PRODUCTION OF CELLULASE USING THERMOPHILLIC YEAST

BY

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CERTIFICATION

This is to certify that this project was carried out by **VICTORY CHINEDU MEGBUZIE** in the Department of Microbiology, Faculty of Life Sciences, University of Benin under my supervision.

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This project work is accepted in partial fulfillment for the award of Bachelor of Science, B.Sc. (Hons.) in the Department of Microbiology, Faculty of Life Science, University of Benin, Benin City

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DEDICATION

This report is dedicated to God Almighty who in his infinite mercy has favoured me during the course of this project.

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ABSTRACT

Large quantities of byproducts generated during the processing of Agro-waste results in an economic and environmental problem due to their high volumes and elimination costs. Corncob waste, banana peel, orange peel and pineapple are undervalued waste materials, unused source of energy that can serve as potential source for cellulase production. This study was conducted to bioconvert agrowaste to cellulase using thermophilic yeast. The thermophillic organism of interest was isolated from hot region of a dumpsite in Benin City, plated using pour plate method and identified based on colonial and sugar fermentation characteristics. Purified isolates were screened for cellulase producing activity and the highest producer was used for further analyses. The standardized organism (*Torulopsis bovina*) was inoculated into each waste medium and incubated at 50^0C over the course of 10 days. pH, viable cell count and cellulase concentration was determined in two days interval. pH was determined using pH meter, viable cell count was determined using pour plate method, while the cellulase concentration was determined using DNS method. The highest cellulase concentration was obtained at day 8 with corncob waste medium; at a pH of 9.70±0.14 and cell count of $4.490\pm0.042 \times 10^{-4}$ cfu/ml. While the least was obtained with banana waste medium at day 8, with viable cell count of 0.730±0.028 cfu/ml and pH of 8.05±0.07. Findings from this study suggest corncob as the best substrate for cellulase production using *Torulopsis bovina.* Hence, the recalcitrance nature of agrowaste in the environment can be salvage through valorization, specifically into cellulase.

CHAPTER ONE

1.0 Introduction

Wastes such as agricultural waste, solid waste, industrial hazardous and non-hazardous waste, animal waste, medical waste, radioactive waste, construction and demolition debris, extraction and mining waste, oil and gas production waste are recalcitrant in the environment. The large quantities of byproducts generated during the processing of some of these wastes such as agro-waste results in an economic and environmental problem due to their high volumes and elimination costs (Verma *et al*., 2011).

Agro-waste is a safer and more nutritious kind of environmental waste utilizable for most industrial bioconversion. The bioconversion of agro-waste can be done by microorganisms. These microbial activity breakdown waste from solid to molten forms (leachate) (Mangesh *et al* 2013).

Microbes that act when wastes are in molten or leachate form are mostly high temperature tolerant microbes such as thermophiles (Singh *et al.,* 2019).

Cellulase is one of the most relevant enzyme produced from waste biodegradation. In order to meet the industrial demand for cellulase which is indirectly influenced by the population demand for industrial products, it is imperative to source for raw materials from which cellulase are produced (Shugaba *et al*., 2005).

Agro-waste can serve as a good substrate which can be utitlized by microorganisms for the production of cellulase. Corncob waste is one of the undervalued agro-waste materials; unused sources of energy that can serve as a potential source for cellulase production (Kumar *et al* 2022)

Batch Experiments have been performed, using agro-waste as a carbon source for cellulase production under solid state and submerged fermentation. The utilization of economically cheap, agro-waste for cellulase production could be an inexpensive, and valuable approach in cellulase production as well as in solid waste management (Marchettini *et* al., 2007).

Cellulose is a biomaterial derived from plants. Plants are the major contributor to the production of cellulose in the biosphere being synthesized through the process of photosynthesis. Thus it is the major constituent of plant biomass. Chemically, cellulose consists of β-D-glucopyranoside units that are linked together via β-D-glucosyl bonds. Despite the fact that cellulose potentially can be used in wide range of applications, majority of cellulose, annual production estimated 1.5 x 1012 tons, is being wasted. For the cellulose to be utilized in various industrial applications it needs first to be converted into its building blocks (Glucose) by the hydrolysis of β-D-(1,4) glucosidic linkages. Naturally, cellulose degradation is mediated by an enzymatic system referred to as cellulases (David *et al* 2021).

Cellulase is a group of three individual enzymes namely endoglucanase (endo-1,4-β-D-glucanase, cellobiohydrolase (exo-1,4- β-D-glucanase, and β-glucosidase (1,4-β-D). These enzymes work synergistically to degrade cellulose to glucose units which can then be used in various biotechnological applications such as textile, paper and pulp industry, laundry industry, biofuel production and amino acids synthesis (Milala *et al* 2005).

Microorganisms are the major contributor to cellulose degradation. Cellulases are synthesized by bacteria, fungi, plants and some animals, and anaerobic microorganisms are known to produce single discrete cellulase system known as cellulosome, more powerful and efficient system for cellulose degradation (Ahmed *et al*., 2018).

In recent years the interest in production of cellulases has increased due to several potential applications, such as the production of bioenergy and biofuels as well as application in the textile and paper industries (Zhou *et al*., 2008). A number of microorganisms; bacteria, fungi, actinomycetes, and yeasts are capable of producing extracellular cellulase enzyme (Kirk *et al*. 2002). Many fungal strains secrete higher amounts of cellulases than bacterial ones. Cellulases from *Trichoderma* and *Aspergillus* spp have been investigated in detail over the past few decades (Fang *et al*. 2008; Hui *et al*., 2010). But due to their long growth cycle, such fungi have a huge spore formation, and this limits their performance in terms of cellulase production and its safe utilization. Yeasts such as *Pichia stipitis*, *Candida shehatae*, and *Pachysolan tannophilus* have the ability to use both C5 and C6 sugars (Agbogbo *et al*., 2008).

This present study investigates the production of extracellular cellulase by yeast using concorb, banana peel, pineapple peel and orange peel as the fermentation medium over the course of 10 days, to reduce the environmental problems caused by these solid biodegradable wastes and provide an inexpensive and valuable means for the production of cellulase.

1.1 Aim and Objectives.

The aim of this research was to produce cellulase from Agro-waste using thermophilic yeast.

The specific objectives were to;

- isolate and identify thermophilic yeast from Agro-waste dumpsite soil leachate
- screen identified yeast for cellulase producing capacity
- determine optimum cellulase production time during fermentation for 10 days
- determine the effects of pH,fermentation time and viable cell count on cellulase production.

CHAPTER TWO

LITERATURE REVIEW

2.1 Environmental waste

There is an increasing acknowledgment of our impact on the environment due to our lifestyle, while the need to adopt a more sustainable approach concerning our consumption habits emerges as of particular significance (Gaidajis *et al*., 2010).

That environmental awareness and a sense that the environment is personally relevant lead to an increased incidence of recycling has been documented in a number of studies. Baldassare and Katz (1992) noted that perceived environmental threat is highest among younger respondents, women, liberals, and Democrats, but that those most likely to recycle perceive environmental waste as a serious threat to their personal health and well-being (Baldassare *et al*., 1992).

