Expression, partial characterisation and utilization of a GH11 xylanase (Xyn2A) from *Trichoderma viride* as an additive in monogastric animal feeds

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by

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Abstract

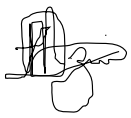
Endo-xylanases (shortly called xylanases) are a group of glycoside hydrolase enzymes that target β-D-1,4-linkages in the xylan backbone, leading to the production of xylooligosaccharides (XOS) of varying degree of polymerization (DP). Xylan is an indigestible non-starch polysaccharide present in monogastric animal feeds which in high amounts leads to increased digesta viscosity, slow movement of digesta in the intestines, malabsorption of nutrients among other challenges. The aim of this study was to investigate the effect of xylanase 2A (Xyn2A) from *Trichoderma viride* on broiler chicken feeds, particularly the hydrolysis of the xylan content, reduction of feed viscosity and the effect of produced XOS on eliciting the growth of gut associated probiotic bacteria. $Xyn2A^E$ was successfully induced with 0.8 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and produced in *Escherichia coli* BL21 (DE3) and $Xyn2A^C$ was expressed in tobacco mosaic plants. For the purification of $Xyn2A^E$, an immobilized metal affinity chromatography (IMAC) column and diafiltration using a 3kDa cut-off Amicon filter membranes were used. $Xyn2A^E$ and $Xyn2A^C$ showed a xylanase active band at a relative weight of 21 kDa. Both enzymes showed high specificity towards soluble wheat arabinoxylan (WAX), with specific activities of 7.61 U/mg for $Xyn2A^E$ and 536.5 U/mg for $Xyn2A^C$. Xyn2A kinetic parameters (V_{max} and K_m) were determined by Michaelis-Menten plots on soluble and insoluble WAX. The V_{max} and K_m values of $Xyn2A^C$ were 1003.01 U/mg and 9.25 mg/mL, 302.89 U/mg and 13.54 mg/mL, respectively. The V_{max} and K_m values of $Xyn2A^E$ for soluble and insoluble WAX were 20.45 U/mg and 12.95 mg/mL, and 8.31 U/mg and 13.15 mg/mL. Xyn2A enzymes displayed optimum activity at pH and temperature parameters of 5.0 and 50°C, respectively, and stability in temperatures ranging between 50 and 80°C and pH 4.0-9.0. Broiler chicken feeds were hydrolysed using $Xyn2A^E$ over a 24 h period and analysed using the dinitrosalicylic (DNS) assay, thin layer chromatography (TLC), viscometry and visualized using scanning electron microscope (SEM). The results showed a release of release of XOS xylotriose, xylopentose and xylohexose; enzyme's ability to decrease the viscosity of the feeds and punched holes of feed surface, which was indicative of xylanase action. XOS produced during hydrolysis was used to study prebiotic effect on selected few bacteria and released short chain fatty acids (SCFAs) were measured. Additionally, SCFAs formation was detected in the presence of XOS as a carbon source for *S. thermophilus* and *L. bulgaricus*, whereas *B. subtilis* formed fewer organic acids in the presence of XOS. The results obtained from this study demonstrated that the supplementation of Xyn2A on broiler feeds has

a positive effect in decreasing feed viscosity. Furthermore, the results of this investigation will assist the South African poultry farming sector to increase profitability in poultry farming and gain stability in the global trade as far as poultry feed is concerned.

Keywords: Animal feeds; GH11 xylanases; monogastric animals; NSPs; prebiotic effect, SCFAs

Declaration

I, Nosabatha Happyness Mzimkulu-Ncoyi, declare that this Master's thesis is my own, unaided work. It is hereby submitted at the Faculty of Science, Rhodes University for Master of Science degree. This work has not been submitted before for any degree or examination at any other institution.

Signature:  Date: 14 February 2022

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Dedication

This work is dedicated to the Kave, Mzimkulu and Ncoyi families at large.

Preface

This thesis consists of five chapters. Chapter one contains a general literature review, problem identification, and aims and objectives of the study. Chapter two covers the expression of a *Trichoderma viride gene* (Xyn2A) which was expressed in *Escherichia coli* and purified on an immobilizable affinity chromatography (IMAC) column. Chapter three consists of partial characterisation of the recombinant xylanase, while chapter four covers the application of the recombinant xylanase in broiler chicken feeds, and the effects of xylooligosaccharides on selected few gastrointestinal tract bacteria. Chapter five contains the general discussion and recommendations for further studies.

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List of Abbreviations

AME	Apparent metabolizable energy
APS	Ammonia persulphate
BCA	Bicinchoninic acid
DNS	3, 4-dinitrosalicylic acid
DP	Degree of polymerization
°C	Degree(s) Celsius
FCR	Feed conversion rate
GH	Glycosyl hydrolase
g	Gram
h	Hour
IPTG	Isopropylthiogalactoside
µL	Microliter
µg	Micrograms
mg	Milligrams
mL	Milliliters
mM	Millimolar
min	Minutes
Mw	Molecular weight
Ni-NTA	Nickel nitrilotriacetic
NSP	Non-starch polysaccharide
OD	Optical density
rpm	resolutions per minute
SCFA	Short chain fatty acids

SDS	Sodium docecyl sulphate
SDS-PAGE	Sodium docecyl sulphate polyacrylamide gel electrophoresis
TEMED	N, N, N, N-tetramethylethylenediamine
Tris	Tris (Hydroxymethyl) aminomethane
TLC	Thin layer chromatography
WAX	Wheat flour arabinoxylan
×g	gravitational force
XOS	Xylooligosaccharides

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Chapter 1: General Introduction and Literature Review

1.1 Introduction

Monogastric animals, such as fowls (e.g. ducks, turkey, and chickens) and pigs, have stomachs with a single compartment. The escalating demand of meat and eggs has led to increased production of monogastric animals across the globe (Sebho, 2016). As a result, the poultry industry alone accounted for 103.5 million tons (34.3% of global meat) of meat production annually (FAO, 2012), thus making it the fastest growing livestock production sector (Mcleod et al., 2009). Correspondingly, Svihus (2014) has reported that there is a constant increase in the production of poultry meat and eggs globally.

The poultry farming industry is divided into three sections, namely traditional poultry farming, subsistence poultry farming and commercial poultry farming (Davids and Meyers, 2017). Traditional poultry farming is largely made up of free-range (scavenging) chickens that mainly feed on environmental insects, worms, snails, house waste and cereal by-products (Ravidran, 1997). On the other hand, subsistent poultry farming consists of free-range crossbred chickens that are fed with on-farming feed mixed rations and yellow maize (NRC, 1994). The commercial poultry farming system is characterised by large stocks of modern strain chickens that are fed with formulated feeds to enhance their growth and development for high production (FAO, 2013).

Poultry and piggery feeds play a key role in the development, health and the quality of the animals. This is due to the nutrients, vitamins, minerals and metabolisable energy which feeds supply monogastric animals with (Alltech, 2014). Cereal grains are commonly used as the basic ingredient in the monogastric feeds. This selection is based on their seasonal availability, production, preparation costs, shelf life, nutritive value, degree of digestibility, low percentage of non-starch polysaccharides (NSPs) and absence of toxins, such as mycotoxins from fungi (Pirgozliev et al., 2019). According to the FAO (2013), animal feeds must contain adequate amount of vital and trace nutrients, such as vitamins, minerals and proteins, which play an important role in animal homeostasis. In addition to these basic feed requirements, supplemental plant and animal proteins are often added to enhance growth performance, general gut-health, and decrease death rate (Pirgozliev and Bedford, 2013; Olukosi et al., 2015; Lamot et al., 2016).

Correspondingly, Gous (2019) reported that enzymes, probiotic microorganisms and/or prebiotic substrates are normally used as feed additives to improve feed digestion in poultry and piggery. Consequently, significant results (i.e. improvement and positive change) have been observed in the digestibility, absorption rate and gut-health of animals (Mokubedi et al., 2019).

It is against this backdrop that researchers concluded that it is important to understand feed composition (e.g., crops), formulations, additives and their impact in animal nutrition (Ncube, 2018; Mokubedi, 2019). The investigation by Bento et al. (2013) demonstrated that different enzymes, such as phytases, cellulases (endo-1,4-β-D-glucanases (EC 3.2.1.4) and exo-1,4-β-D-glucanases (EC 3.2.1.91), amylases and xylanases (EC 3.2.1.8) influence feed digestibility in poultry differently by degrading the NSPs present in feeds at different sites. This is attributed to various abilities of the enzymes to target specific portions in the animal feed. Literature is replete with comprehensive studies that have been conducted across diverse enzyme additives to improve animal feed (Maehara et al., 2017; Pirgozliev et al., 2019). However, there is a gap in the use of xylanolytic enzymes as additives to digest NSPs in monogastric animal feeds. Henceforth, this study sought to investigate the ability of a fungal derived xylanase to release entrapped nutrients from two chicken feed formulations, thereby improving the nutritive value of these animal feeds.

1.2 The gastrointestinal tract (GIT) of chickens

The digestive system of domestic animals is categorized into simple and complex stomach. Monogastric animals have one stomach compartment, while ruminant animals, such as cattle, have four stomach compartments (Alltech, 2014). The gastrointestinal tract of chickens and pigs falls into simple digestive system category (Nielsen, 2004). This implies that it has a lower ability to digest and absorb feed that is dense in lignocellulose, such as grass, while ruminants do that easily (Pirgozliev et al., 2019). An attempt to enhance feed digestibility was to introduce a wide range of feed additives (Coffey et al., 2014). The digestive system of chickens bred, whether broilers (for meat quality) or layers (for high quality and quantity of eggs), had to adjust to better adapt to feed alterations (Svihus, 2014).

The digestive system of chickens is very important when overall performance and product quality is concerned. There is a significant difference between a male and female chicken body weight, and the amount of feed and water each consumes daily (Mokubedi et al., 2019). According to Svihus (2014), a modern strain male broiler chicken is reported to digest more than 7 grams of feed every hour. This imposes a high risk of digestive system disorders and overall health complications to the male chicken.

The poultry industry is fast growing, resulting in advanced modern chicken breeding selection for the best performing and high-quality breeds (Svihus, 2014). However, when considering breed selection, it is essential to understand what constitutes the digestive system of the chicken, and a functional or dysfunctional digestive system. Below is a simple diagram illustrating the digestive system of a chicken (Figure 1).

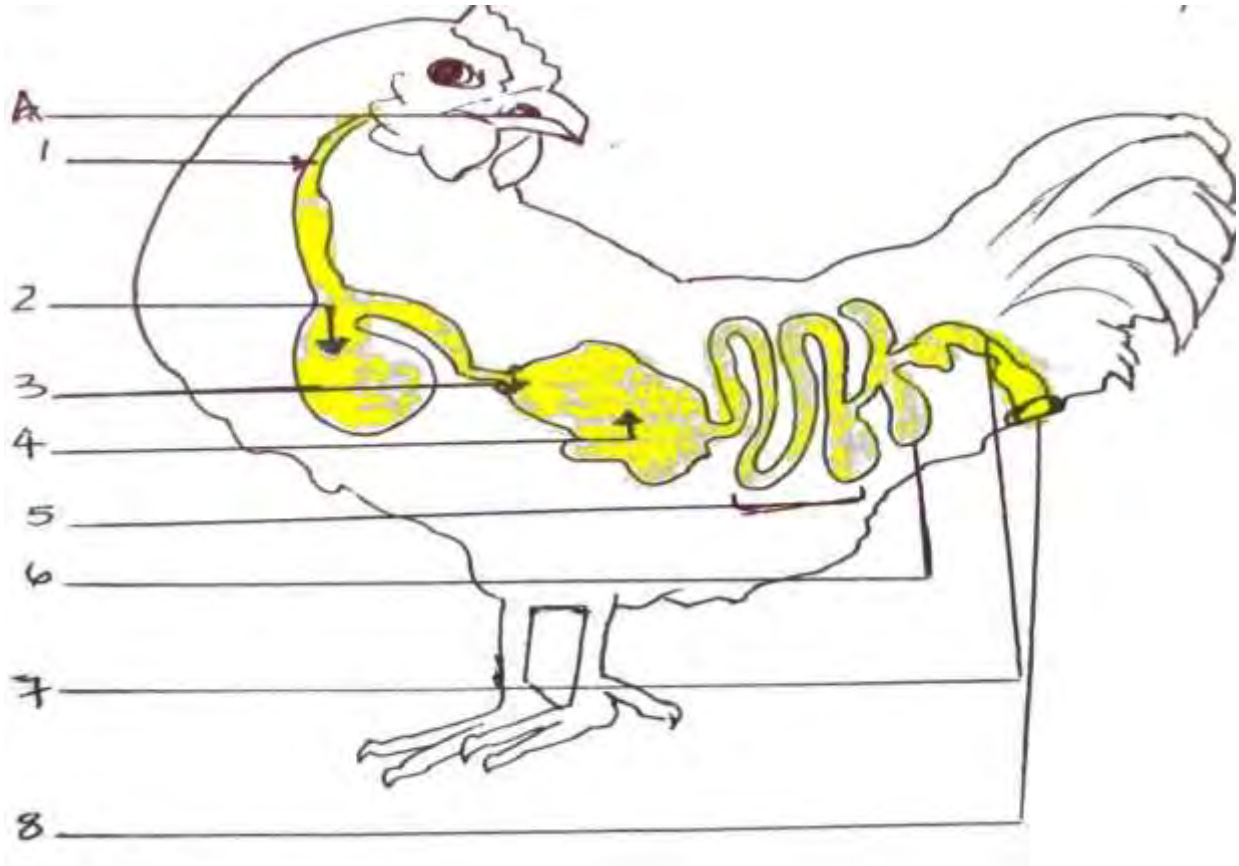

Figure 1: Asimple diagramof a chicken's digestive system. A-mouth/beak, 1- esophagus, 2- crop, 3- proventriculus, 4- gizzard, 5- intestines, 6- caeca, 7- colon and 9- cloaca.

1.2.1 The crop

The mouth (i.e. beak) is the organ through which food is ingested, that is where the process of digestion begins. In the mouth, the food is swallowed as it is, and goes down the esophagus to the crop. In the crop, the food is moisturised and then delivered to the gizzard via the proventriculus (Chaplin et al., 1992; Nielsen, 2004). According to a study conducted by Jackson and Duke (1995), the crop is not the same in all chickens.

The crop is mainly a food storage section. When chickens are eating discontinuously, it is best that chickens be fed at ad libitum (e.g. allowed to feed as they wish) routinely so that feed may bypass the crop (Svihus, 2014). In intermittent fed chickens, the crop stores large quantities of food (Svihus et al., 2013). The function of the crop during feed digestion process varies widely depending on the feed system that the broiler chickens are subjected to (Svihus et al., 2010). For example: ad libitum, continuous, discontinuous or intermittent feeding system.

The pH in the crop varies significantly. The studies done by Svihus et al. (2010) and Svihus et al. (2013) demonstrated that the pH of the crop ranges between 4.5 and 6.5. This is influenced by the time the food stays in the crop. The longer the ingested food stays in the crop, fermentation occurs, resulting in organic acid production (Hilmi et al., 2017). This implies that, the more these organic acids are produced, the more the crop will become acidic. Similarly, Ao et al. (2008) indicated the instability and variation in pH may possibly be attributed to the different times (retention time) which the food takes to pass the crop.

1.2.2 The proventriculus and gizzard

The gizzard, together with the proventriculus, is referred to as the true stomach of birds (Svihus, 2014). This is because this section of the system digestion largely takes place. The gizzard has a storage capacity of 5 to 10 grams (Svihus, 2014) and the time it takes for food to leave the gizzard has been reported to range between 30 to 60 minutes (Danicke et al., 1999). The retention time of food in the gizzard increases when feed is mixed with structural additives (e.g. titanium oxide, chromium-mordanted sunflower hulls, whole or coarsely ground grains) (Svihus et al., 2008). Correspondingly, Jimenez-Moreno et al. (2009) and Rougiere and Carre (2010) reported that the

retention time can increase from 60 minutes to 4 hours depending on the structural additive present in the feed.

On the other hand, the gastric juices secreted by the proventriculus have a pH of 2.0 (Duke, 1986), while studies conducted by Svihus (2011) reported a pH range of 3.5 to 4.5. Furthermore, the form of feed given to the chickens (e.g. crumbs, pellets or mash) tends to influence feed pH in the gizzard (Huang et al., 2006; Frikha et al., 2009). Mashed feed form normally has a short duration due to smaller feed particles while pellets feed form stays longer due to the size of the feed particles size.

1.2.3 The intestines

In the small intestine nutrient absorption and further digestion occurs (Svihus, 2013). The acidic contents that come from the gizzard are mixed with gastric juice, as the duodenal loop refluxes (Rougiere and Carre, 2010). In this segment, feed retention time here is less than 5 minutes (Noy and Sklan, 1995), and the pH rises to more than 6.0 as digestion proceeds (Svihus, 2011). Majority of nutrients are digested and absorbed in the jejunum (Frikha et al., 2009), since it is large, retention time is longer, between 40 – 60 minutes (Rougiere and Carre, 2010). In the last segments of the small intestine (i.e. ileum), water and mineral absorption takes place (Svihus et al., 2008). Additionally, some studies observed that protein, starch and fat are also digested in the ileum (Frikha et al., 2009; Svihus, 2013).

1.2.4 The caeca

A pair of caeca is located on the junction between the ileum and colon and it has a longer feed retention time of over 24 hours (Hinton et al., 2000). Though the functionality of the caeca is influenced by diet, it is significantly known for electrolyte absorption and water re-absorption (Warris et al., 2004).

1.3 Poultry industry

1.3.1 Global poultry

The poultry sector has been reported to be the fastest growing livestock industry, due to the increasing demand of poultry meat and eggs (Mcleod et al., 2009). Poultry meat accounts for 103.5

million tons (34.3% of global meat) of global meat statistics collected annually (FAO, 2012; Pawar et al., 2016). Chicken meat and eggs have become the mostly consumed essential protein source globally (Sebho, 2016). As a result, world poultry trade has gained much significance, rapidly increasing from 8.79 million tons in 2000 to 103.5 million tons in 2012. When compared with the 2000 trade date it had 94% increase 15 years later (GTIS, 2015). Although world poultry industry is growing exponentially, South African poultry farming is struggling to gain stability in the global poultry trade (Nkukwana, 2018).

1.3.2 South African Poultry sector

In the African continent, South Africa is the leading poultry farming country, with poultry meat and products being the mostly consumed animal protein sources (SAPA, 2016). Poultry farming constitutes the largest portion of the agricultural sector in the country, accounting for above 16% of gross domestic product (SAPA, 2016). Correspondingly, the statistics released by the Department of Agriculture, Forestry and Fisheries (DAFF) revealed that poultry farming generated revenue of R37.2 billion between 2014-2015 (DAFF, 2016). The poultry sub-sector has also been reported to utilise more than 3.3 million tons of animal feeds (with 2.1 million tons of maize) per annum (DAFF, 2016).

In the global market, South Africa's inability to compete is due to increased low-cost chicken meat imports even though feed costs escalated by 193% between 2005 and 2015 (Davids et al., 2015). Consequently, this led to serious concerns for the country's long-term sustainability (SAFA, 2016). In South Africa, feeds account for 71.3% of production costs for broiler chicken production (SAPA, 2016). When compared with global broiler industry, the South African broiler industry contributes 1.8% towards global production (Davids and Meyers, 2017). The South African poultry (e.g. broiler chicken) production market is dominated by five largest production firms, namely Rainbow Chickens Ltd, Astral Foods Ltd, Pioneer Foods; Country Bird Holdings Ltd and AFGRI Poultry (Davids 2013). These account for more than 70% of the SA poultry market (Davids and Meyers, 2017).

The main drivers of economy efficiency in the South African poultry production industry are feed costs, carcass quality and consistency (Louw et al., 2011). These market drivers pose a serious challenge for meat producers. In addition, factors that make the South African poultry industry

uncompetitive are large numbers of chicken imports from the Europe (e.g. United Kingdom, Germany and Netherlands) (Davids and Meyers, 2017). These have high carcass quality compared to locally produced ones together with, cheaper prices, and lack of local support towards poultry producers (OECD, 2014).

1.4 Global feed industry history and evolution

The commencement of an animal concentrated feed-system is not clearly indicated in the available literature, hence there are scarce documentations of procedures used in the earlier industry (Coffey et al., 2015). As time went on, practices in animal farming changed from free-range grazing to concentrated housing and animal breeding for better production purposes (Alltech, 2014). There were noticeable changes in agricultural production methods, which led to a ripple effect that initiated the continuous change in the feed industry (Pirgozliev et al., 2019). Animal nutrition and balanced formulations gained significant recognition in the early 1800s. Later, the National Research Council (NRC), which prepares recommendations for animal feed nutrient requirements, was established in the late 1800s.

The data collected by Alltech Global Survey (2014), indicated that the complete feed global supply had significantly increased from 873 million tones (estimated $350 billion) to 980 million tones (estimated $460 billions) between 2011 and 2015. More than a third of the global feed supply is from the Asian continent, which makes it the largest and leading complete feed supplier globally (Svihus, 2014). In few decades ago, the African continent has recently undergone a significant rapid growth in the number of feed mills (Alltech, 2014).

In the livestock feed industry, poultry feeds constitute 46% of the feed market share (Coffey et al., 2015). Following the poultry feed production, pig feeds and pig production has been steadily increasing since 2011 (Alltech, 2014). This contributed to the rapid increase in the GDP, and global population suggests that the industrialised feed production will continue increasing promptly, as demand for staple food grains increases (Coffey et al., 2015).

A major challenge facing feed producing industries is high input costs in purchasing raw materials such as animal protein, hence they are constantly sought to improve feed efficiency (Louw et al., 2011). However, the advancement in science and technology shows promising outcomes in addressing such challenges, particularly in the part of nutrition and feed formulation sector

(Alltech, 2015). These new developments also address monogastric animal's inability to digest raw materials in the feed, presence of toxins in feeds and feed safety for animal consumption (SAPA, 2016). Furthermore, the section of nutritional science in the industry, monitors feed digestibility and the overall health, and weight response of the chickens after feed consumption (Svihus, 2014).

There are many steps involved in feed processing, beginning from feed production to feed consumption. Feed production embroils crop farmers, feed producers, trading companies and livestock farmers, as a result, each phase of feed production adds to the costs and selling price (Pirgozliev et al., 2019). Figure 2 below illustrates the processes involved in feeding production value chain in a simple schematic diagram.

Figure 2: Diagram showing processes involved in animal feed production.

Crop farmers deal largely with the farming practice and the production of the cereal grains (and animal meals in livestock farming). Then feed producers purchase the grains (and meat meals/meat saw dust) from traders or directly from the primary farmers, and livestock farmers (e.g. poultry and pig farmer) mix the basic grains with additives to formulate basal feeds conforming or exceeding the NRC and FAO recommendations. Due to the processes and costs involved in feed production, poultry farmers spend a lot of capital in purchasing feeds. This contributes to the most costs associated with poultry farming (Lee et al., 2017). Consequently, some semi-commercial

farmers have resorted in making their own feed formulations (guided by FDA and NRC feed recommendations), branding and marketing them for returns (Louw et al., 2011).

Cereals are the primary source of energy in feedstuff for rearing poultry and pigs (Awan et al., 2000). The feed producing mills mix and present the feeds in different forms for marketing purposes, ease of digestion, to keep moisture content low and binding additives (Pirgozliev et al., 2019). Although various feed producers use different feed mixing ratios, they are guided by the NRC 1994 recommendations which ensures a better all-round growth performances. Below are some examples of how feeds are presented in the feed market (Figure 3).

