VALORISATION OF ORANGE (*Citrus sinensis* L.) PROCESSING WASTES FOR PECTINASE ENZYMES PRODUCTION USING *Aspergillus brasiliensis*

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A Thesis Submitted to the Graduate School in Partial Fulfilment of the Requirements for the Master of Science Degree in Food Science of Egerton University

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been previously submitted, either in its entirety or partially, for the conferment of any degree at Egerton University or any other academic institution.

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Recommendation

This research proposal has been submitted with our approval as the official university supervisors.

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DEDICATION

To my adorable husband Paschal J. Mazengo and my beloved son and daughter, Innocent and Innes.

Thanks for your prayers, love and support.

God bless you to excel higher!

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ABSTRACT

Orange processing wastes are produced in large quantities about 45% to 50% of the fresh fruits, the wastes are not adequately utilised thus creating environmental challenges. This study focused on conversion of the wastes into pectinase enzymes by optimizing various process parameters, such as temperature, pH, and incubation time through solid-state fermentation using *Aspergillus brasiliensis* isolated from rotted orange peels. Enzymes evaluated on application in juice extraction and clarification. *Aspergillus brasiliensis* strain was isolated and screened using PDA media. The strain was selected for morphological identification and molecular characterisation and used for pectinase enzymes production through solid- state fermentation. The optimum enzymes production conditions were determined by varying temperature, pH and incubation time. Pectinase enzymes application was evaluated in juice extraction and clarification using 0 to 5% v/v concentration. For juice extraction, the volume of juice increased was determined compared to juice without pectinase enzymes, with juice clarity at 660 nm absorbance. The microorganism isolated from rotted orange peels was identified as *A. brasiliensis* using macroscopic, microscopic characteristics and molecular technique of transcribed sequence of ITS1 and ITS4 region for identification. Optimized conditions for maximum production of pectinase enzymes showed that parameters have a significant effect on enzyme production at $P< 0.05$ after data was analysed using Box Behnken Design. Based on enzyme activity the maximum pectinase enzyme activities of polygalacturonase, lyase and esterase were 44.58U/g, 16.53U/g and 74U/g respectively at a temperature of 35℃, pH 4 and 6 days of fermentation. Based on the clear zone diameter the maximum pectin hydrolysis was observed at 31℃, pH 4 and 7 days of fermentation, where the clear zone diameter was 12 mm. The produced pectinase enzymes applied in apple juice extraction and clarification, results showed that it has a significant effect on juice yield and juice clarity. The percentage of juice yield increased from 65 to 84.5% at 4% enzyme concentration, in juice clarity the absorbance was decreased from 0.365 to 0.040, the pH of juice was decreased from 3.55 to 3.35 and pectin content from o.45% to 0.040%, this was due to action of produced pectinase enzyme on pectin in the juice. The findings suggest that this approach could be useful for the industrial-scale production of pectinolytic enzymes for various applications. Further studies are required to characterize the enzyme for potential heterologous expression and its application in upcycling waste into valuable products using indigenous microorganisms through biotechnology.

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LIST OF ABBREVIATIONS AND ACRONYMS

AOAC	Association of Official Analytical Chemists
BSA	Bovine Serum Albumin
CRD	Completely Randomized Design
DNA	Deoxyribose Nucleic Acid
DNS	Dinitrosalicylic
FAO	Food and Agriculture Organization of the United Nations
GHG	Green House Gases
PDA	Potato Dextrose Agar
RCF	Relative Centrifugal Force
UV	Ultra Violet

CHAPTER ONE

INTRODUCTION

1.1 Background information

Oranges are the fourth most common fruits produced in Kenya after bananas, mangoes, and pineapples. From 2017 to 2019, the production of oranges was 72,437 tonnes, 71,671 tonnes, and 69,733 tonnes respectively (FAOSTAT, 2020). About 8% of oranges produced are processed into juice and other products, 65% are consumed as fresh fruit, 0.4% are exported, and 20 - 25% is wasted along the value chain (Babigumira, 2017; FAO, 2019) Orange wastes generated in processing ranges between 45 - 50% of fresh fruit total weight (Olabinjo *et al*., 2017; Ricci *et al*., 2019). Disposal of wastes produced after processing is a major problem. Wastes generated from the food processing industries present environmental pollution due to the lack of appropriate technology to biodegrade the wastes. Most of these agro-industrial wastes contain components that are not easily broken down for example lignin, cellulose, hemicelluloses, and pectin. Orange wastes are rich in pectin by 20.0 - 25.4% (Karimi & Satari, 2018; Rathinavel & Saravanakumar, 2021), cellulose by 18 - 20% (de la Torre *et al*., 2019), lignin by 1.2 - 7.52% (Panwar *et al*., 2019) and hemicelluloses by 8.6 - 21.3% (Li *et al*., 2015). Appropriate technologies need to be applied to enhance the biodegradation of these polysaccharides. One such technology is biological degradation by the use of appropriate microorganisms to synthesize several beneficial compounds for medical and industrial exploitation.

Mostly, these wastes are used as animal feed, for landfilling, and for composting, though they have their limitations. In animal feeding, the wastes have high anti-nutritional components e.g. tannins, phytic acids, saponins, and oxalates (Ani & Abel, 2018), it does not support the performance of animal growth and it has poor palatability to the animals (Abdelhafez, 2014). During landfilling, there is environmental pollution due to the emission of greenhouse gases (GHG), for example methane, and biodegradation by mixed culture most of which do not have proper enzymes to break down polysaccharides (Ortiz *et al*., 2020). In addition, composting orange waste contain polysaccharides that are not utilized in terms of nutrients. The composting orange wastes are a source of soil pH alteration, which affects plants' growth performance. Moreover, it is time-consuming in terms of the decomposition process and it also needs experienced personnel for technical processes *(*Voběrková *et al*., 2020).

Due to the above limitations, the orange processing waste can be valorised into valuable products, for example, essential oil, pectin, natural flavour, and enzymes. Enzymes are proteinaceous molecules with a globular structure that accelerate biochemical reactions (Sewalt *et al*., 2016). Enzymes extracted from

organisms can work independently of their source (Robinson, 2015). Microorganisms are a major source of enzymes due to their faster growth and high yield compared to animal and plant sources, and are easy to control and predict the quality of the desired products (Li *et al*., 2020). Microorganisms can produce enzymes by fermentation either submerged or by solid-state fermentation (Patel *et al*., 2017). Solid-state fermentation is considered superior to submerged culture for the conversion of agro and industrial wastes in producing higher enzyme yields and regulating the composition of enzymes mixtures (Chilakamarry *et al*., 2022). According to Sharma *et al*. (2017), enzymes biosynthesis in solid-state fermentation depends on water activity, pH, temperature, incubation time, moisture content, inoculums size, presence of inhibitor or activator, and carbon and nitrogen source. Moreover, the selection and optimization of substrate and culture is another imperative approach to trim down the industrial costs of enzyme biosynthesis. For pectinase enzymes biosynthesis use of citrus, wastes are significant due to the high quantity of pectin that acts as an inducer to enhance microorganisms to produce pectinolytic enzymes.

Enzymes play a major role in food processing and production (Collados *et al*., 2020). The most common enzymes used in food industries are hydrolases, which break down macromolecules into simple molecules. Examples of these enzymes are amylases, proteases, lactases, lipases, and pectinases among others. Pectinase enzymes are a heterogeneous group of enzymes consisting of polygalacturonase, pectin lyase, and pectin esterase (Kokare & Liu, 2017; Satapathy *et al*., 2020). Pectinase enzymes are produced by microorganisms, these include the most common fungi which can produce pectinase enzymes are *Aspergillus spp.*, *Penicillium spp.*, and *Trichoderma reesei.* Pectinases also can be produced by bacteria such as *Basillus spp.* and *Erwini* are also expressed as efficient pectinase producers (Singh *et al*., 2019). However, the best microbes for the production of pectinases are the various *Aspergillus* species where *Aspergillus brasiliensis* is the most preferred (Takagi *et al*., 2020). The United State Food and Drug Administration (FDA) recognized the products produced by *Aspergillus brasiliensis* and *Aspergillus brasiliensis* itself as "General Regarded As Safe" (GRAS) (Takagi *et al*., 2020). Therefore, the enzymes produced by *Aspergillus brasiliensis* are safe to use in food processing.

Pectinase enzymes hydrolyse pectin substances, complex polysaccharides components of the cell wall of plants with high molecular weight made up of poly-D-galacturonic acid linked by α-(1→4) glycosidic bonds, which form the backbone of the chain and esterifies with methoxy groups and branched with sugars such as L-rhamnose, L-arabinose, and D-galactose (Klinchongkon *et al*., 2017). These pectin substances are found in most fruits and can be degraded by pectinase enzymes to release cell content resulting in increased juice yield, clear sparkling liquid after filtration, and pigmentation by extracting

more anthocyanin (Sharma *et al*., 2017). This study aimed to investigate the valorisation of orange processing wastes into high-value commercial products - pectinase enzymes for industrial utilization e.g. extraction and clarification of juices. The current commercial pectinase enzymes used are imported and are costly, produced by submerged fermentation using pure pectin as substrate.

1.2 Statement of the Problem

Many of the developing countries are agricultural and agro-industrial countries producing huge amounts of agricultural residues leading to disposal problems and pollution. Most of this waste is organic waste and can be used as a source of energy and material generation. In Kenya, there are fruit processing factories that generate a lot of organic waste that could be used for beneficial applications. Among other challenges faced by these industries are waste disposal and the high cost of food processing enzymes. In the orange processing industry, it is estimated that orange wastes generated range from 45 to 50% of the total processed fruit. The current waste management methods employed are not economically viable as they present various challenges. Orange processing wastes are currently used as animal feed, for landfilling and composting. Nevertheless, the orange processing wastes have low palatability and nutritional availability is also limited. As landfilling material, concerns of environmental and air pollution have arisen for example GHG emissions, putrid smells, and soil contamination. Composting as orange wastes management method is limited by the slow-nutrient release from the material, hence it is time-consuming and alters the soil pH, therefore affecting plants' growth performance. On the other hand, the high costs of industrial enzymes make the food products produced by the application of enzymes more expensive. The key factor driving the high cost of enzymes is the increasing demand for enzymes including pectinase enzymes from the food and beverages processing industries. Most of the enzymes are imported from other countries such as North America, Europe, and Asia countries. Orange processing waste contains various nutritional components which can be used as substrates in enzymes production using microorganisms. However, studies on the isolation of microorganisms, their characterization, and their application on enzymes production using agro-industrial wastes are limited in Kenya. This study, therefore, seeks to evaluate orange processing wastes as a substrate in a solid-state fermentation mode for the optimum production of pectinase enzymes using *Aspergillus brasiliensis* which is locally isolated for potential application in juice extraction and clarification.

1.3 Objectives

1.3.1 General objective

To contribute to the circular economy by valorising orange processing wastes into commercial products like – pectinase enzymes using isolated *Aspergillus brasiliensis.*

1.3.2 Specific objectives

i. To characterize pectinases-producing *Aspergillus brasiliensis* strain isolated from rotted orange peels.
ii. To maximize culture conditions for pectinase enzymes production by *Aspergillus brasiliensis* using orange waste as substrate.
    (a) Based on clear zone diameter
    (b) Based on enzyme activity
iii. To evaluate the application of pectinase enzymes produced by isolated *Aspergillus brasiliensis* in apple juice extraction and clarification.

1.4 Hypotheses

i. Characterized *Aspergillus brasiliensis* have no significant effect on pectinase enzyme production.
ii. Production parameters have no significant effect on pectinase enzyme production.
iii. Pectinase enzymes produced by *Aspergillus brasiliensis* have no significant effect on juice extraction and clarification.

1.5 Justification

The use of orange waste as a substrate for pectin extraction or pectinase enzyme production holds great potential due to its high pectin content, which can reach up to 25.4% (Rathinavel & Saravanakumar, 2021). This presents an opportunity to reduce the cost of industrial enzymes, which are essential in the food industry for improving product quality in processes such as juice and wine production. Currently, pectinase enzymes constitute 10% of global enzyme production (Saranraj, 2014) and their demand is projected to grow alongside the global industrial enzyme market, which was valued at $6.3 billion in 2021 (KC *et al.*, 2020). Furthermore, the use of locally sourced food waste, such as orange waste, for enzyme production offers significant economic benefits, especially for countries like Kenya and other African nations. It decreases reliance on imported enzymes and open up possibilities for local industries to participate in the global market. This approach not only addresses cost concerns but also contributes to solving waste management issues in the juice processing industry. Therefore, using solid-state fermentation techniques with orange waste presents a cost-effective, sustainable, and environmentally

friendly method for enzyme production, creating value from waste while promoting industrial growth in the food sector.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Food processing industries produce large amounts of waste worldwide. The wastes are generated from the production process. These wastes increase severe environmental pollution and loss of valuable energy-rich biomass and nutrients (Khedkar & Singh, 2018). Table 2.1 shows the type of waste produced by common food processing industries and the value-added products which can be processed from the waste.

Table 2. 1: Waste Produced from the Common Food Processing Process and the Value-Added Products that can be processed from the Waste

Food type processing	Some of the Processed products	Type of wastes/residues	Valuable products
Cereal grain, legumes, nuts, and seeds	Flour, starch, bread, cookies crackers, cakes, starch, flakes, canned beans, fried nuts, peanut butter	Straw, stem, leaves, husk, cobs, hulls and fibre, bran, germ, gluten, corn steep liquor, pods, shells, skin, defective and under-grade grains/seeds	Feed, organic acids, antibiotics, biofuel, enzymes, microbial lipids
Fruits and vegetables	Fruit and vegetable juice, syrup, dried fruits and vegetables, jams, marmalade, sauerkraut, and salad	Leaves, skins, roots, cores, rotten fruits, stems, seeds, peel, pulp, pomace, non-nutritive fibres, defective and under size fruits and vegetables	Polyphenols, carotenoids, pectin, enzymes, flavours, single-cell proteins, vitamins, pigments (natural food colours)
Fish and seafood	Canned fish, filleted fish, smoked fish, salted fish, processed crustaceans	Scales, fins, shells, bones, gut remains, fish oil	Omega-3 polyunsaturated fatty acids, collagen, gelatin, polysaccharides, minerals,

	and molluscs, fish meal, and fish oil		vitamins, antioxidants, enzymes, and bioactive peptides
Meat	Meat cuts (beef, pork, mutton, lamb), sausages, canned meat	Blood, hides, hair, head and horns, bones, hoof, white and red offal, carcass fat, scraps, meat trimming, feathers, feet, guts, giblets, poultry, and offal	Collagen (emulsifiers in meat products)
Milk	Milk and milk products example fresh milk, cheese, yoghurt, ice cream, butter, milk powder	Whey, wastewater	Protein concentrate, microbial polysaccharide
Beverages	Alcoholic and non-alcoholic beverages, for example, beer, wine, cocoa, tea, and coffee	Solid waste from malting and mashing, shells, pods, and pulp	Feed, enzymes, bioethanol, dietary fibres, phenolic compounds, organic acids
Edible oil and fats	Seed refined oil, hydrogenated fats	Shells of seeds, cakes, rancid oil	Feed, biodiesel, microbial polysaccharides
Sugars	Sugars, purified sugars, confectionary	Molasses, Dilute sugar solutions, bagasse	Ethanol, biogas

Source: Kasi *et al*. (2012)

2.2 Methods of waste management

Landfilling is one of the traditional methods of industrial waste disposal, whereby the waste is transported to the dumping site and then spread and compacted on the land. Wastes that end up in landfills produce a large amount of greenhouse gases such as methane (Picot-Allain *et al*., 2020). Excess amounts of greenhouse gases such as carbon dioxide absorb infrared radiations and heat the earth results causing global warming and climate change. Landfilling is inappropriate waste disposal because may lead to problems for human, animal, and plant health. Some of the wastes may be the source of diseases spread caused by microorganisms and poisonous gases produced by wastes during disposal (Kumar *et al*., 2019). Preventing the use of waste in landfill technology has improved the production of valuable products such as biofuels.