As human needs and activities overload the assimilative capacity of the biosphere, the debate on waste management has become paramount. According to European Union guidelines, the reduction of the present levels of waste generation and the increase in energy and materials recovery represent two of the most important future requirements for environmentally waste management practices (Marchettini *et al*., 2007).

The most common types of waste treatment and final disposal are incineration, composting and managed landfilling. The latter still is the most popular choice; in 2001 in Italy 67.1% of the waste produced was disposed in landfills, 8.7% was burnt in incinerators and the rest was

recycled. It is no longer possible to see landfills as a first choice for disposal as Italian law prohibits it. Rather, landfills must be seen as the final step of waste management, after all possible material and energy recovery has taken place. As the approach to waste management changes, the methodology used to assess its management should change accordingly. The new paradigm forces us to overcome the approach of evaluating only the impact associated with the various waste management systems in favour of considering the options chosen also in terms of the advantages that may be provided by the refuse. It is necessary to quantify the energy and materials saved from different waste management options while using the same unit of measure for energy, money and material costs. For this purpose eMergy analysis is used in this paper. This environmental accounting methodology was developed by H.T. Odum in the 1980s (Odum, 1988, 1996) and makes it possible to convert all of the inputs to a system into solar eMergy joules (Marchettini *et al*., 2007).

2.2 Different Types of Environmental Waste

2.2.1 Agricultural waste

Agricultural wastes also known as agro-wastes are defined as the residues from the growing and processing of raw agricultural products such as fruits, vegetables, meat, poultry, dairy products, and crops. They are the non-product outputs of production and processing of agricultural products that may contain material that can benefit man but whose economic values are less than the cost of collection, transportation, and processing for beneficial use. Their composition will depend on the system and type of agricultural activities and they can be in the form of liquids, slurries, or solids. Expanding agricultural production has naturally resulted in increased quantities of livestock waste, agricultural crop residues and agro-industrial by-products. There is likely to be a significant increase in agricultural wastes globally if developing countries continue

to intensify farming systems. It is estimated that about 998 million tons of agricultural waste is produced yearly. Organic wastes can amount up to 80 percent of the total solid wastes generated in any farm of which manure production can amount up to 5.27 kg/day/1000 kg live weight, on a wet weight basis (Obi *et al.,* 2016).

2.2.1.1 Agricultural Waste Generation

Agricultural development is usually accompanied by wastes from the irrational application of intensive farming methods and the abuse of chemicals used in cultivation, remarkably affecting rural environments in particular and the global environmental in general. The waste generated is dependent on the type of agricultural activities carried out (Gaidajis *et al*., 2010).

Wastes from Cultivation Activities

While tropical climate is favorable for growing crops, it also supports the generation and development of insects and weeds. This situation creates a high demand for pesticides in order to kill insects and protect against the spread of epidemic diseases; this need often lead to the abuse of pesticides by farmers. After using pesticides, most of the bottles and packages holding these pesticides are thrown into fields or ponds. According to an estimate made by the Plant Protection Department (PPD), about 1.8% of the chemicals remain in their packaging. These wastes have the potential to cause unpredictable environmental consequences such as food poisoning, unsafe food hygiene and contaminated farmland due to their potentially lasting and toxic chemicals (Hargreaves *et al*., 2008).

2.2.2 Waste from Aquaculture

The growth in aquaculture has led to an increase in the use of feeds for improved production. The amount of feed used in a system is the most important factor used in determining the quantity of waste generated. The wastes that result from the use of aquaculture feeds. One of the major wastes generated in aquaculture is metabolic waste which could be dissolved or suspended. In a properly managed farm, approximately 30% of the feed used will become solid waste. Feeding rates are dependent on the ambient temperature. Increase in temperature results in increased feeding which gives rise to increased generated waste (Khatiebi *et al*., 2015).

2.2.3 Wastes from Livestock Production

Waste from livestock activities include solid waste such as manure and organic materials in the slaughterhouse; wastewater such as urine, cage wash water, wastewater from the bathing of animals and from maintaining sanitation in slaughterhouses; air pollutants such as H_2S and CH_4; and odors. The pollution caused by livestock production is therefore a serious problem since most of them are usually built around residential areas. Air pollution includes odors emanating from cages resulting from the digestion process of livestock wastes; the putrefaction process of organic matter in manure; animal urine, and/or from redundant foods (Ugwuishiwu *et al.,* 2016).

2.2.4 Municipal solid waste

Everyday items like product packaging, grass clippings, furniture, clothing, bottles, food scraps, newspapers, appliances, paint, and batteries are the composition of municipal solid waste (MSW), sometimes known as trash or garbage. This is produced in our residences, workplaces, schools, and hospitals. The production of solid waste is an inevitable consequence of human

activity, and its management directly impacts the health of the people and environment surrounding it. Globally, people are discarding growing quantities of waste, and its composition is more complex than ever before (Sintana *et al*., 2012). Municipal solid waste (MSW) is largely made-up of kitchen and yard waste (Hargreaves *et al*., 2008). The basic stages in the management of municipal solid waste are: (1) generation of wastes; (2) collection, handling and transfer of waste; and (3) disposal, processing and treatment of waste (Nanda *et al*., 2020).

2.2.5 Medical/Clinical waste

These are the waste generated from the hospitals and nursing homes example, waste generated during medical research, immunization, testing, diagnosis, treatments, etc., culture dishes, gloves, bandages, glassware, needles, scalpels, swabs and tissues. It contains infectious materials. This is also called as biomedical waste (BMW) (Parishmita *et al.,* 2020).

2.2.6 Electronic waste

E-waste or electronic waste are actually the unwanted electronic devices which are discarded for recycling, reuse or refurbishment example, computers, VCRs, DVD players, televisions, stereos, copiers, fax machines etc. The electronic waste has turned out to be a major problem for mankind. When these wastes are dumped into landfills, toxic substances such as mercury, lead, cadmium, etc. leach out into the soil and water and as a result affect the human race and animals

2.3 Microbial Degradation of Waste

Microorganisms, as their name suggests, are very small organisms that are found around us and inside our body and usually require a microscope to observe them. These microorganisms are being categorized into a wide range of category which includes bacteria, viruses, fungi, archaea, protozoa and algae. Some bacteria and fungi are well known for the process of degradation. A common example Microbial degradation of waste is the bio degradation process which involves the catalyzed reduction in complexity of chemical compounds.

2.3.1 Biodegradable and Non-Biodegradable Waste

Biodegradable waste Materials and substances can be termed as biodegradable if they are easily decomposed by bacteria and other natural organisms and do not contribute to pollution. Biodegradable waste is easily found in municipal solid waste like kitchen waste, green waste, food waste, paper waste, etc which are usually degraded by microbes (bacteria, fungi, etc.), abiotic components like temperature, UV, oxygen, etc. They are broken down into carbon dioxide, methane, water and other basic natural mixes by various processes like fertilizing the soil, aerobic digestion, anaerobic processing or comparative ways. It additionally incorporates a few inorganic compounds like gypsum and its products which can be broken down by the microorganisms. Biodegradable waste affects the environment only when they are present in excess. They can generate are large quantity of microbial population around the waste which can cause many communicable diseases to humans, animals, etc., it can generate bad odor, release certain gas on the process of burning, dumping grounds can act as a breeding ground for certain

vectors or carriers like mosquitoes and rats which ultimately can various harmful diseases. Biodegradable waste can also be utilized as a source of heat energy, power and fills by methods anaerobic digestion or burning.