Figure 3: Broiler chicken feed presented in different forms. A- crumbles, B- pellet and C- mash. Accssed on 9 May 2021: https://www.feedstrategy.com/poultry-nutrition/broiler-feed-form-particle-size-assists-performance/

Diverse farmers prefer different feed presentations (or form) for use during animal rearing (Whiting et al., 2017). As shown in Figure 3, mash feeds contain smaller feed particle sizes, hence it is used for ease of digestion and it is commonly preferred feed form for broilers and layers (FAOSTAT, 2013). On the other hand, crumble and pellet feeds forms are bigger in comparison to mash feed and are suitable for piglets. The ingredients of various feed form consist of starch cereals such as yellow maize (Gous et al., 2012). These are then mixed with protein meals which are by-products from oilseed crops (such as soybean, canola, palm kernel, linseed peanut, sesame seed, and sunflower) after oil extraction processes (Baltic, 2017). Animal protein meals such as fish m eal, blood meal, bone meal, meat meal, poultry by-products meal, and feather meal are also used to increase feed nutrition content where high amounts of anti-nutritive fiber have been used (Gous et al., 2012).

In addition to protein meals, fat-soluble and water-soluble vitamins and minerals (such as calcium, zinc, iodine, magnesium, biotin, folic acid, riboflavin and vitamin B12, E, A and K) are added to maintain the high quality and standard of growth performance in poultry and pigs (Baltic, 2017). Furthermore, additives such as DL -methionine, salt, calcium carbonate, oil, dicalcium phosphate, starch, glucose, cellulose, sand, and choline 50 as commonly used (Whiting et al., 2017). To monitor and control the feed quality, the FAO (2013) recommended approved poultry feed additives and doses together with feed formulation and portions for poultry throughout growth stages (NRC, 1994).

Poultry feeds are categorized according to the growing stage of the chicken. These feed groups are namely starter, grower and finisher, and these vary in available energy (kcal/kg) and crude protein (%) (Mokubedi et al., 2019). Starter feed consists of 3200 kcal/kg of available energy and 23% crude protein, making it suitable for chicks that are from day old to 2 weeks. Higher protein content and energy aid in rapid muscle and skeletal development (Coffey et al., 2014). Grower feed consists of 3200 kcal/kg and 20% crude protein and is fed to chicks that are from 2 to 4 weeks, while finisher (3200 kcal/kg energy and 18% crude protein) is administer to chickens that are from 4 to 6 weeks. The decrease in protein content from start to finisher, helps to maintain good muscle and skeletal growth, without accumulating excess fat (FAO, 2013).

In large scale commercial poultry farming, feeds account for approximately 65% to 70% of total production costs (Alqaisi et al., 2017; Pirgozliev et al., 2019). Soya oilcakes and maize accounting for most of the raw ingredients in feeds (Davids and Meyer, 2017). In global feed trading, Argentina, Brazil and USA are the key importers of maize and soya oilcakes at affordable price, while protein meals remain expensive than maize and soya oilcakes (Trade map, 2013). Exchange rate and South American trade polices largely affect soya oilcake prices, as South Africa is among the biggest importers of these ingredients (FAO, 2014). Soya oilcake has been reported to be the main player that determines the growth of South African poultry production (Davids and Meyers, 2017).

1.4.1 South African feeds market

In South Africa, poultry farming composes of two sub-sectors; the poultry meat and egg breeder production (Mokubedi et al., 2019). Poultry farming sector is ranked as the second largest maize

consumer in South Africa (Sihlobo, 2016). South Africa is the main maize producer in the Southern African region (FAO, 2014), producing about 50% of this crop. Maize contributes about R9.4 billion in the country's economy each year (DAFF, 2015). North West, Free State and Mpumalanga province are the leading maize producers in South Africa, accounting for 87% of total maize production country wide (Dike and Jun, 2020).

Unlike maize production, South Africa's soybean production has been lagging behind in the agricultural sector. As a result, large quantities of soybeans imported from South America and then locally crushed into soy oilcakes (Ncube, 2018). Interesting, South Africa's soya crushing capacity has rapidly increased which lead to exponential increase in soybean imports over the past five years (Davids and Meyers, 2017).

1.5 Poultry feeds and probiotics

The demand of livestock products, such as eggs and meat (particularly pig and chicken), has been increasing drastically for the past few years (FAO, 2018). Poultry products hold a second place in world meat production, with 109.02 million tons following pig meat at 112.13 million tons (FAOSTAT, 2013). In order to maintain a constant supply to the increasing demand, Krajmalnik-Brown et al. (2012) reported that poultry nutrition and overall health are of high importance. These are inseparable due to the influence feed has on the gut microbiota and the overall growth performance of the animal (Yirga, 2015). In the gastrointestinal tract (GIT), the harmonious relationship that exists between probiotic and pathogenic bacteria can easily be imbalanced by malnutrition, introduction of new bacteria, and external environment (Svihus, 2011).

The gut microbiota plays an important role in the digestion, storage and distribution of sugars and nutrients provided in the feed (Martínez Cruz et al., 2012). Therefore, it is important that the microbiota be kept stable and balanced for growth and development of the animal (Seal et al., 2018). In order to achieve that, probiotics are used as living microorganisms that can enhance the health of animals via alterations of the gut environment (Yirga, 2015). Among the commonly used probiotics, the lactic acid bacteria (LAB), which are found in many genera such as *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, and *Leuconostoc*, are the most studied due to their ability to alter the intracellular pH (via organic acid formation) and their potential to eliminate/inhibit pathogenic bacterial growth (FAO, 2016; Seal et al., 2018). LAB species have

been reported to produce lactic acid (as a post-hydrolysis product) which has pathogen inhibition elements (Russo et al., 2017). On the other hand, short chain fatty acids (SCFAs) alter the animal cell's intracellular pH, cut the excessive use of cellular energy and disturb the metabolic activities of the pathogens in the gut (Ricke, 2003; Surendran Nair et al., 2017; Zhitnitsky et al., 2017).

In addition, LAB species have also shown the ability to produce ethanol, diacetyl, reuterin and hydrogen peroxide, which can inhibit the growth of pathogens, such as *Salmonela enterica* (*S. enteria)*, *E. coli*, *Listeria monocytogenes (L. monocytogenes)*, *Candida* and *Staphylococcus aureus* (*S. aureus*), to name a few (Wang et al., 2015; Elshaghabee et al., 2016; Helal et al., 2016; Surendran Nair et al., 2017; Singh, 2018). *Lactobacillus bulgaricus* (*L. bulgaricus*) is among LAB species that produce diacetyl through uptake and metabolism of citrate (Singh, 2018). Previous studies have shown that probiotics are host dependent and work in different mechanisms, due to this, probiotics used in poultry and pigs sectors may come from different genera and species, and are specific to different pathogens (Hai, 2015; FAO, 2016; Banerjee and Ray, 2017).

1.6 Challenges in the poultry industry

The increasing demand of cereal grains (such as wheat, corn, rye, sorghum and barley) for human consumption, has led to shortages in supply for poultry feed production (Negassa et al., 2013). Wheat has become an important food security crop (followed by corn) globally. In the African continent, South Africa is among the top wheat producing countries following Ethiopia (FAO, 2017). There are various types of wheat, and bread wheat is in the top of the list of wheat production (Tadesse et al., 2018).

However, the production of these crops (i.e. maize and wheat) faces a wide range of challenges. These challenges include increased input costs (such as fertilizers, irrigation water, pesticides and farm machinery), increased heat-drought intensity, increased diseases (e.g. stresses and outbreaks) and climate change (Shiferaw et al., 2013). Among the above-mentioned challenges, climate change has more severe impact which indirectly affect poultry production (Deke et al., 2001). This includes crop yields, distribution and prices (Gbetibouo and Hassan, 2005). The changes affect local prices, the economy of the country and global trading structure. Although the practice of using plant-oil meal and animal meals as a protein sources in animal feed production is often used,

it has raised some concerns. This is attributed to potential present pathogens and drug residues in animal products, especially with the use of poultry and swine liter (Sapkota et al., 2007).

The desire for producing all round healthy and high-quality products has led to other challenges, such as excess fat accumulation in broiler chickens that are reared for meat (Verardi et al., 2012). Consequently, it has become increasingly noticeable that customers are rejecting poultry meat products with excessive fat (Novele et al., 2008). Furthermore, another major challenge in the poultry industry which affects both the feed producers and the feed consumer who rear broiler is the occurrence of fowl diseases which can be highly infectious and deadly (Haque et al., 2020). Disease causing pathogens include but not limited to *E. coli*, *Campylobacter jejini* (Vieco-Saiz et al., 2019); *Salmonella enterica* - Serovar enteritidis, *Clostridium perfringens* - Necrotic enteritis (Murate et al., 2015). The animals' ability to fight diseases is dependent on immune system development, which is largely influenced by nutrition among other things (Yang et al., 2018). Another documented challenge is the presence of indigestible and anti-nutritive agents in poultry and pig feeds, which results in malnutrition and poor growth performance in large scale commercial farming systems (FAOSTAT, 2013).

1.7 Feed additives

Feed additives (or supplements) as defined by the European Feed Standard Agency (EFSA) are products that recover the bound or lost nutritive portions of animal feeds to improve feed quality. This has a positively affect the animals' general health and growth performance. There are various feed supplements that are used in feeds, such as vitamins and micro minerals, enzymes, prebiotics, antibiotics, mold killers, probiotics, pH stabilizers, pellet binders and growth promoters (Adedokun and Olojede, 2019). On the other hand, there are essential diet elements which the poultry must be supplied with due to slow synthesis or lack thereof by the animal (e.g. vitamins and essential amino acids), there are macro- (calcium and phosphorus) and micro- mineral (zinc and iron) elements (Svihus et al., 2008).

1.8 Lignocellulosic biomass and xylan

1.8.1 Lignocellulosic biomass

Plants like grass, hard and soft wood are used in different industries such as the paper and timber. These plant contain the most abundant plant biomass classified as lignocellulosic biomass (Naidu et al., 2018). Lignocellulosic biomass is made up of three main components: lignin, cellulose and hemicellulose. The presence and amount of these components in biomass is largely dependent on plant age and species (Verardi et al., 2012). Figure 4 below illustrated the structure and main lignocellulosic biomass composition.

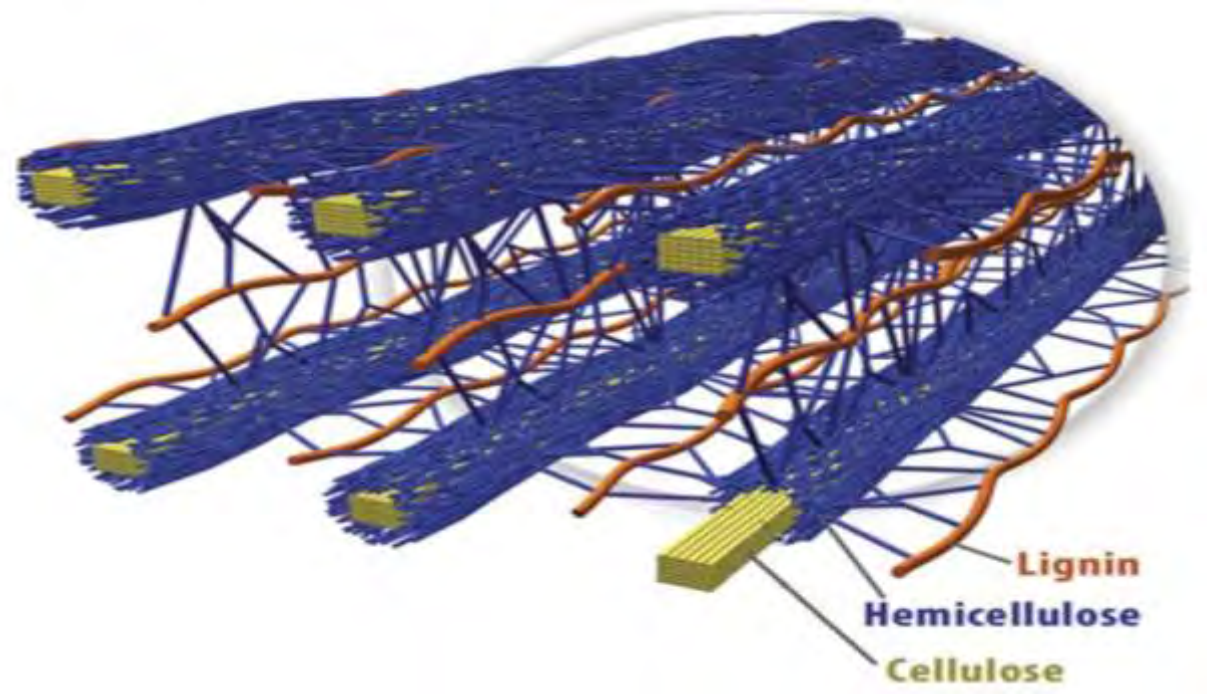

Figure 4: A diagram illustrating lignocellulosic biomass composition (Brandt et al., 2013).

Lignin makes up 10-20% of the lignocellulosic biomass, and largely made up of different types of linkages which holds the phenylpropane units in place, serving as a connective structure between cellulose and hemicellulose (Naidu et al., 2018). On the other hand, cellulose makes up 30-50% of the lignocellulosic biomass, composed of β-1,4-linkages that join together the D-glucose units (Mussatto and Teixeira, 2010). The degree of cellulose's polymerization can range from 10000 to 15000 monomers (long chains) and is the most abundant polymer (Agbor et al., 2011 and Naidu et al., 2018).

1.8.2 Hemicellulose

Hemicellulose makes up 15-35% of the lignocellulosic biomass. Previous conducted investigation reported that this heteropolymer is composed of β-1,4-linked D-pyranoside residues (e.g., xylose, glucose and mannose) (Rose, 2003; Limayem and Ricke, 2012). In comparison to cellulose, hemicellulose has shorter chains (Mussatto and Teixeira, 2010), and it is highly branched and degree of polymerization also depends on plant origin (Naidu et al., 2018). Hemicellulose solubility depends on the sugars present in it which can be pentose and hexose sugars (Naidu et al., 2018). Hemicellulose is composed of complex hetero polymers (e.g. non-starch polysaccharides) such as manna, xyloglucan and xylan. Figure 5 below illustrates a type of hemicellulose.

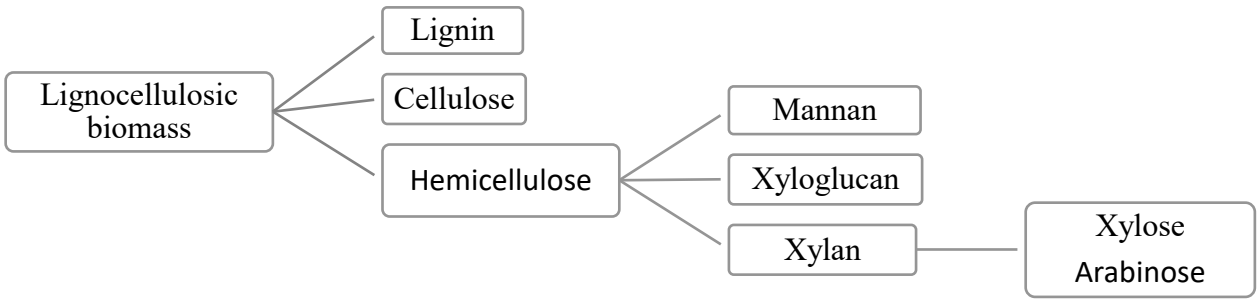

Figure 5: A simple diagram illustrating different types of hemicellulose.

Hemicellulose is divided into sub-groups, namely mannans, xyloglucans and xylans, which contain the pyranoses which are linked via 1,4-linkages (Naidu et al., 2018). Mannans are polymers that are made up of a backbone of mannan units, these mannan polymers are grouped into galactomannan, glucomannans, galactoglucomannans and linear mannans (Yildiz & Oner, 2014; Malgas et al., 2016). The second sub-group, xyloglucans, are characterised by a cellulose-like backbone and are composed of β-1,4- linked glucose units with xylose residues on C6 position

of the glucose (Girio, 2010). Xyloglucans assist the plant by keeping the structure intact and rigid through hydrogen bonding with the cellulose (Girio, 2010; Naidu et al., 2018).

1.8.2.1 Xylan

Xylans are composed of a backbone of β-1,4-xylose units with arabinose, glucuronic acid, 4-O-methyl ether or acetic acid attached to the backbone (Van Dyk and Pletschke, 2012; Bailey et al., 2016; Mafa et al., 2021). The plant origin determines the type of xylan and the branching that goes forth from the main backbone, examples of xylans are: homoxylan, arabinoxylan, glucuronoxylan and arabinoglucuronoxylans, and figure 6 below illustrates these (Naidu et al., 2018; Malgas et al., 2021).

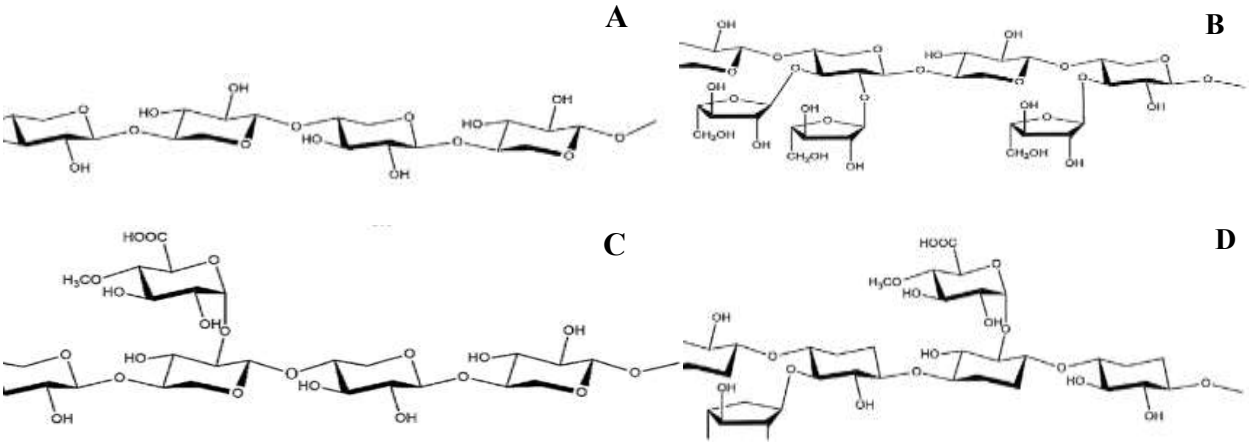

Figure 6: A diagram illustrating different types of xylan. A- homoxylan, B- arabinoxylan, C- glucuronoxylan and D- arabinoglucuronoxylan (Ebringerova, 2005).

Homoxylans are composed of linear or branched xylose units linked with β-1, 4- glycosidic linkages (Chakdar et al., 2016). According to Ebringerova & Heinze, (2000), homoxylans have been reported to exist in seaweed and in algae. Arabinoxylans are composed of backbone of β-1,4-xylose units with mono- or di-substitution of arabinose side residues attached at the C2 or C3 position in the xylose units (Bailey et al., 2016). Arabinose is the abundant but not the only side residue in arabinoxylans, acetyl groups may also be present (Zhou et al., 2016). Glucuronoxylans consists of xylose units which make the backbone and partially have acetyl groups at the C2 and/or C3 positions of xylose units. The backbone is mostly substituted with 4-O-methyl-D-glucuronic acid at every 10th xylose unit (Biely et al., 2016). Arabinoglucuronoxylans are also composed of the main xylan backbone with arabinofuranose (at every 5-12 xylose unit) and glucuronic acid (at

every 5-6 xylose unit) residues attached at the O2 and O3 positions of the xylose units (Yamasaki et al., 2012). Arabinose may have ferulic acid or coumaric acid attached via ester linkages to the C5 position (Yamasaki et al., 2012).

1.9 Xylanases

1.9.1 Introduction to xylanases

Xylanases (endo-xylanases) are a group of glycoside hydrolyase (GH) enzymes (EC 3.2.1.8), that target the xylan backbone at the β-D-1,4-linkage (Shi et al., 2014). There is quite a number of enzymes that can be utilised to depolymerise the complex substituted xylan, such as α-glucuronidase (EC 3.2.1.139), acetylxylan esterase (EC 3.1.1.72), α-L-arabinofuranosidases (E.C.3.2.1.55), *p*-coumaroyl esterase (3.1.1.B10) and ferulic acid esterase (EC 3.1.1.73) (Walia et al., 2017; Malgas et al., 2019; Mafa et al., 2021; Malgas et al., 2021). The hydrolysis of xylan by xylanase produces xylose and xylooligosaccharides (XOS); which are further hydrolysed by xylosidases (EC 3.2.1.37) to xylose (Shi et al., 2014). Xylanases are grouped according to the molecular weight, isoeletric point (pI), similarities in the amino acid sequences, crystal structure, kinetic properties (Biely et al., 1997) and they are categorized into glycoside hydrolase (GH) families and subfamilies. Structural, and genomic data of xylanases is accessible on Carbohydrate-Active enZYmes (CAZy) database. Xylanases belong to the many families such as GH5, GH8, GH9, GH10, GH11, GH16, GH30, GH43, GH44 and GH51, to mention a few (https://www.cazy.org). The families GH7, GH8, GH10, GH11, and GH43 have been reported to have a single unique catalytic domain, whereas the families GH5, GH16, GH44 and GH51 have two catalytic domains Collins et al., 2005).

1.9.2 Xylanase mode of action

The mode of action of xylanases may fall in the two categories, (i) retention: double displacement mechanism with intermediate formation of an oxo-carbonium and an α-glycosyl or (ii) inversion, where there is a single displacement mechanism of the center with aspartate and glutamate catalytic residues (Subramaniyan and Prema, 2002; Lombard et al., 2014). The GH5 family consists of xylanases from many sources, such as *Erwinia chrysanthemi* and *Bacillus subtilis*, which are well documented to hydrolyze linear hemicellulose backbone (Gallardo et al., 2010). Similar to GH5,

the bacterial GH30 family (specifically from subfamily 8) are well established for the enzymes unique glucuronoxylan specificity. The fungal GH30 xylanases, show diverse β-1,4-xylanase-related functional properties (Maehara et al., 2017). The GH30 glucuronoxylanase from *Streptomyces turgidiscabies* displays hydrolytic activity on the 4-*O*-methyl-glucuronic acid (MeGlcA) substituted branches of glucuronoxylan to produce glucuronic acid (GlcA) and XOS (Maehara et al., 2017).

1.9.3 The structure and biochemical properties of GH11 xylanases

The family GH11 xylanases have differing molecular weights even smaller than 30 kDa and some have thermostability ranging from 45-50ºC (He et al., 2019), with others having been reported to be stable at temperatures beyond 70ºC (Zhang et a., 2011) which are classified as thermostable (e.g., 45 – 80 ºC). Several studies have been conducted in an attempt to enhance the thermostability of GH11 xylanases by taking advantage of the structural flexibility found in these enzymes (Gilbert, 2010; Paes et al., 2012; Chadha et al., 2019). The GH11 tertiary structure resembles a closed right hand (Paës et al., 2012 and Chadha et al., 2019) with two primary anti-parallel β-sheets and one single major α-helix that forms 'fingers and a palm' (Figure 7).

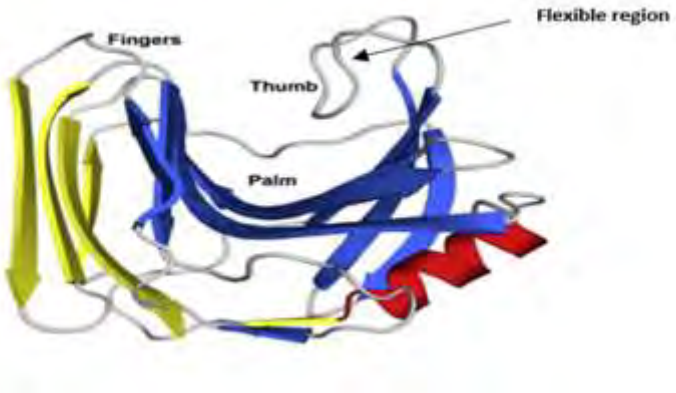

Figure 7: Atertiary structural illustrating the structure of the glycoside hrdolase (GH) family 11 xylanases (Paes et al., 2012).

