Isolation, screening and characterization of cellulase producing bacteria from rumen fluids of ox and sheep samples

By: Abushe Abebe

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfilment for the Requirements of the Degree of Masters of Science in Applied Biology (Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

Adama, Ethiopia

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Advisors: Dr. Mulugeta Kebede

Dr. Tadessa Daba

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APPLIED BIOLOGY DEPARTMENT

APPROVAL OF BOARD OF EXAMINERS

We undersigned, members of the board of examiners of the final open defense by Abushe Abebe have read and evaluated her thesis entitled "**Isolation, screening and characterization of cellulase producing bacteria from rumen fluids of Ox and Sheep samples**" and examined the candidate. This is therefore, to certify that the thesis has been accepted in partial fulfillment of the requirement of the degree of Master of Science in applied Biology with specialization in Biotechnology.

Supervisor/Advisor	Signature	Date
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Chair person	Signature	Date
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Department	Signature	Date
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CANDIDATE DECLARATION

I hereby declare that the work which is being presented in this research entitled as **Isolation, screening and characterization of cellulase producing bacteria from rumen fluids of ox and sheep samples** in partial fulfillment for the requirement of degree of MSc in Biotechnology. It was authentic record of my original work carried out at Holetta Agricultural Research Center, Microbial biotechnology laboratory under the advisors of Dr. Mulugeta Kebede and Dr. Tadessa Daba. The matter embodied in this has not been submitted by other person or me any were for the award of any other degree. All relevant resources of information used in this body have been duly acknowledged.

_____ _____ _____

Candidate Signature Date.

This MSc Thesis has been submitted for examination with the approval of my advisor.

Name:

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Signature:

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Date of submission……

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LIST OF ACRONYMS AND ABBREVIATIONS

ATP	Adenosine triphosphate
CMC	Carboxymethylcellulose
DNS	Dinitrosalicyclicacid
GH	Glycosidehydrolases
HARC	Holetta Agricultural Research Center
MR-VP	Methyl Red Vogues Proskauer
NA	Nutrient Agar
SCFA	Short-chain fatty acids
SmF	Sub-merged fermentation
SSF	Solid State Fermentation
U	Enzyme unit
VFA	Volatile Fatty acids

ABSTRACT

Ruminant animals lack enzymes to break down fibrous feeds but they harbor different microorganisms capable of degrading their feeds. Rumen bacteria contribute a considerable part in the breakdown of cellulose biomass. The purpose of this study was to isolate, screen and characterize cellulase producing bacteria from rumen fluids of domestic ruminants and optimize their growth condition for cellulase production. Samples were collected from five fistulated oxen from Holetta agricultural research center and another sample were collected from three sheep and two goats from slaughter house at Holetta. A total of 85 pure isolates were collected and screened for cellulase activity by using Congo red stain on Carboxymethylcellulose (CMC) agar. Isolates which have shown maximum clear zone around the colony were characterized based on morphological and biochemical characteristics. These isolates were selected for enzyme production and optimization of the fermentation medium for maximum cellulase production. Dinitrosalisyclicacid (DNSA) method was used for measuring cellulase activity at 540 nm. Among all screened isolates, 26 bacterial isolates were found to hydrolyze CMC. From 26 cellulolytic bacterial isolates, 7 isolates have shown maximum clear zone around the colony. In this study, the optimum incubation time, carbon source, pH, temperature, and nitrogen source were found to be 72 hr., wheat straw, 7.0, 39ºC and Ammonium sulphate respectively. These cellulolytic bacterial isolates determine great potential for the study of enzymes in cellulose degradation and for improving the bioconversion of lignocellulose biomass. The current study showed that the 7 isolates were promising for cellulase production at their respective optimum parameters. From cheap lignocellulosic substrates, Wheat straw was found to be best digested substrate by cellulose degrading bacteria compare to Barley and Teff straw. Further characterization of the seven isolates at a molecular level and purification and detailed characterization of cellulase enzyme are recommended for effective utilization in feed industry.

Keywords: Cellulose, Fibrous feed, Rumen bacteria, Ruminants, Rumen fermentation

1. INTRODUCTION

1.1. Background of the study

Ruminant animals are poly gastric herbivores, which feed on plants and roughages rich in cellulose and hemicellulose. Ruminants are artiodactyl mammals that digest their feed in two steps, first by eating the raw material and regurgitating a semi-digested form known as cud from their first stomach, known as the rumen. The process of chewing the cud to break down the plant matter and stimulate digestion is ruminating (Vankessel *et al.*, 2002).

Ruminants lack enzymes capable of breaking down these materials, but they harbor microorganisms capable of degrading their feeds in their digestive system particularly in the rumen. The rumen is the primary site of digestion in ruminant animals and it is home to a vast population of microorganisms that assist in digesting feed by producing specific cellular enzymes. Ruminants can degrade cellulose by the living microorganisms in the rumen which produce cellulase. Therefore, ruminants can eat and digest agricultural by products and degrade huge cellulose biomass, which is widely available in nature (Al-Rawi, 2011).

Cellulose is the most abundant carbohydrate found in plants that provide structural integrity to their cell walls. It composes of linear polymer D-glucose linked by β-1, 4 glycosidic bonds which can be degraded by cellulase enzyme (Karnchanatant *et al*., 2008). Cellulase is an enzymatic complex composed of three major enzyme groups for cellulose hydrolysis in to glucose monomer, namely exoglucanases, endoglucanases and cellobiases. Synergy between these enzymes is important for the hydrolysis process (Ye *et al*., 2017).

Cellulases are produced by a large diversity of microorganisms during their growth on cellulose substrates A large numbers of microorganisms mostly bacteria and fungi in rumen break down plant materials ingested by the host animal and provide the animal with protein, vitamins and assimilable carbon and energy yielding substrates (Maiturare, 2017). Therefore, the efficiency of ruminants to utilize of roughage feeds is due to highly diversified rumen microbial ecosystem consisting of mainly bacteria. Das and Qin (2012) isolated and characterized different superior rumen bacteria.

Manipulation of rumen function through modification of rumen microbial flora is a promising method to enhance feed utilization in ruminants. Previous studies have proved that it is possible to alter rumen function by infusion of specific microbial species. Therefore, digestibility of fiber in ruminants can be improved by the introduction of highly fibrolytic strains of ruminal microbes or their enzyme extracts. Administration of a better fiber degrading anaerobic fungus isolated from goat to sheep resulted in an increased digestibility and intake of lignocellulosic feeds (Lee *et al*., 2000).

Therefore, potent rumen cellulolytic microorganisms can be isolated, characterized and possibly transferred to ruminants to synergistically perform cell wall hydrolysis. Alternatively, enzymes could be extracted and used to treat feed material externally. Enzymes can be used as biological tool to enhance digestion through the action of cellulase, hemicellulase and lignase enzymes resulting in improved animal product. The development of feed additives holds great promise for the improvement of livestock growth and yield for both large commercial and smaller subsistence farmers. AL-Rawi (2011) studied the production of cellulase from rumen cellulolytic microorganisms.

Cellulases have enormous potential in the degradation of cellulose with subsequent conversion in to useful products. Glucose produced from cellulosic substrate can be further used as substrate for subsequent fermentation or other processes, which could yield valuable end products such as ethanol, butanol, methane, amino acid and single cell protein (Cherian *et al*., 2015). Many researchers have used low cost agricultural residues such as sugarcane bagasse, Wheat bran, Rice bran and others, which have been found to be good sources of cellulase production (Techapun *et al.*, 2003).

There are several factors that positively influence the production of cellulase and such factors include; cellulose quality, temperature, incubation period, carbon sources, composition and pH of the medium (Immanuel *et al*., 2006). However, screening of bacteria, optimization of fermentation conditions and selection of substrates are important for the successful production of cellulase (sukumaran, 2005). Therefore, the objective of the present study was to isolate

and identify cellulase producing bacteria from rumen fluids and optimize growth conditions for cellulase production.

1.2. Statement of the problem

Ruminant production is a considerable economic value and supports food security in many regions of the world. However, aspects of ruminant production sector have major challenges because of diminishing of natural resources and increasing of production cost. Traditionally, livestock feed supply primarily depends upon poor pasture lands and crop residues, which are usually inefficient to help cattle production. Crop residues from cereals (Wheat, Teff and Sorghum for example) are the main sources of feed during the long dry season in mixed crop-livestock farming system of Ethiopia. These fibrous by-products and their feeding value limited by their poor voluntary intake, low in protein and have poor digestibility (Mengistu *et al.*, 2017). Hence, improving the utilization of poor quality roughage feeds to provide the required nutrients to the animal has great potential of raising the overall animal production and productivity.

The digestion of plant biomass in ruminant animals strongly depends on rumen micro biota due to the lack of cellulolytic enzymes. These animals harbor potent cellulolytic and hemi cellulolytic microorganisms that supply the host with nutrients acquired from degradation of ingested plant material. Rumen bacteria contribute a considerable part in the breakdown of cellulosic biomass. Cellulase production from bacteria can be an advantage as the enzyme production rate is normally higher due to the higher bacterial growth rate as compared to fungi. High cost of cellulase is mainly due to the substrates used in the production and also the slow growth rate of fungi. Bacteria, which has high growth rate as compared to fungi has good potential to be used in cellulase production (Ariffin *et al*., 2006).

The production of ethanol and other biofuels from lignocellulose biomass has received great attention both in industry and in academic communities worldwide. This biomass can be converted in to ethanol by hydrolysis and subsequent fermentation. In the hydrolysis process, acid reliant hydrolytic processes have been used for many years, but have also been blamed for negative effects on the environment. Thus, enzymatic hydrolysis is a more environmentally

sound approach. In the fermentation process, the conversion of cellulose, the recovery efficiency hydrolysis and the cost depends strongly on the fermentation efficiency of the microorganisms and enzymes (Nikolic *et al*., 2010; Hamelink *et al*., 2005). In line with the above information, this work focused mainly on the isolation of cellulose degrading bacteria from rumen fluids of ruminant animals and assessment of their cellulolytic activity.

1.3. Significance of the study

The symbiotic microbes inhabiting the rumen elaborate enzymes for fermentative digestion of large amounts of fibrous feed consumed by the ruminants. By providing a suitable habitat for these microorganisms, the ruminants are able to utilize the end products of microbial fermentation to meet their nutritional need. Several methods are currently employed to manipulate rumen fermentation to enhance post-ingestion the nutritional value of fibrous forages through use of biotechnology including inoculants of native and recombinant rumen microorganisms and microbial feed enzymes (Flint and Scott, 2000).Therefore, maximizing the efficiency of breakdown and digestion of plant cell walls in the rumen can have a marked effect on animal productivity. So, determining the role of microbes and their enzymes in plant polysaccharides breakdown is fundamental to understanding digestion and maximizing productivity in ruminant animals.