2.3.2 Non-biodegradable waste

Non-biodegradable wastes are waste or materials which cannot be degraded or decomposed by the biological processes or broken down by natural organisms and add up to the pollution are referred to as non-biodegradable waste. This waste cannot be taken care of. It remains on earth for thousands of years without being decomposed. Hence, they are more dangerous than the biodegradable waste. Extreme use of such waste, for example, chemical fertilizers and pesticides makes the soil more acidic or alkaline, thus affecting the growth of plants and the fertility of the soil. From the fields, these harmful chemicals might wash off into the nearby water bodies thereby disturbing the aquatic life and endorsing the algal bloom causing eutrophication. Most of the non-biodegradable waste can enter the food chains or biological cycles and since humans occupy the highest tropic levels at any of these cycles, therefore most of the harmful chemical concentrations are found in the human bodies. A usual example of this is the plastic which is found in every area. In order to give plastics more durability and better outcome, better quality plastics are being used. Other examples include metals, cans, industrial trash, chemicals from agricultural fields, etc. These are the major causes of air, soil and water pollution and causes deadly diseases like cancer (Parishmita *et al.,* 2020).

2.4 Essence of Soil Leachate

As a result of boideterioration and various physical, chemical, and biological reactions occurring in agro-waste, leachate is produced as a by-product. Leachate is a dark brown liquid that is

produced from municipal solid waste and contains soluble and suspended material. Leachate composition consists of inorganic and soluble organic and inorganic compounds, nutrients, suspended particles, heavy metals, and many hazardous chemicals, causing significant damage to both natural and agricultural ecosystems when released in an untreated and uncontrolled manner (Naveen *et al*., 2017). The rate of leachate production, its volume, and its properties depend on various factors, e.g., the composition of waste material, its particle size, waste moisture and temperature, the amount of rainfall, and biochemical reaction that occur in the degradation stages of the municipal solid waste (Rezapour *et al*., 2018).

Several studies have found the negative effects of waste leachate on soil quality due to the presence of high content of nutrients, heavy metals, and soluble salts in the leachate (Arunbabu *et al*., 2017)

2.5 Essence of Thermophiles in Cellulase Production

Enzymes produced by these microorganisms are often being used in commercial industrial processes. Enzymes work best within specific temperature and pH ranges, and sub-optimal conditions can cause an enzyme to lose its ability to bind to a substrate. However, microorganisms such as thermophiles can produce enzymes which can survive and function in extreme conditions, which are generally required for these applications. Often the thermophiles are found in such diverse and harsh environments (Acharya *et al*., 2012).

2.6 Lignocellulosic Biomass and Its Composition

Lignocellulosic biomass is composed mainly of three basic structural components; cellulose, hemicellulose and lignin. The content of these components in biomass varies depending on the biomass type. Woody plant species have tightly bound fibers and are richer in lignin while herbaceous plants have more loosely bound fibers, a fact that indicates lower lignin content. Usually, cellulose, hemicellulose and lignin constitute 40-50 wt.%, 20-40 wt.% and 10-40 wt.% of the plant material respectively (Stylianos *et al*., 2014).

Lignin is as an amorphous, polyphenolic material arising from enzymatic dehydrogenative polymerization of three phenylpropanoid monomers, namely, coniferyl, sinapyl alcohol, and p-coumaryl alcohol. The structural building blocks of lignin are linked by carbon–carbon and ether bonds. Units that are trifunctionally linked to adjacent units represent branching sites which give rise to the network structure characteristic of lignin. Thus lignin consists of complex and diverse structures, including in softwood lignin an eight-member ring configuration (dibenzodioxocin) (Xue-Fei Zhou 2014).

Hemicelluloses constitute roughly one-third of the wall biomass and encompass the heteromannans, xyloglucan, heteroxylans, and mixed-linkage glucan. The fine structure of these polysaccharides, particularly their substitution, varies depending on the plant species and tissue type. The hemicelluloses are used in numerous industrial applications such as food additives as well as in medicinal applications (Pauly *et al*., 2013).

Cellulose, which is the most abundant renewable biological resource, is produced mainly by plant photosynthesis. Cellulose biodegradation mediated by cellulases or cellulolytic microorganisms releases organic carbon in plant, animal, and microbial sediments back to the atmosphere as carbon dioxide and methane. Complete enzymatic crystalline cellulose hydrolysis requires three types of enzymes (endoglucanase, exoglucanase or cellobiohydrolase (CBH), and

b-glucosidase) to work together. Physical heterogeneity of the cellulosic materials and the complexity of cellulase enzyme systems (synergy and/or competition) on solid enzyme-accessibility-limited substrate surfaces present some challenges for cellulase activity assays (Jayasekara *et al*., 2019)

Biomolecules derived from natural resources play a major role in manufacturing products needed for daily use. Enzymes are one of those molecules that are globally recognized for their multifarious applications in industries. For instance, their utility in brewing, dairy products, detergents, food and feed, pharmaceutical production, and paper and pulp industry is huge. One of those most widely used enzymes is cellulose (Pauly *et al*., 2013).

According to recent global cellulase market analysis reports, the demand for this enzyme is exponentially increasing. Cellulose, the substrate of cellulase, is the most abundant polysaccharide present on earth. It is the main substance in plant materials. Anselme Payne was the very first person to discover and isolate this amazing compound from green plants. It happened more than two centuries ago. From the past, cellulosic materials have played a crucial role in daily human life. They used it to fertilize their soil for crop cultivation. It was also fodder for their cattle. It was firewood for cooking, and they were igniting cellulosic material to generate heat whenever they needed to produce energy (Jayasekara *et al*., 2019).

Currently, the role played by cellulose is not that simple. Especially, as it is recognized as a cost-effective raw material, the useful applications of cellulose in the industrial sector have become much more complex. This has laid a huge platform for scientists to do cellulose-based research in multidisciplinary approaches. One such area is hydrolysis of cellulose. In nature, this is usually accomplished by cellulases (Shang *et al*., 2007).

2.7 Cellulase Enzyme

Cellulase is an enzyme that catalyzes the hydrolysis of cellulose. However, cellulase is not a single enzyme. It is a group of enzymes which is mainly composed of endoglucanase and exoglucanases including cellobiohydrolases and β-glucosidase. Fungi, yeast, bacteria, and are recorded to be efficient cellulase enzyme producers in the natural environment. These microorganisms must secrete cellulases that are either free or cell surface bound. Their enzyme production efficiency and the enzyme complex composition are always diverse from each other. The enzyme breaks β-1,4-linkages in cellulose polymer to release sugar subunits such as glucose. (Preeti *et al*., 2014).