The fingers and the thumb close the palm in creating the catalytic cleft, which is lined mostly by conserved nucleophile catalytic residues Arg110 and Gln124. The GH11 xylanase structure has been reported to be compact as it mimics a closed hand (Chadha et al., 2019), though this is the common structure, a GH11 xylanase structure strain XynCDBFV from ruminant *Neocallimastix*

patriciarum fungus was reported to be unique (Cheng et al., 2014). As the chemical properties of xylanases differ, so do the sources of origin, and molecular weight.

The main source of commercially available xylanases is produced in filamentous fungi, particularly from the genera of *Chytriomycota*, *Basidomycota*, *Ascomycota*, *Penicillium*, *Aspergillus*, *Fusarium* and *Trichoderma* (Juturu and Wu, 2011). The *Trichoderma* genus has approximately 160 species (Srivastava *et al.,* 2014), but for the interest of this study the focus was on *Trichoderma viride*. A xylanase from (*T. viride*) was utilised by our group in a synergy study where a cocktail of GH10 and GH11 xylanase were used synergistically to degrade lignocellulosic biomass (Malgas and Pletschke, 2019). Xyn2A has been evaluated by our group in synergy studies and it revealed promising traits such as high specific activity. There is, however, limited literature concerning the application of Xyn2A from *T. viride* in animal feeds. In an attempt to acquire insight into the activity of the enzyme, we partially characterized it and investigated its potential as a stand-alone enzyme in monogastric animal feeds.

1.10 Applications of xylanases in feeds and their advantage

The demand for xylanases increases as different industries discover their usefulness in one way or another, which has resulted in the global xylanase market rapid growth and the global market is expected to increase with shares from $300 million in 2018 to $500 million by year 2023 (Chadha et al., 2019). Xylanases are used in many industries such as baking industry to enhance dough quality, paper and pulp industry to pulp bleaching and de-ink re-cycled paper, food and beverage industry for juice clarification, and animal feed industry to improve feed digestibility (Walia et al., 2017; Fitz et al., 2018; Matthiesen et al., 2020).

Years of collected literature data has revealed that xylanases have a beneficial role in feeds by liberating encapsulated nutrients through the hydrolysis of non-degradable fibres (Santos et al., 2013; Fitz et al., 2018; Arczewska-Wlosek et al., 2019). Early studies by Pettersson and Aman (1989) have reported that GH 11 xylanases effectively degrades insoluble NSPs, which leads to the release of protein, starch and minerals that are encapsulated in the feed. Xylanases have also been reported to improve nutrient digestibility (Chadha et al., 2019), reduced small intestinal relative weight and length (Engberg et al., 2004).

1.10.1 Growth performance (body weight)

A study conducted by Arczewska-Wlosek et al. (2019) reported a great improvement in body weight and feed conversion rate of male Ross 308 broilers (1-21 days old) that were fed rye-based diets supplemented with a xylanase. These findings were similar to those reported by Santos et al. (2013), who observed that xylanase inclusion on rye-based diets resulted in increased growth performance in the first 21 days of a chicken's life. On the other hand, a study that investigated the effect of xylanase supplementation on rye-based diets reported no effect on feed intake rate, but significant impact on body weight increase (Mendes et al., 2012).

1.10.2 Nutrient digestion and absorption

A previous study by Kubis et al. (2020) reported that xylanase supplementation had a positive effect on feed digestibility and absorption. In the previously mentioned study, the crypt deepened on chickens fed xylanase supplemented feed, the deepening of the crypt is indicative of high overall body activity (e.g. fast cell proliferation and increased tissue re-pair demands) (Fan et al., 1997; Iji et al., 2001). The study also reported goblet cell decrease (these cells are responsible for mucus production). As far as goblet cells are concerned, other studies reported an increase in goblet cell production, which correlates with crypt deepening which implies an increased demand for mucus secretion (Langhout et al., 1990 and Silva and Smithard, 2002; Kubis et al., 2020;).

1.10.3 Feed intake and digesta viscosity

Many studies support the claim that adding xylanases improves feed conversion rate (FCR) by 1.5-7% when added to wheat and rye-based diets that are low viscosity or high viscosity diets (Kalmendal and Tauson, 2012; Zhang et al., 2014; Kiarie et al., 2017). There are inconsistent results reported on the effect of xylanase supplemented diets on changes in feed intake of modern broilers (Albuquerque et al., 2008; Classen, 2017 and Nassar et al., 2019). A recent study by Matthiesen et al. (2021) has reported that Ross 308 broiler chickens have the ability to adjust feed intake as per their different nutritional requirements are concerned. Low amounts of apparent metabolisable energy (AME) content likely influence feed intake and body weight (Nassar et al., 2019). Xylanase supplementation in wheat-based diets (~780 g/kg) has been reported to greatly

improve the feed conversion rate (FCR) by 9% (Choct et al., 2014). These findings agreed with findings by Matthiesen et al. (2021), which reported FCR to be significantly improved in diets supplemented with xylanase compared to those without.

Another study showed that xylanase supplementation decreased viscosity of the intestinal digesta (Danicke et al., 1997; Silva and Smithard, 2002; Arczewska-Wlosek et al., 2019). Previous studies have also reported that soluble xylan contents influence digesta viscosity in rye and wheat-based diets, and that soluble NSPs from cereal grains have a high water-holding capacity (Pettersson and Aman, 1989, Bach Knudsen, 2014 and Chadha et al., 2019). Diet composition influences protein digestibility, as studies have reported improved ileal and jejunal digestibility of protein and mineral when xylanases were added in diet (Liu and Kim, 2017; Zhang et al., 2014; Matthiesen et al., 2021).

1.10.4 Gut microbiota and organic acid formation

Addition of xylanase on the feed resulted in organic acid formation increase (Kubis et al., 2020), and previous studies agreed with these findings reporting more short-chain fatty acids (SCFAs) in the caeca than in the small intestinal section of the digestive system (Choct et al., 1999; Koniecza et al., 2019). The increase of SCFAs concentration directly indicated the activity of the gut microbiota as affected by the feed (Kubis et al., 2020).

A study by Lee et al. (2017) investigated the effect of xylanase supplementation on the performance and intestinal contents of fermentable sugars and volatile fatty acids (VFAs) of broilers fed wheat-based diets as they age. The GH11 xylanase used was from *Nonomea flexuosa* (Econase XT25), the minimum estimated xylanase activity was 16000 BXU/kg feed. The recovered xylanase activity from starter feed was 8800 BXU/kg, and from grower feed it was 13 500 BXU/kg (Lee et al., 2017). The results of the study revealed that xylanase supplementation in feed reduced proportion of branched-VFAs in total SCFAs and protein fermentation (Lee et al., 2017). This was suggesting that longer-chain XOS were produced by xylanase supplementation as similar findings were reported by van Craeyveld et al. (2008) which reported that longer-chain XOS (average 61 degree of polymerization (DP)) reduced branched-chain SCFAs significantly when compared to average chain (<3 DP) XOS.

Another study investigated the effect of commercially available XOS supplementation on laying hens' gut microbiota and SCFAs. The results revealed that cecal *Bifidobacterial* enumeration increase linearly, *E. coli* enumeration decreased linearly and there was no effect on *Lactobacillus* enumeration as XOS concentration increased. When it came to SCFAs, there was no noticeable effect on propionic acid, linearly increase in butyrate and acetate as XOS concentration increased (Ding et al., 2018).

1.11 Problem statement

The use of xylanases in feeds was introduced decades ago and gained popularity in the 1980s (Duke, 1986). As a result, adequate quantities of data which demonstrate their role and effect in broiler chicken growth performance and feed digestibility have been documented. Xylanases differ widely in molecular weights, mechanistic, substrate specificity and physico-chemical properties (Mattheisen et al., 2021). Furthermore, the efficacy of xylanases is also determined by the type of cereal grains, solubility of NSPs, species of animals (e.g. chickens, turkey and ducks), and presence of xylanase inhibitors in digestive juices (Bach Knudsen, 2014; Nadsen et al., 2018).

A clear understanding of enzyme characteristics, such as stability and substrate specificity, helps in selection of industrial production process to produce the xylanase of interest (Bhardwaj et al., 2019). Interestingly, unlike cellulose, the xylan backbone is decorated with residues making it a complex branched polymer. The decoration on the xylan affects the hydrolysis mechanism of xylanolytic enzymes, thus the hydrolysis of the backbone is not linear (i.e. zero order reaction rate), and occurs randomly (Schendel et al., 2015; Beily et al., 2016). A study conducted by Malgas and Pletschke (2019), where commercial xylanases from GH11 and 10 families (Xyn2A and XT-6 from *T. viride* and *Geobacillus stearothermophilus*) were used to synergistically hydrolyse beechwood xylan demonstrated that Xyn2A was significantly effective at hydrolysing xylan on its own due to its high activity and substrate specificity. Based on the above-mentioned previous finding by our group, this study pursues to investigate, the use of Xyn2A to enhance the nutritional value of the animal feed by cleaving the xylan backbone to xylooligosaccharides, which contribute to the gut health of animals and decrease digesta viscosity (He et al., 2019). Thereby improving the overall health and the production of chickens and pigs.

1.11 Aim and Objectives

1.11.1 Aim

This study sought to investigate the effects of utilising a GH11 endo-xylanase (Xyn2A) isolated from *T. viride* as an additive in monogastric animal feeds (GenBank accession number: AAS31740).

1.11.2 Objectives

To achieve the aim, the following objectives were addressed:

I. To clone and express Xyn2A from *T. viride* in *E. coli* BL21 (DE3) cells;

II. To purify the recombinant xylanase using an immobilised affinity chromatography (IMAC) column;

III. To conduct comparative biochemical characterisation of the recombinant enzyme (Xyn2A) produced in *E. coli* with another recombinant version expressed in plants,

IV. To perform xylan hydrolysis using Xyn2A and its analysis using thin layer chromatography (TLC) and microscopy (SEM),

V. To evaluate the prebiotic effect of the XOS produced from animal feed hydrolysis by Xyn2A using selected monogastric gut associated probiotic bacteria.

Chapter 2: Expression and Purification of Xyn2A

2.1 Introduction

Protein engineering techniques, such as recombinant protein expression, are increasingly becoming common in the biological science fields and are a fast-growing market (Rosano and Ceccarelli, 2014; Chen et al., 2019). Recombinant protein technology involves inserting a foreign protein gene in a host genome so that it can be expressed along with the host's inhouse proteins (Peebo and Neubauer, 2018). Different organisms have different proteins, which help with keeping the organism well (e.g., maintaining homeostasis); thus, choosing a host to recombinantly express the target protein is very crucial (Gomes et al., 2018). There are several established expression systems available since there is no single host suitable for every expression (Stoger et al., 2002; Chen et al., 2019).

Established hosts range from bacterial cells, fungi (and yeast) and mammalian cells, such as Chinese hamster ovary (CHO) and baby hamster kidney cells (used for monoclonal antibody production, gene therapy vectors, viruses and examining fundamental aspects of protein processing), insect cells for baculovirus expression and plants (Ma et al., 2003; Palomares et al., 2004; Stoger et al., 2005; Ferrer-Miralles et al., 2009). Hiatt et al. (1989) demonstrated the ability of tobacco plants to produce functional antibodies, then later several other studied were published where plants were utilised for vaccine agent production and blood enzymes (Stoger et al., 2002; Claparols et al., 2004, Twyman et al., 2005).

Production of recombinant pharmaceutical proteins in plants is called molecular or plant pharming (Twyman et al., 2005). Molecular pharming was once dismissed at its early development due to low scale production when compared to microbial and mammalian system production (Sabalza et al., 2014). Current research has demonstrated several strategies that combated the previous challenge, such as producing glycoforms of proteins and increasing yields (Aviezer et al., 2009). Plants accomplish complex eukaryotic protein folding, produce no toxins and pharmaceutical proteins can be expressed at a large scale with low costs (Ma et al., 2003; Kunka et al., 2005; and Stoger et al., 2005).

Escherichia coli (*E. coli*) is the commonly used expression system and is a versatile bacterium with several advantageous features as an expression host. These include rapid growth, uses inexpensive carbon sources, possesses an inducible a T7 RNA polymerase for high protein yields, and easy to scale up for fermentation (Olaofe et al., 2010). In the past, several studies were conducted to improve the induction of protein expression in *E. coli*, currently isopropyl-β -D-1-thiogalactopyranoside (IPTG) is the most effective induction method (Srivastava and Mukherjee 2005; Olaofe et al., 2010; Schmideder et al., 2016). The *E. coli* (DE3) strain is advantageous due to them having an ability to repress the leaky expression, reducing cases of expression of toxic proteins in the host (Braind et al., 2016).

Vectors are commonly used to transport the target protein gene into the suitable expression host. There are several vectors used for the cloning and expression processes (Rosano and Ceccarelli, 2000). Cloning vectors are used to amplify the target gene and expression vectors used to translate the target gene to protein (Braind et al., 2016). The pET plasmids are commonly used for their high protein yield, due to the selective T7 RNA polymerase that manipulates the expression cell resources for the target protein expression, leading to more than 50% target protein production within a few hours after induction (del Solar and Espinosa, 2000; Rosano and Ceccarelli, 2014). These plasmids have an ability to repress the target gene before induction and give room to optimise target protein expression.

The target genes are inserted into the plasmid using restriction sites and fusion tags (Gomes et al., 2018). There are various fusion tags (depending on the plasmids), which can be utilized for western blot purification, His-bound resin purification, or protein folding (Braind et al., 2016). In this study, the target protein was expressed using a pET27b(+) vector which contained T7 and T7lac polymerase, HSV tag, 6-His tag for purification using immobilised metal affinity chromatography (IMAC), and kanamycin resistance gene as the selective marker of choice. Recombinant technology is fast improving with companies such as GenScript™ that can design a plasmid construct based on and system of expression, the *xyn*2A expression plasmid was synthesised. The target gene (*xyn*2A) was inserted in the multiple cloning site (MCS) between NotI and EcoRI restriction enzymes (https://www.genscript.org) and expressed in *E. coli* cells (Appendix A). The successful purification of Xyn2A involved 30 and 3 kDa cut-off amicon filters and/or IMAC affinity column. Another Xyn2A was produced via transient expression in tobacco plants by Cape

Bio Pharms (Pty) Ltd. The tobacco plants were grown in indoor hydroponic grown-rooms, syringe-infiltrated for six weeks with the target protein, and plant leaves were harvested, protein extracted and purified (https://www.capebiopharms.com).

Table 1: Biochemical and kinetic parameters of a select few xylanases from bacterial and fungal origin.

Bacterial xylanase									
Xylanase	**Origin**	**Substrate**	**Reaction conditions**	**Km***	**Vmax***	**Kcat**	**Kcat/Km**	**Specific activity***	**References**
SV-34S	*Bacillus sp.*	-	8.0 pH, 55°C	3.7 mg/mL	133.33 IU/mL	-	-	3417.2 IU/mL	Mittal, Nagar, Gupta, 2013
Cpt20	*Actinomadura sp.*	-	5.0 – 10.0 pH, 60 - 90°C	1.55 mg/mL	-	-	-	-	Taibi et al., 2012
CGMCC 1669	*Penicillium sp.*	-	4.5 – 9.0 pH, 40°C	22.2 mg/mL	15 105.7 µM/mg/min	-	-	-	Liu et al., 2010
MTCC 5112	*Enterobacter sp.*	-	9.0 pH, 50°C	3.3 mg/mL	5000 µM/mg/min	-	-	-	Khandeparkar and Bhosle, 2006
-	*Bacillus licheniformis*	-	6.0 – 7.5 pH, 60°C	3.33 mg/mL	1111 µmol/mg/min	-	-	28.71 U/mg	Archana and Satyanarayana, 2003

Fungal xylanase									
TIXyn A	Trichoderma lanuginous		pH 6.0, 50°C	4.38 g/l	400U/mg	7.025 g/L	1.604 L/s/g	221.56 U/mg	Wu et al. 2020
RrXyn 11A S	Rhizophlyctis rosea	Beechwood xylan	pH 7.0, 40°C,	21.63±3.43 mg/mL	219.5 U/mg	106.25 ±10.53 (s^{-1})	-	-	Huang et al. 2018
RrXyn 11A L			pH 7.0, 50°C	18.83 ± 3.56 mg/mL	210.7 U/mg	54.80± 6.21 (s^{-1})	-	-	Huang et al. 2018
XynM	*Talaromycin amestolkiea*	Birchwood xylan	pH 3.0 -4.0, 50°C	5.5 ±1.4 mg/mL	129.0 ±12.6 mg/mL	-	-	-	Nieto-Dminguez et al. 2017
-	*T. viride*	Larchwood xylan	5.0 pH, 53°C	4.5 mg/mL	160 µmol/mg/ min	-	-	152.6 U/mg	Ujiie et al., 1991

* Parameters may share different units. - not available

Xyn2A is a GH11 xylanase which is originally produced by *T. viride* of the *Trichoderma* family of filamentous fungi with a genome ranging from 31-319 Mb (Srivastava et al. 2014). Xyn2A is 20.773 kDa (approximately 21 kDa) in size and consists of 190 amino acids from *T. viride* with a UniParc ID: UPI0000034D34 and GenBank ID: AAS31740 (Appendices-Figure A1).

2.2. Aims and objectives

2.2.1. Aim

To express and purify *T. viride* xylanase (*xyn2*A) in *E. coli* BL21 (DE-3) competent cells in large quantities for research purposes.

2.2.2. Objectives

- To express recombinant Xyn2A with a pET-27b expression system in *E. coli* BL21 (DE-3) competent cells;
- To purify Xyn2A by diafiltration using Amicon filters and affinity chromatography using an IMAC column;
- To conduct Xyn2A protein determination with the Bicinchoninic acid copper assay (BCA);
- To use SDS-PAGE to visualize recombinant Xyn2A protein;
- To confirm recombinant Xyn2A identification by zymography;
- To quantify Xyn2A enzyme specific activity.

2.3 Materials and Methods

The gene coding for Xyn2A (GenBank accession number: AAS31740) was synthesized as a codon optimised gene for expression in pET27b expression vector by GenScript (Piscataway, NJ, USA). *E. coli* BL21 (DE3) competent expression host cells were purchased from ThermoFisher Scientific (Germering, Germany). A second Xyn2A was also expressed and purified in tobacco plants by the Cape Bio Pharms company, Cape Town, Western Cape, RSA(https://www.capebiopharms.com). The xylanase expressed in *E. coli* cells was designated as $Xyn2A^{E}$ and the one expressed in tobacco plants was designated as $Xyn2A^{C}$.

2.3.1 Induction and Expression of recombinant Xyn2A

Freshly cultured *E. coli* BL21 (DE3) cells (ThermoFisher) were transformed with a pET27b(+) plasmid containing the *xyn2A* gene. The *E. coli* cells that contained the target gene were used for the induction study. The induction study was conducted under the following parameters: different conical flasks, each containing 250 mL of 2×Yeast extract Tryptone (YT) broth that had kanamycin to a final concentration of 50µg/mL was inoculated with the *E. coli* cells containing target gene (1% v/v). The inoculated broth was incubated at 37°C with shaking at 150 rpm until the optical density at 600 nm (OD_{600}) reading between 0.5 and 0.8. When the required OD600 was obtained, the isopropyl β-D-1-thiogalactopyranoside (IPTG) at different concentrations (ranging from 0.1 mM - 0.4 mM) was added at temperature 20°C for 2 h.

When the effect of IPTG on protein concentration was observed, a different conical flask with 250 mL of 2× (YT) broth (1.6 % (w/v) tryptone; 0.5 % (w/v) NaCl and 1 % (w/v) yeast extract powder) with 50 µg/mL of kanamycin inoculated with 1% v/v culture. When the desired OD_{600} reading was obtained, IPTG was added at a final concentration of 0.5 mM and incubated at 20ºC with shaking at 150 rpm. Samples were periodically collected every hour from 0 to 8 hours. When the induction study was completed, recombinant Xyn2A was finally expressed (as per above mentioned protocol) at 20ºC, 0.5 mM IPTG with 150 rpm shaking for 5 hours.

2.3.2 Purification of recombinant Xyn2A^{E}

After Xyn2A^{E} induction and expression, the cells were harvested by centrifugation at 15, 316 ×g in 250 mL Beckman centrifuge tubes at 4ºC for 10 minutes. The culture pellet was resuspended in 10 mL of lysis buffer containing 300mM NaCl and 50 mM NaH_2PO_4, 1 mg/mL lysozyme at pH 8.0. The resuspended pellet was sonicated at 40 Hz five times for 10 seconds bursts with 30 second rest intervals in between each burst (Vibra Cell Sonics Materials). The lysate (crude) was filtered using 0.45 µm syringe filters to remove cell debris and diluted in ratio 1:3 (to 30 mL) with lysis buffer.

A cell-free lysate (5 mL) bed volumes were directly loaded onto a nickel (5 mL Ni+-chelating) affinity column to bind the 6× His tagged Xyn2A with incubation for 2 hours at 4ºC with shaking. Recombinant protein was then washed with two bed volumes of cold wash buffer (containing 50 mM NaH_2PO_4; 300 mM NaCl; 10 mM imidazole; pH 7.4. After removing non- specifically bound protein, the 6× His-tagged recombinant protein was eluted with elution buffer (300 mM NaCl, 50

mM sodium phosphate, 200 mM imidazole, pH 7.4) into two elution fractions. The elution fractions were loaded on the Amicon filters with a 30 kDa cut-off and the sample was centrifuged at 13 500 x g for 15 min at 4 °C, then concentrated with 3 kDa cut-off Amicon filters.

2.3.3 Protein determination

The Bicinchoninic acid copper assay (BCA) method was used to determine protein concentration using a standard solution of fifty parts Bicinchoninic acid solution with one-part of copper (II) sulfate solution to make the reagent. Each protein sample (25 µL) was made by mixing the reagent (230 µL) to a total volume of 255 µL. The blank samples were with deionized water (25 µL) and BCA reagent (25 µL). All samples were incubated at 50 ℃ for 30 minutes, covered with foil, and absorbance readings were recorded at 562 nm (BioTeK Epoch 2, USA)

2.3.4 Visualisation of the recombinant protein

2.3.4.1 SDS-PAGE

The total protein produced was visualised on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad Chemidoc XRS. The SDS-PAGE buffers were prepared as follows: A 5× loading 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 (pH 6.8)) was prepared. A resolving gel buffer, 1.5 M Tris-HCl, pH 8.8 (dissolved 18.15 g of tris(hydroxymethyl)aminomethane in 100 mL of dH_2O and was adjusted to the correct pH using 5M NaOH) was made. Stacking gel buffer, 0.5 M Tris-HCl (pH 6.8) was made (dissolved 8 g of Tris base in 100 ml of dH_2O and was adjusted to correct pH using 2M HCl). A 10× tris-glycine (TGB) running buffer was prepared by dissolving 30 g Tris, and 140 g of glycine and 10 g of SDS in 1L of water. From the 10× TGB buffer, 1× TGB running buffer was prepared.

The protein samples were analysed using a 12% resolving gel, and 4% stacking gel which polymerised with the addition of freshly prepared 10% (w/v) ammonium persulphate and tetramethyethylenediamine (TEMED), according to protocol described by Laemmli (1970). The protein samples were mixed with SDS loading buffer in a ratio of 1:4 and incubated at 95°C for 3 min in the presence of 5% (v/v) β-mercaptoethanol) and then loaded on the gel. Electrophoresis

was carried out at 120V (Bio-Rad Power Pac™ Basic), and the gel run for 45 min. For band visualisation, Coomassie brilliant blue R250 staining method was employed according to Fazekas et al. (1963) and the image was captured using a Bio-Rad Chemidoc XRS.

