes, such as beans, lentils, lupins, and peas have also been included in monogastric animal diets as sources of proteins (Navarro et al., 2019; Waldroup et al., 1992). Soybean meal (SBM), canola meal (CNM), sunflower meal (SFM), rapeseed meal (RSM) and cottonseed meal (CSM) are used as protein sources (El Deek et al., 2020). NSPs from legumes are considered more complex than those from cereal grains because they contain pectic substances (Izydorczyk and Biliaderis, 1995). These pectic substances include colloidal polysaccharides (such as polygalacturonic acid, galactan and arabinans) and neutral polysaccharides (such as xyloglucans and galactomannans) (Kocher et al., 2002). Pectinase has been applied to corn-soy diets (Kocher et al., 2002). This project focuses on cereal NSPs (xylans) that can be hydrolysed by xylanases.

1.1.1. Xylans

Xylans are categorised into four major groups based on their substituents. These groups include homoxylan (containing xylose residues linked by β-1,4-linkages), arabinoxylan (AX) (consisting of a β-1,4-xylan main chain substituted with α-1,6-linked arabinose residues), glucuronoxylan (consisting of β-1-4-linked D-xylopyranosyl residues substituted with α-1,2-linked 4-*O*-methyl-D-glucuronic acid residues) and glucuronoarabinoxylan (backbone is linked to arabinofuranose and uronic acid) (Chakdar et al.,2016). Xylan has been reported to have a degree of polymerisation (DP) ranging from 150 to 200 (Chakdar et al., 2016). Xylans are commonly substituted with L-arabinofuranosyl, D-galactopyranosyl, D-glucuronopyranosyl and/or 4-*O*-methyl-D-glucuronopyranosyl units, and sometimes uronic acids derived from glucose and galactose (Pluske et al., 2001).

Cereal AXs are substituted by 3-linked L-arabinofuranosyl (L-Araf) residues, single chains of 2-*O*-linked-α-D-glucopyranosyl uronic acid units, and its 4-*O*-methyl derivatives (depending on the cereal type) (Izydorczyk, 2021) (Figure 1.3). Sorghum AXs were reported to be substituted by two Araf residues linked at position O-3 to the xylan backbone in addition to the features of a neutral arabinoxylan (Saulnier et al., 1995a). In addition to Araf residues, corn cob AXs contain D-xylopyranose (D-Xylp), D-galactopyranose (D-Galp), and α-D-glucuronic

acid (GlcA) or 4-*O*-methyl-β-D-glucuronic acid (4-*O*-Me-α-D-GlcA*p*) residues (Saulnier et al., 1995a) (Izydorczyk, 2021). More complex cereals such as rice, sorghum, finger millet and maize bran contain higher amounts of glucurono(arabino)xylans (2-*O*-linked glucuronic acid residues) substituted on their arabinoxylans compared to wheat (Saulnier et al., 1995b).

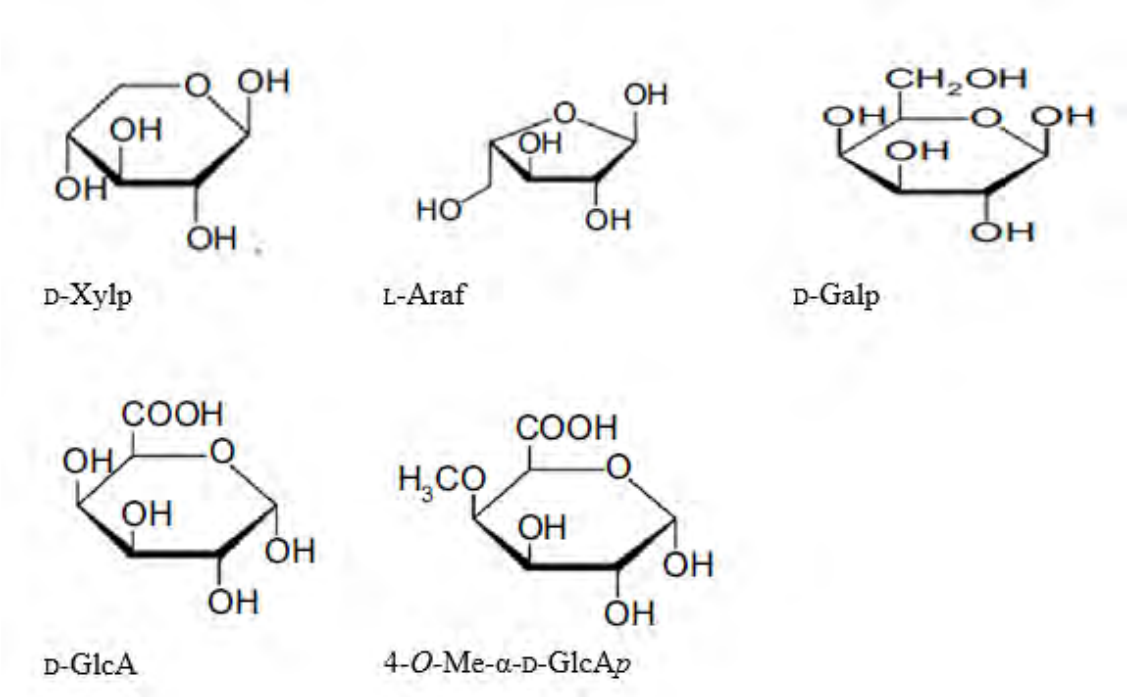

Figure 1.3: Structural features of molecules that can be substituted on cereal arabinoxylan (AX) units. D-Xylp - D-xylopyranose, L-Araf - L-arabinofuranosyl, D-Galp - D-galactopyranose, GlcA - α-D-glucuronic acid, 4-*O*-Me-α-D-GlcA*p* - 4-*O*-methyl-β-D-glucuronic acid.

The amounts of AXs differ depending on the grain cereal type and part (Table 1.1). Rye, wheat barley, oats, rice, sorghum, maize and millet are cereals that contain AXs in the endosperm, aleurone of their cell walls, bran tissues and in the husk, while corn contains AX in their whole-grain, bran and straw fractions as shown in Table 1.1 (Izydorczyk, 2021).

Table 1.1: Average % of arabinoxylan (w/w) (on a dry mass basis) content in different cereal whole grains and tissues *(*Broekaert et al., 2011; Collins et al., 2010; Izydorczyk and Biliaderis, 2007).

Whole grain and tissues	 Wheat	 Barley	 Corn	 Rice	 Oats	 Rye
	Arabinoxylan (%)					
Whole grain	8	5	6	2	2	9
Bran	23	18	4	3	2	15
Flour	2	3			1	3
Straw		12	29			
Husk		20		9	6	

1.1.2. Xylanases, their structure and substrate specificity

Xylanases (β-1,4-D-xylan hydrolases) are hemicellulases that catalyse the hydrolysis of internal β-1,4-glycosidic bonds of xylan (Teplitsky et al., 2004). Xylanases are distributed among bacteria, fungi and actinomycetes (Bagewadi et al., 2016). Xylanases have been classified into glycosyl hydrolase (GH) families based on their amino acid sequence and structure similarities (Paës et al., 2012; Beaugrand et al., 2004). Several families (GH 5, GH 8, GH 10, GH 11, GH 30 and GH 98) have been reported in the literature. However, GH10 and GH11 families have been intensively studied compared to other families (Pollet et al., 2009). GH10 xylanases have been isolated from bacterial species, whereas GH11 family xylanases have been exclusively isolated from fungal species (Chakdar et al., 2016). Three-dimensional structures of xylanases have been reported to vary based on the GH family of the enzyme. GH5 and GH10 family members belong to the same clan (GH-A), which have a three-dimensional structure consisting of an 8-fold alpha/beta (α/β) barrel - also referred to as a TIM-barrel (Figure 1.4 below) (Bhardwaj et al., 2012; Pollet et al., 2009; Subramaniyan and Prema, 2002).

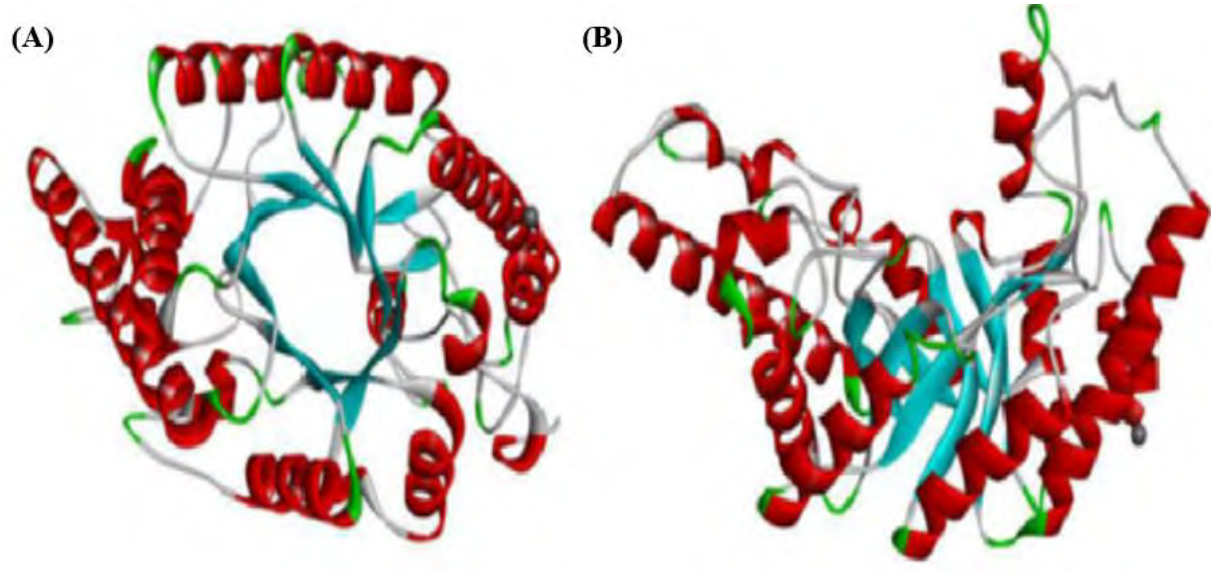

Figure 1.4: The 3D structure of a GH10 xylanase, showing the α/β barrel fold. (A) Top view and (B) side view of the xylanase. Adapted from Bhardwaj et al. (2012).

On the other hand, GH11 family xylanase members consist of a β-jelly roll structure (containing two β-sheets and one α-helix) that resembles a partially closed right hand (Pollet et al., 2009). The three-dimensional structure of GH8 xylanases contains 13 α-helices and 13 β-sheets (Pollet et al., 2009).

During xylan hydrolysis by endo-xylanases, the polymeric backbone is cleaved to produce xylooligosaccharides (XOS) (Collins et al., 2005; Teplitsky et al., 2004). The XOS produced can be substituted or unsubstituted, depending on the xylan and its side chains (Teplitsky et al., 2004). XOS can be broken down further into smaller oligosaccharides (e.g., xylose and xylobiose) by β-D-xylosidase (Biely, 1997). GH11 xylanases are primarily active against insoluble polymeric xylans, while GH10 xylanases are primarily active against soluble substrates (Beaugrand et al., 2004). Various xylanolytic enzymes are required to completely depolymerise the structure of the substrate as demonstrated in Figure 1.5.

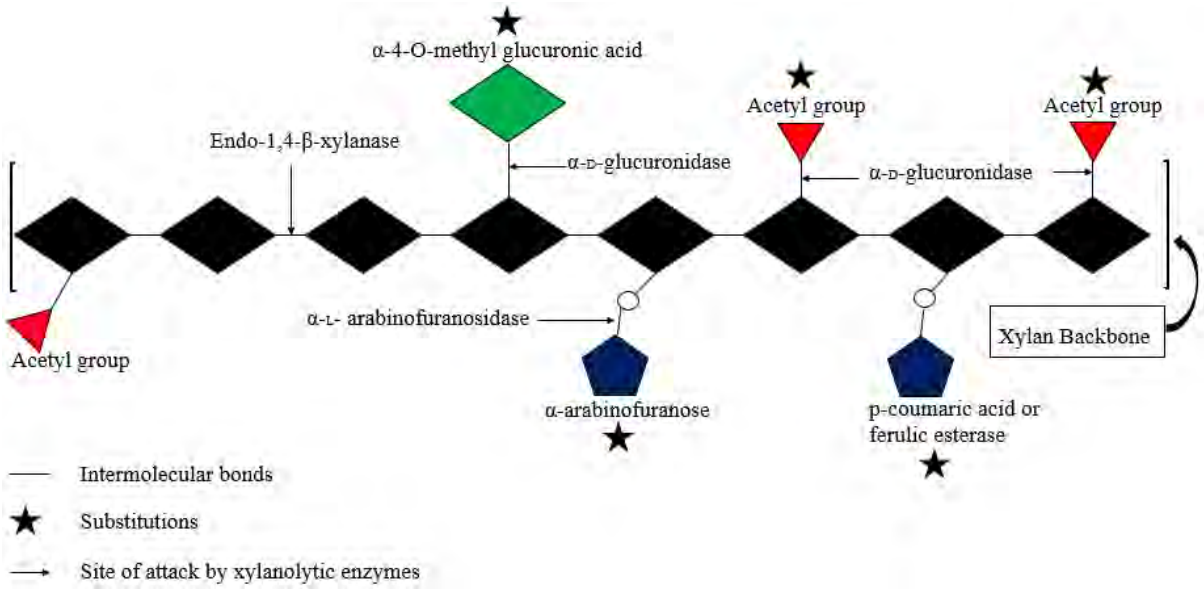

Figure 1.5: Structure of xylan (1,4-β-linked xylose residues) showing different intermolecular bonds, substitutions, and the sites of attack by xylanolytic enzymes. Adapted from Sunna and Antranikian (1997) and Samanta et al. (2015a).

GH10 xylanases attack the glycosidic linkages of xylan on linear (unsubstituted) and substituted xylans and XOS (Malhotra et al., 2018; Paës et al., 2012; Pollet et al., 2009). They prefer two unsubstituted xylose residues between branched residues (Beaugrand et al., 2004). The GH10 xylanases cleave the β-1,4-linkages (Linares-Pastén et al., 2018; Raza et al., 2019). These enzymes bind to arabinose-decorated xylose residues in either the (-3), (-2), or (+1) subsites (Paës et al., 2012).

The GH11 xylanases are considered true endoxylanases; they cleave glycosidic linkages after three consecutive unsubstituted xylose residues (Chakdar et al., 2016; Beaugrand et al., 2004). They cannot attack glycosidic linkages next to a branched xylose and at the non-reducing end (Biely et al.,1997; Pollet et al., 2009). The GH8 xylanases (endo-β-1,4-xylanases and reducing end xylose releasing exo-oligoxylanases) have been reported to have a diverse substrate specificity (Pollet et al., 2009). They cleave glycosidic linkages (β-1,4 linkages) on linear homoxylans and on long-chain XOS (Collins et al., 2005). GH5 xylanases cleaves xylan that is substituted with methyl-D-glucuronic acid (MeGA) on their backbone (Pollet et al., 2009). XOS produced from xylan hydrolysis by GH5 xylanases are always substituted. GH5, GH10 and GH11 xylanases hydrolyse substrates via the double displacement mechanism and are classified as retaining enzymes (Linares-Pastén et al., 2018). Unlike the other families, a GH8 xylanase hydrolyses its substrates via a single displacement mechanism and is classified as an

inverting glycoside hydrolase (Pollet et al., 2009). Xylanases have been applied to several industries, including feed, food processing, plant oils, pharmaceuticals, coffee extraction, chemical production, bread making, brewing, paper and textiles, and bio-bleaching of wood pulps (Beg et al., 2001; Denisenko et al., 2019; Khasin et al., 1993).

1.1.3. GH10 xylanases

Previous studies have reported that GH10 family xylanases have five xylopyranose binding sites with two glutamate moieties that act as the catalytic residues initiating the enzymatic reaction (Bhardwaj et al., 2012). Their active sites consist of glycone subsites (well-conserved), which provide a strong binding affinity to the substrates, and aglycone (less conserved) subsites, with weaker binding affinities (Pollet et al., 2009). These enzymes are highly active on short xylooligosaccharides (Bhardwaj et al., 2012). Several studies on GH10 and GH11 xylanases have indicated that the absence of the N- or C-terminal accessory module can reduce or increase the thermal stability of these enzymes (Chakdar et al., 2016). The stability of the TIM-barrel fold of GH10 enzymes is due to various factors. These include packing of the β-strand residues in the barrel core, folding of TIM barrels by energy minimization, and the amino acid clustering pattern in TIM-barrel proteins (Bhardwaj et al., 2012). These stabilising residues are found in the N- and C-terminal loops. The β-sheets (organized in the cavity of the crescent, forming the interior surface) were stated to be more crucial for the stability of the enzyme's TIM-barrel structure compared to α-helices (arranged in the outer surface of the protein exposed to the solvent) (Bhardwaj et al., 2012; Hegazy et al., 2019). The location of polar uncharged, charged, and non-polar amino acids, which are conserved among members of the GH10 family, affect their biochemical properties (Saksono and Sukmarini, 2010).

The thermostability of GH10 xylanases is due to several distinct features (Leggio et al., 1999). These include six short helices that shorten the connecting loops, introduce an additional hydrogen bond on a proline residue found on α-helix 6 and 7, resulting in efficient packaging of the hydrophobic core and stabilisation of the helices (Natesh et al., 1999; Leggio et al., 1999). The presence of additional Arg and His residues and fewer Asn residues in Xyn10A (from *Thermoascus aurantiacus*) contributes to its tolerance to high pH (Linares-Pasten et al., 2014). The GH 10 xylanase/s have been reported to have higher molecular weights (approximately 40 kDa) and high pI values compared to GH11 xylanases (about 20 kDa) (Subramaniyan and

Prema, 2002). The optimum activity of endo-xylanases occurs at temperatures between 40℃ and 80℃ and a pH range of between 4 and 12 (Liew et al., 2019; Li et al., 2008; Verma and Satyanarayana, 2012). The GH10 enzymes are unaffected by *Triticum aestivum* xylanase inhibitor (TAXI)-like proteinaceous inhibitors found in cereals - unlike GH11 xylanases that are affected (Beaugrand et al., 2004).

1.1.4. Production of XOS from NSPs and their effect on gut microflora

NSPs form part of the group of prebiotics (Ding et al., 2018), which are defined as small molecules that promote the growth of probiotics (beneficial bacteria) upon consumption (Yadav and Jha, 2019). Prebiotics should be able to be fermented by probiotics, stimulate the growth of intestinal bacteria, be stable in an acidic environment (i.e. gastric acidity), should not be absorbed in the upper gastrointerstinal (GI) tract and should not be hydrolysed by GI tract secreted enzymes (Aachary and Prapulla, 2011). Due to their indigestibility in the intestine, NSPs are fermented by the gut microflora and maintain gastrointestinal health by improving the growth of probiotics. NSPs decrease the pH of the gut and increase the availability of substrates for degradation by gut microbiota (Murphy et al., 2009). Prebiotic compounds can be produced via the enzymatic degradation of cereal cell walls (Zhang et al., 2018). Literature has also reported that oligosaccharides (produced from NSP degradation) such as xylooligosaccharides (XOS), mannooligosaccharides (MOS) and fructooligosaccharides (FOS) form part of the prebiotic group of compounds (Ding et al., 2018; Zhang et al., 2018). These oligosaccharides may promote the proliferation of probiotics by influencing calcium and magnesium availability, which reduces intestinal pH and increases mineral absorption (Ding et al., 2018).

The hydrolysis of NSPs by xylanases produces XOS, which are constituted of repeating xylose units linked by β-1,4-glycosidic bonds (Figure 1.6). Xylobiose, xylotriose, xylotetrose, xylopentose and xylohexose are the most commonly produced XOS from agricultural by-products (Carvalho et al., 2017; Chakdar et al., 2016; Samanta et al., 2015a). As in the case of MOS and FOS, XOS with a degree of polymerisation (DP) of 2 to 5 (X2-X5) have been reported to be utilised by probiotic microorganisms in the gastrointestinal tract (Ding et al., 2018).

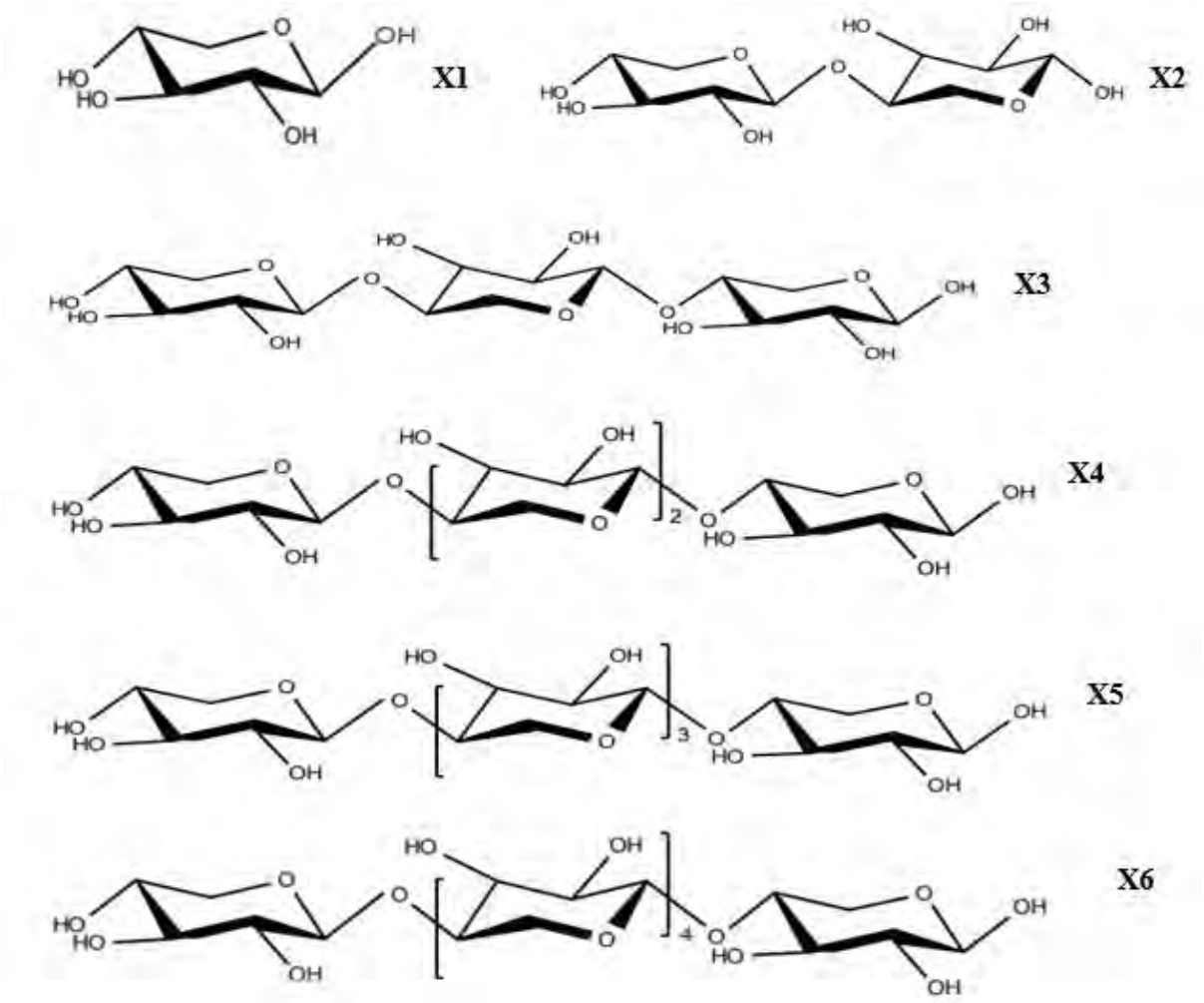

Figure 1.6: Chemical structures of xylooligosaccharides. X1- xylose, X2- xylobiose, X3-xylotriose, X4-xylotetrose, X5-xylopentose and X6-xylohexose. Modified from Carvalho et al. (2013).