Cellulase is one of the most useful enzyme in industrial processes. In industries these enzyme have found novel applications in the production of fermentable sugars and ethanol, organic acids, detergents and other chemicals. Moreover, applications of cellulase in the animal feed industry have received considerable attention because of their potential to improve feed value and performance of animals. Furthermore, cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization (Wen *et al*., 2005). Information that obtained from this study is useful in knowing the potential of rumen cellulase producing bacteria and the common genera of bacteria involved in cellulase enzyme production. It will add some contributions to the knowledge of cellulase enzyme production using lignocellulose as a substrate to reduce the enzyme production cost. In addition it will be used as a reference for the coming study on this issue.

1.4. Objectives of the study

1.4.1. General objective

The general objective of this study was to:

- Isolate, screen and characterize cellulase producing bacteria from rumen fluids three fistulated ox and sheep samples and determine their optimum growth parameters for cellulase production

1.4.2. Specific objectives

The Specific objectives of this study were to:

- Isolate and screen cellulose degrading bacteria
- Examine the morphological and biochemical characteristics of cellulolytic bacteria isolated from rumen fluid.
- Produce and extract crude cellulase enzyme from potential isolates
- Determine best performing lignocellulose substrate for cellulase production
- Determine the optimum growth condition for the best cellulase producing bacterial isolates.

2. LITERATURE REVIEW

2.1. Ruminants and their digestive tract

The relationship between humans and ruminants dates back to prehistoric times where hunter-gatherers realized they could increase their food supply by domesticating ruminant animals and subsequent farming communities resulting in stable social communities (Russell and Rychlik, 2001). In fact, meat and milk that we consume today come from domestic ruminants. The term ruminant comes from the Latin word ruminare which means to chew the cud (Collins *et al*., 1986). Cud chewing, even-toed, hooved mammals consists of four-chambered stomach and categorized under the suborder Ruminantia are known as true ruminants (Dobson and Dobson, 1986).

Diets that ruminants consume are diverse. Nowadays, doubtlessly cattle are the most important livestock species throughout the world. About 41% of the earth's land surface is covered by dry land systems (Lund, 2007). Ruminant animals beneficially digest and utilize this complex biomass by converting apparently indigestible carbohydrates and proteins to use full products with the help of rumen microbes. When ingested feed is chewed, plant material is broken down to be mixed with the saliva and forms a bolus that is swallowed into the reticulo-rumen. Through this process, particle density is increased and salivation induces a buffering action in the rumen pH (Church, 1979).

Rumination starts after an inactive period upon the first meal, when the animal has collected enough feedstuff. Rumination is the process of regurgitating ingesta from the reticulo-rumen into the mouth, where the bolus is masticated again and mixed with saliva for 30 to 60 seconds and then re-swallowed. This mechanism reduces the particle size of the feedstuffs, exposing the cell walls for the microbial digestion and sorting particles according to their density (Dehority, 2003). The duration of rumination can vary from periods of 15 to 30 minutes, up to 2 to 6 hours (Saras-Johansson, 2011).

One of the main differences between ruminants and monogastric are the anatomical differences of their gastrointestinal tracts. Ruminants possess a compartmentalized stomach

consisting of four chambers: rumen, reticulum, omasum, and abomasum. These four sac-like structures vary in size and their epithelial structure and differ for various activities such as absorption of nutrients (Van Soest, 1994).

Rumen: is the largest of the four compartments and is divided in to several sacs. It can hold 94 liters or more of materials depending on the size of the cow and act as storage or where the major fermentation processes are held (Figure 1). Enzymes present in the rumen are produced by microorganisms. These enzymes are used to digest and ferment food eaten by ruminants. Thus, the rumen is considered as a fermentation vat (Hungate, 1966)

The rumen microorganisms are also digested and absorbed in the small intestine of the dairy cow as the main protein source for milk production providing up to 70–90% of a cow's protein requirements. The ability of rumen microorganisms to produce the enzymes necessary for fermentation processes allows ruminants to efficiently obtain the energy contained in forages (Duskova and Marounek, 2001). The end products of rumen fermentation include volatile fatty acids (VFAs) mainly, acetate, propionate and butyrate and gases includes CO_2 and CH_4 Volatile fatty acids (VFAs) are the main source of carbon and energy to the animal. This fermentation process results in many intermediate products such as lactate, succinate, formate, H_2, and NH_3. The steady supply of food and constant removal of digested feed material and end-products allow a dense population of microorganisms to grow inside the rumen (Hungate, 1966).

There is a symbiotic relationship between the rumen inhabiting microorganisms and the host animal. The host animal provides the substrates to the microbes by consuming forage, which is then digested and fermented by the rumen microbes to form VFAs, CO_2 and CH_4 (Hungate, 1966). Therefore, rumen microorganisms can be viewed as a combination of working units, which attack and break down feed particles in the rumen.

Reticulum: It is the second stomach compartments and a small pouch-like organ, distinctive of the rumen, with epithelial surface resembling a honeycomb. The function of the reticulum is transferring feed particles to the esophagus and to the mouth during the rumination process (Russell, 2002).

Omasum: is the third compartment of the ruminant stomach. Its epithelium is organized in longitudinal laminae that act as a filter. During muscular contractions of the reticulo-rumen, bacteria and small particles can either pass through the reticulo-omasal orifice to the omasum or to flow to the abomasum, while larger feed particles remain in the rumen to be further reduced (Church, 1979).

Abomasum: It is called as the gastric stomach but, its pH is not as low as in the stomach of non-ruminant species (Russell, 2002). In the abomasum, digesta and microbes undergo the first phases of protein digestion and then they are transferred to the small intestine where additional enzymatic breakdown is carried out.

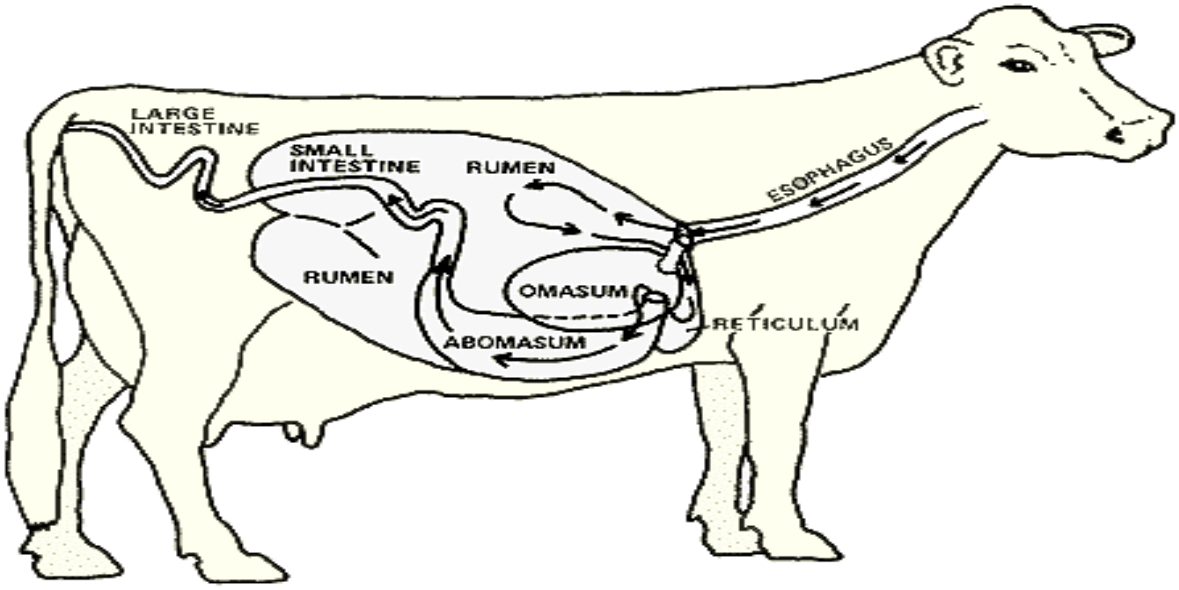

Figure 1: Anatomy of the adult digestive tract (Source: Rush, 2009)

2.2. Physiochemical properties of the rumen

The major factors influencing the growth and activity of ruminal microbial populations are temperature, pH and buffering capacity. These factors are determined by environmental conditions. The temperature of the rumen is important for bacterial fermentation. Most microorganisms require an optimal temperature range in order to maintain steady growth. If rumen temperature varies from being optimal, rumen microbes become less active, reducing

the efficiency of digestion through bacterial fermentation. Bacterial fermentation has been shown to be at its optimum at temperatures between 38°C and 40°C and may increase up to 41^{o}C immediately after the animal eats because the fermentation process generates heat (Dehority, 2003; Brock *et al*., 1982). Generally, as fermentation increases, a rise in rumen temperature is observed with heat produced by fermentative reactions.

The pH depends on the production of saliva, the generation and absorption of short-chain fatty acids (SCFA), the type and level of feed intake and the exchange of bicarbonates and phosphates through the ruminal epithelium (Aschenbach *et al.,* 2011). Thus, these factors determine both pH and buffering capacity in the reticule ruminal environment. The pH constantly changes, but it usually remains in the range of 5.5 to 7.0 (Krause and Oetzel, 2006).

During bacterial fermentation, VFA such as acetic, propionic and butyric acid are produced. An accumulation of these VFA leads to decrease in rumen pH, which in turn has an inhibitory effect on bacterial fermentation. To regulate rumen pH, ruminants produce large quantities of saliva. Saliva is chiefly made up of sodium bicarbonate which acts as a buffer to neutralize accumulating VFA (Hungate, 1975). Cellulolytic bacteria are sensitive to low pH, so it is essential that fluctuations in rumen pH are avoided if optimum fiber digestion is to be achieved (Wallace, 1992).

2.3. Rumen microbial ecosystem

The rumen is an open ecosystem, where many microbial species have an opportunity to grow. Members of the three domains of life, bacteria, eukarya (protozoa and fungi), and archaea, are all found in high abundance in the rumen. Of the three domains, bacterial numbers in the rumen are the highest and bacteria play a significant role in all facets of ruminal fermentation in this complex ecosystem (Weimer *et al.*, 2009).

The efficiency of ruminants to utilize feeds is due to highly diversified rumen microbial ecosystem consisting of bacteria about 10^{10}–10^{11}viable cells per gram of content that represents about 200 species, variety of ciliate protozoa 10^{4}–10^{6}per gram about 25 genera, anaerobic fungi of zoospore population 10^{2}–10^{4} per gram, about 5 genera (Miron *et al*.,

2001).The capability for breaking down plant fiber rests relatively with a number of bacterial, fungal and protozoan species. It is best to address the role of each ruminal bacteria, protozoa and fungi separately for clarity of purpose.

2.3.1. Fibrolytic ruminal bacteria

The majority of rumen bacteria are strictly anaerobic. However, facultative anaerobic bacteria as well as some aerobes are also found in smaller numbers (Miron *et al*., 2001). The plant cell wall incorporates 35-80% of the cells carbohydrates, with cellulose as the dominant component (Jung and Allen, 1995). The ability to degrade cellulose depends mainly on the type of forage, crop maturity and the members of the cellulolytic bacterial communities (Fondevila and Dehority, 1996). To ensure maintenance and growth of cellulolytic bacteria, optimal ruminal conditions are required.