2.3.4.2 Silver stain

After running the gels (as per the SDS-PAGE protocol), instead of staining with Coomassie brilliant blue, silver stain (Pierce Silver stain kit, Thermo Scientific, Rockford, IL 61105 USA) was used. Gels were rinsed for 5 minutes (two times) with deionized water and fixed in 30% ethanol:10% acetic acid solution for 15 minutes (twice). After the time has lapsed, the gels were washed (twice) for 5 minutes with deionized water, and then sensitized with ready-made sensitizer solution (1:500 ratio of sensitizer: water) for 1 minute after which they were washed twice for 2 minutes. The gels were then stained with a ready-made stain solution (1:50 ratio of enhancer: stain) for 30 minutes, after which the gels were developed in a developer working solution (1:50 ratio of enhancer: developer) for 1-3 minutes until bands appeared. The development reaction was stopped with 5% acetic acid for 10 minutes and the image was captured using a Bio-Rad Chemidoc XRS.

2.3.4.2 Activity gel (zymography)

The Native-PAGE was prepared as described in 2.4.1 with a few modifications (no SDS in loading buffer and gels, total concentration of 0.1% wheat flour arabinoxylan). The gels were incubated in 2.5% Triton X-100 for 60 min after electrophoresis, then incubated at 50°C in sodium citrate buffer (pH 5.0) for 30 min. The gels were stained with 0.1% Congo Red stain (0.1 g Congo Red powder in 100 mL deionized water) for 15 min, and destained in a 1M sodium chloride solution (8.55 g NaCl in 500 mL deionized water), then fixed in 5% acetic acid.

2.3.5 Activity assay

Substrate solution (of the various substrates) of final concentration of 10 mg/mL was prepared by mixing 300 µL of substrate (sodium citrate, pH5.0, 50 mM) and 100 µL of enzyme to make a 0.01 mg/mL protein concentration. The sample reactions were prepared while working on ice and then incubated with shaking at 50ºC with sharking at 150 rpm. The negative control was 400 µl of

buffer, the substrate control was 300 µL of substrate with 100 µL of buffer, and the enzyme control was 300 µl of buffer mixed with 100 µl of enzyme.

The reaction was stopped after 30 minutes by incubation at 100°C for 5 minutes, reducing sugar content was determined using dinitrosalicylic (DNS) assay (Miller, 1959). The 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) was added to develop the reducing sugars. From the reactions, 150 µL of the sample and 300 µL DNS reagent was mixed and boiled at 100°C for 8 minutes, then cooled for absorbance values to be recorded at 540 nm (BioTeK Epoch 2). A unit of xylanase activity was defined as the amount of enzyme liberating 1 µmol of reducing sugar equivalent per minute (U) under standard conditions and regression equation (i.e. D-xylose used to construct the curve) was used to convert concentrations.

2.4 Results

2.4.1 Induction study

2.4.1.1 Induction with different IPTG concentrations

The induction study was conducted under the following parameters until culture OD_{600} reading reached 0.8. The assessed parameters for induction were: IPTG concentration ranging from 0.1 mM - 0.4 mM at a temperature of 37°C with a shaking speed of 150 rpm for 2 h. The expressed protein concentration was determined at the induction conditions (Figure 8).

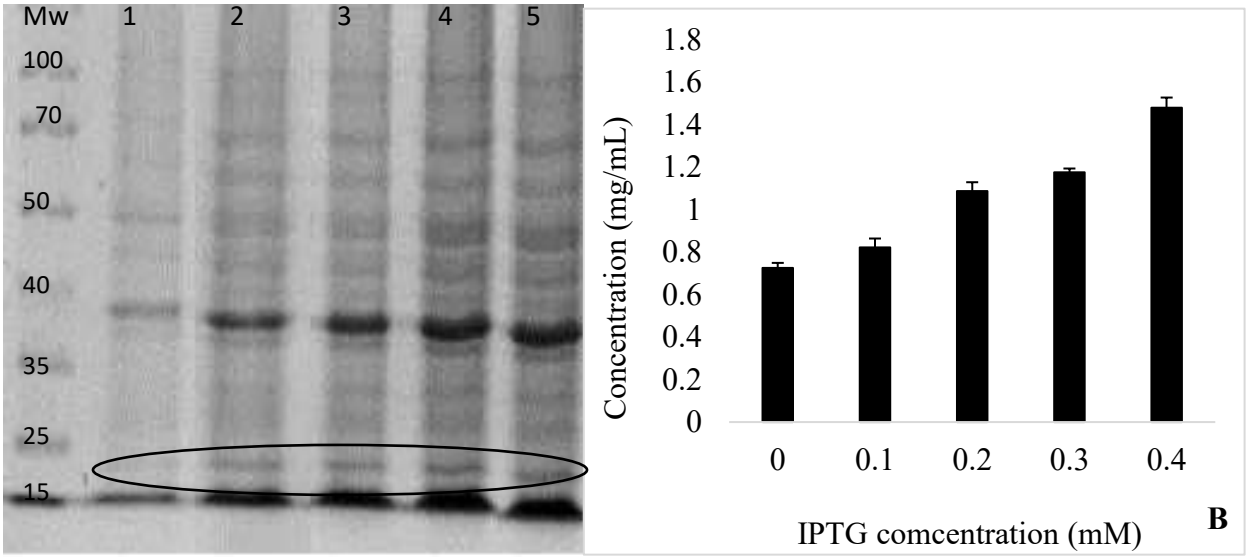

Figure 8: Induction of transformed cells to express rcombinant $Xyn2A^E$ at diffrent IPTG concentrations (mM) for 1 h, 12% SDS-PAGE analysis stained with Coomassie Brilliant blue. molecular weight marker-Mw (Spectra multicolor broad range protein ladder, cat no. 26634), Lane 1- uninduced, Lane 2: 0.1 mM, Lane 3: 0.2 mM, Lane 4: 0.3 mM, Lane 5: 0.4 mM. Xyn2AE marked with a black circle. B- Protein estimation using BCA standard curve. Values presented as mean±SD. n=3.

Induction of BL21 *E. coli* competent cells with different IPTG concentrations for expression showed that protein concentration increased generally when compared to the uninduced cells. As it was expected, $Xyn2A^E$ bands appeared with the co-expressed proteins from the expression host cell which also were increasing in concentration as IPTG concentrations were increasing. The co-expressed host-cell protein bands seemed darker than the recombinant $Xyn2A^E$ bands. The uninduced sample had 0.73 mg/mL, 0.1 mM sample had 0.82 mg/mL, 0.2 mM sample had 1.09 mg/mL, the 0.3 mM sample had 1.18 mg/mL and the 0.4 mM sample had 1.48 mg/mL of total

protein, respectively. Xyn2A^{E} band was also getting darker with the increasing IPTG concentration.

2.4.1.2 Expression of recombinant enzyme at constant IPTG concentration over different time duration

A time study was conducted to attempt optimising expression time for recombinant protein. At a constant IPTG concentration of 0.5 mM, recombinant protein was expressed at different time intervals (h). Figure 9 (A & B) below shows that the recombinant protein was getting darker, implying increase in protein concentration the longer the expression time.

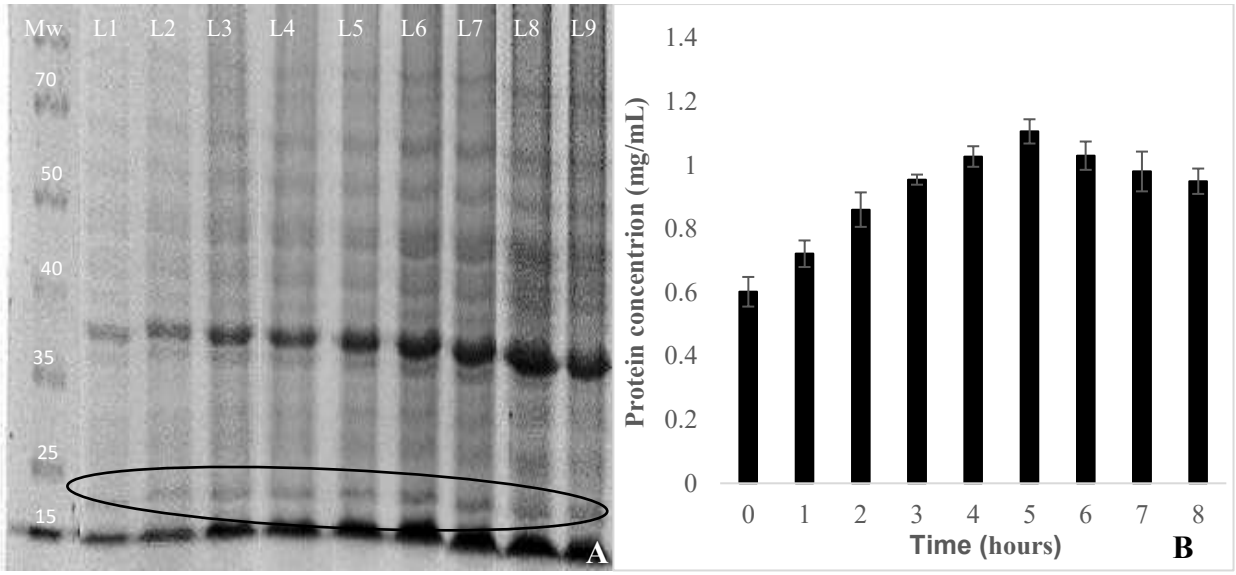

Figure 9: Expression of recombinant Xyn2A^{E} at a constant IPTG concentration overtime (0.5 mM) 12 % SDS-PAGE analysis stained with silver stain (Pierce Silver stain kit, Thermo Scientific, QL224435). Mw: molecular weight marker (Spectra multicolor broad range protein ladder, cat no. 26634), Lane 1- uninduced, Lane 2: 1 h, Lane 3: 2 h, Lane 4: 3 h, Lane 5: 4 h, Lane 6: 5 h, Lane 7: 6 h, Lane 8: 7 h and Lane 9: 8 h. Xyn2A^{E} marked with a black circle. (37°C, with 150 rpm shaking). B: Protein estimation after expression using BCA standard curve. Values presented as mean ± SD, n=3.

The longer the expression samples were left to run in the incubator with shaking (150 rpm), the protein was expressed as the estimation with BCA assay shows in figure 9B, the standard was generated with bovine serum (appendix B-figure B1). The results show that the optimal expression time was anywhere between 4 to 6 hours, with the best expression time at 5 hours. The expression of recombinant Xyn2A appeared to be increasing with the increasing incubation time, even though

the target protein was lightly expressed in contrast to the host-cells proteins. The uninduced a total protein of 0.60 mg/mL, at 1 h the concentration was 0.72 mg/mL, at 2 h it was 0.86 mg/mL, at 3 h it was 0.95 mg/mL, at 4 h it was 1.03 mg/mL, at 5 h it was 1.11 mg/mL, at 6 h it was 1.03 mg/mL, at7 h it was 0.98 and at 8 h 0.95 mg/mL.

2.4.1.3 Zymography

Confirmation of the recombinant Xyn2A bands was achieved by the use of native PAGE activity gels (zymography). $Xyn2A^{C}$ and $Xyn2A^{E}$ was run alongside. The molecular marker lane was cut and stained with silver stain, while the rest of the gel was stained with 0.3% Congo red stain (Figure 10).

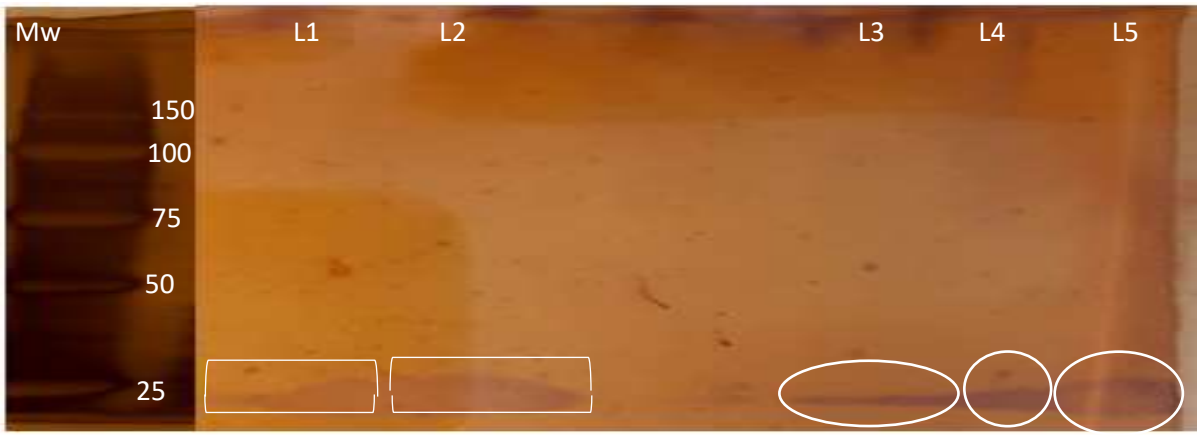

Figure 10: 12% SDS-PAGE stained with silver stain. Confirmation of recombinant enzyme with Native PAGE activity gels (zymography). Mw: molecular weight marker, Lane 1 and 2: $Xyn2A^{C}$, Lane 3, 4 and 5: $Xyn2A^{E}$ zones of clearance (digestion). Marker stained with silver stain; rest of the gel stained with 0.3% Congo Red stain.

The results above show zones of clearance (digestion) at a position a little lower than 25 kDa for both $Xyn2A^{C}$ (i.e., L2 and L3) and $Xyn2A^{E}$ (i.e., from L3, L4 and L5). The zones of clearance in the above-mentioned positions suggest that the recombinant enzymes are active and for the recombinant $Xyn2A^{E}$ (L3 to L5), the lightly expressed bands approximately at 21 kDa are the protein of interest.

2.4.2 Purification of recombinant Xyn2A^{E} and purification table

A diluted cell-free lysate (5 mL) was loaded on the IMAC column for purification and the eluted fractions were subjected to ultrafiltration with 30 kDa cut-off and the sample was centrifuged at 13 500 x g for 15 min at 4 °C, and the centrifuged again at the same conditions in 3 kDa Amicon filters to concentrate the sample. All these concentrated recombinant Xyn2A samples were run on SDS-PAGE and protein determined as described in section 2.3 (Figure 11).

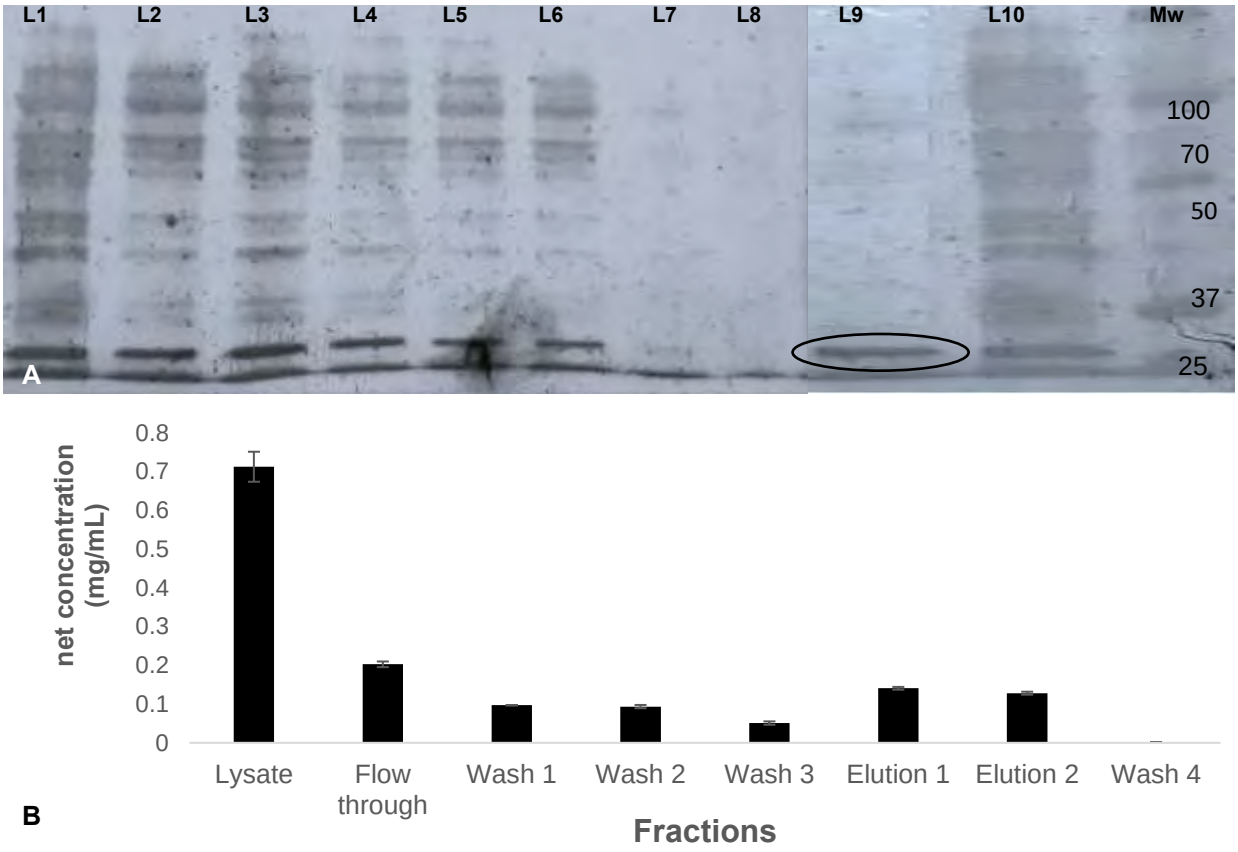

Figure 11: **A**: 12% SDS-PAGE showing purification fractions using nickel (IMAC) column. Lane 1: Lysate crude, Lane 2: Flow through, Lane 3: Wash 1 sample, Lane 4: Wash 2 sample, Lane 5: Wash 3 sample, Lane 6: Elution 1 sample, Lane 7: Elution 2 sample, Lane 7: Wash 4 sample, Mw: molecular weight marker, Lane 9: concentrated elution fractions (3 kDa), Lane 10: concentrated sample (from IMAC-30 kDa-3 kDa) sample. **B**: Protein estimation of purification fractions ran in SDS-PAGE in the above figure (BCA assay). Lysate (0.71 mg/mL), flow through (0.20 mg/mL), wash 1 (0.097 mg/mL), wash 2 (0.093 mg/mL), wash 3 (0.051 mg/mL), elution 1 (0.15 mg/ml), elution 2 (0.13 mg/mL) and wash 4 (0.0013 mg/mL). Values presented as mean ± SD, n = 3.

The results above show the different purification fractions from the IMAC column (lane 1 to lane 9) and the sample from ultrafiltration using Amicon filters with 30 kDa cut-off to remove the

higher molecular mass protein bands and the concentration of the sample (from IMAC - 30 kDa) with 3kDa Amicon filters to remove excess buffer salts. Xyn2A^{E} band is denoted by the black circle, and there are host-cell co-proteins that are present in the samples. The recombinant protein seemed to lightly/weakly bind to the nickel column, such that it was mostly present in the washes (lane 3 to lane 5). Ultrafiltration with the Amicon cut-off filters seemed to yield a better recombinant protein band (lane 10) than the IMAC column (lane 9).

A purification table was constructed to assess the purity of Xyn2A^{E}. There was protein lost in every purification step, roughly 45% loss in flow through and washes. The elution fractions were ultrafiltered with 30 kDa cut-off Amicon membrane filters to remove large bands, while the sample volume decreased, the protein concentration increased, especially after concentrating the sample with 3 kDa cut-off Amicon membrane filters from 0.39 to 0.52 mg. The fold purity score attests to the increased protein specific activity after concentrating the enzyme via ultrafiltration from 5.13 to 7.60 U/mg (Table 2).

Table 2: Protein purification table for Xyn2A^{E} enzyme. Values represented as mean ± SD (n = 3).

Sample	**Volume (mL)**	**Protein (mg)**	**Total protein (mg)**	**Activity (U)**	**Total activity (U)**	**Specific activity (U/mg)**	**Fold purity**	**Yield (%)**
Lysate	30.0	0.71	21.45	1.92	57.60	1.99	1	100
Ni^{2+} affinity	15.0	0.39	5.89	1.46	21.93	5.13	2.6	38.06
Ultrafiltration	11.8	0.52	6.19	2.07	24.37	7.60	3.8	42.31

Soluble wheat arabinoxylan was used as a substrate to check for activity (citrate buffer 50mM, pH 5.0) incubated for 30 minutes at 40°C and calculations carried out (DNS assay standards).

2.5 Discussion and conclusion

2.5.1 Discussion

The induction study of the expression showed that all the proteins present in the sample were increasing in concentration as the IPTG concentration was increasing, including the recombinant $Xyn2A^{E}$ protein (i.e. protein concentration in general increased). The low level of target protein production after induction could be indicating protein toxicity, thus a change of expression vectors to lac-based promoter vectors, adding glucose or pLysS/pLysE strains in T7 systems could solve the problem (Rosano and Ceccarelli, 2014). Furthermore, the increase in incubation time at a constant IPTG concentration directly resulted in a linear increase of target protein expression. This was expected as previous studies also reported gradual increase in protein expression as inducer (IPTG) concentration increases until the cells reach full capacity where no further increase could be observed (Brain et al., 2016).

Microorganisms produce a low basal level of xylanase in the absence of an inducer (Bala and Singh, 2019). Previous studies have indicated that low protein production activity after the exponential and stationary phases were likely due to carbon deficiency, and extracellular inducer concentration toxic effect (Bentley et al., 1991 and Duan et al., 2000). Due to carbon deficiency and toxicity, the cell's metabolic activity decreases, which leads to a decrease in protein expression. A reduction in growth rate and production rate was likely to have occurred in IPTG concentrations above 0.1 mM. Previous studies by Wood et al. (1991) and Srivastava and Mukherjee (2005) indicated that competent cell growth rate and plasmid stability decreases at high inducer concentrations (>1.0 mM), as the cell tried to balance protein production and cell growth. However, other studies indicated no change in cell growth rate and protein production after IPTG addition from 40 – 1000 mM (Dedhia et al., 1994; Durany et al., 2004; Olaofe et al., 2010). $Xyn2A^{E}$ was expressed and partially purified using IMAC, and the partially purified $Xyn2A^{E}$ xylanolytic activity was confirmed in activity gels (zymography).

Zymography is a very common approach that is used to identify protein bands exhibiting hydrolase activity in protein gels. The presence of digested zones on the native PAGE gel relatively at 21 kDa gave indication of the presence and size of $Xyn2A^{E}$ and $Xyn2A^{C}$ (Figure 12). The approximate band was also seemingly equivalent with the isolated protein as shown in figure 13.

The commercial xylanase, marketed by Sigma Aldrich as Xyn IIA (same as Sigma V) was reported to have a relative molecular weight of 22 kDa (Ujiie et al., 1991). In this study, however, Xyn2A was confirmed to have relative molecular weight of 21 kDa (20.773 kDa), as calculated in www.uniprot.org (UniParc ID: UPI0000034D34, GenBank accession no.: AAS31740) (Appendix B- Figure B1 (A-D)).

Low molecular weight xylanases are common in fungi species. Previous authors have identified low molecular weight xylanases from the *Trichoderma* species, such as 20.1 kDa from *Trichordema sp* K9301 (EX2), 18 and 20 kDa from *Trichoderma harzianum* strain (C and E58), 21.5 kDa from *Trichoderma koningii* strain (G-39), and 17.8 and 22 kDa *T. viride* strain (Onozuka and Sigma V) (Hunag et al. 1991, Ujiie et al. 1991; Wong and Saddler, 1992; Cheng et al. 2009). Purity score of a protein affects its activity and application. $Xyn2A^{E}$ seemed to poorly bind to the nickel column, however, ultrafiltration increased the purity score (Table 3). This is attributed to the structure of xylanases and/or ununiformity of the membrane pores that allow enzyme to pass through membrane, concentrating the enzyme (Nathan et al., 2019).

