ASSESSMENT OF EXTRACELLULAR BETA-GALACTOSIDASE PRODUCTION POTENTIAL OF FUNGI ISOLATED FROM DAIRY EFFLUENT

BY

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DEPARTMENT OF MICROBIOLOGY

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY ZARIA, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN MICROIOLOGY

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JUNE, 2018.

DECLARATION

I declare that the work in this Dissertation entitled "ASSESSMENT OF EXTRACELLULAR BETA-GALACTOSIDASE PRODUCTION POTENTIAL OF FUNGI ISOLATED FROM DAIRY EFFLUENT" has been carried out by me in the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree at this or any or institution.

_____ _____

Abdullahi Dabban IDRIS Date

(P14SCMC8011)

CERTIFICATION

This dissertation entitled "ASSESSMENT OF EXTRACELLULAR BETA-GALACTOSIDASE PRODUCTION POTENTIAL OF FUNGI ISOLATED FROM DAIRY EFFLUENT" by Abdullahi Dabban IDRIS (P14SCMC8011) meets the requirements for the award of Master of Science degree in Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation

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ABSTRACT

Dairy wastewater is increasingly becoming an environmental concern. There is a widespread interest in the use of beta-galactosidase (EC 3.2.1.23) to hydrolyze lactose in milk and dairy products which aid in improving their functional and digestive properties. An attempt was made to isolate fungi from dairy effluent using standard culture techniques. Physicochemical characteristics and proximate composition of the dairy effluent was analyzed. The fungi isolated were subsequently screened using Ortho-nitrophenol-beta-D-galactopyranoside (ONPG) discs method for potential to produce beta-galactosidase. The isolate showing beta-galactosidase activity was selected and used to produce beta-galactosidase under submerged fermentation using the dairy effluent as a substrate. A total of nine (9) fungi were isolated from the dairy effluent belonging to the Genera *Aspergillus*, *Rhizopus*, *Rhizomucor* and *Penicillium* with *Aspergillus* having the highest frequency of occurrence (67%). Physicochemical analyses of the raw dairy effluent revealed the effluent had a pH of 6.8 (±0.20), electrical conductivity of 645µs/cm (±1.00) and total dissolved solids of 324mg/L (±2.00). The dissolved oxygen and biochemical oxygen demand were 200mg/L (±1.00) and 100mg/L (±1.70) respectively while the nitrates, phosphates and sulfates were $1.4x10^{-3}$% (±$1.0x\ 10^{-4}$), 54.94mg/L (±0.79) and 13.66mg/L (±0.61) respectively. The proximate composition of the dairy effluent revealed ash, lipid, protein and carbohydrate contents were 0.15% (±0.01), 5.85% (±0.02), 0.88% (±0.01) and 13.13% (±0.00) respectively. Only *Rhizopus* species showed beta-galactosidase activity and was used to produce extracellular beta-galactosidase. A maximum beta-galactosidase activity of 4.65U/mL (±0.02) was obtained with the selected *Rhizopus* species. It was concluded that *Rhizopus* species could be a potential fungal strain for beta-galactosidase production using the dairy effluent as substrate.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

A dairy is a business enterprise established for the harvesting of animal milk, mostly from cows or goats, but also from buffalo, sheep, or camels for human consumption (Sreemoyee and Priti, 2013). Among the major industries in the world, dairy is one of the industries producing wastewater rich in organic matter and thus leading to creation of odorous and high Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) containing water (Harush *et al*., 2011). Dairy industry produces washwater (a high strength waste) as a byproduct of cleaning the milking facility after each milking event to maintain sanitary operations. Generally, dairy wastes contain large quantities of milk constituents such as casein, lactose, fat, inorganic salts besides detergents and sanitizers, fresh water and sometimes animal waste which contribute largely towards high BOD and COD (Marwaha *et al*., 2001; Sweet, 2009).

Milk, a vital nutrient for all living beings, contains lactose, proteins, fat, vitamins and minerals such as calcium and phosphorus. Among these, lactose, as the main carbohydrate in milk, is the major carbon source during early stage of mammalian life (Mahoney, 2005). Lactose (4-ortho-β-D-galactopyranosyl-D-glucose), an abundant disaccharide found in milk is composed of the two simple sugars D-glucose and D-galactose (Geilman, 1993; Fennema, 1996; Ustok, 2007; Panesar *et al*., 2016). It is the principal carbohydrate and the natural sweetener of mammalian milk (Holsinger and Kligerman, 1997).

Beta-galactosidase is an important enzyme for organisms, as it aids in the production of energy and a source of carbons through the breakdown of lactose to galactose and glucose (Salehi *et al*., 2009). In

vivo, β-D-galactosidase is secreted by intestinal villi which catalyze two different reactions in organisms. In one, it can go through a process called transgalactosylation to make allolactose, creating a positive feedback loop for the production of β-galactosidase. It can also hydrolyze lactose into galactose and glucose which will proceed into glycolysis (Juers *et al.*, 2012; Li *et al.*, 2013; Panesar *et al.*, 2016).

Microorganisms especially fungi are producers of beta-galactosidase using lactose as the sole source of carbon and energy (Ahmed *et al.*, 2016). The production of beta-galactosidase by microorganisms is influenced by cultural parameters such as composition of the medium, temperature, pH, aeration and agitation, type of fermentation etc. (Ahmed *et al.*, 2016).

The enzyme is widely distributed in nature and is produced by plants, animals and microorganisms (Somyos and Phimchanok, 2009; Kumari *et al.*, 2011). Among these, microbial sources offer several advantages, such as easy handling, high production yields and the ability to grow on low cost agro-industrial wastes which minimizes the cost of production (Vermaa *et al.*, 2012; Panesar *et al.*, 2016).

Beta-galactosidase is produced by a widely diverse population of microorganisms such as yeasts, e.g. *Kluyveromyces lactis, K. fragilis K. marxianus, Candida kefyr* and *C. pseudotropicalis*; bacteria, such as *Escherichia coli, Lactobacillus bulgaricus, Streptococcus lactis, Pyrococcus woesei, Bifidobacterium infantis, Bifidobacterium longum, Enterobacter cloacae, Geobacillus stearothermophilus, Thermus sp.* and *Bacillus* sp; and molds, such as *Aspergillus foetidus, A. niger, A. oryzae* and *A. Phoenecia, Alternaria alternata, Trichoderma reesei, T. viride, T. harzianum, Rhizopus stolonifera, Penicillium simplicissimum* and *Penicillium expansum* (Holsinger, 1997; Almeida and Pastore, 2001; Asraf and Gunasekaran, 2010).

1.2 Statement of Research Problem

The dairy industry is one of the most polluting industries in terms of volume and characteristics of effluent generated (Adeoye *et al.*, 2009). It generates about 0.2–10 liters of effluent per liter of processed milk with an average generation of about 2.5 liters of wastewater per liter of the milk processed (Balannec *et al.*, 2005). Whey and other related wastes from the dairy industries with content of lactose poses serious environmental problems (Princely *et al.*, 2013). The main environmental problems related to milk production affect the pollution of water, air and biodiversity. They often cause a growth of algae and bacteria that consume oxygen through a process called eutrophication (Bharati and Shinkar, 2013). Due to high pollution load of dairy wastewater, the milk- processing industries discharging untreated or partially treated wastewater cause serious environmental problems (Ganapathy, 2011). Nutrients present in dairy effluent such as nitrogen can lead to eutrophication of receiving waters (Kushwaha *et al.*, 2011).

Majority of the world population (70%) encounter problems due to their consumption of foods containing high amount of lactose (Sieber *et al.* 1997; Daniel *et al.,* 2002). Hence, lactose intolerance is a common health concern causing gastrointestinal symptoms such as bloating, pain or cramps, gurgling or rumbling sounds in the stomach, gas, loose stools or diarrhea, vomiting and avoidance of dairy products by afflicted individuals (Suchy *et al.,* 2010).

Since milk is a primary source of calcium and vitamin D, lactose intolerant individuals often obtain insufficient amounts of these nutrients which may lead to adverse health outcomes, especially reduced bone mineral density and fractures (Enattah *et al.*, 2005). Beta-galactosidase is essential for the complete digestion of whole milk; it breaks down lactose, a sugar which gives milk its sweetness. With lack or shortage of beta-galactosidase in small intestine, lactose cannot be

hydrolyzed; hence a person consuming dairy products may experience symptoms of lactose intolerance (Paige and Davis, 2005).

The uses of whey, a lactose-rich effluent from cheese industries, have been reviewed, although much of the nutritious protein of whey is recovered, the lactose portion is largely unutilized and its disposal represents a serious environmental concern (Moulin and Galzy. 2004).

Beta-galactosidase can be synthesized from many sources including animals, plants, bacteria, fungi, and yeast (Husain, 2010). Although bacterial sources can efficiently produce beta-galactosidase, they are not considered safe for use in food production due the risk of microbial contamination. Another limitation is the high cost of additional technique required to extract the intracellular enzyme (Oliveira *et al.*, 2011; Panesar *et al.*, 2016).

Several researchers have attempted in search of native potent beta-galactosidase producers from their vicinity using cheap and readily available sources of substrates (Amal *et al.*, 2012; Muzumder *et al.*, 2012). Rice husk, wheat bran and other agricultural wastes are often used as substrates for production of beta-galactosidase under solid state fermentation. These substrates contain little or no lactose. As a result, pure lactose is supplemented in the medium for production of the enzyme. Lactose which is the main substrate for production of beta-galactosidase is obtained mainly from animal sources (dairy) which is in low supply in its pure form. Submerged fermentation for production of beta-galactosidase has been faced with challenges such as microbial contamination which tend to reduce quality of the fermentation product.

1.3 Justification of the Study

Hydrolysis of lactose in milk by beta-galactosidase may increase the digestibility, solubility, taste and may improve functional properties of dairy products (Grueger *et al.*, 1994) hence, food with low lactose contents, ideal for lactose-intolerant consumers, is thus obtained (Kardel *et al.*, 1995;

Pivarnik *et al.*, 1995; Mahoney, 1997; Kastsianevich, 2017). It also favours consumers who are less tolerant to crystallized dairy products, such as milk candy, condensed milk, frozen concentrated milk, yoghurt and ice cream mixtures, (Kardel *et al.*, 1995; Mahoney, 1997; Kastsianevich, 2017).

Many adult humans lack the beta-galactosidase enzyme, so they are not able to properly digest dairy products. Hence, β-galactosidase is important for this lactose intolerant community as it is responsible for making lactose-free milk and other dairy products (Salehi *et al.*, 2009). Lactose hydrolyzed products decrease the lactose intolerance problem (Milichova and Rosenberg, 2006). Since lactose is a disaccharide composed of glucose and galactose, it can be hydrolyzed into these monosaccharides using either a β-glucosidase or β-galactosidase and consumption of lactose-free milk, produced as a result of adding the enzyme (β-glucosidase or β-galactosidase) directly to milk, provides a means of maintaining good health while avoiding the symptoms of lactose intolerance (Li *et al.*, 2013).

Whey and other related dairy wastes as well as other lactose rich foods can be hydrolyzed in both cases by the action of beta-galactosidase to monosaccharides (glucose and galactose) and galactooligosaccharide (GOS) which are formed simultaneously (Princely *et al.*, 2013; Kastsianevich, 2017). Galacto-oligosaccharides (GOS) are used as prebiotic food ingredients. These compounds are indigestible, acting as dietary fiber. They promote the growth of intestinal *Bifidobacteria*, with the subsequent healthy effect in the intestine and the liver of human and animals (Grosová *et al.*, 2008; Kastsianevich, 2017).

The enzyme is industrially important because it can be used to avoid lactose crystallization in sweetened, condensed and frozen dairy products such as ice creams and condensed milk and also solve problems associated with whey utilization and disposal (Artolozaga *et al.*, 1998; Ahmed *et al.*, 2016).

Commercially available β-galactosidase is obtained from microorganisms of different genera in particular from *Kluyveromyces, Candida, Trichoderma, Aspergillus, Bacillus* spp. and *E. coli* (Pinheiro *et al.,* 2003; Panesar *et al.*, 2006; Panesar *et al.*, 2016). Although various microbial species have been exploited for beta-galactosidase production, still beta-galactosidase from fungus is of special interest as the enzyme synthesized is extracellular and thermostable by nature (Panesar *et al.*, 2016). Extracellular enzymes are of great economic significance, since the production cost arising from the additional techniques to extract the enzymes is low as compared to intracellular enzymes. The selection of suitable substrate along with the microorganism giving the higher yield of the enzyme, and efficient processing techniques are the prime factors determining the cost of the enzyme production (Panesar *et al.*, 2016).

Keeping the above in view, the present study was carried out to isolate local fungal strains from dairy effluent with the capacity to produce beta-galactosidase. The production of this important enzyme using fungi either on small scale or industrial basis can be achieved through the following aim and objectives:

1.4 Aim of the Study

The aim of this research was to screen some fungi isolated from dairy effluents for extracellular beta-galactosidase production potentials.

1.5 Objectives of the Study

Objectives of this research were to:

1. Isolate and characterize fungi from the raw dairy effluent.
2. Determine the physicochemical properties and proximate composition of the dairy effluent.
3. Screen and select fungal isolates with the potential for producing extracellular beta-galactosidase by submerged fermentation.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Dairy Industry

Dairy industry is of crucial importance to Nigeria. Milk production in Nigeria has developed significantly in the past few decades. The various activities of the Nigerian dairy industry such as milk production, importation, processing, packaging, marketing and consumption have been going on in Nigeria for many years (Olaloku, 2006). However, these activities are not organised except for the relatively few processing firms that produce and market reconstituted milk products from imported powdered milk. Despite the unorganized nature of the industry, the dairy industry represents an important component of the agricultural sector of the economy with great economic, nutritional and social implications (Olaloku, 2006).

In recent years, considerable attention has been paid to industrial wastes, which are usually discharged on land or into different water bodies (Porwal *et al*., 2014). Rapid growth of dairy industries has not only enhanced the productivity but also resulted in release of potential toxic substances into the environment, creating health hazards which have seriously affected normal operations of ecosystems, flora and fauna (Porwal *et al*., 2014). Dairy industry is one of the large scale food production industries which play an important role in causing water pollution (Sreemoyee and Priti, 2013). Dairy effluent in the form of wastewater has to be treated by taking various parameters into consideration. This is achieved by assessment of several physicochemical parameters of dairy wastewater with raw waste which includes determination of pH, temperature, acidity, alkalinity, total dissolved solids (TDS), Biochemical Oxygen Demand (BOD) out of which BOD is the most important parameter (Sreemoyee and Priti, 2013). The pH of dairy wastewaters usually varies in the range of 4.7–11 (Passeggi *et al*., 2009).

Kohle and Powar (2011) analyzed treated and untreated effluents samples from dairy industry for physicochemical parameters like pH, temperature, dissolved oxygen (DO), biochemical oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS), total suspended solids (TSS), chlorides, sulphate, oil and grease and found vast differences between two groups of effluents for various parameters. Dairy effluent in the form of wastewater is released into the environment and this is hazardous to human, flora and fauna. It is estimated that about 2% of total milk processed is wasted into drains (Munavalli and Saler, 2009)

Uaboi-Egbenni *et al.* (2009) analyzed physicochemical parameters like pH, electrical conductivity, TDS, COD, BOD and oil levels in industrial effluents from 5 different dairy industries in Lagos, Nigeria. Results obtained revealed that the main drain (MD) had the highest pH, electrical conductivity as well as total dissolved solids of 10.43, 1961μs, and 977 mg/l respectively. Studies by Adeoye *et al.* (2009) on the effect of short-term storage on basic properties of dairy wastewater carried out in Minna, Niger state, Nigeria, revealed that the iron content of the effluents sample ranged from 0.41mg/l to 0.52mg/l. The sulphate, phosphate and nitrate concentrations of the samples ranged between 11.8-35.6mg/L, 26.0-43.4mg/L and 19.4-41.2mg/L respectively. The BOD, COD and DO measured in mg/l had high values ranges of 40-75 mg/L, 43-67 mg/L and 880-1174 mg/L respectively. Also, during aeration, the odour was eliminated. The research demonstrated that the dairy wastewater could be used as soil amendment on a plot of land. Several limitations of physicochemical methods including partial treatment, higher cost, secondary pollutants generation, higher quantity solids and use of chemical agents make the biological methods a favorable alternative for the removal of pollutants (Kushwaha *et al.*, 2011).