According to recent enzyme market reports, the key areas of the industry where cellulase enzyme is increasingly being applied are healthcare, textile, pulp and paper, detergent, food, and beverages. Its wide application in coffee processing, wine making, and fruit juice production is related to food and beverage segment. In other industrial applications, it is broadly used to produce laundry detergents and cleaning and washing agents.

Cellulase is also being highly recognized as an effective alternative to available antibiotics for treatment of biofilms produced by Pseudomonas. Therefore, the potential of cellulases to fight against antibioticresistant bacteria is an amazing trend which will overcome problems in the

healthcare sector. Application of microorganisms or microbial enzymes for pretreatment of lignocellulosic material is currently earning a huge attention of the industry (Xing-hua *et al.,* 2007). This is a result of growing interest about depletion of fossil fuel resources in the world which have inspired the production of bioethanol from lignocellulosic biomass through enzymatic hydrolysis. Lignocellulosic biomass is one of the best options as a low-cost, readily available, eco-friendly raw material. However, it is not found alone. Cellulose is forming lignocellulose in combination with hemicellulose and lignin which finally becomes a compact network structure. Moreover, it has a crystalline structure which is hard to break down. Therefore, cellulose is insoluble in water and causes limitations in hydrolysis. That is why it is essential to pretreat lignocellulosic material in industries like bioethanol production. During pretreatment, it will loosen up the crystalline structure and facilitate the degradability to release fermentable sugar forms. There are several methods available for pretreatment of lignocellulose. Physical, chemical, and biological methods. Biological pretreatment using cellulolytic microorganisms and their enzymes is found to be the best way of addressing this problem. By all means, cellulase is an enzyme which can cause a huge economic impact. However, there are some considerable bottlenecks of utilizing this enzyme in the industry. For example, the higher cost of cellulase and less catalytic efficiency are especially understood. Another important point is less understanding of the relationship between hydrolysis mechanisms and molecular structure of the enzyme. This knowledge is important to carry out further improvements in the enzyme to enhance its catalytic activity (Jayasekara *et al*., 2019).

2.8 FERMENTATION AND ITS TYPES

2.8.1 Submerged Fermentation

Submerged fermentation (SmF) is a process in which the growth of microorganisms takes place in liquid broth medium which is optimized with required nutrients to have a better cultivation of microorganisms. This involves growing carefully the selected microorganisms in closed reactor containing the fermentation medium and a high concentration of oxygen. SmF has well-established equipment that makes use of the existing microorganisms. Bacteria are commonly used as a source in this process as it requires high moisture content (Doriya *et al*., 2016).

SmF utilizes free flowing liquid substrates, such as molasses and broths. The bioactive compounds are secreted into the fermentation broth. The substrates are utilized quite rapidly; hence need to be constantly replaced/supplemented with nutrients. This fermentation technique is best suited for microorganisms such as bacteria that require high moisture content. An additional advantage of this technique is that purification of products is easier. SmF is primarily used in the extraction of secondary metabolites that need to be used in liquid form (Subramaniyam *et al*., 2012).

2.8.2 Solid state fermentation (SSF)

Solid state fermentation has long been applied to the food industry. SSFs are processes carried out with microbes growing on nutrient impregnated solid substrate with little or no free water. Solid state fermentation (SSF) can be directly carried out with abundant low-cost biomaterials (starch, cellulose, lignin, hemicellulose, chitin, etc.) with minimal or no pretreatment, and thus is relatively simple, uses less energy than submerged fermentation (SmF). Solid state fermentation provides unique microenvironments conducive to microbial growth and metabolic activities bacteria (Shang *et al*., 2007).

SSF is extensively used in various processes such as bioremediation, bio-detoxification of various hazardous compounds, production of various therapeutic enzymes and secondary metabolites. SSF uses agricultural wastes and industrial residues that are cheap and readily available material as source of growth for microorganism for the production of low volume–high-cost products (Doriya *et al*., 2016).

2.8.3 Enzymes from Solid state fermentation

Historically, enzymes have long been produced from SSF. Several reviews of the production of enzymes from SSF have been published in recent years. Recordings of SSF can be found in Asia starting from thousands of years ago. Evidently, the SSF process originated from food fermentation and production of enzymes. In theory, all the enzymes that are presently known and produced by any means are able to be produced under SSF. The microorganisms involved can be filamentous fungi, yeasts, or bacteria (Shang *et al*., 2007).

2.9 Global demands for enzymes

Enzymes are known to be very useful in many industrial processes. Their broad applicability has created a significant market demand in the recent years. According to market reports on world enzyme demand (2017), they have recognized several key factors which lead to huge consumer demand for enzymes. Some of them are completely bound with economical advances. For example, increased per capita income in developing countries causes huge growth in consumer-

related industrial applications. A recent industry study done by Freedonia in January 2018 on "Global Industrial Enzymes" reveals that global demand for industrial enzymes is projected to grow 4.0% per year to $5.0 billion in 2021. This report also emphasizes the gains in personal incomes in developing countries as the key factor which is supporting growth in demand for enzymes. The development of scientific research on enzymes is mainly based on disciplines such as biotechnology, molecular biology and genetics. Continued advances in these areas of research, particularly related to DNA manipulation and sequencing, result in extensive increases in enzyme demand worldwide. Cellulase is one such enzyme which earns consecutively increasing demand. Therefore, collection of knowledge about this enzyme is essential for further development of fundamental and applied research on cellulase and for consequent application in human life (Jayasekara *et al*., 2019).

2.9.1 Applications of Microbial Cellulases

For many decades, cellulases have played a crucial role as biocatalysts. They have shown their potential application in a large number of industries. Textile, paper and pulp, laundry and detergent, agriculture, medicine, and food and feed industries are some of the major industries which employ microbial cellulases. According to Coherent Market Insights, the textile industry is the dominant market for cellulases in 2017. According to most of the enzyme market research reports published in 2018, food and beverages, textile industry, animal feed, and biofuels have been reported to be the major areas of applications

According to another Global Cellulase (CAS 9012-54-8) Market Research Report published in 2018, Asia-Pacific is the largest consumer of cellulase, with a revenue market share nearly 32.84% by 2016. Furthermore, the reported data showed 29.71% of the cellulase market demand in animal feed, 26.37% in food and beverages, and 13.77% in the textile industry in 2016. This

same report forecasts that the applications of cellulases will reach 2300 million USD by the end of 2025, growing at a compound annual growth rate (CAGR) of 5.5% during the 2018–2025 period. These data suggest that the application of cellulases in industries is drastically rising annually. Novozymes and DuPont from Denmark are key cellulase enzyme producers supplying these enzymes to the global market for industrial applications. From this point forward, in this chapter, our major effort was to discuss about the current applications of cellulases in major fields that have been listed above. The novel biotechnological trends emerging in those fields while understanding the key areas of research where further studies required also surfaced to an extent.