When anaerobic digestion occurs, wastes produce energy along with other forms of power generation, such as solar and wind. Anaerobic biotechnology is a sustainable technology because it produces renewable bioenergy and biofuels from wastes that preserve the environment and energy objectives (Kasi *et al*., 2012).

Some of the food industrial wastes are used in animal feeding. Wastes can be used directly or processed and then fed to livestock without any additional costs. This application is higher compared to other methods which incur extra costs and it has the advantage of reducing landfill pressure and reducing methane emissions, good for the economy as the farmers reduce the cost of buying animal feed (Dou *et al*., 2017). Apart from advantages the use of waste for animal feed has its advantages if the precaution will not be considered such as the availability of nutrients and antinutrient factors, and physical, chemical, and microbial safety of the waste.

Another application of industrial waste is used as fertilizer after decomposition. Wastes are rich in micro-organisms, micronutrients, carbon, and other nutrients such as potash, nitrogen, calcium, sulfur, magnesium, and phosphate that make appropriate utilization as fertilizer although agro-industrial waste contains components which not easily degraded and cause slow release of nutrients.

Incineration also is the traditional method of waste disposal in which the heating of the waste generates energy such as steam for processing purposes. Some of the incineration processes are done without energy recovery, the wastes are burnt to produce ash. This method of waste disposal is less applied due to environmental and human health impacts. The other method of traditional waste disposal is pyrolysis, whereby high temperatures are used to break down the hydrocarbon-containing wastes with no (pyrolysis) or less oxygen than incineration (Kasi *et al*., 2012).

2.3 Valorization of wastes

Waste valorisation is the process of converting waste materials into high-value products or extracting useful components from waste (Kartal & Otles, 2018) . Presently, technology has grown whereby waste is used to produce valuable products such as chemicals, materials, and fuels. Biomolecules like protein, lipids, starch, vitamins, mineral, fibre, and antioxidants present in food waste and by-products from industries can be separated individually using the extraction technique or used directly to make nutritional food products. The Valorisation process depends on the nature of the wastes and the type of value-added product to be obtained. Agro wastes are pre-treated and treated by the use of physical, chemical, or biological processes to convert organic matter into new value-added products or extract components of interest (Almaraz-Sánchez *et al*., 2022). This method of waste management is sustainable and efficient for food industries due to the large amount of waste produced by food industries.

Despite these good perspectives, the valorisation of agro-waste materials is also challenging for several reasons, the different stability of the added-value components during processing, the technological difficulty in large-scale production, the low energy efficiency and high costs of conventional extraction processes, and the use of non-food grade solvents during the conventional extraction processes. In this context, more research is needed to discover the cheapest ways of waste conversion resulting in high-quality value-added products (Socas-Rodríguez *et al*., 2021).

2.4 Biotechnological utilization of food industrial waste

Biotechnological processes play a big part in the economical ways of food waste bioconversion into valuable products (Sarfraz *et al*., 2023). Biotechnologically food wastes are used as components of microbial media in the production of value-added products, some of the products are cellular biomass of yeasts, biofuels, microbial polysaccharides, single cell protein, lipids, carotenoids, enzymes, antibiotics, organic acids (Kot *et al*., 2020).

2.4.1 Production of organic acids

Organic acids are chemical compounds widely used in the food, chemical, agriculture, and pharmaceutical industries. Citric acid, succinic acid, lactic acid, itaconic acid, lactobionic acid, gluconic acid, propionic acid, fumaric acid, oxalic and acetic acid are the major type of organic acids produced biotechnologically by microbial activity (Ahmad *et al*., 2023). Food waste can be used as the substrate for organic acid production through the fermentation process. Cabbage was used by Li *et al*. (2023) to produce lactic acid through anaerobic fermentation with the addition of carbon sources (glucose,

fructose, sucrose, and molasses). The results revealed that the addition of fructose and molasses increases lactic acid production due to carbon source enriched the acid-producing bacteria such as *lactobacillus* species.

2.4.2 Production of yeast biomass

Yeasts are microorganisms used in the production of fermented food such as bread, wine, and beer. The demand for yeast grew exponentially due to the increased manufacture of fermented food and the production of yeast protein concentrate. Biotechnology leads to an efficient and profitable process of yeast biomass using cheap substrates such as food waste. Palm oil mill wastewater was used in the production of yeast biomass using yeast isolated from stale palm oil mill effluent and palm oil mill effluent dump site, among 7 isolates obtained *Saccharomyces* from stale palm oil mill effluent produce the highest amount of biomass of 4.4 g/L after 96 hours of fermentation (Iwuagwu and Ugwuanyi, 2014).

2.4.3 Production of single-cell protein

Single-cell protein is the protein source from the microbial culture used as a protein supplement in human food or animal feed. Due to the high demand for protein worldwide, microorganisms like algae, fungi, yeast, and bacteria can be grown using cheap substrates like food waste and utilize the carbon and nitrogen present in the waste for the production of high-quality protein. Wastes like citric wastes, yam peels, and whey concentrate have been reported in the production of single-cell protein. Orange and lemon wastes were used by Mahan *et al*. (2018) for single-cell protein production. The maximum cell dry weight and total protein yield of 3.55 g/l and 1.77 g/l were obtained respectively after 48 hours of fermentation using orange agro waste as the substrate. This shows that the utilization of food industrial waste is applicable in the production of single-cell protein at minimum cost.

2.4.4 Production of lipid

Microbial lipids also are known as single-cell oil, microorganisms such as algae, fungi and bacteria are sources of microbial lipids. Lipids produced by microorganisms can be used for nutrition or fuel. Industrial food waste is mostly used as cost-effective substrates for fermentation during the production of lipids. Waste utilization in the production of microbial lipids reduces the current industrial production of bio fuel relies mainly on vegetable oil and hence can increase food security. After fermentation the extraction of the lipid is done by disruption of microbial biomass and solvent extraction applied. According to Lee (2017), lipids were successfully produced from corn bran hydrolysates by *Trichosporon oleaginosus* with lipid yields of more than 200 mg/g.

2.4.5 Production of microbial polysaccharides

Microbial polysaccharide is a polysaccharide produced by microorganisms such as archaea, fungi, bacteria and algae through the fermentation process. Microbial polysaccharides such as xanthan, dextran, alginate, gellan, polluan and curdian are used in food industries as an emulsifier, stabilizers, thickeners, texturizers, film formers, coating and gelling agents in food products like bread, sauce, syrup, ice cream, beverages and ketchup (Giavasis, 2013). Apart from application in food industries, these polysaccharides can be used in cosmetics and pharmaceutical industries. Food waste in the form of liquid such as syrup, whey, oil mill waste water or solid forms such as lignocellulosic biomass and fruit pomace can be used in the production of polysaccharides (Siddeeg *et al*., 2019). According to Alsudan (2021), Czapex Dox Broth medium contains different concentrations of banana powder as a source of carbon. *Trichoderma reseei* was used for fermentation, production of polysaccharides was maximum in 40 ml/l banana peels concentration the amount obtained was 0.54g/100 ml.

2.4.6 Production of carotenoids

Carotenoids are natural pigments that can be extracted from plants and algae or synthesized by various microorganisms including bacteria, algae, yeast and fungi through the fermentation process. After fermentation, carotenoids are usually obtained by disruption, solvent extraction and concentration. Carotenoids are mostly used in the food, feed and pharmaceutical industries. Cheap industrial by-products used in the production of microbial carotenoids. Carotenoids contain biological properties such as antioxidant, anti-inflammatory, antitumor and pro-vitamin A (Cardoso *et al*., 2017).

2.4.7 Production of antibiotics

Antibiotics are secondary metabolites products that inhibit the growth of bacteria or kill bacteria. Food wastes such as cassava peels, corn pomace, corn cob, sugarcane bagasse, rice husk and rice stem have been used as substrates for antibiotics production by microorganisms. Antibiotics such as tetracycline and streptomycin are among the common antibiotics produced through microbial processes (Sadh *et al*., 2018).

2.4.8 Production of biofuels

Biofuels are energy delivered from natural sources such as bio-based raw materials. The most common biofuels produced using agro wastes are biogas and bioethanol. These biofuels are mostly used for heating and electricity generation. Due to the high cost and environmental pollution of conventional energy sources (petroleum fuels) that are delivered from crude oil most countries around the world preferred the use of biofuels (Yafetto, 2022). Agro wastes such as bagasse, fruits and vegetable wastes,

kitchen waste, straws, and livestock manure can be converted into biofuels. The process of waste conversion into biofuels can be done through anaerobic and aerobic digestion and microbial fermentation (Zeng *et al*., 2022).

2.5 Food waste used in enzyme production

It is possible to obtain enzymes by a fermentation process using a wide range of waste materials. Food wastes are good sources of nutrients needed for microbial growth. Production of enzymes from food wastes by fermentation such as fruits peels, bagasse, sugar beet pulp, or coffee pulp can be applicable because they contain large amounts of lignocellulosic substances such as cellulose, hemicelluloses, and pectin which could be inducers for cellulases, xylanases, and pectinases respectively (Jaiswal & Ravindran, 2016). According to Jahan *et al*. (2017), five agro-industrial wastes (apple peels, orange peels, lemon peels, potatoes peels and wheat bran) were used for the production of Polygalacturonases enzymes by *Bacillus licheniformis*. These agro-industrial wastes were selected because of their easy availability and can be obtained in large quantities at low cost. Among these agro-industrial wastes studied, fermentation broth having wheat bran 1% as the substrate produced a high yield of Polygalacturonases enzymes. The medium also was supplemented with nitrogen sources in a combination of $NaNO_3$ and yeast extract while KH_2PO_4 was selected as a suitable micronutrient.

2.6 Biotechnological application fruits waste

Items removed from fruits are leaves, peels, pomace, skins, rinds, cores, pits, pulp, stems, seeds, twigs and spoiled fruits. These parts account for more than 50% of fresh fruits and contain a higher amount of nutrients than final products (Torres-León *et al*., 2018). Most fruits waste contains bioactive compounds such as carotenoids, polyphenols, dietary fibres, vitamins, enzymes and essential oils which can be extracted and used as food ingredients or pharmaceutical compounds. Fruits peels are one of the wastes which can be used as substrate in enzymes production. Fruits producing a high amount of wastes make an interest to use the materials in fermentation process. Pectin found in the fruit wastes such as peels acts as an inducer for the production of pectinase enzymes. Therefore, when the fruits waste is used as substrate there is no need for the addition of pectin in the substrate. Mango waste from the industry was used by Purnachandra Reddy and Saritha (2015) in the production of pectinase enzymes a researcher select the mango waste as the waste contains enough amount of pectin. Fruits waste mainly can be used alone and can be mixed with other agro-industrial in solid-state fermentation for enzyme production (Patidar *et al*., 2018). Some of the works of literature show that fruit waste can be mixed with grain waste such as rice or wheat bran in enzyme production. According to Aggarwal *et al*. (2020),

4 g of orange peels and 1g of wheat bran were mixed and used as substrates in the production of pectinase enzymes from *Candida* isolated from textile mill effluent. Ortiz *et al*. (2017) observed that the use of three types of agro-industrial residues (wheat bran, Orange and lemon peels) were mixed and used as a substrate for pectinase enzyme production by *Aspergillus giganteus*. The results obtained showed that there are positive results of enzyme production when the wastes were mixed in solid-state fermentation.

Citrus wastes are the ones used in the production of enzymes due to the high amount of nutrients which support the growth of microorganisms and enzyme production. The following table 2.2 shows the composition of orange processing wastes.

Table 2. 2: Composition of Orange (*Citrus sinensis* L.) Waste in Percentage of Dry Basis

Component	**Value %**	**References**
Crude protein	7.7 - 16.5	de la Torre *et al*. (2019)
Total sugar	24. 1 - 43.1	Zema *et al*. (2018)
Pectin	20.0 - 25.4	Satari and Karimi (2018), Rathinavel and Saravanakumar (2021)
Cellulose	18 - 20	de la Torre *et al*. (2019)
Hemicelluloses	8.6 - 21.3	Li *et al*. (2015)
Lignin	1.2 - 7.52	Panwar *et al*. (2019)
Fat	1.98 - 4.4	Satari and Karimi (2018)
Ash	1.2 - 5.51	Olabinjo *et al.* (2017), Abdelhafez (2014)

2.7 Enzymes and their application in food industries

Enzymes are biological catalysts which catalyse and control all chemical reactions in living cells. Facilitate the chemical reaction by reducing activation energy hence the rapid formation of products and they are specific in their reactions (Nielsen, 2017). Sources of enzymes are plants, animals and microorganisms. Microbial enzymes take part as important living cells in the production of enzymes due to high yield, sustainability of enzyme production, low cost, easily predicted and controlled non-toxic and environmentally friendly. Microbial enzymes are easily isolated and produced in large quantities because microorganisms have a high growth rate compared to plants and animals, easily manipulated to increase their activities in enzyme production (Anbu *et al*., 2015). According to Satapathy *et al*. (2020), pectinase enzymes is the one which is produced by microorganisms. It consists of hydrolases and lyase enzymes that break down pectin at specific sites as shown in figure 2.1.

Classification of pectinase enzymes based on their mode of action during hydrolysis of pectin. The structure of pectin is very complex. Pectin structure consists of a D-galacturonic acid chain, which is linked together by α (1–4) and the carboxylic group of galacturonic acids are partially esterified with methanol (Nighojkar *et al*., 2019).