Probiotics are live microorganisms that can confer health benefits to a host by balancing microbial distribution in the host intestine (Liao and Nyachoti, 2017). Examples of commercialised probiotics include *Bacillus*, *Bifidobacterium, Streptococcus, Lactobacillus* and *Enterococcus* spp. (Li et al., 2019; Khan et al., 2020). Probiotics that have been applied to animal products include *Bacillus* spp. (such as *Bacillus subtilis*, *B. coagulans*, and *B. licheniformis*) (Abd El-Hack et al., 2020; Kabir, 2009; Khan et al., 2020). Advantages of probiotics include that they are able to produce stable spores at high temperatures and under harsh environments, allowing them to endure gastrointestinal conditions during feed digestion and benefit host health (Abd El-Hack et al., 2020). These probiotic species have been used in pig diets, where they improve the growth performance of pigs (Tsukahara et al., 2013). Therefore, supplementing pig diets with *B. subtilis* advocated that the probiotics affect gut morphology, microbiota compositions and immune function (Lee., et al. 2014). In pigs, a high

percentage of NSPs in cereal feeds were found to impact the gut's microbial composition by increasing the population of *Lactobacillus*, *Ruminnococcus* and *Prevotella* species (Murphy et al., 2009). Furthermore, supplementing broiler diets with *B. coagulans* was hypothesised to improve the feed conversion ratio (FCR) and positively modulate the composition of the microflora in the gastrointestinal tract, which can increase the population of lactobacilli and lower coliform bacteria composition (Hung et al., 2012; Li et al., 2019).

XOS has been reported to stimulate the growth of *Bifidobacterium* spp. and *Lactobacillus* spp which are considered beneficial microflora (Ding et al., 2018; Samanta et al., 2015b). The product from the consumption of XOS by one microorganism may serve as a nutrient for other organisms (Samanta et al., 2015a). Potential pathogenic bacterial species, such as *Escherichia coli*, *Enterococci* spp., *Clostridium difficile,* or *Clostridium perfringens*, cannot utilise XOS (Samanta et al., 2015a). XOS from birchwood xylan have antimicrobial activity against *Bacillus cereus*, *Staphylococcus aureus* and *Helicobacter pylori* (Christakopoulos et al., 2003). Animal intestines are colonized by several diverse microorganisms that have essential effects on gut health. With antibiotic-free diets being prefered in the livestock production industry, prebiotics and probiotics have been suggested as an alternative to antibiotics (Ding et al., 2018; Samanta et al., 2015b).

1.1.5. Chicken gut physiology

The gastrointestinal tract (GIT) is the most important part of a monogastric animal's digestion system (Scanes and Pierzchala-Koziec, 2014). The GIT contains a population of microorganisms, including probiotics (beneficial) and pathogenic bacterial species. The host animal has a symbiotic relationship with the microbiota in the GIT (Aimutis and Polzin, 2011). The microbial species ferment endogeneous mucosal secretions, NSPs and exfoliated epithelial cells as nitrogen and carbon sources (Borda-Molina et al., 2018; Svihus, 2014). At the same time, they release short-chain fatty acids and lactate, which get absorbed by the host animal (Aimutis and Polzin, 2011). The imbalance of microbiota in the GIT leaves the host animal susceptible to diseases; therefore, a balanced GIT system needs to be maintained for the animal to remain healthy (Diaz Carrasco et al., 2019). The digestion of feeds in chickens goes through a number of stages as illustrated in Figure 1.7.

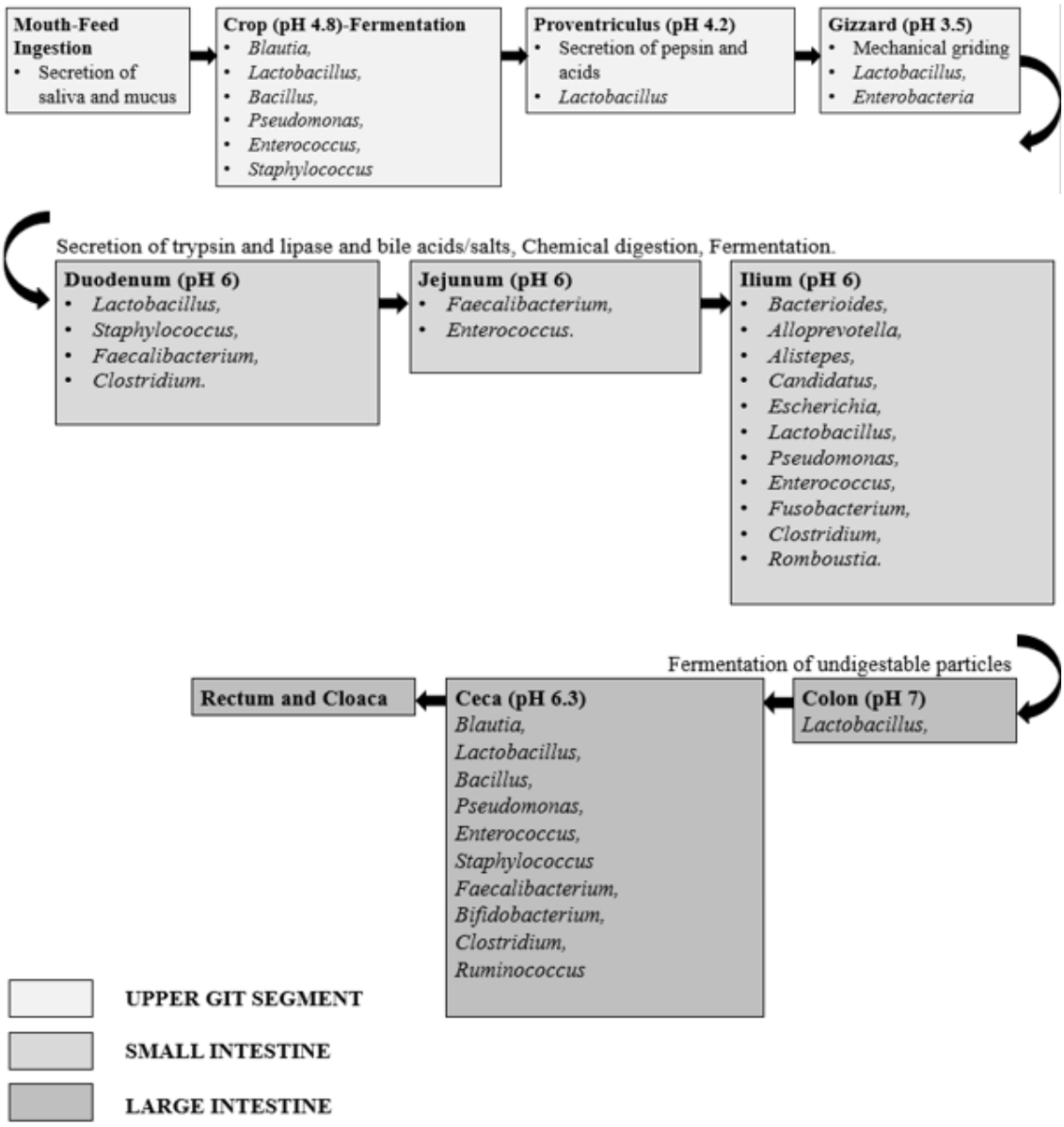

Figure 1.7: The overview of the gastrointestinal tract of poultry showing the flow of feed digestion, significant components of the GIT, and microbiota diversity in different segments.

Microbial populations and profiles differ throughout the GIT from the upper segment (crop, gizzard), small intestine (ileum, jejunum and duodenum) and the large intestine (ceca, colon and rectum) (Diaz Carrasco et al., 2019; Khan et al., 2020). In the upper GIT, upon ingestion, the feed is combined with secretions such as saliva (containing amylases that break down starch) and mucus (a lubricant repels some pathogenic bacterial species) (Scanes and Pierzchala-Koziec, 2014). These secretions help to soften the feed (Nkukwana et al., 2015). The mixture of feed and these secretions is called a bolus. The bolus is transferred from the mouth through the oesophagus to the crop. During transportation, the bolus gets mixed with

more mucus (Svihus, 2014). The crop contains several bacterial species such as *Lactobacillus* sp., *Bifidobacterium* sp. and members of the *Enterobacteriaceae* family (Khan et al., 2020; Singh et al., 2012; Witzig et al., 2015). *Lactobacillus* is the most abundant species found in the crop (Hammons et al., 2010). The crop's bacterial species are responsible for producing lactic acid, acetate, propionate, and butyrate, resulting in an acidic environment (pH 4.8) in the crop (Borda-Molina et al., 2018; Scanes and Pierzchala-Koziec, 2014).

From the crop, the bolus is transported through the proventriculus to the gizzard. Pepsins (which break down proteins into simple peptides aiding with their absorption) and acids are secreted by the proventriculus, which further lower the pH of the bolus (Svihus, 2014). Many *Lactobacillus* species and low numbers of *Clostridium* have been found in the proventriculus and the gizzard (Khan et al., 2020). The feed particles are ground into smaller pieces for easier and more rapid transportation to the small intestine (Svihus, 2014). In the small intestine, the bolus is exposed to other digestive enzymes, including trypsin (breakdown of proteins), lipases (breakdown of lipids) and bile acids/salts (aid in the digestion of lipids and proteins) (Scanes and Pierzchala-Koziec, 2014; Singh et al., 2012). The environmental pH in the small intestine is higher (pH 6) in comparison to that in the gizzard (pH 3.5) (Khan et al., 2020). In addition to the bacterial species found in the upper GIT, more bacterial species such as *Enterococcus*, *E.coli*, *Streptococcus*, *Bacteroides, Alloprevotella, Alistepes, Candidatus, Pseudomonas, Fusobacterium,* and *Romboustia* are in abundance in the small intestine (Craig et al., 2020; Khan et al., 2020; Witzig et al., 2015).

Fermentation of undigestable feed particles in the upper and small intestine occurs in the large intestine (Scanes and Pierzchala-Koziec, 2014). Complex substrates, including NSPs, are fermented by the gut microbiota (Khan et al., 2020). Different sets of species are found in the large intestine (Borda-Molina et al., 2018). These include coliforms, *Bifidobacterium*, *Eubacteria, Bacteroides, Clostridiaceae, Lachnospiraceae*, *Lactobacillaceae, Enterococcus* species and other species that have not yet been identified (Borda-Molina et al., 2018). The large intestine has also been characterised by high levels of SCFAs (Khan et al., 2020). The large intestine has the longest feed retention time of about 12–20 hours compared to the upper GIT and the small intestine (Singh et al., 2012). From the large intestine, the undigestable feed particles are transported to the rectum and cloaca, which then get excreted as droppings.

An unhealthy gut system in poultry is associated with a lot of undigested feeds in droppings (Raza et al., 2019). Inefficient nutrient digestion and absorption is linked with low metabolizing

energy (ME), low growth rate, and increased infections in animals (Aimitis and Polzin, 2010). These effects can be due to an imbalance of gut microbiota caused by a number of factors such as the size of the feed pellet, solubility, and viscosity of the feed particles, which slow down the gut transit time (GTT) (Jozefiak et al., 2007). Several exogenous enzymes such as amylase, phytase, proteases, xylanases and glucanases have been supplied to the monogastric animal industry to improve feed digestion (Scanes and Pierzchala-Koziec, 2014). A suitable enzyme or supplement should function in the gut environment (at the relevant gut pH) and withstand the digestive enzymes and acids secreted by the host animal and the gut microbial population (Raza et al., 2019).

1.1.6. Feeds for monogastric animals

About 60% to 70% of the livestock and poultry rearing expenses derive from feed cost (El-Deek et al., 2020; Imran et al., 2016). The production of eggs and meat from poultry utilises large amounts of energy and protein sources (Khan et al., 2020). Therefore, it is vital to access the nutritive value of the feeds to maintain the profitability of the animal product. When it comes to the rearing of monogastric animals, farmers focus on four factors: organic mineral nutrition, efficiency in digestion, absorption of nutrients, and maintaining the nutrition value of feeds (Khan et al., 2020). These factors have been addressed by formulating diets containing different components such as specific amino acids, mineral supplements, energy, protein sources, and vitamins, improving nutrient digestion and absorption (Khan et al., 2020). However, due to the cost of these supplements, the production costs outweigh the value of animal products. Hence the industry has opted to use agricultural wastes as feeds. These contain large amounts of NSPs and ANFs that cannot be broken down by monogastric animals' endogenous enzymes (Baker et al., 2021; Bedford, 2018). An unbalanced feed supplement ratio has been associated with the inefficient digestion of feeds, resulting in high amounts of valuable nutrients (such as nitrogen, phosphorus (P), calcium (Ca) and zinc) being excreted during defecation and urination (Khan et al., 2020). About 15-25% of the feed is excreted as waste due to the indigestibility of NSPs and ANFs (Imran et al., 2016). Since the monogastric animals do not have enzymes that can digest the NSPs and ANFs, the feed efficiency is decreased, and the digestive system becomes distressed, resulting in low productivity (Pluske et al., 2001). Therefore, to minimise these costs, enzyme additives have been utilised as supplements in feeds

to enhance the production of meat per animal or to maintain productivity in a cheaper and faster way.

In the animal production industry, animals are usually fed a mixture of starch and proteins (Navarro et al., 2019). The most commonly used grains and legumes are corn and soybeans. The type of grain used differs in different regions, mainly due to their level of production in that specific region and the costs involved (El-Deek et al., 2020). The concentration and structure of non-digestible carbohydrates in diets fed to pigs and poultry depend on the age of the animal and the type of feed ingredients included in the mixed diet to feed the animal (and the type of production that is required from that animal - for example, egg laying or meat production). Therefore, the relative percentage of ingredients required for young chicks, adult egg layers and meat production in poultry differ (Abdelaziz, 2021; Kassim and Suwanpradit., 1996; Waldroup et al., 1992).

A low-cost, effective way to reduce production costs and maximize profit is practised by interchanging the protein and starch content in a feed mixture in the poultry industry (Kassim and Suwanpradit., 1996; Waldroup et al., 1992). The feed's protein content is reduced as the chicken grows (Kassim and Suwanpradit, 1996). Reduced protein content is an advantage because, as the protein content in feed decreases, the cost of the feed decreases (El-Deek et al., 2020; Saleh and Watkins, 1997). Several diets that differ in protein content are used at different stages, including starter feed, grower feed, and finisher feed. Initially, the broiler chickens are fed with a starter diet. A starter diet contains high protein content and low energy content required by the chicks to reach optimum growth (Waldroup et al., 1992). Starter diet particles are smaller in size; therefore, it is easier for the chicken to swallow and has a large surface area, thus maximizing nutrient absorption. After 6 to 8 weeks, the chicken diets are shifted to grower feeds (Waldroup et al., 1992). Grower diets have reduced protein content and high energy contents. The grower diet is more pelleted compared to the starter diet, this increases feed consumption, body weight gain, and feed conversion ratio in poultry (Abdelaziz, 2021). The finisher diets have an even lower protein content than starter and grower feeds (El-Deek et al., 2020). For this project, starter and grower feeds from a local poultry farm were used as substrates.

1.1.7. Enzymes supplemented to chicken feeds and their effects

Due to the negative impact of NSPs from cereals, different types of enzymes (which target specific substrates from the NSPs) have been applied to break down the NSPs. These enzymes include amylases, β-glucanases, phytase, pectinase and pentosanases (xylanases) and are applied in the feed industry for different reasons (Raza et al., 2019). The addition of xylanases to wheat-based animal feeds decreases intestinal viscosity, and increases nutrient absorption and digestion (Nian et al., 2011). Enhanced nutrient absorption leads to an improved gastrointestinal tract micro-ecosystem, which increases broilers' metabolizing energy, and growth, leading to high productivity in the meat industry (Nian et al., 2011). Other studies have predicted that the hydrolysis of NSPs by xylanases can indirectly modify the microbial populations in the gut of monogastric animals (Choct et al., 1999). However, literature also indicates that the addition of endo-1,4-β-xylanase to animal feed increases animal growth rates via an improvement in nutrient digestion and improves the quality of animal litter (no undigested nutrients and low phosphate levels) (Zhang et al., 2018).

The addition of amylase to chicken feeds improves the degradation of excessive starch (Choct, 2006; Khattak et al., 2006). The addition of glucanases to barley-based feeds improved the degradation of glucan, improving barley market nutritive value (Torok et al., 2008). This has been linked to the reduction of excretion of undigested nutrients and ammonia production in chicken manure (Choct, 2006). Supplementation of phytase in monogastric animals has been associated with improved phytate (organic phosphorus) utilisation, enhanced growth performance, feed conversion, and decreased phosphate levels excreted in broilers excreta. The introduction of xylanases in the feed industry has been hypothesized to improve digestion of nutrients and improve rye and wheat values (Nian et al., 2011). The addition of an endo-1,4-β-xylanase to broiler wheat-based diets was reported to decrease ileal digesta viscosity, and improve growth performance and apparent ileal digestibility of nutrients (Liu and Kim, 2017). Supplementation of xylanase has also been reported to have beneficial effects on intestinal morphology and microfloral balance, leading to reduced NH_3 levels in the excreta, resulting in reduced odour emissions from broiler faeces (Liu and Kim, 2017).

These enzymes have also been used in combinations to enhance the breakdown of excess NSPs. For example, supplementation of xylanases in addition to β-glucanases removes the encapsulation of nutrients, improving nutrient digestion (Bedford et al., 1991). This effect contradicts the finding of Cowieson and colleagues, who reported that xylanase and glucanase

may compete for similar substrates, leading to no beneficial effect on the chickens (Cowieson et al., 2006). Therefore, a different combination of enzymes has been studied in an attempt to address most of the problems in animal feeds. Enhanced nutritive values of grains (rye, corn, wheat, barley, and oats) in broiler chickens were reported after the addition of xylanases, amylase, proteases and β-glucanases (Dalfonso, 2003). In addition to the digestion of cereal NSPs in animal feeds by different enzymes, pectinases have been reported to increase the ME value of monogastric animal feeds (Kocher et al., 2002).

Several authors recently reported other examples of enzyme combinations. Supplementation of phytase, xylanase or phytase, xylanase and protease can improve body weight gain and food conversion ratio in broilers (Walk and Poernama, 2019). This was comparable to a combination reported by Munyaka and colleagues who reported an improved body weight gain, food conversion ratio, starch digestibility and jejunal digesta viscosity of broiler chickens fed a corn and a wheat-based diet supplemented with xylanase and β-glucanase (Munyaka et al., 2016). The combination of xylanase, amylase and phytase improved the nutrient digestibility in broilers compared to the individual use of each of the enzyme supplements (Singh et al., 2019). In pigs, xylanase alone could improve the digestibility of nutrients, while the addition of phytase showed improvement in phosphorus digestibility (Olukosi et al., 2007). A contradictory observation was also reported in pigs where xylanase supplementation in pigs reduced feed intake (l'Anson et al., 2013). However, a combination of xylanase, amylase, protease and phytase was shown to improve piglets' performance (Olukosi et al., 2007).

1.2. PROBLEM STATEMENT AND JUSTIFICATION FOR THE STUDY

Monogastric animals do not produce most of the enzymes required to hydrolyse NSPs (Baker et al., 2021; Bedford, 2018). Therefore, the presence of soluble NSPs in the animal diet has an adverse impact on nutrition, such as lowering energy availability and increasing digesta viscosity (Raza et al., 2019). On the other hand, insoluble NSPs produce a "cage effect" (entrapment of nutrients in the insoluble parts), resulting in the inaccessibility of nutrients by digestive enzymes, leading to a reduction in nutrient digestion and absorption by the animal (Cowan, 1995). Indigestibility of nutrients is associated with interference with the gut microflora, leading to an imbalance of beneficial and pathogenic bacterial species, leaving the animal susceptible to diseases. Indigestibility of nutrients is also associated with undigested feed being observed in poultry droppings (Liu and Kim, 2017). This has been linked to an increase in the excretion of undigested nutrients and ammonium production in chicken manure, which causes unpleasant odour emissions (Liu and Kim, 2017).

To combat this, xylanase addition to cereal animal feed diets can enhance NSP hydrolysis in the intestinal tract. In addition, commercial xylanases from *Trichoderma viride* and *Geobacillus stearothermophilus* have been deemed effective ingredients in enzyme cocktail formulations for degrading lignocellulosic biomass (Malgas and Pletschke, 2019). The production of digestible oligomers through NSP hydrolysis by xylanases may increase the population of beneficial bacteria in the digestive system and reduce the risk of diseases (Abd El-Hack et al., 2020; Kabir, 2009). The addition of xylanases to animal feeds will likely bring cost-effective ways to increase feed digestibility, leading to sustainable feed sources. It is known that digestible oligomers improve the normal gut microbiota in monogastric animals, enhancing their resistance to infections. This attribute decreases the desperate use of antibiotics in animal breeding and subsequently reduces antibiotic-resistance in bacteria globally.

1.3. RESEARCH HYPOTHESIS, AIM, AND OBJECTIVES

1.3.1. Hypothesis

The addition of a GH10 xylanase, XT6, from *Geobacillus stearothermophilus* T6 to chicken feeds is effective in NSP hydrolysis, leading to the generation of XOS.

1.3.2. Aim

The main aim of this study was to clone, express, biochemically characterise and apply a GH10 xylanase, XT6, from *Geobacillus stearothermophilus* T6 as an chicken feed additive. The following objectives were addressed to achieve the aim of the study:

1.3.3. Objectives

- To clone a gene sequence encoding *xt6* xylanase from *G. stearothermophilus* T6 into *E. coli* DH5α cells using a pET28a(+) vector;
- To express the xylanase, XT6, in *E. coli* BL21 (DE3) cells using pET28a(+);
- To purify XT6 by nickel-affinity chromatography and ultrafiltration;
- To biochemically characterise the purified XT6 enzyme;
- To evaluate the effect of XT6 on chicken feed digestibility related properties;
- To evaluate the prebiotic effects of xylo-oligosaccharides produced during the degradation of arabinoxylan in chicken feed.

Chapter 2: Cloning, expression and purification of a GH10 xylanase (XT6) from *Geobacillus stearothermophilus* T6

2.1. INTRODUCTION

Recombinant protein expression is a standard method for producing proteins that are used for research in the biological sciences. Several organisms are used to express recombinant proteins; these include bacteria, yeast, insect cells and human cells (Lanza et al., 2014). Heterologous gene expression enables non-native biological molecules from other hosts to be produced in different organisms (Lanza et al., 2014). The *E. coli* expression system is commonly used to produce stable globular proteins in large quantities (Chen, 2012).

However, the recombinant protein expression in *E. coli* may be hindered by non-frequent codon usage in a specific genome. Due to different host physiologies, codon optimization is necessary to improve heterologous gene expression by replacing rare codons with more frequently occurring codons to match the host organism's codon usage (Lanza et al., 2014). The principle behind codon usage is linked to the distribution of the non-random 64 unique DNA codons. Different codon sets may encode for the same amino acid (Gustafsson et al., 2004). The arrangements of these codons differ within a genome and among organisms. These occurrences have resulted in both rare and abundant codon distribution (Gustafsson et al., 2004). Different organisms have different codon distributions, which leads to hosts having specific codon usage bias (Plotkin and Kudla, 2011). Therefore, a particular gene's codons need to be optimized before cloning to produce the protein of interest.

Xylanases have been successfully cloned in *E. coli* DH5-α and expressed using various vectors (Beg et al., 2001; Gat et al., 1994; Subramaniyan and Prema, 2002). Recently, xylanases have been purified using ion-exchange, gel and affinity chromatography, ammonium sulfate precipitation and ultrafiltration (Bagewadi et al., 2016; Liew et al., 2019; Xiao et al., 2014). The most commonly used mode of affinity chromatography is immobilised metal affinity chromatography (IMAC) (Mongkorntanyatip et al., 2017). The IMAC method yields over 95% recombinant purity in a single purification step (Bornhorst and Falke, 2000). The principle

behind IMAC is based on the interaction between the metal ions immobilized on the resin/beads and the specific tag on the protein to be purified (Porath, 1992). This tag can be fused to the N or C terminus of the protein of interest (Block et al., 2009). Histidine (His) is commonly exploited because it has the most robust interaction with the immobilized metal ion matrices, forming strong bonds with the beads (Block et al., 2009). This interaction does not affect the protein function or its biological activity (Bornhorst and Falke, 2000), which allows the protein of interest (containing the His-tag) to bind to the IMAC column beads. The unbound proteins/contaminants are easily washed off, leaving the protein containing the His-tag to be eluted by the addition of free imidazole in the elution buffer or adjusting the buffer's pH (Bornhorst and Falke, 2000).

Following purification, protein can be quantified via colorimetric assays, which include Coomassie Blue G-250 dye-binding (Bradford assay), the bicinchoninic acid assay (BCA), ninhydrin assays, and ultraviolet (UV) absorbance. UV absorbance measures the number of tyrosine and tryptophan residues in proteins (Gill and Hippel, 1989). This is achieved by measuring absorbance at 280 nm (A_{280nm}) and using the molar extinction coefficient at 280 nm ($\varepsilon 280$) of a specific protein to calculate its concentration (Gill and Hippel, 1989).