It was also observed that the specific activity for the $Xyn2A^{E}$ was very low compared to Xyn2A and lower than the other xylanases from the same family with similar number of amino acid residues such as B-xylanase (XYN2) from *T. reeseei* (La Grange, Pretorius and Van Zyl, 1996). The huge difference in specific activities may be due to $Xyn2A^{E}$ folding incorrectly and lack of glycosylation during the post-translational modifications in *E. coli* BL21 (DE3) cells. According to Kesidis et al. (2020), eukaryotic expression systems have added advantages compared to prokaryotic expression systems, including advantages such as correct folding of complex proteins, glycosylation, phosphorylation and disulphide bond formation which prokaryotic systems cannot do (Green and Moehle, 1999).

Prokaryotes such as *E.coli* are different from eukaryotes such as yeast (*Saccharomyces cerevisidae*, *Pichia pastoris*, mammalian cells) and protein expression occurs slightly differently in each group, and these are generally the systems used for target protein expression (Kesidis et al., 2020, Bill and von der Haar, 2015). The differences in protein expression of the two groups include sites/compartments involved in protein production which greatly affect post-translation processes such as folding and activity of the target protein (Bill and von der Haar, 2015).

E. coli is a commonly used bacterial expression system due to its fast growth rate, high expression efficiency, and flexibility to genetic manipulation (Bill, 2015). *S. cerevisiae* and *P. pastoris* on the other hand have added advantages over the prokaryotic expression systems due to their ability to express complex large target proteins which require correct folding, glycosylation, disulphide bond formation and phosphorylation, which prokaryotic systems cannot do, they are not costly, and are easy to culture. These post-translational modifications are of vital importance for protein activity (Kesidis et al., 2020). The challenges of toxicity and/or post-translational modification could be overcome by choosing another *E. coli* strain such as C41 (DE3)/C43 (DE3) which are known to reduce toxicity (Snijder et al., 2016). Alternatively, yeast expression systems such as *P. pastoris* could be used.

2.5.2 Conclusion

In conclusion, the aim of this chapter was to express and purify recombinant Xyn2A^{E}, and the partial purified Xyn2A^{E} was obtained and confirmed with zymography. The aim was achieved with a yield of 42% partially purified enzyme and 7.6 U/mg specific activity on soluble WAX. The subsequent chapter deals with Xyn2A^{E} partial characterisation.

Chapter 3: Comparative partial biochemical characterisation of Xyn2A expressed in bacteria and tobacco plants

3.1 Introduction

Xylanases are increasingly becoming important in different industries for different reasons, particularly for lignocellulosic biomass degradation (Kumar et al., 2017 and Lee et al., 2016). However, the production of xylanases is costly, and this is among major challenges in lignocellulosic biomass conversion (Botto et al., 2019 and Wei et al., 2016). The search for more xylanolytic enzymes and improving ways to combat these challenges are ongoing (Ali et al., 2017, Lara et al., 2014). Molecular pharming on the other hand seems to be a promising large scale production system for recombinant proteins in plants and offers possible advantages (Avierzer et al., 2009; Claparols et al., 2004 and Salbaza et al., 2014).

Xylanases are carefully studied for different applications in different industries (Singh, 2019). The characterisation of an enzyme includes studying its optimal conditions for activity, estimated molecular weight, substrate specificity, thermal and pH stability, effect of proteases on enzyme activity, inhibitors and mechanism of action of substrate hydrolysis (Alvarez-cervantes et al., 2016). There are many model and raw substrates that can be used to identify the substrate to which the enzyme has affinity towards (Botto et al., 2019). For xylanase activity determination, substrates include (but are not limited) to soft wood xylan (e.g. pinewood), hard wood xylan (e.g. birch and beechwood) and wheat flour xylan. McCleary and McGeough (2015) reported an increasing demand for the traditional birchwood 4-O-methyl glucuronoxylan which has led to high costs, opened a gap for searching alternatives of which many are in stores already such as beechwood xylan, and wheat flour.

Model substrates are pretreated, and the xylan separated from the rest of the biomass. These are considered good substrates for characterizing xylanase activity as they afford xylanases potentially high chances of activity (Botto et al., 2019; McCleary and McGeough, 2015). Xylanases (depending on their source of origin, and structure) have different physico-chemical properties. GH11 endo-1,4-β xylanases cleave internal glycosidic bonds of the linear xylose backbone randomly at 1,4-β-linkages (He et al., 2019).

T. viride, a filamentous fungus, produces enzymes such as cellulases and xylanases (Nathan et al. 2017). Xyn2A from *T. viride* is a GH11 xylanase which has been reported to have a low molecular weight, high thermostability and catalytic activity (Shi et al., 2014; Malgas and Pletschke, 2019). Previous studies which performed characterisation of xylanases from *T. viride*, observed that the enzyme was most active at acidic pH (2.5 to 5.5), high temperature (45 to 60°) (Taibi et al., 2012; He et al., 2017) and stable at temperature above 50°C (Nieto-Dominguez et al., 2017 and Liu et al., 2010). A study by Luo et al. (2009) demonstrated that alkaline xylanases were commonly suitable for paper/pulp industry whereas acidophilic xylanases were commonly utilised in food and animal feed industry. When xylanases are added in monogastric animal feeds, they are being subjected to gastric fluids while hydrolysis of xylan takes place (Svihus, 2014). Gastric juices also called gastrointestinal fluids are secreted in the proventriculus by mucosal or glandular cells (Bell, 2002 and Svihus, 2014). Gastric juices are composed of hydrochloric acid which mixed with feed and lowers the pH to activate pepsinogen to pepsin, and pepsin which digests protein (Bell, 2002; Svihus, 2014; Gonzalez-Ortiz et al., 2021).

3.2 Aim and objectives

3.2.1 Aim

The aim of the study is to comparatively characterize the in-house recombinantly expressed Xyn2A to the plant expressed version supplied by Cape Bio Pharms.

3.2.2 Objectives

- To determine the substrate specificity of the recombinant protein;
- To investigate the suitable recombinant protein dose for optimum hydrolysis;
- To investigate the effect of pH, temperature on the activity of the recombinant protein, and
- To investigate the effect of proteolytic enzymes on recombinant enzyme's activity.

3.4.1 Effect of pH and temperature on Xyn2A activity

pH optimum and pH stability

Soluble AX, at a final concentration (10 mg/mL), was dissolved in citrate buffer 50 mM (pH 2.0-7.0) and universal buffer 50 mM (pH 8.0 -9.0) and the enzyme (0.05 mg/mL) was incubated at

different pH (2.0 – 7.0). Enzyme + substrate reactions were incubated at 40°C for 30 minutes with 150 rpm shaking, the reaction was stopped by heating at 100°C for 5 min. Enzyme pH stability over incubation time was also investigated. Enzyme and substrate were incubated separately at different pH range (2 – 9) for 60 minutes, 4 °C, then enzyme + substrate reactions (n = 3) were incubated 40°C for 30 minutes, 150 rpm shaking. Activity was reported as relative activity. All values presented as mean ± SD, n = 3.

Temperature optimum and temperature stability

A range of temperatures (20 – 90 °C) was used in order to determined enzyme optimum temperature (citrate buffer 50 mM, pH 5.0, incubated for 30 minutes), and activity reported as percentage relative activity in reference to highest activity. To determine temperature stability, enzyme was incubated at different temperature range (20 – 90°C) with substrate for 60 min at 40 °C with 150 rpm shaking. When the time lapsed, reaction was stopped at 100°C, cooled on ice and centrifuged at 13 500 × g for 5 min at 4°C. DNS assay was used to determine reducing sugars released (Appendix B). Activity was reported as relative activity. All values presented as mean ± SD, n = 3.

3.4.2 Simulated gastrointestinal stability of Xyn2A

The proteases used were trypsin (0.005 mg/mL) and pepsin (0.025 mg/m), bile salts (10 μM) and mucins (0.05 mg/mL) effect were also investigated. The enzyme was incubated in the different proteases for 60 min prior to reaction at 4°C. The substrate used was the soluble wheat flour arabinoxylan at the final concentration of 10 mg/mL as previously described in section 2.3.5, positive control was a normal reaction with enzyme in sodium citrate buffer + substrate and negative control was enzyme in dioinised water. DNS assay was used to determine reducing sugars, and all reactions were in triplicates. Activity was reported as relative activity and values presented as mean ± SD, n = 3.

3.5 Results

3.5.1 Substrate specificity

Substrate specificity of Xyn2A was evaluated in both soluble and insoluble wheat arabinoxylan (WAX). The substrate final dose was 10 mg/mL in citrate buffer (50 mM, pH 5.0), reducing sugars were determined as described in section 2.3.5 and enzyme specific activity was calculated (Table 3).

Table 3: Substrate specificity (U/mg) of $Xyn2A^E$ in soluble and insoluble WAX substrates.

Substrate	Enzyme	
	$Xyn2A^C$	**$Xyn2A^E$**
Wheat flour arabinoxylan (soluble)	536.47 ± 8.8814	7.61 ± 0.3511
Wheat flour arabinoxylan (insoluble)	210.55 ± 11.2752	3.71 ± 0.1183

U = 1µmol of product released per mg of enzyme per min. Values reported as Mean ±SD, n =3.

$Xyn2A^C$ was significantly active in both substrates when compared to $Xyn2A^E$. Both enzymes showed more affinity towards the model WAX substrates. To better understand the substrate affinity of the enzymes, the kinetic parameters of $Xyn2A^E$ and $Xyn2A^C$ were investigated using Lineweaver-Burk plot to determine the K_M and V_{max} values at optimal pH and temperature (Table 4).

Table 4: Kinetic parameters of $Xyn2A^E$ and $Xyn2A^C$ enzymes. Values presented as mean SD, n = 3.

Enzyme	**Substrate**	**V_{max} (U/mg)**	**K_M (mg/mL)**
$Xyn2A^C$	Soluble AX	1003.01	9.2513
	Insoluble WAX	302.891	13.54
$Xyn2A^E$	Soluble AX	20.4477	12.1494
	Insoluble WAX	8.3123	13.149

The K_m and V_{max} values were determined by Lineweaver–Burk plots Xyn2A^C had a V_{max} value of 1003.01 (U/mg) and Xyn2A^E had a value of 302.891 (U/mg) in soluble WAX and 20.4477 (U/mg) and 8.3133 (U/mg) in insoluble WAX respectively.

3.4.2 Hydrolysis overtime

A time study (0 to 24 h) was conducted on the model soluble and insoluble WAX substrates using Xyn2A^E and Xyn2A^C enzymes. Figure 12 shows the reducing sugars released after xylan hydrolysis over 24 h.

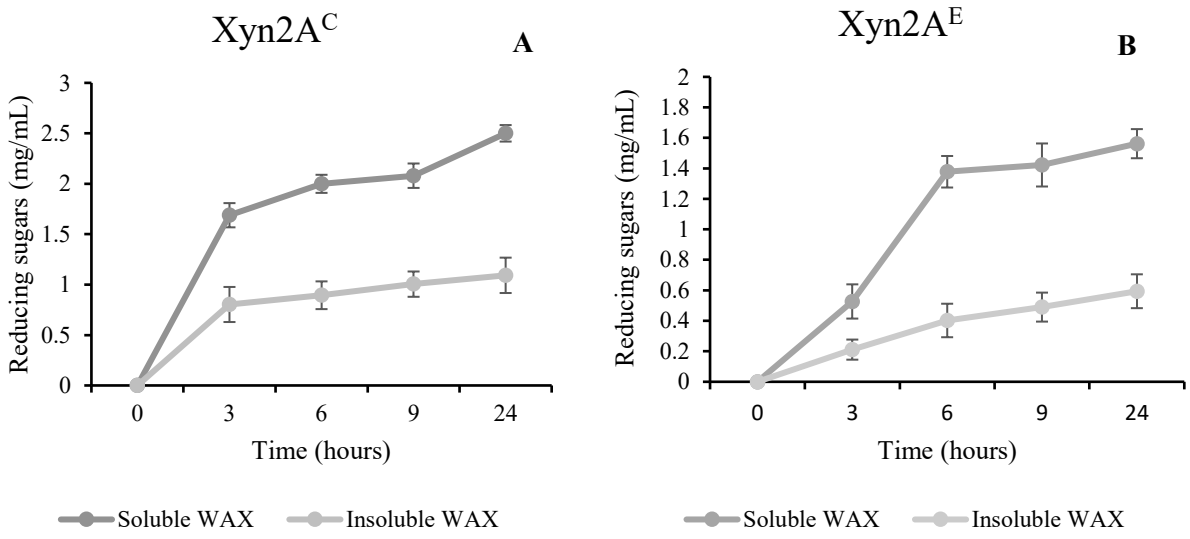

Figure 12: Hydrolysis of soluble and insoluble arabinoxylan overtime with Xyn2A^C (A) and Xyn2A^E (B). Values presented as mean ± SD, n =3, $p<0.05$.

There was a statistically signinificant difference ($p<0.05$) between the activity of enzymes in the substrates, with soluble WAX more favoured than the insoluble WAX overtime (h). The enzyme activities increased with the increasing reaction incubation time (Figure 12). There was quite a significant difference ($p<0.05$) in the concentration of reducing sugars released initially and sugars released 3 h later (both enzymes). Figure 12 shows that sugars released were increasing linearly for both soluble and insoluble substrate. Between 6 and 9 h there no much difference in the concentration of reducing sugars released, but at 24 h both enzymes produced significantly higher concentration of reducing sugars.

3.4.3 Effect of pH and temperature on enzyme

The optimum reaction pH of Xyn2A^{E} and Xyn2A^{C} and pH stability was investigated using soluble WAX as substrate in 50 mM citrate buffer at different pH range (2.0 – 9.0) and overtime (60 min) for pH stability, temperature optimum, and temperature stability over 60 min (figure 13).

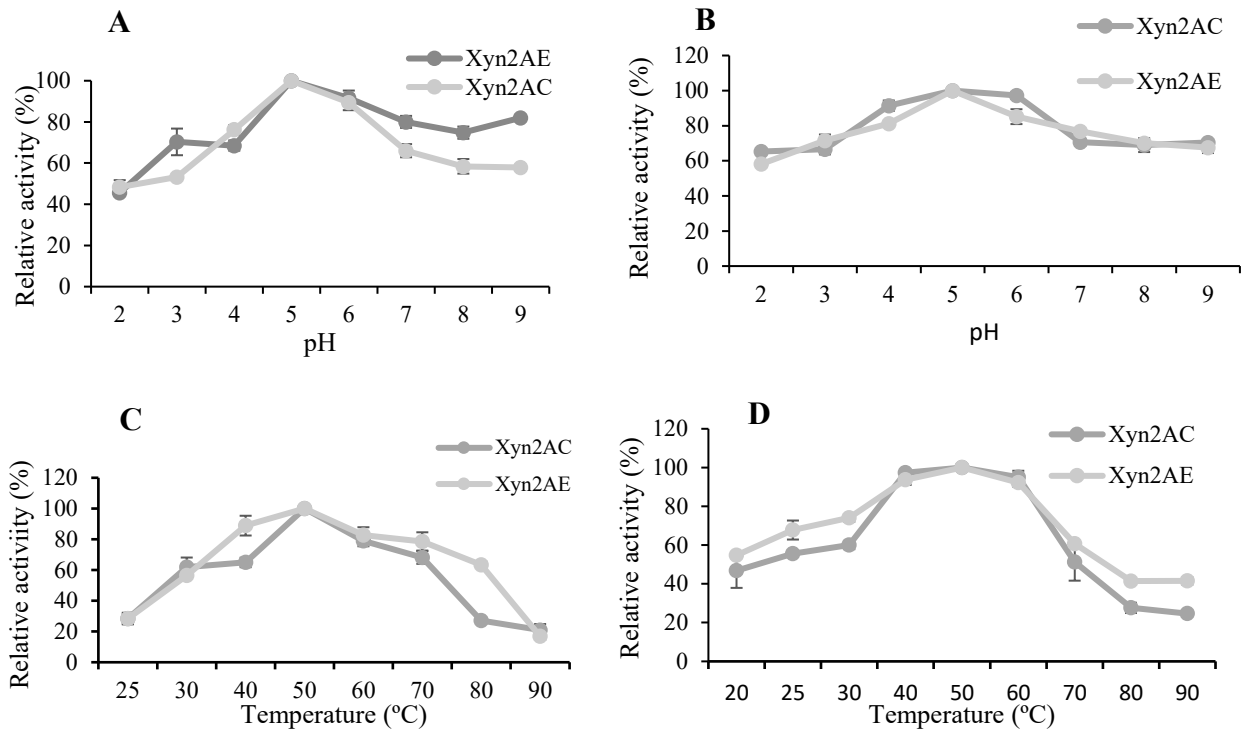

Figure 13: **A**-- (pH optimum): Soluble WAX substrate was dissolved in citrate buffer 50 mM (pH 2.0 – 7.0) and universal buffer 50 mM (pH 8.0 -9.0) and the enzyme was incubated at different pH (2.0 – 7.0). Enzyme + substrate reactions (n = 3) were incubated at 40°C for 30 minutes with 150 rpm shaking. **B**- (pH stability): Enzyme and substrate were incubated separately at different pH range (2 – 9) for 60 minutes, 4 °C, then enzyme + substrate reactions (n = 3) were incubated 40°C for 30 minutes, 150 rpm shaking. **C**- (temperature optimum): A range of temperatures (20 – 90 °C) was used in order to determined enzyme optimum temperature (citrate buffer 50 mM, pH 6, incubated for 30 minutes). **D**- (temperature stability): Enzyme was incubated at different temperature range (20 - 90°C) for 60 minutes, then substrate was added and reaction was incubated at 40 °C for 60 minutes with 150 rpm shaking.Activity was determined using DNS assay, and reported as percentage relative activity. Values reported as mean ± SD (n = 3).

Xyn2A^{E} and Xyn2A^{C} showed optimum activity at pH 5.0 and remained active with more than 50% activity between pH 6.0 to 9.0. Both enzymes were most active at temperature 50°C and had more activity in temperatures between 50°C and 70°C, then activity dropped below 50% from 80°C to 90°C (Figure 13).

The effect of bile salts (10 µM), mucin (0.05 mg/mL) from porcine stomach and proteases (pepsin: 0.025 mg/mL, trypsin: 0.005 mg/mL) on the Xyn2A^{E} and Xyn2A^{C} activity was investigated. The enzymes retained more that 50% of their activity in the presence of all substances in question (Figure 14).

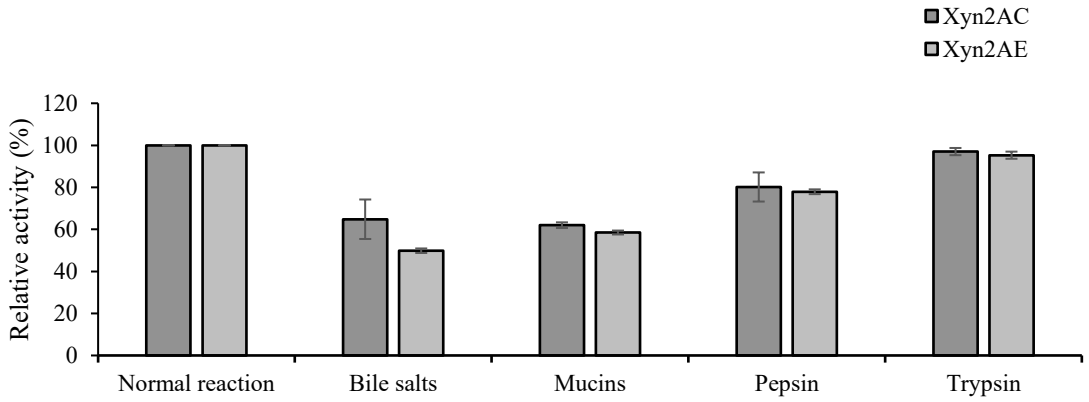

Figure 14: Effect of digestive salts and proteolytic enzymes on xylanase activity. Normal reaction was soluble WAX + enzyme and was taken as 100%. All values presented as percentage mean ± SD, n=3.

Pepsin and trypsin had no significant effect on both enzymes' activity as both enzymes lost approximately 10-15% of activity after incubation in these proteases. A similar trend was observed in the effect of digestive salts on enzymes, the enzymes retained 50% and more activity after incubation. Mucins and bile salts caused a loss of about 50% of Xyn2A^{E} activity, whereas Xyn2A^{C} lost about 40% activity (Figure 14).

3.6 Discussion and conclusion

Both Xyn2A^{E} and Xyn2A^{C} showed higher specific activity towards the soluble WAX than the insoluble WAX substrate. The K_m values for both enzymes (Xyn2A^{E} and Xyn2A^{C}) towards soluble WAX were the slightly high (i.e. 12.1494 and 9.2513 mg/mL), which indicated that both enzymes have affinity towards the substrate. The results also indicated that the enzymes needed slightly more substrate to achieve maximum velocity. Xyn2A^{C} had specific activity of 536 U/mg, whereas Xyn2A^{E} had a specific activity of 7.61 U/mg. This was most likely due to the purity of the enzyme,

as Xyn2A^{C} had more than 80% purity (data not shown) and the inhouse recombinant version had about 40% purity score. These findings agree with the commercial Xyn2A from our previous study (Malgas and Pletschke, 2019) which showed high specific activity on wheat flour arabinoxylan with specific activity of 502.1 U/mg. The commercially available Xyn2A (XynIIA) was reported to have specific activity of 165.2 U/mg with a k_m of 4.4 mg/mL in Larchwood xylan (Ujiie et al., 1991 and Nathan et al., 2017).

To determine the best incubation time for sufficient release of reducing sugars, a time study was conducted. The trend observed showed that the longer the incubation time, the more reducing sugars released by both enzymes in respect to soluble WAX. There was quite a significant difference in the concentration of reducing sugars released initially and sugars released 3 h later (both enzymes). This implied that within 30-60 min a sufficient concentration of sugars would have been released for characterisation purposes. These finding were in line with the observations in the previous study (Malgas and Pletschke, 2019) that when model substartes were incubated for at least 15 to 30 min there was reasonable hydrolysis of model substrate. In both figures, the sugars released were increasing linearly for both soluble and insoluble substrate. Between 9 and 24 h there was not so much difference in the concentration of reducing sugars released, but 24 h had the highest reducing sugars concentration for both enzyme in both substrates. The difference observed in the reducing sugars released did not differ that much, this could be due to the flactuating activity among the different batches of Xyn2A^{E} which indicated a fast degradation over short time spans, thus a shelf-life study may be conducted. Our group identified the optimal activity of commercial Xyn2A at pH 6.0 and 50°C, with thermal stability at 37°C and 50°C during a 24 h period (Malgas and Pletschke, 2019). These findings agree with the findings of this study, with both enzymes showing thermal stability at 50 to 90°C and pH range 5.0 to 9.0. Bala and Singh (2019) also reported high thermal stability of low molecular weight enzymes such as *Thermomyces lanuginosus* and *Trichoderma* sp to be 45 and 50°C. There are various things that can inhibit enzyme from performing at its best, such as proteolytic enzymes.

Proteases have a proteolytic activity on proteins that cannot be digested by endogenous enzymes of the monogastric animals (Wang et al., 2011). In this study, it was observed that pepsin and trypsin had no negative effect on xylanase activity. The aim of this chapter was to partially characterize the Xyn2A^{E} alongside with Xyn2A^{C}. Both enzymes showed optimum activity at pH

5.0 and 50°C temperature, and thermal stability at temperatures above 50°C. These findings agreed with the previously reported findings on most xylanases from bacterial and fungal sources (Nathan et al., 2017: Bala and Signh, 2019; Chadha et al., 2019; Malgas and Pletschke, 2019). Pepsin and trypsin had no significant hinderance on both enzymes activity . These results agreed with the results reported by Duarte et al. (2019) which observed that there was no negative effect on xylanase by protease but synergistic effect in digestion of weaned piglet feeds.