2.9.2 Textile industry

The textile industry is one of the largest industries in the world. The customer demand for fashion is increasing as they want uniqueness in styles, colors, and the clothes they wear. There was a significant growth in this industry during the last few decades as a result of this increasing customer demand. This enzyme has now become the third largest group of enzymes used in these applications. This creates a very competitive market platform for manufacturers that are always looking for environmentally friendly approaches of giving their products a unique look. Cellulase is used for many purposes in the industrial sector. Especially for textile wet processing, biostoning of denim fabric, biopolishing of textile fibers, softening of garments, and removal of excess dye from the fabrics are some of the major applications of this enzyme in the industry. Fungal cellulases from Trichoderma reesei are the mostly applied enzyme in the textile industry. Apart from that, actinomycetes from the genera Streptomyces and Thermobifida and other genera of bacteria, such as Pseudomonas and Sphingomonas, are some of the sources of enzymes

to be used for decolorization and degradation of textile dyes. Biostoning and biopolishing are well known for the best applications of cellulases in the current textile industry.

2.9.3 Paper and pulp industry

This is one of the largest industrial sectors in the world. According to the World Wildlife Fund (WWF), the pulp and paper industry, which includes products such as office and catalog paper, glossy paper, tissue, and paper-based packaging, uses over 40% of all industrial wood traded globally. On the other hand, the latest paper industry statistics reveal China, the United States, and Japan as the three countries where the largest paper production occurs in the world. Half of the total paper manufacture of the world is done by these three countries. However, Germany and the United States are the world's leading paper importers and exporters. Moreover, the United States is reported to be the largest consumer of papers. Papers and pulp are renewable resources. Therefore, recycling and reusing are two popular concepts related to this industry. Application of microbial cellulases is usually utilized for this purpose. The application of cellulases in this industry is broader. Starting from the 1980s up to now the possible applications are branching toward many areas. For instance, deinking, pulping, bioremediation of industry wastes, bleaching, and fiber enhancement can be taken.

2.9.4 Pulping

The drawbacks in mechanical pulping processes of woody raw materials such as refining and grinding resulted in pulps with higher amounts of fines, bulk, and stiffness. On the other hand, the process was high energy consuming which was not a profitable option for an industry. The substantial energy saving is reported around 20%–40%. During the refining process, it generates small particles of the pulps. These particles reduce the drainage rate during the paper-making

process. These particles can be readily degraded by cellulases in order to increase the drainage ability of the pulp. Mixtures of cellulases (endoglucanases I and II) and hemicellulases have also been used for bio-modification of coarse pulp material to improve fiber properties. It is strengthening the hand sheets. On the other hand, biological pulping has the potential to improve the quality of pulp and properties of the paper while reducing energy costs and environmental impact.

2.9.5 Laundry and detergent industry

The application of enzymes in manufacturing enzymatic washing agents or biological detergents dates back to the 1960s. Using enzymes in detergent formulae is a common practice today. In fact, according to market reports, by 2014, the detergent industry was the largest single market for enzymes at about 25–30% of total sales. Another market research report published in 2017 on laundry detergent market stated that its global market size valued at 133.3 billion USD in 2016. The latest trend in the industry is to use alkaline enzymes in large amounts. For instance, protease, cellulase, α-amylase, lipase, and mannanase are broadly applied in heavyduty laundry and automatic dishwashing detergents.

The capability of enzymes to remove stains is the major focus of using them in manufacturing detergents. Cellulases are available in the market in different brands. For instance, Celluzyme® and Carezyme® are two main brands applied in detergent blends. These detergent blends are mainly applied in washing fabrics made of cotton and cotton blends. These detergents are making fiber modifications in the fabric in order to improve color brightness, softness, and particulate soil removal.

2.9.6 Agriculture

The application of cellulases in agriculture is usually reported in enhancement of crop growth and a control agent of plant diseases. For this purpose, combinations of cellulases, hemicellulases, and pectinases are broadly applied. Certain fungal cellulases are with the ability to degrade cell wall of plant pathogens. There are lots of details about application of bacteria such as plant growth-promoting rhizobacteria (PGPR) to improve plant performance. It is reported that these bacteria play a major role in reducing application of chemical fertilizers increasing plant development and also controlling potential plant pathogens and protecting plants from diseases. Moreover, many fungi including Trichoderma sp., Geocladium sp., Chaetomium sp., and Penicillium sp. enhance seed germination, support rapid plant growth, accelerate flowering, improve the root system and increase the crop yield. However, exact mechanisms behind these reactions are not yet clearly understood. But all these organisms have the ability to produce cellulase and related enzymes which may have a direct participation in these reactions. Some reports are about possible synergisms between bacterial cellulase production and bacterial antibiotic production against plant pathogenic fungi. According to available information, it is evident that cellulolytic microorganisms are participating in many processes such as; rhizosphere soil decomposition, increasing the availability of nutrient for the plant, controlling plant pathogens, facilitating root colonization, and penetration of cereal crops improving yields and nutritional contents. However, as there are no solid evidence to prove the mechanisms behind these, this area needs further research. The studies should be performed in order to characterize and improve applications of microbial cellulases in this field. During traditional agriculture practices, especially in countries like Sri Lanka, farmers used to add straw and Gliricidia leaves like cellulosic materials into their fields. They observed that the incorporation of these types of plant material not only improved the quality of the soil but also increased the yield due to added

nutrients. Therefore, it is obvious that in this type of processes cellulolytic microorganisms must have a direct contribution.

2.9.7 Medical applications

Medical pharmacology is currently a very active field of research that novel discoveries are coming into action. By the way, humans are not cellulase producers, but the recent research on health and medicine reveals the benefits of consuming blends of enzymes including cellulase. As a result of global demand for enzyme blends, cellulase produced by the natural fermentation process of *Trichoderma reesei* and *Bacillus licheniformis* has been included in commercially available enzyme blends. This type of enzyme blends target collective digestion of cellulose-rich fibrous substances such as fruits and vegetables, cereals, legumes, bran, nuts and seeds, soy, dairy, healthy greens, sprouts, and herbs along with fats (lipids), sugars, proteins, carbohydrates, and gluten. One such example is VeganZyme®. Apart from that digestive aids (e.g., Digestin, P-A-L Plus Enzymes, Polyenzyme Plus, etc.) to treat people suffering from metabolic disorders are evolving as a promising strategy in medicine. In some records, the direct and indirect applications of cellulase in medicine have been mentioned apart from using it as consumable enzyme blends. Indirect applications of cellulases for medical purposes Cellulase of fungal origin in combination with chitinases and lysozymes has a reported use in chitosan degradation. To obtain chitosan, a partial degradation of chitin must take place. As cellulose, chitin is a structural polysaccharide present in animals such as marine animals like shrimp and insect exoskeleton as well as participates in the formation of some parts of fungal cell walls. Chitin is a poly-ß1,4-N-acetyl-D-glucosamine, conforming crystalline microfibrils. This polysaccharide provides structural integrity, stability, and protection to animals. Chitosan is the most important semicrystalline derivative form of chitin. This is obtained by partial deacetylation of chitin

(around 50%, soluble in aqueous solution) under alkaline conditions or enzyme hydrolysis. Chitosan and its derivatives have many medical applications, viz., surgical sutures, bone rebuilding, production of artificial skin, anticoagulant, antibacterial agent, hemostatic dressings, anticancer and antidiabetic agents (in combination with metals), hypocholesterolemic effectors, elaboration of cosmetics, production of biopharmaceutics, and encapsulation of diverse materials.