The Somogyi-Nelson method and 3,5-dinitrosalicylic acid (DNS) assays are commonly used to determine the amount of reducing sugars (RS) produced by carbohydrase activities against different polysaccharides in a reaction (Miller, 1959; Quero-Jimenez et al., 2019). The DNS assay has been used to analyze reducing sugars (RS) formed during enzyme hydrolysis (Deshavath et al., 2020; Miller, 1959; Wood et al., 2012). RS are produced when an enzyme cleaves the glycosidic bond between two carbohydrates or a carbohydrate and a noncarbohydrate moiety (Bailey et al., 1992). With the DNS assay, 3,5-dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicylic acid (Deshavath et al., 2020). This oxidation process produces a shift in the solution's color from a yellow to dark brown, which can absorb light at 540 nm (Miller, 1959). It is assumed that the more reducing sugars there are in a mixture, the higher the color intensity that is produced (Miller, 1959).

2.2.AIM AND OBJECTIVES

2.2.1. Aim

To clone, express and purify GH10 xylanase (XT6) from *G. stearothermophilus* T6 in sufficient quantities and with a sufficiently high purity.

2.2.2. Objectives

- To obtain the gene sequence encoding for the *xt6* xylanase;
- To clone XT6 into *E. coli* DH5α cells using a pET28a(+) vector;
- To express XT6 into *E. coli* BL21 (DE3) cells using a pET28a(+) vector;
- To purify XT6 using metal nickel affinity column chromatography; and
- To construct a protein purification table for the purification of XT6.

2.3. METHODOLOGY

2.3.1. Bioinformatic analysis

The protein sequence of the GH10 xylanase, XT6, from *Geobacillus stearothermophilus* was retrieved from the NCBI website with the accession number [AB149951.1] (Appendix Figure A). The amino acid sequence was reverse-translated to a nucleotide sequence using Sequence Manipulation Suite (SMS) (Stothard, 2000) (Appendix Figure B). The plasmid DNA sequences were obtained from the Addgene website (Anon., 2021a). The cloning and expression vectors contained selection markers used to confirm the insert (Appendix Figure C).

2.3.2. Competent cells preparation

A protocol described by Mandel and Higa (1970) was used, where *E. coli* BL21(DE3) and *E. coli* DH5α competent cells were grown overnight at 37℃ on 2× yeast extract tryptone (YT) agar plates (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5% (w/v) agar (w/v)). The competent cell stocks were prepared by inoculating 5 ml of 2× YT broth with a single colony and grown overnight at 37℃. The overnight culture was transferred into fresh 2× YT broth and grown to OD_{600nm} readings of between 0.4 and 0.6. The cultures were then centrifuged at 5000 ×*g* for 10 minutes using an Avanti J-E Centrifuge (Life Sciences division, Indianapolis, United States) at 4℃. The supernatant was discarded, while the pellet was resuspended in 5 ml of $MgCl_2$ (0.1 M) and incubated on ice for 30 minutes. Centrifugation was repeated at 5000 ×*g* for 10 minutes at 4℃, and the supernatant discarded. The pellet was resuspended in 5 ml of $CaCl_2$ (0.1 M) and incubated on ice for 4 hours. The suspension was centrifuged as described previously. The pellet was resuspended in a 3 ml solution comprising 0.07 M $CaCl_2$ and 30% (v/v) glycerol. While working on ice, 200 µl aliquots of cell suspension were aliquoted into pre-chilled Eppendorf tubes and stored at -80℃ (Mandel and Higa, 1970).

2.3.3. Cloning of XT6

The codon-optimized *xt6* gene sequence was directly cloned into a pET28a(+) expression vector using *BamHI* (restriction site GGATCC) and *SalI* (restriction site GTCGAC) restriction enzymes (Promega, Wisconsin, United States). The vector containing the insert (pET28a(+)/*xt6*) was transformed into *E. coli* DH5α cells (Thermo Fischer Scientific, Massachusetts, United States). The pET28a(+)/*xt6* plasmid was extracted using the Gene JET Plasmid Miniprep Kit (Thermo Fischer Scientific) according to the manufacturer's instructions.

2.3.4. Transformation of pET28(+)/*xt6* vector into *E. coli* BL21(DE3) cells

The pET28a(+)/*xt6* plasmid DNA (2 µl) was added to an aliquot of 100 µl of *E. coli* BL21(DE3) competent cells. The cells were incubated on ice for 30 minutes, heat-shocked at 42℃ for 45 seconds, and immediately placed on ice for 10 minutes. About 900 µl of super optimum broth with catabolite repression (SOC) media was added and incubated at 37℃ for 1 hour. The cells were transferred to 2× YT plates containing 50 µg/ml kanamycin and were incubated overnight at 37℃.

2.3.5. Restriction enzyme digestion

Restriction endonuclease digestion of the pET28a(+)/*xt6* plasmid DNA (Table 2.1) was conducted using *BamHI* and *SalI* (Promega Corporation, Madison, USA). Briefly, the samples were incubated at 37℃ for 1 hour. The reaction was terminated by incubating the samples at 65 ℃ for 15 minutes. The samples were electrophoresed on a 1% (w/v) agarose gel prepared in 1 M Tris-acetate-EDTA (TAE) buffer (400 mM Tris, 0.1% (v/v) glacial acetic acid, 10 mM EDTA) at 120 V, and the DNA was visualized under ultraviolet light using a Bio-Rad Chemidoc XRS (Bio-Rad Laboratories, South Africa).

Table 2.1: Restriction enzyme digestion to confirm the insertion of XT6 into pET28a(+) vector

Reagents	Tube 1 (control)	Tube 2 (*Bam HI*)	Tube 3 (*SalI*)	Tube 4 (*SalI* and *Bam HI*)
H_2O	16 µl	15 µl	14 µl	13 µl
Buffer	2 µl	2 µl	2 µl	2 µl
DNA	2 ng	2 ng	2 ng	2 ng
Enzyme	-	1 µl	2 µl	3 µl
Total	20 µl	20 µl	20 µl	20 µl

2.3.6. pET28a(+)/*xt6* plasmid DNA sequencing

After the plasmid extraction using the Gene JET Plasmid Miniprep Kit (Thermo Fischer Scientific, Massachusetts, United States), plasmid samples were sequenced at Inqaba Biotechnologies (Johannesburg, South Africa) using universal T7 primers (promoter- 5'-TAATACGACTCACTATAGGG-3' and terminator 5'-GCTAGTTATTGCTCAGCGG-3'). Plasmid sequences were analyzed using Snapgene (4.3.7_win.exe) viewer software. Sequence alignments were performed using the NCBI ClustalW website (Anon., 2021b).

2.3.7. Induction and expression of XT6

E. coli cell colonies containing the pET28a(+)/*xt6* vector were cultured in 5 ml of 2× YT broth and incubated 37℃ overnight. The overnight culture was transferred into fresh 2× YT broth and grown at 37℃ until the mid-log phase reached a $OD_{600\ nm}$ reading of between 0.4 and 0.6. Protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Induction studies were performed by collecting bacterial cell culture samples every hour for up to 5 hours and then again 24 hours post-induction. The OD_{600nm} readings of the bacterial cells were recorded each hour. Collected samples were centrifuged at 16 060 × *g* using a desktop centrifuge-biofuge pico Heraeus (San Diego, California, United States) for a minute. The supernatant was discarded, and the pellet resuspended in 2× SDS sample buffer (0.004% (v/v) bromophenol blue, 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 4% (v/v) SDS and 0.125 mM Tris-HCl). The amount of sample buffer used to resuspend the pellet was

obtained using the formula: [resuspension volume (ml) = (OD_{600nm})/6]. Samples were boiled for 5 minutes and then incubated on ice before SDS-PAGE analysis.

2.3.8. Purification of the recombinant XT6 enzyme

Two-column volumes of the supernatant (2 ml on a Ni^{2+} charged resin) were directly loaded onto the column containing the IMAC resin. The mixture was incubated at 4℃ for 2 hours with shaking to allow XT6 to bind to the resin. After incubation, the supernatant was decanted and kept as the flow-through fraction (unbound protein sample). To remove all non-specifically bound protein on the resin, two-column volumes of wash phosphate-buffered saline (PBS) (50 mM NaH_2PO_4, 50 mM Na_2HPO_4, 300 mM NaCl, 0.03% (w/v) sodium azide, and 10 mM imidazole, pH 8.0) were added to the resin, carefully decanted and retained as the wash (W) solution. This step was repeated three times (W1-W3). Two-bed volumes of elution buffer (50 mM NaH_2PO_4, 50 mM Na_2HPO4, 300 mM NaCl, 0.03% (w/v) sodium azide, and 250 mM imidazole, pH 8.0) were added to the resin, decanted, and kept as the eluate (E). This elution step was repeated three times to elute any remaining protein (E1-E3). A final wash step (W4) was conducted to remove residual imidazole from the column using the wash buffer. The elution fractions (E1 to E3) were then pooled and concentrated by centrifugation (4000 × *g* at 4℃ for 20 minutes) (Megafuge 1.OR Heraeus) using 30 kDa Amicon centrifugal filter units. The concentrate was then re-constituted in a final glycerol concentration of 20% (v/v) for XT6 stabilization during storage at -20℃.

2.3.9. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis was conducted to determine which fraction contained the majority of the polyhistidine-tagged XT6 protein. Firstly, a 12% resolving gel (distilled water, acrylamide (30% acrylamide/bisacrylamide [37.5:1] stock), 1 M Tris-HCl buffer (pH 8.8), 10% (v/v) SDS, 10% (v/v) ammonium persulphate (APS)and N,N,N',N'- tetramethylethylenediamine (TEMED)) was prepared. The stacking gel (4%) was then prepared by mixing distilled water and acrylamide (30% acrylamide/bisacrylamide [37.5:1] stock), 0.5 M Tris-HCl buffer (pH 6.8), 10% (v/v) SDS, 10% APS (v/v) and TEMED). The polymerizing gel solutions were

immediately poured to set between gel casting plates. The gels were then placed in a Mini-Protein® 3 cell tank with 1× SDS running buffer (25 mM Tris base; 192 mM glycine; 1% (w/v) SDS). All the fractions were mixed in a 1: 5 ratio with 5× sample buffer [50 mM Tris HCl (pH 6.8); 40% (w/v) glycerol; 3% (w/v) SDS; 0.14% (w/v) bromophenol blue; 5% (w/v) β-mercaptoethanol)]. The samples were boiled at 100℃ for 5 minutes using a Labnet AccuBlock™ digital heating block and centrifuged at 16 060 × *g* for 5 minutes. Approximately 16 µl of protein sample was added to each gel well. Gel electrophoresis was conducted at a constant voltage (120 V) and 240 amps for 90 minutes with a Bio-Rad Power Pac™ Basic. The gels were stained with Coomassie staining solution [(0.1% (w/v) Coomassie Brilliant Blue G250; 20% (w/v) methanol and 15% (w/v) glacial acetic acid)] for 30 minutes. The gels were then destained sequentially with destain solution 1 (45% (v/v) methanol and 10% (v/v) glacial acetic acid) and destain solution 2 (5% (v/v) methanol and 7% (v/v) glacial acetic acid). Protein bands on the gels were visualized using a Bio-Rad Chemidoc XRS.

2.3.10. Protein quantification using absorbance at 280 nm

About 250 µl of the protein samples were aliquoted into a 96-well plate. Absorbance readings were measured at 280 nm using a plate reader (BioTeK Epoch 2, Winooski, VT, USA). XT6 protein concentration was determined using a molar extinction coefficient (ε280) of 80 790 $M^{-1}cm^{-1}$ at 280 nm and a theoretical molecular weight of 46 763.45 Da.

2.3.11. Enzyme activity assay

Xylanase activity assay was performed using a total reaction volume of 400 µl (containing 100 µl of the enzyme (10 µg/ml) and 300 µl of 1.33% (w/v) wheat-flour arabinoxylan in a citrate buffer at pH 5. All reaction mixtures were performed in triplicate and incubated at 40℃ (using the Labnet AccuBlock™ digital dry bath) for 30 minutes. Xylanase activity was estimated by measuring the amount of reducing sugars produced in a reaction (estimated using DNS standard curve). A reducing sugar standard curve was prepared using 0.1-1 mg/ml xylose prepared in 50 mM citrate buffer (pH 5). A volume of 150 µl of each standard was mixed with 300 µl DNS reagent [1% (w/v) NaOH; 1% (w/v) dinitrosalicylic acid; 20% (w/v) sodium potassium tartrate;

0.2% (w/v) phenol and 0.05% (w/v) sodium metabisulphite]. The samples were boiled at 100℃ for 8 minutes and allowed to cool down. The samples (250 µl) were added to a 96 well microtiter plate and the absorbance was measured at 540 nm (BioTek Epoch 2). A standard curve was constructed (see Appendix Figure D).

2.4. RESULTS

2.4.1. Bioinformatics analysis

The amino acid sequence of XT6 was translated into a nucleotide sequence (Appendix Figure B). The nucleotide sequence was codon-optimised (Figure 2.1) and the resulting sequence was analysed using the Protein Basic Local Alignment Search Tool (BLAST) (Anon., 2021c). The sequence analysis confirmed that the xylanase was from *G. stearothermophilus* T-6 (Appendix Figure E). A pair-wise alignment using ClustalW2 showed that the optimised *xt6* was complementary to the *xt6* sequence extracted from NCBI (Appendix Figure F).

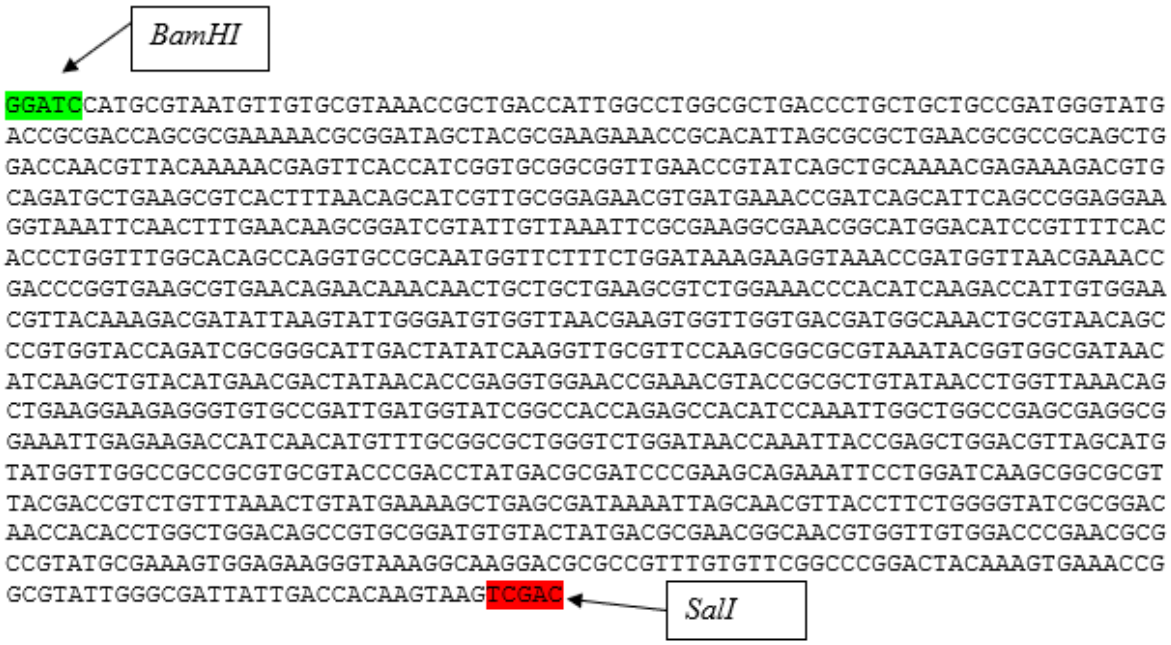

Figure 2.1: The codon-optimized XT6 gene obtained from GenScript

The *xt6* gene was then inserted into a pET28a(+) vector between the *SalI* and *BamHI* restriction sites (Appendix Figure G). The vector also contained a 6× His tag region that allowed for IMAC purification of the expressed recombinant protein (see Appendix Figure C).

2.4.2. Transformation efficiency and insert integrity analysis

The *E. coli* DH5α competent cells transformed with the pET28a(+)/*xt6* plasmid selectively grew on 2× YT agar plates containing 50 µg/ml kanamycin (Figure 2.2).

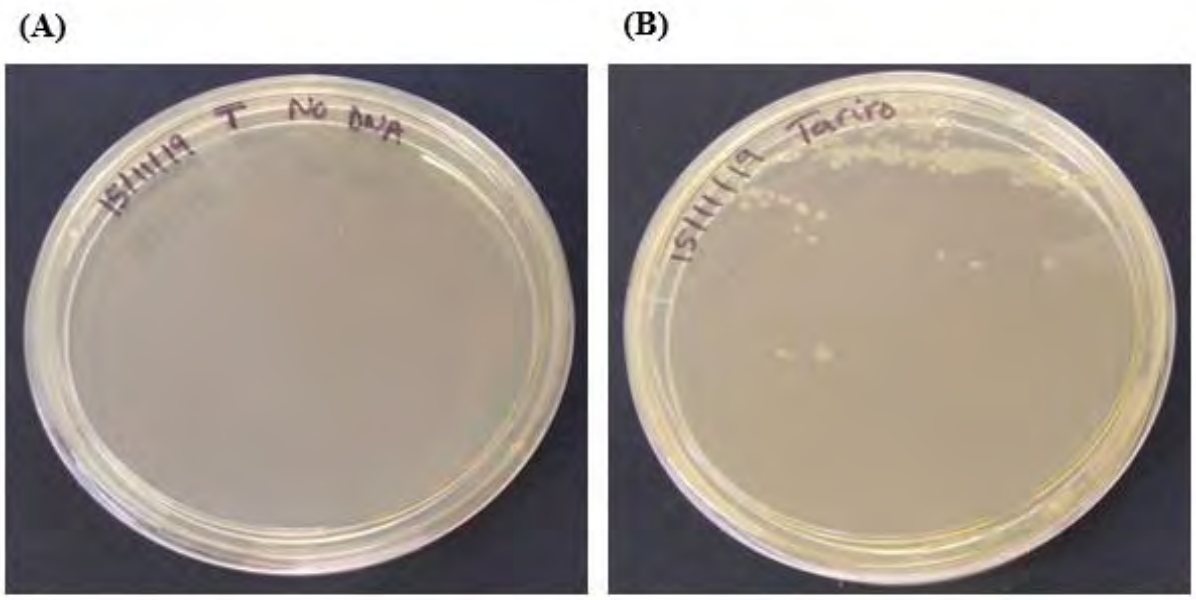

Figure 2.2: The 2× YT agar plates showing the *E. coli* DH5α cells containing the pET28a(+)/*xt6* after transformation and overnight incubation. (A) Negative control (untransformed *E. coli* cells), (B) *E. coli* containing pET28a(+)/*xt6*.

No colonies were observed on the control plate with the untransformed *E. coli* cells (Figure 2.2 A). However, several isolated colonies were observed on the plate with transformed *E. coli* DH5α cells (Figure 2.2 B). These results suggested that the transformation was successful. The presence of *xt6* was further confirmed by sequence alignment between the optimised *xt6* from GenScript and pET28a(+)/*xt6* extracted from *E. coli* DH5α cells (see Appendix Figure H).

Restriction enzyme digestion was performed using *SalI* and *BamHI* restriction enzymes to confirm plasmid integrity (section 2.3.6 and Table 2.1). The restriction digests were analysed by agarose gel electrophoresis (AGE) (Figure 2.3)

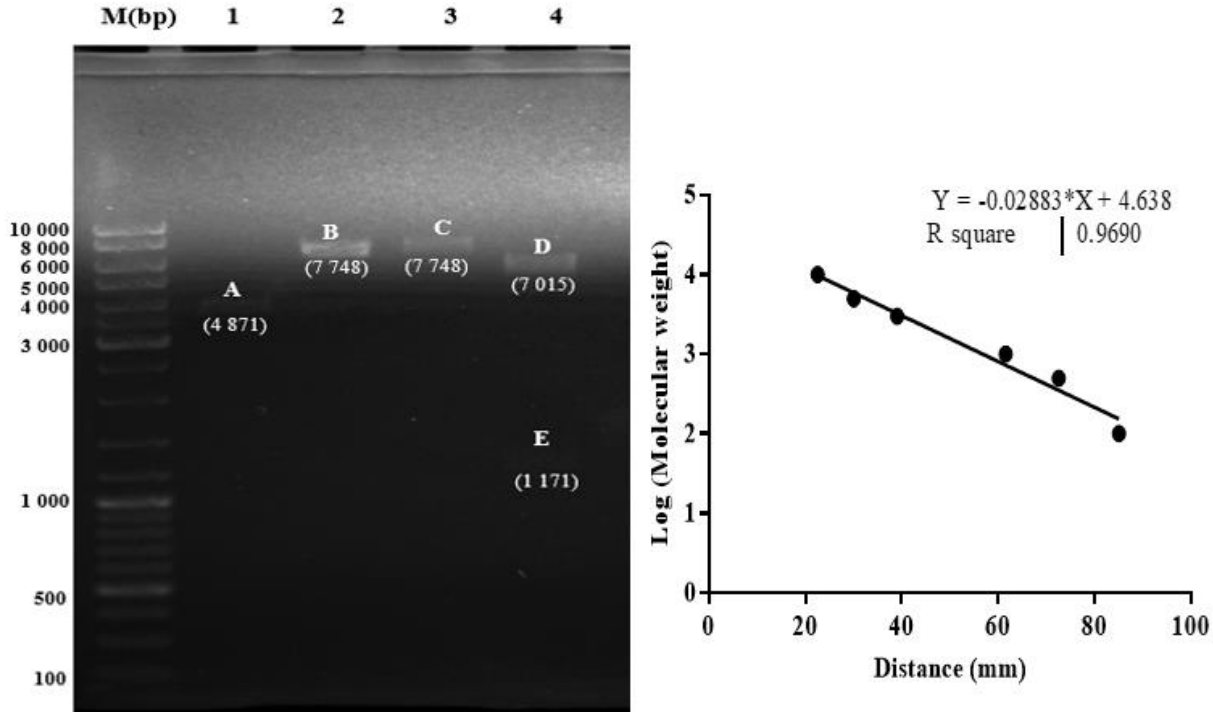

Figure 2.3: Restriction enzyme digestion of pET28a(+)/*xt6* by *BamHI* and *SalI*. M(bp) - Molecular weight marker (Base pairs), Lane 1 - uncut pET28a(+)/*xt6* plasmid, Lane 2 - Single digestion by *BamH1*, Lane 3 - Single digestion by *Sal1* and Lane 4 - double digestion by *BamH1* and *Sal1*.

The pET28a(+)/*xt6* was observed in a supercoiled DNA form (Figure 2.3, lane 1, band A). Single digestion by either *BamHI* or *SalI* restriction enzymes produced single linear conformation DNA (Figure 2.3, lanes 2 and 3, bands B and C respectively). Double digestion by *BamHI* and *SalI* produced two linear conformations (Figure 2.3, lane 4). This was expected because band D is predicted to be the linearised vector and E is the linearised insert (*xt6*) of 1171 base pairs.

2.4.3. Heterologous expression and XT6

The extracted pET28(+)/xt6 plasmid DNA was transformed into the T7 expression cells, *E. coli* BL21(DE3) for expression studies. The induction and expression studies were conducted at 25℃, and the protein expression profile is shown in the SDS-PAGE results in Figure 2.4.