In this study, mucins (0.05 mg/mL) and bile salts (10 µM) caused a loss of about 50% of $Xyn2A^{E}$ activity, whereas the $Xyn2A^{C}$ lost about 40% activity. Mucins are characterized by *O*-glycans which account for 50% of total mass, and they form mucus membrane which coats epithelial cells of the GIT protecting against pathogenic and commensal bacteria. Bile salts, secreted in the duodenum assist in fat digestion (Abudabos, 2014). Bile salts had no significant impact on $Xyn2A^{C}$, whereas $Xyn2A^{E}$ lost about 30% of its relative activity. In a study conducted by Mohamed et al. (2020), where bile salts were supplement with enzyme on a broiler chicken feed, bile salts had a positive impact on intestinal enzyme activity and overall growth performance of chicks aged 1-21 days, showing a synergistic relationship with xylanase rather than inhibition. Another study reported decreased intestinal *E. coli* and *Salmonella* sp invasion when broiler chicks where fed feed supplemented with bile salts and enzyme (Hassan et al., 2010).

In light of the main aim of this chapter, both Xyn2A were successfully comparatively characterised, as far as substrate specificity is concerned. The enzymes displayed the highest activity on polymeric xylan (soluble WAX). The enzymes had optimum activity at pH 5.0 and 50°C, and displayed stability at pH range 4.0 to 7.0, and retained activity in temperatures above 50°C. Both Xyn2A enzymes showed strong resistance to degradation by proteolytic enzymes (trypsin and pepsin), and bile acids and agglutination by mucins. The high activity in acidic pH and high specific activity as depicted by the purer version of Xyn2A (e.g. Cape Bio pharms) clearly supports the suitability of this enzyme for animal feeds industry. Lastly, different expression systems seem to have to lead to no difference in physicochemical properties such as the optimum conditions, stability and substrate specificity.

Chapter 4: Application of Xyn2A in animal feeds

4.1 Introduction

Monogastric animals that are raised for meat production have been fed wheat, corn, and soybean meal-based diets for many years (Hu et al., 2019). Due to the complexity and indigestibility of the agricultural residues fed to the animals and the absence of NSP degrading enzymes in monogastric animals' gut, a significant amount of nutrients is lost as indigestible non-starch polysaccharides (NSPs) (Lee et al., 2017). Xylans are repeats of xylose that are linked together by β-1,4-linkages forming a polymer. The loss of indigestible fiber negatively affects digestion and leads to malnutrition, frequent bone fracture, and gut microbiota imbalance in monogastric animals (Bach et al., 2012). Most poultry and pig farmers have resorted to antibiotics as growth promoting supplements for better animal growth performance (Al-Khalaifah, 2018). Excessive use of antibiotics has led to the increase in antibiotic resistance both in animals and humans (Al-Khalaifah, 2018).

The search for alternatives for improving monogastric animal gut microbiota began with the use of NSP degrading enzymes, such as xylanases (Angelakis, 2017). Xylanases have been used to degrade xylan containing NSPs, such as arabinoxylan, to xylooligosaccharides (XOS) which have a prebiotic effect on animal gut microbiota (Chadha et al., 2019). Previous studies show a positive correlation between enzyme mediated hydrolysis of xylans to XOS and an increase in beneficial gut bacteria (Angelakis, 2017; Lee et al., 2017 and Nathan et al., 2017; Chadha et al., 2019). In monogastric animals, it has been observed that digestion is strongly connected with the feed digestibility, nutrient absorption and gut normal flora (Yang et al., 2015; Chadha et al., 2019). *Lactobacillus*, *Streptococcus, Klebsiella*, *Salmonella*, *Enterococcus*, to mention a few, are some of the gut bacteria that have been studied in poultry and pig production (Yang et al., 2015). Other authors have also shown a relationship between the increase in *E. coli* and increased susceptibility to *Salmonella* infection in poultry, whereas an increase in *Streptococcus* and *Lactobacillus* species tended to control and decrease *Salmonella* infections (Yang et al., 2015).

Researchers have shown that utilizing some xylanase as an additive to pigs dietary fiber liberates the nutrients and decreases the viscosity of the feed for efficient use of nutrients (Wang et al.,

2015), which led to increased feed intake, significant weight gain and better growth performance (Angelakis, 2017). Due to their complexity, the xylans in animal feeds require more than one set or family of enzymes to completely degrade (Biely et al., 2016 ;Lee et al., 2017). Xylanases are costly to produce, thus a need to search for new and improved ways of producing such enzymes is what many researchers are focusicng on (Botto et al., 2019). There are, however, limited studies reporting on the use of Xyn2A on monogastric animal feeds to liberate the encapsulated nutrients and produce oligosaccharides. XOS produced from the degradation of xylan by Xyn2A were utilised to evaluate their prebiotic effect on beneficial GIT bacteria. De Maesschalck et al. (2015) reported that fermentation of XOS resulted in volatile fatty acids (VFAs) which positively affect GIT overall functionality.

4.2 Aim and Objectives

4.2.1 Aim

- To investigate the prebiotic effect of xylooligosaccharides (XOS) produced by hydrolysis of broiler chicken feeds, on both probiotic and pathogenic gut bacteria commonly found in poultry and pigs.

4.2.2. Objectives

- To determine xylanase activity on broiler chicken feeds;
- To analyse xylanase-mediated hydrolysis products (i.e. XOS) using TLC;.
- To analyse feed viscosity before and after hydrolysis with xylanase;
- To investigate change in bacterial culture pH after incubation with XOS and
- To establish the prebiotic effect of XOS on beneficial monogastric animal gut bacteria

4.3 Materials and methods

Broiler chicken feed starter and grower were purchased from a local poultry farmer and feed supplier (Epol(Pty)Ltd, Berlin General, Ntabozuko). A recombinant *Trichoderma viride* (Xyn2A, GH11) xylanase was expressed using (*E. coli*) BL21 (DE3) competent cells. Beneficial bacterial strains were cultured inhouse which were *Lactobacillus bulguricus*, *Streptococcus thermophilus*

and *Bacillus subtilis.*These bacterial strains were kept in glucose and refridgirated at -80ºC and the carbonsource refreshed every month to keep them viable.

4.3.1 Xylanase 2A-mediated substrate hydrolysis

Enzyme effect on broiler chicken feeds was carried out as described in section 2.3 with a few modifications. A substrate volume of 300 µl, suspended in 50 mM citrate buffer (pH 5.0) at a loading of 1.33% (w/v), was mixed with 100 µl of $Xyn2A^E$, and then incubated at 40°C for 24 hours with sharking at 150 rpm. When 24 hours had elapsed, the reaction was terminated by incubation at 100°C for 5 minutes in a dry heating block (Labnet AccuBlock™ digital dry bath) and centrifuged at 13 500 × g for 5 minutes. Reducing sugars were estimated using DNS assay.

4.3.2 Substrate viscosity measurements

A semi-micro viscometer (size 150, serial number C764) (Cannon Instruments, State College, USA) was used to determine the change in viscosity of the samples post-hydrolysis. Viscosity measurements were taken in triplicate and the values (cSt/s) was determined by multiplying the time taken for the liquid to move from the upper meniscus to the lower one with the viscometric constant (0.03146 mm^2/s^2 (cSt/s)).

4.3.3 Analysis of hydrolysis products using Thin Layer Chromatography (TLC)

The broiler chicken feed starter and grower were hydrolysed by $Xyn2A^E$. About 5 µl of the samples were loaded on a silica gel 60 F254 TLC plate (MERCK, Darmstadt, Germany). To run the samples on the plate, 1-butanol: acetic acid: water (2: 1: 1, v/v/v) was used as mobile phase and stained in Molisch's Reagent (0.3% (w/v) α-naphthol dissolved in sulfuric acid: methanol solution (5: 95, v/v)). The plates were developed twice. To visualise the sugars, the plate was incubated at 110 °C for 10 minutes in an oven.

4.3.4 Scanning electron microscopy(SEM)

Substrate hydrolysis was performed as previously describes in section 2.3. The pellet was dried at 25°C for 24 h in the incubator. Dried samples were mounted on iron pins on top of double sticky tape and coated with gold The samples were then analysed under scanning electron microscope (SEM) at different magnification to investigate change of feed with and /or without xylanase supplementation.

4.3.5 Prebiotic effect of XOS on selected few broiler chicken GIT bacteria and pH analysis

Bacterial strains of beneficial bacteria (i.e. *B. subtilis*, *L. bulgaricus*, *S. thermophilus*) were cultured by aliquoting 100 µL(1.0 OD_{600}) of each bacteria in 5 mL of Luria broth . The cultures were done in triplicate and incubated at 37°C for 24 hours, with shaking at 200 rpm. After 24 h had elapsed, 1 mL from each culture was aliquoted in Eppendorf tubes centrifuged at 13 500 × g for 5 minutes, the supernatant was discarded, and pellet resuspended in phosphate buffer to take optical density (OD_{600}) readings at 600 nm. The culture was then diluted from the obtained absorbance to absorbance of 0.1 using 1× M9 minimal media. Negative controls contained varuos bacterial culture + 1× M9 media, positive controls contained various bacterial culture + (1 mg/mL) glucose, experiments contained various bacterial culture + XOS (from starter feed or grower feed). The cultures with XOS from broiler chicken feed starter and grower were incubate for 7 hours and then the pH of the bacterial cells was measured with a pH meter. Resazum reagent (20 µL) was mixed with cell culture (50 µL), incubated at 37°C until colour changes and then absorbance taken at 590 mn to monitor cell viability. All reactions were in triplicates with glucose and XOS final concentation of 1 mg/mL.

4.3 6 Extraction of short fatty acids

The procedure was followed as described above (section 4.3.6), instead of resazun reagent the volatile fatty acids were extracted as follows. Each sample (1 mL) was mixed with 100µL of hydrochloric acid (HCl, 0.25 M) and vortexed for 15 sec, then diethyl-ether (5 mL) was added. Samples were gently mixed for 20 min and centrifuged at 13 500 × g for 5 min at 4°C, supernatant separated to a new tube while pellet was further mixed with 0.5 mL sodium hydroxide (NaOH, 0.4

M) and more (5 mL) diethyl-ether. The samples were centrifuged again at the same conditions, the supernatants were mixed with HCl (0.25 M) at a ratio of 1:1 and taken for absorbance reading at 210 nm. All reaction were in triplicates. (Appendice B).

Values were analyzed in triplicates and presented as mean ± SD, using analysis of variance (one-way ANOVA) with software GraphPad version 5.0. Statistical significance was considered at $p < 0.05$

4.4 Results

4.4.1 Enzyme activity on broiler chicken feed and TLC analysis

After hydrolysis of broiler chicken feeds (starter and grower) by $Xyn2A^E$, the supernatant was separated from pellet and then analysed for the degree of polymerisation of the produced XOS (Figure 15).

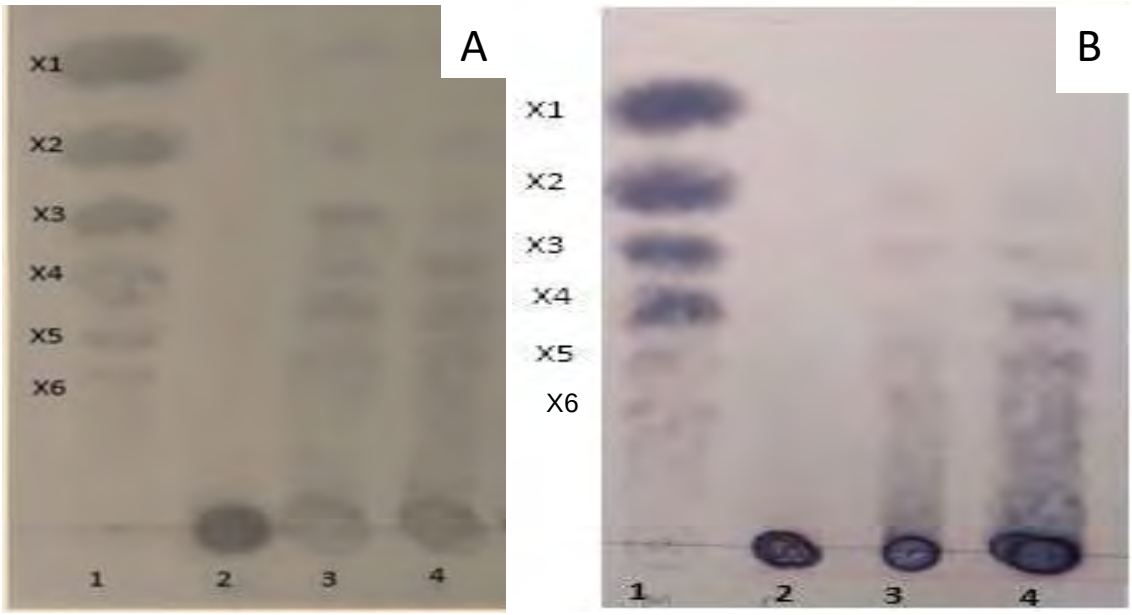

Figure 15: Thin layer chromatography (TLC) analyses of hydrolytes (XOS) produced by enzymatic hydrolysis starter and grower feeds. X1-xylose, X2-xylobiose, X3-xylotriose, X4-xylotetraose, X5-xylopentose, and X6-xylohexose (both A and B). A: Lane 1-xylose standard, Lane 2-starter control, Lane 3 and 4- starter experiments ($Xyn2A^E$). B: Lane 1-xylose standard, Lane 2- grower control, Lane 3 and 4- grower experiments ($Xyn2A^E$). The substrates (starter and grower) were incubated with $Xyn2A^E$ in sodium citrate buffer (50 mM, pH 5.0) for 24 hours at 50ºC.

In both the broiler chicken starter feed (A) and grower feed (B), the substrate control samples had no visible release of XOS. In figure 15A, Lane 3 to 6, there was more xylotriose to xylopentaose sugars, than xylobiose. The same released reducing sugars' pattern was observed for both enzymes in broiler chicken grower feed, with the absence of xylobiose.

4.4.2 SEM analysis

The effect of Xyn2A^{E} supplementation on broiler chicken feeds (starter and grower) was investigated. The difference between feed supplemented (feed + Xyn2A^{E}) and feed not supplemented with enzyme (feed + no Xyn2A^{E}) was analysed by SEM (Figure 16).

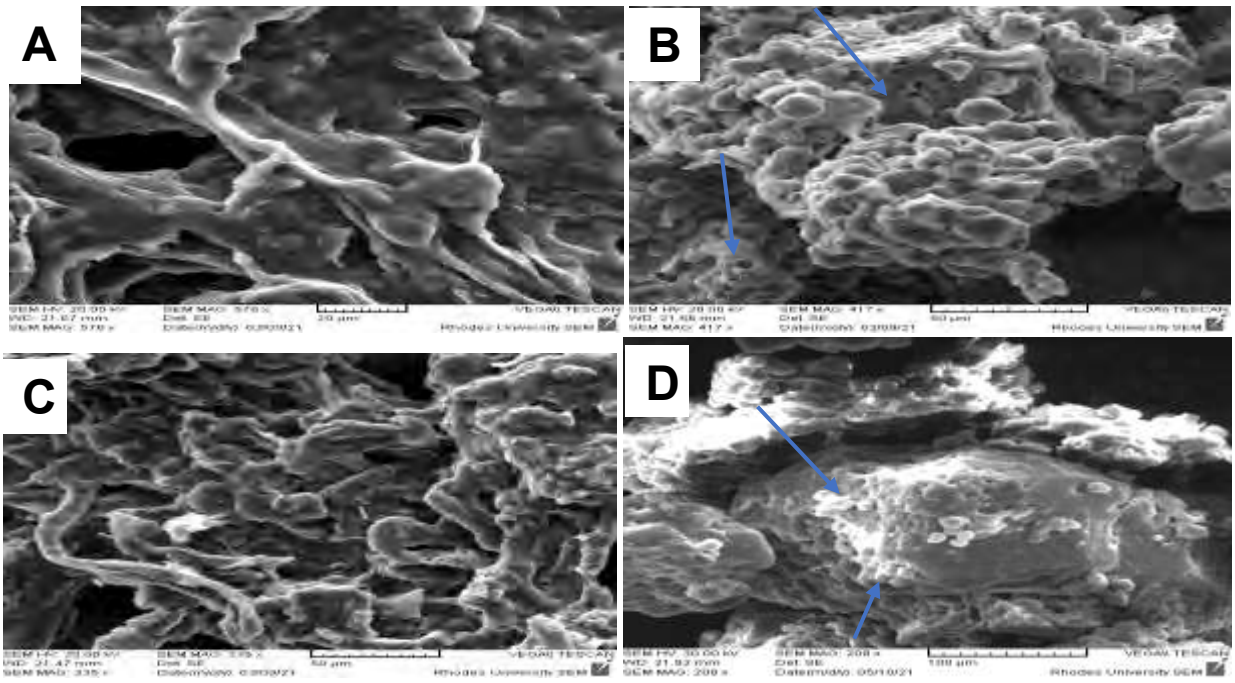

Figure 16: Microscopic analysis of hydrolysed broiler chicken feeds. A: starter feed control, B: Xyn2A^{E} supplemented starter feed, C: grower feed control and D: Xyn2A^{E} supplemented grower feed. Blue arrows pointing to the holes on sample surface post-hydrolysis.

The broiler chicken feeds contain different ingredients other than cereal grains (such as wheat or corn), thus it is only expected that xylanase will hydrolyse xylan at the specific site (1,4-β-linkages). Picture 16A shows a sufficiently hydrated, sample where there was no Xyn2A^{E} supplementation. Then picture 16B above shows significant change on the feed surface when Xyn2A^{E} was present in the feed compared to picture 16A. The sample seemed to be less viscous, and some feed particles appear separately. It was noted that in picture B and D, Xyn2A^{E} supplementation resulted in small holes punched in the feed surfaces (blue arrows).

4.4.3 Viscosity analysis

The viscosity of substrates and broiler chicken feeds with/without Xyn2A^{E} supplementation was analysed, and results are presented below (Figure 17).

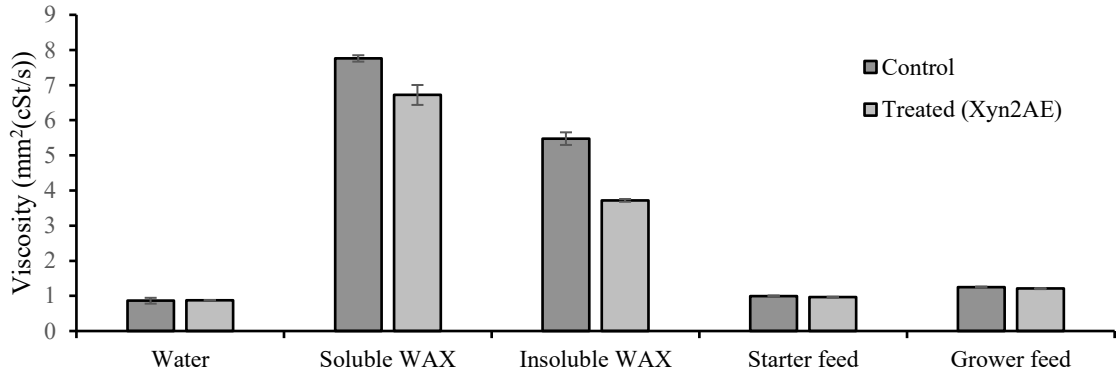

Figure 17: Effect of Xyn2A^{E} on substrate viscosity. All values presented as mean ± SD, n=3.

Water has low viscosity as it moves faster without much surface resistance, for this reason water was the reference value for post-hydrolysis viscosity change. In model substrates, Xyn2A^{E} supplementation reduced viscosity from 7.8 to 6.7 mm^2/s (soluble WAX), and 5.4 to 3.7 mm^2/s (insoluble WAX). However, in broiler feeds, there was minute change in viscosity observed after Xyn2A^{E} supplementation.

4.4.4 Prebiotic effect

The investigation of the prebiotic effect of XOS obtained from the hydrolysis of broiler chicken feed starter and grower on a selected few monogastric animal gut bacterial species (Figure 18). Monogastric animal gut bacteria were incubated with XOS from broiler chicken feed starter and grower for 7 hours and then the optical density and viability of the bacterial cells was measured (OD600). Glucose was incubated with the various bacterial cells as positive control, while 1x M9 salt media was incubated with various bacterial cells as a negative control as described in methods section 4.3.5.

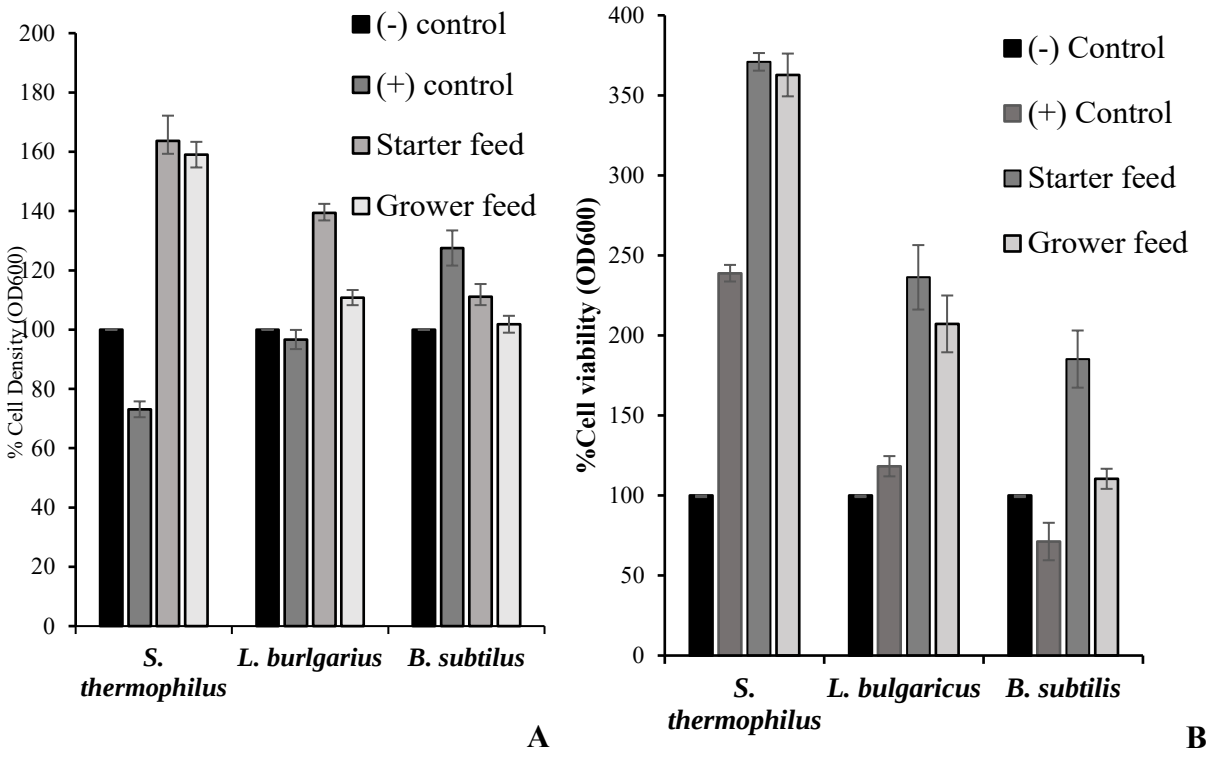

Figure 18: XOS effect on bacterial cell density (A) and cell viability (B), (-) control was the respective bacteria with 1× M9 media, (+) controls were the respective bacteria with glucose. (-) = negative, and (+) = positive. All values are presented as mean ± SD, n=3.

After hydrolysis, the supernatant containing reducing sugars (XOS) was utilised as carbon source to investigate its impact on selected few bacteria that are commonly isolated in monogastric GIT. In order to investigate where or not it affects monastic animal microbiota, bacterial cell density and cell viability (i.e., cell growth) in the presence of XOS was monitored. The above figure 18B showed that cell viability of all bacterial cells increased when XOS was present, in comparison with the presence of glucose. Previous research has reported that probiotic bacteria hydrolyse and ferment XOS releasing organic acids (Lee et al., 2017).