Cellulolytic bacteria found in the rumen most commonly include: Gram positive bacteria from the Firmicutes phylum, such as *Ruminococcus albus* and *Ruminococcus flavefaciens*, as well as the Gram negative *Fibrobacter succinogenes* from the Fibrobacteres phylum. These cellulolytic bacteria are able to adhere to ingested plant polymers providing access for cell-associated enzymes to act in the enzymatic breakdown of fibrous plant material. Enzymes produced by these cellulolytic bacteria include, endo and exo-cellulases, β-glucosidases, hemicellulases, pectin methyl esterase, pectin lyase and polygalacturonases (Krause *et al.*, 2003). These enzymes work synergistically to hydrolyze the β-1, 4 glucosidic linkages of structural carbohydrates to produce digestible fermentation products; predominately pyruvate, succinate, lactate and glucose.

Hemicellulose degrading bacteria; after cellulose, the second most abundant polysaccharide in plant material is hemicellulose, which is a branched heterogeneous polymer composed primarily of pentose and hexose monomers with xylose as the major carbohydrate (Saha, 2003). In the rumen, hemicellulose is primarily degraded by *Prevotella* species, which produce enzymes capable of degrading the hemicellulose polymer as well as the various di- and monosaccharide subunits. From the rumen *Prevotella* species, *Prevotella ruminicola* and *Prevotella bryantii* have been the most extensively studied. The end products of hemicellulose

degradation are further utilized as substrates in the production of VFAs such as acetate, butyrate, formate and propionate.

Lipolytic bacteria; the rumen bacterial population also contributes to the metabolism of plant lipids. Bacteria such as *Butyrivibrio fibrisolvens* and *Anaerovibrio lipolytica* hydrolyze dietary esterified lipids to glycerol, free fatty acids and small amounts of mono- and di-glycerides. The unsaturated double bonds of these free fatty acids are rapidly hydrogenated by rumen bacteria, producing saturated or mono-unsaturated fatty acids (Buccioni *et al.*, 2012). Amino acids released from fermentation are deaminated by proteolytic rumen bacteria such as *P. ruminocola* and *Ruminobacter amylophilus*, with ammonia and the acidic carbon skeleton (can be VFA's and branched chain VFA's) being produced. Ammonia released from proteolysis is subsequently utilized for microbial protein synthesis (Pengpeng and Tan, 2013).

It is clear that rumen bacteria play an essential role in the digestion of plant matter through a process of anaerobic fermentation. The resultant production of VFAs, essential amino acids and microbial protein, are key precursors for cellular growth and maintenance in ruminants. Non fibrolytic bacteria in the rumen have indirect effect on plant tissue degradation by utilizing the intermediate and end products produced by plant cell wall degrading microbes. For example, *Selenomonas ruminantium* is incapable of fermenting cellulose but will utilize the cellodextrins produced during hydrolysis of cellulose by *F. succinogens* (Russel, 1985). Also succinate produced by many plant cell wall degrading bacteria, never accumulate in the rumen. It is utilize by other bacteria such as *selenomonas ruminantium* to produce propionate, which is essential to the ruminant because it is the only intermediate used in gluconeogenesis (Wolin and millar, 1988).

2.3.2. Fibrolytic ruminal fungi

Ruminal fungi where first observed when fungal rhizoids where seen attached to plant tissue in rumen contents (Orpin, 1975). The anaerobic fungi in the rumen are a group of zoosporic fungi occupying a unique niche in the digestive tract of ruminants (Theodore *et al*., 1996). Ruminal fungi have been reported to play a fundamental role as the initial colonizers of plant

fiber in the rumen and able to produce enzymes that hydrolyze cellulose and xylans (Lee *et al*., 2000).

Their enzymatic activity is variable, depending on their phylogenetic origin and especially their rhizoidal structure, but it has been postulated that some species, such as *Neocallimastix frontalis*, *piromyces joyonii* and *Orpinomyces communis* can more efficiently digest structural polysaccharides than cellulolytic bacteria species in monoculture (Bernalier *et al*., 1992).

The fungal activity helps the ruminal digestion of the plant cell wall. The production of zoospores by chemo taxis allows rapid adhesion to the particles, then the fungi fracture zones of lignified tissues by mechanical action and the non lignified plant tissues are rapidly degraded. Thus, ruminal fungi are particularly important when the ruminant consumes many lignified substrates. For example, *N. frontalis* has the ability to solubilize small lignin fractions of the plant cell wall allowing the bacteria access to the cellulose (Bernalier *et al*., 1992).

2.3.3. Ruminal protozoa

The other important population in the rumen is the rumen protozoa. Protozoa are the first microorganism discovered from the rumen in 1843 by Gurby and Delafond (Hungate, 1966). There are about10^6 protozoal cells per gram of rumen content (Dehority, 2003). Both ciliate and flagellate protozoa are found in the rumen; however the ciliates are both the largest and most important in terms of rumen function (Williams and Colema, 1988).

Estimates of the number of ciliates protozoa in the rumen between 10^5-10^6 ml^{-1} and are thought to be responsible for 25-33% of the plant tissue degradation in the rumen (Hungate, 1966).Two major groups of ciliate protozoa have been described; the *Entodiniomorphs* and *Holotrichs*. More than 100 species of *Entodiniomorph* protozoa have been found in the rumen. All are strict anaerobes which feed mainly by engulfing particulate matter, including other ruminal microorganisms (Williams and Colema, 1988).

The *Entodiniomorph* lack the abundant cilia which cover the surface of *Holotrichs*, but have involved specialized bands of syncilia (fused ciliary bands), which function both for movement and food ingestion (Hungate, 1996). Ruminal *Holotrichs* protozoa possess cilia

over the entire body surface which inserted singly and not fused with others except in the region of mouth (Hungate, 1966). *Holotrichs* protozoa are mainly involved in the utilization of nonstructural carbohydrate and soluble sugars and have only limited ability to degrade plant structural polysaccharides (Demeyer, 1981).

2.3.4. Ruminal archae or methanogens

Archae classified in to three depending up on their ability to adapt very extreme environments such as thermophilic, methanogenic and halophilic. The majority of Archaea present in the rumen belong to the methanogenic group. Methanogen are responsible for regulating the overall fermentation in the rumen by removing H_2 during methane production, encouraging the activity of H_2 producing species and altering their metabolism towards higher yielding pathways (Leahy *et al*., 2010).

Methane is the final product of breaking organic materials down in to the simple methanogenic substrates and thus, methanogens complete the last step in the digestion of organic matter. These ruminal microorganisms utilize the CO_2 and H_2 produced by the protozoa, fungi and bacteria from the catabolism of hexoses to produce CH_4 and generate ATP (Ferry *et al*., 2010). Because they are dependent on the organisms for provision of substrate and the establishment of reducing conditions, methanogens are found in a symbiotic association with ruminal bacteria, protozoa and fungi (Leahy *et al*., 2010).

2.4. Manipulation of rumen fermentation

Manipulation of rumen microbial ecosystem is one of the major thrust research areas of rumen microbiologists and ruminant nutritionists to maximize the efficiency of fibrous feed utilization to increase ruminant production and productivity (Nagaraja *et al*., 1997). Biotechnology has been used to modify ruminal fermentation by promoting certain fermentation processes leading to a higher efficiency in animal productivity. Some application of biotechnology for the improvement of rumen fermentation is listed below.

1) Management of diet and modification of fermentation profile

Some diet changes may improve the microbial fermentation profile. For example, the inclusion of legumes to the ruminant diet can have positive results in terms of microbial activity and fermentation end products. Because of leguminous plants is a good source of protein, rich in amino acids, vitamins, and minerals and good substrates of cellulolytic microorganisms for growth and enzyme function.

On the other hand, forages of low nutritional value exist which contain high amounts of lignin, cellulose, low concentrations of fermentable sugars and proteins of low quality; these characteristics affect the microbial activity and thus the profile of the fermentation products. For example, the use of rice straw decreases cellulolytic, proteolytic, and amylolytic bacterial populations, if these agricultural byproducts are treated with urea, the microorganism can use it as a substrate, leading to an increase of these populations and improvement of the fermentation profile (Wanapat, 2000).

2) Feed transformation before consumption

Feed transformation before consumption involves all of the physical and chemical treatments applied to feed before being consumed by the ruminant. Physical treatments include heating and varying the particle sizes (Schadt *et al*., 2012). Chemical treatments are the best used methods, including the use of enzymes such as cellulases and xylanases which are purified from fermentation cultures of both bacteria and fungi (Beauchemin *et al*., 2003). Researchers have shown that supplementing the cattle diet with fibrolytic enzymes significantly improves the use of nutrients and increases ruminant efficiency. Several of these fibrolytic enzymes have been evaluated as additives in ruminant diets and were originally developed as additives in silage, hay and agricultural by-products (Tang *et al*., 2008).

For example, Tang et al. (2008) proved that fibrolytic enzymes supplementation increased in vitro digestibility of dry matter and in vitro organic matter digestibility of maize Stover, Rice straw and Wheat straw. Cellulose and hemicellulose are insoluble polysaccharides but in the presence of enzymes, they are converted to soluble sugars so that they can be used by ruminal

microorganisms. The improvement in ruminant efficiency due to the use of fibrolytic enzymes is attributed to improved ruminal forage digestion, which results in an increase in dry matter digestibility and voluntary intake increasing the digestible energy that is used by the ruminant (Beauchemin *et al* ., 2003).

3) Use of microorganisms fermentation activators

Probiotics are microorganisms that are not of ruminal origin but can be adapted to ruminal conditions and improve the ruminal fermentation process. The probiotics used in ruminant feed mainly include fungi and bacteria, which have replaced conventional antibiotics as growth promoters (Lila *et al.,* 2004). Specific strains of bacterial species of the genera *Lactobacillus* and *Bifidobacterium* have been shown to have good results as probiotics in ruminants, increasing levels of weight gain and efficiency. Furthermore, it has been seen that these lactic acid bacteria are able to reduce the risk of enteric disease caused by *E. coli* and *Salmonella* spp. (Stephens *et al*., 2007). The fungi *Aspergillus oryzae* and *Saccharomyces cerevisiae* have been used as probiotics as well as food additives to improve the ruminal fermentation process (Marrero *et al.,* 2011).

2.5. Cellulose: Structure and microbial break down

Cellulose, the most abundant polysaccharide on earth and a major component of ruminant diets and can be broken down into metabolites that can be absorbed by the host to use as a source of energy (Naas *et al.,* 2014). The efficiency of a ruminant ability to convert plant biomass into meat, milk or wool products is a growing area of interest and further knowledge of this process could be useful in developing strategies to meet a growing food demand. Furthermore, cellulose is not the only substrate metabolized by ruminal microorganisms. Substrates such as starch, fat and protein are also metabolized by ruminal microbiota into utilizable by-products for host absorption. In fact, the majority of the host's daily protein requirements are fulfilled through absorbed microbial proteins resulting from bacterial cell lysis (Yeoman and White, 2014).