Waste materials associated with the dairy industry usually contains sludge, heavy organic matter, fats, oil and grease, fatty acids, nitrogenous compounds etc (Healy *et al.*, 2012). The dairy and other

food-related sectors have one of the highest consumptions of water and among the biggest producers of effluents per unit of production (Ramjeawon, 2000). Dairy wastewater deserves special attention since its levels of potential contaminants mostly exceed those levels considered hazardous for domestic wastewater (Meul *et al.*, 2009). Dairy industry is found all over the world, but the manufacturing process varies tremendously (Porwal *et al.*, 2014). This sector generates huge volume of wastewater and its pollution is primarily organic (Briao and Tavares, 2007; Kushwaha *et al.*, 2011).

A major utility in dairy industry is water and this results in insignificant effluent volumes being generated; hence the challenge of its disposal cannot be ignored. The dairy industry on an average has been reported to generate 6–10 liters of wastewater per liter of the milk processed (Kolhe and Powar, 2011). Raw dairy wastewater is characterized by high concentrations and fluctuations of organic matter and nutrient loads (Farizoglu and Uzuner, 2011). The composition varies depending on the operations and products (Khojare *et al.*, 2002). The wastewater of dairy contain large quantities of milk constituents such as casein, lactose, inorganic salt, besides detergents and sanitizers used for washing (Kolhe *et al.*, 2009).

The use of industrial effluents rich in organic matter and plant nutrients for agriculture are considered as cheaper way of disposal (Porwal *et al.*, 2014). Gaikar *et al.* (2010) studied the impact of various concentrations (viz.10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%) of dairy effluent on seed germination and early seedling growth of soybean. It was observed that increase in effluent concentration led to a corresponding decrease in percentage germination but seedling growth gradually increased at 50% effluent concentration. Whereas 10% dilution of effluent enhanced seed germination and 100 % effluent completely inhibit both the seed germination and seedling growth, suggesting that the effluent could be used as a liquid fertilizer with up to 50% dilution rate.

Dairy effluents contain dissolved sugars and proteins, fats and possibly residues of additives and these are the main contributors to the organic load of these wastewaters (Castro and Mendes, 2004). Due to the presence of high organic load, dairy effluents degrade rapidly and deplete the dissolve oxygen (DO) level of the receiving streams and become the breeding ground for mosquitoes and flies carrying malaria and other perilous disease such as dengue fever and yellow fever (Kumar and Desai, 2011).

The wastes are also characterized by strong butyric acid odor and heavy black flocculated sludge masses (Briao and Tavares, 2007). The dairy industries produce effluents rich in fats, oils and greases (FOGs) and these can have negative impacts on wastewater treatment systems as they often cause foul odors, blockage of pipes and sewer lines (Porwal *et al*., 2014). Presence of nitrogen in dairy effluent is another major problem that once converted may contaminate groundwater with nitrate (Ulery *et al*., 2004).

Indigenous microorganisms can be isolated from contaminated dairy wastewater (Kushwaha *et al*., 2011). It is therefore, very important to know the wastewater micro-biota composition and biochemical properties, as well as the optimum metabolic activity of the indigenous micro-biota and physicochemical conditions of the dairy wastewaters (Janczukowicz *et al*., 2007).

2.2 Lactose Hydrolysis

Lactose contributes up to 40% of the energy consumed by human infants during nursing. In order to utilize this energy, the lactose must be hydrolyzed to the constituent monosaccharides (Fennema, 1996). Hydrolysis of lactose in milk by beta-galactosidase to its constituent monosaccharides (glucose and galactose) enhances the sensorial and organoleptic properties such as increase in the digestibility, solubility, sweetness and decrease in the fermentation of dairy products (Grosová *et al*., 2008; Husain, 2010; Panesar *et al*., 2010). Lactose hydrolysis can be performed either chemically

by treatment with acids and/or resins at higher temperatures (150°C), or enzymatically using β-galactosidase enzyme that permits milder operating conditions of both temperature and pH (Grosová *et al.*, 2008).

2.2.1 Chemical hydrolysis

Chemical hydrolysis of lactose can be performed with mineral acids or cation exchange resins. The acid hydrolysis is characterized by severe conditions required to completely hydrolyse lactose into glucose and galactose at a temperature of 100-150°C under acidic conditions of pH 1-2 (Abdelrahim, 1989). Coughlin and Nickerson (1975) showed the feasibility of using a temperature range as of 50-70°C with strong mineral acids (pH < 1.0) to efficiently hydrolyse lactose. Homogeneous reaction is used to achieve acid hydrolysis where the acid is free in solution. Heterogeneous reaction on the other hand is catalysed by hydrogen ions in an acid cation exchange resin. The hydrolysis of lactose from whey using catalytic resins is of great interest, since capital and production costs are relatively low and the process does not appear to have technological difficulties. Nowadays, cation exchange resin is regarded as the method of choice, since demineralization is not required (Grosová *et al.*, 2008).

The chemical hydrolysis of lactose is simple, rapid, and inexpensive. However, it has some undesirable features, such as: denaturation of milk proteins due to high temperatures and acidic conditions; presence of salts in whey which causes the deactivation of acids; Millard reaction, i.e. the interaction between amino acids from proteins hydrolysis and sugars from lactose hydrolysis; cost of specific materials needed for plant construction to resist the chemically corrosive conditions and; the loss of valuable essential nutrients of milk and the formation of undesirable by-products (Abdelrahim, 1989). The industrial acid hydrolysis has been achieved with pure lactose solutions,

but the production of pure lactose is expensive and the hydrolysed lactose syrup cannot compete with the starch-based sweeteners which are sweeter and less expensive (Panesar *et al.*, 2010).

2.2.2 Enzymatic hydrolysis

There are a number of advantages in using enzymes as industrial catalysts (Daniel *et al.*, 2002). They are highly specific, active at very low concentrations, and undesirable side reactions associated with chemical catalysis are significantly minimized because of the mild conditions of pH and temperatures (Vermaa *et al.*, 2012). Enzymes are more efficient than inorganic catalysts (Vermaa *et al.*, 2012). Beta-galactosidase is capable of breaking down the β-1,4-glycosidic linkage of lactose and consequently give rise to the constituent monosaccharides glucose and galactose (Carminatti, 2001; Panesar *et al.*, 2016). The enzymatic hydrolysis of lactose depends on the properties and the characteristics of the enzyme used as they differ widely according to the enzyme source. The enzymatic hydrolysis of lactose using beta-galactosidase is now the most widely used technique for the production of dairy products low in lactose (Mahoney, 2005). Enzymatic hydrolysis of lactose offers some benefits mainly in three major areas: health, food technology and environment (Grosová *et al.*, 2008).

2.2.2.1 *Health*

Consumption of milk and other dairy products is limited for the majority of the world's adult population (75%). These people are unable to digest lactose present in these products because of the insufficient β-galactosidase in the mucosa of the small intestine (Suchy *et al.*, 2010). Consuming milk products causes abdominal pain, diarrhea, cramps, or flatulence (Suchy *et al.*, 2010). Lactose intolerance can be the result of damage to the intestinal lining by viral, bacterial, or autoimmune inflammatory responses (Cichoke, 2006). It may also be due to genetic factors resulting in a decrease or total absence of beta-galactosidase production. A beta-galactosidase gene has been identified,

including a "wild-type" that is characterized by beta-galactosidase non-persistence – a physiological decline in intestinal beta-galactosidase activity that often results in lactose intolerance (Lomer *et al.*, 2008). This problem is circumvented if lactose in the products is hydrolyzed by beta-galactosidase to the readily utilizable sugars, glucose and galactose (Sieber *et al.*, 1997; Grosová, 2008). Also, enzyme supplementation is an established method to treat a number of digestive conditions (Holsinger and Kilgerman, 1997).

The simultaneous formation of galacto-oligosaccharides (GOS) used as prebiotic food ingredients is another advantage of enzymatic lactose hydrolysis. These compounds act as dietary fibre due to their indigestible nature. Diets enriched with GOS significantly increase the population of intestinal *Bifidobacteria, Lactobacillus* and some species of *Streptococcus* (Grosová *et al.*, 2008). The beneficial roles of these organisms especially *Bifidobacteria* for the host organism are to produce vitamins to assist in digestion and absorption, prevent growth of putrefactive bacteria and to stimulate the immune response (prebiotic properties) (Grosová *et al.*, 2008). This change in the intestinal flora (bifido effect) composition has been proposed to be responsible for the decrease in putrefactive products in the faeces, low blood cholesterol content (Chonan *et al.*, 1995; Kikuchi *et al.*, 1996), higher Ca^{2+} absorption, and lower incidence of colon cancer (Rowland, 1997). Nowadays, the demand for GOS production, as well as the development of an effective and inexpensive GOS has increased significantly (Tuohy *et al.*, 2003).

2.2.2.2 *Food technology*

Milk and milk products with high lactose content such as sweetened, condensed and frozen dairy products, leads to excessive crystallization of lactose. The resulting product tends to have a sandy or gritty texture (Grosova *et al.*, 2008). Low solubility, tendency to crystallize, and low sweetness makes lactose a less attractive food sugar (Grosova *et al.*, 2008). Enzymatically hydrolyzed lactose

offers a better alternative for commercial use in food products (Ashish *et al.*, 2014). This is because the use of β-galactosidase to process such products reduces lactose concentrations to acceptable values, and so improve some technological and sensorial quality of dairy foods; e.g. increasing the digestibility, softness, creaminess, and decrease in fermentation period (Husain, 2010; Panesar *et al.*, 2010; Kastsianevich, 2017). Also, Trans-oligosaccharides (TOS), a product of beta-galactosidase are non-cariogenic sugars widely used as additives in several infant formulations, and in the manufacture of candy, pastry, bread and jams because of their heat stability (Matsumoto, 1995; Husain, 2010).

2.2.2.3 *Environment*

Whey is a waste in the cheese industry and this causes several economic and environmental problems. Approximately 47% of whey produced annually worldwide is disposed-off (Grosova *et al.*, 2008). The problems arise because lactose is associated with the high biochemical and chemical oxygen demand and because of lactose uncertain solubility (Grosova *et al.*, 2008). Hydrolysis of lactose present in whey converts whey into very useful sweet syrup, which can be used in the dairy, confectionery, baking and soft drinks industries (Grosova *et al.*, 2008). In addition, several studies demonstrate the opportunity to degrade whey lactose using β-galactosidase for manufacturing of galacto-oligosaccharides (Foda and Lopez-Leiva 2000; Novalin *et al.*, 2005; Kastsianevich, 2017). The enzymes most commonly used in the industry are β-galactosidases, which are generally inhibited as glucose and galactose concentration increases (Husain, 2010).

2.3 Beta-Galactosidase

The enzyme β-D-Galactosidase (EC 3.2.1.23, β-D-galactosidehydrolase, Beta-galactosidases, galactohydrolase, lactase) is a glycoside hydrolase that catalyzes the hydrolysis of β-galactosides into monosaccharides through the breaking of a glycosidic bond (Panesar *et al.*, 2016; Kastsianevich,

2017). Beta-galactosides include carbohydrates containing galactose where the glycosidic bond lies above the galactose molecule. Substrates of different β-galactosidases include ganglioside GM1, galactosylceramides, lactose, and various glycoproteins. The enzyme catalyzes the terminal residue of beta-lactose galactopyranosyl bond and produce glucose and galactose (Juers, *et al.*, 2012; Kastsianevich, 2017). It may also cleave fucosides and arabinosides but with much lower efficiency. It is an essential enzyme in the human body. Deficiencies in the protein can result in galactosialidosis or Morquio B syndrome (Panesar *et al.*, 2006). In *E. coli*, the gene of β-galactosidase known as the *lacZ* gene, is present as part of the inducible system *lac* operon which is activated in the presence of lactose when glucose level is low (Harvey *et al.*, 2003).

Beta-galactosidase has many homologues based on similar sequences. A few are evolved beta-galactosidase (EBG), 6-phospho-beta-galactosidase, beta-mannosidase, and lactase-phlorizin hydrolase. Although they may be structurally similar, they all have different functions (Salehi *et al.*, 2009). The enzyme is inhibited by L-ribose, non-competitive inhibitor iodine, and competitive inhibitors phenylethylthio-beta-D-galactoside (PETG), D-galactonolactone, isopropyl thio-beta-D-galactoside (IPTG), and galactose (Juers, *et al.*, 2003). The enzyme is essential for the digestion of bovine milk which contains an average of 4.8% lactose. Deficiency for the gene coding for β-galactosidase leads to mal-absorption of lactose and subsequent fermentation of lactose by the gut flora (Swallow, 2003; Suchy *et al.*, 2010).

The β-galactosidase assay is used frequently in genetics, molecular biology and other life sciences (Krivtsov and Armstrong, 2007). The enzyme also has many industrial and medicinal applications like cleavage of blood group A and B glycotopes, biosensors for lactose determination and enzymatic hydrolysis of lactose (Asraf and Gunasekaran, 2010; Panesar *et al.*, 2016; Kastsianevich, 2017). Hydrolytic activity of this enzyme is important for applications in the food industry in

reducing the lactose content in milk, and increases the solubility and sweetness in dairy products (Otieno, 2010). Beta-galactosidase is used in dairy products such as yoghurt, sour cream, and some cheeses which are treated with the enzyme to break down any lactose before human consumption (Kastsianevich, 2017).

2.4 Structure of Beta-galactosidase

Beta-galactosidase is a tetramer of four identical polypeptide chains, each of 1,024 amino acids (Fowler and Zabin, 1978; Kalnins *et al.*, 1983; Juers *et al.*, 2000; Juers *et al.*, 2012). The crystal structure was initially determined in a monoclinic crystal form with four tetramers in the asymmetric unit (Jacobson *et al.*, 1994). Subsequently, the structure was refined to 1.7A resolution in an orthorhombic crystal with a single tetramer in the asymmetric unit (Juers *et al.*, 2000). The latter form is technically superior and has been used for subsequent structural and functional studies.

The 1,024 amino acid of *E. coli* β-galactosidase was first sequenced in 1970 by Fowler and Zabin, and its structure was determined twenty-four years later in 1994. The protein is a 464-kDa homotetramer with 2, 2, 2-point symmetry. Each unit of β-galactosidase consists of five domains; domain 1 is a jelly-roll type barrel, domain 2 and 4 are fibronectin type III-like barrels, domain 5 is a β-sandwich, while the central domain 3 (residues 334–627) is a so-called triose phosphate isomerase (TIM-type barrel) or $\alpha_8\beta_8$ barrel with the active site forming a deep pit at the C-terminal end of this barrel (Jacobson *et al.*, 1994). The third domain contains the active site. The active site is made up of elements from two subunits of the tetramer (Juers *et al.*, 2012).

Critical elements of the active site are also contributed by amino acids from elsewhere in the same polypeptide chain as well as from other chains within the tetramer. Hence, disassociation of the tetramer into dimers removes these critical elements of the active site. Fig 2.1 shows the overall structure of beta-galactosidase.