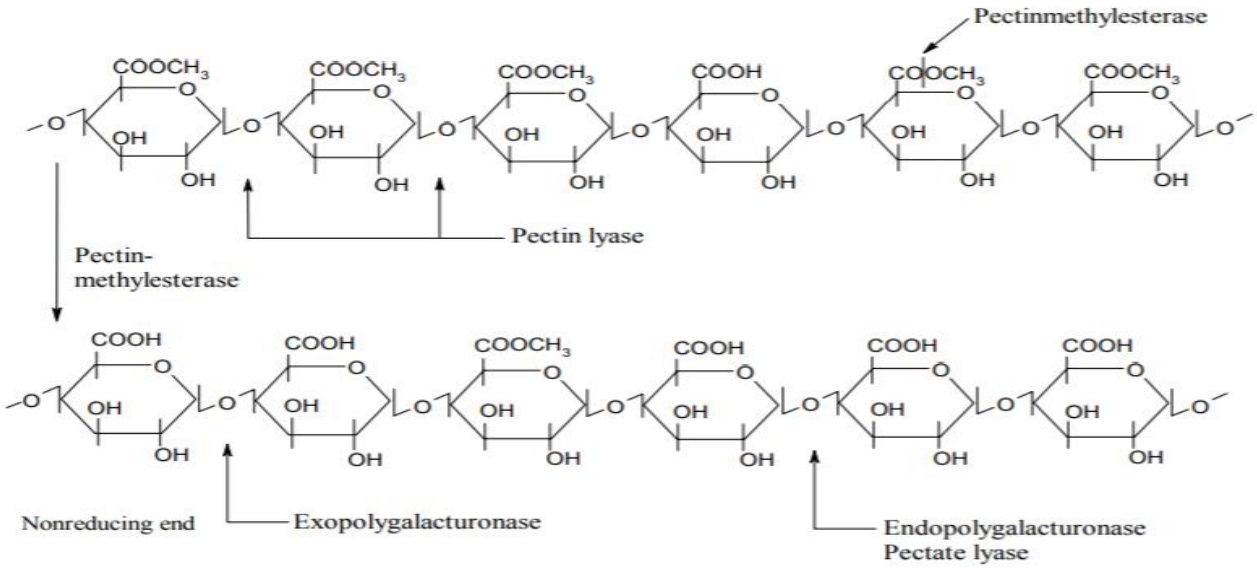


Figure 2. 1*: Action of Pectinolytic Enzymes on Different Sites of Pectin Substance*

Source: Garg *et al.* (2016)

Microbial enzymes have a wide range of applications in different industries such as food industries; can be used in baking, dairy, and alcoholic and non-alcoholic beverages. Other sectors are pharmaceuticals, waste management, organic synthesis, detergents, cosmetics, textile, leather, biopolymer, feed processing, paper and pulp (Singh *et al*., 2016). Table 2.3 shows the application of enzymes by percentages in different industries.

Table 2. 3 : Global Enzymes' Market Share by Application (2015)

Application	Food and beverages	Cleaning agents	Animal feed	Agro fuel	Others
Market share %	35	25	20	10	10

Source: Guerrand (2018)

The growth of global industrialization leads to an extensive increase in enzymes utilization in food processing industries. Food and beverage application accounts for 35% of the global enzymes market and the market is continuing to grow by more than 7% annually (Tolkacheva *et al*., 2018). Most of the enzymes in the market are produced in North America, Europe and Asia countries. In Africa, there is no evidence of enzyme production for business or industrial applications. Most of the enzymes are imported from outside countries.

Pectinase enzymes mostly are used in the juice and wine industries. According to Ahmed *et al*. (2016), the pectinase enzymes account for 25% of food enzymes sales worldwide. Pectinase enzymes are produced by microorganisms that degrade pectic polymers. Degradation of the pectin found in fruit pulp resulted in maximum juice extraction and clarification. Pectinase enzymes are produced by both prokaryotic and eukaryotic microorganisms. Bacteria, fungi and yeasts have been discovered for the production of industrial pectinolytic enzymes. Alkaline and acid pectinases are mostly produced by prokaryotic and eukaryotic respectively. By eukaryotic microorganisms; fungi are used to synthesize acid pectinases. Yeast has also been found in the production of these enzymes, that was proved Daskaya-Dikmen *et al*. (2018) pectinolytic activity of 0.76- 1.73 U/ml was produced by yeast isolates (*Rhodosporidiobolus*, *Cystofilobasidium* and *Yamadazyma* at a temperature of 15℃. According to Ahmed and Sohail (2020), pectinase enzymes were prepared from *Geotrichum candidum* AA15. The activity of the enzymes was characterized. The highest activity was obtained when enzymes were incubated at 35°C for 25 minutes at a pH of 5.

Optimum pectinase enzyme production from microorganisms varied greatly with the type of strains, environmental parameters and the nutritional composition of the substrate used. Microorganisms can synthesize pectinolytic enzymes with different action mechanisms and biochemical properties. Suitable strains for high pectinolytic activities can produce efficient pectinase enzymes for industrial applications. The study was conducted by Heerd *et al*. (2014) using two strains of *Aspergillus sojae*, *Aspergillus sojae* ATCC 20235 showed 6.9 times higher pectinolytic enzyme production than *Aspergillus sojae* CBS 100928 under optimized condition. In most cases, the microorganisms with pectinolytic activities are isolated from the soil and decomposed organic materials like rotten fruits.

Among them, soil samples are commonly used in fungi isolation. 55 fungi isolated from 20 soil samples by Kc *et al*. (2020) were primarily screened to obtain 14 isolates. Among 14 isolates 4 strains of *Aspergillus* species showed pectinolytic activities after secondary screened.

Aspergillus brasiliensis is useful in food industries for a long time and safety examination show that the products produced are Generally Regarded As Safe (GRAS) by the United States Food and Drug Administration (Frisvad *et al*., 2018). *Aspergillus brasiliensis* strains have efficient machinery for enzyme secretion which release into culture medium hence easily isolated. *Aspergillus brasiliensis* can produce several enzymes such as lipase, prolyl endopeptidase, mannose, xylanases, glucoamylase, pectinase, phospholipase, lysozyme and naringinase (Li *et al*., 2020). By submerged fermentation in shake flasks for 10 hours *Aspergillus* can produce up to 7.24g/l of pectinase enzymes (Ahmed *et al*., 2016). Although *Aspergillus brasiliensis* is Generally Regarded As Safe, mycotoxins produced by *Aspergillus brasiliensis* lead to needing much attention during the production of enzymes (Frisvad *et al*., 2018). Therefore, it is necessary to screen non-mycotoxins-producing strains for the production of food enzymes. Another method that can be applied is blocking or deleting the gene cluster responsible for mycotoxins biosynthesis (Li *et al*., 2020).

Production of pectinase enzyme can be either by solid-state fermentation or submerged fermentation. Utilization of low-cost substrate, simplicity of the process, increased yield, low contamination risk and energy requirement make solid-state fermentation preferable to submerged fermentation (de Castro & Sato, 2015; Kapoor *et al*., 2016). Maximum pectinase production and activity depend on the parameter maintained during the fermentation process. The final yield of the pectinase enzymes depends on process variables such as type of strain used, pH, temperature, salt concentration, Particle size, moisture content, aeration, time of incubation, type of substrate used, additional nutritional source and inducers (Amin *et al*., 2019). El-Bakry *et al*. (2015) stated that the initial parameters of the substrate may vary depending on the type of substrate and microorganism used. *Aspergillus sojae* was used in the production of pectinase enzymes showed that at 37 °C temperature and moisture of 62% and fermentation time of 4 days pectinase enzymes activity obtained was 536U/g. The incubation period can be monitored for maximum production of enzymes. Maximum yield of alpha-amylase (0.281 U/ml) was observed on the 3rd day of the incubation period followed by days 4, 5 and 6 according to studies conducted by Behailu and Abebe (2018) *Aspergillus brasiliensis* FAB-211 was used and the best production parameters were initial moisture content of 60%, pH of 5 and 30°C. Ruiz *et al*. (2012) used the same condition but change the moisture content to 70% using the lemon peels as the substrate the pectinase enzyme activity obtained was more than 2000 U/L. Depending on the type of substrate and

parameters of the fermentation process the maximum production and activities of the pectinase enzyme can be achieved.

2.8 Fruit juice extraction and clarification.

Fruit juice extraction is one of the processing methods of fresh fruits which makes the availability of fruit products throughout the year. Most of the fruits are produced seasonally and are perishable thus need processing effort to avoid their loss. Production of fruit juice requires a proper method for extraction, clarification and stabilization (Sharma *et al*., 2017). Traditional methods of juice extraction are mechanical crushing and pressing. Mechanical crushing of the flesh of the fruit rich in pectin results in viscous juice from which it is difficult to extract the maximum amount of the juice by pressing. The juices remain in the residues after pressing (Shiv, 2015). When crushed, the fruit pulp treated with enzymes like pectinase enzymes extraction yield, taste and colour of the product will be improved. A cocktail of enzymes can be used during juice extraction and clarification to increase juice yield. Apart from different enzyme combination, the juice extraction and clarification rate depend upon incubation time, temperature, enzyme concentration, agitation and pH. Evaluation for juice extraction and clarification of plum, peach, pear and apricot juices was conducted by Joshi *et al*.(2011) using pectinase enzyme produced by *Aspergillus brasiliensis* in solid-state fermentation of apple pomace. The results obtained show that the juice increased significantly from 52% to 78% in plum, 38% to 63% in peach, 60% to 72% in pear and 50% to 80% in apricot. Colour, total soluble solids (TSS), titratable acidity and total sugars also increased with the addition of the pectinase enzyme. The best concentration of the enzyme used was 2.5% (Nighojkar *et al*., 2019). Most factors affecting juice clarification are incubation time followed by temperature and enzyme concentration respectively. This is according to Ahmed and Sohail (2020) who studied the optimum clarity of orange juice and the results obtained was 61% when enzymatic treatment of juice performed with 4% (v/v) crude pectinase was prepared from *Geotrichum candidum* AA15 for 180 minutes at 30°C. According to Ajayi *et al*. (2014), the investigation revealed that the enzymes produced in the laboratory using *Aspergillus brasiliensis* are needed in minimum volume for juice clarification than the commercially produced pectinase enzymes**.**

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The research was conducted in Nakuru County. Laboratory activities were conducted at the Department of Dairy and Food Science and Technology, Microbiology and chemistry laboratories of Egerton University.

3.2 Objective 1: Characterization of pectinase-producing *Aspergillus brasiliensis* strain isolated from rotted orange peels.

3.2.1 Rotted orange peel sampling.

The oranges (Valencia variety) were sourced from the market and then peeled. The peels were stored inside a sterile polythene bag for a period of seven days at room temperature (ranges 18°C -22°C) until they rot.

3.2.2 Isolation and characterization of *Aspergillus brasiliensis*

The rotted orange peel samples were cut into small pieces (1 cm) with a sterile scalpel and placed into the sterile petri dishes with sterilized and solidified Potato dextrose agar (PDA). The PDA was prepared by weighing 39 grams and addition of distilled water up to 1000mls. The media was then incorporated with chloramphenicol (0.25g/l) to hinder the growth of bacteria. The plates were incubated at 30°C for 5 days. The growth of the fungal colonies was observed on the petri dishes after the stipulated incubation period. Different colonies were selected and sub-cultured onto two different PDA petri dishes and incubated at 30˚C for 5 days. Pure cultures of fungi were streaked on freshly prepared PDA media and later stored at 4°C for further study (Amilia *et al*., 2017).

3.2.3 Morphological identification

Characteristics based on morphology were used in identifying the *Aspergillus brasiliensis* microorganism. For microscopic study, the fungal isolate was identified under the humascope premium microscope using a 400× total magnification, after being placed on a glass slide using transparent seal tape in the presence of lactophenol cotton blue dye. Other petri dishes containing isolated fungal strain were stored at 4°C for further molecular characterization (Alsohaili & Bani-Hasan, 2018).

Table 3.1: Macroscopic and Microscopic Characteristics for Fungi identification

Macroscopic characteristics	Initially colony Growth	Surface colony colour	Reverse colony colour	Colonies diameter range	Colony Texture
Microscopic characteristics	Conidiophore	Vesicle	Phialide	Metulae	conidia

3.2.4 Molecular characterization

Spores of *Aspergillus brasiliensis* were prepared from a fully sporulated (7 days old) PDA media. About 30-100 mg (wet weight) if the fungal cells that were re-suspended in up to 200ul of the isotonic buffer (Phosphate Buffered Saline) were added to a bashing bead lysis tube (0.1 mm and 0.5 mm). Thereafter, 750ul bashing bead buffer was added to the tube. The resultant was secured in a bead beater that is fitted with a 2ml tube holder assembly and processed at maximum speed for 2-5 minutes. The bashing bead was centrifuged in a micro centrifuge at 10000xg for 1 minute. The centrifuged bashing bead was transferred up to 400ul supernatant to a Zymo Spin III filter in a collection tube and then centrifuged at 8000 x g for 1 minute. About 1200 ul of the genomic lysis buffer was added to the filtrate in the collection tube from the previous step. The mixture (800ul) was then transferred from the step above to a Zymo Spin IICR column 3 in a collection tube and centrifuged at 10000 x g for 1 minute. About 200ul of DNA pre-wash buffer was added to the new collection tube and centrifuged at 1000 x g for 1 minute. The g- DNA wash buffer (500ul) was added to the Zymo Spin IICR column and transferred to a clean 1.5 ml micro centrifuge and 100ul (min 35ul) of DNA elution buffer was added directly to the column matrix. The resultant was then centrifuged at 1000 x g for 30 seconds to elute the DNA (Angebault *et al*., 2020). The DNA samples were then sent to Inqaba Biotechnical Industries Ltd, Pretoria, South Africa, for DNA amplification and internal transcribed sequence (ITS) (5.8S rRNA-ITS gene regions for the fungal isolates using the primer pairs; ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for identification (Muriithi *et al*., 2022). For phylogenetic analysis, the partial rRNA gene nucleotide sequences were analyzed and determined using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi.). Consensus sequences were aligned and deposited in the GenBank database and they were assigned unique accession numbers.

3.3 Objective 2a: To optimize parameters for pectinase enzyme production by *Aspergillus brasiliensis* using orange waste as substrate.

3.3.1 Substrate preparation

The orange fruit waste was produced in a food processing plant. Oranges (Valencia variety) were squeezed to produce juice and the remaining wastes were cleaned by distilled water to remove pulp and seeds, chopped in small particles and dried at 60°C for 48 hours. The portion of dried orange waste was ground into powder, and stored in air tight container for future use.

3.3.2 Proximate analyses for Nutritional Composition of orange peels

The proximate compositions of the orange waste powder were determined using standard methods to know the nutritive properties. All measurements were done in triplicates and values were presented in percentages.

Moisture content

The moisture content of the dried orange wastes was determined using the air oven method as per AOAC (2005), method number 967.19. About 2 g of grounded orange wastes samples were weighed and then placed on the shelf of the oven involving a single-stage air oven drying at 105˚C for 3h. Total moisture loss was calculated using the equation below and expressed as a percentage of the original sample weight.

$$\text{Moisture content (\%)} = \frac{weight\ before\ drying - weight\ after\ drying}{weight\ before\ drying} \times 100 \quad \text{..... Equation 3.1}$$

Ash content

The ash content of dried orange wastes was determined using AOAC (2005), method number 930.05. About 2 g of dried orange waste samples were weighed and placed into a dry crucible. The samples were then charred with a flame to decompose all the organic components. The samples were then ashed into a muffle furnace and incinerated at 550°C for 6 h. The samples were cooled to room temperature in a desiccator and then weighed.

$$\%\ Crude\ ash = \frac{Weight\ of\ ash}{original\ weight\ of\ sample} \times 100 \quad \text{..... Equation 3.2}$$

Crude fat

The crude fat was determined by Soxhlet extraction according to AOAC (2005), method number 930.09. Approximately 4 g of Orange wastes samples were weighed and placed into an extraction thimble then covered with cotton wool. Petroleum ether (Hydrocarbons, C6, Isoalkalines, <5% n-Hexane) was added into a clean dried flat-bottomed flask (receiver flasks). The thimbles and contents were placed into the Soxhlet extractor and the receiver flasks were connected to the extractor. Petroleum ether was added to fill the Soxhlet extractor and left to drain down the flasks and more solvent was added to the Soxhlet extractor about half full. Condenser was connected to the Soxhlet extractor and placed the whole set up to the heating system. After 6 hours the thimbles were removed and the solvent (petroleum ether) in a receiver flask was evaporated by drying in an air oven at 105˚C for 30 mins, cooled in a desiccator then weighed and recorded.