Cellulose is a linear polymer of glucose linked by β-1, 4-glycosidic bonds, having a simple primary and complex tertiary structures and its repeating unit is cellobiose. A single molecule of cellulose can be comprised of hundreds or up to thousands of monomeric glucose subunits, making it one of the longest organic polymers found in nature (Lee *et al*., 2014).

Its chain length can vary between 100 and 14000 glucose residues, which form microfibrils (Figure 2). The microfibrils consist of highly ordered crystalline regions interspersed by more disordered amorphous regions. The crystalline regions of cellulose are rigid and not easily accessible to endo-acting cellulases while the amorphous regions are easily attacked by dilute acid, endoglucanases or exoglucanases (Sinitsyn *et al*., 1991) In the primary cell walls of plants, cellulose can be assembled into long, rigid microfibrils that allow the plant to control cell expansion and serves as a key component in growth and development (Thomas *et al*., 2013).

Enzymes responsible for cellulose breakdown have yet to be identified in any animal genome, but they are expressed by bacteria, protozoa, and fungi. In the rumen, a number of different cellulases involved in the hydrolysis of cellulose have been characterized and most ruminal cellulases belong to a broad family of enzymes known as glycoside hydrolases (GH). Cellulase is an enzymatic complex composed of endo-1, 4-β-D-glucanases or endoglucanases, exo-1, 4-β-D-glucanases or cellobiohydrolases and 1, 4-β-D-glucosidases, which act on cellulose to produce glucose (Ye *et al*., 2017).

In bacteria, these three types are expressed as extracellular enzymes that work synergistically in the conversion of long polysaccharide chains to D-glucose subunits (Krause *et al*., 2003). As feed is ingested by the host ruminant, polymers such as cellulose become available to ruminal microorganisms. Endocellulases function as the initial biochemical tools in cellulose breakdown, by cutting of β, 1-4 glycosidic bonds at random positions within the cellulose polymer, yielding cellu-oligosaccharides possessing new chain ends. The non-reducing ends of the newly formed cellu-oligosaccharides then become available for the active site in exocellulases (Krause *et al*., 2003). Typically, exocellulases can cleave the β, 1-4 glycosidic

bond 2-4 subunits from the non-reducing end, forming cellobiose as their primary product (Zverlov *et al*., 2005).

Cellobiose is a disaccharide consisting of two D-glucose monomers linked by a β, 1-4 glycosidic bonds that can subsequently be cleaved by β-glucosidases to release the monomeric glucose subunits (Krause *et al*., 2003). Soluble glucose can then be absorbed by the host or it can be transported across bacterial membranes for intracellular fermentation into VFAs (Thauer *et al*., 2008).

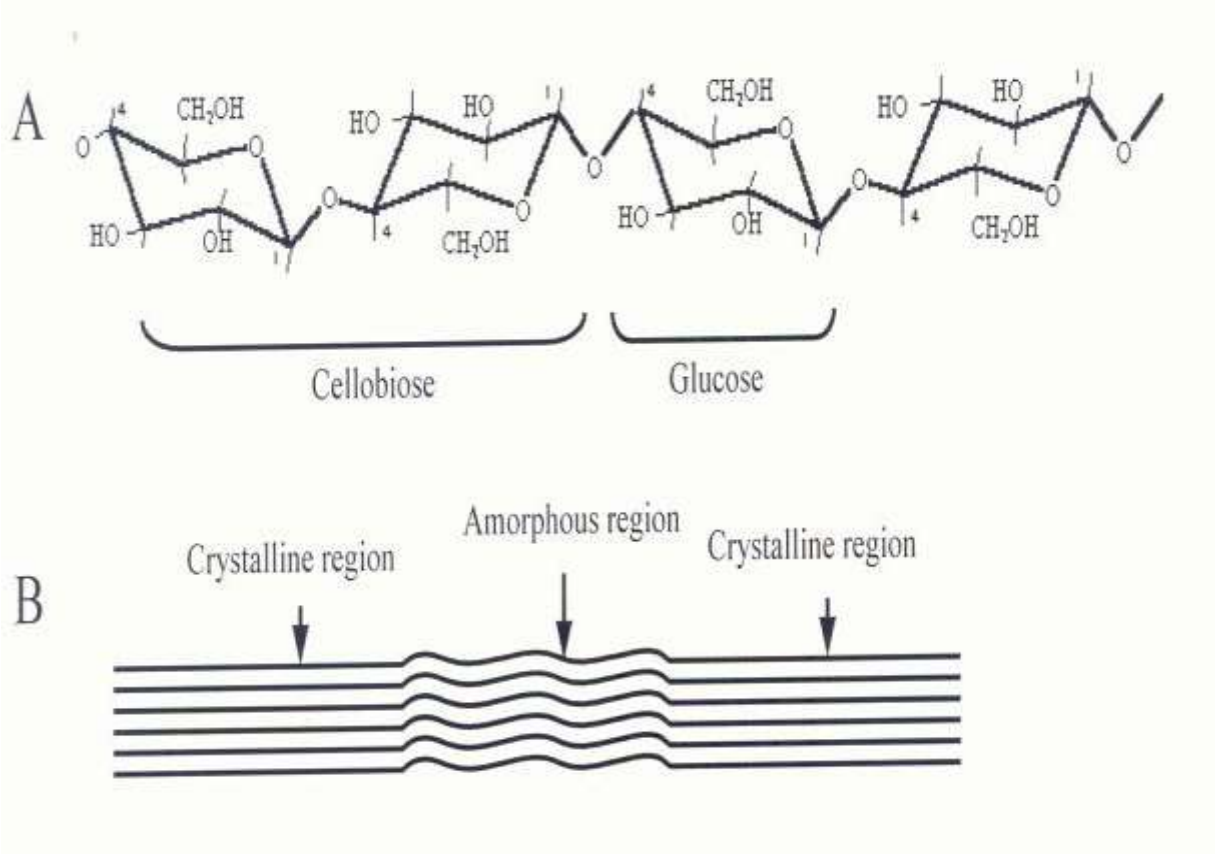

Figure 2: The structure of cellulose showing: A. Cellobiose and glucose sub-units B. Cellulose microfibrils showing crystalline and amorphous regions (Beguin and Aubert, 1994).

2.6. Methods of enzyme production

Submerged fermentations (SmF) and solid-state fermentations (SSF) are the two methods widely employed for the production of enzymes.

2.6.1. Submerged fermentation

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms such as bacteria and fungi. Fermentation is the primary technique for the production of various enzymes. Both bacteria and fungi yield an important array of enzymes when fermented on appropriate substrates. Submerged fermentation (SmF) is commonly implemented in case of bacterial enzyme production, due to the requirement of higher water potential (Subramaniyam and Vimala, 2012).

Submerged fermentation utilizes free flowing liquid substrates, such as molasses and broths. This involves growing carefully selected microorganisms in closed vessels containing a rich broth of nutrients or the fermentation medium and a high concentration of oxygen. As the microorganisms break down the nutrients, they release the desired enzymes into solution. The substrates are utilized quite rapidly; hence need to be constantly replaced/supplemented with nutrients. This fermentation technique is best suited for microorganisms such as bacteria that require high moisture (Babu and Satyanarayana, 1996).

An additional advantage of this technique is that purification of products is easier. SmF is primarily used in the extraction of secondary metabolites that need to be used in liquid form. Currently industrial demand of most enzymes is met by production using SmF employing genetically modified strains. SmF is intrinsically less problematic, heat transfer is better and homogeneity is much better (Pandey *et al*., 2000).

2.6.2. Solid state fermentation

Solid state fermentation (SSF) is resembles the natural environment of the fungi, which require lesser water potential and preferred when enzymes have to be extracted from fungi. SSF is defined as any fermentation process performed on a non soluble material that acts both

as physical support and source of nutrients in absences of free flowing liquid. SSF holds tremendous potential for the production of enzymes. The hyphal mode of fungal growth and their good tolerance for low water activity and high osmotic pressure conditions make fungi efficient and competitive in natural microflora for bioconversion of solid substrates (Pandey *et al.*, 2000).

SSF offers numerous opportunities in processing of agro-industrial residues. SSF utilizes solid substrates, like wheat straw, bran, bagasse, and paper pulp. The main advantage of using these substrates is that nutrient-rich waste materials can be easily recycled as substrates. In this fermentation technique, the substrates are utilized very slowly and steadily, so the same substrate can be used for long fermentation periods. Hence, this technique supports controlled release of nutrients. SSF is best suited for fermentation techniques involving fungi and microorganisms that require less moisture content. However, it cannot be used in fermentation processes involving organisms that require high water activity, such as bacteria (Babu and Satyanarayana, 1996). The most significant problem of SSF is the high heterogeneity, which makes difficult to focus one category of hydrolytic processes, and lead to poor trials of modeling, labour intensive and lack of uniformity in the substrate (Pandey, 1992).

2.7. Determination of enzyme activity

Enzyme assay means laboratory methods for measuring enzymatic activity. Assays for determining cellulase activity have been classified into three main groups (Zhang *et al*., 2006). The first group involves assays in which accumulation of products after the hydrolytic process is targeted, the second groups are assays in which the reduction of the substrate concentration is monitored and the third groups are for assays in which change in physical properties of the substrate are monitored. The first group of assays is preferred when measuring individual cellulase activities in a short time and the third group is preferred for total enzyme activities within a given time (Wu *et al*., 2006; Zhang *et al*., 2006).

For soluble enzyme the sample is filtered or centrifuged to remove solids and the filtrate or the supernatant is analyzed for enzyme activity, while for cell bound enzymes, the cells are homogenized in an appropriate buffer such as 0.05M citrate, pH 4.8 (Ghose *et al*., 1987).

Assay activities may not reflect potential saccharification performance since other factors such as end product inhibition, addition of cellobiase activity, reactor process configuration come into play in commercial cellulose hydrolysis. These factors vary with different cellulase systems and significantly affect the conversion efficiency (Ghose *et al*., 1987).

2.8. Application of cellulase enzyme

Cellulases have been a potential candidate for research by both academic and industrial research groups because of the diversity of their application. Many researches have been conducted to find out industry beneficiary strain to increase the yield of cellulase production. Microbial cellulases find application in the following industries.