2.9.8 **Food and feed industry**

Food processing industry Food is essential for all living organisms to obtain nutritional support for their growth and well-being. The huge demand for food has laid a path to a very complex, interconnected global business that supplies most of the food consumed by the world's population. Food biotechnology nowadays considers cellulases as an invaluable resource due to their increased applicability in a broad range of processes. Fruit and vegetable juice clarification, reducing the viscosity of nectars, concentrating purees, alteration of fruit sensory properties, carotenoid extraction, olive oil extraction, and the quality improvement of bakery products are among the various processes in food biotechnology that cellulase is exploited worldwide. The cloudiness which is usually present in fruit and vegetable juices is a result of floating polysaccharide materials such as cellulose, hemicellulose, lignin, pectin, starch, metals, proteins, and tannins. The presence of these materials in the juice makes it low quality and draws less consumer demand. "Rapidase pomaliq" is a commercially available enzyme preparation composed of cellulase, hemicellulases, and pectinases obtained from Trichoderma reesei and Aspergillus niger. The application of this product in fruit juice clarification was beneficial to a considerable level. It is also reported that cellulase produced by bacteria such as Bacillus and Paenibacillus in combination with other enzymes such as pectinases and hemicellulases carries out the fruit and vegetable juice clarification. Apart from that, treatment of nectars and purees

also found to be efficiently carried out by this enzymatic process. Rheological parameters such as viscosity of these products are brought down to a commercially acceptable level. Modification of sensory parameters of food is another important area where application of cellulases is highly recommended. The aroma properties, flavor, and texture of fruits are some sensory properties which play a crucial role in food biotechnology. The infusions of pectinases and cellulase enzymes have been found to be effective in altering the sensory properties of fruits and vegetables. These enzymes are also applied in degradation of grape fruit peels to release sugars. These sugars will be used in many industries including food production. Another important application of cellulase is extraction of phenolic compounds from grape pomace. During extraction of olive oil, malaxing (mixing) is an indispensable step. This period allows the tiny oil droplets to attach with bigger ones and increase the oil yield which is coming from the olive paste. The use of cellulases alone or in combination with other hydrolytic enzymes like pectinases in this step has been found to have an enhancing effect on the extraction as well as the quality of olive oil. The enzymatic treatment of olive oil at the extraction stage causes significant enhancements in phenolic content and antioxidant activity of olive oil, thereby ultimately improving its quality. The enzyme cocktails of cellulases with other hydrolytic enzymes such as amylases, proteases, and xylanase result in increased loaf volume, improvement in bread quality, and production of softer crumb. Enzyme cocktail containing cellulases, hemicellulases, amylases, lipases, and phospholipases results in dough conditioning with improvement of flavor, prolonged shelf life, and increase in volume after baking (Jayasekara *et al*., 2019).

CHAPTER 3

METHODOLODY

3.1 Sample collection and preparation

Soil sample was collected from the hot region of agro-waste dump-site containing decayed organic matter, dripping leachate and plated using yeast extract peptone dextrose agar at 50 ^{0}C for 0-10 days.

3.2 Isolation of Thermophillic yeast

3.2.1 Serial dilution

5 g of soil sample was added to conical flask containing 45 ml of sterile peptone water. The conical flask was shaken after which 1ml was 1ml aliquot was transferred into a testube

containing 9ml of sterile peptone water. From here, a four-step serial dilution was performed. The last test-tube contained inoculum dilution of 10^{-4}. 1ml of inoculum was aseptically pipetted into sterile Petri-dish for cultural analysis using pour plate method with yeast extract medium (YPD). After pouring of the medium, the plates were rocked gently and allowed to solidify after which they were incubated at 50 ^{0}C for 24 to 48 hr.

After incubation period, pure / discrete colonies were subcultured into prepared YPD agar plate using quadrant streak plate method. After 24 hr, pure colonies were identified based on colonial, microscopic characteristics and sugar fermentation test.

3.3 Screening for Cellulase Production

Purified isolates were subjected to cellulase screening. Medium containing yeast extract ($1gl^{-1}$), carboxylmethylcellulose (CMC 1%), peptone ($2gl^{-1}$) and dihydrogen phosphate ($0.5gl^{-1}$) was used. Concentration of cellulase produced was determined after 48hrs. The medium containing the isolate with the highest cellulase concentration was selected for fermentation experiment.

3.3.1 Inoculum Preparation

The selected yeast isolate from screening was aseptically liquefied in 10ml of sterile water in a testube. From here a solution comparable to Macfarland standard $1.5x10^8$ $cfuml^{-1}$ was prepared using turbidimetric method. Determination of inoculum size was achieved using the formula

$C_1 V_1 = C_2 V_2$.

Where:

C_1= initial concentration ($1.5x10^8 cfuml^{-1}$)

V_1=initial volume (x)

C_2=final concentration ($1x10^6 cfuml^{-1}$)

V_2=final volume. (200ml)

The calculated inoculum size (150 µl) was extracted using a pipette and transferred into each fermentation medium. The inoculated samples were transferred into an incubator set at 50 ^{0}C.

3.3.2 Preparation of Agro-Waste Substrate Medium for Cellulase Production.

Corncob, banana peel, pineapple peel and orange peel wastes were dried completely. Dried wastes were then grinded to fine powder. 20g of powder was dissolved in 200ml of deionized water (containing 0.6g, $NaNO_3$; 0.1g, KCl; 0.2g, KH_2PO_4; 0.1g, $MnSO_4$. $7H_2O$; 0.002g, $FeSO_4.7H_2O$), standardized to pH 5.0 in a 500ml capacity bottle. This was done for each substrate, and the samples were prepared in duplicates for fermentation period of 10 days. The sample bottles were sterilized at 121^{0}C for 15min in an autoclave.

3.4 Inoculation of Fermentation Medium

Preparation of 1% solution of anhydrous $BaCl_2$ and 1% solution of sulfuric acid solution (H_2SO_4) (equivalent to 0.5 Mcfarland i.e 1.5×10^8 cfu/ml of yeast concentration) in a test tube with

deionized water was done, and then vortexed to form a turbid suspension. A second sterile deionized water of same quantity as the aforementioned solution was inoculated with pure and discrete colony of the screened isolate until same turbidity was achieved as that of the standard sulfuric acid and barium chloride mixture when placed against white light. Inoculum size was calculated using the formula;

$C_1 V_1 = C_2 V_2$.

Where:

C_1= initial concentration ($1.5x10^8 cfuml^{-1}$)

V_1=initial volume (x)

C_2=final concentration ($1x10^6$ $cfuml^{-1}$)

V_2=final volume. (200ml)

The calculated inoculum size was aseptically pipetted into fermentation vessel and incubated at 27^OC.