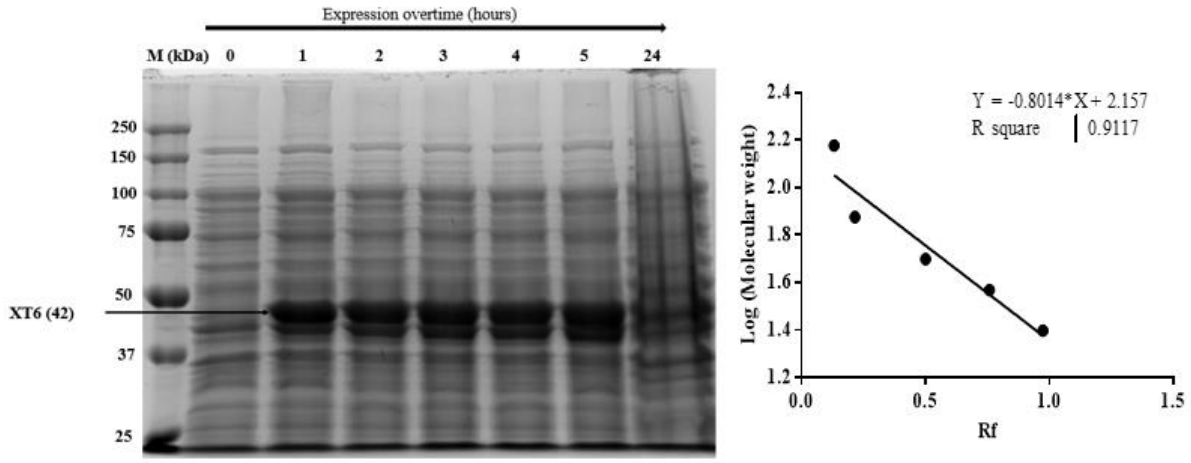

Figure 2.4: SDS-PAGE gels showing expression and induction studies of the recombinant XT6 at 25℃ in *E. coli* BL21(DE3) cells. Lane M - protein marker (kDa), Lane 0 - Pre-induction samples, Lane 1 to 5 - Post-induction samples from 1-5 hours.

Upon induction with IPTG, two bands were observed between 37 and 50 kDa, which may suggest that XT6 was co-expressed with another housekeeping protein. Within the induction study, Lane 0 showed a faint lower band between 37 and 50 kDa before the addition of IPTG, signifying no leaky expression. There was an increase in protein band intensity during the first 3 hours of expression, and then no apparent change in band intensity after this time.

2.4.4. Purification of the recombinant produced XT6 enzyme

The expressed protein was partially purified using IMAC. The relative protein content in each purification step was visualized via SDS-PAGE analysis (section 2.3.9) and quantified by measuring the absorbance at 280 nm (section 2.3.10). The SDS-PAGE profile and total protein contents of the various fractions during the protein purification are shown in Figure 2.5.

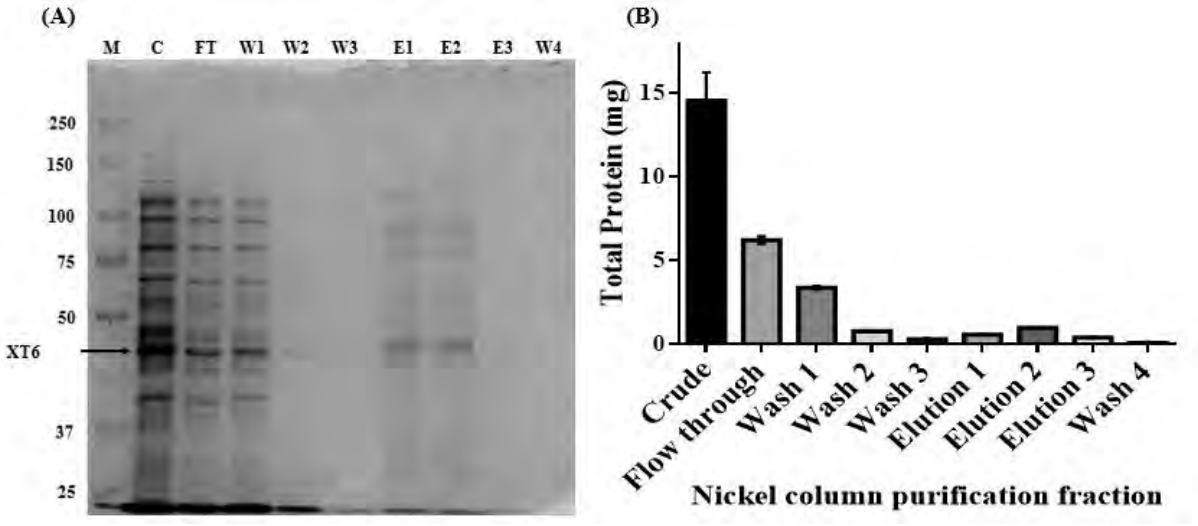

Figure 2.5: Recombinantly produced XT6 purification fractions profile analysis on nickel columns. (A) SDS-PAGE profile, and (B) protein quantification. M - protein precision marker (kDa), C - crude, FT - flow-through, W1 - wash 1, W2 - wash 2, W3 - wash 3, E1 - elution 1, E2 - elution 2, E3 - elution 3 and W4 - wash 4.

Multiple bands were observed in the crude, flow-through and wash 1 fractions. Single faint XT6 bands were observed in washes 2 and 3. Multiple faint bands were observed in elution 1 and elution 2 fractions, with the XT6 bands being more pronounced than those in washes 2 and 3. No bands were observed in the elution 3 and wash 4 fractions. The presence of several protein bands in the flow-through were expected as it contained native proteins that do not bind to the resin. Some of the XT6 protein appeared to be in the flow-through and the washes (Figure 2.5 A). This observation may be due to the weak interactions between the protein tag and the beads. The protein was eluted as a reasonably clean fraction in elutions 1 and 2, although some was lost in the flow-through and washes.

After purification, elutions 1-3 were pooled together and concentrated using the 30 kDa Amicon centrifugal filter units. A purification table was combiled, shown in Table 2.2.

Table 2.2: Purification table of XT6 purification

Purification step	Total volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Activity (U/ml)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Fold purity
Crude	10.0	2.3	23	6.3	62.7	2.7	100.0	1.0
Ni^{2+} affinity column	30.0	0.2	4.7	1.5	45.6	9.8	72.7	3.6
Ultrafiltration	5.0	0.2	0.9	8.4	41.9	45.9	66.8	16.8

The specific activity increased from the crude fraction to the purified enzyme and concentrated enzyme fractions (ultrafiltration). The yield of the purified XT6 was 66.8% after the Ni^{2+} affinity column and ultrafiltration steps, with a purification of 16.8-fold (Table 2.2).

2.5. DISCUSSION

Xylanase-encoding genes have previously been cloned and expressed in bacterial and fungal systems using various vectors (Gat et al., 1994; Subramaniyan and Prema, 2002). The GH10 xylanase-encoding genes have been cloned and expressed successfully in *E. coli* cells (Gat et al., 1994; Liew et al., 2019). In this project, the amino acid sequence (Appendix Figure A) was confirmed to be that of an extracellular xylanase XT6 precursor using BLAST analysis; it was 81% identical to *Geobacillus stearothermophillus* with a maximum score of 863, total score of 863, 99% query cover and an E-value of zero [Accession number AB149951.1]. The *xt6* gene with a size of 1236 base pairs coding for 379 amino acids was inserted into the pET28a(+) vector using *BamHI* and *SalI* restriction enzymes. The pET28a(+)/*xt6* was cloned into *E. coli* DH5α and the positive construct was confirmed by restriction enzyme digestion (Figure 2.3). Cloning of the recombinant XT6 was successful because single digestion of the pET28a(+)/*xt6* by *BamHI* or *SalI* produced one DNA band with a linear conformation (Figure 2.3 lanes 2 and 3). Double digestion by *BamHI* and *SalI* produced two bands with linear conformations (Figure 2.3 lane 4).

Different DNA conformations and sizes gave different migration profiles on agarose gel electrophoresis (Figure 2.3). Our findings concurred with those of Liew and collegues who reported a similar restriction digest profile after GH10 xylanase cloning into *E. coli* BL21(DE3) cells using *EcoR*I and *Not*I restriction enzymes (Liew et al., 2019). Chromosomal DNA exists in three conformations: linear, circular and supercoiled (Drlica and Bendich 2003; Fologea et al., 2007). The different conformations are due to cellular processes such as DNA packaging, recombination and environmental stress (Fologea et al., 2007). The supercoiled conformation is double-stranded DNA giving rise to a more compact topology (De Mattos et al., 2004). The circular form occurs when there is a breakage on one strand on the double-stranded DNA (Drlica and Bendich, 2003). The linear conformation occurs when both DNA strands break at the same position (De Mattos et al., 2004). On agarose gel electrophoresis, these conformations

give different migration profiles where the supercoiled conformation migrates the fastest, followed by circular and then linear DNA (Fologea et al., 2007).

The induction and expression of XT6 were studied using a BL21(DE3) strain of *E. coli* cells grown on LB media (Figure 2.4 A). The addition of 1 mM IPTG induced the expression of XT6. The rate of expression increased until 3 hours, after which it leveled off. As the xylanase enzyme was produced intracellularly with the host native proteins, the cells were lysed and the target protein was purified by IMAC (Figure 2.5).

The specific activity of the purified XT6 was 41.9 U/mg with a 16.8-fold purity and a yield of 66.8%, as depicted in Table 2.2. The yield obtained from this study was low as the IMAC is expected to yield approximately 95% of recombinant proteins (Bornhorst and Falke, 2000). Despite the low yield, the purified XT6 was active and in sufficient quantity to be used for the remainder of the project. The low yield may have been due to the low protein affinity to the beads and the binding capacity of the column having been exceeded. The yield of the purified *xt6* in this study was higher than that of a GH10 xylanase produced by Li and collegues (Li et al., 2008). The XT6 protein displayed a molecular mass of approximately 43 kDa (Hegazy et al., 2019); this corresponded with the predicted or expected XT6 size in our study, which was approximately 42 kDa. The XT6 molecular weight was comparable to that of other cloned GH10 xylanases from *Bacillus stearothermophilus*-T6 and *Rhodothermaceae bacterium RA* (Gat et al., 1994; Liew et al., 2019; Khassin et al., 1993). The size of the recombinant XT6 was smaller compared to that of a GH10 xylanase *Geobacillus strain* DUSELR13 of approximately 45 kDa (Bibra et al., 2018).

2.6. CONCLUSION

The *xt6* gene product, xylanase XT6, from *Geobacillus stearothermophilus* T6, was successfully cloned and expressed in *E. coli* BL21(DE3) cells. The recombinant protein was partially purified with a relatively good yield. This partially purified XT6 was characterised as described in the next chapter, Chapter 3. Ultimately, the activity of the recombinantly produced XT6 has the potential to be applied as an additive in chicken feeds.

Chapter 3: Biochemical characterisation of a recombinant XT6 enzyme from *Geobacillus stearothermophilus* T-6

3.1. INTRODUCTION

Cereal by-products used as feeds contain high quantities of non-starch polysaccharides (NSPs) and anti-nutritive factors (ANFs) (Yuan et al., 2017). Several studies have reported that these NSPs in animal feeds limit nutrient digestibility (dos Passos and Kim, 2014). Wheat is made up of hemicellulosic polysaccharides such as arabinoxylans (which form part of the NSPs) (Parsaie et al., 2007). Arabinoxylans are either soluble or insoluble in water (Parsaie et al., 2007). Hydrolysis of xylan by endo-xylanases has improved the processability and product quality of cereals in many biotechnological applications (Goesaert et al., 2004). Xylanases are classified into glycosyl hydrolase (GH) families based on their sequences and similarities (Beaugrand et al., 2004). Xylanases from different families vary in structure, catalytic mechanism, biochemical properties, substrate specificity and hydrolysis products (Velasco et al., 2019). The recombinant XT6 produced in this project belongs to the GH10 family and *G. stearothermophilus* T6 species. *Geobacillus* species have been characterised as thermophilic organisms with an optimum temperature of 55–65℃ (Burgess et al., 2017). They are Gram positive and form spores that can withstand high temperatures (Burgess et al., 2017). *Geobacillus* species are found in different environments, which include food and dairy manufacturing plants, hot-springs and cool soils (Burgess et al., 2017). This family is known to hydrolyse xylan from agricultural residues (Schramm et al., 2017; Sethy et al., 2015; Zhang et al., 2018).

Several authors have shown that members of the GH10 family display thermostability and activities over a broad range of temperature and pH values (Gat et al., 1994; Liew et al., 2019; Li et al., 2008). Several *Geobacillus* species such as *G. stearothermophilus*, *Geobacillus thermodenitrificans*, *Geobacillus sp.* strain WSUCF1 and *Geobacillus thermolevorans*, are known to produce thermostable xylanases; however, the degree of their thermostability varies (Bibra et al., 2018). The *Geobacillus sp.* strain WSUCF1 was found to produce the most stable

xylanase with a half-life ($t_{1/2}$) of 12 days at 70℃ (Bibra et al., 2018). However, when the same gene was expressed in *E. coli*, the thermostability changed from 12 days to only 20 minutes at 70℃ (Bibra et al., 2018). The thermostable *Geobacillus stearothermophilus* derived T-6 xylanase, XT6, is stable over a wide pH range and has a high catalytic efficiency with an optimum temperature of 75℃ (Hegazy et al., 2019; Saksono and Sukmarini, 2010). The two catalytic residues, Glu159 and Glu265, along with several aromatic and hydrophilic residues beside the crescent cavity, form the active site of the thermostable XT6 enzyme (Hegazy et al., 2019; Collins et al., 2005). The xylanase from *G. stearothermophilus* T6 retains approximately 60% activity at pH 10, with an optimum activity at pH 6.5 (Beg et al., 2001) (Khasin et al., 1993). This enzyme has been reported to have V_{max} and K_M values of 288 U/mg and 1.63 mg/ml on oat spelt xylan (Khasin et al., 1993). A similar observation was made regarding the XT6 enzyme's specific activity of 273.6 U/mg on soluble wheat flour arabinoxylan (Malgas and Pletschke, 2019).

In this project, partial biochemical characterization of the recombinantly produced XT6 was conducted using a model substrate, wheat-flour arabinoxylan. In addition, the effect of gut-derived proteases, mucin and bile salts on the XT6 was investigated to determine if the enzyme was able to withstand conditions associated with the gut.

3.2. AIMS AND OBJECTIVES

3.2.1. Aim

To biochemically characterise the purified recombinantly produced GH10 xylanase (XT6) from *G. stearothermophilus* T6.

3.2.2. Objectives

- To determine the substrate specificity of the recombinant *in vitro* recombinant produced XT6;
- To determine the temperature optimum and thermostability of XT6;
- To determine the pH optimum and stability of XT6;
- To determine the kinetic parameters of XT6 with wheat-flour arabinoxylan;
- To determine the effect of (commercial) porcine gut-derived proteases, mucins and bile salts on the activity of the recombinant XT6;
- To determine the shelf-life of the recombinantly produced XT6.

3.3. METHODOLOGY

3.3.1. Substrate specificity

The total reaction volume for all activity assays contained 100 µl of the enzyme (10 µg/ml) and 300 µl of 1.33% (w/v) wheat-flour arabinoxylan. Enzyme hydrolysis was performed on both soluble and insoluble wheat-flour arabinoxylan suspended in citrate buffer at pH 5. All reaction mixtures were performed in triplicate and incubated at 40℃ (using the Labnet AccuBlock™ digital dry bath) for 30 minutes.

3.3.2. Temperature optimum and thermo-stability study

Temperature optimum and thermostability studies for the recombinant xylanase (XT6) were conducted over a range of different temperatures (30-90℃). To determine the temperature optimum of XT6, enzyme hydrolysis was performed on soluble wheat arabinoxylan at different temperatures for 30 minutes. For the temperature stability study, XT6 was mixed with 50 mM sodium citrate buffer (pH 5) and incubated at different temperatures (on Labnet AccuBlock™ digital dry bath) for an hour before adding the substrate. Enzyme activity was determined using the DNS method with all assays being performed in triplicate (section 2.3.11).

3.3.3. pH optimum and stability studies

Buffers with a range of pH values (pH 3 - pH 9) were used to determine the pH optimum and pH stability for the recombinant xylanase XT6. Buffers used were 50 mM citric acid/Na_2HPO_4 (pH 3 - 7) and 50 mM Trizma/Tris-base (pH 8 - 9). The pH optimum was determined by first dissolving the substrate in buffer at different pH values. The hydrolysis was then performed by mixing the XT6 enzyme and substrate. The reaction samples were then incubated at 40 ℃ (using the Labnet AccuBlock™ digital dry bath) for 30 minutes. The pH stability was determined by incubating XT6 in buffers (pH 3 - 9) at 4℃ for an hour. The soluble commercial substrate was added, and hydrolysis was performed at 40℃ (using the Labnet AccuBlock™

digital dry bath) for 30 minutes. Residual enzyme activity was determined using the DNS method (section 2.3.11) with all assays being performed in triplicate.

3.3.4. Kinetics studies

Different substrate concentrations (0 - 20 mg/ml) were prepared for both the soluble and insoluble wheat-flour arabinoxylan. The enzyme concentration of XT6 was maintained at 0.01 mg/ml (0.021 µM) and added to all samples. Enzyme hydrolysis was performed at 40℃ (using the Labnet AccuBlock™ digital dry bath) for 30 minutes, with all assays conducted in triplicate. The enzyme activity was determined using the DNS assay (section 2.3.11). Kinetic parameters were estimated by fitting the hyperbolic Michaelis-Menten equation to the experimental data using GraphPad Prism 6.05 software (San Diego, CA, USA).

3.3.5. Shelf-life studies

XT6 was incubated in the fridge (4℃) and at room temperature (25℃) for nine weeks. Enzyme hydrolysis and activity assays were performed (using the procedure in section 2.3.11) each week to monitor the enzyme's stability over this period.

3.3.6. Inhibition studies

A 1:1 ratio of the purified recombinant XT6 from *Geobacillus stearothermophilus*-T6 and commercial gut-derived proteases (trypsin, pepsin), mucin or bile salts from porcine (Sigma-Aldrich, St. Louis, USA) were mixed (separately) and incubated at 40℃ (using the Labnet AccuBlock™ digital dry bath) for 1 hour. The substrate (soluble wheat-flour arabinoxylan) was added to the samples. Enzyme hydrolysis was performed at 40℃ for 30 minutes and all assays were performed in triplicate using the DNS method (section 2.1.11).

3.3.7. Statistical Analysis

One-way analysis of variance (ANOVA) was used for statistical analysis. A *p*-value of less than 0.05 was considered statistically significant. Data were analyzed using GraphPad Prism 6.05 software (San Diego, CA, USA).

3.4. RESULTS

3.4.1. Substrate specificity

To evaluate the specific activity of the recombinantly produced XT6, the enzyme was applied to soluble and insoluble wheat arabinoxylan, and xylanase activity was measured (Figure 3.1).

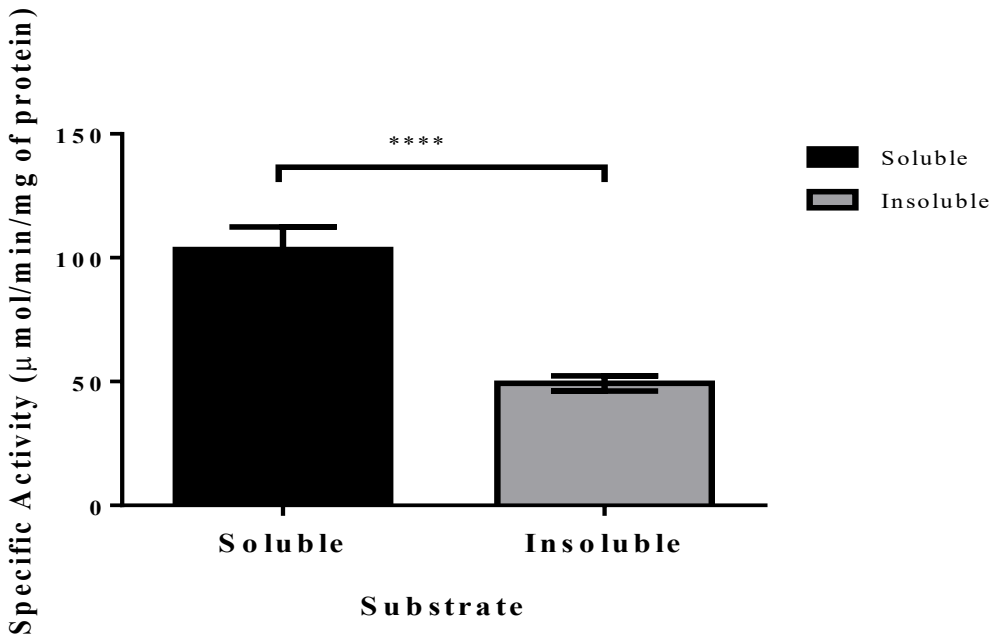

Figure 3.1: Xylanase specific activities during the hydrolysis of soluble and insoluble wheat-flour arabinoxylan. Soluble and insoluble wheat-flour arabinoxylan were hydrolysed using recombinant XT6. ANOVA analysis was conducted to evaluate the significance of difference in reducing sugars produced by the various wheat flour arabinoxylans (**-*p*-value < 0.0001 and data points represented the mean values ±SD (n=3). A *p*-value smaller than 0.05 shows 95% confidence (significant difference).**

XT6 showed significantly higher specific activity (p-value < 0.0001) on soluble wheat-flour arabinoxylan (110.9 µmol/min/mg of protein) compared to the insoluble wheat-flour arabinoxylan (63.98 µmol/min/mg) (Figure 3.1).

3.4.2. Temperature optimum and thermo-stability study

Xylanase activity was measured on soluble wheat-flour arabinoxylan to determine the enzyme's temperature optimum and thermostability (Figure 3.2).

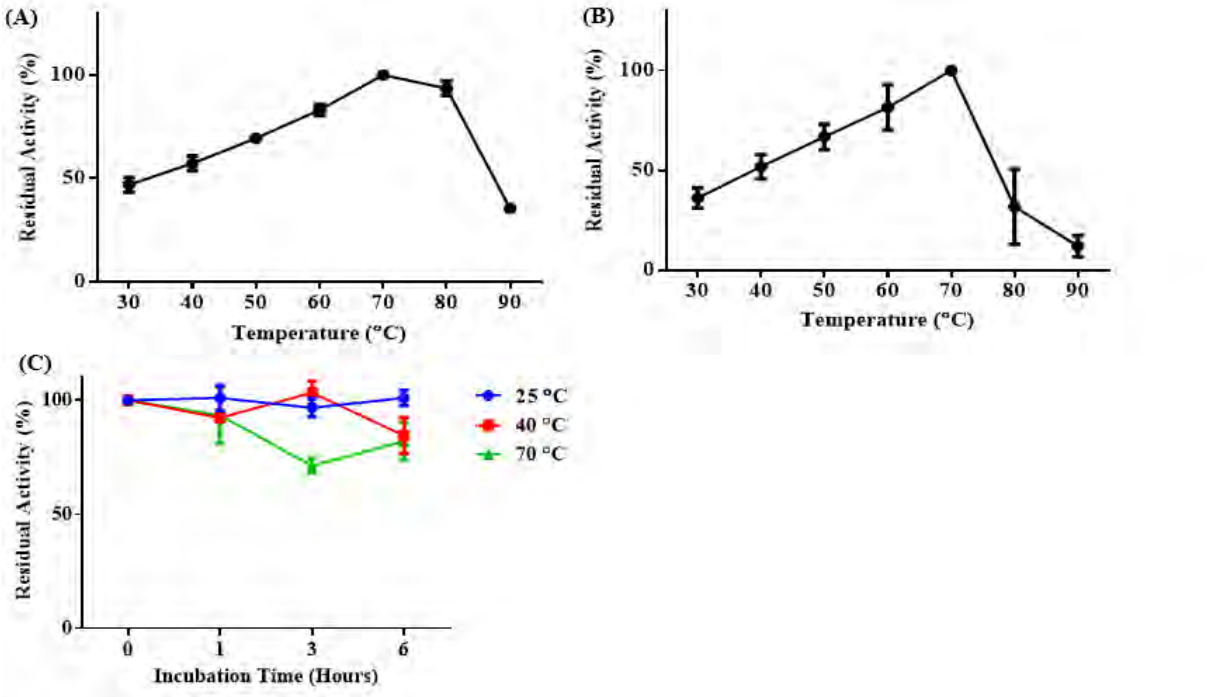

Figure 3.2: Temperature profile of the recombinant produced XT6. (A) Temperature optima study, (B) temperature stability study where the enzyme was incubated at different temperatures for 1 hour before hydrolyzing the soluble wheat arabinoxylan at 40℃. A stability study over longer periods was also performed at (C) 25℃, 40℃ and 70℃. Data points represent the mean values ±SD (n=3).