2.8.1. Cellulases in the textile industry

The textile industry has been revolutionized by introduction of enzymes that are slowly replacing the conventional chemical processes, which are generally severe and lead to fiber damage (Bhat, 2000). Cellulases have the ability to modify cellulosic fiber in a controlled and desired manner thus improving the fabric quality (Mojsov, 2012). They are mostly used during wet processing to improve fabric properties. Processes that involve cellulase activity include bio stoning of jeans and bio polishing cellulosic fibers. Denim is heavy grade cotton and when dyed, the dye is mainly adsorbed on the surface of the fiber. When cellulases are used during the biostoning process, they break off small fiber ends on the yarn surface to loosen the dye, which is consequently easily removed during the wash cycle by mechanical abrasion. This enzyme based treatment replaced pumice stone bio stoning hence less damage to the fiber, increased productivity and a safe working environment (Schimper *et al*., 2006)

Fading can be achieved without loss of fabric strength. Fabrics made from cellulosic fibers such as cotton, linen, viscose and lyocell are normally characterized by short fibers protruding from the surface (fuzz formation) and pilling, loosened fuzz attached to the surface. This often decreases their market value and in order to prevent this, a process called biopolishing is done. Biopolishing is usually done during the wet processing stage and includes desizing, scouring, bleaching, dyeing and finishing. Cellulase mixtures usually rich in endoglucanases are used in

this process to remove the small protruding fibers from the fabric surface without using chemicals. The fabric attains a smooth and a glossy appearance, improved brightness and uniformity. Biopolishing is a key procedure in the production of high quality garments (Bhat, 2000).

2.8.2. Cellulases in the detergent industry

Recent innovations in the detergent industry have seen the incorporation of enzymes such as cellulases, proteases and lipases in detergents (Singh *et al*., 2007). Due to repeated washing, cotton and cotton blend fabrics become dull and fluffy due to the presence of detached micro fibrils. Cellulase containing detergents are capable of degrading the cellulose microfibrils to restore a smooth surface and original colour to the garment. In addition, the degradation softens the fabric and removes dirt particles trapped in the microfibril network (Sukumaran *et al*., 2005; Singh *et al*., 2007). Cellulase preparations that are active under mild alkaline conditions (pH 8.5-9) and temperatures over 50^{o}C are added to detergents. Such cellulases active under alkaline conditions increase the cleaning capacity of detergents by selective contraction fibers hence facilitating the removal of oil from inter fiber space (Karmakar and Ray, 2011).

2.8.3. Cellulases in pulp and paper industry

Application of enzyme preparations comprising cellulases, xylanases and lignases in the pulp and paper industry has increased in the last decade (Karmakar and Ray, 2011). Pulping starts with the conversion of woody raw material into a flexible fiber that can be made into paper. Depending on the application of the paper, various methods of pulping can be used (Bajpai, 2012). Mechanical pulping usually involves mechanical grinding of the woody material to give fibers that can be used in the production of different grades of paper. This method is usually characterized by high energy consumption and gives paper with incompletely ground fiber bundles, low strength and tends to yellow with time due to little removal of lignin a weakness associated with the process (Bhat, 2000).

Bio pulping using cellulases and associated enzymes reduces the energy required to achieve the desired strength and freeness of the pulp hence it's a better alternative to mechanical pulping (Karmakar and Ray, 2011). Cellulases containing enzyme mixtures are also useful in the hydrolysis of fines small particles produced during refining of primary or secondary fibers. These particles usually reduce the drainage rate of pulp during the paper making process. Deinking process is crucial during paper recycling. Enzymatic deinking using cellulases reduces the need for deinking chemicals and also results to little or no loss in paper strength. Enzymatic deinking is usually combined with mechanical agitation in order to improve the efficacy of the process (Karmakar and Ray, 2011).

2.8.4. Cellulases in feed industry

Applications of cellulases and hemi cellulases in the animal feed industry have received considerable attention because of their potential to improve feed value and performance of animals. The enzymes can also eliminate anti nutritional factors present in the feed grains, degrade certain feed constituents to improve the nutritional value and provide supplementary digestive enzymes such as proteases, amylases and glucanases. Most low-quality feedstuffs contain higher concentrations of cellulose, small amounts of protein and fat and relatively high ash contents when compared with high-quality feedstuffs (Sharada *et al*., 2014).

Cellulases can be used to improve silage production for cattle feeding, which involves enhancement of the digestibility of grasses containing large amounts of potentially total digestible nutrients and energy values together with only small amounts of water-soluble carbohydrates. Enzyme preparations containing high levels of cellulase, hemicellulase, and pectinase have been used to improve the nutritive quality of forages (Sharada *et al*., 2014)

2.8.5. Cellulases in the food industry

Industries producing fruit juices in the 1930s encountered challenges such as low yield and a poor clarity of the product (Uhlig, 1998). Research on industrially suitable enzymes such as cellulases, hemicellulases and pectinases from food grade microorganisms such as *Aspergillus niger, Trichoderma sp* and increased knowledge of fruit components led to the overcoming of

these challenges and led to improved methods of extraction, clarification and stabilization (Singh *et al.*, 2007).

Cellulases along with xylanases and pectinases are the macerating enzymes that serve to increase the yield and process performance without any additional cost. Macerating enzymes are usually used in two steps; after crushing, the fruit pulp is macerated to either partial or complete liquefaction. After the extraction, pectinases are then used for its clarification and this lowers viscosity of fruit juice prior to its concentration and further increases the filtration rate and the stability of the juice. Macerating enzymes also improve the cloud stability, texture, decrease viscosity and facilitate easy concentration of nectars and purees (Grassin and Fauquembergue, 1996).

There is a growing demand for natural pigments for food colorants such as carotenoids. In their natural state, carotenoids remain bound to proteins thus preventing pigment oxidation. When solvents are used to extract carotenoids, they disrupt that association thus making the pigments insoluble in water and oxidation. This can be prevented by use of enzymatic methods. Cellulases hydrolyze cellulose in the cell walls hence the structural rigidity is interfered with exposing intracellular materials for extraction. These pigments remain bound to proteins and are more stable than those obtained through traditional methods that involve use of solvents (Bassi *et al*., 1993).

2.8.6. Cellulases in the biorefinery

Bioconversion of lignocellulosic biomass to produce biofuel is the most popular area of cellulase application being investigated recently (Sukumaran *et al*., 2005). Potential lignocellulosic feed stocks sources include agricultural crop residues such as stover and straw, the perennial prairie grass, municipal waste, packaging and construction debris, agricultural or forest processing by products e.g. food processing residues, pulping liquor from paper mills and forest woody biomass either logging residues from conventional harvest operations or removal of excess biomass from timberlands. Lignocellulosic biomass consists of cellulose tightly linked to lignin and hemicellulose (Kuila *et al*., 2011). Cellulose must be separated from lignin and hemicelluloses in order to make it more accessible to the hydrolytic enzymes

through pretreatment. Pretreatment methods used can be physical, chemical or biological but the latter are not yet intensely developed as the physical and chemical methods (Kuila *et al*., 2011). Conversion takes place in two phases; hydrolysis of cellulose into fermentable reducing sugars by cellulases and fermentation of the sugars to ethanol, a process carried out by yeast or bacteria. The cost of ethanol production from lignocellulosic materials is relatively high with the main challenges being low yield and a high cost of hydrolysis (Sun and Cheng, 2002).

3. MATERIALS AND METHODS

3.1. Description of the study site

This research work was carried out at Holetta Agricultural Research Center (HARC), microbial biotechnology laboratory from April to October, 2018. Holetta is located at 34 km west of Addis Ababa in the Oromia Special Zone Surrounding Finfinne, Oromia Region. Geographically, HARC is located at a latitude, longitude, and an altitude of 9 °3' N, 38 ° 30 ' E and 2391 meters above sea level, respectively. Typically it has a bimodal rainfall pattern with a mean annual precipitation of about 1100 mm. The main rainy season extends from June to September while the short rainy from February to April. Mean annual maximum and minimum temperatures are 21°C (ranging from 20°C to 27°C) and 6°C (ranging from 2°C to 9°C), respectively. Mixed crop/livestock farming is the major agricultural production system practiced in the area with the major crops comprising Teff, Wheat, Barley, Chickpea and Faba bean. The predominant soil type in the area are nitosols and vertisols varying between reddish brown to dark reddish brown clays (www.eiar.gov.et).

3.2. Sample collection and processing

Five rumen samples were collected from fistulated oxen before morning feeding in to clean sterile and pre gassed with CO_2 glass bottle. Another five rumen samples were collected from sheep and goats in the morning immediately after slaughtering from slaughter house at Holetta in clean sterile and pre gassed with CO_2 glass bottle. All samples were labeled and immediately transported to the laboratory and further gassed with CO_2 to enhance anaerobiosis and the bottles were sealed with parafilm and aluminum foil and placed in an incubator set at 39°C till inoculation. The rumen digesta and fluid samples from each sample was then gently squeezed by sterilized cheese cloth in to sterilized fulken tube with continuous supply of CO_2.

3.3. Culture media preparation

For the preparation of one liter of the media, 28g of nutrient agar was added to one litter of distilled water as prescribed by the producer. During isolation, nutrient agar media used was enriched with 5ml mineral 1 solution and 0.4 ml mineral 2 solutions (Tilley and Terry, 1963).

The composition of nutrient agar media, mineral 1 and mineral 2 solution used in this study is are listed in Table 1.

Table 1: Composition of nutrient agar (NA) media, mineral 1 and mineral 2 solutions

Component (NA)	Amount g/l
Peptone	5
Sodium chloride	5
Meat extract	1.5
yeast extract	1.5
Agar	15
Mineral 1 solution	
K_2HPO_4	0.6
Mineral 2 solution	
K_2HPO_4	0.6
$(NH_4)_2\ SO_4$	0.6
NaCl	1.2
$MgS0_4.7H_20$	0.245
$CaCl_2.\ 2H_20$	0.159

3.4. Isolation of cellulase producing rumen bacteria

Bacteria were isolated from rumen sample collected from different domestic ruminants. Serial dilution was undertaken by taking 1ml of rumen fluid in 9ml of normal saline (sterilized 0.85% NaCl) with successive dilution up to 10^{-6}. The samples from 10^{-3}-10^{-6} were inoculated in to nutrient agar (NA) media enriched with mineral 1 and mineral 2 solutions by spread plate technique with continuous flushing of CO_2 based on Paul and kamara (2004). Nutrient agar plates with inoculum were incubated at 39°C for 24 hr. After incubation period, colonies formed on the surface of the medium. These bacterial colonies were purified by taking single

colony each time in a streak plate method on the same media repeatedly until plate contained one type of colony. The purified colonies were checked for cellulolytic activity, according to the method described by Teather and wood (1982).

3.5. Screening of cellulase producing bacteria

1% of Carboxymethylcellulose (CMC) agar were prepared by enriching with nutrient agar (g/l: 5 peptone, 5 NaCl, 1.5 meat extract, 1.5 yeast extract and 15 agar) (Ariffin *et al*., 2006). Totally eighty five pure isolated bacterial isolates (54 from five fistulated oxen, 20 from three sheep sample and 11 from two goats sample) were screened for cellulose degrading activity by Congo red test. After incubation, for three days the media plates were flooded with 0.1% Congo red dye for 15 minutes. The dye was discarded and plates were flooded with 1M NaCl for 15 min. NaCl was discarded and then cellulase activity was observed as a clear zone around the colonies (Apun *et al*., 2000).