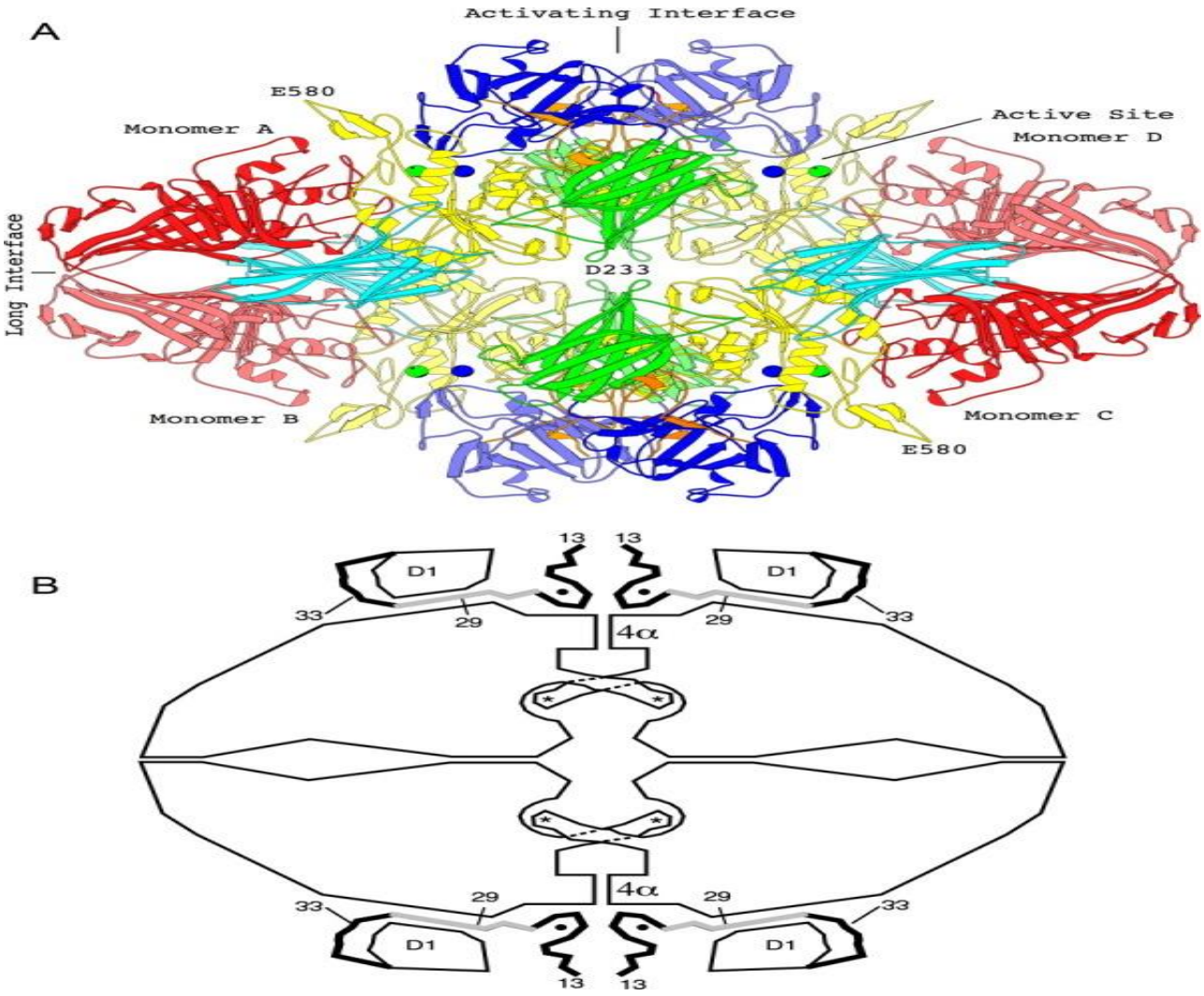

Figure 2.1: Tetrameric Structure of Beta-galactosidase (Juers *et al.*, 2012)

2.5 Mechanism of Action of Beta-galactosidase

Beta-galactosidase has three enzymatic activities (Figure 2.2). First, it can cleave the disaccharide lactose to form glucose and galactose, which can then enter glycolysis. Secondly, the enzyme can catalyze the transgalactosylation of lactose to allolactose, and, thirdly, the allolactose can be cleaved to the monosaccharides (Juers *et al.*, 2012). It is allolactose that binds to *lacZ* repressor and creates the positive feedback loop that regulates the amount of β-galactosidase in the cell (Juers *et al.*, 2012). The active site of β-galactosidase catalyzes the hydrolysis of its disaccharide substrate via "shallow" (nonproductive site) and "deep" (productive site) binding (Juers *et al.*, 2012). Galactosides such as phenylethylthio-beta-D-galactoside (PETG) and isopropylthio-beta-D-galactoside (IPTG) bind in the shallow site when the enzyme is in "open" conformation while transition state analogues such as L-ribose and D-galactonolactone will bind in the deep site when the conformation is "closed" (Juers *et al.*, 2012).

2.6 Sources of Beta-galactosidase

Many studies have been carried out on beta-galactosidase and as such data on the enzyme is quite voluminous. Beta-galactosidase is widely distributed in nature. The enzyme has been found to be produced by plants, animals and microorganisms (Somyos and Phimchanok, 2009; Kumari *et al.*, 2011; Althaf *et al.*, 2012; Panesar *et al.*, 2016).

The β-galactosidases most widely used in industry are mesophilic in nature obtained from the fungi *Aspergillus* spp. and yeast *Kluyveromyces* spp. (Oliveira *et al.*, 2011). Almonds, apricots, apples, peaches, seeds of soybeans, alfalfa, and coffee are some of the plants that have been shown to contain beta-galactosidase (Gopalakrishnan *et al.*, 2014).

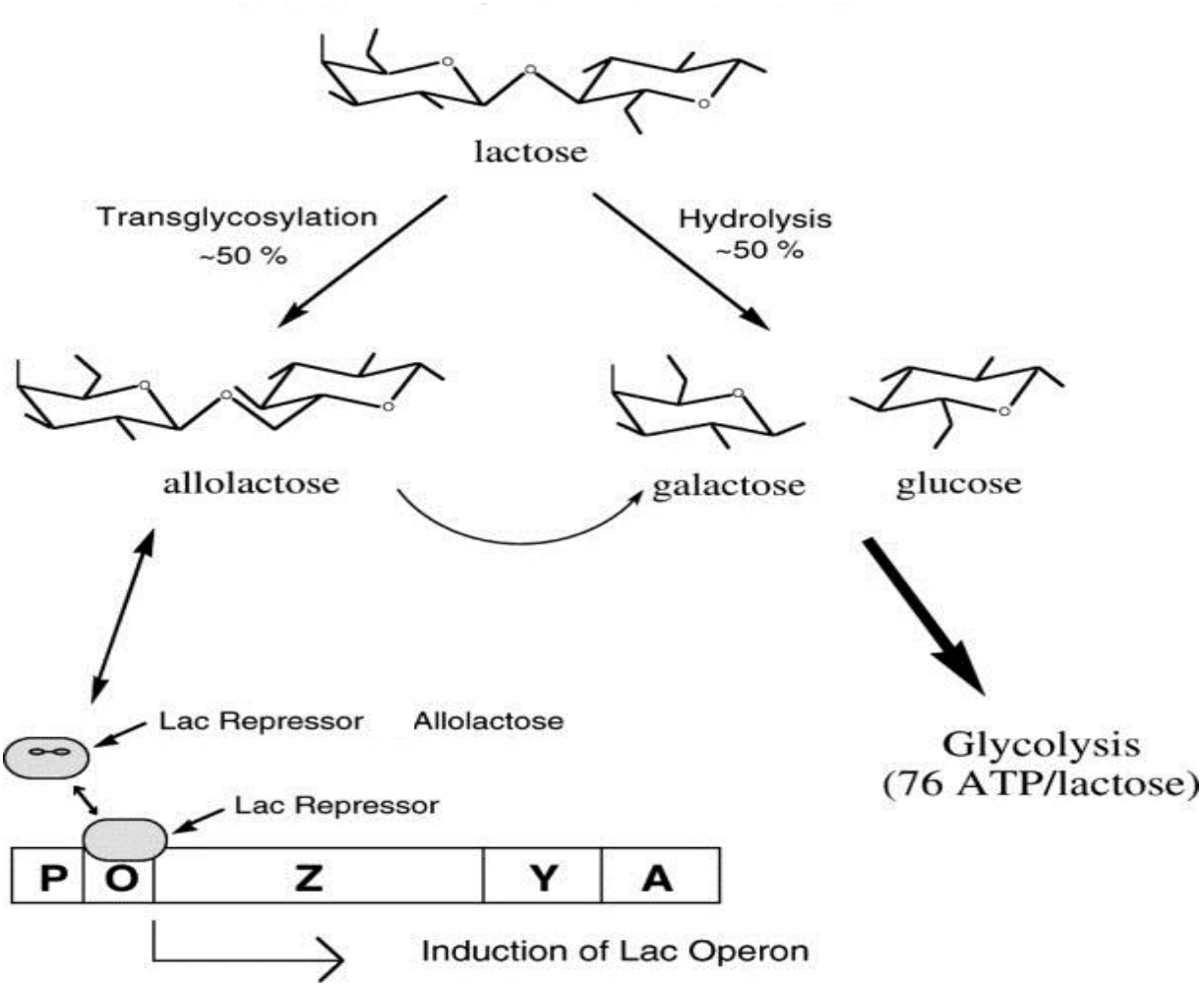

Figure 2.2: Generalized Scheme for Enzymatic Hydrolysis of Lactose by Beta-Galactosidase (Juers *et al*., 2012)

Key: P-Promoter; O-Operator; Z-β-galactosidase; Y-Lactose permease; A-Galactosidase transacetylase; ATP- Adenosinetriphosphate

The enzyme is also found in animals such as snails, the intestines of dogs, rabbits, calves, sheep, rats, and it is a normal constituent of the human intestinal secretion, brain, placenta and testis (Soares *et al*., 2012; Gopalakrishnan *et al*., 2014; Alikkunju *et al*., 2016; Panesar *et al*., 2016).

Beta-galactosidases are produced by most microorganisms including many bacteria, yeast, and moulds, as well as their mutant strains which are induced to produce large amounts of the enzyme (Nevalainen, 1981; Holsinger and Kligerman, 1997; Almeida and Pastore, 2001; Asraf and Gunasekaran, 2010; Sen *et al*., 2012). The commercial beta-galactosidases currently available in the market are derived mainly from yeasts and moulds. In 1996, the Food and Drug Administration (FDA) amended its regulations to affirm that beta-galactosidase preparation derived from *Candida pseudotropicalis* for use in milk and milk-derived products to hydrolyze lactose is generally recognized as safe (GRAS) (Asraf and Gunasekaran, 2010).

Increased industrial demand for beta-galactosidase requires cost-effective production methods to ensure the economic viability of lactose hydrolysis at commercial scale (Nor *et al*., 2001). Beta-galactosidase is now produced commercially from *Kluyveromyces fragilis, Saccharomyces lactis* or *Aspergillus niger* (Nduka, 2007). In an attempt to improve production of beta-galactosidase, several researchers have made investigations to select microorganisms that have abilities to produce enzyme with characteristic high activity and production yields (Manera *et al*., 2008). Fermentation parameters such as substrates, defined optimized fermentation conditions and nutrient requirements for any chosen microorganism of study are continuously being evaluated (Ramírez, 2003; Domingues *et al*., 2004).

The most common fungal sources of beta-galactosidases are *Kluyveromyces lactis, Kluyveromyces fragilis* (*Sacchoramyces fragilis*), and some *Aspergillus* species which are accepted as ‘‘generally recognized as safe’’ (GRAS) by Food and Drug Administration (FDA) (Saqib *et al*., 2017).

Aspergillus oryzae produces extracellular beta-galactosidase with optimal pH and temperature of 5 and 50^0C respectively with galactose as a competitive inhibitor and glucose a noncompetitive inhibitor (Saqib *et al.*, 2017). Beta-galactosidase from this fungus has found application in whey utilization and it is used in commercial scale (Saqib *et al.*, 2017). *Aspergillus niger* originated beta-galactosidase are usually involved in the removal of the galactose residues from oligosaccharides and polysaccharides derived from plants (Kazemi *et al.*, 2016).

In order to find an alternative fungal source, Seyis and Aksoz (2004) isolated 13 different fungi (*Aspergillus, Trichoderma*, *Penicillium*, *Rhizopus* and *Fusarium* sp.) cultured in lactase production medium at 30°C and 150 rpm for 6 days. Results showed that *Trichoderma viride* ATCC 32098 has maximum beta-galactosidase specific activity, followed by *Trichoderma harzianum* 1073 D3.

Vishwanataha *et al.* (2012) made an attempt on β-galactosidase synthesis from fungal origin due to its stability in action as well as industrial application. Twenty fungi were isolated from black and red soil samples in different regions of Karnataka (India) using serial dilution method. Twenty fungal isolates were screened using silica gel thin-layer chromatography where n-butanol, acetic acid, diethyl ether and water were used as solvent systems in the ratio of 9:6:3:1. Four fungal isolates *Trichoderma* sp., *Aspergillus niger, Rhizopus stolonifera* and *Fusarium* sp. showed positive results. These strains were employed for the production of β-galactosidase through submerged fermentation. Strain *Rhizopus stolanifera* showed maximum 2250IU extracellular β-galactosidase activity.

Studies carried by Kaur *et al.* (2015) to utilize whey for the production of beta-galactosidase using both yeast and fungal cultures. The yeast *Kluyveromyces marxianus* WIG2 and various fungal strains were used in the study. Different disruption techniques were investigated for the extraction of the intracellular enzyme produced by the yeast cells. Among the different methods tested for the disruption of yeast cells, sodiumdodecylsulfate (SDS)-chloroform showed the maximum beta-

galactosidase activity. For fungal isolates tested, *Aureobasidium pullulans* NCIM 1050 was observed to be the maximum extracellular enzyme producer.

Recently, a study was carried out by Panesar *et al*. (2016) to isolate beta-galactosidase producing fungal strains from food as well as agricultural wastes. More than 100 fungal cultures were examined for their potential for enzyme production. All the fungal strains were screened using X-gal (5-bromo-4-chloro-3 indole-β-D-galactopyranoside) and IPTG as inducers in modified Czapek Dox agar medium. Among the various isolated fungal strains, *Rhizomucor pusillus* strain exhibited the highest enzyme activity and was chosen for further phenotypic and genotypic characterization on the basis of 5.8s RNA gene sequencing data. Table 2.1 shows some of the most widely used microbial sources of beta-galactosidase.

2.7 Properties of Beta-galactosidase

Beta-galactosidases produced by the different microorganisms vary considerably in their properties, but the specificity of the enzyme towards lactose is the same, regardless of the different Km values. The Km value which is known as the apparent Michaelis-Menten constant is a kinetic parameter used to designate the enzyme affinity towards its substrate (Dutra *et al*., 2015). The beta-galactosidase preparations from different microorganisms are not identical with respect to their structure, size, molecular weight and the optimum conditions for lactose hydrolysis. Also, the optimum pH and temperatures of various commercially available sources of beta-galactosidase are different (Abdelrahim, 1989).

One of the advantages of differences in the optimum pH and temperature is that the industrial processes can be developed using a particular source for beta-galactosidase depending on the requirements dictated by the nature of the process, substrate and the desired final product (Jelen, 1993).

Table 2.1: Some Common Microbial Sources of Beta-galactosidase

Bacteria	Fungi	
Escherichia coli	Yeast	Moulds
Streptococcus cremoris	*KIuyveromyces fragilis*	*Aspergillus niger*
Lactobacillus acidophilus	*KIuyveromyces lactis*	*Aspergillus oryzae*
Lactobacillus bulgaricus	*KIuyveromyces marxianus*	*Aspergillus parascicoda*
Lactobacillus planetarium	*Candida pseudotropicalis*	*Aspergillus flavus*
Streptococcus thermophilus	*Candida kefyr*	*Aspergillus fumigatus*
Streptococcus lactis		*Aspergillus palmi*
Bacillus megaterium		*Aspergillus foetidus*
Bacillus coagulans		*Aspergillus phoenecia*
Bacillus acidocaldarius		*Trichoderma reesei*
Bacillus stearothermophilus		*Trichoderma viride*
Thermus aquaticus		*Trichoderma harzianum*
Pyrococcus woesei		*Penicillium simplicissimum*
Bifidobacterium infantis		*Penicillium expansum*
Bifidobacterium longum		*Alternaria alternate*
Enterobacter cloacae		*Mucor pusillus*
Geobacillus stearothermophilus		*Scopulariosis sp.*
Leuconostoc citrovorom		*Rhizomucor pusillus*

(Abdelrahim, 1989; Seyis and Aksoz, 2004; Grosova *et al.*, 2008; Manera *et al.*, 2008; Nizamuddin *et al.*, 2008; Asraf and Gunasekaran , 2010; Althaf *et al.*, 2012; Princely *et al.*, 2013; Amal *et al.*, 2012; Ashish *et al.*, 2014; Gopalakrishnan *et al.*, 2014; Dutra *et al.*, 2015; Alikkunju *et al.*, 2016; Bosso *et al.*, 2016; Panesar *et al.*, 2016).