3.5 Determination of pH

pH was determined using pH meter (Mettlar Toledo). At each sampling time i.e day 0, 2, 4, 6, 8 & 10, 4ml of fermentation medium is aseptically drawn from the four different wastes and separated in well labeled beakers. Then zeroed pH rod is dipped in the medium and the pH is read and written.

3.6 Viable cell count

This was determined using the pour plate method. Fermentation medium samples were serially diluted in a four step setup. The sample bottle was shaken after which 1ml from each sample bottle was pipetted out into the first testube and serially diluted.

No of colonies in cfu/ml = no of colonies counted × dilution factor ÷ amount of sample (1ml)

3.7 Determination of Cellulase Concentration

Procedure

The appropriate sample vessels were identified and selected for processing. The bottles were placed in a water bath set to 65°C for 15 min. Following this, the sample was transferred to centrifuge tube and spun for 10 min at 4000 rpm. Carefully, the upper portion of the solution (containing the free microbial cells or supernatant) was decanted out, and 1 ml extracted from the middle portion of the tube and transferred into a clean, dry test tube. The bottom layer contained the substrate residue. Equal volume of CMC substrate solution (1 ml) was added to the test tube and mixed thoroughly. The test tube was kept at a temperature of 40° C for 10 min after which 4 ml DNS - Lactose solution was added, and the tube was placed in boiling water for 5 min. After 5 min, the tube was taken out, and allowed to cool, and colour was maintained by adding 2 ml of sterile, filtered deionized water. The tube was cooled, and the absorbance of the solution was determined with a spectrophotometer at 540m against a blank (sterile, filtered deionized water).

Absorbance readings for the standard concentrations (mg/L) were obtained by repeating the steps above but omitting CMC substrate solution.

3.8 Data Analysis

All result values were analyzed using spss statistical package version 16.0. Values were analyzed in duplicates and expressed as mean± standard deviation.

CHAPTER 4

RESULTS

Torulopsis bovina a thermoohilic yeast which was isolated from hot region of agro-waste dumpsite was used in this study for the production of cellulase enzyme.

Table 4.1 depicts the cultural and biochemical characteristics of yeast isolates obtained from agrowaste dumpsite soil leachates.

Table 4.2 shows screening of the identified isolates for cellulase production capacity. Where *Torulopsis bovina* had a cellulase concentration of 2.5489±0.008gL^{-1} and *Candida auris* had a cellulase concentration of 0.8819±0.006gL^{-1}.

Table 4.3 shows the pH of various agro-waste fermentation media over 10 days. The highest pH recorded was 9.70 on day 8 by corncob medium while the lowest pH recorded was 5.00 on day 0 for every medium. At peak cellulase production (day 8) the lowest pH recorded was 5.20 (by orange peel medium) while the highest recorded was 9.70 (by corncob medium).

Table 4.4 shows the viable cell count recorded from the various fermentation media. The highest count was recorded on day 8 by corncob medium while the lowest recorded on day 0 by banana peel medium. On cellulase peak production day, the highest cell count recorded was $4.490x10^{-4}$ cfu/ml by corncob medium while the lowest recorded was $0.730\ x10^{-4}$cfu/ml by banana peel medium.

Tahle 4.5 shows cellulase concentration recorded from the various fermentation media. The highest cellulase concentration was recorded by concorb medium on day 8, while the lowest cellulase concentration was recorded by banana peel medium on day 10. On the peak production day highest cellulase concentration recorded was $1.180gl^{-1}$by corncob medium while the lowest recorded was $0.016gl^{-1}$ by banana peel medium.

Table 4.1: Cultural and Biochemical Characterisation of Yeast Isolates Obtained From Soil Leachates.

Isolate Codes	Cultural Characteristics	Sugar Fermentation (L, X, M, G)	Identified Isolates
1	Cream, wavy, raised, lobate	**-, +, +, +.**	*Torulopsis bovina*
2	Cream, wavy, flat,	**-, +, +, +.**	*Candida auris*

	undulate

Table 4.2: Cellulase Screening of Identified Yeast Isolates for Cellulase Production Capacity.

Isolate codes	Isolate identified	Cellulase Concentration
1	*Torulopsis bovina*	2.5430±0.008 gL^{-1}
2	*Candida auris*	0.8776±0.006 gL^{-1}

Table 4.3: pH Values of Various Agro-Waste Substrate Media Fermented for 10 Days (Mean±S.D).

Time	Corncob		Pineapple		orange		banana		*P*-value
	Control	Test	Control	Test	Control	Test	Control	Test	

Day 0	5.00±0.00	5.00±0.00[b]	5.00±0.00	5.00±0.00[b]	5.00±0.00	5.00±0.00[b]	5.00±0.00	5.00±0.00[b]	0.000
Day 2	5.25±0.07	6.20±0.00[a]	5.15±0.07	5.85±0.07[a]	5.00±0.00	5.50±0.00[a]	5.30±0.00	6.60±0.00[a]	0.000
Day 4	5.35±0.07	8.65±0.07[a]	5.25±0.07	7.30±0.14[a]	5.15±0.07	7.05±0.07[a]	5.85±0.07	5.60±0.14[a]	0.000
Day 6	5.55±0.07	8.85±0.07[a]	5.35±0.07	8.00±0.14[a]	5.25±0.07	7.85±0.07[a]	6.10±0.14	7.2±0.00[a]	0.000
Day 8	5.75±0.07	9.70±0.14[a]	5.45±0.07	8.60±0.14[a]	5.20±0.00	8.55±0.07[a]	6.65±0.07	8.05±0.07[a]	0.000
Day 10	5.80±0.14	9.05±0.07[a]	5.45±0.07	8.30±0.14[a]	5.25±0.07	7.90±0.00[a]	6.75±0.07	7.65±0.07[a]	0.001

KEY: a= significant difference, b= no significant difference

Table 4.4: Viable Cell Count of Various Agro-Waste Substrate Media Fermented for 10 Days (Mean ±S.D x 10^{-4} cfu/ml)

Time	Corncob		Pineapple		orange		banana		*p*-value
	Control	Test	Control	Test	Control	Test	Control	Test	0.000

Day 0	-	0.307±0.017 [a]	-	0.160±0.014 [a]	-	0.110±0.014 [a]	-	0.095±0.007 [a]	0.000
Day 2	-	1.085±0.092 [a]	-	0.610±0.014 [a]	-	0.440±0.02 [a]	-	0.255±0.007 [a]	0.000
Day 4	-	2.655±0.091 [a]	-	1.155±0.078 [a]	-	0.855±0.049 5 [a]	-	0.337±0.024 [a]	0.000
Day 6	-	3.880±0.056 [a]	-	2.080±0.098 [a]	-	1.810±0.007 [a]	-	0.569±0.028 [a]	0.000
Day 8	-	4.490±0.042 [a]	-	2.435±0.106 [a]	-	2.265±0.033 [a]	-	0.730±0.028 [a]	0.000
Day 10	-	3.835±0.021 [a]	-	1.995±0.077 [a]	-	1.912±0.016 [a]	-	0.4725±0.074 [a]	0.000

KEY: a= significant difference, b= no significant difference

Table 4.5: The Cellulase Concentration of Various Agro-Waste substrate Media Fermented for 10 Days (gL^{-1}).