3.6. Characterization of rumen cellulolytic isolates

Selected bacterial isolates were characterized on the basis of their morphological features, biochemical characteristics and Grams reaction.

1.3.6. Gram staining

Gram staining was done to identify bacteria according to their Gram character (Gram positive or Gram negative). Bacterial culture broth was placed on a glass slide with sterilized loop. ASmear was then prepared by mixing and spreading the bacteria by means of a circular motion of the loop. The smear was then allowed to air dry followed by heat fixation. The smear was flooded with crystal violet and let stand for 1 minute. Then, the smear was gently washed with tap water. It was then flooded again with the gram's iodine mordant and allowed to stay for 1 minute followed by gentle wash with tap water. After that, the smear was decolorized with acetone alcohol and gently washed with tap water. Finally, it was counterstained with safranin for 1 minute and gently washed with tap water. Finally, the glass slide was washed with water, dried and observed under microscope (Smith and Hussey, 2005).

3.6.2. Morphological characterization of the bacteria

Selected pure isolates were inoculated in to nutrient agar enriched with mineral 1 and 2 solution media under sterile condition to obtain isolated discrete colonies. The plates were then incubated at 39°C for 24 hr. After incubation, the bacterial colonies were evaluated for shape, color, margin, elevation and surface of colony.

3.6.3. Biochemical characterization of the bacteria

Several biochemical tests were carried out in order to have a presumptive identification of the bacteria chosen before. The biochemical tests performed were carbohydrate fermentation (Sucrose, glucose, fructose, xylose, mannitol, cellobiose, arabinose, lactose and mannitol), H_2S production, Indole test, Methyl red test, Citrate utilization test, Urease test and Catalase test. Each test was conducted with three replicates for each isolates. All tests were addressed for identification according to the method given by Berge's Manual of determinative bacteriology (Bergey, 1984)

Carbohydrate utilization test

Peptone; 10 g/l, carbohydrate; 5 g/l, NaCl; 15 g/l and phenol red 0.0189 g/l were prepared by autoclaving at 121°C for 15 minutes in separate test tubes. Using sterile technique, the experimental bacteria from 24 hr. pure culture was inoculated into the broth. All the tubes were incubated for 24 hr. at 37°C. The development of a yellow color indicated positive result (Bergey, 1984).

H_2S gas production

Triple sugar iron agar media was prepared in separate test. Overnight cultured bacteria was inoculated in to already prepared tubes of triple sugar iron agar and incubated at 37°C for 48 hr. After 48 hr. of incubation period, the tubes were observed for H_2S production (black coloration) (Bergey, 1984).

Indole test

Pure bacterial isolates were inoculated in to Tryptophan broth prepared in separate test tube using sterile technique. Test tube with inoculum were incubated for 48 hr. at 37°C. In order to test for Indole production, 5 drops of kovac's reagent was added directly into the tubes. The appearance of a red color indicated positive result (Bergey, 1984).

Methyl red test

MR-VP broth of 5 ml in each test tube were prepared and autoclaved at 121°C for 15min. Aseptically, small amount of the experimental bacteria from 24 hr. pure culture was inoculated into the tubes and the tubes were incubated for 24 hr. at 37°C. After 24 hr. five drops of methyl red indicator solution was added to observe the immediate development of a red color. The appearances of a red color indicated a positive result, while yellow indicated negative (Bergey, 1984).

Citrate utilization test

Simmons citrate agar slant were prepared and using sterile technique, the bacteria from 24 hr. pure culture was inoculated by means of a streak inoculation method with an inoculating needle and incubated for 48 hr. at 37°C, the appearance of a blue color indicated positive result, citrate was utilized but the presence of the original green color indicated a negative result, citrate was not utilized (Bergey, 1984).

Catalase test

A microscopic slide was placed inside a petri dish. Under sterilized condition pure bacterial isolates from 24 hr. culture was placed on to the microscopic slide by using sterilized loop. One drop of 3% H_2O_2 was placed on to the bacteria on the microscopic slide using a dropper and observed for immediate bubble formation. Positive test was indicated by bubbling and the absence of bubbling indicated negative test (Bergey, 1984).

Urease test

The isolates were inoculated in a urease agar slant and incubated at 37°C for 48 hr. After 48 hr. of incubation time, the development of red pink color indicated positive result (Bergey, 1984).

3.7. Source and preparation of growth substrates for cellulase production

The growth substrate like Teff straw, Wheat straw, Barley straw, carboxymethylcellulose (CMC) and mineral salts were obtained from Holetta Agricultural Research Center (HARC) and from market. The lignocellulosic substrates were oven dried in order to free all substrates from moisture for ease of grinding. After that all substrates were ground by using a blender separately. Then all substrates were sieved through a 1mm size mesh and the powder passed through 1mm size mesh was used for submerged fermentation.

3.8. Production of cellulase enzyme

3.8.1. Inoculum development

Pure cultures of the selected bacterial isolates were inoculated in nutrient broth and incubated at 37°C for 24 hr. of fermentation period. After 24 hr., the cultures were used as inoculum source for the production medium (Irfan *et al.,* 2012).

3.8.2. Submerged fermentation process

Media used for submerged fermentation were arranged in separate Erlenmeyer flasks of 250 ml capacity containing each 10 g of a single type of lignocellulosic substrate and 10 g of carboxymethylcellulose as a control. The respective substrates were submerged in 50ml basal medium consisting of (g/l):1; K_2HPO_4, 0.5; $MgSO_4$, 0.01; $FeSO_4.7H_2O$, 0.5; yeast extract, 0.5; $NaNO_3$ with a final pH of 7.0. The media was autoclaved at 121°C for 15 min and cooled. Sterilized production medium was inoculated with 2.5 ml of culture. Experiments were performed in duplicate. The flasks were incubated at 39°C on shaker incubator at 120 rpm for 72 hr. (Shaikh *et al*., 2013).

3.8.3. Preparation of crude enzyme

According to the method described by Shaikh et al. (2013) the fermented broth was centrifuged at 6000 rpm for 15 minutes at 4°C. After centrifugation the clear supernatant was collected to serve as crude enzyme source from bacteria and utilized for determination of enzymatic activity.

3.9. Cellulase enzyme assay

Cellulase activity was estimated using 1% solution of carboxymethylcellulose (CMC) in 0.05M sodium citrate buffer (pH 4.8). The reaction mixtures contained 1 ml of substrate solution (1% CMC dissolved in citrate buffer) and 1 ml crude enzyme solution. The blanks contained all assay components without enzyme. The reaction was carried out at 50°C for 30 minutes. After 30 minutes of incubation at 50°C, the reaction was stopped by adding 2ml DNS reagent followed by boiling for 5 minutes and cooling. Absorbance was measured at 540 nm against a reagent blank by a UV-Spectrophotometer. The cellulase activity was determined by using a calibration curve for glucose. One unit of enzyme activity is defined as the amount of enzyme that produced 1μmol of reducing sugars in 1min under assayed condition (Miller, 1959). The amount of cellulase produced in Submerged fermentation was expressed as U/ml (Ghose, 1987).

3.10. Determination of optimum parameters for maximum cellulase production

The optimum parameters were determined for cellulase production from the efficient isolates. The cellulase fermentation was carried out at different ranges of parameters included temperature, pH, incubation period, carbon source and nitrogen sources. After fermentation at different parameters, the crude enzyme sample was collected from each to check the enzyme activity.

3.10.1. Effect of lignocellulose substrate on the production of cellulase

The basal medium was supplemented with various carbon sources. These carbon sources were: Wheat straw, Teff straw, Barley straw and carboxymethylcellulose (CMC) as a control.

After 3 days of incubation at 39°C, the amount of the cellulase was assayed as mentioned in section 3.8.

3.10.2. Effect of growth time on the production of cellulase

The effect of incubation period was determined in order to achieve maximum cellulase production. Selected bacterial isolates were grown on the basal liquid medium containing carboxymethylcellulose (CMC) and incubated at 39°C for 24-96 hr. and sample were harvested at 24 hr. time intervals. The amount of cellulase was measured as described in section 3.8.

3.10.3. Effect of pH on the production of cellulase

pH is another factor affecting the microbial growth as well as enzyme production. After preparation of the basal liquid medium containing carboxymethylcellulose (CMC), the media were adjusted at pH 4. 5, 6, 7, 8 and 9 using 0.1M NaOH and 0.1N HCl buffer system. After 3 days of incubation at 39°C, the amount of cellulase was assayed as mentioned in section 3.8.

3.10.4. Effect of temperature on the production of cellulase

Temperature is an important factor in a bioprocess for the production of extracellular enzyme. For the selection of optimum temperature for cellulase production by selected isolates, the inoculated media in separate flasks was incubated at different temperatures (30°C, 35°C, 39°C, 45°C and 50°C) for 3 days. After 3 days of incubation, the amount of cellulase was assayed as mentioned in section 3.8.

3.10.5. Effect of nitrogen sources on the cellulase production

Different nitrogen sources were used to see their inductive effect on the enzyme production. These include Ammonium sulphate, yeast extract and peptone. After 3 days of incubation at 39°C, the amount of cellulase was assayed as mentioned in section 3.8.

3.11. Data analysis

Microsoft office Excel worksheet 2010 was used to analyze data and presented in tables and figures

4. RESULT AND DISCUSSION

4.1. Isolation and primary screening of cellulolytic rumen bacteria

Out of ten rumen samples collected from different ruminants, eighty five pure isolates were obtained from all rumen samples (Table 2). Among them, twenty six isolates were found to be cellulase producers. From these, the better clear zone producing seven isolates around the colony are chosen (Figure 3) and preceded for identification through morphological and biochemical test.

Table 2: Cellulase producing rumen bacterial isolates from the collected rumen samples

No.	Sample name	Total no. of pure isolates	No. of clear zone forming isolates	No.of selected isolates	Code
1	Fistulated Ox1	13	7	3	C90,C91,C94
2	Fistulated Ox2	5	2	1	C53
3	Fistulated Ox3	7	-	-	
4	Fistulated Ox4	21	13	2	C50, C51
5	Fistulated Ox5	8	-	-	
6	Sheep 1	6	-	-	
7	Sheep 2	1	-	-	
8	Sheep 3	13	4	1	C5
9	Goat 1	6	-	-	
10	Goat 2	5	-	-	
	Total	85	26	7	

Degradation of cellulose materials is a complex process requiring the activities of microbial enzymes. Habitats that contain these substrates are the best sources in which to find these microorganisms (Shanmugapriya *et al*., 2012). Rumen contains a variety of microorganisms

that can secrete cellulase. Therefore, rumen was selected as a source for obtaining desirable cellulase producing bacteria.

Figure 3: Clear zone on nutrient agar supplemented with 1% of CMC media after flooded with 0.1% Congo red solution. The formation of clearing zone around the colonies confirms the secretion of extracellular cellulase.