Calculations were done using the formula below;

$$\%\ Crude\ fat = \frac{(W_1 - W_2)}{W_1} \times 100 \quad \text{..... Equation 3.3}$$

Where;

W_1= Weight of sample before extraction

W_2= Weight of sample after extraction

Crude protein content

The crude protein content of the orange wastes was determined by the Kjeldahl method according to AOAC (2005), method number 978.04. About 0.2 g of orange waste samples were weighed into a test tube and digested using concentrated H_2SO_4 in the presence of selenium catalyst until the colour change is observed (blue). The digest later underwent steam distillation using 40% NaOH. Ammonia was released and then trapped in a solution of boric acid. The distillate was collected and about 60mL was titrated with 0.1 M HCl in the presence of methylene blue indicator until the colour change was observed (orange). The protein content was determined by multiplying the per cent nitrogen content by 6.25 (see equations 3.4 and 3.5).

$$\text{Nitrogen (\%)} = M\text{HCL} \times \frac{Corrected\ Acid\ volume}{Weight\ of\ sample} \times \frac{14gM}{Mol} \times 100 \quad \text{.....Equation 3.4}$$

$$\text{Protein (\%)} = \text{Nitrogen (\%)} \times 6.25 \quad \text{..... Equation 3.5}$$

Where:

Corrected acid volume = (volume of acid sample – volume of acid blank),

M HCl = Molarity of HCl,

14g= atomic weight of nitrogen

6.25 = the conversion factor (on the assumption that the sample contained 16% nitrogen).

Crude fibre

The crude fibre was determined using AOAC (2000), method number 987.10. About 2 grams of orange waste samples were weighed and placed in a beaker. About 100 ml of hot water was added before adding 25 ml of 2.04 N H_2SO_4 and then volume was increased with hot water to 200 ml and residues were boiled for 30 minutes, volume was kept at 200 ml by constantly adding hot water. The residues were removed and filtered using a stick packed with glass wool. The contents were washed three times and 100 ml of hot water was added before adding 25 ml 0f 1.78 N KOH then the volume increased to 200 ml with hot water, and boiled for 30 minutes while kept at 200 ml by constantly adding hot water. The filter stick remained in the solution with the residues. The residues were filtered and washed three times with hot water. Residues and glass wools were transferred into 75 ml porcelain. The residues were washed with ethyl alcohol to remove pigments like chlorophyll and carotene. Glass wools were pushed out into a crucible and remained samples were wiped out using glass wool soaked with ethyl alcohol. The crucibles were dried with their residues in an oven at 105 ℃ for 2 hours, cooled in a desiccator and weighed accurately. The crucible with residues was placed in a muffle furnace maintained at 550°C for four hours, cooled to about 100 ℃ before being cooled in a desiccator to room temperature (22°C) and weighed.

$$\%\ Crude\ fibre = \frac{(W_1 - W_2)}{W} \times 100 \quad \text{..... Equation 3.6}$$

Where;

W_1= Weight of acid and alkali digested sample

W_2= Weight of the incinerated sample after acid and alkali digestion

W= Weight of sample

Note:

W_1 = (Crucible +Residue weight) - Crucible

W_2 = (Crucible + Ash weight) – Crucible

Total carbohydrate

Total carbohydrate content was obtained by difference. (Ani & Abel, 2018).

% carbohydrate = 100% - (% moisture + % protein + % fat + % ash + % crude fibre)……Equation 3.7

3.3.3 Fermentation process

About 30g of dried orange waste and distilled water were added to adjust moisture to 70% in 500 ml media bottles. Bottles containing orange wastes were sterilized at 121°C for 15 minutes and cooled at room temperature. Inoculation was done aseptically with 3 ml of spore suspension mixed well and incubated at different temperatures 25°C, 30°C and 35°C. In each substrate, the initial pH of the substrate was adjusted to 4.0, 5.0 and 6.0 using 0.1 M NaOH. Each bottle was monitored for 4, 6 and 8 days (See Appendix A). After incubation enzymes were extracted using 0.05% tween 80 1:1 (w/v) ratio. Then the mixture was clarified by filtration through a cotton cloth, and then taken into the centrifuge at 232 RCF for 30 minutes. The clarified supernatant obtained was used as crude pectinase enzymes (Salim *et al*., 2017).

3.3.4 Pectin Agar plate assay

The pectinase activity of the enzyme was determined by the plate method described by Amanat *et al*. (2019). About 6mm size wells were made on a solidified medium composed of 1.5g agar and 0.2g pectin dissolved and boiled in 100ml of distilled water. Wells were filled with 60 µL of enzyme and incubated for 24 hours at room temperature (21°C). After incubation, the Petri dishes were flooded with iodine solution as a maker of the clear zone. The results of clear zone diameter (mm) around the wells were recorded and expressed as pectinase activity.

3.3.5 Experiment design

The Response Surface Methodology (RSM) based Box- Behnken design (BBD) approach was used to optimize three significant factors for enzyme production (Nikku *et al*., 2008), temperature (25, 30 and 35 °C), time interval (4, 6 and 8 days), and pH (4.0, 5.0 and 6.0) were independent variables and enzyme activity was the dependent variable. A total number of 45 experiments of 15 runs with 3 replicates were carried out to estimate pectinase enzyme production (see Appendix A).

The statistical model was a second-order polynomial equation as shown:

$Y= \beta_0+ \beta_1x_1+\beta_2x_2+ \beta_3x_3 +\beta_{11}x_1^2 +\beta_{22}x_2^2+ \beta_{33}x_3^2+ \beta_{12}x_1x_2 + \beta_{13}x_1x_3 + \beta_{23}x_2x_3$

Where:

Y - Response (pectinase yield)

β_0 - model intercept

x_1x_2 and x_3 - independent factors under study (temperature, pH and time)

β_1, β_2, and β_3 - linear coefficient

β_{11}, β_{22}, β_{33} - quadratic coefficients

β_{12}, β_{13} and β_{23} - interaction coefficients

3.4 Objective 2b: To determine the activity of pectinase enzymes produced by isolated *Aspergillus brasiliensis* on pectin.

3.4.1 Polygalacturonase enzyme assay

Polygalacturonase enzyme activity was determined by a spectrophotometer using a Dinitrosalicylic acid (DNS) reagent (Miller, 1959). This method was used to determine the amount of reducing sugar released from the pectin substrate. 1 ml of crude enzymes was mixed with 1 ml of 1% citrus pectin in 0.2 M acetate buffer (pH 4.5) in a sterile tube and incubated at 35°C for 30 minutes. After incubation 0.5 ml of reaction mixture was added with 0.5 ml of DNS reagent (75 g of Sodium potassium tartrate, 4 g of sodium hydroxide and 2.5 g of DNS in 250 ml of distilled water) to stop the hydrolysis reaction. The mixture was shaken to homogenize the content and placed in a boiling water bath for 5 minutes for colour development, then absorbance was read at 540 nm spectrophotometrically. D-(+) - galacturonic acid monohydrate was used as a standard whereby one unit of polygalacturonase activity (Equation 3.8) was defined as the amount of enzyme that liberates 1 µmol of D galacturonic acid per minute at aforementioned.

$$\text{Polygalacturonase activity} = \frac{Galacturonic\ acid\ concetration \times Dilution\ factor}{212.15 \times 15} \quad \text{.....Equation 3.8}$$

3.4.2 Pectin esterase assay

The titrimetric method was used for the determination of pectin esterase activity based on the determination of the carboxyl group released due to the action of the enzyme on the pectin substance (Rouse and Atkins, 1955). About 1 ml of crude pectinase enzymes was added to 15 ml of 0.5% citrus pectin in 0.1M NaCl, the pH was adjusted to 4 with 0.1 M sodium acetate buffer. The reaction was carried out at 45°C for 60 minutes and then held in a boiling water bath for 3 minutes to stop the reaction. The mixture was cooled to room temperature before being titrated with 0.01 M NaOH using methyl red as an indicator. One unit of pectin esterase was defined as the amount of enzyme that releases 1 µmol of carboxyl groups per minute (Equation 3.9).

$$\text{Pectin esterase activity} = \frac{ml\ NaOH \times Molarity\ of\ NaOH}{Time \times ml\ of\ sample} \quad \text{.....Equation 3.9}$$

3.4.3 Pectin lyase assay

Pectin lyase enzyme activity was determined by the method of coloured derivation. The following was the procedure for pectin lyase determination. A 0.5 mL of the enzyme solution was added to 0.5 mL of substrate solution (1% pectin solution) prepared in acetate buffer pH 6 and incubated in the water bath at 50 °C for 10 minutes. Then 100 µL of 1 N Sodium Hydroxide (NaOH) was added to the 1 mL reaction mixture and incubated for 5 minutes in a water bath at 80 °C and then cooled to room temperature. A 1.2 mL of 1 N Hydrochloric acid (HCl) and 1 mL of 0.04 M thiobarbituric acid solution were added to the cooled reaction mixture, incubated for a second time at 80 °C for 5 minutes and cooled before measuring the absorbance at 550 nm by using a UV-VIS spectrophotometer. One unit of pectin lyase activity was defined as µmol of unsaturated uronides formed per ml of supernatant (Babagil & Nadaroglu, 2021).

The same procedure was done to commercial pectinase enzyme under the optimum conditions as instructed by the producer. Commercial pectinase enzyme activity was used as a control to compare with the specific activity of crude pectinase enzymes under optimum conditions.

3.4.4 Protein quantification

Total protein was quantified by the Bradford method using Bovine Serum Albumin (BSA) as standard. Bradford reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol and 100 ml 85% phosphoric acid was added. The mixture was diluted to 1 litre when the dye was completely dissolved and filtered using Whatman paper number 1. About 0.1 ml of each stock solution prepared with different concentrations of 0.1 to 0.6 mg/ml of BSA standard were added to 3

ml of Bradford reagent, mixed gently and put into a spectrophotometer at 595 nm absorbance and measured after 40 minutes. The standard curve of concentration versus absorbance was plotted (see Appendix C). Tubes with 0.1 ml of buffer (without protein sample) added with 3 ml of Bradford reagent were used as blank (Chang & Zhang, 2017). About o.1 ml of crude enzymes were added to 3 ml of Bradford reagent, mixed and put into a spectrophotometer at 595 nm absorbance. The Absorbance values obtained were used to calculate protein concentration from the bovine serum standard curve.

3.4.5 Experimental design

The Response Surface Methodology based Box- Behnken design (BBD) approach was used as explained by John and Soloman (2020) where temperature (25, 30 and 35°C), time interval (4, 6 and 8 days), and pH (4, 5 and 6) are the independent variables and enzymes activity (polygalacturonase, pectin esterase and pectin lyase) are the dependent variables. A total number of 135 experiments of 15 run with 3 replicates for 3 types of pectinase enzymes was carried out to estimate pectinase enzyme activity (see Appendix B).

The statistical model was a second-order polynomial equation as shown:

$$Y= \boldsymbol{\beta}_0+ \boldsymbol{\beta}_1\mathbf{x}_1+\boldsymbol{\beta}_2\mathbf{x}_2+ \boldsymbol{\beta}_3\mathbf{x}_3 + \boldsymbol{\beta}_{11}\mathbf{x}_1^2 + \boldsymbol{\beta}_{22}\mathbf{x}_2^2+ \boldsymbol{\beta}_{33}\mathbf{x}_3^2 +\boldsymbol{\beta}_{12}\mathbf{x}_1\mathbf{x}_2 + \boldsymbol{\beta}_{13}\mathbf{x}_1\mathbf{x}_3 + \boldsymbol{\beta}_{23}\mathbf{x}_2\mathbf{x}_3$$

Where:

Y - Observation (polygalacturonase, pectin esterase and pectin lyase activity)

$\boldsymbol{\beta}_0$ - model intercept

$\mathbf{x}_1$, $\mathbf{x}_2$ and $\mathbf{x}_3$ - independent factors under study (temperature, pH and time)

$\boldsymbol{\beta}_1$, $\boldsymbol{\beta}_2$, and $\boldsymbol{\beta}_3$ - linear coefficients

$\boldsymbol{\beta}_{11}$, $\boldsymbol{\beta}_{22}$, $\boldsymbol{\beta}_{33}$ - quadratic coefficients

$\boldsymbol{\beta}_{12}$, $\boldsymbol{\beta}_{13}$ and $\boldsymbol{\beta}_{23}$ - interaction coefficients

3.4.6 Characterization of polygalacturonase, pectin esterase and pectin lyase

Effect of pH and temperature on enzymes activities

The optimum pH of the enzymes was determined by measuring the polygalacturonase, pectin esterase and pectin lyase activity at different pH (3, 4, 5, 6, 7and 8) using 0.1 M sodium acetate buffer with

pectin as substrate. For temperature, the enzyme activity of each enzyme was investigated at various temperature ranges of 20 to 70 °C.

3.5 Objective 3: To evaluate the application of pectinase enzymes produced by isolated Aspergillus brasiliensis in apple juice extraction and clarification

3.5.1 Juice yield

Apples were purchased from the Nakuru market, washed and cut into small pieces. The pieces were mixed with distilled water in a 1:2 ratio (w/v) and blended in a blender. Centrifuge tubes with 0, 1, 2, 3, 4 and 5% crude enzyme were added with apple pulp up to 10 ml, immediately the tubes were incubated for 3 hours at 40°C then the reaction was stopped by boiling the reaction mixture for 3 minutes. The pulp was pressed and filtrated using cotton cloth to remove impurities, juice yield was determined as shown in equation 3.10 (Gani *et al*., 2021).

$$Juice\ yield = \frac{Amount\ of\ juice\ recovered}{Amount\ of\ pulp\ taken} \times 100 \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \text{Equation 3.10}$$

3.5.2 Juice clarity

About 0, 1, 2, 3, 4 and 5% of crude enzymes were added into 10 ml of extracted juice in centrifuge tubes, Tubes were left in the water bath for 3 hours at 40℃, and reaction mixtures were stopped by boiling for 3 minutes in boiling water then centrifuged at 1957 RCF for 15 minutes to remove impurities. Absorbance of the juice was determined at 660 nm absorbance using a spectrophotometer and distilled water was used as a reference (Shiv, 2015).

The pH of the juice

The experiment was done to test if there were changes in the pH of juice after the addition of different concentrations produced pectinase. pH of the juice was measured using a pH meter (AOAC 2005).

Pectin determination

The pectin content of clarified juice was determined using the gravimetric method (Wang *et al*., 2021). About 4g of each clarified juice was added with 2ml of NaOH left for 1 hour then 1 ml of 1N Acetic acid was added before the addition of 1 ml of 1M Calcium chloride left for 1 hour then the mixture with precipitate boiled in the water bath. The mixtures were filtered and washed using filter paper and dried in the oven at 105 ℃ overnight before weighing. Pectin content was expressed by the weight of the Calcium pectate formed after precipitation with $CaCl_2$. Calculations were done using the following formula.

$$X = \frac{(M-m)}{m0\ (100-w)} \times 100$$ ……Equation 3.11

Where X is the pectin content in percentage, M is the total weight of dried filter paper containing calcium pectate precipitate in (g), m is the weight of dried filter paper, m_0 is the weight of the sample (g) and w is the moisture of the sample.