The operational pH range is one of the primary parameters that determine the application of a given beta-galactosidase (Bosso *et al.*, 2016). One of the advantages of these differences in the optimum pH and temperature is that the industrial processes can be developed using a particular source for beta-galactosidase depending on the requirements dictated by the nature of the process, substrate and the desired final product (Jelen, 1993; Bosso *et al.*, 2016).

The fungal enzymes from *Aspergillus niger* and *Aspergillus oryzae* have an optimum pH range of 3.0-5.0 with relatively high optimum temperatures (Nizamuddin *et al.*, 2008). A broad optimum pH range is advantageous in view of the fact that food processors often have little control over the pH of the food (Nduka, 2007).

Microbial beta-galactosidase is of much interest because of their thermo-stability, acidophilic or basophilic properties and these enzymes are known to catalyze both hydrolytic and transglycosylation reactions (Asraf and Gunasekaran, 2010; Alikkunju *et al.*, 2016). The commercial beta-galactosidases now available in the market are derived mainly from the yeast, *Kluyveromyces lactis*, and the molds, *Aspergillus niger*. These two commercial enzymes differ widely in their properties, particularly in their pH optimum (Oliveira *et al.*, 2011). Fungal sources, with acidic pH-optima, are effective for hydrolyzing lactose in whey, while yeast sources, with neutral pH-optima, are more effective for hydrolyzing lactose in milk (Husain, 2010). Also, β-galactosidase produced from molds is very stable and does not require metal ion cofactors for its action (Gonzalez and Monson, 1991).

Fungal enzymes have pH optima in the range of 3 to 5. Therefore fungal enzymes are suitable for processing acid whey and permeate. They also have relatively high optimum temperature which is between 55-60 °C. As it is known, combination of low pH and high temperature discourages microbial growth (Mahoney, 2005). Studies carried out by Seyis and Aksoz (2004) on stability of

beta-galactosidase in the pH range of 3.0–7.5 at the temperature between 20 and 70°C. It was observed that the activity of beta-galactosidase produced by *T. viridae* ATCC 32098 was above 90% in the pH range of 3.0–7.5 at the temperature between 20 and 60°C, and even 66% at 70°C. It was concluded that *Trichoderma* sp., especially *T. viridae* ATCC 32098, could be used as an alternative for the production of beta-galactosidase in industrial scale. The thermostable beta-galactosidase from *Aspergillus niger, Bacillus stearothermophilus, Pyrococcus woesei, Thermus sp* are relatively stable at 35–80°C.

Bosso *et al*. (2016) carried out studies on commercial beta-galactosidase (E.C. = 3.2.1.23) from *Kluyveromyces lactis* (liquid) and *Aspergillus oryzae* (lyophilized) for their hydrolysis potential in lactose substrate, UHT milk, and skimmed milk at different concentrations (0.7, 1.0 and 1.5%), pH values (5.0, 6.0, 6.5 and 7.0), and temperature (30, 35, 40 and 55ºC). High hydrolysis rates were observed for the enzyme from *K. lactis* at pH 7.0 and 40ºC, and from *A. oryzae* at pH 5.0 and 55ºC. The enzyme from *K. lactis* showed significantly higher hydrolysis rates when compared to *A. oryzae*. The effect of temperature and β-galactosidase concentration on the lactose hydrolysis in UHT milk was higher than in skimmed milk, for all temperatures tested. With respect to the thermal stability, a decrease in hydrolysis rate was observed at pH 6.0 at 35ºC for *K. lactis* enzyme, and at pH 6.0 at 55ºC for the enzyme from *A. oryzae*. They concluded that the properties of the β-galactosidases from *K. lactis* and *A. oryzae* enable its use most efficiently to control the enzyme concentration, temperature, and pH in many industrial processes and product formulations.

Although pasteurization conditions denature many enzymes, several thermostable beta-galactosidases have been identified from a variety of sources (Synowiecki *et al*, 2006; Chen *et al*., 2008; Park and Ho, 2010). The hydrolysis by beta-galactosidase has been found to be affected by the presence of some mineral ions naturally occurring in milk. The most important activators are

magnesium and manganese, whereas sodium and calcium have a negative effect on the enzymes' activity. Also the activity of beta-galactosidase was also found to be hampered by phytic acid which is present in soybean proteins, an important factor, since milk is incorporated with vegetable proteins in many food formulations (Burgess and Shaw, 1983; Inagawa *et al.*, 1987; Abdelrahim, 1989; Mahoney, 2005).

2.8 Applications of Beta-galactosidase

2.8.1 Production of Milk/Milk Products (Dairy)

Nutritional, technological, and environmental problems are associated with lactose. The usefulness of hydrolyzed lactose to its constituent monosaccharide using beta-galactosidase infers an added value gained by the product (Burgess and Shaw, 1983). The enzymatic hydrolysis of lactose by beta-galactosidase can result in; the production of lactose-reduced milk and dairy products for patients with beta-galactosidase deficiency (Kardel *et al.*, 1995; Pivarnik *et al.*, 1995; Mahoney, 2005; Kastsianevich, 2017); pre-hydrolysis of lactose to accelerate acid production and ripening of cheese and yoghurt; prevention of crystallization in ice-cream and concentrated milk; reduction of hygroscopicity in dairy products and modification of the properties of lactose such as increased solubility and sweetness, high reducing power and fermentability (Grueger and Grueger 1994).

The products of hydrolysis are superior to lactose itself, being more sweet, soluble and digestible than lactose, and offer a number of opportunities for the end product. Consequently, these changes in the physical and chemical properties of milk and whey are of interest to dairy processors (Holsinger and Kligerman, 1997). Panesar *et al.* (2007) carried out trials to overcome the problem of enzyme extraction and poor permeability of cell membrane to lactose. Permeabilized *K. marxianus* NCIM 3465 cells were used for the production of lactose-hydrolyzed milk. The ethanol-permeabilized yeast cells gave 89% hydrolysis of milk lactose under optimized conditions.

In addition, lactose hydrolysed milk (LHM) can be used in flavored milk products such as chocolate (Mahoney, 2005), sweetened, condensed and frozen milk products such as ice cream in which the sandiness is reduced while viscosity, whipping ability, over-run, and organoleptic properties are improved (Artolozaga *et al.*, 1998).

2.8.2 Medical applications

Beta-galactosidase with its transgalactosylation property finds prominent medical applications such as cleavage of blood group A and B glycotopes, disease diagnosis, in treatment of digestive disorders and development of digestive supplements (Grosová *et al*., 2008; Asraf and Gunasekaran, 2010). Anderson *et al.* (2005) isolated an endo beta-galactosidase from *Clostridium perfringens* ATCC 10543 capable of liberating both the A trisaccharide and B trisaccharide from glycol-conjugates containing blood group A and B glycotopes, respectively. Recombinant EABase damaged the blood group A and B antigenicity of human type A and B erythrocytes and also released A-Tri and B-Tri from blood group A+- and B+-containing glycoconjugates. The exceptional specificity of this beta galactosidase should make it useful for studying the structure and function of blood group A- and B-containing glycol-conjugates**.**

Liu and Roffler (2006) examined the expression of *E. coli* beta-galactosidase in muscle fibers and concluded that repeated intramuscular injections of beta-galactosidase can encourage strong immune responses in immune-competent animals and cause abolition of transduced muscle fibers by inflammatory cells.

In 2009, a recombinant endo-beta-galactosidase (ABase), which releases A/B antigen was developed. It removed 82% of A antigen and 95 % of B antigen in human A/B red blood cells, and concealed anti-A/B antibody binding and complement activation effectively. It was also found to remain active at 4°C. In-vivo infusion into a blood type A demonstrated a marked reduction of A

antigen expression in the glomeruli of kidney (85% at 1h, 9% at 4h and 13% at 24h) and the sinusoids of liver (47% at 1h, 1% at 4h and 3% at 24h) without grave adverse effects. This substitute approach might be useful for minimizing antibody removal and anti-B cell immunosuppression as an adjuvant therapy in ABO incompatible kidney, liver and possibly heart transplantation (Kobayashi *et al.*, 2009).

Beta-galactosidase has been researched as a potential treatment for lactose intolerance through gene replacement therapy where it could be placed into the human DNA so individuals can break down lactose on their own (Ishikawa *et al.*, 2015). The primary commercial use of β-galactosidase is as supplement to break down lactose in milk to make it suitable for people with lactose intolerance (Ishikawa *et al.*, 2015; Kastsianevich, 2017). A study conducted on 48 healthy Guatemalan preschool children examined the efficacy of two different microbe-derived beta-galactosidase preparations to prevent symptoms of lactose intolerance after consumption of whole cow's milk or milk prehydrolyzed with beta-galactosidase (Barillas and Solomons, 1987). One enzyme preparation was derived from the yeast, *Kluyveromyces lactis* (3,250 neutral lactose units), the other from *Aspergillus oryzae* (6,635 FCC lactose units). Pre-hydrolyzed milk was used as a standard of reference for effective lactose digestion. Each child, after ingestion of 240 mL whole milk containing 12g lactose, was tested for degree of lactose via a hydrogen breath test. Although 27 of 48 children could not adequately digest whole milk, when given prehydrolyzed milk with beta-galactosidase, 25 of the 27 lactose-intolerant children showed no signs of mal-digestion (Mario, 2008). Recently, a beta-galactosidase from the mesoacidophilic fungus *Bispora* sp. MEY-1 under simulated gastric conditions, has shown greater stability (100%) and hydrolysis ratio (>80%) toward milk lactose than the commercially available beta-galactosidase from *A. oryzae* ATCC 20423. Thus,

this beta-galactosidase may be a superior digestive supplement for alleviating symptoms associated with lactase deficiency (Wang *et al.*, 2009).

2.8.3 Food formulations/Non diary

Beta-galactosidase is used industrially to obtain the hydrolysates of lactose from milk and milk whey for utilization in bakery products, ice creams, animal feed and as a sugar source for several fermentation products (Grosova, *et al.*, 2008). Saad (2004) demonstrated that submerged culture of *Aspergillus japonicus* produced β-D-galactosidase, with 2.95 U mg-1 protein specific activity, when developed on cheese whey permeate fortified with 0.5% yeast after 4 days incubation at 28°C. the rate of lactose hydrolysis in whey was about 55%, after 4h incubation at 45°C. This enzyme was found suitable for obtaining fermentable sugars from whey wastes.

Enzymologists have recently attributed great importance to the galactosyltransferase activity of β-galactosidases to obtain the synthesis of oligosaccharides with 2 or more galactose units starting from lactose (Princely *et al.*, 2013). Solutions with high lactose concentrations, such as evaporated milk whey, submitted to the action of beta-galactosidase from some microorganisms suffer a transgalactosylation reaction, producing a galactooligosaccharide mixture or transgalactosylated oligosaccharides (TOS) which act as functional foods, with several beneficial effects for their consumers (Grosová *et al.*, 2008).

Whey has been utilized for the production of exopolysaccharide and single cell protein by employing beta-galactosidase from microorganisms like *Aspergillus oryzae, Kluveromyces lactis, Kluveromyces marxianus, Lactobacillus delbrueckii subsp. bulgaricus, Saccharomyces cerevisiae* (Asraf and Gunasekaran , 2010). Transglycosylation and transgalactosylation properties of beta-galactosidase from *A. niger, Bacillus megaterium, Beijerinckia indica, Bifidobacterium infantis, Bifidobacterium longum, Enterobacter cloacae, Geobacillus stearothermophilus, K. marxianus, Lactobacillus sp,*

Lactobacillus reuteri, *Penicillium expansum* have been utilized for production of glucose, galactose, heteropolysaccharide, galacto-oligosaccharides (Asraf and Gunasekaran , 2010).

The lactose-hydrolysed whey can also be concentrated to produce syrups of 70-75% solids which may provide a source of functional whey proteins and sweet carbohydrates to be used as food ingredients in ice cream, bakery, confectionary products, alcohols, bakers' yeast, fermented beverages, and soft drinks (Husain, 2010). Both lactose-hydrolysed milk and lactose-hydrolysed whey give a wide spectrum of uses which emphasize the importance of the enzyme beta-galactosidase as a key industrial enzyme capable of minimizing the problems associated with lactose (Abdelrahim, 1989).

Rodriguez *et al*. (2006) constructed and analyzed two hybrid proteins from the beta-galactosidase of *K. lactis*, intracellular, and its *A. niger* homologue that is extracellular. One of the hybrid proteins obtained was found to have interesting properties for its biotechnological utilization that increases the yield of the protein released to the growth medium. Changes introduced in the construction, besides to improve secretion, conferred to the protein biochemical characteristics of biotechnological interest. Thus, beta-galactosidase plays a key role in food and other allied industries.

Beta-galactosidase has found applications in the production of Galactooligosaccharides (GOS). Much investigation has been carried out in the field of pro- and prebiotics as functional foods (Asraf and Gunasekaran, 2010). Galactooligosaccharides (GOS) are used as non-digestible, carbohydrate-based food ingredients in human and animal nutrition. Much of the research has been focused upon microorganisms that produce beta-galactosidases with improved quality for production of galacto-oligosaccharides (Asraf and Gunasekaran, 2010). The synthesis of GOS with a high yield of 55% from 275 g/L lactose at 50°C for 12h was performed using transglycosylating beta-galactosidase producing *Enterobacter cloacae*. The enzyme showed an extensive range of acceptor specificity for

transglycosylation and catalyzed glycosyl transfer from ONPGal to various chemicals resulting in novel saccharide yields from 0.8% to 23.5 % (Lu *et al*., 2009).

Cheng *et al*. (2006) in a study used *Bacillus* spp. for the production of low-content GOS from lactose that resulted in the highest yield of trisaccharides and tetrasaccharides. GOS production was improved by mixing beta-galactosidase with glucose oxidase. The low content GOS syrups, produced by beta-galactosidase was subjected to the fermentation by *K. marxianus*, whereby glucose, galactose, lactose and other disaccharides were at a low level, resulting in up to 97% and 98% on a dry weight basis of high-content GOS with the yields of 31% and 32% respectively. Hence, beta-galactosidase plays a significant role in production of galacto-oligosaccharides which can be used as food and feed for human-beings and animals respectively (Li *et al*., 2008).

2.8.4 Biosensor

Industrially, microbial beta-galactosidases have a prominent position in terms of their role in production of various industrially relevant products like biosensor-compounds (Asraf and Gunasekaran, 2010). The β-galactosidase assay is used frequently in genetics, molecular biology and other life sciences. An active enzyme may be detected using X-galactosidase, which forms an intense blue product after cleavage by β-galactosidase, and it is easy to identify and quantify (Krivtsov and Armstrong, 2007). It is used for example in blue white screen where its production may be induced by a non-hydrolyzable analog of allolactose, IPTG, which binds and releases the lac repressor from the lac operator, thereby allowing the initiation of transcription to proceed (Juers *et al*., 2012).

Marrakchi *et al*. (2008) developed a biosensor associating two distinct enzymatic activities, that of the beta-galactosidase and that of the glucose oxidase, in order to apply it for the quantitative

detection of lactose in commercial milk samples. To eliminate interferences with glucose, a differential mode of measurement was used in this biosensor.

2.8.5 Whey utilisation

Another important application of beta-galactosidase is the role it plays in whey utilization (Kastsianevich, 2017). Whey has enormous therapeutic applications due to its composition in terms of proteins, lactose, minerals and valuable milk nutrients. The disposal of whey remains a major problem for the dairy industry especially in developing countries where a relatively insignificant part of whey is used for production of protein concentrates or permeates and a significant part of it is disposed-off into the water streams causing severe water pollution resulting in high BOD and 5–6% dissolved solids (Moulin and Galzy. 2004).

Bansal *et al.* (2007) carried out studies related to beta-galactosidase production using whey containing 4.4% (w/v) lactose inoculated with *K. marxianus* MTCC 1389 and alleviated water pollution problems caused due to its disposal into the water streams. The major options for treatment or bioconversion of whey into commercially important products, ethanol and β- galactosidase which finds an increasing use because of growing lactose intolerant population. Thus, microbial beta-galactosidases production is an important area in whey utilization (Asraf and Gunasekaran, 2010).