Screening of cellulase production by the 59 isolated strains showed no clearing zone after staining with Congo red. This might indicate that they are not cellulase producers (due to the absence of CMCase activity). As for 26 isolated strains that showed a clearing zone when placed on carboxymethylcellulose (CMC) media in all Petri dishes prepared indicates the ability of producing cellulase enzymes. The findings of this study is comparable to the work of Arun et al. (2007) who isolate *Bacillus circulans* and from Fish gut for the production of cellulase. The same organisms were also identified by Ariffin et al. (2006), who used local *Bacillus pumilus* to produce cellulase. This study is in line to that of Shanker (2011) screened as cellulolytic bacteria from mid-gut of the popular composting *Earth worm*.

4.2. Characterization of efficient cellulolytic bacteria

The morphological and biochemical characteristics of the isolates were studied as shown in Table 2 and Table 3. Based on the morphological and biochemical characteristics, all the selected isolates were confirmed *Enterobacter species* using Bergey's manual of systematic bacteriology (Bergey, 1984). So far, different species of cellulolytic rumen bacteria, such as *Butrivibrio fibrisolvens, Streptococcus sp., Clostridium sp., Cellulomonas sp., Pseudomonas* sp., *Bacillus sp., Micrococcus sp., Fibrobacter succinogenes, Ruminoccus albus and Enterobacter sp.* were isolated from rumen fluid and reported as they have better cellulase enzyme activity (Das and Qin, 2012; Al- Rawi, 2011; Ye *et al*., 2017). The majority of effective rumen bacteria are anaerobic, but some are aerotolerant and facultative gram negative and gram positive cellulolytic bacteria were reported previously from rumen sample and fresh cow dung (Faridha *et al*., 2013; Sari *et al*., 2017; Seo *et al*., 2013). The cellulolytic bacteria found in this study were found to be gram negative facultative anaerobes *Enterobacter* species.

4.2.1. Morphological characteristics of isolated bacteria

The morphological characteristics or colonial appearance of seven selected isolates shown in Table 2. The isolates C50, C90, C51 and C53 were yellowish in color and all circular in shape. Similarly, C94, C91 and C5 appeared to be white colony with circular shape (see appendix Figure 1.)

Table 3: The colony characteristics of seven cellulase producing isolates.

Cultural characteristics	Representative isolates						
	C91	C94	C90	C53	C50	C51	C5
Colony surface	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Colony color	White	White	Yellow	Yellow	Yellow	Yellow	White
Colony shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Elevation	Raised	Raised	Raised	Raised	Raised	Raised	Raised

4.2.2. Biochemical characterization of isolates

All the seven selected isolates were observed under microscope. The gram staining results indicated that all selected bacteria were appeared to be gram negative bacteria. Most of them excihibeted the same biochemical characteristics. Briefly, all the seven isolates showed positive for citrate utilization test, catalase test, urea hydrolysis test, sucrose test, glucose test, fructose test, cellobiose test, arabinose test, lactose test, mannitol test and negative to xylose test (Table 4).

Table 4: Biochemical characteristics of seven selected cellulase producing isolates

Biochemical characteristics	Representative isolates				
	C91	C94	C90	C53	C50
Gram stain	-ve	-ve	-ve	-ve	-ve
Citrate utilization test	+ve	+ve	+ve	+ve	+ve
Urease test	+ve	+ve	+ve	+ve	+ve
H_2S production	+ve	-ve	+ve	+ve	-ve
Catalase test	+ve	+ve	+ve	+ve	+ve
Indole test	+ve	-ve	+ve	+ve	-ve
Methyl red test	+ve	-ve	-ve	-ve	-ve
Carbohydrate test					
Sucrose	+ve	+ve	+ve	+ve	+ve
Glucose	+ve	+ve	+ve	+ve	+ve
Fructose	+ve	+ve	+ve	+ve	+ve
Xylose	-ve	-ve	-ve	-ve	-ve
Cellobiose	+ve	+ve	+ve	+ve	+ve
Arabinose	+ve	+ve	+ve	+ve	+ve
Lactose	+ve	+ve	+ve	+ve	+ve
Mannitol	+ve	+ve	+ve	+ve	+ve

Note: +ve = positive; -ve = negative

4.3. Production of cellulase from lignocellulosic substrates under submerged fermentation

Production of cellulase by seven isolates grown on different substrates like, Wheat straw, Teff straw, Barley straw and carboxymethylcellulose (CMC) as a control under submerged fermentation were evaluated and the results are summarized in Figure 4. Each substrate was used as nutrient sources in submerged media. However, all isolates produced maximum cellulase enzyme on media supplemented with carboxymethylcellulose as a carbon source. As reported by Das et al. (2010), utilization of carboxymethylcellulose (CMC) as carbon source is best for cellulase production. This finding is in agreement with the report of Das et al. (2010). It is assumed that this is due to its less complexity and easy assimilation by the isolated microbe (Wood and Bha, 1988).

According to the results here obtained, from lignocellulose substrate, the highest enzyme production was obtained from wheat straw by C51, C94 and C53 isolates. Liand et al. (2014) used wheat straw as a carbon source for cellulase production and reported to be good carbon source for cellulase production. So the result obtained from this study is in correlation with the report of Liang et al. (2014). This result indicates, wheat straw is digested by different rumen cellulolytic bacterial species. Likewise, the enzyme yield using Teff straw as a substrate was found to be low. From this result it can be concluded that,mainly cellulase produced by C51, C94 and C53 isolates could be used in animal feed treatments in order to improve nutritional value and digestability of fibrous feeds. In general, to reduce the cost of enzyme production, lignocellulosic substrate is better instead of synthetic cellulose due to their reasonable cost and high enzyme production capacity. Therefore, identifying inexpensive raw materials for enzyme production is a solution to make the enzyme production process cost effective.

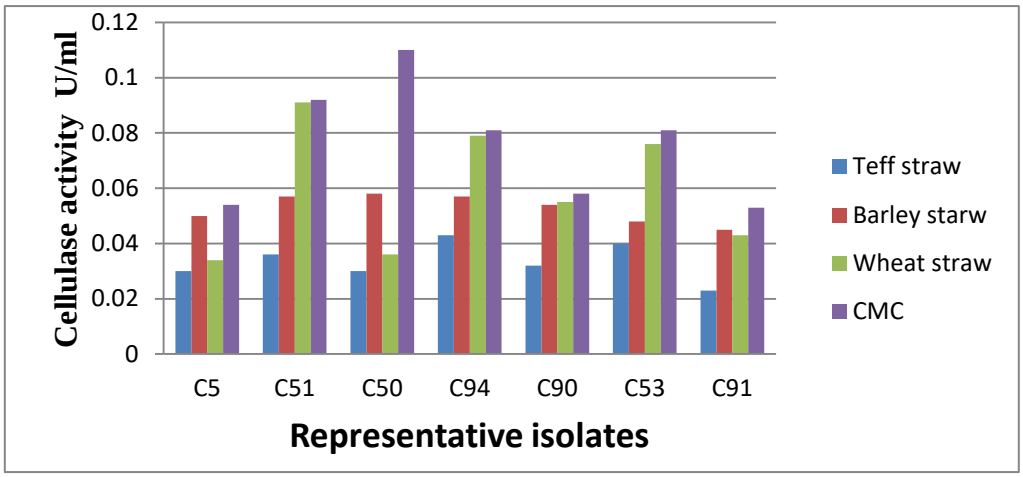

Figure 4: Cellulase production by seven selected bacteria grown on lignocellulosic substrates.

4.4. Optimization of culture conditions for cellulase production

Optimization of different growth parameters is an important aspect to be considered in the development of fermentation technology.

4.4.1. Determination of optimum incubation period for cellulase production

Optimum growth time for selected microbes for cellulase production was investigated and maximum production was observed at 72 hr. by C50 at which cellulase activity reached 0.09 U/ml as shown in Figure 5. The activity of cellulase increases gradually and reached the optimum activity at 72 hr. by C50, C90, C91, C5 and C51. This result is in agreement with the finding of Lokhande and Pette (2017). They reported that, maximum cellulase was observed when fermentation time was carried out for three days. While C53 and C94 reached optimum activity at 48 and 96 hr., respectively. However, at 96 hr. of incubation time, the enzyme activity decreased in most isolates (Figure 5). Hydrolysis rate declines with time may be due product inhibition and enzyme inactivation (Andriani and park, 2010). Incubation time had asignificant effect on cellulase production by each isolates. From the application point of view, faster production of an enzyme could be advantageous, which may allow appreciable reduction in the production cost of the enzyme and product can be obtained in short period of time.

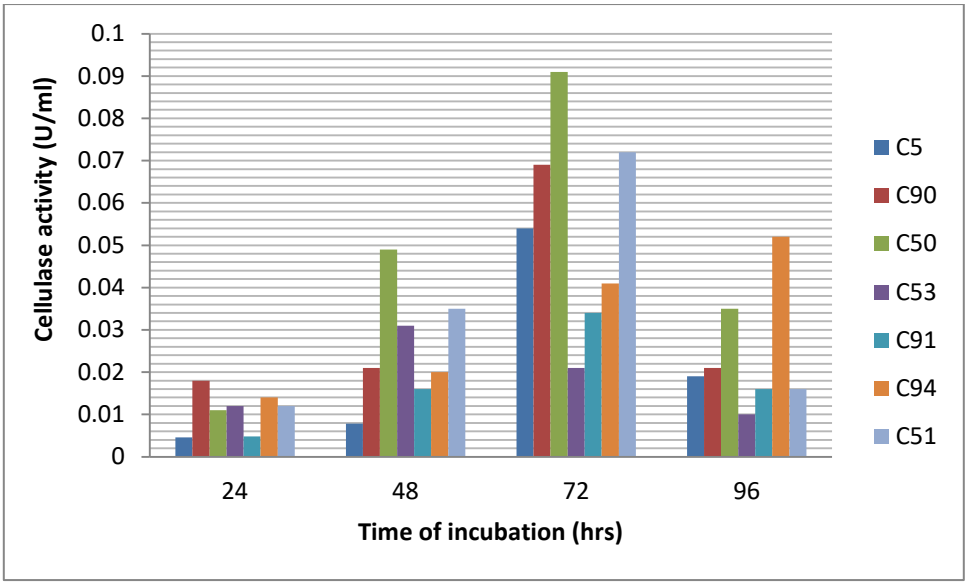

Figure 5: Effect of incubation time on cellulase production

4.4.2. Determination of optimum pH for cellulase production

In this study, cellulase production under submerged fermentation was monitored over a pH ranging from 4-9. The results revealed that optimum of pH 7 to be the best for all the seven isolates for cellulase production. However, a significant level of cellulase production was observed by four isolates (C51, C90, C53 and C50) at a pH range of 5-7. The result of the study suggested that, the pH of the fermentation medium influences the cellulase enzyme production (Figure 6). Hence, the maximum production for cellulase was observed at pH 7 by C50 (0.18 U/ml).