There was a gradual increase in xylanase activity as the temperature increased from 30℃ to 70℃; however, the XT6 activity started decreasing from 80℃ (Figure 3.2A). The optimum temperature of the enzyme was observed at 70℃ (Figure 3.2A). A similar profile was observed after pre-incubating the XT6 enzyme at different temperatures for 1 hour (Figure 3.2B). XT6 remained stable after being exposed to different temperatures for an hour. Stability studies were conducted at different temperatures for 6 hours where enzyme aliquots were withdrawn, and substrate hydrolysis was performed. At 25℃, XT6 displayed stability (approximately 100% activity) (Figure 3.2C). At 40℃, XT6 retained more than 80% activity (Figure 3.2C). At 70℃, XT6 activity decreased as the incubation time progressed; however, 60 to 80% activity was still retained after 6 hours (Figure 3.2C).

3.4.3. pH optima and pH stability study

The enzyme was incubated in buffers of different pH values to determine the pH optimum and pH stability profiles of the recombinant XT6 enzyme (section 3.3.3), and enzyme hydrolysis was performed using soluble wheat arabinoxylan (Figure 3.3).

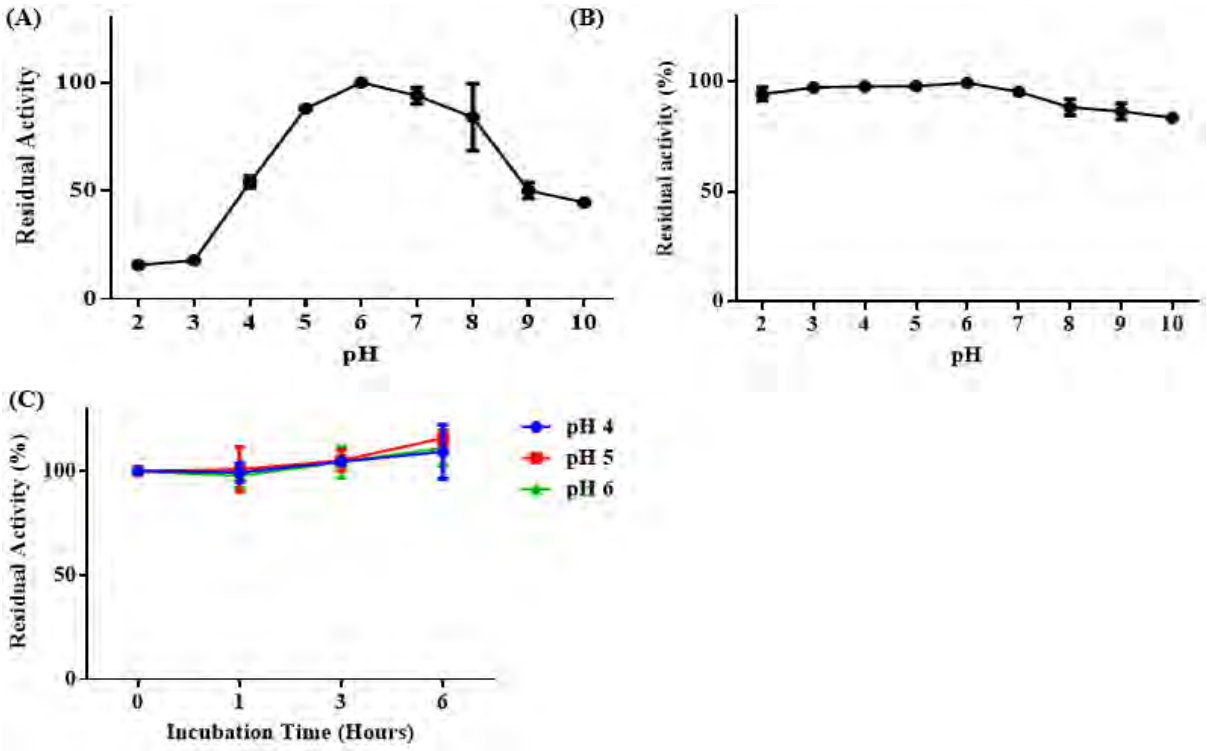

Figure 3.3: pH profile of the recombinantly produced XT6. (A) pH optimum study, (B) pH stability study where the enzyme was pre-incubated in buffers of different pH values for 1 hour, then the enzyme was used to hydrolyse the soluble wheat arabinoxylan at 40℃. Stability studies were performed at different pH values; (C) pH 4, pH 5 and pH 6. Data points represent the mean values ±SD (n=3).

There was a gradual increase in xylanase activity as the buffer pH increased from pH 3 to pH 6; however, the XT6 activity decreased at pH values above 8 (Figure 3.3A). The maximum activity of XT6 was observed at pH 6. Similar profiles for optimum xylanase activity were reported by Khasin and colleagues (Khasin et al., 1993). After incubating the recombinant XT6 in buffers with different pH levels for 1 hour, the enzyme retained approximately 100% activity at all pH values (Figure 3.3B). The pH stability studies were conducted at pH different values, where the enzyme was incubated for up to 6 hours. The recombinant XT6 displayed a stable profile at pH 4 (Figure 3.3C), pH 5 (Figure 3.3C) and pH 6 (Figure 3.3C), with the relative activity remaining above 100% for up to 6 hours. Khasin and collegues also reported that

xylanase from *Bacillus stearothermophilus* T-6 could retain activity over a wide range of pH (Khasin et al., 1993).

3.4.4. Kinetic studies

The kinetic parameters of the recombinantly produced XT6 enzyme were determined by conducting the enzyme reaction with varying soluble and insoluble wheat arabinoxylan substrate concentrations (0 to 20 mg/ml). The experimental data were modelled on the Michaelis-Menten equation (GraphPad Prism v6.05). (see Table 3.1 below).

Table 3.1: Kinetics parameters of the reaction catalysed by the recombinant XT6 on wheat flour arabinoxylans.

Substrate	Soluble AX	Insoluble AX
V_{max} (µmol/min/mg)	231.6	99.0
K_M (mg/ml)	2.8	5.1
k_{cat} (s^{-1})	42.3	18.1
k_{cat}/K_M ($M^{-1}s^{-1}$)	2300.4	536.5

On soluble wheat arabinoxylan, the V_{max} and K_M values of the recombinant XT6 were 231.6 µmol/min/mg and 2.8 mg/ml, respectively (Table 3.1). On the insoluble wheat arabinoxylan, the recombinant enzyme's V_{max} and K_M values were 99.0 µmol/min/mg and 5.1 mg/ml, respectively (Table 3.1). The V_{max} and K_M values were used to determine the turnover number (k_{cat}) and the catalytic efficiency of the recombinant XT6 (k_{cat}/K_M) (Appendix Figure I, Table 3.1). The recombinantly produced XT6 had a lower K_M value (2.8 mg/ml) on the soluble substrate than the insoluble substrate (5.1 mg/ml), which suggests that the enzyme has a slightly higher substrate affinity for soluble substrates. This observation was also in agreement with the findings for the substrate specificity study in Figure 3.1.

3.4.5. Enzyme inhibition studies

The effect of gut-derived proteases (trypsin and pepsin), mucin and bile salts on the activity of recombinantly produced XT6 was investigated at 40℃ for 1 hour to simulate the physiological temperature and conditions within the chicken gut (Figure 3.4).

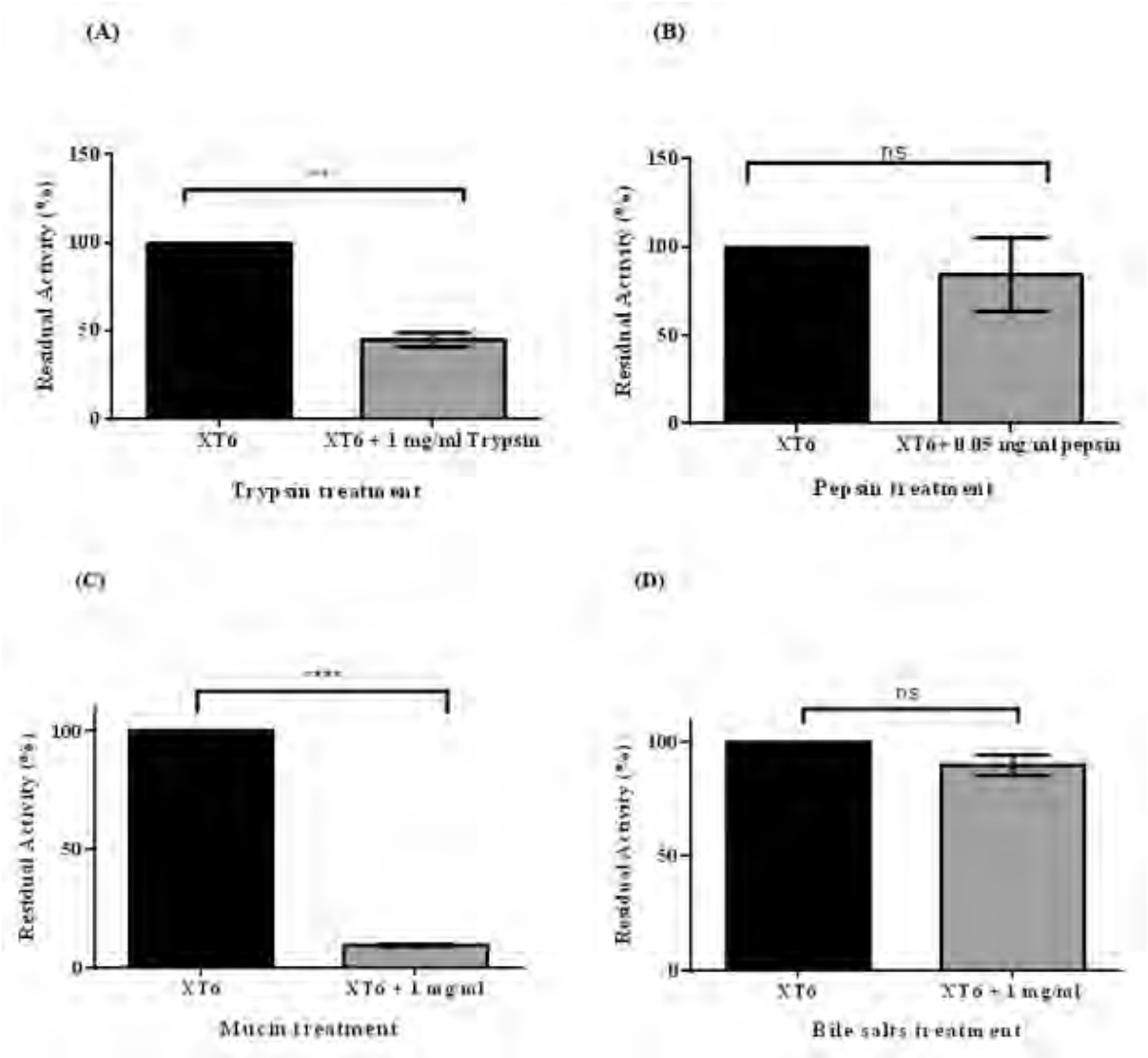

Figure 3.4: The effect of gut-derived proteases, mucin and bile salts on the activity of the recombinant XT6 enzyme. To determine the residual xylanase activity of XT6, commercial soluble wheat arabinoxylan was hydrolysed by (A) XT6:trypsin, (B) XT6:pepsin, (C) XT6:mucin, and (D) XT6:bile salts. Data points represent the mean values ±SD (n=3). (**- p - value <0.0001, **- p - value <0,0025, ns - not significant).**

The addition of trypsin (Figure 3.4A) had a significant effect on XT6 activity, compared to the addition of either pepsin or bile salts (Figure 3.4B and and 3.4D), which had a minor (non-signficant) effect on XT6. Addition of mucin significantly reduced XT6 activity (Figure 3.4C).

3.4.6. Shelf-life studies

The stability of the recombinantly produced XT6 was investigated over an extended period (1-9 weeks) at 4℃ and 25℃ by measuring the residual xylanase activity on soluble wheat-flour arabinoxylan.

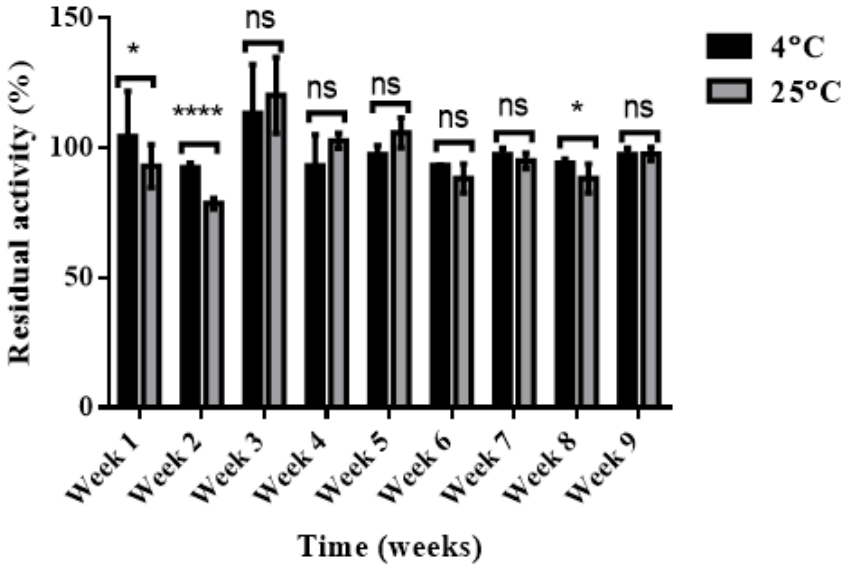

Figure 3.5: The effect of time on the recombinant XT6 activity using commercial soluble wheat arabinoxylan. The enzyme has a stable shelf life at 25℃ and at 4℃. Data points represented the mean values ±SD (n=3). (*-p value < 0.05, **-p value < 0.0001 and ns- not significant).**

XT6 remained active over the 9 week experimental period (Figure 3.5) for both the samples in the fridge and at room temperature. The residual activity at 25℃ was slightly lower than the residual activity in the fridge (4℃), but the difference was not significant (Figure 3.5).

3.5. DISCUSSION

Catalytic efficiency and enzyme stability are some factors that have an effect on the efficient formation of products (You et al., 2019). Some enzymes lack favourable enzymatic properties such as high catalytic efficiency and excellent stability over a range of pH and temperatures. The exposure of xylanases to different temperatures and pH for XOS production from xylan has been studied using various members of the GH10 family (You et al., 2019). The stability of these enzymes under these conditions has attracted a great deal of interest in several industries; for example, hydrolysis of xylan at high temperatures reduces the reaction mixture viscosity, thus lowering mass transfer (Linares-Pasten et al., 2014). This is generally observed when the substrate has a high molecular weight, which causes it to be less soluble in aqueous solutions (Linares-Pasten et al., 2014). Another advantage of good thermostability in enzymes is an increase in their half-lives, leading to efficient hydrolysis and high product yield (Linares-Paten et al., 2018).

In this chapter, the recombinant XT6 enzyme was partially characterised using soluble wheat-flour arabinoxylan. The activity of the recombinant XT6 at various pH and temperatures was determined. XT6 was found to perform better at acidic to neutral pH values. XT6 showed stability over a wide range of temperatures, with maximal activity observed at 70℃. A similar trend in the temperature optima of XT6 was previously reported for a commercial XT6 (Malgas and Pletschke 2019). The temperature optimum was similar to that of the GH10 xylanases investigated by various researchers (Karlsson et al., 2018; Li et al., 2008; Linares-Pasten et al., 2014) but was higher than that reported for the same xylanase (Khasin et al., 1993; Liew et al., 2019; Mongkorntanyatip et al., 2017) and lower than that of a *Geobacillus* strain reported by Bibra and colleagues (Bibra et al., 2018). Other GH10 xylanases such as those from *Aspergillus nidulans*, *Caldicellulosiruptor bescii*, *Micrococcus roseus*, *Saccharopolyspora pathumthaniensis* S582 and S*treptomyces coelicolor* A3 have also been reported to be stable around the same temperature range (Li et al., 2008). The stability of the recombinant XT6 enzyme was further confirmed by shelf-life studies that showed that the enzyme was stable at 4℃ and room temperature (25℃) over an extended period of time (Figure 3.5).

The recombinant XT6 enzyme was tested using model arabinoxylan substrates. It was observed that the XT6 enzyme exhibited high specific activity on soluble wheat arabinoxylan (110.9 µmol/min/mg of protein) compared to insoluble wheat arabinoxylan (63.98 µmol/min/mg of

protein). A similar trend was reported for a GH10 xylanase (SfXyn10) from *Streptomyces fradiae* (Li et al., 2008). The difference in activity may be due to the fact that, GH10 likes short and highly substituted AXs that are naturally soluble in nature (Kiszona et al., 2013; Malgas et al., 2020). Solubility is linked to the arabinose to xylose ratio on AX side chains, where the more arabinose residues there are on the substrate, the more soluble the AX is in water (Navaro et al., 2019; Sethy et al., 2015). Ordaz-Ortiz and colleagues demonstrated that disubstitution on the O-3 led to a large variation in arabinose to xylose ratios on both soluble and insoluble wheat arabinoxylans (Dervilly et al 2000; Ordaz-Ortiz et al., 2005). This was further confirmed by Delcour and collegues who reported a decrease in substitution associated with an increase in the arabinose to xylose ratio (Delcour et al., 1999). Therefore, the higher the degree of substitution, the higher the arabinose to xylose ratio (Kiszona et al., 2013).

Our findings also concurred with literature stating that xylanases from the GH10 family prefer soluble substrates to insoluble substrates (Sethy et al., 2015; Vega-chacón et al., 2018). The kinetic parameters reported on soluble wheat arabinoxylan were comparable to those reported for oat spelt xylan for the same enzyme (Khasin et al., 1993). The V_{max} of the recombinant XT6 was lower than that of a GH10 XynRA1 reported by Liew and colleagues, however, XT6 k_{cat} (42.3 s^{-1}) was higher than the XynRA1 k_{cat} (33.72 s^{-1}) (Liew et al., 2019). The turnover number of our XT6 enzyme was significantly higher than the k_{cat} (0.2 s^{-1}) reported by Khasin and colleagues (Khasin et al., 1993). The catalytic efficiency of XT6 was found to be 2300.36 $M^{-1}s^{-1}$ (Table 3.1). The difference in kinetic parameters could be due to the difference in amount of total arabinoxylan and water soluble arabinoxylan in wheat and oats (Roye et al., 2020;) where wheat has more soluble arabinoxylan.

The monogastric animal digestive system contains various biological entities that assist with nutrient digestion; these include proteases and bile salts (Khan et al., 2020). The recombinant XT6 activity was significantly reduced after an hour of incubation in the presence of trypsin (Figure 3.4A). This observation contradicts an observation that SfXyn10 from *Streptomyces fradiae* var. k11 was resistant to the action of trypsin and other neutral to alkaline proteases (Li et al., 2008). As shown in Figure 3.4B, the enzyme XT6 was also slightly reduced after incubation for 1 hour with commercial pepsin. This was comparable to XynA from *S. olivaceoviridis,* which retained about 95% of its activity after being exposed to pepsin (Zang et al., 2003). However, the effect of pepsin on the recombinant XT6 enzyme was in contrast to that reported for SfXyn10, which ultimately lost activity after being exposed to pepsin for a short period (Li et al., 2008). Mucin significantly decreased XT6 activity (Figure 3.4C), while

XT6 displayed stability in the presence of gut-derived bile salts (Figure 3.4D). The ability of the recombinant XT6 to retain some activity upon exposure to gut-derived proteases and bile salts demonstrated its potential even though it was quite strongly inhibited by mucin and trypsin.

3.6. CONCLUSION

The recombinantly produced XT6 could hydrolyse both soluble and insoluble xylan substrates, with higher activity observed on soluble arabinoxylan. The enzyme also showed stability over a wide range of temperatures, with maximum activity observed at 70℃. It was also stable over a wide range of pH, with maximal activity observed at pH 6. The XT6 retained activity upon exposure to the protease pepsin and bile salts, but lost activity upon exposure to trypsin. Mucin, however lowered XT6 activity quite dramatically. The enzyme was very stable at room temperature and at 4℃ over an extended period (9 weeks). Several characteristics of the XT6 enzyme, such as its thermo- and pH-stability, its high activity and catalytic efficiency on substrates, and stability to pepsin and bile salts, make it suitable for industrial application.

Chapter 4: Application of XT6 from *Geobacillus stearothermophilus* T-6 to chicken feeds

4.1. INTRODUCTION

Over the past few decades, antibiotics have been applied in the animal feed industry to reduce infections, prevent diseases and promote animal growth (Ding et al., 2018). However, the overuse of antibiotics has led to the development of antibiotic-resistant bacterial infections in animals, resulting in these pathogens transferred to humans through food consumption (Aliu and Sulaj, 2014; Samanta et al., 2015b). Prebiotics have been introduced to animal diets as an alternative to antibiotics in order to curb this problem (Ding et al., 2018). Prebiotics are small molecules that promote beneficial bacterial growth upon consumption (Yadav and Jha, 2019). Several authors have reported the effect of prebiotics on several bacterial species (Ding et al., 2018; Li et al., 2020; Shin et al., 2019). Commonly studied probiotic bacteria include *Bacillus*, *Lactobacillus*, *Bifidobacterium*, *Enterococcus* and *Streptococcus* (Liao and Nyachoti, 2017; Yadav and Jha, 2019).

Xylo-oligosaccharides (XOS) produced by the hydrolysis of NSPs by xylanases are the prebiotics used in animal feeds (Ding et al., 2018). XOS are oligosaccharides made up of repeating xylose units linked by β-(1–4)-linkages - examples are xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose (Malgas and Pletschke, 2019; Teng and Kim, 2018). XOS have been reported to promote the growth of beneficial bacteria such as *Bifidobacterium* spp., *Lactobacillus brevis* and *Streptococcus* spp. (Liao and Nyachoti, 2017; Ding et al., 2018). An increase in the growth of *Lactobacillus* spp. was reported in poultry that were fed a wheat-based diet (Yadav and Jha, 2019). Lactic acid bacteria (LAB), such as *Lactobacillus* spp., *Bifidobacterium* spp. and *Bacillus* spp., have been reported to inhibit or kill pathogens in the gastrointestinal tract and improve the microbial balance in pig intestines (Yang et al., 2015). An increase in *Bifidobacterium* spp. has been reported to reduce the population of pathogenic bacterial species such as *E. coli* (Ding et al., 2018). In addition to *E. coli*, other pathogenic bacterial species reported in chicken gut include *Salmonella* spp., *Clostridium perfringens* and *Campylobacter jejuni* (Borda-Molina et al., 2018).

Thin-layer chromatography (TLC), high-pressure liquid chromatography (HPLC) and fluorophore-assisted carbohydrate electrophoresis (FACE) are methods used to qualitatively separate samples of different sizes in a mixture (Jork et al., 1990; Qing et al., 2013). HPLC can be applied to quantify the compounds in a solution (Jork et al., 1990). HPLC employs detectors (such as refractive index (RI) and diode array detector (DAD)) for the determination of total component monosaccharides produced after hydrolysis (Qing et al., 2013). The addition of xylanases to cereal chicken feed diets can enhance NSP hydrolysis and the production of prebiotic xylo-oligomers.

In this study, the effect of xylan hydrolysis using the recombinantly produced XT6 xylanase was determined, the product profiles were analyzed using chromatographic techniques and the effect of these products on probiotic microbes (*Streptococcus thermophilus, Bacillus subtilis* and *Lactobacillus bulgaricus*) were examined.

4.2. AIMS AND OBJECTIVES

4.2.1 Aim

The aim of this study was to investigate the *in vitro* benefits of applying the purified recombinant GH10 xylanase (XT6) from *Geobacillus stearothermophilus* T6 to local chicken feeds.

4.2.2. Objectives

- To hydrolyse chicken feeds with XT6;
- To qualitatively analyze chicken feed hydrolysis products using TLC;
- To quantitatively analyze chicken feed hydrolysis products using HPLC;
- To analyze the morphology of the hydrolysed chicken feed using scanning electron microscopy (SEM);
- To determine the viscosity of the soluble NSP fraction of chicken feeds upon hydrolysis;
- To determine the effect of chicken feed hydrolysis products (XOS) on probiotics.

4.3. METHODOLOGY

4.3.1. Hydrolysis of model substrates and chicken feeds using the recombinantly produced XT6

The total reaction volume for all activity assays contained 100 µl of the enzyme (10 µg/ml) and 300 µl of 1.33% (w/v) wheat-flour arabinoxylan or chicken feeds. Enzyme hydrolysis was performed on both wheat-flour arabinoxylan (soluble and insoluble) and chicken feeds (starter and grower) suspended in citrate buffer at pH 5. All reaction mixtures were performed in triplicate and incubated at 40℃ (using the Labnet AccuBlock™ digital dry bath) for 30 minutes (wheat-flour arabinoxylan) and 24-hours for chicken feeds.