Oberoi *et al.* (2008) found that *K. marxianus* NCIM 3465 showed greatest beta-galactosidase activity of 1.62 IUmg-1 dry weight using whey and cauliflower waste. Although a minor increase in enzyme production was seen by incorporating 5% to 10% cauliflower waste in whey, nearly 15% increase in beta-galactosidase production was observed when cauliflower waste level was increased to 20%. Supplementing whey with 20% cauliflower waste also lowered the production time. Lactose concentration in whey, mainly responsible for increasing the BOD of the effluent water, decreased

from 4.2 % to nearly 0% at 24 h. Thus, the study established that both these by-products / residues could be effectively used for beta-galactosidase production at commercial scale.

2.8.6 Production of Ethanol

Several mathematical models have been developed for ethanol production from whey using beta-galactosidase. In 2007, mathematical models for semi-continuous ethanolic fermentation in a whey medium employing co-immobilized *S. cerevisiae* strain and β-D-galactosidase was developed. Kinetic parameters of biomass growth, experimentally determined fluxes of ethanol and water, kinetic constants of ethanol separation, the degree of sugar utilization and ethanol productivity, the time of ethanol separation were predicted using this model (Staniszewski *et al.*, 2007).

Ozmihci and Kargi (2007) used lactose utilizing yeast strain, *K. marxianus* DSMZ-7239 for ethanol production from cheese-whey powder (CWP) solution in batch experiments and developed a kinetic model describing the rate of sugar utilization and substrate inhibition as function of the initial substrate and the biomass concentrations. Oda and Nakamura (2009) found that *K. marxianus* converted lactose in media containing 20% (w/v) sugar cheese whey most economically to ethanol.

2.9 Future Prospects of Beta-galactosidase

An intensive research on beta-galactosidases is ongoing, because of their potentially useful role in solving nutritional, environmental, medical and technological problems related to lactose in dairy products and cheese whey treatment (Torres *et al.*, 2010; Princely *et al.*, 2013). Although there are many commercial beta-galactosidases produced, mainly from yeast and fungi (Seyis and Aksoz, 2004; Oliveira *et al.*, 2011), the practical application of these enzymes is still faced with many technical problems which are summarized below:

i. Most of the known beta-galactosidases with optimum temperatures above 30^{O}C do not have good activity for hydrolysing lactose at low temperatures of 0-10^{O}C at which milk is usually kept and stored to prevent spoilage (Asraf and Gunasekaran, 2010).

ii. The beta-galactosidases produced have the problem of being sensitive to metal ions like zinc, magnesium, sodium, and calcium as well as galactose which are all present in milk (Carminatti, 2001; Juers *et al.*, 2012).

iii. The microorganisms used to produce the beta-galactosidases have varying nutritional requirements (Dutra *et al.*, 2015) hence, they produce enzymes other than beta-galactosidases such as proteolytic and lipolytic enzymes which can produce inferior organoleptic properties or other quality defects in milk/milk products (Amal *et al.*, 2010). The subsequent enzyme purification needed to isolate beta-galactosidases from other enzymes is expensive, since it includes many separation steps involving various types of chromatography (Panesar *et al.*, 2010). Each step of the purification process has many column variables that must be carefully chosen in order to obtain the desired resolution and separation of the required protein (Mowery and Seidman, 2005).

iv. Confirmatory tests on the enzyme efficiency and activity are performed on chromogenic substrates such as *O*-nitrophenyl-β-D-galactopyranoside (ONPG) or methyl-β-D-galactopyranoside (Nizamuddin *et al.*, 2008; Althaf *et al.*, 2012; Amal *et al.*, 2012; Princely *et al.*, 2013; Ashish *et al.*, 2014; Gopalakrishnan *et al.*, 2014). The complicated composition of milk and whey is mostly neglected and this may lead to deviations from the ideal estimated enzymatic properties (Murugan, 2013). Consequently, there is a need for new microbial sources that are capable of producing economic quantities of beta-galactosidases with the ability to function efficiently at high or low temperatures (Vishwanataha *et al.*, 2012).

Research and development in the beta-galactosidase will help to address the problems faced in the food and allied industries that look for enzymes with novel properties like acidophilic, basophilic, cold-stability and thermo-activity (Asraf and Gunasekaran, 2010). Novel galacto-oligosaccharides production by beta-galactosidase will pave a way for development of prebiotics that can be used as food supplement. Dairy wastewater utilization by beta-galactosidase will help to reduce the water pollution caused by lack of downstream-processing and lead to production of products like bioethanol and lactose-hydrolysed milk. Individual molecular study of beta-galactosidase has shown the various unknown kinetic properties of beta-galactosidase. Thus, research and development of beta-galactosidase finds application in several industries (Asraf and Gunasekaran, 2010).

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Collection of Sample

Effluent sample was collected from the sedimentation tank of the Dairy unit, National Agricultural Production Research Institute (NAPRI) Shika, Kaduna State, Nigeria. The effluent sample was collected in clean 10litres container and transported immediately to the Department of Microbiology, Ahmadu Bello University Zaria. The sample was stored in the refrigerator for further analyses.

3.2 Isolation and Characterization of Fungi

3.2.1 Preparation of media

Sabouraud Dextrose Agar (SDA) was prepared according to manufacturer's instruction, supplemented with streptomycin and sterilized at 121^{o}C for 15minutes. Twenty millilitres of the prepared sterile agar was poured aseptically into Petri-plates and Bijou bottles and incubated at room temperature for 2-3 days to ensure sterility.

3.2.2 Serial dilution

Using a sterile pipette, 9ml of sterile distilled water was dispensed into a series of five test tubes. One ml of the sample was transferred aseptically using a sterile syringe to the first test tube and mixed thoroughly to give a dilution of 10^{-1}. Also, one ml was transferred aseptically from the first test tube to the second test tube to give a dilution of 10^{-2}. This process was repeated until a dilution of 10^{-5} was obtained (Rohilla and Salar, 2012; Onyeze *et al.*, 2013).

3.2.3: Culture of fungi

Using direct plating technique, 0.5ml of the sample was aseptically inoculated on the prepared SDA and spread evenly by gently rotating the plate. Also about 0.5ml of the diluted sample from the 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions was aseptically inoculated separately on the prepared sterile SDA medium. All inoculated plates were incubated at ambient temperature (25-27^{o}C) for 5-7days. Organisms obtained after the incubation period were sub-cultured to obtain pure fungal isolates. Stock cultures of the isolates were obtained by sub-culturing the pure isolates on agar slants for future use (Nizamuddin *et al*, 2008).

3.2.4: Characterization and identification of fungal isolates

The fungal isolates was characterized and identified based on macroscopic and microscopic analysis using taxonomic guidelines and standard procedures as described by Daniel *et al.* (2014). Macroscopic characteristics of 6-7day old fungal isolates were examined such as colonial growth, colonial color, presence or absence of aerial mycelium, nature of mycelia, texture, growth pattern, presence of wrinkles and furrows, pigment production.

Microscopic examination was carried out using Lactophenol cotton blue staining and culture slide techniques as described by Barnett and Hunter (1999). For Lactophenol cotton blue staining technique, the pure fungal isolates were sub-cultured on SDA plates and incubated at room temperature for 2-3days. After the incubation period, a pinch of the mycelia of the sub-cultured fungal isolates were transferred aseptically from the culture plate using a sterile inoculating needle and gently spread on a drop of water on clean, grease-free glass slides and teased. This was followed by addition of 2-3 drops of lactophenol cotton blue. The slide was then covered with a clean coverslip and examined under the microscope using x40 objective and compared with a mycological atlas by Larone (2002).

For slide culture technique, Sabouraud Dextrose Agar was prepared then a sterile knife was used aseptically to cut small rectangular (3cm by 2cm) blocks of the agar. Using the sterile knife, the rectangular agar blocks were aseptically removed and transferred onto sterile clean grease-free glass slides. The slides with the agar blocks were placed into clean sterile petri-dishes containing moist cotton wool spread at the base of each petri dish to prevent contamination and preserve moisture during incubation. Using a sterile inoculating needle, a pinch of each fungal isolate was transferred aseptically from a 3day old stock culture in slant bottles to the agar blocks on the slides. After inoculation, clean cover slips were placed aseptically on the fungi inoculated agar blocks. The slide cultures were then incubated at room temperature for 3days. After incubation period, the slides were examined under the microscope using x10 and x40 objectives and compared with an atlas by Larone (2002).

3.3 Determination of Physicochemical Properties of the Raw Dairy Effluents

The physicochemical parameters that were analyzed included: pH, Temperature, Electrical Conductivity (EC), Total Dissolved Solids (TDS), Dissolved Oxygen (DO), Biochemical Oxygen Demand (BOD), Nitrate, Phosphate, and Sulphate, by using the Standard methods as described by APHA (1995). Analyses were carried out in triplicates.

3.3.1 Determination of pH, temperature, electrical conductivity and total dissolved solids

The pH, temperature, electrical conductivity and total dissolved solids of the effluent were determined at the point of collection using the HANNA combo tester (H198130, Denver, USA), a water proof tester that offers high accuracy of pH, EC, TDS and temperature in a single test. Following the manufacturer's instructions, the electrodes connected to each meter was submerged in a clean container containing the sample. The values displayed for each parameter by the tester were recorded.

3.3.2 Dissolved oxygen and biological oxygen demand

Dissolved Oxygen (DO) and Biochemical Oxygen Demand (BOD) were determined using HANNA instrument (HANNA 3200, Denver USA). Dissolved Oxygen (DO) was determined by inserting the instrument into the sample at the point of collection while the BOD was carried out by transferring the sample into a BOD bottle and incubated for five days at 25^0C in the dark. After the incubation period, the instrument was inserted into the incubated sample and the level of residual oxygen was recorded. The value of residual DO was then subtracted from the initial DO value to obtain the BOD_5 of the samples (Radojavic and Bashkin, 1999)

3.3.3 Nitrate determination

The sample (5mL) was transferred into a distillation tube and 2g of Devada alloy was added to the tube, this was followed by the addition of 2g Magnesium oxide. To a conical flask, 3 drops of indicator was added. Ten millilitres of 2% Boric acid was added to the conical flask to make a total volume of 10mL with indicator. The tube containing the sample mixture was fixed into the distillation chamber of the distiller and the conical flask containing the indicator was fixed at the receiving point. The mixture was distilled and the distillate was collected in the boric acid-indicator mixture until it turned green. The solution in the receiving flask was titrated with 0.01M hydrochloric acid. The percentage nitrate was calculated using the formula below:

$$\text{Nitrate (\%)} = \frac{0.014 \text{ X } 0.1 \text{ X } 100}{V}(T-B)$$

Where;

0.014 = Molecular mass of Nitrogen, 0.1 = Molar Conc. of HCl, V = Volume of sample used, T = Titre value, B = Blank (APHA, 1995).

3.3.4 Sulphate determination

Fifty millilitres (50mL) of the sample was dispensed into a clean glass beaker and 30mL of nitric hippochloric acid in a ratio of 2:1 was added to the sample as a clarifying agent. The mixture was placed on a digestion chamber for 1h. After digestion, the digested sample was transferred into a 50mL volumetric flask. The mixture was allowed to stand for 30mins. Using a pipette, 10ml of the sample was transferred into a 25mL volumetric flask and 1ml gelatin Barium chloride reagent was added. The sample was diluted to 25mL mark, swirled and allowed to stand for 30min for color development. The sample was read at 420nm using a spectrophotometer. Sulfate was determined using the formula below:

$$\text{Sulfate (mg/L)} = \frac{Df \times Dv \times R}{V}$$

Where,

Df = Dilution factor, Dv = Digestion volume, R = Absorbance Reading (at 420nm), V=Volume of sample used (APHA, 1995).

3.3.5 Phosphates determination

Preliminary sample treatment

One drop of phenolphthalein indicator (0.05ml) was added to 100ml of the sample and concentrated hydrochloric acid solution was added drop wise until the colour turns pink.

Color development

One hundred millilitres (100mL) of treated sample was dispensed into a flask and 4.0ml of molybdate reagent was added and mixed thoroughly. One millilitre (1mL) of stannous chloride reagent was added drop-wisely. Distilled water was used to prepare a blank in the same way. After

10minutes, the absorbance of the colour developed was measured at 690nm using a spectrophotometer. The phosphate content of the samples was read off the standard curve and the concentration was then calculated using the formula:

$$\text{Phosphate (mg/L)} = \frac{\text{mg } PO_4 \text{ x Absorbance at 690nm x 1000}}{\text{Volume of sample analyzed}}$$

3.4 Proximate Composition of the Sterilized Dairy Effluent

The raw dairy effluent was filtered using a muslin cloth. Sterilization of the filtered dairy effluent was carried out by autoclaving at 121^0C for 15min. The proximate composition of the sterilized dairy effluent determined includes moisture content, ash content, crude fat, crude protein and carbohydrate content as described by Association of Official Analytical Chemists (AOAC) (2010). Analyses were carried out in triplicates.

3.4.1 Determination of Moisture Content

Crucible was washed and dried to a constant weight in an oven at 100°C. It was later removed, cooled in a desiccator and weighed (**W1**). Five milliliters (5mL) of the sample was placed in the weighed crucible and weighed (**W2**). The crucible containing the sample was kept in an oven for 3hrs, the sample was then removed and cooled in the desiccator and weighed (**W3**) (AOAC, 2010). The crucibles containing the sample was dried in an oven for 24hrs, cooled in desiccators and weighed to a constant weight.

The % of the moisture content was calculated as: $\frac{\mathbf{W2 - W3}}{\mathbf{W2 - W1}} \times 100$

3.4.2 Determination of Ash Content

Crucible was cleansed and dried in an oven, after which it was cooled in a desiccator and weighed (**W1**). Five milliliters (5mL) of the effluent sample was placed in the crucible and weighed (**W2**). It

was then transferred into a muffle furnace (SXL Model) set at 550^{o}C for 2hrs. Thereafter, the crucible was removed, cooled in a desiccator and weighed (**W3**) (AOAC, 2010).

The % Ash of the sample was calculated as: $\frac{\mathbf{W3 - W1}}{\mathbf{W2 - W1}} \times 100$

3.4.3 Determination of crude fat (CF)

The crude fat content of the sample was estimated following the standard AOAC (2010) procedure. Eleven milliliters (11mL) of the sample was placed in labeled Gerber tubes. Ten milliliters (10mL) of 90% H_2SO_4 (Gerber Acid) was added to the tube and followed by the addition of 1ml n-amyl alcohol. The tube was properly corked. This was followed by vigorous shaking of the tubes. The tube was allowed to stand in a boiling water bath for 45minutes for proper fat separation. The separated fat was read from the graduated part of the Gerber tube which is in percentage.

3.4.4 Determination of crude protein (CP)

The crude protein was determined by Kjeldhal method. The nitrogen content in each sample was determined by using Kjeltech apparatus based on AOAC Method 46-10 (AOAC, 2010). The percentage protein was calculated by multiplying nitrogen with the conversion factor i.e. 6.25.

Digestion

Two milliliters (2mL) of the sample was placed into a Kjeldahl flask and a catalyst (Kjeltabs Cu/3.5 containing 3.5g of K_2SO_4 and 0.4g of $CuSO_4$. $5H_2O$) was added followed by 15ml of concentrated Sulphuric acid. The tube was placed in a digestion chamber and the fume cupboard closed. The content of the tube was heated until the solution assumes a green color and then cooled. Thereafter, each of the digest was transferred with several washings into 100ml with distilled water.

Distillation

The digest was first steamed through the Markham distillation apparatus for about 15mins. Under the condenser, a 100ml conical flask containing 10ml of 4% boric acid was placed and 2drops of the indicator added. About 10ml of 40% NaOH was added to 10ml of the digest after which the NaOH-sample mixture was distilled and the distillate was collected in the boric acid-indicator mixture until it turned green. This was then titrated with 0.1N hydrochloric acid.

Titration

The solution in the receiving flask was titrated with 0.01N hydrochloric acid. The Nitrogen content and hence the protein content of the sample was calculated.