The rumen pH depends on the production of saliva, the generation and absorption of short-chain fatty acids (SCFA), the type and level of feed intake (Aschenbach *et al.*, 2011). Krause and Oetzel (2006) reported that, due to the buffering capacity of saliva and rapid absorption of VFA and ammonia through the ruminal wall, pH is maintained mostly between 5.5 to 7.0 The finding in this study comparable to most suitable pH for the production of cellulase at pH between 5 to 7 (Figure 6). The least cellulase production was recorded at pH 4. The rumen pH declined, when cattle fed too much rapidly digestible starch or carbohydrates (Ash and Dobson, 1963).

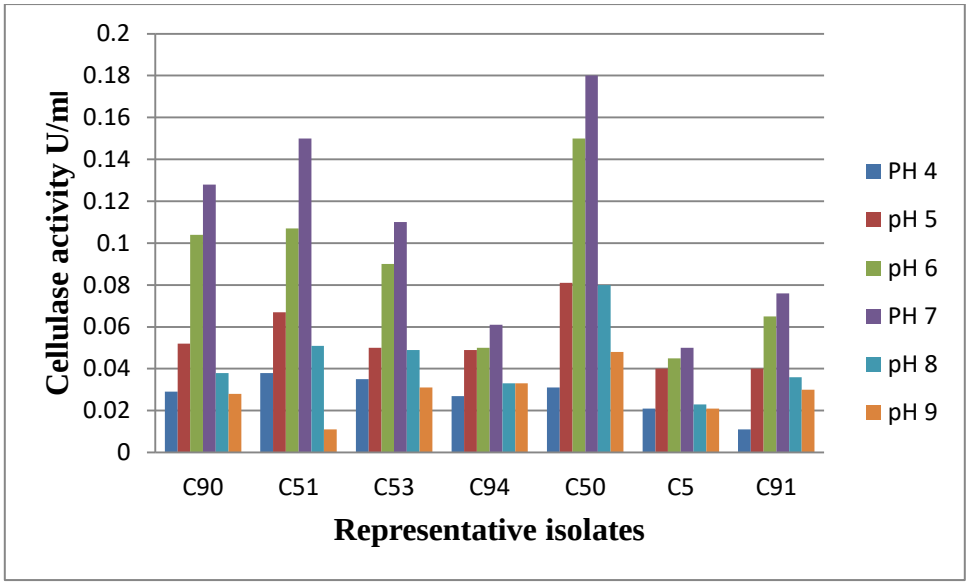

Figure 6: Effect of pH on cellulase production

4.4.3 Determination of optimum temperature for cellulase production

Temperature is a critical factors affecting enzyme production. The production of cellulase was determined at different temperatures. In the rumen, temperature was optimum (39°C to 41°C) for many enzyme activities. The rumen temperature is controlled mainly by the animal's homoeothermic mechanisms and partly due to the heat generated during fermentation. The result of the present study indicated that, from all isolates, C53, C50 and C90 had the ability to produce cellulase with the fermentation temperature at 39°C with activity of 0.17 U/ml, 0.15 U/ml and 0.12 U/ml, respectively.

The highest activity is achieved at 39°C, where the isolates start to produce enzyme at 30°C with an activity of 0.10 U/ml, 0.05 U/ml and 0.063 U/ml respectively (Figure 7). Then, the production of cellulase enzyme increases with increasing temperature until 39°C. From all, C53 produced maximum of 0.17 U/ml. These result nearly similar with the finding of Faridha et al. (2013) who found that maximum cellulase production at 40°C. According to the report by Ray et al. (2007), the minimum cellulase yield was observed when fermentation was carried out at 45°C, while maximum yield was obtained at 40°C by *Bacillus subtilis*.

Nandimath et al. (2016) reported that maximum cellulase production by *Pseudomonus sp.* at 30^{o}C.

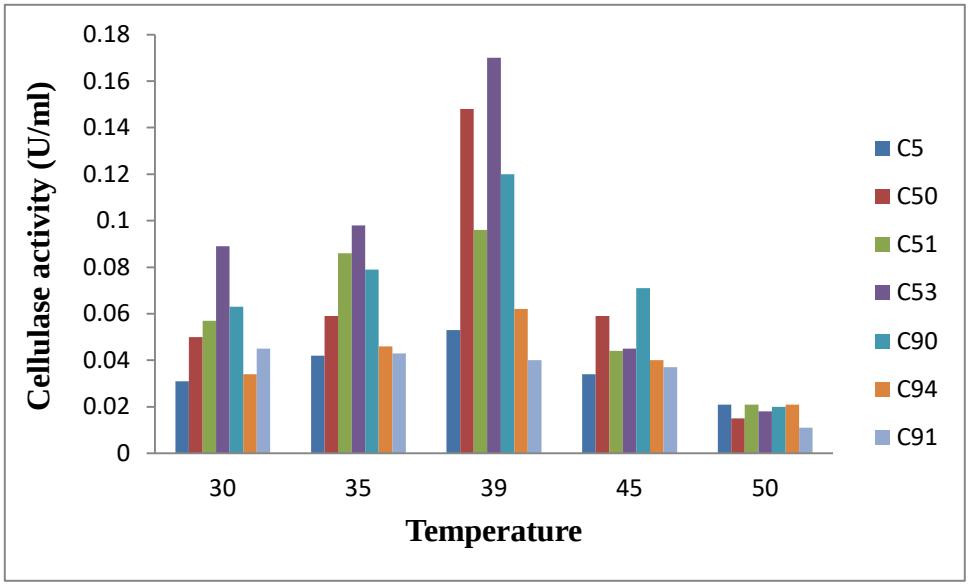

Figure 7: Effect of temperature on cellulase production

4.4.4. Determination of optimum nitrogen sources for production of cellulase

Different nitrogen sources used in submerged fermentation medium were evaluated and the result is summarized in Figure 8. It was found that all the nitrogen sources, which were used in the present study, supported cellulase enzyme production. However, among the various nitrogen sources tested, ammonium sulphate was found to be the best nitrogen source for cellulase production with the highest activity of 0.13 U/ml by C51.

Ruminal ammonia is the major nitrogen source used for protein synthesis by rumen microbes (Mandels, 1975). All isolates except C5 in this study produce the highest cellulase activity at media supplemented with ammonium sulphate salt as a nitrogen sources. Nitrogen is one of the major cell proteins and stimulation of cellulase production by ammonium sulphate salt might be due to their direct entry in protein synthesis (Mandels, 1975).

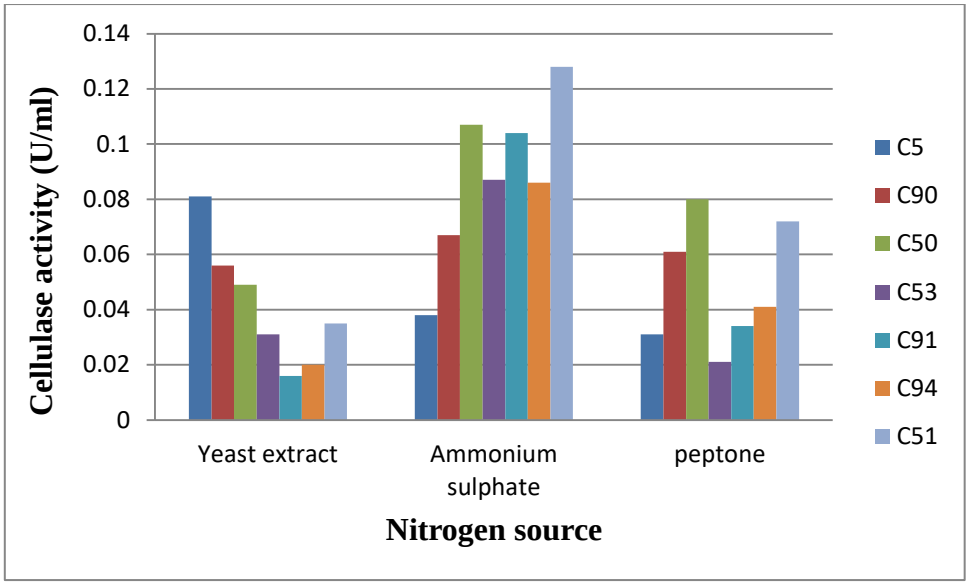

Figure 8: Effect of nitrogen on cellulase production

5. CONCLUSION AND RECOMMENDATION

5.1. Conclusion

Based on the result, potent cellulolytic bacteria were successfully isolated and screened from rumen fluids of different animals and efforts were made to identify based on morphological and biochemical tests. Based on morphological and Biochemical tests, the current isolates were confirmed as *Enterobacter* species. Important enzyme like cellulase, can be produced using agricultural biomass. Isolates (C51, C94 and C53) from two fistulated oxen are good producers of cellulase using lignocellulose substrate as a carbon source, especially, wheat straw than Barley and Teff straw. As determination of optimal production condition is very important for enzyme production, cellulase production was found to be higher at 72 hr. of incubation period by C50, C90, C5, C51 and C91 whereas higher at 48 and 96 hr. by C53 and C94 respectively. The maximal pH and incubation temperature were 7.0 for seven selected isolates and 39°C for C53, C50, C51 C5, C94 and C90 respectively. The media supplemented with Ammonium sulphate was found to be the most preferred nitrogen source for all isolates except C5 for cellulase production. The result indicates that favorable fermentation conditions and the selection of a suitable growth medium for cellulolytic bacteria played a key role in the production of cellulase.

5.2. Recommendation

The following recommendations were made on basis of the finding of current investigation:

- Further molecular level identification of the isolated rumen bacteria is required.
- Optimization of cellulase production conditions should be done for industrial level production.
- Further purification of the extracted crude enzyme need to be addressed.
- Further research is recommended on the use of cheap lignocellulose substrate for cellulase production.

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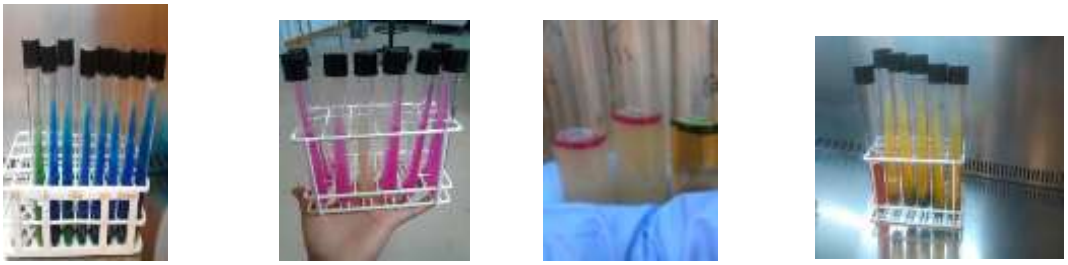
7. APPENDICES

Figure 1**:** Initial colony obtained from nutrient agar media enriched with mineral 1 and mineral 2 solution.

Figure 2: Pure isolates on nutrient agar media enriched with mineral 1and mineral 2solution.

Biochemical test

Citrate test urease test Indole test H_2S Production Test



Carbohydrate test