3.5.1 Experimental design

Juice extraction and clarification of the CRD experiment with one factor which varies and 3 replicates were used as explained by Rana *et al*. (2017).

Statistical model

$y_{ij}=\mu + \tau_i+\varepsilon_{ij}$

Where: y_{ij} - observation of the i^{th} treatment and j^{th} replication

τ_i - effect of i^{th} level of treatment (crude enzyme concentration)

μ - Overall mean

ε_{ij} - random error term

3.6 Data Analysis

Statistical analysis was carried out in SAS software (2006) version 9.4 for objective 2 and objective 3 in t-test mean comparison. Minitab16 was used for objectives 2 and 3. Study hypotheses were tested by performing an Analysis of variance (ANOVA). The level of significance was established at $p < 0.05$ confidence level.

CHAPTER FOUR

RESULTS

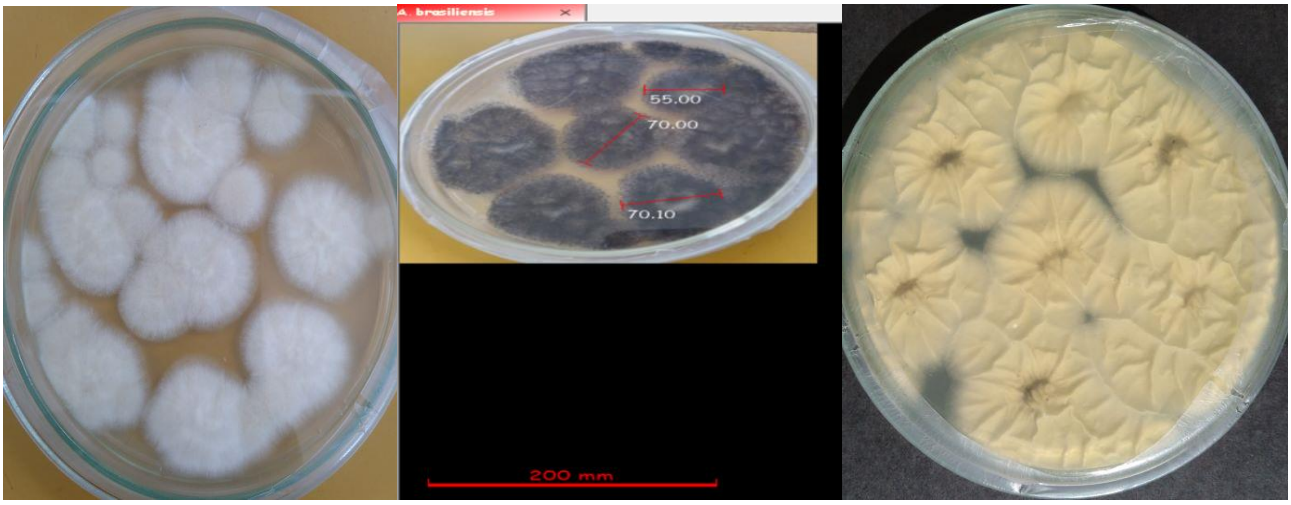
4.1 Macroscopic and microscopic features for *Aspergillus brasiliensis* identification

On PDA media fungi colonies were initially appear white, this was observed between the first to the third day of sub-culturing. On the surfaces of colonies hair-like structures were observed and fourth day the black spores started to sprout and increased as the number of incubation days increased. The Reverse of the colonies appeared cream and wrinkled (see figure 4.1).

Microscopic observation was conducted under a microscope for *Aspergillus brasiliensis* identification. A walled conidiophore was observed smooth and hyaline, ending in a bulb shape head called a vesicle covered with phialide and metule. Conidia spores were globular and radiated with a rough surface (See figure 4.2). All characteristics for *Aspergillus brasiliensis identification* summarizes in table 4.1.

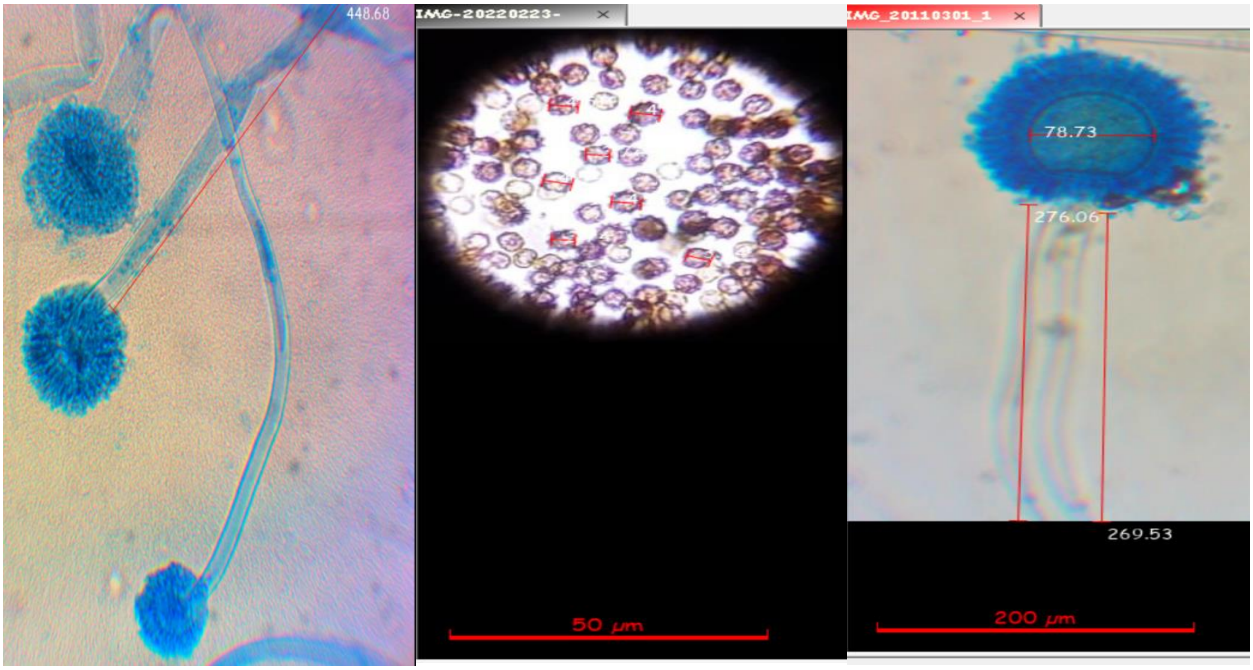
Table 4. 1: Morphological Characterization for *Aspergillus brasiliensis* Identification

Macroscopic characteristics	Initially colony Growth	Surface colony colour	Reverse colony colour	Colonies diameter range	Colony Texture
	White	Black	Cream and wrinkled	55-70 mm	Hairy like velvet
Microscopic characteristics	Conidiophore Long structure, walled, hyaline and smooth surface, length ≈ 269-448μm	Vesicle Bulb shape attached at the end of conidiophore, diameter ≈ 79 μm covered with phialide and metulae.	Phialide Biseriate	Metulae Attached with phialide	conidia Globular shape, diameter ≈ 4-5 μm, rough surface and radiate



A B C

Figure 4. 1: *A- Initial Growth of the Isolate on the third day, B- Growth of the Isolate on the Seventh Day with Black Spores (dimensions indicated), C-The Reverse of the Colonies.*



A **B** **C**

Figure 4. 2 : *A- Microscopic Views of the Isolate, B – Conidia with diameter scales, C- Vesicle and conidiophore dimensions.*

4.2 Molecular characterization of the isolated strain

Based on phenotypic characterization, the fungal isolates were presumptively identified as *A. brasiliensis.* For a precise identification to species level, the 5.8 S rRNA-Internal Transcribed Sequence

(ITS) were compared to sequences available in the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) in Gene Bank and the Ribosomal Database Project (RDP) database. The isolate's sequences exhibited high similarity to sequences in the database that enabled the identification of the isolates. The results showed 100% similarity to *Aspergillus brasiliensis* species and the consensus gene sequences were aligned and then deposited into the GenBank database with accession number OQ748632.

BASE COUNT 86 A 158 C 146 G 116 T

ORIGIN

1 CAACCTCCCA TCCGTGTCTA TTGTACCCTG TTGCTTCGGC GGGCCCGCCG CTTGTCGGCC

61 GCCGGGGGGG CGCCTCTGCC CCCCGGGCCC GTGCCCGCCG GAGACCCCAA CACGAACCCT

121 GTCTGAAAGC GTGCAGTCTG AGTCGATTGT TTGCAATCAG TTAAAACTTT CAACAATGGA

181TCTCTTGGTT CCGGCATCGA TGAAGAACGC AGCGAAATGC GATAACTAAT GTGAATTGCA

241 GAATTCAGTG AATCATCGAG TCTTTGAACG CACATTGCGC CCCCTGGTAT TCCGGGGGGC

301 ATGCCTGTCC GAGCGTCATT GCTGCCCTCA AGCCCGGCTT GTGTGTTGGG TCGCCGTCCC

361CTCTCTCCGG GGGGACGGGC CCGAAAGGCA GCGGCGGCAC CGCGTCCGAT CCTCGAGCGT

421 ATGGGGCTTT GTCACATGCT CTGTAGGATT GGCCGGCGCC TGCCGACGTT TTCCAACCAT

481 TCTTTCCAGG TTGACCTCGG ATCAGG

Sequence of Amplified ITS Region

Based on both macroscopic and microscopic characters the fungal isolate was identified as *A. brasiliensis* and molecular characterization by sequencing results confirmed the isolate to be *A. brasiliensis.*

4.3 Nutritional composition of dried orange peels

Nutritional composition of orange peels samples including moisture, crude protein, crude fat, Ash, Crude fiber and carbohydrate, were found to be 9.37%, 15.5%, 3.56%, 5.6%, 11.62% and 54.35% respectively.

The results show orange wastes contain organic matter mainly crude protein, fats and carbohydrates which are a good source of nutrients. The highest percentage of the nutrient was carbohydrates at 54% and the lowest percentage of fats at 3.6%

4.4 Optimization of parameters for pectinase enzymes production by *Aspergillus brasiliensis* using orange waste as substrate based on clear zone diameter

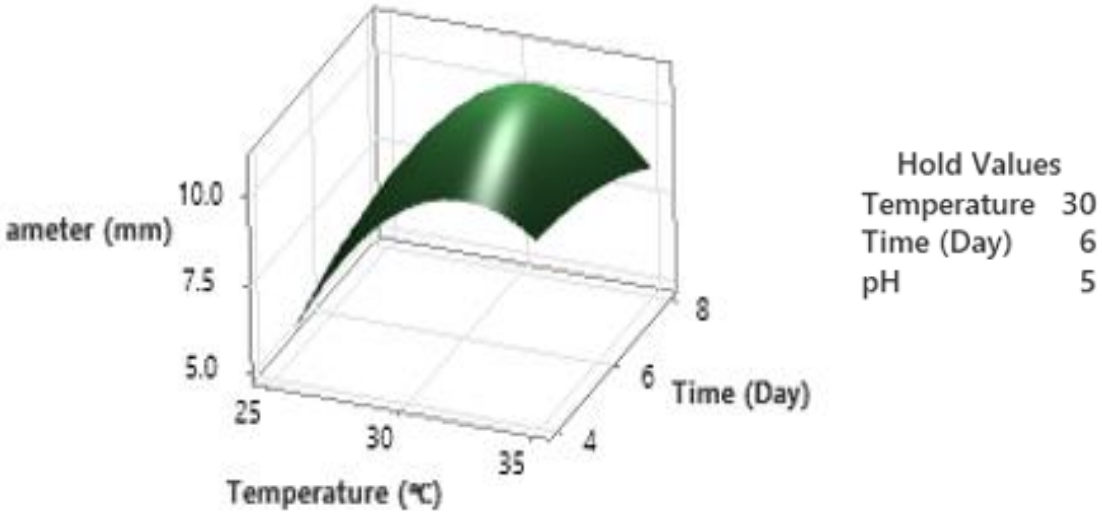
From the ANOVA results shown in Table 4.2, the model was significant. It was also observed that the three major factors and almost all interactions significantly affected the production of pectinase enzymes in terms of clear zone diameter except time in linear and in quadratic time and pH were insignificant (P> 0.05) on pectinase enzyme production. The interaction of temperature and pH also was insignificant (P> 0.05).

Table 4. 2: Analysis of Variance for Clear Zone Diameter

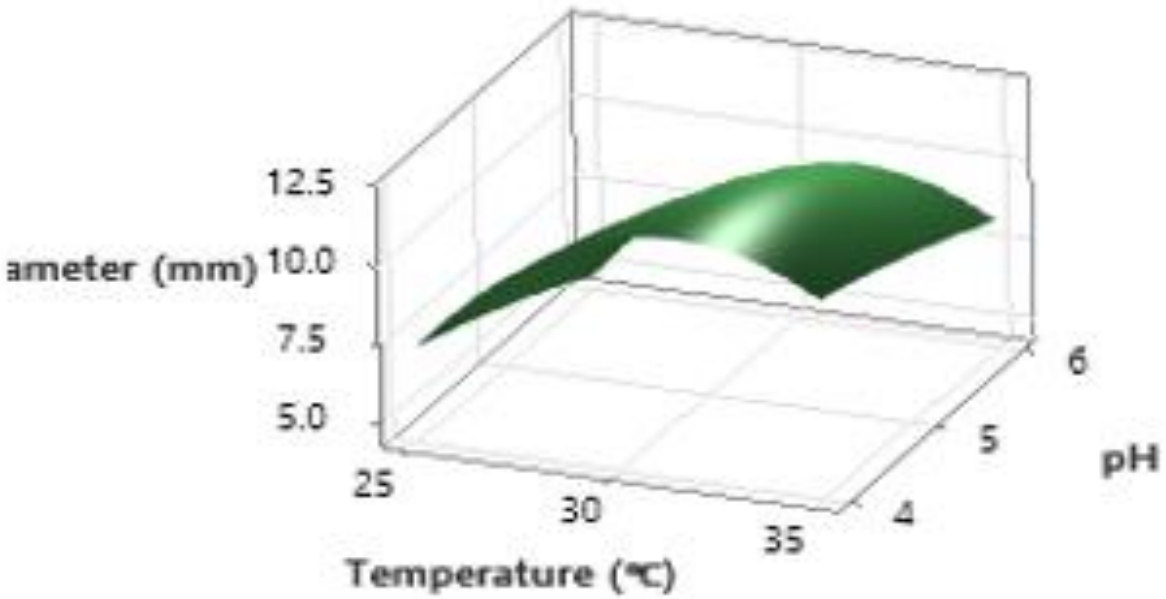
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	199.498	22.1665	15.12	0.000
Linear	3	106.061	35.3536	24.12	0.000
Temperature	1	70.727	70.7267	48.25	0.000
Time	1	0.050	0.0504	0.03	0.854
pH	1	35.284	35.2837	24.07	0.000
Square	3	79.713	26.5709	18.13	0.000
Temperature*Temperature	1	79.427	79.4270	54.18	0.000
Time *Time	1	1.379	1.3785	0.94	0.339
pH*pH	1	0.259	0.2585	0.18	0.677
2-Way Interaction	3	13.725	4.5750	3.12	0.038
Temperature*Time	1	7.521	7.5208	5.13	0.030
Temperature*pH	1	0.041	0.0408	0.03	0.868
Time*pH	1	6.163	6.1633	4.20	0.048
Error	35	51.305	1.4658		
Lack-of-Fit	3	6.122	2.0408	1.45	0.248
Pure Error	32	45.182	1.4119		
Total	44	250.803			