4.3.2. Analysis of xylan hydrolysis products by TLC

Model substrates and chicken feeds were hydrolysed and the hydrolysis products were analysed. A 5 µl aliquot of the sample was applied to Silica Gel 60 F254 TLC plates (Merck, Darmstadt, Germany). The plates were then developed twice with a mobile phase composed of 1-butanol: acetic acid: water (2: 1: 1, v/v/v). The plates were then stained by soaking in Molisch's Reagent (0.3% (w/v) α-naphthol dissolved in sulfuric acid: methanol solution (5: 95, v/v)). The sugars developed on the plates were finally visualized by heating the plates at 110℃ (using the oven) for 10 minutes. Xylooligosaccharides standards [xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentose (X5) and xylohexose (X6)] were purchased from Megazyme, Ireland.

4.3.3. Analysis of xylan hydrolysis products by HPLC

The supernatant fractions from the hydrolysates were filtered using a 0.2 µm filter. The xylooligosaccharides in the samples were quantified with a Shimadzu RID-20 HPLC system (Shimadzu Scientific Instruments, North America) using a Oligo CarboSep CHO 411 column (Transgenomic, Inc., Omaha, North America) at 80℃ with a mobile phase flow rate of 0.3

ml/minute and analysed with a refractive index (RI) detector. Filtered distilled water was used as the mobile phase. Xylooligosaccharides standards [xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentose (X5) and xylohexose (X6)] were purchased from Megazyme, Ireland.

4.3.4. Microscopic visualisation of hydrolysed feeds by scanning electron microscopy (SEM)

A 24-hour hydrolysis experiment was performed on model substrates and chicken feeds using the recombinant XT6. The samples were centrifuged and the supernatants were discarded. The pelleted samples were dried at 40℃ for 2 days. The dried samples were mounted onto a metal stub and dried using the critical point-drying process, then coated with a thin layer of gold and analysed by scanning electron microscopy (SEM) using a JOEL JSM 840 electron microscope.

4.3.5. Viscosity measurement of hydrolysed biomass

Model substrates and chicken feeds were hydrolysed (section 4.3.1), and the hydrolysates were filtered using a 0.2 µm filter. The viscosity of the samples was determined using a Cannon-Manning Semi-Micro viscometer (size 150, State college, PA, USA). Measurements to estimate the cSt/s of the samples were performed by multiplying the time with the constant (0.03146 mm^2/s^2 (cSt/s)) in triplicate.

4.3.6. Prebiotic effect of chicken feed hydrolysates

The prebiotic activity of xylooligosaccharides was tested using three probiotics; *S. thermophilus*, *B. subtilis* and *L. bulgaricus*. Each bacterial culture was inoculated into Luria Broth media (1% (w/w) tryptone, 0.5% (w/w) yeast extract and 1% (w/w) NaCl) incubated in a shaking incubator overnight at 37℃. After incubation, 1 ml of the culture was centrifuged at 16 060 ×*g* for 5 minutes. The supernatant was discarded and the pellet resuspended in 250 µl phosphate buffer (2% (w/w) Na_2HPO_4 and 0.3% (w/w) NaH_2PO_4) pH 7). Absorbance readings

were taken at 600 nm. Samples were diluted to 0.1 mg/ml using 1× minimum media (M9) [3.34% (w/w) $KHPO_4$, 1.5% (w/w) KH_2PO_4, 0.5% (w/w) NH_4Cl, and 0.25% (w/w) NaCl)]. A volume of 1 ml (2 mg/ml) of the starter or grower feed-derived reducing sugars were mixed with 4 ml of the diluted culture (OD 600 of 0.1). Glucose was used as a positive control, while M9 media on its own was used as a negative control. The 5 ml sample (in 10 ml test tubes covered with foil) was incubated at 37℃ for 7 hours. After incubation, cell viability was determined by mixing 100 µl of the culture with 40 µl of 27 mM resazurin, followed by incubation at 37℃ until the colour changed to bright pink and then readings were taken at 560 nm. The absorbance readings for the cell viability study were converted to percentages using the negative control as a reference (100%). Cell density was determined by centrifuging 1 ml of each cell culture at 16 060 ×*g* for 5 minutes, discarding the supernatant, resuspending the pellet in PBS buffer, and taking OD600 readings. The OD600 readings were converted to percentages in relation to the negative control (100%). pH readings were taken of the remaining cultures. All assay reactions were performed in triplicate.

4.4. RESULTS

4.4.1. Analysis of xylan hydrolysis products by TLC

To visualize the degree of polymerization (DP) of XOs produced during the hydrolysis of the model substrates and local chicken feeds by the recombinant XT6, thin-layer chromatography was performed (Figure 4.1). After 30 minutes of hydrolysis, 0.9 mg/ml and 0.38 mg/ml reducing sugars were produced from soluble and insoluble substrates, respectively. A 24-hour hydrolysis of the chicken feeds produced 0.2 mg/ml and 0.16 mg/ml reducing sugars from the starter and grower feeds, respectively.

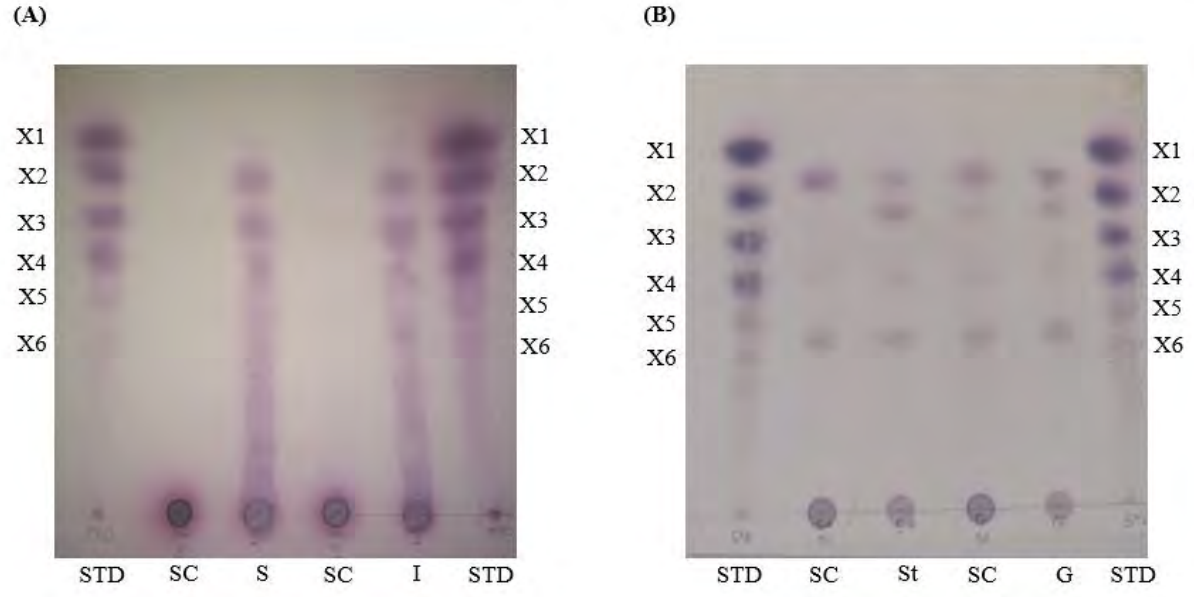

Figure 4.1: TLC profile of xylooligosaccharides produced from wheat arabinoxylan and chicken feed hydrolysis by XT6. XT6 was used to hydrolyse (A) model substrates and (B) chicken feeds. STD - Xylo-oligosaccharides standards, SC - substrate control (No XT6), S - soluble wheat arabinoxylan, I - insoluble wheat arabinoxylan, St - starter feed, and G - grower feed. X1 - Xylose; X2 - Xylobiose; X3 - Xylotriose; X4 - Xylotetraose; X5 - Xylopentaose; X6 - Xylohexaose.

Hydrolysis of both model substrates (soluble and insoluble wheat-flour arabinoxylans) by the XT6 enzyme produced three xylooligosaccharides that corresponded to xylobiose, xylotriose and xylotetraose (Figure 4.1A). There was some streaking or smearing observed around xylohexaose and below. This observation is most likely due to other higher DP oligosaccharides that could not properly be resolved by TLC. Hydrolysis of the chicken starter and grower feeds by the recombinant XT6 produced xylooligosaccharides which migrated between xylose, xylobiose, xylotriose, xylotetraose and xylohexaose (Figure 4.1 B). The

substrate controls of the chicken feeds displayed some faint spots that corresponded to those of the hydrolysed samples. This may have been due to decomposition of the substrates during termination of the reaction (heating at 100℃).

4.4.2. Analysis of xylan hydrolysis products by HPLC

To determine the concentration of the xylooligosaccharides produced after hydrolysis of the model substrates and local chicken feeds using the XT6 enzyme, HPLC analysis was performed (Figure 4.2).

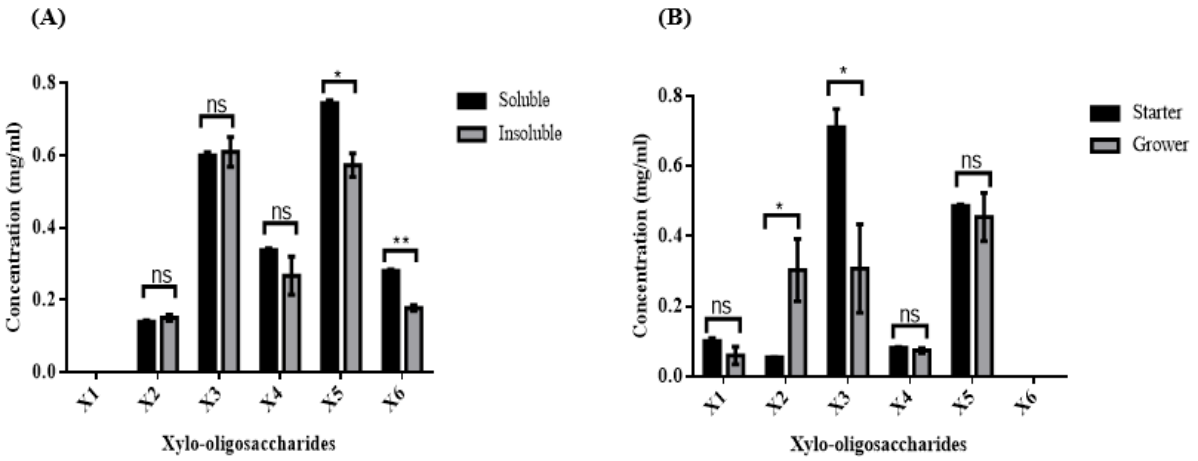

Figure 4.2: HPLC profiles on xylo-oligosaccharides produced by model substrates and chicken feed hydrolysis. XT6 was used to hydrolyse (A) model substrates (commercially available soluble and insoluble wheat arabinoxylans) and (B) chicken feeds (starter and grower). Data points represent the mean values ± SD (n=3). (* - p value < 0.05, ** - p value < 0.036 and ns - not significant). X1 - Xylose; X2 - Xylobiose; X3 - Xylotriose; X4 - Xylotetraose; X5 - Xylopentaose; X6 - Xylohexaose.

In addition to xylobiose, xylotriose and xylotetraose observed on TLC (Figure 4.1A), hydrolysis of model substrates by the XT6 enzyme also produced xylopentaose and xylohexose (now detectable on HPLC) (Figure 4.2A). Both soluble and insoluble wheat arabinoxylan produced the same product profiles, where X5 and X3 were produced in the highest amounts, followed by X4, X6 and X2. However, insoluble wheat arabinoxylan hydrolysis resulted in slightly lower amounts of products [X5 (0.59 mg/ml), X3 (0.6 mg/ml), X4 (0.2 mg/ml), X6 (0.18 mg/ml) and X2 (0.15 mg/ml)] compared to soluble wheat arabinoxylan [X5 (0.7 mg/ml), X3 (0.6 mg/ml), X4 (0.3 mg/ml), X6 (0.28 mg/ml) and X2 (0.14 mg/ml)] (Figure 4.2A). The arabinoxylan yields (%) from the model substrates were similar - however, the soluble wheat arabinoxylan substrate (1.3%) displayed a slightly higher percentage arabinoxylan yield than

the insoluble substrate (1.2%) (Appendix Table B). In contrast to model substrates, hydrolysis of starter and grower feeds by the XT6 enzyme produced xylose but did not produce xylohexaose (Figure 4.2B). This may be as a result of the xylohexaose already being degraded to smaller XOS. The starter feed produced the highest amounts of X3 (0.7 mg/ml), followed by X5 (0.5 mg/ml), X1 (0.09 mg/ml), X4 (0.08 mg/ml), and X2 (0.05 mg/ml), respectively (Figure 4.2B). The grower feed produced X5 (0.49 mg/ml) in the highest amount, followed by X3 (0.36 mg/ml), X2 (0.21 mg/ml), X4 (0.07 mg/ml), and X1 (0.05 mg/ml), respectively (Figure 4.2B). The chicken feeds showed a profile of XOS more closely resembling the insoluble substrates.

4.4.3. Viscosity measurement of hydrolysed biomass

To investigate the effect of the recombinant XT6 on substrate viscosity, the model substrates and chicken feeds were hydrolysed, and their viscosities were measured using a Cannon-Manning Semi-Micro viscometer (Figure 4.3).

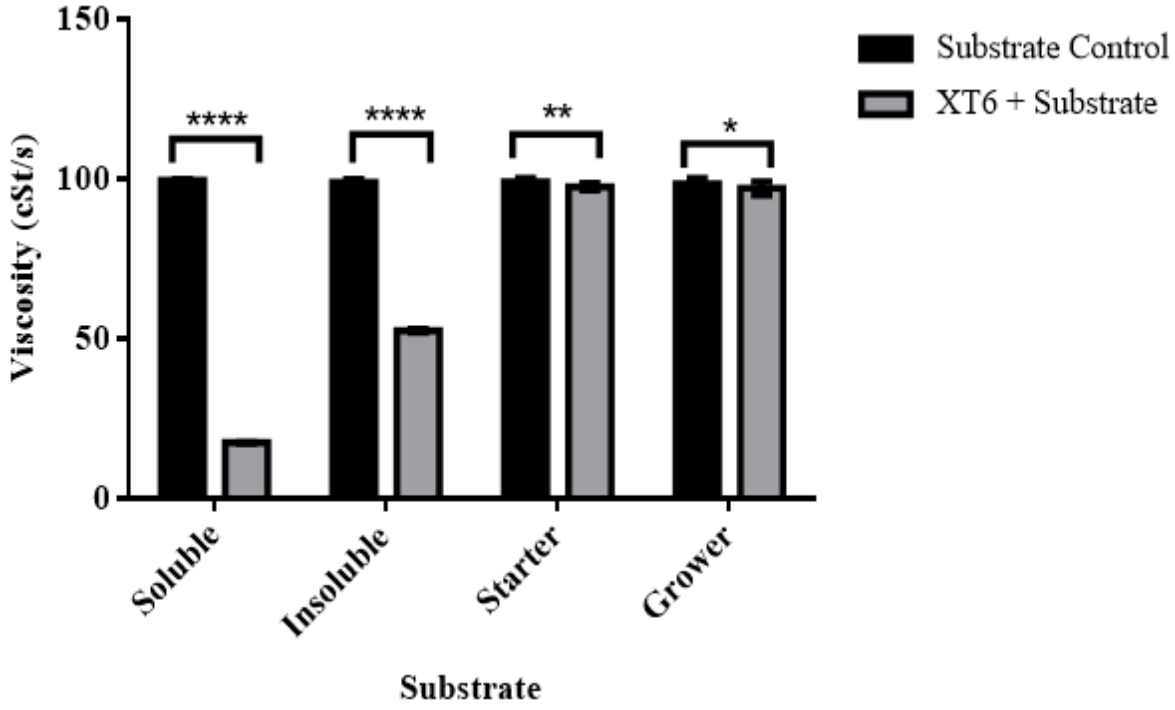

Figure 4.3: The effect of the recombinant XT6 on the viscosity of the model substrates and chicken feeds. Data points represent the mean values ± SD (n=3). (*-p value < 0.05, (-*p*-value < 0.04 ****-p value < 0.0001 and ns- not significant).**

Hydrolysis of wheat flour arabinoxylan by the XT6 showed a significant reduction in viscosity, with soluble wheat flour arabinoxylan resulting in a 70-80% decrease in viscosity. The viscosity of the insoluble wheat flour arabinoxylan was decreased by about 50%. There was a small, but significant reduction in viscosity for both starter and grower feeds (Figure 4.3).

4.4.4. Microscopic visualization of hydrolysed feeds by scanning electron microscopy

The effects of the XT6 enzyme on the morphology of the structures of insoluble wheat-flour arabinoxylan and chicken feeds were visualised using scanning electron microscopy (SEM), and the results are depicted in Figure 4.4.

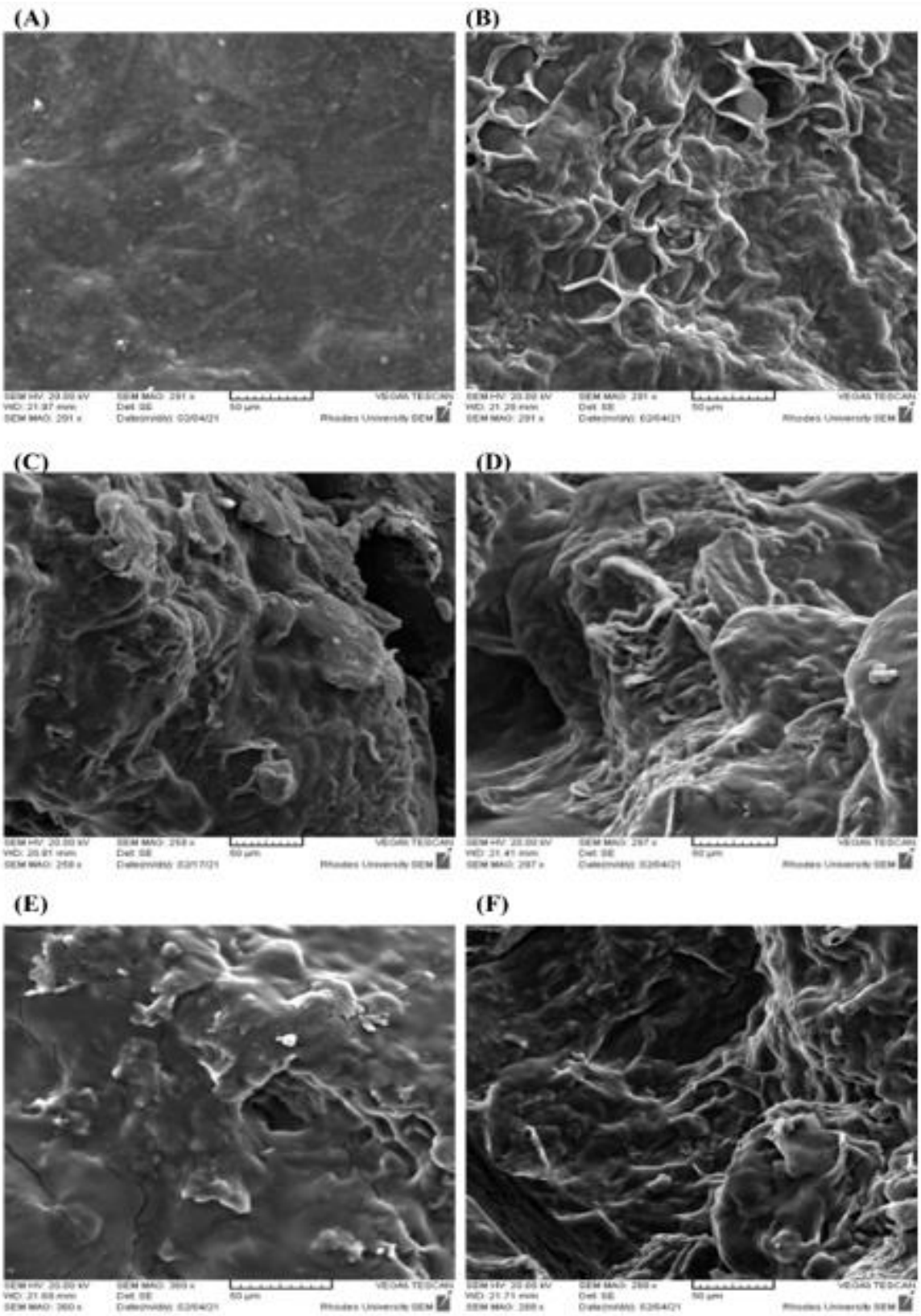

Figure 4.4: Tracking morphological changes on insoluble arabinoxylan and chicken feeds by SEM upon XT6 hydrolysis. The substrates were hydrolysed by the XT6 enzyme for 24 hours. SEM micrographs of (A) unhydrolysed insoluble wheat arabinoxylan, (B) hydrolysed insoluble wheat arabinoxylan (C) unhydrolysed starter feed (D) hydrolysed starter feed, (E) unhydrolysed grower feed, and (F) hydrolysed grower feed. MAG: (A) 291×, (B) 291×, (C) 258×, (D) 297×, (E) 360× (F) 288×.

A slight difference in structure was observed between the controls (unhydrolysed samples) (Figure 4.4 A, C, and E) and samples hydrolysed by the XT6 enzyme (Figure 4.4 B, D, and F). All the unhydrolysed substrates showed a compact, rigid structure (Figure 4.4 A, C and E), whereas the samples hydrolysed by XT6 displayed less compressed structures (Figure 4.4 B, D and F).

4.4.5. Prebiotic effects of XT6-derived XOS from chicken feeds

The effect of XOS produced from the hydrolysis of chicken feeds by the XT6 enzyme was tested on probiotics that are commoly used in the chicken. Three bacterial strains were used to conduct prebiotic studies namely *S. thermophilus, B. subtilis,* and *L. bulgaricus* (see Figure 4.5). Lower cell density (lower OD600 readings) on negative controls compared to positive contols were expected since the cells were not provided with a carborn source required for cell growth. Supplementation of XOS (produced from the hydrolysis of starter and grower feeds by the XT6 enzyme) as the carbon source in the bacterial growth media produced slightly higher cell density (high OD600 readings) for *B. subtilis* than media supplemented with glucose in the positive (+) control. Furthermore, the negative (-) control (carbon-free growth media) for *B. subtilis,* and *L. bulgaricus* strains displayed lower cell density when compared to the positive control, except for *S. thermophilus* which showed the same reading for (+) and (–) controls (Figure 4.5 A).

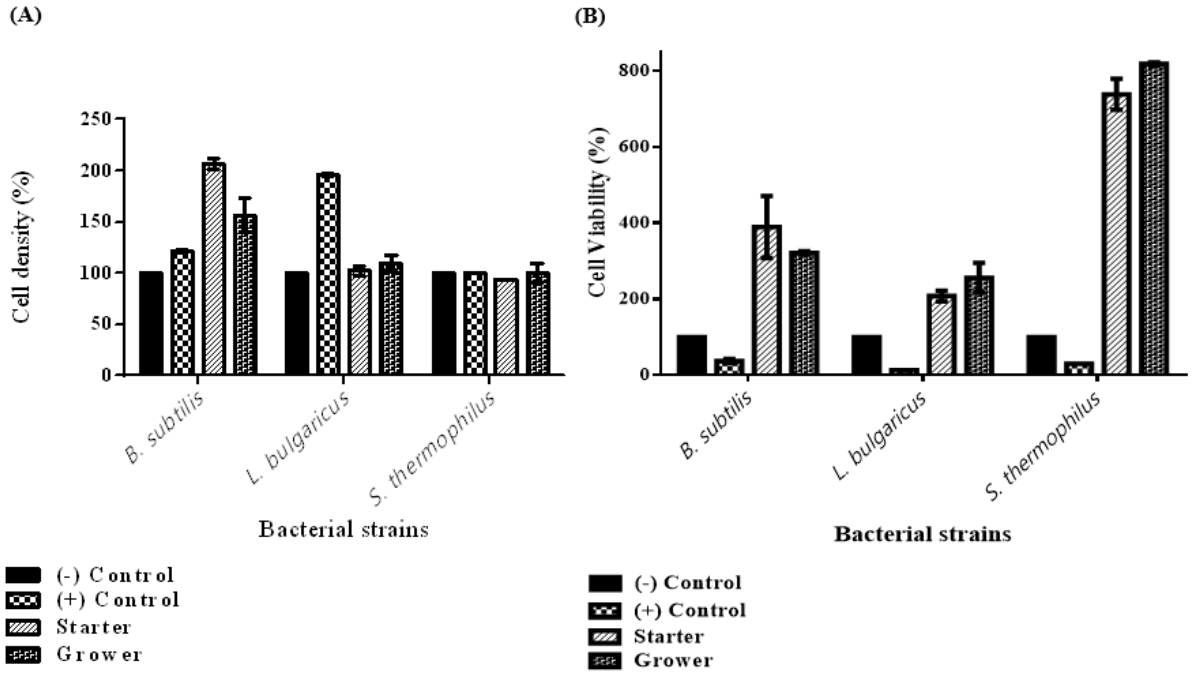

Figure 4.5: The effect of XOS on probiotics. XOS produced from the hydrolysis of chicken feeds were supplemented into various bacteria cell cultures and incubated for 7 hours. (A) Cell density (OD600) and (B) Cell viability was measured after incubation. Data points represent mean values ± SD (n=3). (-) Control = M9 media, (+) Control = Glucose, Starter, and Grower - local chicken feeds.