The % protein content was calculated as:

$$\frac{\text{Final reading} - \text{Initial reading} \times \text{standard number of nitrogen (1.4)}}{\text{Initial weight} \times \text{Standard number of protein (6.38)}}$$

3.4.5 Determination of carbohydrate (CHO)

Carbohydrate content was obtained by subtracting all other fractions of the proximate analyses from 100% i.e.

CHO = 100-(%Moisture + %Ash + %Protein +%Fat)

3.5 Screening of Fungal Isolates for Production of Beta-galactosidase.

Ortho-Nitrophenyl-β-galactopyranoside (ONPG) disc method was used as described by Gopalakrishnan *et al.* (2014) with some modifications. The main modification was the use of lactose broth as opposed to lactose agar used for production by Gopalakrishnan *et al.* (2014). Lactose broth (containing g/L: Lactose - 20.0, $NaNO_3$ - 2.0, K_2HPO_4 - 1.0, KCl - 0.5, $MgSO_4$. $7H_2O$ - 0.5, $FeSO_4.5H_2O$ - 0.01) pH 6.8 was prepared. Thirty milliliters (30mL) was dispensed aseptically into sterile 100mL conical flasks. The fungal isolates were inoculated aseptically into the medium using a

sterile wire loop. The medium was incubated at ambient temperature for 7days. After the incubation period, the broth was filtered using a double layered muslin cloth. The filtrate was then centrifuged at 4000rpm for 30min. The supernatant was separated from the pellet using a Pasteur pipette and transferred into sterile clean test tubes. One ONPG (Sigma-Aldrich) disc each was inserted aseptically into another series of sterile clean test tubes using a sterile inoculating needle and 0.5mL of the enzyme solution from the fermentation medium was transferred into the test tubes using Pasteur pipette. This was carried out in duplicates. A set of test tubes were incubated at room temperature while another set was incubated at 35°C for 24hrs. The test tubes were observed at an interval of 1hr for the first six hours during the incubation period. A change in the color of the solution from colourless to yellow indicates presence of the enzyme.

3.6 Production of Beta-galactosidase under Submerged Fermentation using Dairy Effluent as Substrate.

3.6.1 Inoculum preparation

The selected isolate was subcultured on SDA slants and incubated at room temperature for 5days. Using a sterile syringe, 5.0mL of 2.5% sterile Tween 80 was aseptically dispensed into the SDA slants containing the isolates. The spores were dislodged by shaking and 1mL of the spore suspension was diluted in 99ml of sterile distilled water to give an inoculum size of 3.0×10^6 spores per mL, this was determined by counting in Neubauer chamber (Adhikari and Shrestha, 1989). This was used as inoculum for the fermentation experiment (Seyis and Aksoz, 2004; Nizamuddin *et al*, 2008; Antoine *et al.*, 2015).

3.6.2 Production of beta-galactosidase

Sterile dairy effluent was used as production medium. Using a sterile syringe, 2.5mL of the inoculum was aseptically inoculated into 500mL Erlenmeyer flask containing 250mL of the sterile production medium i.e. sterile dairy effluent. The broth was incubated at room temperature for 7days (Akinola *et al.*, 2012). After incubation period, the broth was filtered through a double layered muslin cloth and the filtrate was centrifuged at 10000 rpm for 10minutes at 4^{o}C. The supernatant was separated from the pellet using a clean Pasteur pipette into clean test tubes. The solution was then tested for beta-galactosidase activity (Murugan, 2013).

3.6.3 Determination of enzyme activity

A preliminary beta-galactosidase activity was tested on the enzyme solution using ONPG disc (Sigma-Aldrich) as described earlier. Enzyme activity was then assayed using ortho-Nitrophenyl-β-galactopyranoside (ONPG) (Sigma-Aldrich) as substrate (Park *et al.* 1951; Nevalainen, 1981, Mowery and Seidman, 2005; Nizamuddin *et al.*, 2008; Althaf *et al.*, 2012; Amal *et al.*, 2012; Murugan, 2013; Princely *et al.*, 2013; Ashish *et al.*, 2014; Gopalakrishnan *et al.*, 2014; Antoine *et al.*, 2015; Panesar *et al.*, 2016).

Using a sterile syringe, 0.2ml, 0.4ml, 0.6ml, 0.8ml and 1 ml of sterile distilled water was added to a series of 5 test tubes and 0.1 ml of the enzyme solution was added to the tubes except for the test tube containing 1.0 ml distilled water which served as blank. The volume was made up to 1 ml in all the other test tubes. Diluted aliquots of the enzyme solution was incubated with 1.0ml of 2.0mM ONPG (Sigma-Aldrich) solution in 100mM phosphate buffer at pH 7.0 and a temperature of 35°C for 10 min. After the incubation period, the reaction was stopped by the addition of 3.0 ml of 0.1M NaOH. The O-nitrophenol (ONP) liberated was estimated at 420 nm with a spectrophotometer and the activity of the enzyme was calculated using the formula

$$\text{Beta-galactosidase activity (U/ml)} = \frac{A \times Trv}{0.380 \times Ev \times T}$$

Where; A= Absorbance at 420nm; Trv = Total reaction volume; 0.380 = Constant value, a function of the molar extinction coefficient of O-nitrophenol; Ev = Enzyme volume; T = Incubation time.

One unit of beta-galactosidase activity is defined as the amount of the enzyme required to liberate 1µmole of O-nitrophenol (ONP) per minute under assay conditions (Mowery and Seidman, 2005; Cruz *et al.*, 1999; Seyis and Aksoz, 2004; Nizamuddin *et al.*, 2008; Althaf *et al.*, 2012; Murugan, 2013; Antoine *et al.*, 2015; Panesar *et al.*, 2016).

3.7 Statistical analysis

The data obtained were expressed as Mean ± SD for duplicate and triplicate results.

CHAPTER FOUR

4.0 RESULTS

4.1 Isolation and Characterization of Fungal Isolates from Dairy Effluents

A total of nine (9) fungi were isolated from the raw dairy effluent. All fungal isolates were obtained in pure cultures. The results of the macroscopic (cultural) and microscopic examination confirmed 5 isolates to be *Aspergillus flavus*. Other isolates were confirmed to be *Aspergillus niger*, *Rhizopus* spp., *Rhizomucor* spp., and *Penicillium* spp. Table 4.1 (Appendix I - V) shows the summary of macroscopic and microscopic characteristics of the fungal isolates.

4.2 Distribution of fungi isolated from raw dairy effluent

Table 4.2 shows the occurrence of the fungal isolates from the sample analysed. The most prevalent genus among all the isolates was *Aspergillus* species. *A. flavus* had the highest occurrence (5). Others include *Aspergillus niger* (1), *Rhizopus* spp. (1), *Rhizomucor* spp. (1) and *Penicillium* spp. (1). Hence, the percentage occurrence of the fungal isolates from the analyzed samples was calculated as *Aspergillus flavus* (56%), *Aspergillus niger* (11%), *Rhizopus* spp. (11%), *Rhizomucor* spp. (11%) and *Penicillium* spp. (11%).

4.3 Physicochemical Analyses of Raw Dairy Effluent

Table 4.3 shows the physicochemical characteristics of the dairy effluent. The temperature was found to be 26.2^{o}C while the pH was found to be 6.8. The electrical conductivity of the effluent was recorded as 645µs/cm while the total dissolved solid was found to be 324mg/L. The dissolved oxygen and biological oxygen demand were found to be 200mg/L and 100mg/L respectively while the nitrates, phosphates and sulfates were found to be 0.0014%, 54.94mg/L and 13.66mg/L respectively.

Table 4.1: Macroscopic and Microscopic Characterization of Fungal Isolates

S/No.	Macroscopic examination	Microscopic examination	Inference
1	Light green color with white boundary, powdery, irregular colony shape, reverse milky brown in color.	Rough conidiophores, spiny, phialides covers entire vesicle and points out in all directions.	*Aspergillus flavus* (Asf1)
2	Light green to gray in color with white boundary, wooly, circular colonies, reverse is milk to brown in color.	Rough conidiophores, pitted, phialides point out in all directions and covers entire vesicle.	*Aspergillus flavus* (Asf2)
3	Green to gray in color with white boundary, granular and slightly wooly, irregular colony shape, reverse is milky.	Slightly rough conidiophores, spiny, phialides covers entire vesicle and point out in all direction.	*Aspergillus flavus* (Asf3)
4	Dark green to gray in color, white boundary, wooly, colony shape is irregular, reverse is milky brown.	Rough conidiophore, phialides cover entire vesicle and point out in all directions.	*Aspergillus flavus* (Asf4)
5	Black in color, yellow to white boundary, powdery, slightly wooly, round to irregular colony shape, reverse milky brown.	Long smooth conidiophores, phialides form radiate head and covers entire vesicle.	*Aspergillus niger* (Asn1)
6	Brown in color, cotton-like, dense growth that covers entire agar surface quickly, reverse is brown.	Broad hyphae, no septa, long unbranched connected sporangiophores, dark round sporangium containing oval spores.	*Rhizopus* spp. (Rsp1)
7	Light green to yellow in colour, powdery with white wooly boundary, reverse is white to brown.	Rough conidiophore, phialides cover entire vesicle and point out in all directions.	*Aspergillus flavus* (Asf5)
8	Velvety, slightly powdery, very fluffy, whitish black, brown to chocolate in colour, boundary is white, circular to irregular colony shape, reverse is milk.	Smooth branched rhizoids, branched sporangiophores, round sporangia, rhizoids at points on the stolon between the sporangiophores.	*Rhizomucor* spp. (Rzm1)
9	Dark bluish green in colour, white boundary, and colony is circular in shape, reverse is reddish brown.	Septate hyphae, branched and unbranched conidiophores with secondary branches (metulae). Flask-shape phialides are arranged o the metulae in whorls. Phialides bear unbranched chains of round conidia. Entire structure forms a brush appearance.	*Penicillium* spp. (Pen1)

Table 4.2: Distribution of Fungi Isolated from the Dairy Effluent

Isolate	Frequency	Occurrence (%)
Aspergillus flavus	5	56
Aspergillus niger	1	11
Rhizopus spp.	1	11
Rhizomucor spp.	1	11
Penicillium spp.	1	11

Table 4.3: Physicochemical Analyses of the Dairy Effluent

Parameter	Mean Values (±SD)	FEPA (1991) Limit	USEPA (1999) Limit	SON (2007) Limit
Temperature (^{o}C)	26.2 (±0.10)	NS	NS	NS
pH	6.8 (±0.20)	6-9	6.5-8.5	6.5-8.5
Electrical Conductivity (µs/cm)	645(±1.00)	NS	NS	NS
Total Dissolved Solids (mg/L)	324 (±2.00)	2000	500	500
Dissolved Oxygen (mg/L)	200 (±1.00)	NS	NS	NS
BOD (mg/L)	100 (±1.70)	50	NS	NS
Nitrates (%)	$4.0x10^{-3}$ ($\pm 1.0x10^{-4}$)	20	10	50
Phosphates (mg/L)	54.94 (±0.79)	5	NS	NS
Sulfates (mg/L)	13.66 (±0.61)	500	250	100

BOD: Biological Oxygen Demand, NS: Not Specified, FEPA: Federal Environmental Protection Agency, USEPA: United States Environmental Protection Agency, SON: Standard Organisation of Nigeria

4.4 Proximate Analysis of the Dairy Effluent

Result of the proximate composition of the treated dairy effluent revealed the moisture content to be 80%, while the ash content was found to be 0.15%. The lipid content was found to be 5.85% while the protein and carbohydrate were found to be 0.875% and13.125% respectively. The result is summarized in Table 4.4.

4.5 Screening of Fungal Isolates for Production of Extracellular Beta-Galactosidase using ONPG Discs

The nine pure fungi isolated from the raw dairy effluent were further screened for beta-galactosidase activity using ONPG discs method. From the colour change observed in the test tubes as shown in Plate I, beta-galactosidase activity i.e. intense yellow coloration which represents the product release was observed in only isolates number 6 i.e. *Rhizopus* species (Rsp.1). This isolate was selected for further studies. The result is summarized in table 4.5

4.6 Production of Extracellular Beta-Galactosidase by *Rhizopus* spp. under Submerged Fermentation using Dairy Effluent as Substrate.

The selected fungal isolate (*Rhizopus* spp.) was grown in sterile dairy effluent used as fermentation medium. The preliminary activity carried out on the fermentation product using ONPG discs showed a change in the color of the solution which is an indication of presence of the enzyme. Furthermore, determination of the enzyme activity using ONPG as substrate revealed that the organism was able to utilize the effluent to produce the enzyme. Beta-galactosidase produced by *Rhizopus* spp. was found to have an activity of 4.65UmL^{-1}.

Table 4.4 : Proximate Composition of the Dairy Effluent

Parameter (%)	Percentage (±SD)
Moisture	80.00 (±0.03)
Ash	0.15 (±0.01)
Crude Fat	5.85 (±0.02)
Crude Protein	0.88 (±0.01)
Carbohydrate	13.13 (±0.00)

Table 4.5: Screening of the Fungal Isolates for Beta-galactosidase Production Potential

Fungal Isolate	Beta-galactosidase activity (ONPG Discs)	Beta-galactosidase activity (U/mL) (±SD)
Aspergillus flavus (Asf1)	-	0.00
Aspergillus flavus (Asf2)	-	0.00
Aspergillus flavus (Asf3)	-	0.00
Aspergillus flavus (Asf4)	-	0.00
Aspergillus flavus (Asf5)	-	0.00
Aspergillus niger (Asn1)	-	0.00
Rhizopus spp. (Rsp1)	+	4.65 (±0.02)
Rhizomucor spp.(Rzm1)	-	0.00
Penicillium spp.(Pen1)	-	0.00

CHAPTER FIVE

5.0 DISCUSSION

5.1 Isolation and Characterization of Fungal Isolates from the Dairy Effluent

A total of nine fungal strains were isolated from the raw dairy effluent using direct plating technique of diluted and undiluted samples of the raw dairy effluent. From the sample analyzed, the most prevalent genus among all the isolates was *Aspergillu*s spp. The percentage occurrence of the fungi isolates from the analyzed samples were *Aspergillus flavus* (56%), *Aspergillus niger* (11%), *Rhizopus* spp. (11%) *Rhizomucor* spp. (11%) and *Penicillium* spp. (11%). *A. flavus* had the highest occurrence (56%). Its dominance could be due to the fact that the spores of *Aspergillus* spp. are present in abundance in the atmosphere. As such, this group of fungi is known to be a major contaminant of food and other products. It is important to note that *Aspergillus* spp. includes diverse lineage producing toxins in cereals and other food products. Other fungal isolates encountered could be present in the sample as transient organisms. This result agrees with the work of Onyeze *et al.* (2012), Rohilla and Salar (2012), Vishwanataha *et al.* (2012), Gopalakrishnan *et al.* (2014), Oviasogie and Oviasogie (2014) and Nwadiaro *et al.* (2015) who in their separate researches, isolated similar organisms from various sources such as contaminated soil, garden soil, food and agro-industrial wastes.

This finding disagrees with the work of Hedayati and Mirzakhani (2009) in Iran where 326 fungal colonies from wastewater treatment plants. The fungal species isolated in their study included the genus *Geotrichum* (59.5%), *Cladosporium* (13.8%), *Alternaria* (11.3%) and *Penicillium* (10.7%). *Geotrichum* species were the most prevalent with 59.5% occurrence. The

difference could be attributed to nature of sample, sample location and technique used, or method of isolation used.

5.2 Physicochemical Analyses of the Dairy Effluent

The raw dairy effluent was characterized by an offensive odour and a milky black colour which may be due to biological decomposition of organic matter or presence of various aromatic and volatile organic compounds such as carbondioxide and methane in the dairy effluent. The mean temperature of the raw dairy effluent was 26.2^{o}C. This is within the mesophilic temperature range. The temperature of the raw dairy effluent could be due to the weather and climatic condition during which the research was undertaken i.e. rainy season. It could also be due to continuous introduction of wastewater from the dairy industry.

A consequence of this temperature value is existence of a wide range of microorganisms especially mesophilic bacteria, yeast and moulds. Also, most biological activities occur when the water temperature is between 10-30° C (Saxena *et al.*, 2014). This agrees with the work of Saxena *et al.* (2014) who observed a temperature of 18^{O}C in India while working with dairy wastewaters. The findings in this study disagrees with the works of Adeoye *et al.* (2009), Sreemoyee and Priti (2013) and Shivsharan *et al.* (2013) who recorded 36^{O}C in Minna (Nigeria), 33^{O}C in Jaipur (India), and 34^{O}C in Pune (India) respectively. These variations in temperatures could be due to differences in geographical location.