Clear Zone Diameter = -120.2+7.187 Temperature + 5.20 Time (Day) + 2.12 pH -0.1071 Temperature* Temperature-0.0882 Time (Day)*Time (Day)-0.153 pH* pH - 0.0792 Temperature*Time (Day) + 0.0117 Temperature*pH- 0.358 Time (Day) * pH

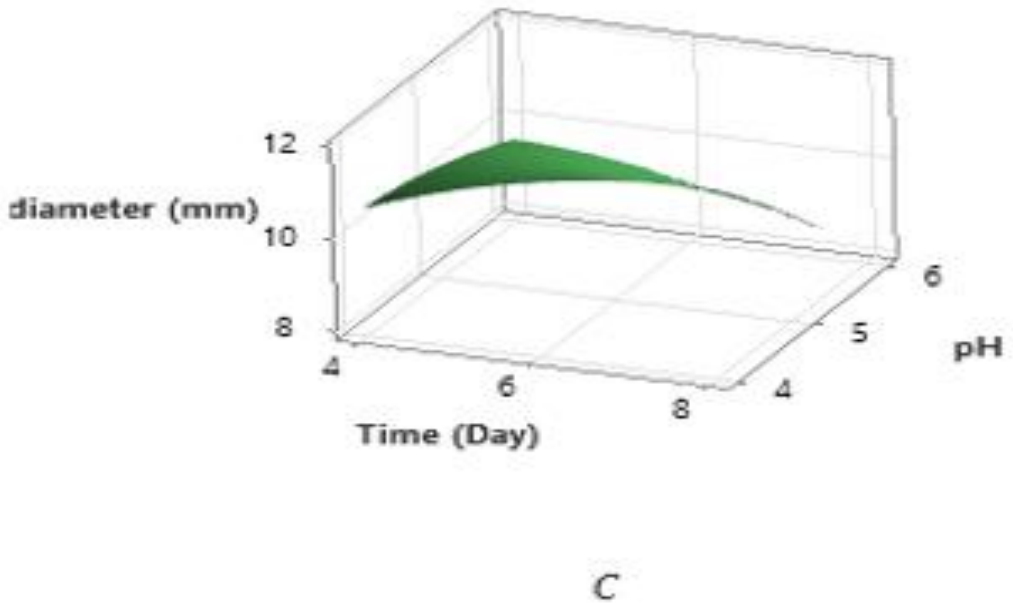
Regression Equation of Clear Zone Diameter……Equation 4.1


ameter (mm)
10.0
7.5
5.0
25
30
35
Temperature (℃)
4
6
8
Time (Day)
Hold Values
Temperature 30
Time (Day) 6
pH 5

a


ameter (mm)
12.5
10.0
7.5
5.0
25
30
35
Temperature (℃)
4
5
6
pH

b



C

Figure 4.3: *Surface Plot showing the interaction of (a) temperature and time (b) temperature and pH (c) time and pH on Clear Zone Diameter*

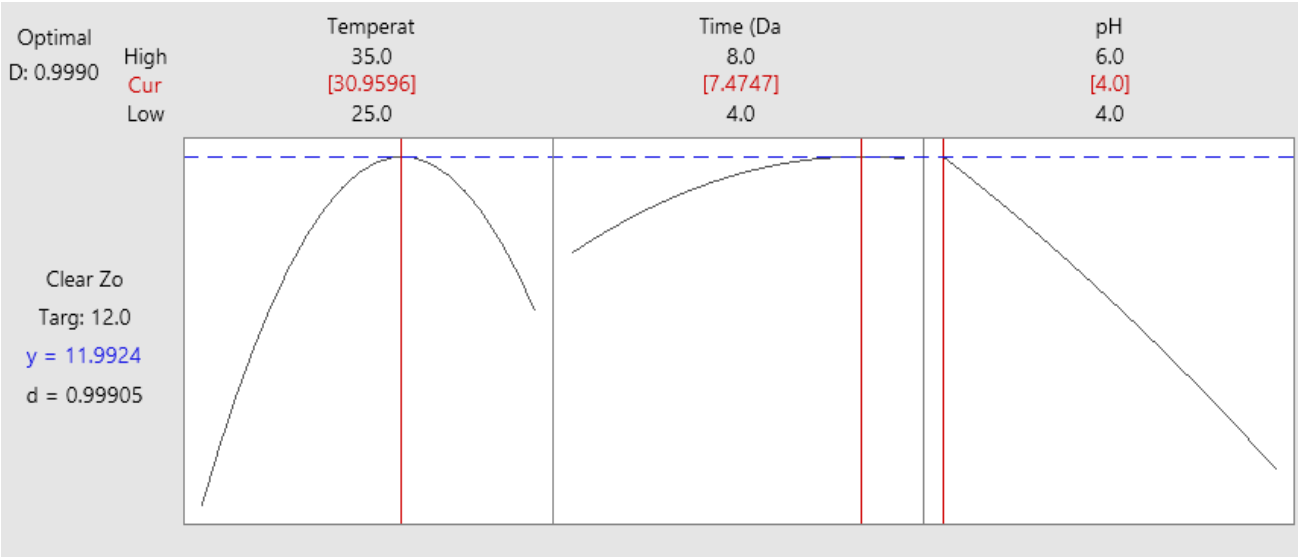


Figure 4. 4: *Optimization of Enzyme Production Based on Clear Zone Diameter*

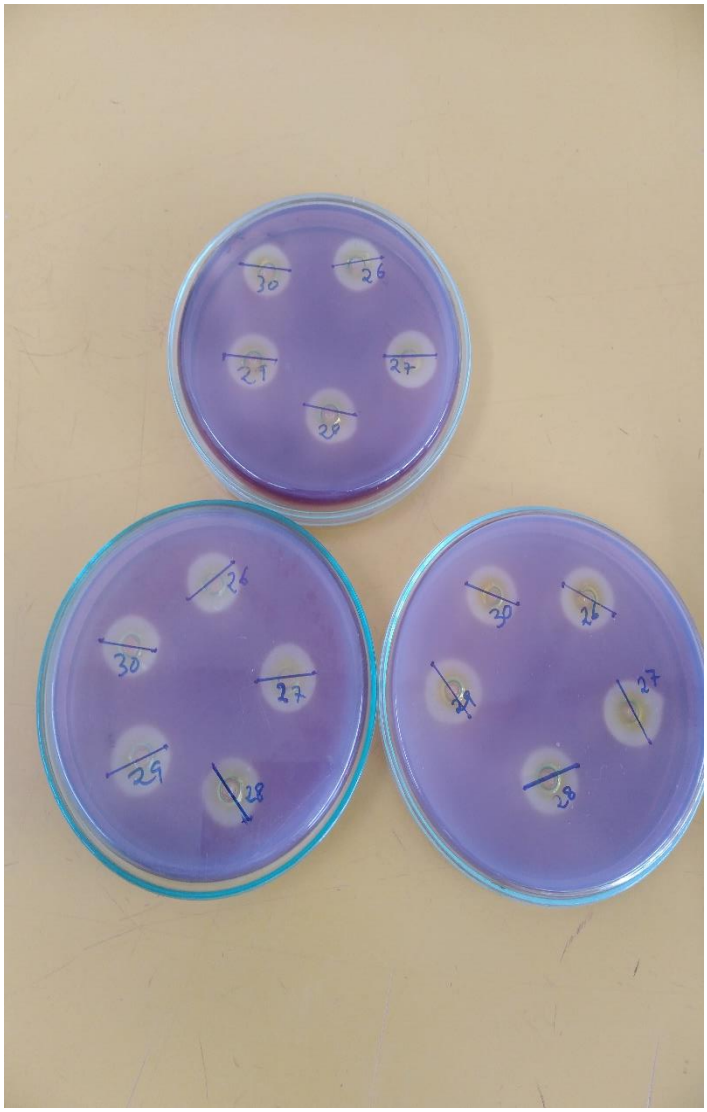


Figure 4. 5: *Plate Indicating Clear Zone by Produced Pectinase Enzyme*

4.5 Optimization of fermentation condition for maximum pectinase enzymes production based on polygalacturonase, pectin lyase and pectin esterase enzymes activity

Fermentation conditions including temperature, initial pH and incubation time are important for the growth of *Aspergillus brasiliensis.* Conditions were optimized using the box Behnken design. Analysis of Variance was performed to find the significant effect of the independent variables on the responses. From Table 4.3 the model was statistically significant at P value < 0.05 confidence level for all responses, which is desirable. For linear, quadratic and interaction of independent variables, there were significant effects on responses (enzyme activities) at the same level of significance. For polygalacturonase, all variables show a significant effect on enzyme activity except for the quadratic term of pH. For Pectin lyase, the quadratic term of pH and time, the interaction of temperature and time together with pH and time were found to be insignificant (P>0.05) on enzyme activity. For pectin

esterase, quadratic term of pH and time, the interaction of temperature and time were found to be insignificant ($P>0.05$) on enzyme activity.

Table 4. 3: Analysis of Variance for Polygalacturonase, Pectin Lyase and Pectin Esterase

		Polygalacturonase				Pectin Lyase				Pectin Esterase			
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Model	9	2587.41	287.49	23.07	0.000	261.602	29.067	10.4	0.000	3297.27	366.36	33.85	0.000
Linear	3	265.31	88.44	7.10	0.002	191.341	63.780	23.78	0.000	2340.97	780.32	72.10	0.000
Temp	1	65.17	65.17	5.23	0.033	135.373	135.373	50.48	0.000	376.07	376.07	34.75	0.000
pH	1	137.42	137.42	11.03	0.003	42.315	42.315	15.78	0.001	1794.79	1794.79	165.82	0.000
Time	1	62.73	62.73	5.03	0.036	13.653	13.653	5.09	0.035	170.11	170.11	15.72	0.001
Square	3	763.33	254.44	20.42	0.000	42.396	14.132	5.27	0.008	156.24	52.08	4.81	0.011
Temp *Temp	1	596.96	596.96	47.91	0.000	33.536	33.536	12.51	0.002	105.50	105.50	9.75	0.005
pH*pH	1	53.38	53.38	4.28	0.052	6.333	6.333	2.36	0.140	9.45	9.45	0.87	0.361
Time *Time	1	87.65	87.65	7.03	0.015	7.363	7.363	2.75	0.113	29.47	29.47	2.72	0.115
2-Way Interaction	3	1558.78	519.59	41.70	0.000	27.866	9.289	3.46	0.036	800.06	266.69	24.64	0.000
Temp *pH	1	707.07	707.07	56.74	0.000	16.274	16.274	6.07	0.023	676.75	676.75	62.53	0.000
Temp*Time	1	130.09	130.09	10.44	0.004	0.000	0.000	0.00	0.998	45.94	45.94	4.24	0.053
pH*Time	1	721.62	721.62	57.91	0.000	11.592	11.592	4.32	0.051	77.38	77.38	7.15	0.015
Error	20	249.21	12.46			53.635	2.682			216.47	10.82		
Lack-of-Fit	3	66.31	22.10	2.05	0.144	12.528	4.176	1.73	0.199	47.95	15.98	1.61	0.224
Pure Error	17	182.90	10.76			41.107	2.418			168.52	9.91		
Total	29	2836.63				315.238				3513.74			

Key: Temp= Temperature

Regression equations for polygalacturonase, pectin lyase and pectin esterase

From the regression equations the polygalacturonase activity was increased as temperature decreased, pH and time increased. For pectin lyase and esterase, the enzyme activities were increased as temperature, pH and time increased.

Polygalacturonase =9.7- 14.19 Temperature+60.9 pH +20.99 Time
+ 0.3596 Temperature*Temperature + 2.69 pH*pH- 0.861 Time*Time
-1.880 Temperature*pH+ 0.403 Temperature*Time - 4.749 pH*Time

Regression Equation of Polygalacturonase……..... Equation 4.2

Lyase = -162.7 + 7.12 Temperature + 19.80 pH
+ 5.55 Time - 0.0852 Temperature *Temperature - 0.926 pH*pH
-0.250 Time *Time - 0.285 Temperature *pH
-0.0001 Temperature *Time - 0.602 pH*Time

Regression Equation of pectin lyase…… …. Equation 4.3

Esterase = -390.2 + 20.68 Temperature + 42.6 pH+ 10.60 Time
- 0.1512 Temperature *Temperature + 1.13 pH*pH + 0.499 Time *Time
- 1.839 Temperature*pH - 0.240 Temperature *Time - 1.555 pH*Time

Regression Equation of pectin esterase…….…. …….. Equation 4.4

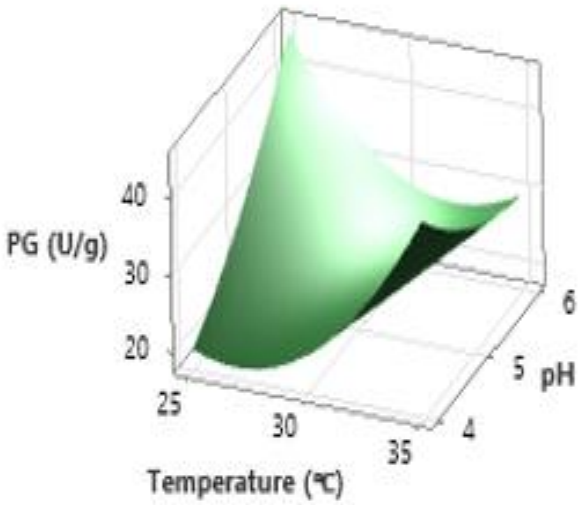
Surface plots for polygalacturonase, pectin lyase and pectin esterase

Surface plots for polygalacturonase, pectin lyase and pectin esterase (Figure 4.6 - figure 4.8) are showing how the interaction of the independent variable (temperature, pH and time) can affect responses (polygalacturonase, pectin lyase and pectin esterase activities). From Figure 4.6, all interactions significantly affect the polygalacturonase enzyme activity at $P<0.05$. The interaction between temperature and pH, pH and time affected negatively the production of polygalacturonase enzyme. At the lowest temperature and pH, there was the highest polygalacturonase enzyme activity the same to pH and time at the lowest pH and shortest time the polygalacturonase enzyme activity was highest. On the other side, the interaction of temperature and time affected positively the polygalacturonase enzyme activity, at the highest temperature and longest time the higher the polygalacturonase enzyme activity.