On the viability study, the samples supplemented with glucose ((+) control) produced less viable cells compared to the (-) control. These findings suggested that the positive control was not effective. The XOS produced from the starter and grower feeds produced more viable cells compared to both the (-) and (+) controls on all bacterial strains (Figure 4.5B). This suggested that the XOS may have promoted the cell viability of all the bacterial strains. For both starter and grower feeds, the cell viability of *S. thermophilus* was approximately double that for *B. subtilis* and *L. bulgaricus* (Figure 4.5B). However, XOS from the grower feed showed slightly higher cell viability for *S. thermophilus* and *L. bulgaricus* than the XOS produced from the starter feed (Figure 4.3B). In addition, *B. subtilis* had a higher cell viability than *L. bulgaricus* (Figure 4.3B).

In the gastrointestinal tract of chickens, the utilisation of XOS by the gut microbiota leads to the production of short-chain fatty acids (SCFAs) such as acetate, propionate, butyrate and lactate (Vázquez et al., 2002). Production of SCFAs reduces the environmental pH leading to an acidic environment (Courtin et al., 2009). The acidic environment has been reported to be unfavourable to pathogenic bacteria (Wang et al., 2009). Therefore, the pH values of the media

in which bacterial cultures were grown, were measured after the incubation and are indicated in Figure 4.6.

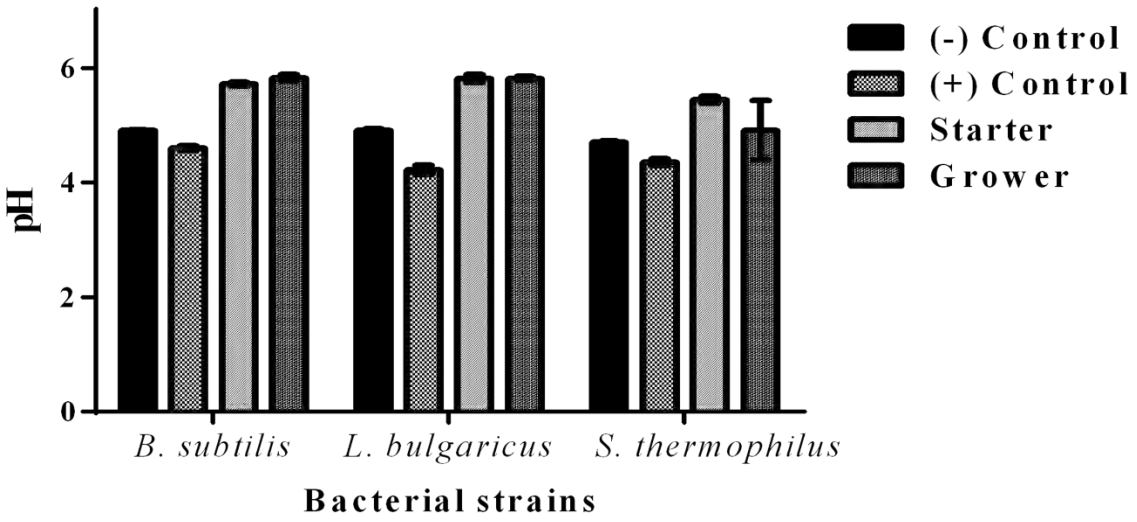

Figure 4.6: Cell culture pH study. Xylo-oligosaccharides produced from the hydrolysis of local chicken feeds using the recombinant XT6 enzyme were evaluated for their ability to alter bacterial media pH. (-) Control = M9 media, (+) Control = Glucose, Starter, and Grower = local chicken feeds. Data points represent the mean values ± SD (n=3).

Supplementation of the XOS from starter and grower feeds to bacterial cell cultures resulted in a higher pH than the positive and negative controls for all bacterial species. However, the pH remained acidic (below pH 6). The reason for this is unknown.

4.5. DISCUSSION

In this project, hydrolysis of model substrates and chicken feeds by the recombinant XT6 enzyme produced XOS with X3 and X5 being the major products (Figures 4.1 and 4.2). Hydrolysis of wheat-flour arabinoxylan predominantly produced X5 (0.7 mg/ml and 0.59 mg/ml) and X3 (0.6 mg/ml and 0.6 mg/ml) from soluble and insoluble wheat arabinoxylan, respectively (Figure 4.2A). This indicated that the recombinant XT6 is a true endo-xylanase as it released XOS with a degree of polymerization between 2 and 6. Hydrolysis of starter and grower feeds produced XOS that migrated inbetween the standards on TLC (Figure 4.1B). This observation may have been due to the substitution of the arabinoxylan in the feeds, leading to

the production of XOS with specific substitutions. The same profile was previously noted by Malgas and Pletschke (2019). The starter and grower feeds contain corn which is known to be substituted by L-arabinofuranose, D-xylopyranose, D-galactopyranose and D-glucuronic acid (Izydorcyk, 2021; Karlsson et al., 2018). Hydrolysis of starter feed by XT6 produced more X3 (0.7 mg/ml) than X5 (0.5 mg/ml) compared to those produced by hydrolysis of grower feed, which produced 0.49 mg/ml of X3 and 0.36 mg/ml of X5 (Figure 4.2B). The amount of XOS produced by the hydrolysis of the feeds by XT6 were slightly lower compared to those produced upon hydrolysis of the wheat flour arabinoxylan. This suggested that the feed arabinoxylan content may be very low. This can be supported by the fact that corn contains low amounts of arabinoxylans compared to wheat (Idzydorczyk, 2021; Navarro et al., 2019). In addition, corn has been reported to be resistant to enzymatic degradation due to its insoluble form, heavily branched side chains and phenolic linkages (Ward, 2020). This may have also been due to the production of xylose from the feeds, which has been reported to have an inhibitory effect on the production of XOS (Puchart and Biely, 2008; Vázquez et al., 2002).

No xylose was observed on TLC for the hydrolysates of both wheat-flour arabinoxylans (Figure 4.1A). This observation agrees with a study by Huang and colleagues, which reported that pure samples produce little of xylose and a higher contents of xylobiose (X2) and xylotriose (X3) (Huang et al., 2019). The absence of xylose in our study was advantageous as xylose production has been associated with the inhibition of XOS production (Chapla et al., 2012; Rahmani et al., 2019). Hegazy and colleagues also reported non-competitive end product inhibition by xylose of a *G. stearothermophilus* derived xylanase, XT6, with a K_i of 12.2 mM (Hegazy et al., 2019).

The effect of the XT6 enzyme on the viscosity of model substrates and feeds was studied. After 30 minute hydrolysis of model substrates and 24-hour hydrolysis of feeds by the XT6 enzyme, viscosity of the remaining arabinoxylan was measured (Figure 4.3). A significant reduction in viscosity was observed in the soluble and insoluble model substrates. However, there was little significant change in the viscosities of both starter and grower feeds. This was contradictory to SEM studies on the hydrolysed samples which showed changes in these stuctures (Figure 4.4). This may be due to the fact that corn has less arabinoxylan compared to wheat (Navarro et al., 2019). Arabinoxylan solubility is linked to the arabinose to xylose ratio on AX side chains; the more arabinose residues there are present on the substrate, the more soluble the AX is in water (Navaro et al., 2019; Sethy et al., 2015). Corn has been identified as one of the cereals that is insoluble in water (Mathew et al., 2017; Pluske et al., 2001). The wheat-flour arabinoxylans were more viscous compared to the chicken feeds. This was expected, because, upon

suspension of feeds in buffer, no soluble xylans were released. Therefore, the exposure of the enzyme to insoluble feed fractions is not expected to have a significant effect on viscosity, because viscosity is associated with xylan solubility (Navaro et al., 2019; Sethy et al., 2015). Additionally, wheat has been reported to contain a large ratio of arabinose to xylose, which makes it viscous and more soluble in water, compared to corn which has more xylose than arabinose (Mathew et al., 2017).

XOS produced from the hydrolysis of the starter and grower feeds by the recombinant XT6 were used as a carbon source for the growth of three probiotic bacterial species (Figures 4.5A). Cell density study demonstrated that XOS from the hydrolysis of both starter and grower feeds by XT6 promoted the growth of *B. subtilis* but had little to no effect on *L. bulgaricus* and *S. thermophilus* (Figure 4.5A). The reason for why increased cell viability was observed for *S. thermophilus* (see Fig. 4.5B), despite the fact that the cell density for this organism was low (Fig. 4.5A) is unknown, therefore further studies should be performed to investigate this phenomenon. The growth of *B. subtilis* and *L. bulgaricus* was further supported by the production of viable cells (Figure 4.5B). A similar observation was reported for *Lactobacillus* species (Chapla et al., 2012; Ding et al., 2018). However, growth of *L. brevis* and *L. fermentum* was reported to increase in the presence of XOS (Chapla et al., 2012; Lin et al., 2016; Moura et al., 2007).

The cell viability study showed that viable cells were produced by probiotic bacterial strains after the supplementation of XOS in the media, which implies that they did utilise XOS as carbon source (Figures 4.5B). It has been reported that different bacterial species utilise XOS and glucose differently (Chapla et al., 2012). Some strains, such as *Bifidobacterium*, prefer less substituted XOS to highly substituted XOS (Aachary and Prapulla, 2011). XOS from starter feeds enhanced the growth of *B. subtilis*, however the growth of *S. thermophilus* and *L. bulgaricus* were not enhanced, as their cell density percentages were lower than those of the positive control (Figure 4.5). XOS from the grower feed also promoted the growth of *B. subtilis* and not that of *L. bulgaricus* or *S. thermophilus.* A similar observation was reported for XOS from corncob by Chapla and colleagues, who reported that *Lactobacillus* species preferred glucose as a carbon source over XOS (Chapla et al., 2012). These species have been reported to lack β-xylosidase activity, which plays a vital role in the degradation of XOS (Chapla et al., 2012).

The interest in XOS production has been linked to the ability of XOS to stimulate the growth of *Bifidobacteria*. *Bifidobacteria* have been reported to suppress the growth of enterobacteria species due to short-chain organic acid production, leading to a decreased pH in the digestive tract (Carvalho et al., 2017; Vázquez et al., 2002). Growth of all bacteral species increased the environmental pH of the cultures compared to those of the controls (Figure 4.6). This indicated a low amount or the absence of short chain fatty acid (SCFA) production (Carvalho et al., 2017; Ding et al., 2018). This observation corresponded with a study by Choct and collegues who reported that broilers fed with diets supplemented with xylanase showed decreased amounts of SCFAs, compared to those without xylanase supplementation (Choct et al., 1999; Choct et al., 1996). A similar observation was also reported by Józefiak and collegues, where supplementation of xylanase to rye-based diets decreased the concentration of SCFAs (Józefiak et al., 2007). Hydrolysis of chicken feeds by XT6-produced XOS with a DP between 2 and 6, which gives them the potential to exert prebiotic effects on gut microbiota (shown by the growth of the probiotics). This further supports the application of XT6 to chicken feeds as it may improve chicken gut health.

4.6. CONCLUSION

Hydrolysis of chicken feeds by XT6 led to the production of XOS with a DP of 2-6, with xylotriose and xylopentaose being the major products. In addition, XT6 demonstrated effective degradation of the water-soluble fraction of xylan, as shown by a reduction in viscosity and in SEM. Supplementation of XOS as a carbon source significantly enhanced the growth of *B. subtilis,* while there was no significant change in growth of *L. bulgaricus* and *S. thermophilus*. However, *S. thermophilus* produced more viable cells compared to *B. subtilis* and *L. bulgaricus*. Growth of bacterial species increased the culture pH, which indicated a decrease in SCFAs production. The ability of XT6 to produce XOS from chicken feeds makes it attractive for application in the chicken feed industry.

Chapter 5: General discussion

In the meat production industry, the diets of animals play an important role. In addition to animal health, feeds play a huge role in the revenue and profit of producers in the poultry industry (Kalmendal and Tauson, 2012). With the ongoing demand in feed ingredients, the animal feed industry focusses on alternative additives that maximise nutrient absorption, enhance utilisation of feeds and improve animal health and immunity. Exogeneous enzymes have been used to break down NSPs, improve feed utilisation by animals and increase substrate availability for fermentation by gut microbiota (Bedford, 2018). Several enzymes, including xylanases, have been used as supplements to monogastric animal diets. Most studies have been performed on the effect of xylanases on wheat and barley based diets, but not much work has been reported on corn based diets. The addition of an endo-1,4-β-xylanase to broiler wheat-based diets was reported to confer a number of advantages, such as decreasing ileal digesta viscosity, improving growth performance, increasing animal growth rates and apparent ileal digestibility of nutrients via improvement of nutrient digestion and absorption (Liu and Kim, 2017; Nian et al., 2011). Similar effects of improvement in nutrient digestibility were reported upon xylanase supplementation in pig diets (Olukosi et al., 2007). Supplementation of xylanase has also been reported to have a beneficial effect on intestinal morphology and microfloral balance, leading to reduced NH_3 levels in the excreta, resulting in reduced odour emissions from broiler faeces (Liu and Kim, 2017; Zhang et al., 2018). The introduction of xylanases in the feed industry has been hypothesised to improve digestion of nutrients and improve the nutritive value of rye and wheat (Choct et al., 2004).

The composition of South African feeds for monogastric animals (chicken and pigs) includes corn as the main energy source and soybean is the main protein source (Biasato et al., 2018). Starter and grower feeds for broilers have been reported to have varying corn and soybean ratio formulations, with starter feeds having more soybean and less corn compared to grower feeds (El-Deek et al., 2020; Saleh and Watkins, 1997). This study was conducted to evaluate the recombinantly produced recombinant xylanase on local chicken feeds. The cloning, expression and partial purification of a *G. stearothermophilus* T6 GH10 xylanase, XT6, was successfully accomplished. The partially purified enzyme showed thermostability at different temperatures with an optimum temperature of 70℃. The enzyme also showed stability over nine weeks at 4℃ and 25℃ with approximately 100% residual activity. These characteristics make XT6

suitable for long-term storage under different conditions, as either a dry or liquid formulation. XT6 remained stable after treatment with buffers ranging from pH 3 to 9 at 40℃ for 6 hours, which may contribute to its resistance to drastic reductions in pH (in acidic environments), caused by the digestive secretions in the chicken's digestive system. The activity of XT6 was not affected by bile salts, thus the enzyme was able to resist the presence of bile salts in the intestinal tract. Exposure of the enzyme to trypsin for an hour dramatically reduced its activity, while its incubation with pepsin led to a less severe reduction in activity. The XT6 enzyme may, therefore, be able to retain a significant amount of activity in the first part of the digestive system and only be severely impacted upon later in the small intestine, where it becomes exposed to additional digestive enzymes such as trypsin (Singh et al., 2012). Further studies on the effect of trypsin on XT6 over time should be conducted to confirm whether the presence of trypsin completely inhibits the activity of XT6. XT6 activity was significantly reduced in the presence of mucin; this may be a disadvantage because mucin is considered to be the first line of host defence, a signal to destroy the enzyme that may be sent before the feeds are hydrolyzed (Scanes and Pierzchala-Koziec, 2014). Even though mucin reduced XT6 activity, the enzyme may not be directly exposed to the mucin. It is part of the bolus or chyme along with the feed, so the substrate may protect the enzyme, allowing it to continue its activity on the feed (Scanes and Pierzchala-Koziec, 2014; Singh et al., 2012).

Probiotics have been used as feed additives due to their ability to balance microbial populations in the gut, with *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, *Bacillus* and *Enterococcus* spp. having been used as probiotics (Abd El-Hack et al., 2020; Kabir, 2009; Khan et al., 2020). Hydrolysis of starter and grower feeds by XT6 produced xylose, xylobiose, xylotriose, xylotetraose and xylopentaose. The production of XOS makes XT6 more relevant for the chicken feeds industry, because XOS are considered to be one of the groups of oligosaccharides that exert prebiotic effects by enhancing the growth of intestinal microbiota. To test these prebiotic effects, XOS produced from the hydrolysis of chicken feeds were supplied to probiotic bacterial strains as a carbon source. All the probiotic strains evaluated could utilise XOS as carbon source. However, some strains grew more readily than others. *B. subtilis* and *L. bulgaricus* displayed the highest growth rates respectively, while *S. thermophilus* presented more viable cells compared to the other strains. Effects associated with the proliferation of probiotics in the gut includes the production of metabolites (such as SCFAs which lower the luminal pH), formation of ecological niches in the gut to competitively exclude colonization of pathogenic bacterial species and antimicrobial compounds which may eliminate pathogenic

bacterial species (Abd El-Hack et al., 2020; Kabir, 2009). In this project, XOS were supplied to the individual bacterial species and resulted in the growth of the probiotics; however, the environmental pH of the culture supplemented with XOS suggested a reduction in SCFAs. Therefore, the concentrations of SCFAs in baterial cultures should be confirmed. In addition, the effect of probiotics on pathogenic bacteria should be investigated to better understand their mechanism of interaction.

There are many ways in which XT6 can be applied to the feed industry:

Firstly, we suggest mixing the enzyme with the feed before ingestion by the animal. This will allow feed hydrolysis at the same time of ingestion, allowing the production of XOS as soon as the feed is ingested. The excess enzyme will not go to waste as hydrolysis will still continue in the gastrointestinal tract. This can be achieved, because XT6 showed a high degree of stability at different temperatures and was able to withstand the action of pepsin and bile salts. However, the presence of mucin and trypsin may possibly adversely affect XT6 activity later in the digestion process as indicated in our study.

Secondly, we suggest that hydrolysis of feeds can be done externally and that the produced XOS can be collected and administered to the animal by mixing these XOS with water or feed (De Maesschalck et al., 2015; Pan et al., 2019). This will directly result in the proliferation of probiotics throughout the digestion process. In this project, the effect of gut-derived products were only tested on XT6 *in vitro* and not on the produced XOS – therefore, the effect of gut-derived proteases, bile salts and mucin on the XOS should be tested as well.

Conclusion

South African animal feed manufactures have reported that most available commercial enzymes that are used as additives in animal diets are manufactured abroad. Therefore, due to the use of different feed formulations in SA compared to other countries, these enzyme formulations may not be suitable for application to South African animal feeds. Hence, this study was conducted to address this challenge.

This study involved the successful cloning and heterologous expression of a GH10 xylanase (XT6) in the pET28a(+) vector system. The recombinant XT6 was partially purified using IMAC, followed by its functional characterisation. XT6 showed high specific activity on

soluble wheat flour arabinoxylan compared to the chicken feeds and was stable at different temperatures and pH, with an optimum activity at 70℃ and pH 6. This enzyme's ability to tolerate different temperatures and pH makes it suitable for application in different industries. XT6 retained its activity upon exposure to pepsin, indicating that it may survive the gastrointerstinal digestive juices of the animal. XT6 retained activity after 9 weeks of storage at room temperature and 4℃. XOS produced from both starter and grower chicken feeds were utilised by probiotics (*S. thermophilus, B. subtilis* and *L. bulgaricus*). Therefore, the application of XT6 as an additive to chicken feeds could improve animal gut health, circumventing the use of antibiotics, leading to the high production of animal products (eggs and meat), thus improving profits in the monogastric animal industry.

Future studies

This project indicated that XT6 could hydrolyse the starter and grower chicken feeds, producing XOS with potential prebiotic effects and lowering the digesta viscosities. In future, certain limitations to our study may be addressed; these include:

- Probiotics utilised XOS as carbon sources, however, the effect of the probiotics on pathogenic bacteria was not tested. Therefore, the antimicrobial effect of probiotics on pathogenic bacterial strains could be investigated by directly applying probiotic bacterial strains to an environment containing pathogenic bacteria.
- Chemical composition analysis of the feeds, together with the structural analysis of the produced XOS by mass spectrosctrometry (MS) and nuclear magnetic resonance spectroscopy (NMR), are recommended. This may help quantify the content of arabinoxylan, arabinose to xylan ratio and characterise the produced XOS and their degree of substitution, which play an important role in their solubilities and prebiotic effects.
- Hydrolysis rates of feeds were low compared to wheat flour arabinoxylan. This may be addressed by incorporating XT6 with xylanases from other GH families and other enzymes such as glucanases and pectinases, phytases and mannanases to achieve complete degradation of NSPs.

- Protease and mucin inhibition studies should be expanded to determine at which concentration the proteases will start to affect XT6 activity. The period of exposure (of the XT6 enzyme to the proteases and mucin) should also be considered in these studies.

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Appendices

Table A :List of Reagents

50 mM Citrate Buffer pH 5 • 10.5 g citrate • 980 ml dH_2O • 0.03 % (0.3 g) sodium azide • Adjust to pH 5 with 2 M NaOH.	BCA Reagents • 12.5 ml reagent A (Bicinchoninic acid) • 250 µl reagent B (Copper (ii) sulfate solution).	DNS Reagent • 10 g Dinitrosalycylic acid • Dissolve in (2 % w/v) NaOH • 40 g potassium sodium tartrate tetrahydrate • 0.4 g phenol • 0.1 g sodium metabisulfite • Top up to 200 ml with dH_2O • Store at room temperature covered with foil.	10 mg/ml Kanamycin • 0.1 g kanamycin powder in 10 ml dH_2O. • Filter sterilize using 200 nm filter and store at -20 ℃
1 M IPTG • 2.38 G IPTG in 10 ml dH_2O water • Filter sterilize using 0.22 µM filter • Store at -20 ℃	50 mM Sodium phosphate buffer (PBS). • 13.4 g Sodium phosphate dibasic heptahydrate (MW 268 g/mol • Make up to 800 ml with dH_2O • Adjust to pH 6.5 with HCL.	2× YT media • 1.6 % (w/v) Tryptone • 1 % (w/v) yeast extract • 0.5 % (w/v) NaCl • 1.5 % (w/v) Agar (for plates).	50 X TAE • 242 g Tris-base • 57.1 ml acetate (acetic acid) • 100 ml 0.5 M sodium EDTA • Add dH_2O up to 1 L.
0.5 M EDTA pH 8 • 18.61 g EDTA disodium salt • Dissolve in 80 ml dH_2O • Adjust pH using NaOH pellets	2 M Glucose • 18 g glucose • Make up to 50 ml with dH_2O.	2 M $MgCl_2$ • 9.52 g $MgCl_2$ • Make up to 50 ml with dH_2O	SOC media • 2 g tryptone • 0.5 g yeast extract • 0.05 NaCl • 1 ml 2M glucose • 0.5 ml 2 M $MgCl_2$ • Make up to 100 ml with dH_2O. • 1.5 Bacteriological Agar (for plates)
1 M CaCl2 • 11.1 g anhydrous $CaCl_2$ • Top up to 100 ml with dH_2O • Filter sterilize using 0.22 µM filter.	Stripping Buffer pH 7.4 • 20 mM NaH_2PO_4 • 20 mM Na_2HPO_4 • 0.5 M NaCl • 50 mM EDTA	Binding Buffer • 20 mM NaH_2PO_4 • 20 mM Na_2HPO_4 • 0.5 M NaCl • 10 mM Imidazole	30 % Bis-Acrylamide • 146.1 g Acrylamide • 3.9 g Bis acrylamide • Make up to 500 ml with dH_2O.
5× sample buffer • 4 ml 100 % glycerol • 1.5 ml 20 % SDS • 2,8 ml 0.5 % Bromophenol blue	Lysis buffer (NP1-10) • 50 mM NaH_2PO_4 • 300 M NaCl • 10 mM Imidazole	Wash buffer (NP1-20) • 50 mM NaH_2PO_4 • 300 M NaCl • 20 mM Imidazole	Elution Buffer (NP1-250) • 50 mM NaH_2PO_4 • 300 M NaCl • 250 mM Imidazole

• 1.7 ml of 0.5 M Tris-HCl pH 6.8	• Adjust to pH 8 using NaOH • Filter sterilize using 0.2 µM filter.	• Adjust to pH 8 using NaOH • Filter sterilize using 0.2 µM filter.	• Adjust to pH 8 using NaOH • Filter sterilize using 0.2 µM filter.
TLC Staining dye • 0.03 g 1-naphthol • 5 ml sulphuric acid • 95 ml methanol	M9 media • $KHPO_4$, • KH_2PO_4, • NH_4Cl • NaCl		

Table B: Table showing the yield (%) of arabinoxylan after the hydrolysis of soluble and insoluble wheat flour arabinoxylan by the recombinant XT6.