The mean pH of the raw effluent was found to be 6.8 which indicate a near neutral pH and within the recommended limits set by FEPA (1991), USEPA (1999) and SON (2007). This result could also be due to continuous supply of wastewater from the dairy industry which neutralizes any change in the pH due to biological activities. A consequence of this pH value may be the

presence and abundance of a wide range of microorganisms. This wastewater could be employed for the irrigation of soils. Adeoye *et al.* (2009), Segun and Kehinde (2010) reported in their separate researches, similar pH values of dairy wastewater to be 6.4 in Minna (Nigeria) and 6.5 in Ibadan (Nigeria) respectively. However, the findings revealed in this research disagrees with the works of Olorunfemi *et al.* (2012), Sreemoyee and Priti (2013) and Shivsharan *et al.* (2013) who recorded pH values of 4.7 in Ibadan (Nigeria), 8.5 in Jaipur (India), and 9.9 in Pune (India) respectively. The variations could be attributed to the difference in the activities of the dairy industries.

The mean electrical conductivity of the raw dairy effluent was 645µs/cm. This could be due to a high concentration of ions in the effluent such as chlorides, sulphates, carbonates, nitrites, calcium, magnesium, sodium and potassium ions. Sources of some of these ions could be from the metallic wash basins and other utensils used in the industrial processes. It could also be from the detergents used for washing. These important ions may impart conductivity in water and thus, impart hardness to water. The finding of this work is in agreement with the works of Adeoye *et al.* (2009) and Porwal *et al.* (2014) who reported an electrical conductivity of 460µs/cm and 436µs/cm respectively. However, the EC revealed in this study disagrees with the findings of Uaboi-Egbenni *et al.* (2009) and Segun and Kehinde (2010) who recorded an EC of 1961µs/cm and 14µs/cm respectively. This disagreement could be due to difference in geographical location, time or season and methodology used in the research.

The Total Dissolved Solids (TDS) of the raw dairy effluent was 324mg/L which is within the recommended limits set by FEPA (1991), USEPA (1999) and SON (2007). The presence of this level of dissolved solids could be due to organic and inorganic matter present in the effluent. A large number of solids can be found dissolved in natural and artificial water bodies, the common

ones include carbonate, bicarbonates, chlorides, sulfates, phosphates and nitrates of calcium, magnesium, sodium, potassium, iron, magnesium etc. (Porwal *et al.*, 2014). A high content of TDS reduces the utility of water for drinking, irrigation and industrial purposes. This agrees with the works of Sreemoyee and Priti (2013) and Segun and Kehinde (2010) who recorded TDS values of 132 mg/L and 300mg/L respectively which are within the recommended limits set by FEPA (1991), USEPA (1999) and SON (2007). The findings revealed in this study disagrees with the findings of Adeoye *et al.* (2009), Uaboi-Egbenni *et al.* (2009), Segun and Kehinde (2010), and Porwal *et al.* (2014) who recorded TDS values of 2400mg/L, 977mg/L, 1600mg/L, and 1715mg/L respectively which exceeds the recommended limits set by FEPA (1991), USEPA (1999) and SON (2007). This disagreement could be due to difference in the activities or the dairy industries.

The dissolved oxygen (DO) of the raw dairy effluent was 200mg/L which is significantly high, hence may allow presence and survival of microorganisms and other possible life forms. This may be due to continuous supply of wash-water from the industry. Without free dissolved oxygen, there will be no survival of aquatic life forms in any water body. Water bodies simply become inhabitable to most aquatic life without free dissolved oxygen (Porwal *et al.*, 2014). At normal temperature water is said to be saturated with oxygen at 9mg/L. This saturation value decreases rapidly with increasing water temperature (Adeoye *et al.*, 2009). This finding agrees with the work of Adeoye *et al.* (2009) who also reported a high DO value of 880mg/L. This result disagrees with the work of Segun and Kehinde (2010), Shivsharan *et al.* (2013), Olorunfemi *et al.* (2012), Saxena *et al.* (2014) who reported significantly low DO values of 17mg/L, 1.2mg/L, 10mg/L and 2.3mg/L respectively.

The result of this study revealed the biochemical oxygen demand (BOD) to be 100 mg/L. This value exceeds the recommended limit set by FEPA (1991). This is a reflection of a high organic load and pollution potential. The presence of organic matter can promote anaerobic processes leading to the accumulation of toxic compounds in water bodies. This result agrees with the work of Noorjahan *et al.* (2004) and Segun and Kehinde (2010) who reported a range of similar BOD values of 100-200mg/L and 75-95mg/L respectively. This finding disagrees with the works reported by Olorunfemi *et al.* (2012), Vishakha *et al.* (2013) and Porwal *et al.* (2014) where BOD values (range) of 7.2mg/L, 500-800 mg/L, and 1268mg/L respectively were reported.

A negligible value of 0.0014% was found for concentration of nitrate in the raw dairy effluent and this is within the acceptable limits set by FEPA (1991), USEPA (1999) and SON (2007). This may be due to absence of organic contaminants in the raw dairy effluent. This finding agrees with the work of Oviasogie and Oviasogie (2014) and Nwadiaro *et al.* (2015) who reported concentrations of nitrates as 0.053% in Benin and 0.013% in Jos respectively. This finding disagrees with the works of Noorjahan *et al.* (2004), Olorunfemi *et al.* (2012) and Saxena *et al.* (2014) who reported much higher values (range) for concentration of nitrates as 3-52mg/L, 85mg/L and 24mg/L respectively. This could be attributed to the difference in environment and activities of the dairy industries.

The concentrations of phosphates and sulfates were found to be 54.94mg/L and 13.66mg/L respectively. Phosphate content of the dairy effluent exceeds the limit set by FEPA (1991) while concentration of sulfate is within the limits set by FEPA (1991), USEPA (1999) and SON (2007). The high values of phosphates and sulfates encountered in this study could be as a result of the detergents used to wash the utensils in every stage of the production process. The presence of these ions may impart hardness to water and make it unsuitable for some industrial purposes

(Porwal *et al.*, 2014). The phosphates and sulphates concentration revealed in this study agrees with the findings of Adeoye *et al.* (2009) who reported similar values for concentration of phosphates and sulfates as 37.6mg/L and 11.6mg/L respectively. However, the findings of this study disagrees with the separate researches of Noorjahan *et al.* (2004), Olorunfemi *et al.* (2012) and Saxena *et al.* (2014) who reported values of phosphates and sulfates as 23mg/L and 114mg/L, 98.5mg/L and 96mg/L, and 3.4 and 240mg/L respectively. Vishakha *et al.* (2013) and Porwal *et al.* (2014) also reported higher values for concentration of sulfates as 245mg/L and 84mg/L respectively. This disagreement could be due to difference in the type and concentrations of detergents used for washing utensils in the different dairy industries.

5.3 Proximate Composition of the Dairy Effluent

The result of the proximate composition reveals the moisture content of the dairy effluent to be 80.00%. This could be attributed to continuous supply of wash water to the pond. The ash content was found to be 0.150%, which is an indication that the mineral content of the effluent is low. This could be attributed to the fact that less than 2% of total milk processed is wasted into drains (Munavalli and Saler, 2009). Hence, the mineral content from the 2% lost will be significantly low. The crude fat and crude protein was recorded as 5.850% and 0.875% respectively. The low fat content may be due to lipase enzymes respectively present in detergents used to wash residual milk or milk products from the basins and other utensils while the low protein content may be attributed to utilization of protein and amino acids by indigenous microorganisms present in the dairy effluent. Carbohydrate was found to be 13.125%. This could be due to accumulation of residual milk from the wastewater introduced into the drains. The major carbohydrate found in milk is lactose (Mahoney, 2005). A consequence of the presence of lactose in this amount is that the sterile dairy effluent could be a potential source of substrate for

production of beta-galactosidase. This result agrees with studies carried out by Anvari and Khayati (2011) in Iran and Kaur *et al.* (2015) in India who in their separate studies reported lactose contents of 4.9% and 5.00%, protein contents of 0.9% and 0.48%, and fat contents of 0.05% and 0.18% respectively. The findings in the study disagrees with the work of Victor *et al.* (2014) who reported 425g/L carbohydrate content, 7% protein content and 3.79% ash. The findings in this study also disagrees with the work of Tikariha and Omprakash (2014) in India who reported a range of carbohydrate, protein and fat contents as 0.1007%- 0.2958%, 13.78%-72.12%, and 0.01%-0.06% respectively. This disagreement could be due to the difference in the nature of the sample analyzed or difference in methodology used.

5.4 Screening of Fungal Isolates for Extracellular Beta-Galactosidase Production

Rhizopus species was selected for beta-galactosidase production because it was the only fungal isolate that showed beta-galactosidase activity, i.e. intense yellow coloration, when screen using ONPG discs. This could be due to the presence of the gene responsible for beta-galactosidase in only *Rhizopus* species. The finding in this study is in agreement with the works of Vishwanataha *et al.* (2012) and Panesar *et al.* (2016) as *Rhizopus* spp. also showed highest activities when screened using thin-layer chromatography and 5-bromo-4-chloro-3-indole-β-D-galactopyranoside (X-gal) respectively. However, the findings of Murugan (2013) and Kaur *et al.* (2015) disagree with the findings of this research. Murugan (2013) screened three fungi using milk agar method and reported *A. niger* and *A. flavus* to have the highest activities. Kaur *et al.* (2015) reported *Aureobasidium pullulans* to have the highest activity when screened on whey medium for beta-galactosidase activity. This disagreement could be due to the variations in the screening method or absence of beta-galactosidase gene in the fungal isolates screened.

5.5 Production of Extracellular Beta-galactosidase by *Rhizopus* spp. under Submerged Fermentation using Dairy Effluent as Substrate

The beta-galactosidase activity of 4.65U/mL (±0.02) produced by *Rhizopus* spp. shows that the organism could utilize dairy effluent as substrate to produce beta-galactosidase. A consequence of this finding is that the sterile dairy effluent has a potential to be used as an alternative substrate for production of beta-galactosidase with possible need for supplementation. The beta-galactosidase activity revealed in this study agrees with the work of Panesar *et al.* (2016) who got similar range of beta-galactosidase activities (0.022-2.14IU/mL) produced by a variety of fungi isolated from various food and agro-industrial wastes using lactose broth as production medium. However, the findings of this research disagrees with the work of Vishwanataha *et al.* (2012) who found extracellular and intracellular beta-galactosidase activities of 2250IU and 2477IU respectively from *Rhizopous stolanifera* strain incubated with 2% lactose for 18hours. This disagreement could be due to the methodology, organism or substrate used for the enzyme production.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

A total of Nine (9) fungi were isolated from the raw dairy effluent which include *Aspergillus flavus*, *Aspergillus niger*, *Rhizopus* spp., *Rhizomucor* spp., and *Penicillium* spp. *Aspergillus flavus* was found to have the highest occurrence of 56%.

The dairy effluent was characterized by a near neutral pH (6.8), mesophilic temperature (26.2^0C), high values of Electrical Conductivity (645µs/cm), Dissolved Oxygen (200mg/L), Biological Oxygen Demand (100mg/L) and Phosphates (54.94mg/L). Values of Total Dissolved Solids (324mg/L), nitrates ($1.4x10^{-3}$) and sulfates (13.66mg/L) were found to be below the acceptable limits set by FEPA (1991), USEPA (1999) and SON (2007).

The dairy effluent had a high moisture content (80.00 ±0.03) considerably amount of carbohydrate (13.13% ±0.00), low ash (0.15% ±0.01), lipid (5.85% ± 0.02) and protein contents (0.88% ±0.01)

Rhizopus species was found to be the only fungal isolate with beta-galactosidase activity from the nine isolates screened and the beta-galactosidase produced had an activity of 4.65U/mL (±0.02) using the sterilized dairy effluent as substrate.

6.2 Recommendations

1. Fungi isolated from this study should be exploited for production of other compounds of industrial importance.

2. The dairy effluent should be utilized for beneficial purposes such as soil amendment, isolation of potential beneficial microorganisms, extraction of elements of industrial importance and use as substrate for beta-galactosidase production.
3. ONPG disc method should be employed for screening of different groups of microorganism with potential to produce beta-galactosidase.
4. Further studies on purification and characterization of this industrially important enzyme should be carried out.