Previous studies have identified gut inhabiting bacterial species from *Streptococci* and *Lactobacilli* as probiotic bacteria due to the role they play in improving gut health (Ding et. al, 2018). In this study, *S. thermophilus, B. subtilis* and *L. bulgaricus* were selected for prebiotic investigation. All bacteria were expected to grow since XOS are a carbon source. Figure 18 showed a general higher growth for *S. thermophilus* and *L. bulgaricus* in the broiler chicken feed starter than the grower.

Table 5: Effect of XOS on the pH of bacterial cell cultures.

Sample	*S. thermophilus*	*L. bulgaricus*	*B. subtills*
Negative control	5.11±0.282	5.43±0.005	5.30±0.045
Positive control	4.41±0.028	4.13±0.343	4.30±0.262
Starter feed	6.12±0.017	5.97±0.005	5.35±0.078
Grower feed	5.61±0.022	5.23±0.022	5.46±0.069

All values were presented as mean ±SD, n=3.

When the pH is compared with the negative controls, there was a slight change in the pH of the bacterial cell cultures that were incubated with XOS from broiler chicken feed starter and grower, although it remained in the acidic end. These observations were correlating with the results reported in Table 6, which suggested a slight increase in the organic acids produced as the XOS were fermented by the bacteria.

Table 6: Short-chain fatty acids released through XOS fermentation.

Sample	*S. thermophilus*	*L. bulgaricus*	*B. subtills*
Negative control	ND	ND	0.000423± 0.000315
Positive control	0.0742±0.00134	0.0421±0.000113	0.0161± 0.0021
Starter feed	0.0812±0.00129	0.0233±0.00217	0.05116± 0.00125
Grower feed	0.0797±0.00141	0.0214±0.00310	0.0734± 0.00412

ND- not detected, values presented as mean ±SD, n=3.

The results obtained above indicated that beneficial commensal bacteria formed SCFA in the presence of glucose (positive control), and more organic acid formation was detected in the presence of XOS (i.e., *S. thermophilus* and *L. bulgaricus*) whereas *B. subtilis* formed less organic acid in the presence of XOS.

4.5 Discussion and conclusion

The GH11 family of xylanases (EC 3.2.1.8) are known to randomly hydrolyse xylan at 1,4-β-linkages, depending on degree of polymerization (DP), xylan length and substitutions present (Alokika and Singh, 2019). Xylan degradation results in high yields of XOS which have numerous beneficial properties (Li et al., 2016). Studies conducted by Li et al. (2016) and Rantanen et al. (2007) reported that GH11 xylanase produce long chained (≥ X4) XOS upon xylan degradation. Several researchers have reported that due to absence of NSP-degrading enzymes in monogastric

animals, XOS derived from xylan degradation pass the monogastric GIT without degradation (Patel and Goyal, 2011; Rashid and Sohai, 2021). XOS possess β-bonds in its chemical structure which enables them to be stable in acidic environments (Rashid and Sohai, 2021). Ding et al. (2018) reported that XOS influenced the availability of minerals such as magnesium and calcium, and reduce the intestinal pH thereby increasing mineral solubility through increased absorption and intestinal cell proliferation (Lopez et al., 2000).

GH11 xylanases were reported to produce more longer-chain XOS (i.e., X4-X6) when compared to GH10 (X1-X3), and this could be due to inability of GH11 xylanases to cleave substituted xylan regions (Li et al., 2017). Rantanen et al. (2004) reported X4 to be the shortest XOS produced by GH11 xylanases. The finding of this study concurs with the previous finding which reported little X1 and X2 amounts, with more X3-X6 post-hydrolysis by Xyn2A (Malgas and Pletschke, 2019). XOS are not digested by monogastric animal due to the lack of suitable enzymes (Gibson et al., 2017), thus they are fermented by gut microbiota (Naidu et al., 2018). There also was a change observed on feed when Xyn2A was present compared to without Xyn2A (Figure 16A and B and Figure 16B and D). Xyn2A supplementation resulted in small holes punctured in the feed surface. Xyn2A supplementation reduced viscosity from 7.8 to 6.7 mm^2/s (soluble WAX), and 5.4 to 3.7 mm2/s (insoluble WAX). However, in broiler feeds, there was minute decrease in viscosity after Xyn2A supplementation (figure 17). Xylan degradation has been reported to induce cell wall degradation (Beaugrand et al., 2017). Thus, through disassembly of cell wall network, the viscosity of digesta is reduced. Arczewska-Wlosek et al. (2019) reported decreased digesta viscosity when xylanase was supplemented in wheat-based diets. The presence of xylanase in feed resulted in holes punctured on the surface of the feed, and the viscosity of the feed slightly decreased.

Gut microbiota plays a vital role, as it acts as an anti-infectious barrier by suppressing the growth of pathogenic bacteria (Diaz Carrasco et.al, 2019). In search for better ways of increasing animal feedstuff value and digestibility, Gibson et al. (2004) came up with the prebiotic concept to describe the relationship observed between enzyme-degraded non-digestible fiber and gut microbiota activity. Previous studies have reported on the positive correlation between arabinoxylan oligosaccharides (AXOS) presence and high lactic acid bacteria production levels, and how ryegrass XOS and AXOS suppress *Enterobactiaceae* growth in pigs' gut (Yacoubi et al., 2018).

Evaluation of the effect of XOS on a selected few monogastric animal microbiota commonly found in the gut was carried out utilizing XOS generated from broiler chicken feed starter and grower using Xyn2A. Cell density experiment results showed that *S. thermophilus* grew multiplied significantly followed by *L. bulgaricus*, after incubation with XOS from the broiler chicken feeds for 7 hours (Figure 18). Bacteria require a carbon source to remain viable and multiple. The increase in cell density suggested that both the probiotic bacteria were utilizing the XOS, with the probiotic bacteria utilizing more XOS and rapidly increasing in total percentage viability. Numerous studies have reported on the ability of *Lactoballi* species to enhance weight gain in fowls (Million et al., 2012; Drissi et al., 2017).

These results were consistent with the findings by Yang et al. (2014) and Stecher et al. (2010), who reported that XOS positively affected the growth of *Streptococcus thermophilus* and *Lactobacillus* bacteria, which tend to control *Salmonella* infections by suppressing *E. coli* growth which correlates with high susceptibility to *Salmonella* colonization. The effect of XOS on chicken gut microbiota is an on-going debate with other findings contrary to the commonly reported findings, where *Lactobacilli* species were associated with low growth and productivity and *Enterobacteria* presence associated with high productivity (Ocejo et al., 2019). These inconsistencies on the effect of XOS on gut microbiota is most likely associated with the degree of diversity of the gut bacteria which promotes overgrowth of certain class of bacteria over the other.

The change in the pH of bacterial cultures after incubation with XOS for 7 hours was monitored after incubation time. The results in Table 6 showed that there was slight change in the pH of experiments when compared to the negative control, thought the pH remained in the acidic end. This agreed with the results reported by De Maesschalck et al. (2015), where no change was observed in monogastric animals' stomach pH post xylanase mediated NSP degradation (Svihus et al., 2015). The beneficial bacterial cells produce SCFAs as they fermented XOS, and organic acid production was detected at 210 nm wavelength (Table 6). When it came to SCFAs, there was no noticeable effect on the acids. This is similar with the findings which reported that as commercial XOS concentration increased no increase in propionic acid, a linear increase in butyrate and acetate which were determined by gas chromatography from cecal digesta samples (Ding et al., 2018).

Literature has reported that different bacteria produce one and/or the other type of organic acid because of sugar fermentation, acids such as but not limited to acetic acid, propionic acid, formic acid, butyric acid and lactic acid (Zhitnitsky et al., 2017). Lactic acid is produced by *lactobacilli* as a post-hydrolysis product, which has pathogen inhibition elements (Russo et al., 2017). *Lactobacilli* have been reported as promoters of acidic environment through lactic acid production, which was observed to enhance nutrient digestion and feed conversion efficiency (Wang et al., 2015 and Yang et al., 2015; Angelakis, 2017). These observations suggested that in the acidic environment, lactic bacterial species were multiplying suppressing the growth of enteric pathogens. Some studies reported that enzyme-mediated hydrolysis of NSPs contributed positively to commensal gut microbiota growth which lower the large intestine pH and favors the inhibition of enteric pathogens (Bach et al., 2012). The results shown in Figure 18 for cell viability are consistent with the observations in Figure 18 and Table 5, suggesting that the peaks observed for cell density came from viable/live cells.

The aim of this chapter was to evaluate the application Xyn2A in monogastric animal feeds (i.e., broiler feeds) and the effect of the generated XOS on selected gut microbiota species, commonly found in broiler chicken. This study showed that there was a positive effect of XOS from broiler chicken feeds on *S. thermophilus* and *L. bulgaricus*. The results obtained from this study also support a previous finding (Hu et al., 2019, Bach et al., 2012) and encourages the application of xylanases in animal feedstuffs to increase production performance.

Chapter 5: General discussion and further recommendations

5.1 Summary

The poultry and pig farming industries are growing rapidly. In poultry production, animal feeds are responsible for approximately 65-70% of operating costs (Pirgozliev, 2017). Animal feeds are composed of cereal grains which contribute as the major metabolizable energy, and to lesser extent protein and vitamin sources such as soybean meal (Gous et al., 2012). The cereal grains that are commonly found in the monogastric feed-mix are wheat, corn, rye, barley, sorghum and millet (to mention a few). The cereal grains are chosen for different reason in different parts of the world, for an example, seasonal (as well as regional) availability and production costs play an important role in grain selection (Gous et al., 2012). Grains like wheat and barley are reportedly cheaper to produce than corn, however, they are most commonly more viscous than corn (Aftab and Bedford, 2018). Since a highly viscous grain (i.e. digesta) usually indicates a significantly high NSP content, which in high amounts poses challenges in poultry farming (Singh et al., 2021).

Cereal grains are an important source of starch and proteins, however, a considerable amount of NSPs is found in their cell walls, limiting the utility of nutrients (Yaghobfar and Kalantar, 2017). The cell wall of plants is made up of hemicellulose, the most abundant polysaccharide in plant cell walls (Li et al., 2017). The hemicellulose is mostly made up of heteroxylans (i.e., NSPs) that are polymers with β-1, 4- linked xylose units (Peng et al., 2012; Li et al., 2017) substituted with side chains of sugars (such as arabinose) and sugar acids (such as glucuronic acid) (Li et al., 2017). NSPs can be classified as soluble and insoluble due to their chemical structure and complexity, which influence their dispersibility in water (Singh et al., 2021). Monogastric animals lack enzymes that can hydrolyse the complex NSPs and this results in nutrient loss, gut microbiota imbalances and negatively affects growth performance of animals (Singh et al., 2021). To combat these challenges, hydrolytic enzymes such as xylanases are used as feed supplements to unravel the cage-like effect of NSPs on nutrients and to lower NSP viscosity (Gonzalez-Ortiz et al., 2021).

Xylanase supplementation has been reported to have positive effects on monogastric animal feeds, where feed nutrients encapsulated in NSPs were liberated, digesta viscosity reduced and prebiotic sugars produced (Gonzalez-Ortiz et al., 2021 and Lee et al., 2017). Xylanases are added to hydrolyse the xylan into smaller fermentable sugars which promote the growth of beneficial gut

microbiota, and the beneficial microbiota produces organic SCFAs upon fermenting these sugars (Cai et al., 2011). Some studies have reported that though some xylanases fall in the same family by classification, their activity may not be the same when subjected to the same or different substrates (Lee et al., 2017). In this study, a xylanase (Xyn2A) originating from *T. viride* was utilised to investigate its effect on monogastric animal feeds (such as broiler chicken feeds).

This study investigated the following:

- Cloning and expression of Xyn2A, a GH11 xylanase from *T. viride*, in *E. coli* BL21 (DE3) competent cells;
- Partial comparative characterization of the recombinant enzyme (Xyn2A), which included substrate specificity and optimum conditions of activity;
- Performing post-hydrolysis analysis of substrates by thin layer chromatography (TLC), viscometry and microscopy (SEM);
- Analysis of the prebiotic effect of the XOS produced from broiler chicken starter and grower feeds hydrolysed by Xyn2A on monogastric gut associated bacteria and SCFAs formation.

There were different findings reported on the highest IPTG dose that can be suitable for protein expression, without causing negative effects on the expression system and host. One study reported that as IPTG concentration increased the target protein concentration linearly increased (Wood et. al., 1991). Another study by Srivastava and Mukherjee (2005) indicated that competent cell growth rate and plasmid stability decreases at high inducer concentrations (<1 mM), as the cell tried to balance protein production and cell growth. However, other studies indicated no change in cell growth rate and protein production after IPTG addition from 40 – 1000 mM (Dedhia et al., 1994; Durany et al., 2004; Olaofe et al., 2010). For this study a dose of 0.8 mM IPTG was used to express the recombinant enzyme in *E. coli* BL21 (DE3) competent cells. The recombinant enzyme was partially purified with IMAC and ultrafiltration as described in section 2.3.3. The efficacy of purification was not good as less than 50% of partially purified enzyme was recovered.

Data concerning Xyn2A in https://www.uniprot.org, reported that the recombinant Xyn2A endo-β-1,4 xylanase with accession number AAS31740.1. The enzymes (i.e., $Xyn2A^{E}$ and $Xyn2A^{C}$) were 21 kDa, falling in the low molecular weight xylanases category with the commercially

available Xyn2A reported as 25.1 kDa (Malgas and Pletschke, 2019). These finding were consistent with the commercially available GH11 endo-β-1,4 xylanases, such as Sigma V (Ujie et al., 1991). Fungal xylanases have different physico-chemical properties as outlined in table 4, and the GH 11 family has been reported to have low molecular weight xylanases (Singh and Bala, 2019; Winger et al., 2014). Xyn2A^{C} had a specific activity of 536 U/mg, whereas recombinant Xyn2A^{E} had 7.61 U/mg, this may have been due to incorrect folding and lack of post-translational modifications in the expression system used (*E. coli*) in the production of Xyn2A^{E} which renders it unfunctional. These findings agree with the commercial Xyn2A from our previous study (Malgas and Pletschke, 2019), which showed high specific activity on wheat flour arabinoxylan with specific activity of 502.1 U/mg. There are quite a few things that affect enzyme purity, as far as the recombinant enzyme is concerned, namely; purification technique and physico-chemical properties of the enzyme (Rosano and Ceccarelli, 2014). Xyn2A^{C} had more than 80% purity (data not shown) and Xyn2A^{E} had about 40% purity score, the level of purity seemed to affect the enzyme activity as seen in Table 3. Xyn2A^{C} was more active in all substrates when compared to Xyn2A^{E}.

In this study, both enzymes were resistant to pepsin and trypsin as they retained more than 50% of their activity when incubated with the proteolytic enzymes. The ability of the xylanase to resist proteolytic enzymes was previously reported by Morgavi et al. (2001) and Luo et al. (2009), indicating that enzymes from *Trichoderma* can survive the proteolytic environments of the intestines. According to Olukosi et al. (2015), a cocktail of xylanases and proteases increased effects on poultry digestibility and general growth performance. Another study conducted by Duarte et al. (2012) showed that protease had no negative effect on xylanase when both enzymes were supplemented on weaned piglet diets, instead they enhanced body weight gain and gut morphology by increasing villi length, decreasing crypt depth and cell proliferation (Knudsen et al., 2012). For these reasons, investigating the interactions and effect of proteolytic and hydrolytic enzymes cocktails is increasing, as recent studies have shown some interest in the use of exogenous proteases as additives (Walk et al., 2018 and Pirgozliev et al. 2019).

The digestive tract of chickens is mostly covered by the mucus membrane which has goblet cells that secret mucins. This membrane acts as a protective barrier against pathogen invasion of the host (Hasson et al., 2010), and for this reason, the effect of bile salts and mucin on enzyme activity

was investigated. The bile salts and mucin did not have any significant impact on enzyme activity. Another study by Mohamed et al. (2020) and Hasson et al. (2010) suggested that bile salts had a positive effect on xylanase when the two were supplemented together in broiler chicken feeds, and the overall growth of broiler chickens was positively impacted. This agrees with the report released by Abudabos (2014), suggesting that bile salts play a major role in fat digestion.

The GIT pH of the commonly bred broiler chicken species Ross 308 was studied, and the results showed that the crop was 4.8, proventriculus was 4.39, gizzard was 3.22, duodenum was 5.89, jejinum was 6.2 and ileum was 6.64, respectively (Mabelebele et al., 2017). Figure 18 indicated that both enzymes had reasonably high activity under a low pH of 4.0, reaching optimal activity at pH 5.0. Interestingly, these findings suggest that Xyn2A is an acidophilic enzyme like Xyn10 from *Penicillium* which tends to exhibit optimal activity at acidic pH (Cai et al., 2011). Acidophilic xylanases contain a higher ratio of acidic residues (Asp and Glu) compared to basic residues (Lys and Arg) on the surface of the enzyme structure (Cai et al., 2011). For this study, the chemical structure of the recombinant enzyme was not analysed to investigate the ratio of residues responsible for its response to pH and temperatures. It is worth noting that the optimum pH of Xyn2A suggested it to be suitable for use in the food and feed industry. An acidic pH enhances acidophilic xylanase activity and mineral salt absorption in the intestines (Lou et al., 2009; Cai et al., 2011; Incharoen, 2013). In broiler chickens, hydrochloric acid is secreted in the proventriculus by glandular epithelial cells and the acid aids in feed digestion (Svihus, 2014).

Xylanase supplementation in monogastric animals has been reported to result in a release of prebiotic XOS, increased feed intake, decrease in digesta viscosity, increased weight gain, balance in gut microbiota and improvement in overall performance (Alusoki et al., 2015). A study by de Freitas et al. (2019) reported that the longer the reaction time for XOS production, the higher the yield of XOS and the 24 h incubation released more sugars. Thus, through disassembly of cell wall network, the viscosity of digesta is reduced. Arczewska-Wlosek et al. (2019) reported decreased digesta viscosity when xylanase was supplemented in wheat-based diets.

In this study, it was observed that there was a slight change in the pH of the bacterial cell cultures that were incubated with XOS from broiler chicken feed starter and grower, although it remained in the acidic spectrum. The findings were corelating with the findings reported in Table 6 for organic acids. Svihus et al. (2010 and 2013) demonstrated that the pH of the crop (i.e., fowl) ranges

between 4.5 and 6.5, the gizzard pH ranges between 3.5 to 4.5, whereas in the intestines it can rise to 6.0 (Svihus, 2011). This is influenced by the time the food stays in the crop; implying that the long retention time in the crop, fermentation occurs and SCFAs are formed making the environment more acidic (Hilmi et al., 2017). XOS have received increasing attention since the discovery of their health benefits in food industry (Mohanty et al., 2018 and de Faris et al., 2019). There are different reports on the effect of XOS on SCFAs, like in a study done by Ding et al. (2018), which suggested there was no noticeable effect on propionic acid amount, linearly increase in butyrate and acetate as XOS concentration increased. *S. thermophilus* and *L. bulgaricus* showed more rapid growth in the presence of XOS than all other bacteria in this study. Yang et al. (2014) and Stecher et al. (2010), reported that XOS positively affected the growth of *S. thermophilus* and *Lactobacillus* bacteria which tend to control *Salmonella* infections by suppressing *E. coli* growth which correlates with high susceptibility to *Salmonella* colonisation. The animals' ability to fight diseases is dependent on immune system development, which is largely influenced by nutrition among other things (Yang et al., 2018). On the other hand, SCFAs alter the animal cell's intracellular pH, they cut the excessive use of cellular energy and disturb the metabolic activities of the pathogens in the gut (Surendran Nair et al., 2017; Zhitnitsky et al., 2017).

5.2 Conclusion

Xyn2A was partially (42%) with 7.6 U/mg specific activity; and displayed the highest activity on polymeric xylan (soluble WAX). The enzymes had optimum activity at pH 5.0 and 50°C, and displayed stability at pH range 4.0 to 7.0, and retained activity in temperatures above 50°C. Both Xyn2A enzymes showed strong resistance to degradation by proteolytic enzymes (trypsin and pepsin), and bile acids and mucins. The high activity in acidic pH and high specific activity as depicted by the purer version of Xyn2A (Cape Bio pharms) clearly supports the suitability of this enzyme for animal feed industry. Xyn2A supplementation reduced viscosity of the soluble and insoluble WAX model, and there was minute change in viscosity observed in broiler feeds. A positive effect of XOS from broiler chicken feeds on *S. thermophilus* and *L. bulgaricus* was observed, as they had higher growth compared to glucose as a carbon source. Based on these results, Xyn2A could be a beneficial additive in monogastric animal feeds, depending on the cereal grain and content on NSPs in the feeds.

5.3 Future recommendations

Based on the results obtained from this study, the following future recommendations are suggested:

- The enzyme in this study was partially purified using an immobilized metal affinity chromatographic column. Clearly, the purer the enzyme was, the more was its activity in different substrates as was seen with $Xyn2A^{C}$ and $Xyn2A^{E}$. Different purification techniques are used for the purification of all enzymes. For bacterial, enzyme one-step techniques can be used but for the purification of eukaryotic enzymes, researchers have used a mixer of techniques. Chromatographic techniques are the commonly used ones, with electrophoresis. To get a purer enzyme, a mixture or other purification techniques can be used. The enzyme in its pure form showed promising potential for use in feed-processing.

- The focus of this study was to utilise the enzyme in monogastric animal feeds; therefore, its characterisation was done partially. There seems to be limited literature available on this enzyme (Xyn2A), whereas other enzymes from same fungal family have been intensely studied (e. g. *T. reseei, T. harizunum*). A use of other expression systems, further structural and biochemical study of this enzyme would be beneficial for deeper understanding of the enzyme's substrate recognition and genetic improvement, as well as upscale production for industrial application. A study of biochemical properties (i.e. gene structure and mutations) could shed light on enzyme performance when gene mutations and their effect of enzyme activity are investigated.

- The enzyme showed high activity on model substrates, which tend to be purer than the lignocellulosic biomass (agri-residues) that composes a greater part of the animal feeds. It is well established that due to the complexity of the lignocellulosic biomass, the addion of protein and fat from different sources, makes the feed more complex (though nutritious) for a single enzyme to degrade it. Some researchers have reported cocktails of xylanolytic enzymes, cellulolytic enzymes and proteolytic enzymes synergistically degrade the biomass and yielding better results. In this study, Xyn2A was resistant to degradation by proteolytic enzymes. For this reason, a synergy study on the two (i.e. cellulolytic and proteolytic enzymes) could be conducted at different doses.