Substrate	Total XOS (mg/ml)	Arabinose (mg/ml)	Xylose (mg/ml)	Yield (%)
Soluble	2.02	0.77	1.25	1.29
Insoluble	1.72	0.60	1.25	1.2

MRNVVRKPLTIGLALTLLLPMGMTATSAKNADSYAKKPHISALNAPQLDQRYKNEFTIGAAVEPYQLQNE
KDVQMLKRHFNSIVAENVMKPISIQPEEGKFNFEQADRIVKFAKANGMDIRFHTLVWHSQVPQWFFLDKE
GKPMVNETDPVKREQNKQLLLKRLETHIKTIVERYKDDIKYWDVVNEVVGDDGKLRNSPWYQIAGIDYIK
VAFQAARKYGGDNIKLYMNDYNTEVEPKRTALYNLVKQLKEEGVPIDGIGHQSHIQIGWPSEAEIEKTIN
MFAALGLDNQITELDVSMYGWPPRAYPTYDAIPKQKFLDQAARYDRLFKLYEKLSDKISNVTFWGIADNH
TWLDSRADVYYDANGNVVVDPNAPYAKVEKGKGKDAPFVFGPDYKVKPAYWAIIDHK

Figure A: Amino acid sequence of an GH10 extra-cellular xylanase T-6 fron Geobacillus stearothermophilus (XynA;XynA2;HUS_CDS38). GenBank accession number: ABI49951. Retrieved from https://www.ncbi.nlm.nih.gov/protein/ABI49937.

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>reverse translation of AAC98140.1 extra-cellular xylanase [Geobacillus
stearothermophilus] to a 1221 base sequence of most likely codons.
atgcgcaacgtggtgcgcaaaccgctgaccattggcctggcgctgaccctgctgctgccg
atgggcatgaccgcgaccagcgcgaaaaacgcggatagctatgcgaaaaaaccgcatatt
agcgcgctgaacgcgccgcagctggatcagcgctataaaaacgaatttaccattggcgcg
gcggtggaaccgtatcagctgcagaacgaaaaagatgtgcagatgctgaaacgccatttt
aacagcattgtggcggaaaacgtgatgaaaccgattagcattcagccggaagaaggcaaa
tttaactttgaacaggcggatcgcattgtgaaatttgcgaaagcgaacggcatggatatt
cgctttcataccctggtgtggcatagccaggtgccgcagtggtttttttctggataaagaa
ggcaaaccgatggtgaacgaaaccgatccggtgaaacgcgaacagaacaaacagctgctg
ctgaaacgcctggaaacccatattaaaaccattgtggaacgctataaagatgatattaaa
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tatcagattgcgggcattgattatattaaagtggcgtttcaggcggcgcgcaaatatggc
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gcgctgtataacctggtgaaacagctgaaagaagaaggcgtgccgattgatggcattggc
catcagagccatattcagattggctggccgagcgaagcggaaattgaaaaaaccattaac
atgtttgcggcgctgggcctggataaccagattaccgaactggatgtgagcatgtatggc
tggccgccgcgcgcgtatccgacctatgatgcgattccgaaacagaaatttctggatcag
gcggcgcgctatgatcgcctgtttaaactgtatgaaaaactgagcgataaaattagcaac
gtgacctttttggggcattgcggataaccatacctggctggatagccgcgcggatgtgtat
tatgatgcgaacggcaacgtggtggtggatccgaacgcgccgtatgcgaaagtggaaaaa
ggcaaaggcaaagatgcgccgtttgtgtttggcccggattataaagtgaaaccggcgtat
tgggcgattattgatcataaa
```

Figure B: The possible DNA sequence of the extra-cellular xylanase, XT6, from *Geobacillus stearothermophilus* obtained from bioinformatics tool. https://www.bioinformatics.org/sms2/rev_trans.html

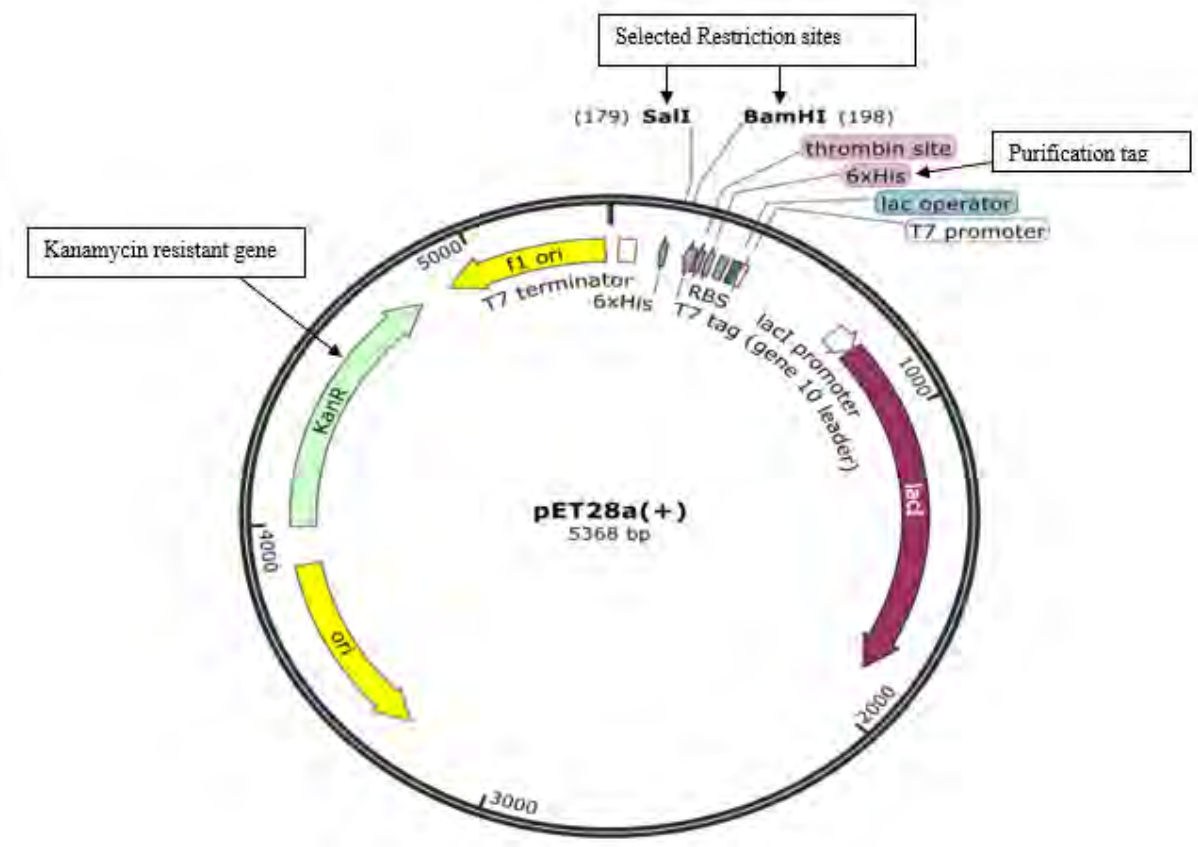


Figure C: Cloning vector showing the selection markers and the selected restriction sites for cloning.

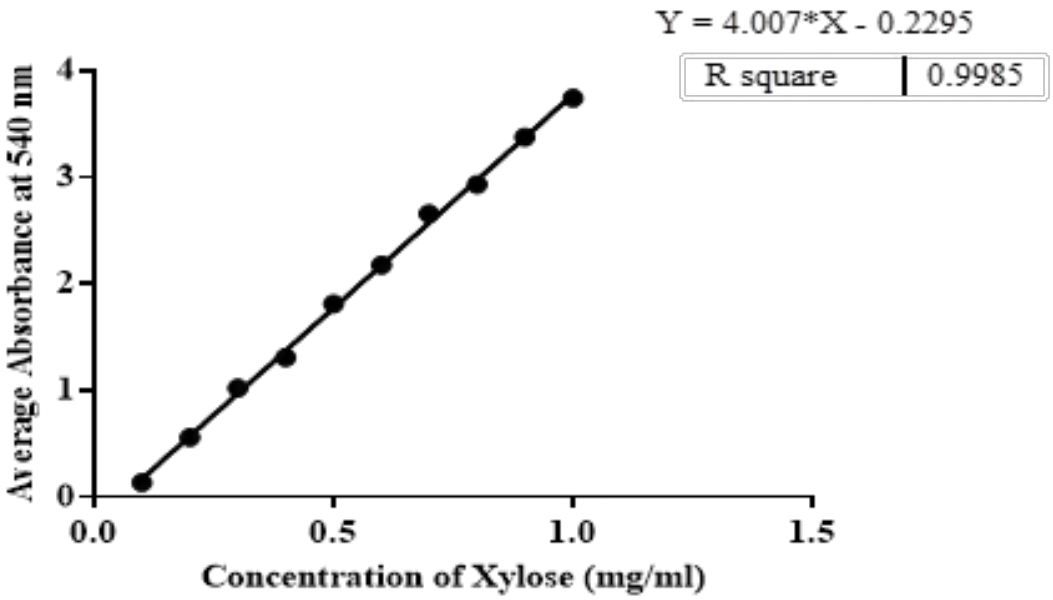


Figure D: DNS Standard curve

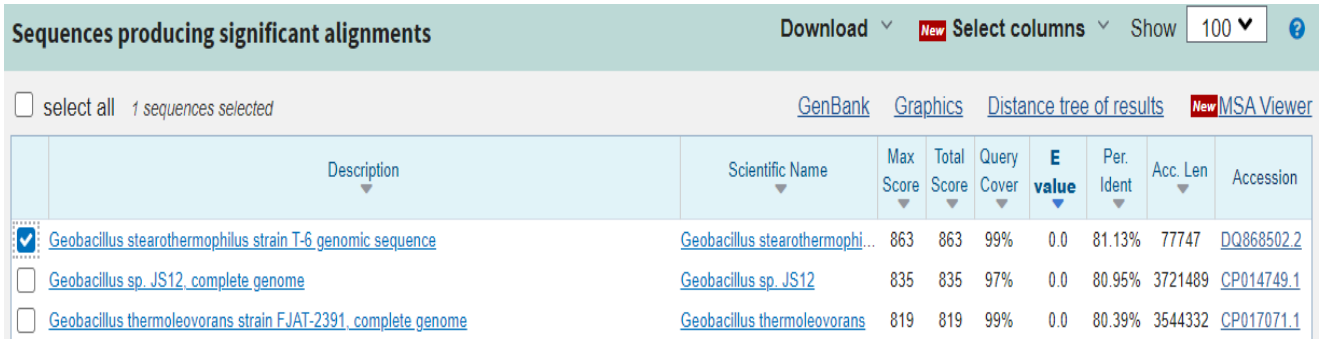


Figure E: Basic local alignment search tool results for the optimized *xt6* gene sequence.

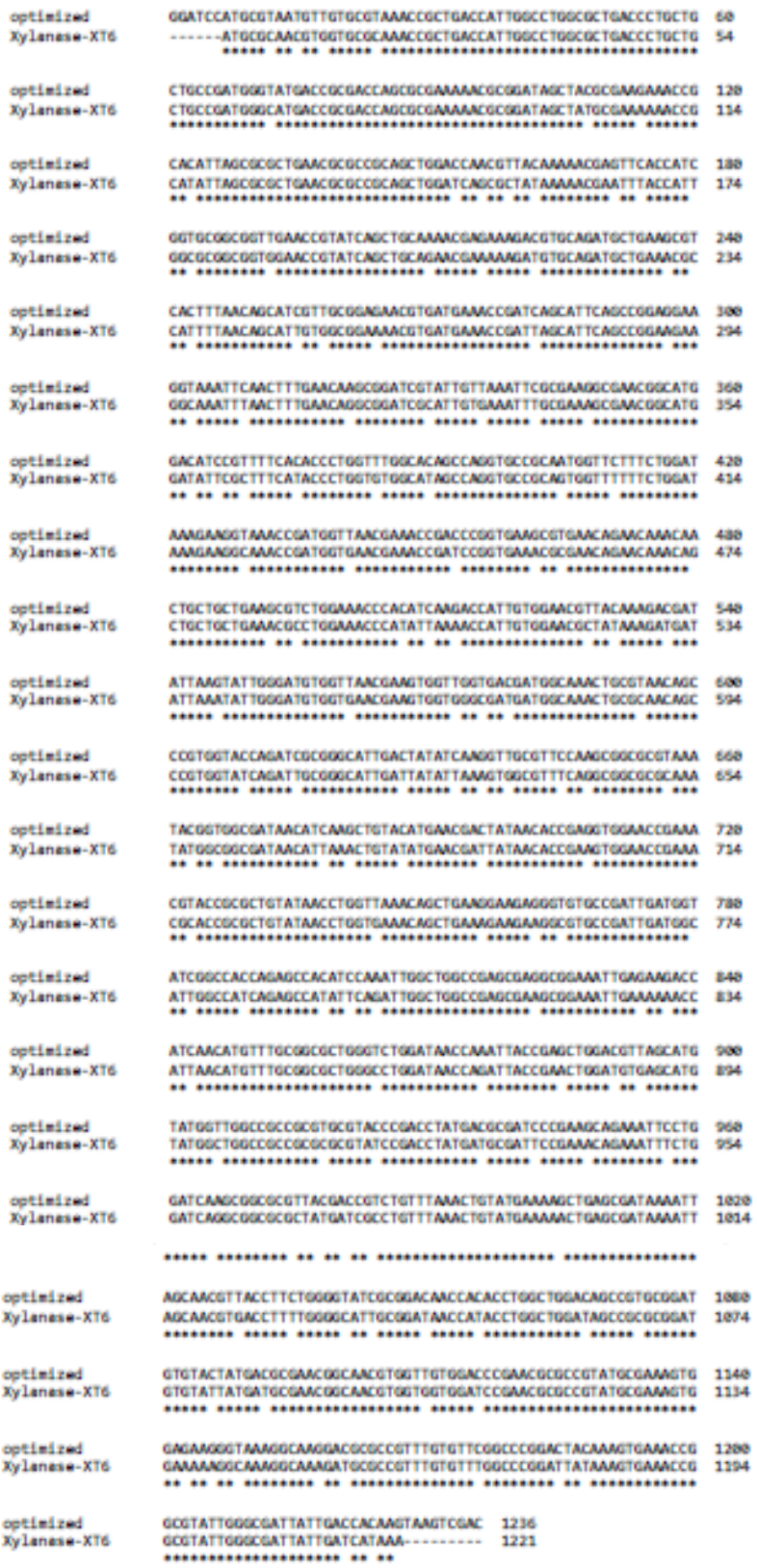


Figure F: Pair-wise sequence alignment of the optimised sequence from GenScript and the *xt6* nucleotide sequence from NCBI using ClustalW2

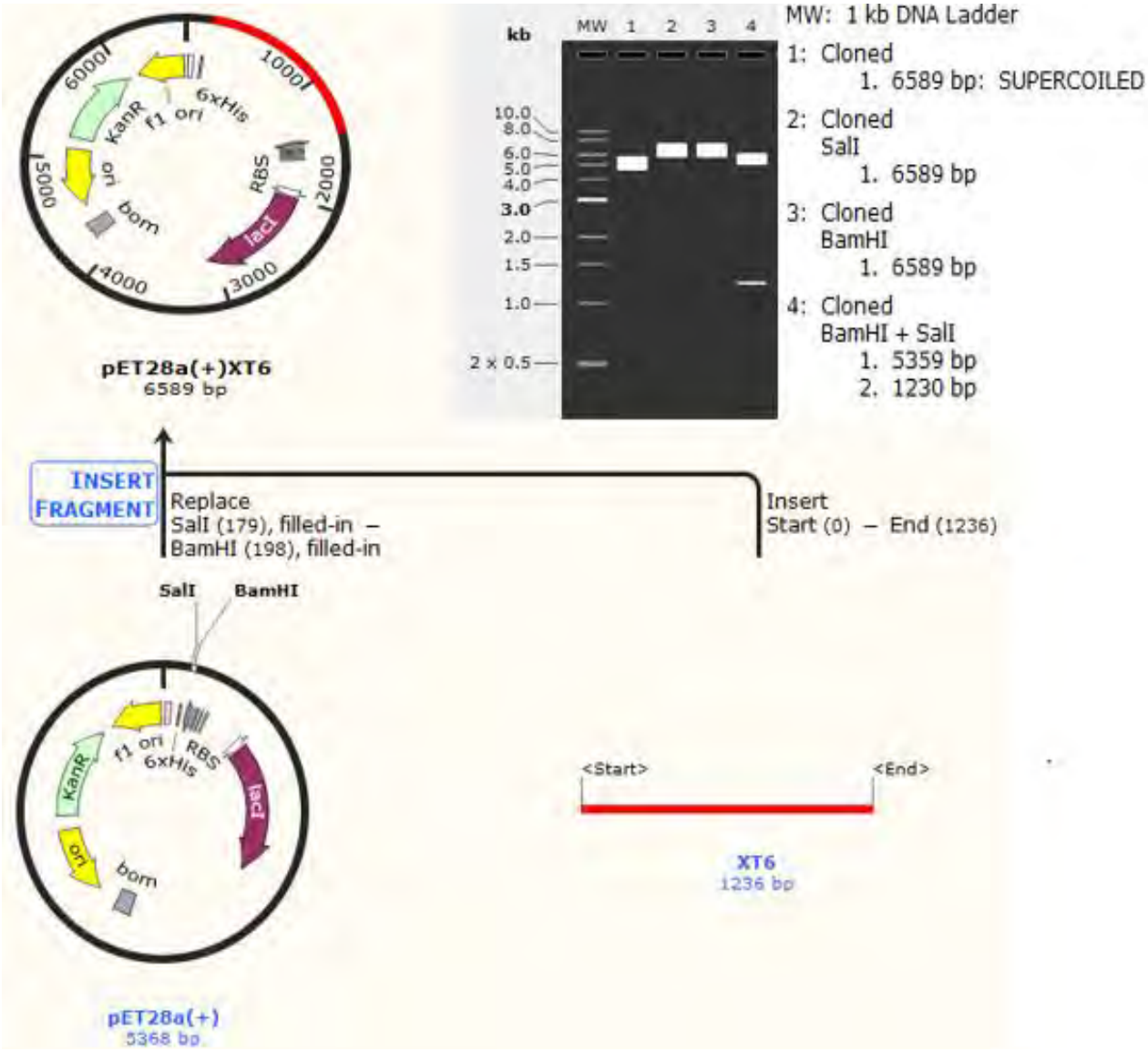


Figure G: Cloning of *xt6* gene into pET28a(+) and agarose gel electrophoresis profile prediction by Snap view

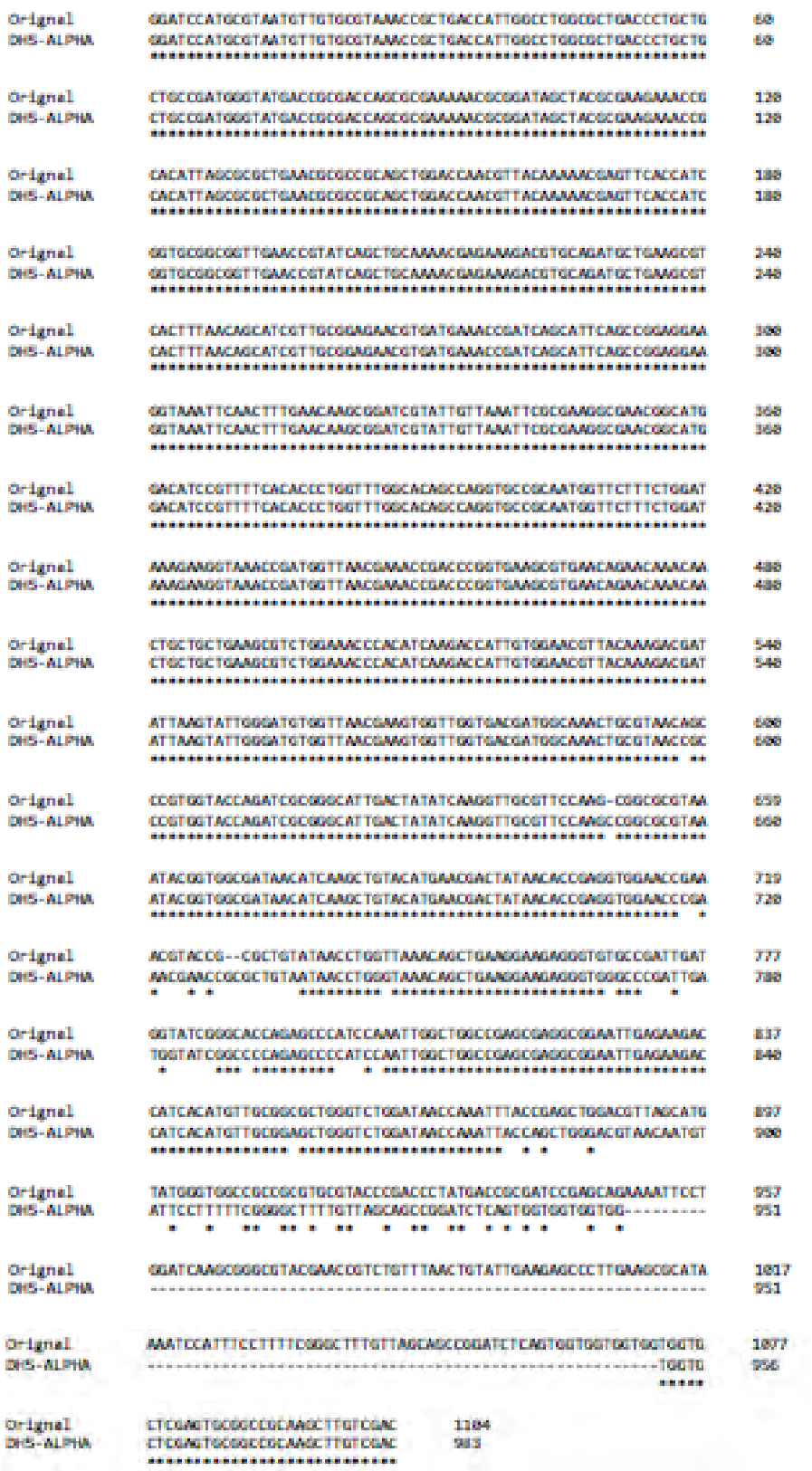
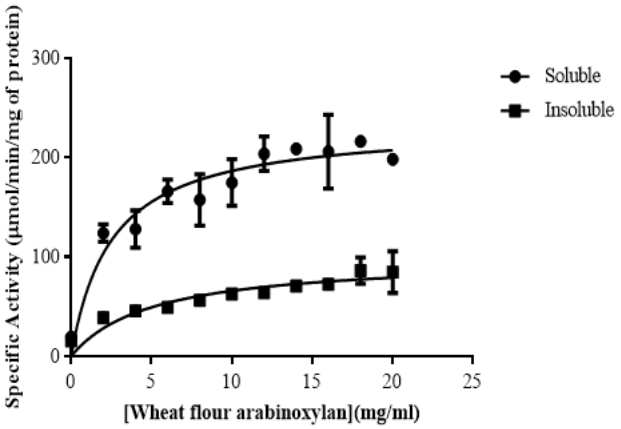


Figure H: Pair-wise sequence alignment of the optimised sequence from GenScript (original) and the xt6 cloned in *E.coli* DH5-ALPHA cells using ClustalW2.



In-house produced XT6	Soluble	Insoluble
Vmax	231.6 µmol/min/mg	99.0 µmol/min/mg
Km	2.8 mg/ml	5.1 mg/ml

Figure I: Michaelis-Menten plot showing kinetics parameters of the recombinant XT6 on soluble and insoluble wheat flour arabinoxylan. Data points represent the mean values ±SD (n=3).