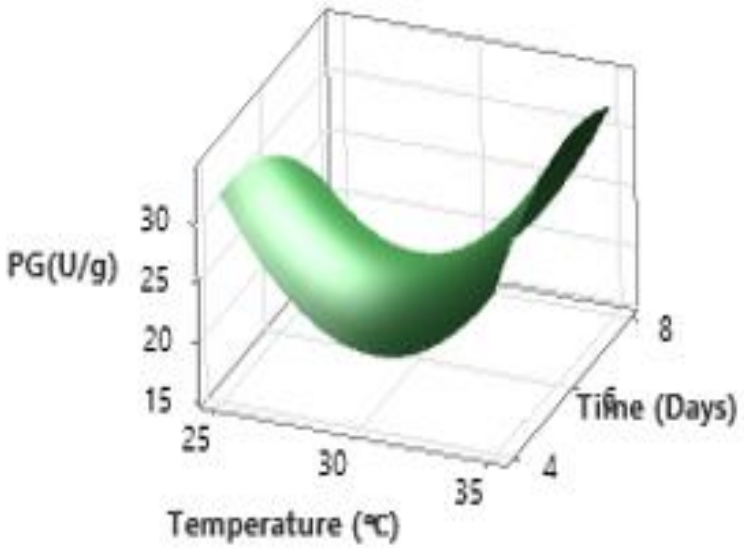
From figure 4.7, only interactions of temperature and time significantly affected the pectin lyase enzyme activity at $P<0.05$. The interaction between temperature and pH affect negatively the production of pectin lyase enzyme. At the lowest temperature and pH, there was the highest pectin lyase enzyme activity.

From figure 4.8, interactions between temperature and time, pH and time significantly affected the pectin esterase enzyme activity at $P<0.05$. All interactions affect negatively the production of pectin esterase enzyme. At the lowest temperature and pH, there was the highest pectin esterase enzyme activity the same to pH and time at lowest pH and shortest time the pectin esterase enzyme activity was highest.

Hold Values	
Temperature (℃)	30
pH	5
Time (Days)	6



a



b

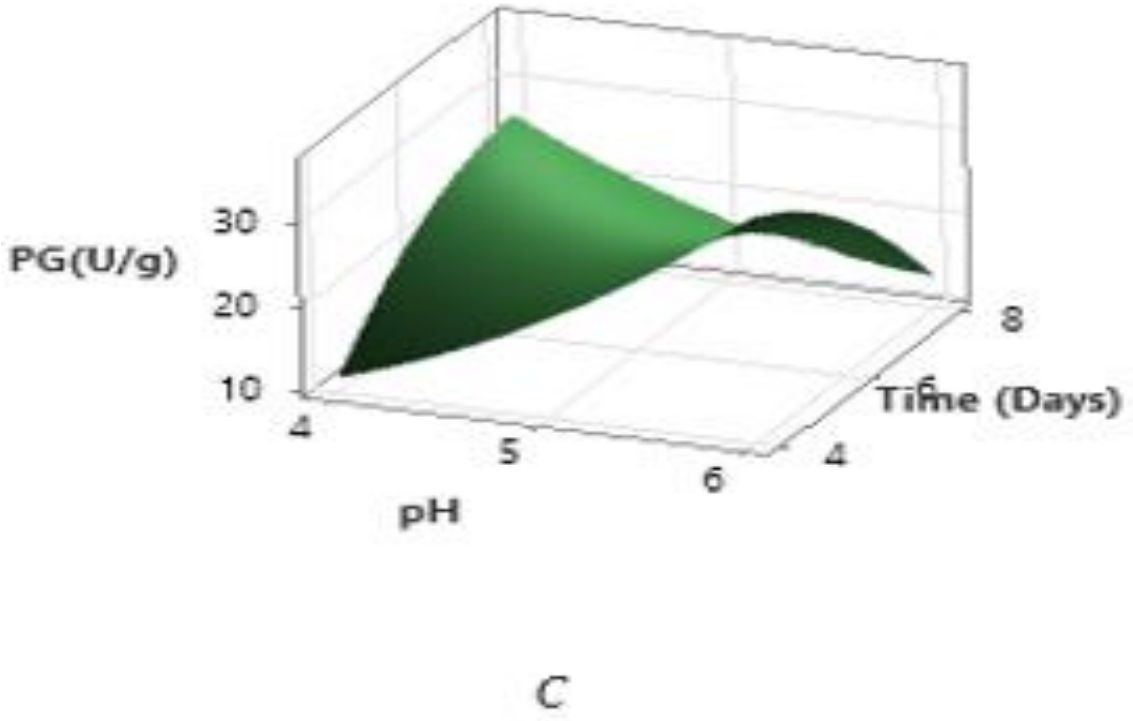
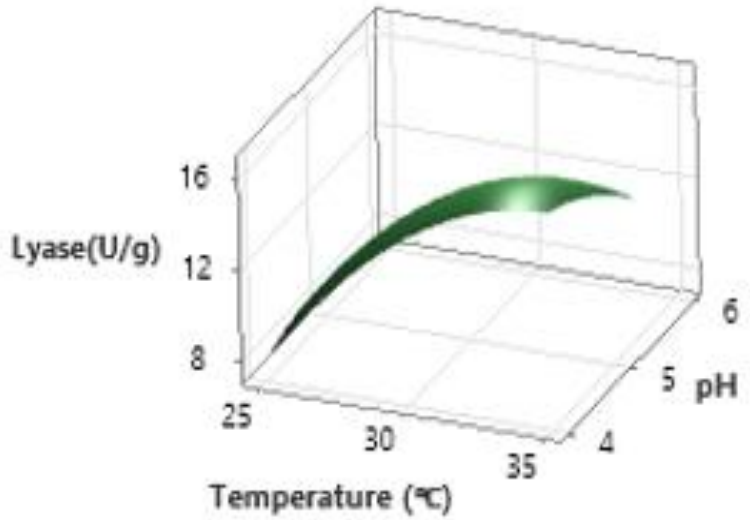
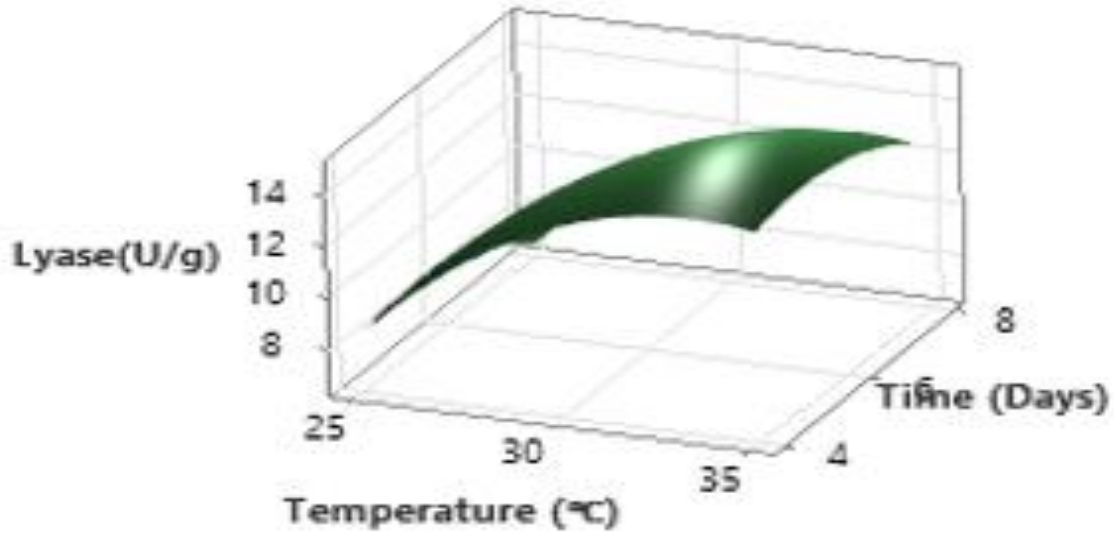


Figure 4. 6: *Surface plots showing the effect of (a) temperature and pH (b) temperature and time (c) pH and time on polygalacturonase (PG) enzyme activity*

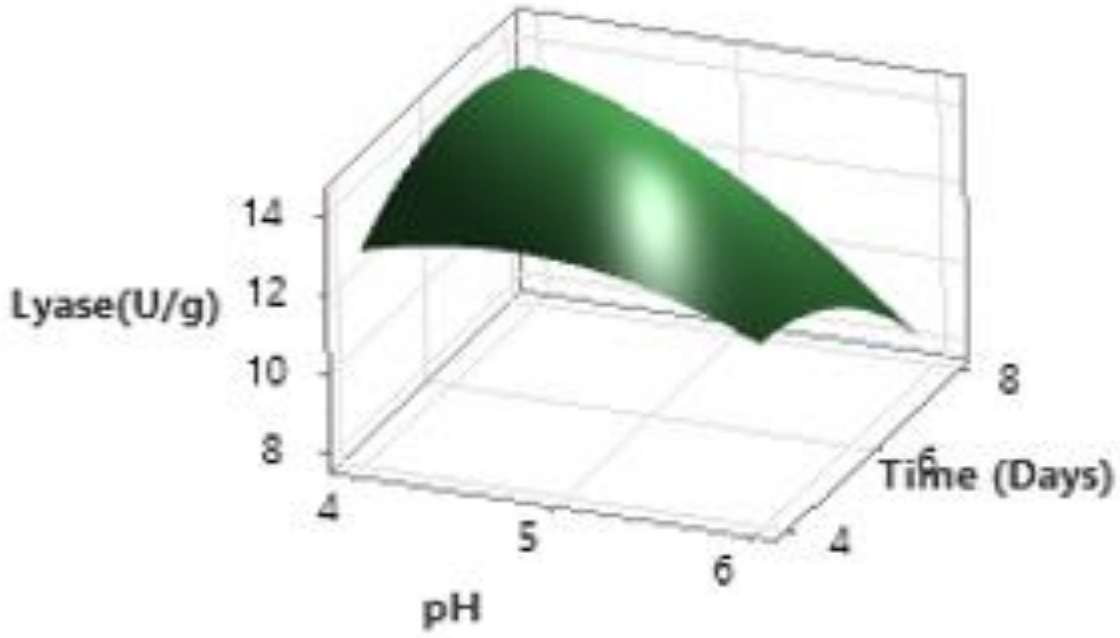
Hold Values	
Temperature (℃)	30
pH	5
Time (Days)	6



a

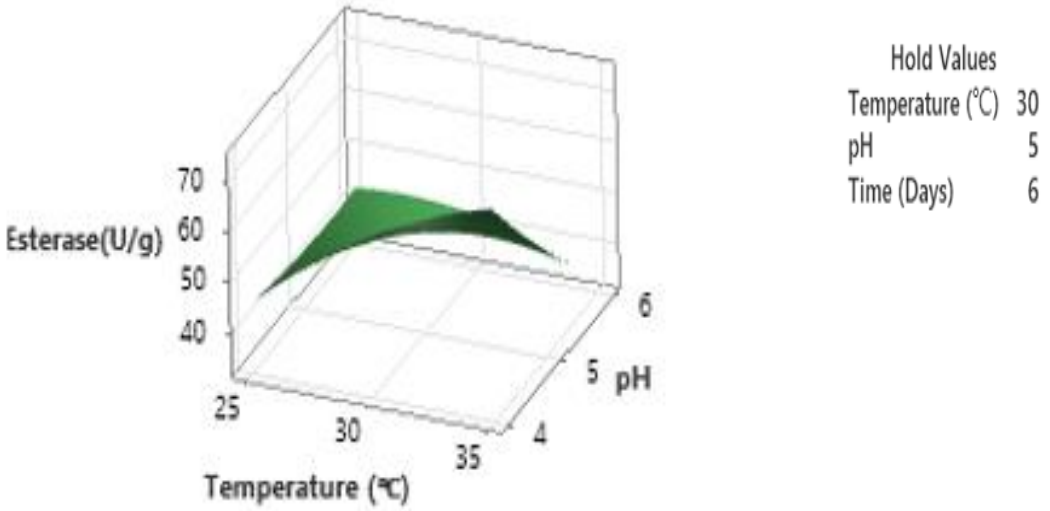


b

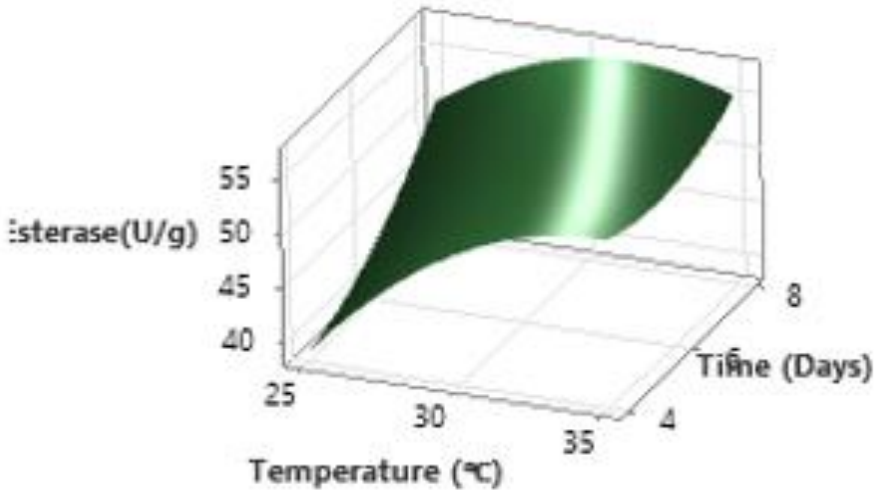


C

Figure 4. 7: *Surface plots showing the effect of (a) temperature and pH (b) temperature and time (c) pH and time on pectin lyase enzyme activity*


Hold Values
Temperature (℃) 30
pH 5
Time (Days) 6
Esterase(U/g)
70
60
50
40
25
30
35
Temperature (℃)
4
5
6
pH

a


Esterase(U/g)
55
50
45
40
25
30
35
Temperature (℃)
4
6
8
Time (Days)

b

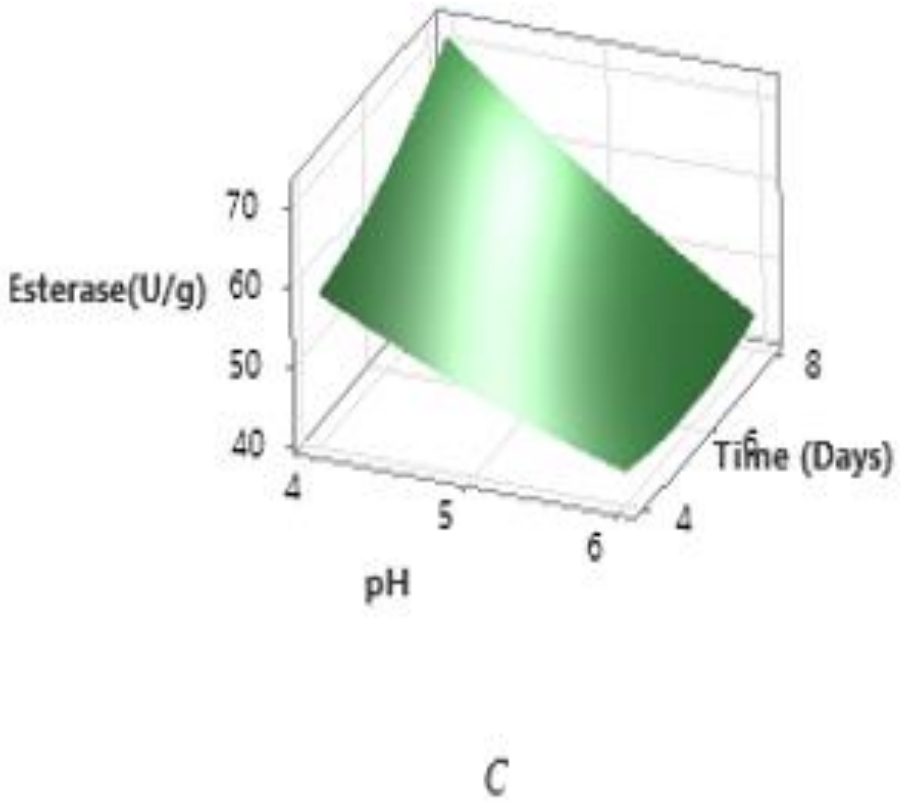


Figure 4. 8: *Surface plots showing the effect of (a) temperature and pH (b) temperature and time (c) pH and time on pectin esterase enzyme activity*

Optimization of polygalacturonase, pectin lyase and pectin esterase production

The maximum polygalacturonase, pectin lyase and pectin esterase activity were 44.58 U/g, 16.53 U/g and 74U/g respectively as shown in Figure 4.9. At the temperature of 35 ℃, pH 4 and 6 days of fermentation as shown in figure 4.9.