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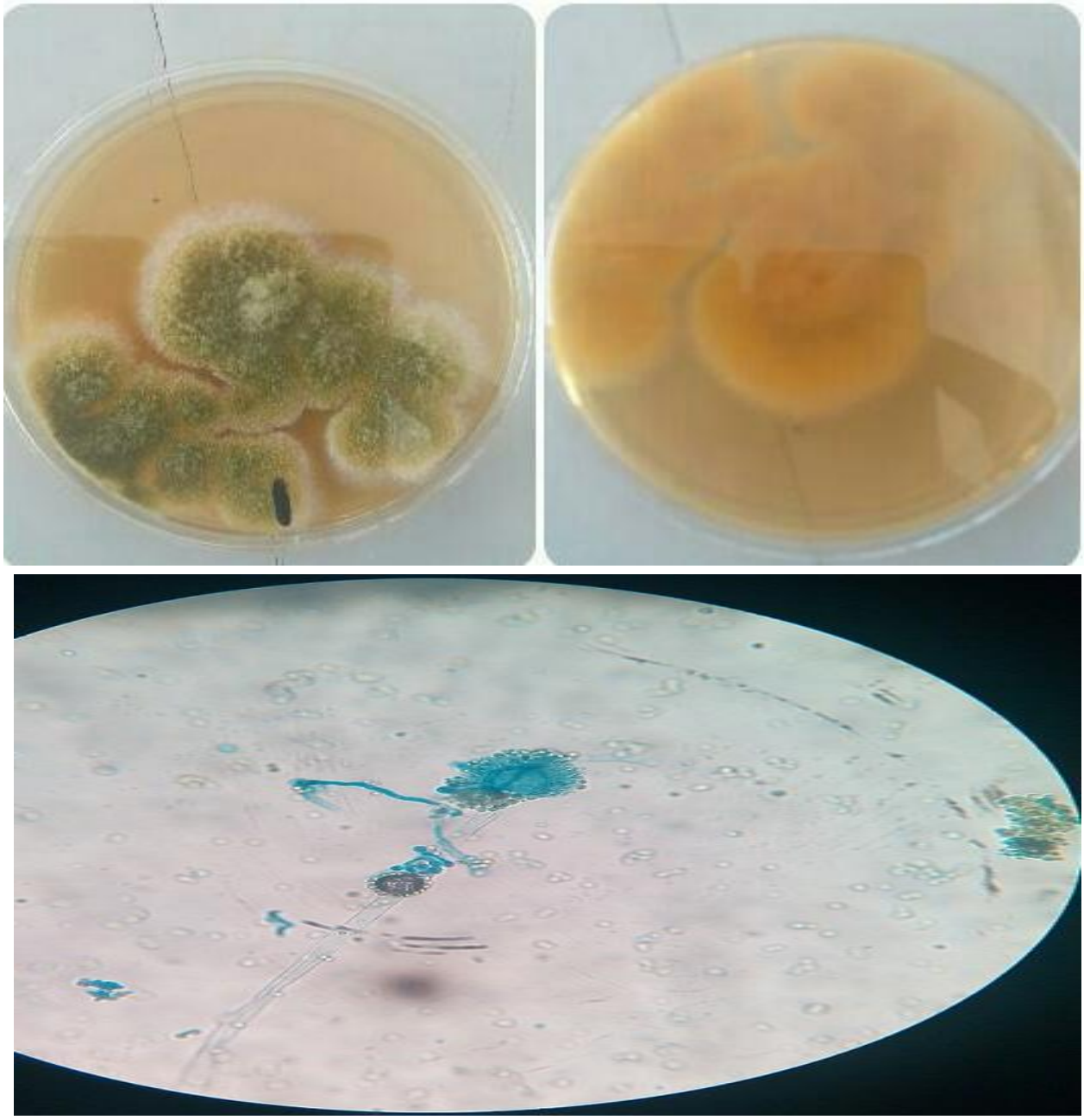
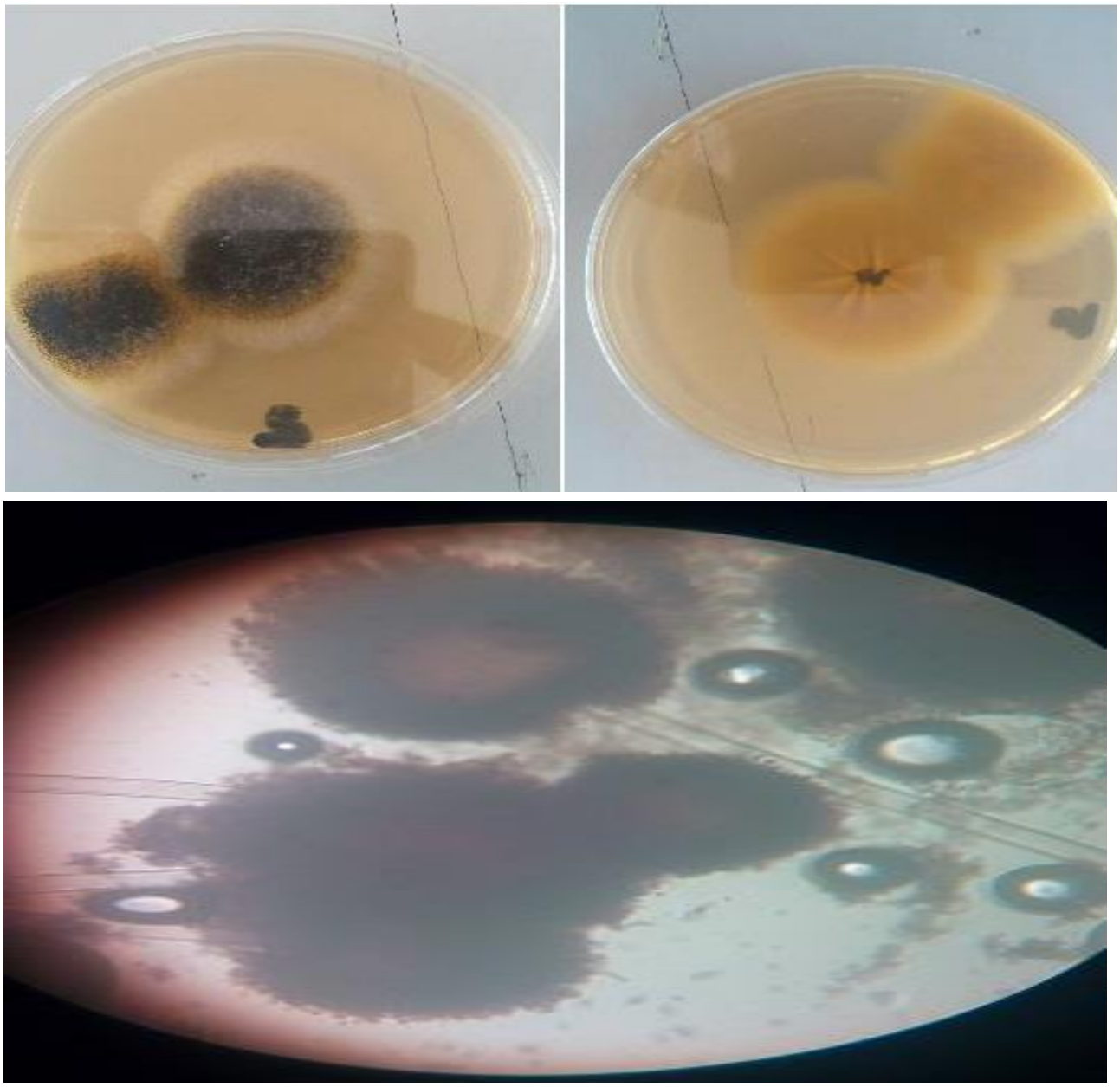
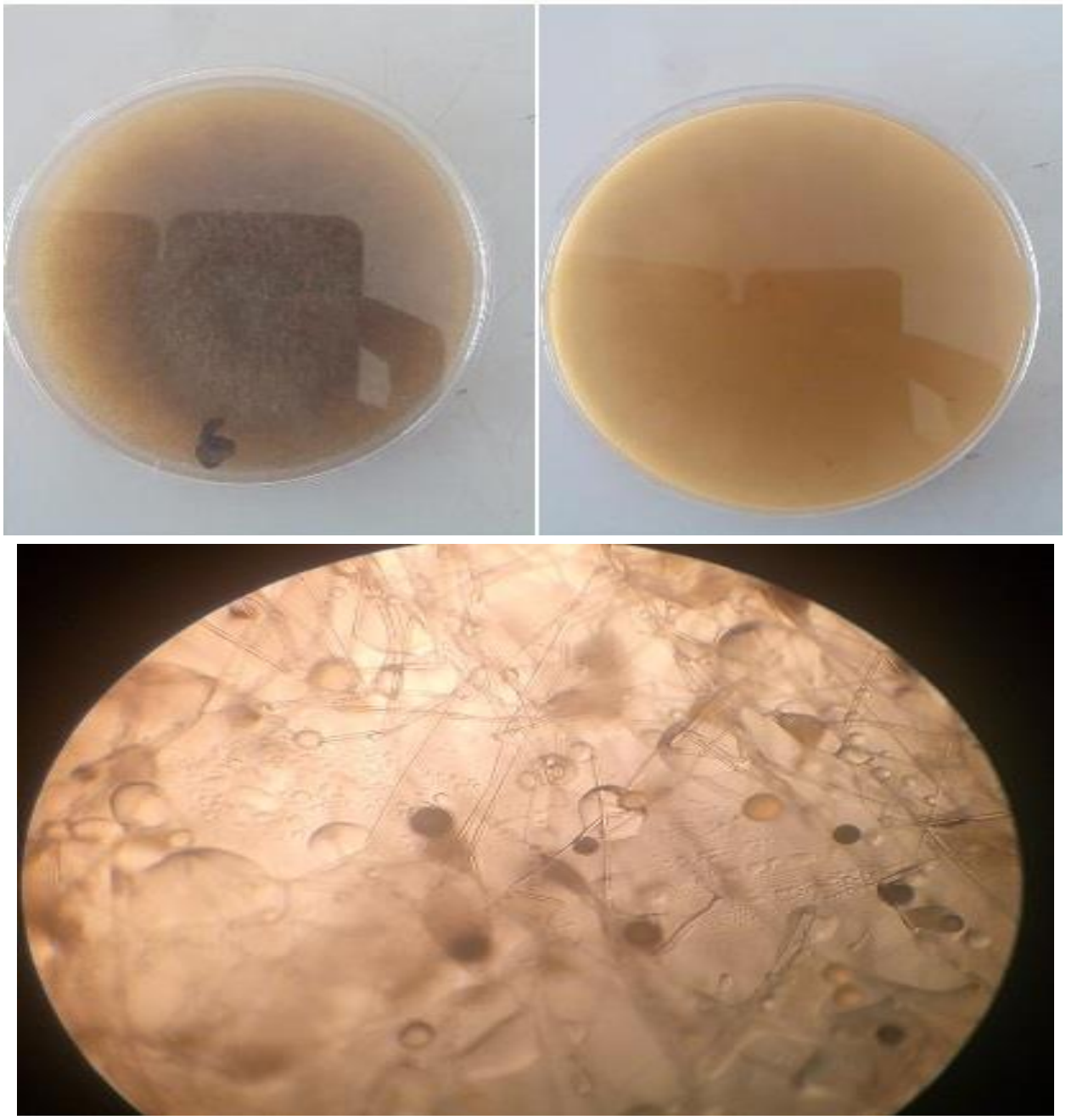
APPENDIX



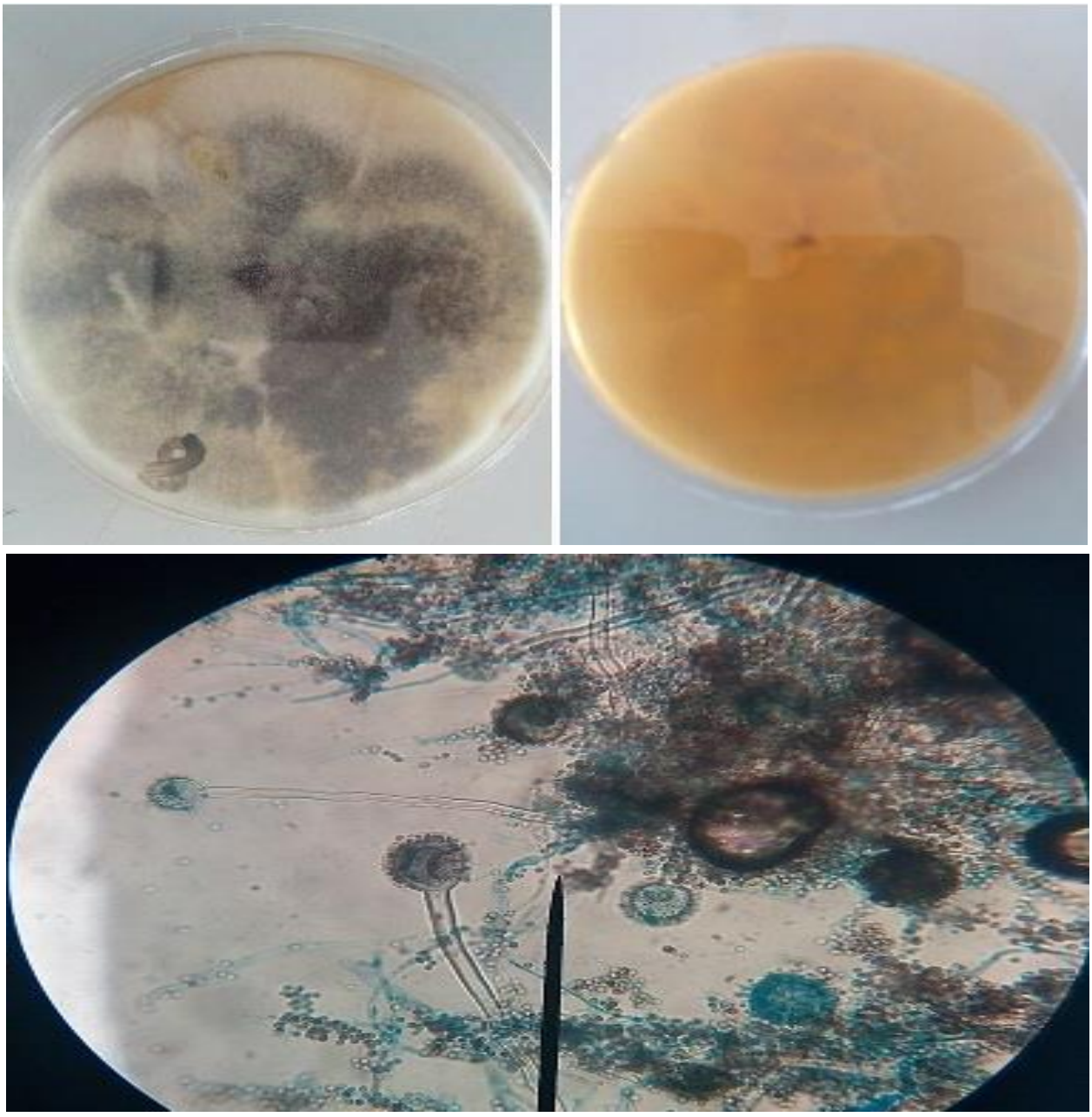
Appendix I: Cultural and Microscopic Characteristics of *Aspergillus flavus*



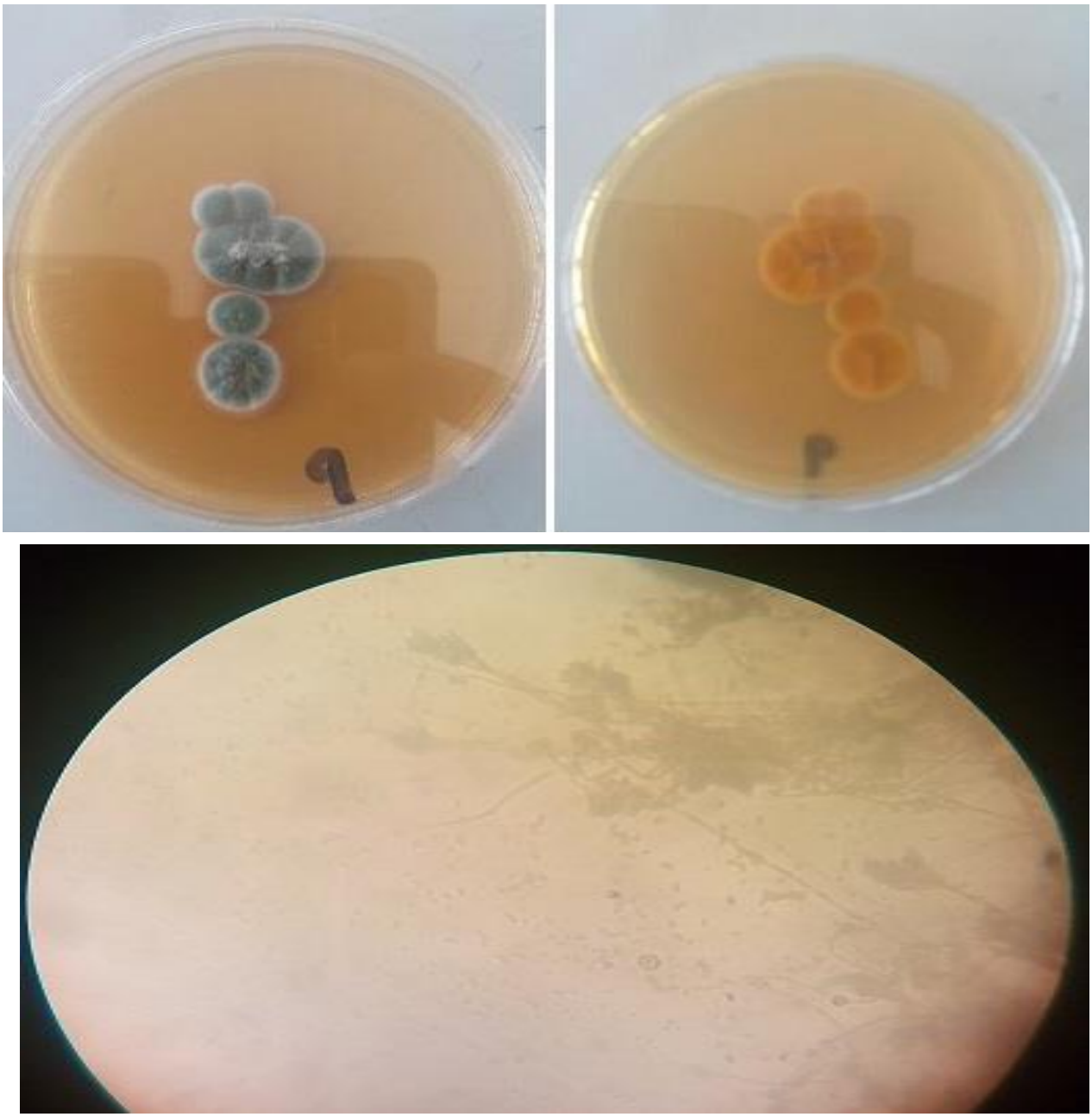
Appendix II: Cultural and Microscopic Characteristics of *Aspergillus niger*



Appendix III: Cultural and Microscopic Characteristics of *Rhizopus* spp.



Appendix IV: Cultural and Microscopic Characteristics of *Rhizomucor* spp



Appendix V: Cultural and Microscopic Characteristics of *Penicillium* spp.

Appendix VI: Fungal isolates screened for beta-galactosidase potential using ONPG discs

KEY: Yellow Color- Enzyme Activity; Colourless-No Enzyme Activity; Isolate 1, 2, 3, 4, 7-*Aspergillus flavus*; 5-*Aspergillus niger*; 6-*Rhizopus* species; 8-*Rhizomucor* species; 9-*Penicillium* species.