6.2 References

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Appendices

APPENDICE A: List of chemicals and suppliers

(D)-Glucose	Saarchem (Cat. No. 2676020)
(D)-Xylose	Sigma-Aldrich (Cat No. X3877)
α-Naphtol	Sigma-Aldrich (Cat No. N1000)
2-mercaptoethanol	Fluka (Cat. No. 63700)
3, 4-dinitrosalicylic acid	Sigma (Cat No. D0550)
Acetic acid	Sigma (Cat No. A6283)
Acrylamide	Sigma (Cat No. A8887)
Ammonium persulphate	Sigma Aldrich (Cat No. A3678)
Bacteriological agar	Biolab (Cat. No. BX1)
Bicinchoninic acid copper	Sigma (Cat No. BCA1 – 1KT)
Bovine serum albumin	Sigma (Cat No. A4503)
Butanol-1	Merck
Bradford's reagent	Sigma (Cat. No. B6916)
Bromophenol blue	Sigma (Cat. No. B8026)
Citric acid	Merck (Cat. No. 1.00244)
Congo Red stain	Sigma (Cat. No. C6767)
Coomassie brilliant blue R250	Merck (Cat. No. 1.12553)
Di-sodium hydrogen orthophosphate	Saarchem (Cat. No. 5822860)
Ethanol	Merck (Cat No. 1.00980)

Glacial acetic acid	Merck (Cat No. 1.00063)
Glycerol	Saarchem (Cat. No.2676520)
Glycine	Merck (Cat. No. 1.04169)
Hydrochloric acid	Saarchem (Cat. No. 3063040)
Imidazole	Merck (Cat. No. 1.04716)
IPTG	Calbiochem (Cat. No. 420322)
Kanamycin	Roche (Cat. No. 106801)
Lysozyme	Fluka (Cat. No. 62971)
Methanol	Merck (Cat. No. 8.22283)
N,N,N',N'-tetramethylethylene diamine	Sigma Aldrich (Cat. No. T9281)
Nickel (II) sulphate	Merck (Cat. No. 1.06727)
Pepsin	Merck (Cat. No. 1.07185)
Potassium phosphate dibasic	Merck (Cat. No. 1.05104)
Potassium phosphate monobasic	Merck (Cat. No. 1.04877)
p-Nitrophenol	Aldrich (Cat. No. 42575-3)
Resazum	ThermoFisher Scientific
Silver Stain kit	ThermoFisher Scientific
Silica Gel HPTLC plates	Merck (Cat. No. 1.00390)
Sodium azide	Sigma-Aldrich (Cat. No. S2002)
Sodium chloride	Merck (Cat. No. SAAR5822320EM)
Sodium dodecyl sulfate	Merck (Cat. No. 8.1170314)
Sodium hydroxide	Merck (Cat. No. SAAR5823180EM)
Sulfuric acid	Merck (Cat. No. 1.01833)

Trypsin	Sigma
Trytone	Merck-Biolab (Cat. No. HG000BX4)
Wheat arabinoxylan	Megazyme
Xyn2A (*T. viride*)	(Cape Bio Pharms)
Yeast extract	Merck-Biolab (Cat. No. HG000BX6)

Appendix B: Xyn2A gene data and plasmid construction

In search for Xyn2A in the Carbohydrate Active Enzymes database (https://www.cazy.org), the following (Figure A1-A) was seen with Xyn2A written as shown with a black circle, with UniProt ID: Q7M519 and GenBank ID: A44594, which later became obsolete and other GenBank ID were provided in its stead. The UniProt ID refered user to https://uniprot.org, a database for proteins which gives them a stable unique identifier. The obsolete UniProt ID for the protein in question referred the user to UniParc where the current UniParc ID was UPI0000034D34 (Figure B1-B and B1-C), and for this study the Xyn2A with GenBank ID: AAS31740 was used. The reference for the proteins (PUBMED) refers user to the study by Ujiie et al., 1991 (as indicated with a black arrow). The calculated molecular weight was given as 20 773 Daltons (Da) and 190 amino acid length (Figure A1-D).

Protein Name	EC#	Reference	Organism	GenBank	Uniprot	PDB/3D
unclassified						
xylanase	3.2.1.8	URL	Trichoderma sp. SY	AAN77423.1	Q8J0T4	
xylanase IIa	3.2.1.8	pubmed	Trichoderma viride	A44594 AAS31740 ABH92532.1 ACK26692.1 ACQ19022.1	Q7M519	
xylanase	3.2.1.8	pubmed	Trichoderma viride	CAB60757.1	Q9UVF9	
Type 3 (fragment)	3.2.1.8	pubmed	uncultured bovine rumen ciliate	AEQ16452.1	G5DDC0	
Type 4 (partial)	3.2.1.8	pubmed pubmed	uncultured bovine rumen ciliate	AEQ16453.1	G5DDC1	
Type 4 (partial)	3.2.1.8	pubmed pubmed	uncultured bovine rumen ciliate	AEQ16453.1	G5DDC1	
xylanase R8 (XynR8) (partial)	3.2.1.8	pubmed	uncultured Neocallimastigales	AAY18781.1	Q3KT08	
xylanase (XynG)	3.2.1.8	pubmed	Verticillium dahliae	ABE02800.1	Q0ZHI9	

Figure 19: **B1-** Xyn2A data extracted from https://www.cazy.org and https://www.uniprot.org.

Figure 20: **B2-** Xyn2A data extracted from https://www.cazy.org and https://www.uniprot.org. GenScript company codon optimised the gene and constructed the plasmid.

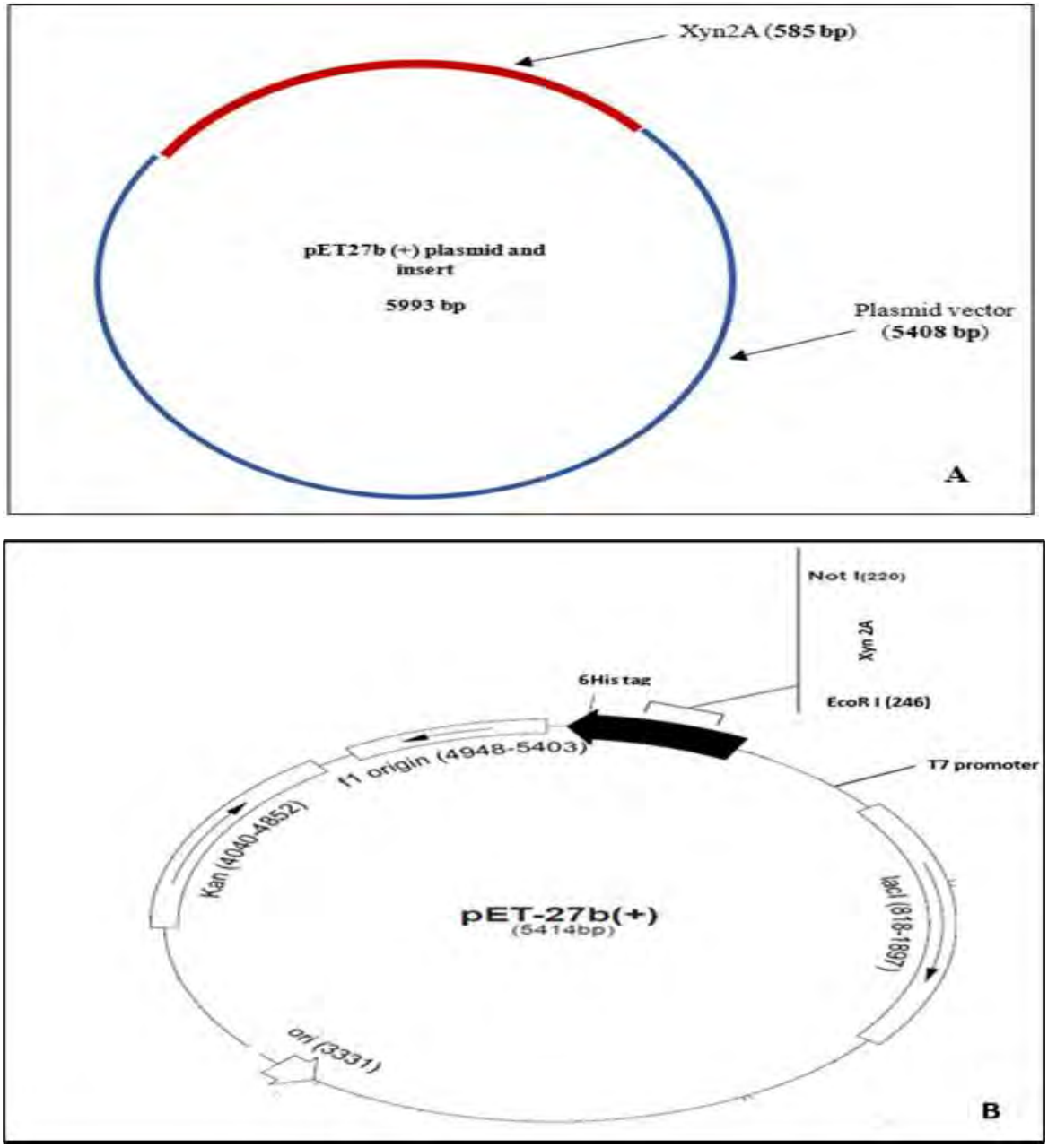


Figure 21: **B3**- pET27b (+)-Xyn2A plasmid construction.

Appendix C: Standard curves

Standard curves were generated to estimate the absorbance or concentration of the substances of interest.

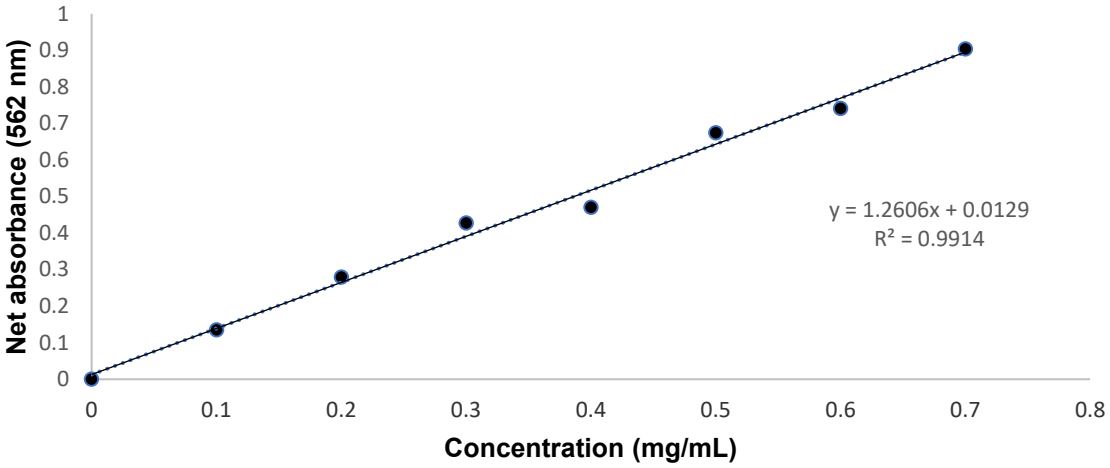


Figure 22: **C1**- BCA standard curve with bovine serum albumin as standard. Values presented as mean±SD, n=3.

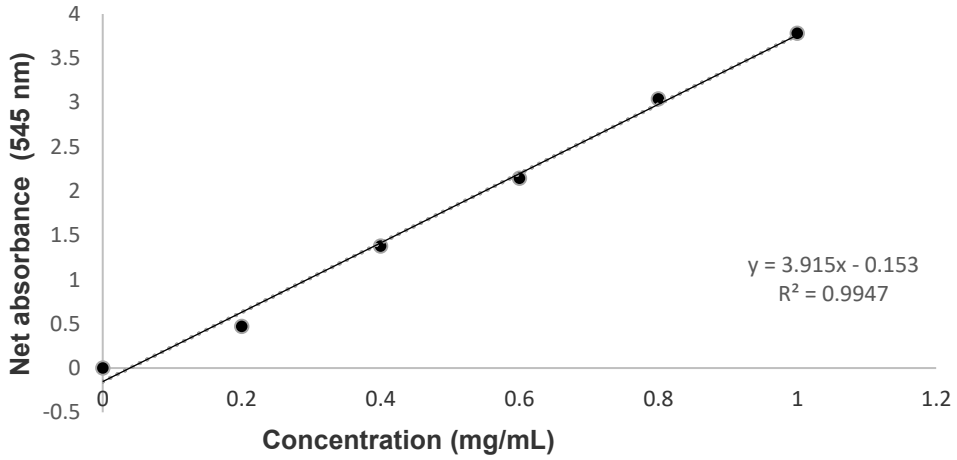


Figure 23: **C2-** DNS standard curve with D-xylose as standard**.** Values presented as mean ± SD, n = 3.

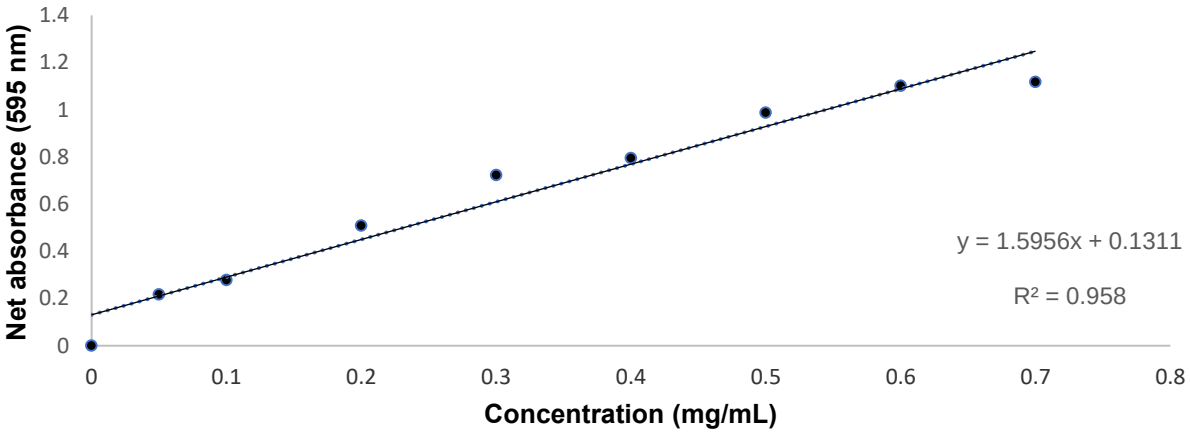


Figure 24: **C3-** BSA standard curve with Bovine serum albumin as protein standard. Values presented as mean ± SD, n = 3.

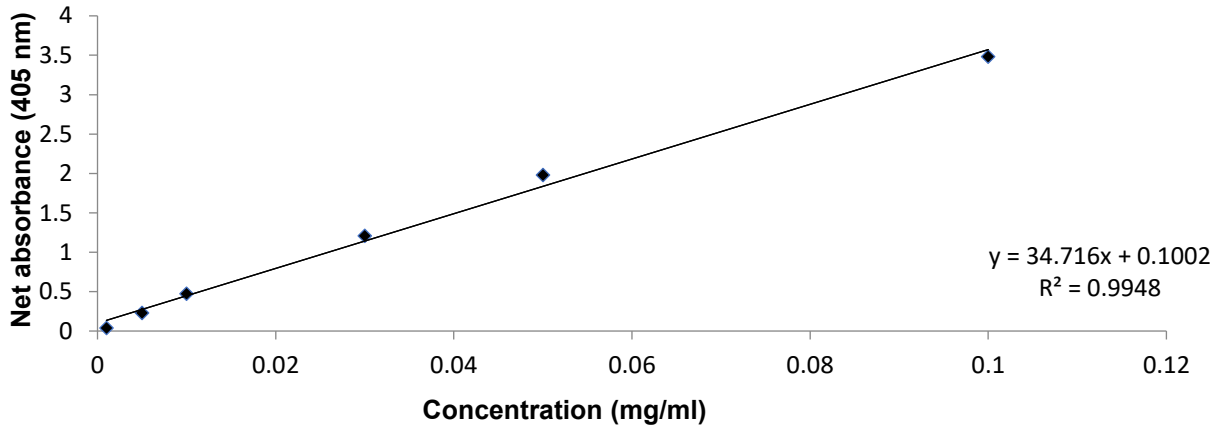


Figure 25: **C4-** p-Nitrophenol standard curve**.** Values presented as mean±SD, n=3.

Standard curves for volatile fatty acids were conducted, lactic acid, acetic acid, butyrate, propionate and formic acid were used as standards. Each standard was mixed 0.25 M HCl, and absorbance was measured at 210 nm.

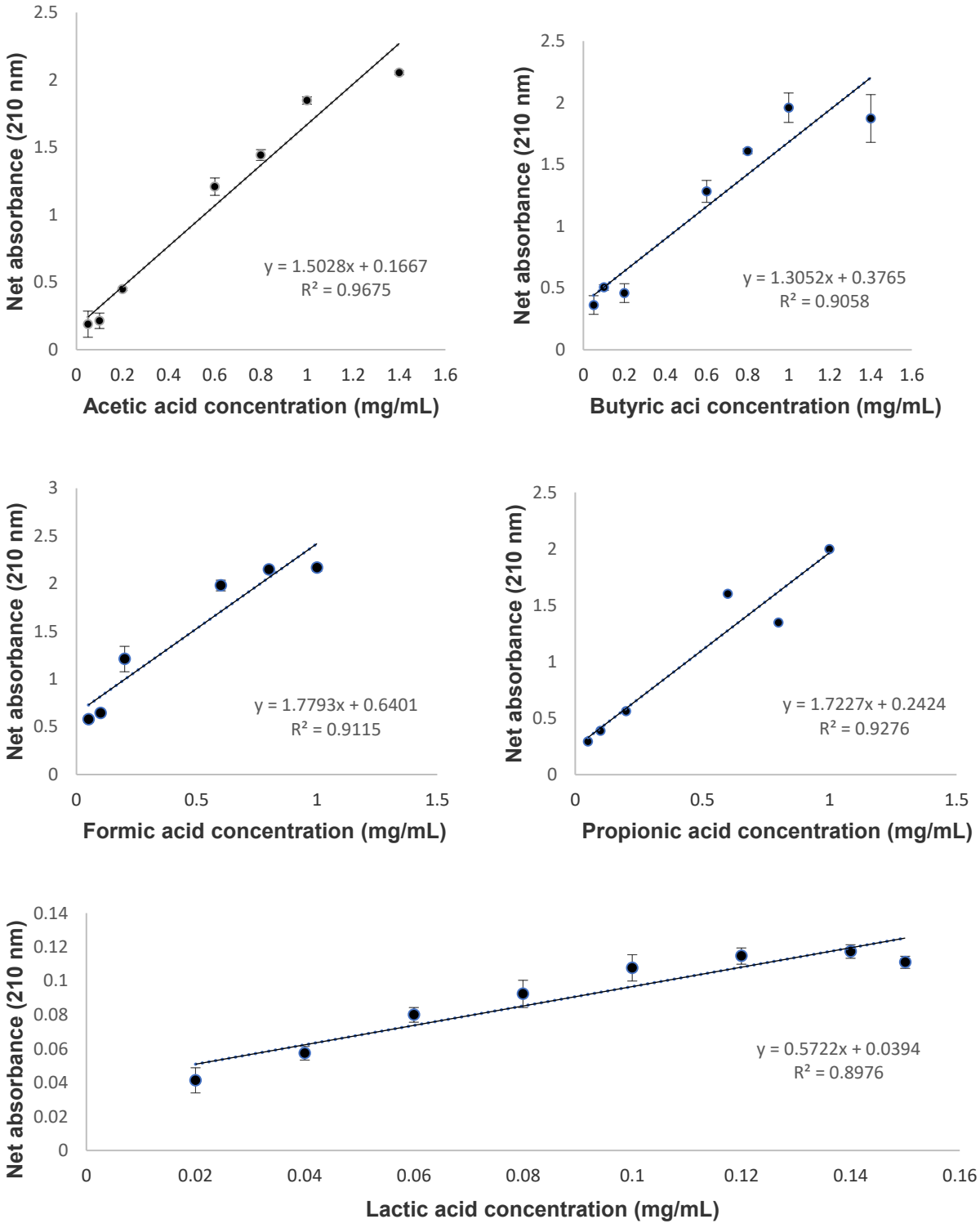
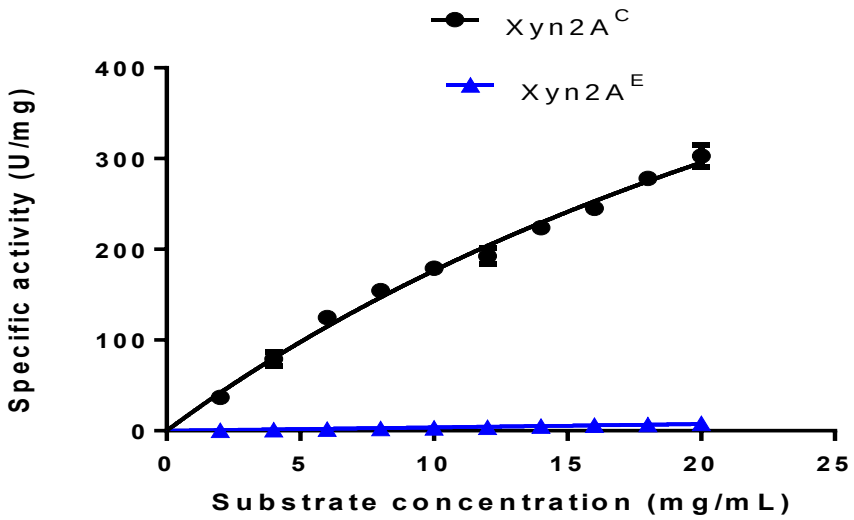
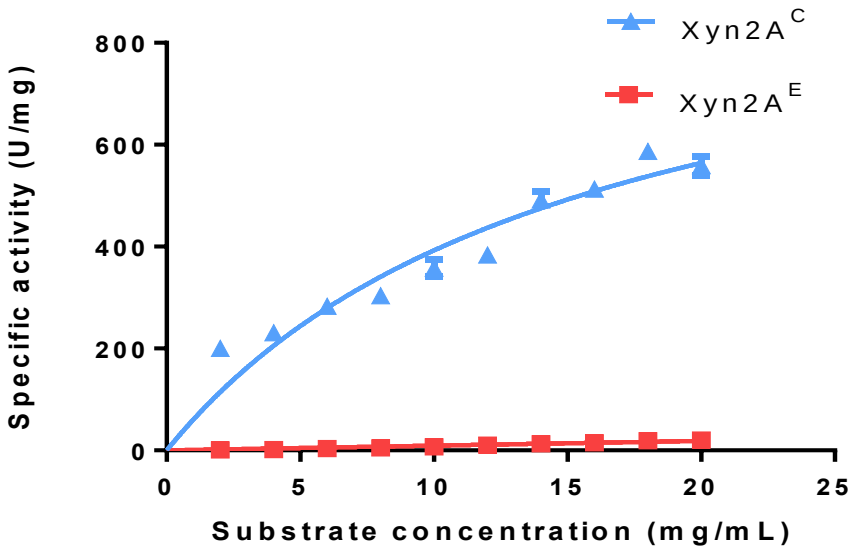


Figure 26: **C5-** Short chain fatty acid (SCFA) standards. Values represented as mean ± SD, n=3.

APPENDICE D: Michaelis-Menten graphs



A



B

Figure 27: D1- Michaelis-Menten plot showing activity of the xylanases in the presence of A- soluble and B- insoluble wheat flour arabinoxylan substrate.

Appendix E: Buffers and media

1× M9 media

This media was prepared in 1L by dissolving:

8.0 g of sodium chloride (NaCl) + 200 mg of potassium chloride (KCl) + 1.44 g of (Na_2HPO_4) + 245 mg (K_2HPO_4) chloride (NaCl) + 200 mg of potassium chloride (KCl) + 1.44 g of (Na_2HPO_4) + 245 mg (K_2HPO_4) in distilled MilliQ water

- 10 x SDS-PAGE Running buffer:

30.3 g Tris(Hydroxymethyl)Aminomethane + 144.0 g Glycine + 10.0 g sodium dodecyl sulphate (SDS) dissolved in distilled MilliQ water to 1 L, and stores at 4°C.

Working buffer:

10 × running buffer was diluted to a 1 × running buffer by diluting 50 ml 10 ×

running buffer with 450 ml distilled MilliQ water.

- 10% SDS Stock Solution

1 g SDS was dissolved in 10 ml distilled water

- 10% Ammonium Persulphate (APS) Stock solution

0.1 g APS was dissolved in 1ml distilled water (was prepared freshly before)

- 30% Acrylamide Stock Solution (100ml)

29.8 g Acrylamide + 0.2 g Bis-Acrylamide dissolved in MilliQ water

- Stacking gel buffer (0.5M Tris-HCl, pH 6.8)

6 g Tris(Hydroxymethyl)Aminomethane (Tris) was dissolved in 100 ml of

distilled MilliQ water and adjusted the pH of the buffer to 6.8 with hydrochloric acid (HCl).

- Resolving gel buffer (1.5M Tris-HCl, pH 8.8)

18.15 g Tris was dissolved in 100 ml distilled MilliQ water, and the pH adjusted to 8.8 with hydrochloric acid (HCl)

- DNS reagent

  2% (w/v) Sodium hydroxide + 2 g Dinitrosalicylic acid (DNS) + 40 g Sodium potassium tartrate (Roschelle salts) + 0.1g Phenol + 0.1 g Sodium Metasulfite in 100 ml Distilled MilliQ water. Store at room temperature.