Time	Corncob		Pineapple		orange		banana		*P*-Value
	Control	Test	Control	Test	Control	Test	Control	Test	0.000

Day 0	0.150±0.0 00	0.194±0. 001[a]	0.050±0. 000	0.173±0.0 00[a]	0.055±0. 006	0.154±0. 000[a]	0.0445±0. 000	0.183±0.0 02[a]	0.000
Day 2	0.172±0.0 03	0.583±0. 001[a]	0.037±0. 003	0.313±0.0 01[a]	0.056±0. 000	0.294±0. 000[a]	0.040±0.0 00	0.293±0.0 00[a]	0.000
Day 4	0.210±0.0 04	0.753±0. 003[a]	0.042±0. 000	0.417±0.0 01[a]	0.053±0. 000	0.369±0. 000[a]	0.033±0.0 02	0.305±0.0 01[a]	0.000
Day 6	0.439±0.0 28	0.925±0. 002[a]	0.068±0. 000	0.478±0.0 00[a]	0.045±0. 000	0.447±0. 000[a]	0.027±0.0 00	0.311±0.0 00[a]	0.000
Day 8	0.306±0.0 1	1.180±0. 002[a]	0.112±0. 00	0.661±0.0 01[a]	0.038±0. 000	0.551±0. 001[a]	0.016±0.0 00	0.406±0.0 01[a]	0.000
Day 10	0.187±0.0 15	0.911±0. 003[a]	0.103±0. 002	0.420±0.0 00[a]	0.036±0. 000	0.403±0. 002[a]	0.014±0.0 02	0.297±0.0 02[a]	0.000

KEY: a= significant difference, b= no significant difference

CHAPTER 5

DISCUSSION

The improper handling of agro-wastes causes unfavorable effects, making them a significant environmental hazard. Taking the burden off waste disposal services will be beneficial, not only

to ameliorate staff and resource burden, but also to provide additional sources of income generation. This intervention can exist by way of converting these agro-waste products into usable forms. One such way is via microbial utilization. Agro-wastes are rich sources of starch, lipids, proteins cellulose and hemicellulose that can sustain microbial growth and expression. Production of bio-enzymes using agro-waste is an economic and cost effective method of converting these wastes into useful products. The present study ascertained the production of cellulase from thermophillic yeast (*Torulopsis bovina*) using corncob, banana, pineapple and orange peels as fermentation substrate.

In this study, *Candida auris* and *Torulopsis bovina* were isolated from agro-waste dumpsite soil leachate. Similar studies conducted by (Capriott., 1961) *Torulopsis* spp was isolated from soil sample from Hyytiala in the Province of Tavastia Australis, Finland. *Candida* sp. from dumpsites. (Hui *et al*., 2012) isolated *Candida* sp from soil sample collected on Hainan Island in China. Certain *Candida* sp. are thermophillic, and can survive in temperature ranges of 37 to 51^{O}C. Certain *Toruplosis* spp such as *Torulopsis pintolopesii, Torulopsis bovina*, survive extreme thermophilic temperature. Therefore, these organisms can substain high temperature condition and their usage could help salvage the heat-microbial challenges experienced in batch valorization of waste. (Shin *et al.,* 2001).

After screening for cellulose hydrolysis ability, *Torulopsis bovina* produced the higher concentration of cellulose (2.5548 gL^{-1}). *Candida auris* recorded 0.8776 gL^{-1}. However, Tolupsis which is a better choice for cellulase production at 50 degree Celsius could be as a result of the fact that it naturally proliferate at such temperature range under natural condition (Capriott., 1961). The thermophilic yeast *Torulopsis bovina* was further used for the inoculation of the fermentation substrates and at each sampling time i.e day 0, 2, 4, 6, 8 & 10 the pH, viable cell

count and cellulase concentration was determined. This study is in correlation with the work done by (Vyas *et al*., 2017). Where yeast strains were isolated from soil samples rich in cellulose wastes such as decaying leaves, vegetable wastes etc. Yeast colonies were further subjected to Congo red screening on a CMC 238 medium. Out of four selected cultures, two yeasts were found to produce a zone of clearance, which 239 indicated that the yeasts were expressing cellulolytic activity. Further, the selected yeasts were subjected 240 to qualitative analysis for lipase. One of the two yeast isolates showed a 241 zone of clearance on a tributyrin agar plate. At the end of the screening, successful isolation of one yeast 242 strain was done. Isolation of oleaginous cellulolytic yeast with both intracellular and extracellular lipase 243 activity has been attempted for the first time in literature. The yeast identified was named *Cystobasidium oligophagum* (Vyas *et al*., 2017).

In determining the optimal conditions for cellulase production, using the isolated organism (*Toruplosis bovina*) corncob produced the highest amount of cellulase on day 8 which was the peak production day with a cellulase concentration of $1.180gl^{-1}$ while the lowest cellulase concentration recorded was $0.016gl^{-1}$ by banana peel medium. (Coojasola and Jilan 2008) also gave similar time course reports of maximum cellulase using *Tricoderma longibrachiatum* produced the highest amount on day 7, *Aspergillus niger* on day 5 and *Saccharomyces cerevisiae* with a cellulase concentration of $0.68\pm0.03mg\ mL^{-1}$ on day 3 for orange peel and albedo with a cellulase concentration of $0.60\pm0.09\ mg\ mL^{-1}$ and day 5 for orange pulp with cellulase concentration of $0.76\pm0.09\ mg\ mL^{-1}$. Effect of Ph on cellulase production from the four waste substrates by *Toruplosis bovina;* at peak cellulase production (day 8) the lowest pH recorded was 5.20 (by orange peel medium) while the highest recorded was 9.70 (by corncob medium). Corncob was found to be a the most suitable raw material compared to other substrates for

cellulase production by *Torulopsis bovina* under submerged fermentation, which might be due to the presence of its high cellulose component, which significantly induces the cellulase production. Similar work was done by (Verma *et al*., 2011) where pea peel waste was utilized for by *Trichoderma reesei* for cellulase production. A cellulase concentration of 2.86±0.10 lU ml^{-1} was recorded on the peak production day with a pH of 5.0 (Verma *et al*., 2011).

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CONCLUSION

After decades of research on lignocellulosic biomass utilization, it is now considered that enzyme based technologies for biomass conversions are most efficient, cost effective and environment friendly. Considerable progress has been made in search of thermophiles, yet their true diversity, has not yet been fully explored. Thermostable cellulases isolated from these

organisms have shown their potential under conditions that are appropriate for bioconversion processes which have role in industries. The future challenges for cellulases production include technologies for cellulosic biomass pretreatment for better microbial attack, processes for cost effective production of cellulases and finally organism development strategies to improve the properties of enzyme to increase their specific activities, process tolerance and thermal stability.

RECOMMENDATION

The production of cellulase using fruit wastes in large scale should be encouraged as this will have positively reduce environmental pollution and also serves as a good source of income in its application or use. Research should be carried out on how these agricultural wastes can be harnessed for the production of other useful products.

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