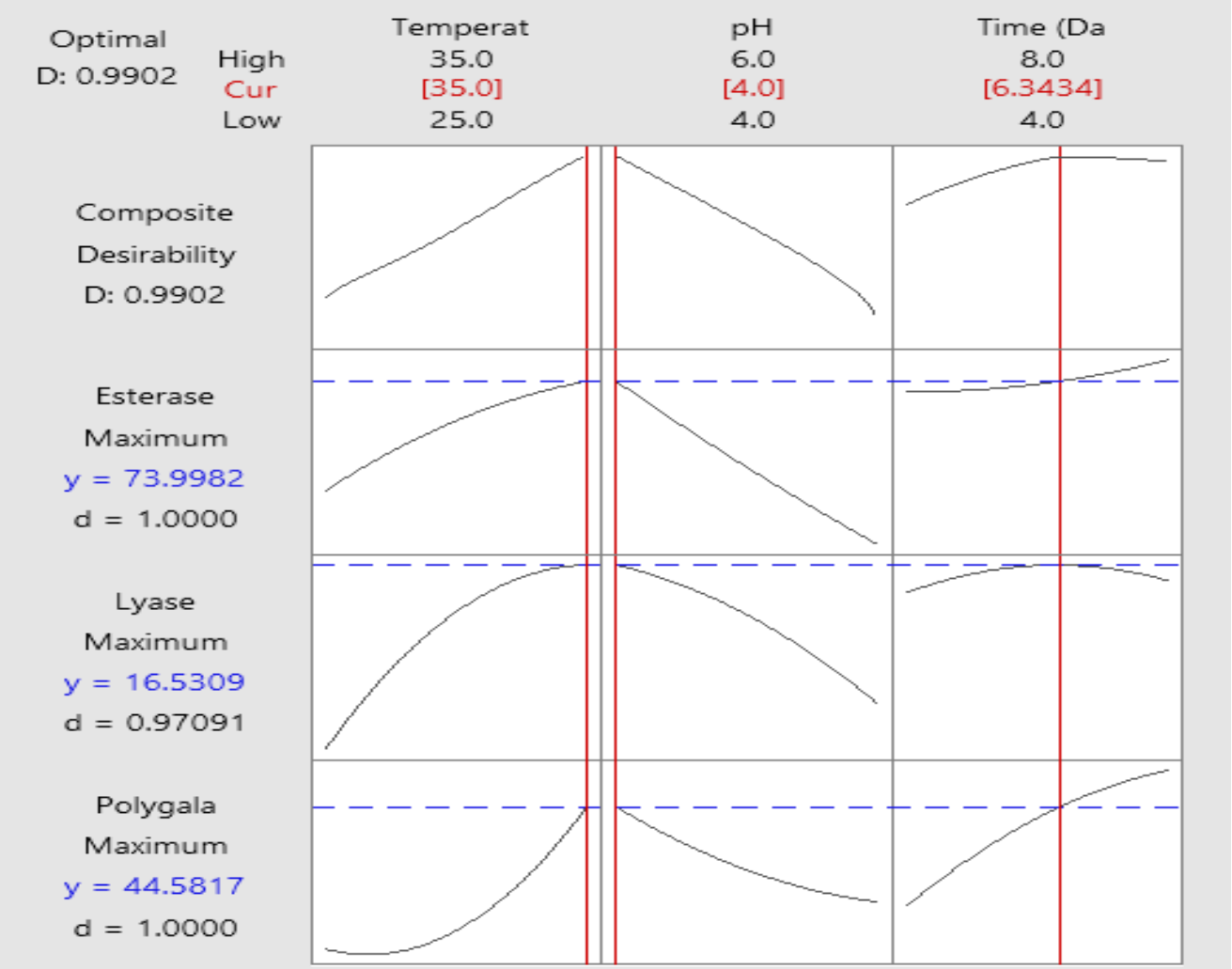


Figure 4. 9: *Optimization for Polygalacturonase, Pectin Lyase and Pectin Esterase*

4.6 Comparison of commercial and optimized pectinase enzymes activity

The results shown in Table 4.4 are the comparison of commercial and optimized polygalacturonase, pectin lyase and pectin esterase enzyme activity. From the results, there were significant differences in all enzyme activity between commercial and produced pectinase enzyme at P value < 0.05.

Table 4. 4: Comparison of Commercial and Optimized Pectinase Enzymes Activity

	Polygalacturonase (U/g)	Pectin lyase (U/g)	Pectin Esterase (U/g)
Commercial pectinase enzyme	25.19±0.30[b]	10.08±0.05[b]	0.00±0.00[b]
Optimized pectinase enzyme	43.88±0.76[a]	16.62±0.30[a]	73.00±0.66[a]
t-value	-32.26	-30.74	-156.98
P-value	0.001	0.001	0.0001

4.7 Characterization of polygalacturonase, pectin esterase and pectin lyase

Figures 4.10 and 4.11 illustrate the effect of temperature and pH on enzyme activity using pectin as a substrate. Different incubation temperatures from 20 to 70 ºC were investigated for their effect on the crude pectinase enzyme. The results illustrated in Fig. 4.11 showed that the activity of polygalacturonase increased gradually at a temperature ranging from 20°C up to 30 ℃ after that the polygalacturonase enzyme activity decreased to 7.87 U/g at a temperature 70℃. Therefore, the optimum temperature for maximum polygalacturonase enzyme activity was 30℃ whereby the enzyme activity was 41.13 U/g. For Pectin lyase 40℃ was the best for enzymes activity the same as the pectin esterase enzyme activity as shown in Fig.4.11 and Fig. 4.12 respectively. The enzyme activities were 13.17 U/g and 74.69 U/g.

Optimum pH of the crude pectinase enzyme activity was performed under same conditions. The polygalacturonase, pectin esterase and pectin lyase activity was determined at different pH of 3, 4, 5, 6, 7 and 8 using 0.1 M sodium acetate buffer with pectin as substrate. These results show that the enzymes do very well at a pH range of 4.0 to 5.0 and if the purification can be done, it could be applied industrially at this pH range. Pectin esterase was more active at the lowest pH of 4 whereby the enzyme activity was 71.45 U/g while polygalacturonase and pectin lyase did well at the pH of 5 and the enzyme activities were 44.73 U/g and 10.89 U/g respectively.

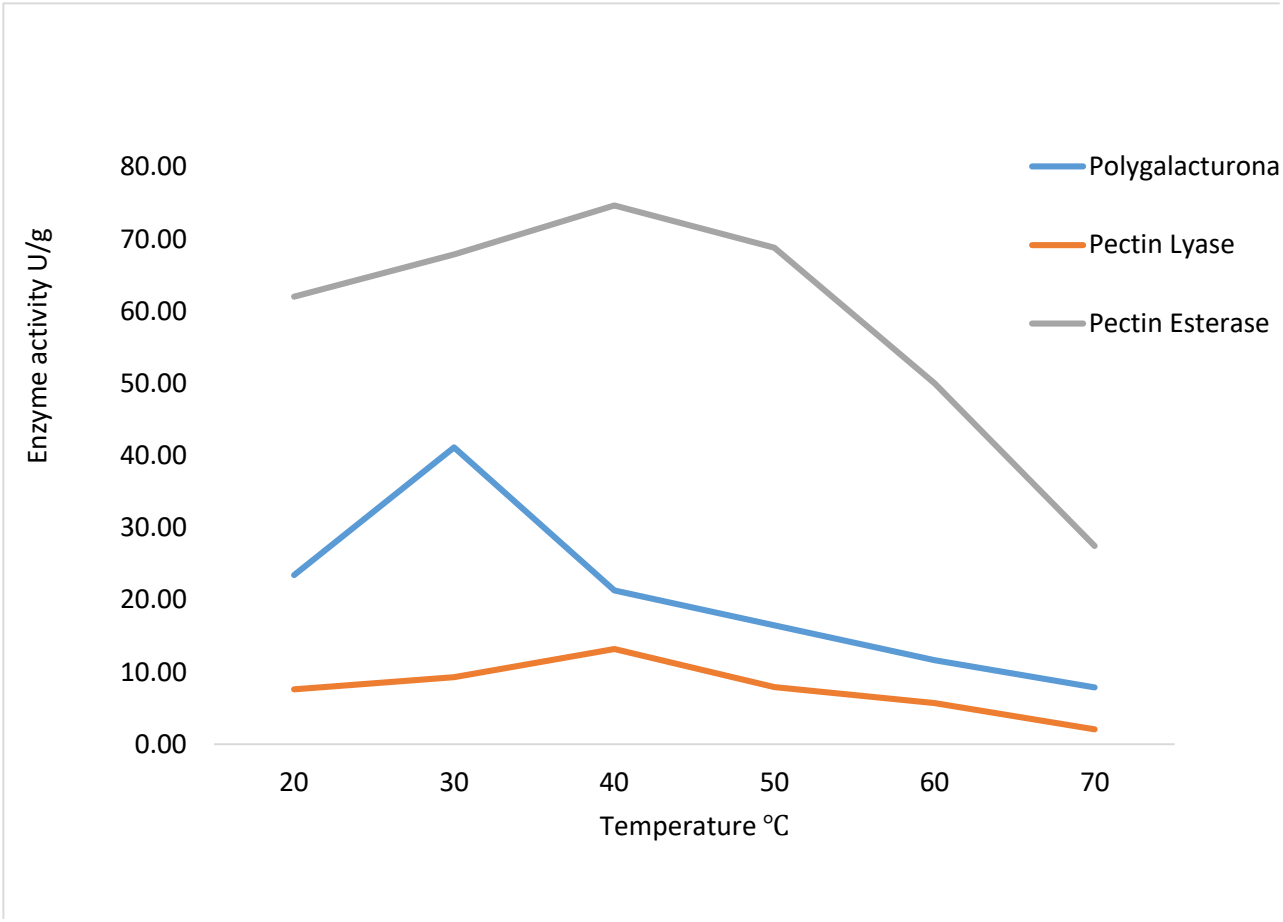


Figure 4.10: *Effect of Temperature on Enzyme Activity*

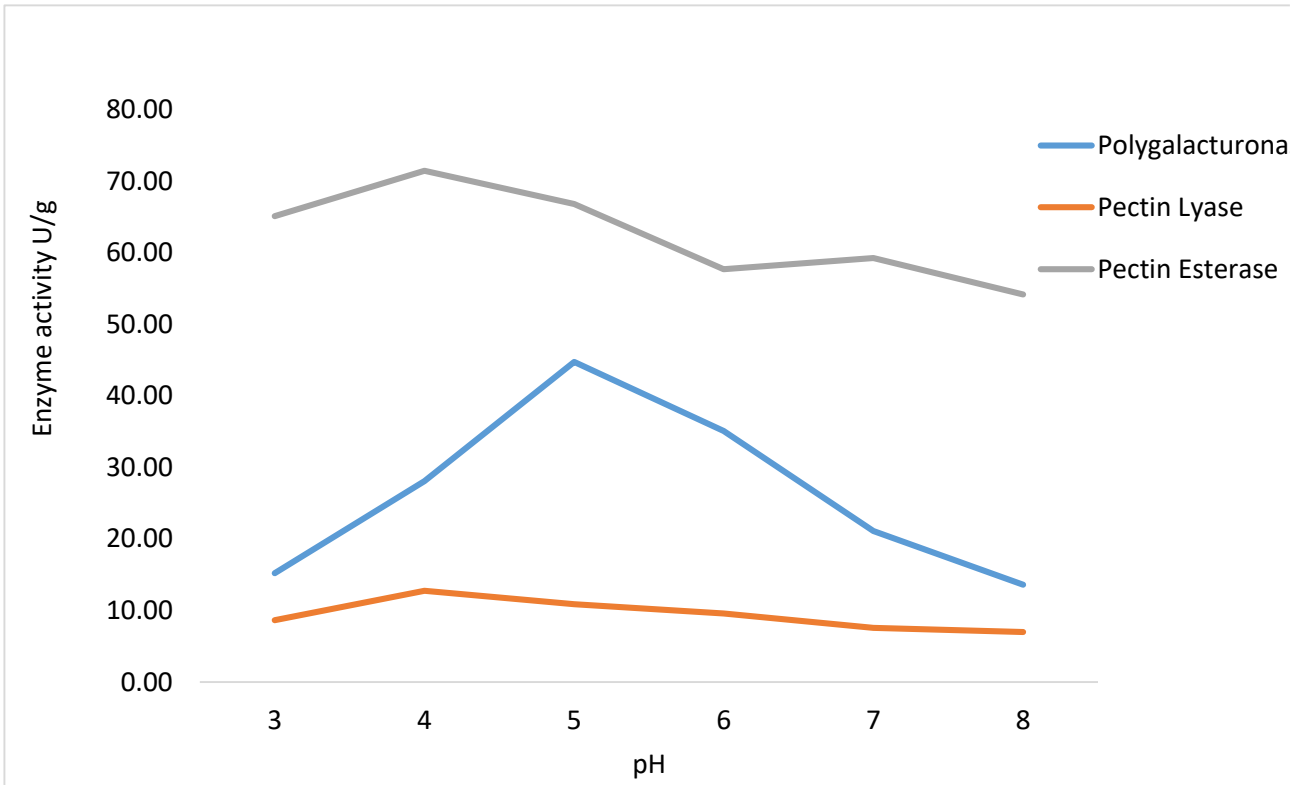


***Figure 4. 11**: Effect of pH on enzyme activity*

4.8 Application of pectinase enzymes produced by isolated Aspergillus brasiliensis in apple juice extraction and clarification

Figures 4.12 to 4.15 explained the effect of enzyme concentration on juice yield, Absorbance, pH and pectin content. The results showed that there was a significant effect of enzyme concentration on juice yield, Absorbance, pH and pectin content but enzyme concentration with the same letters is not significantly different in the corresponding responses. From Figure 4.12 as the amount of enzyme increased the juice yield was increased from 65% of the apple juice without pectinase enzymes to 84.55% of the apple juice treated with 5% pectinase enzymes concentration, there is a significant effect on juice yield at 1% and 2% enzymes concentration but there is no significant effect on juice yield at a concentration of 2% and 3%, 3% and 4%, 4% and 5%.

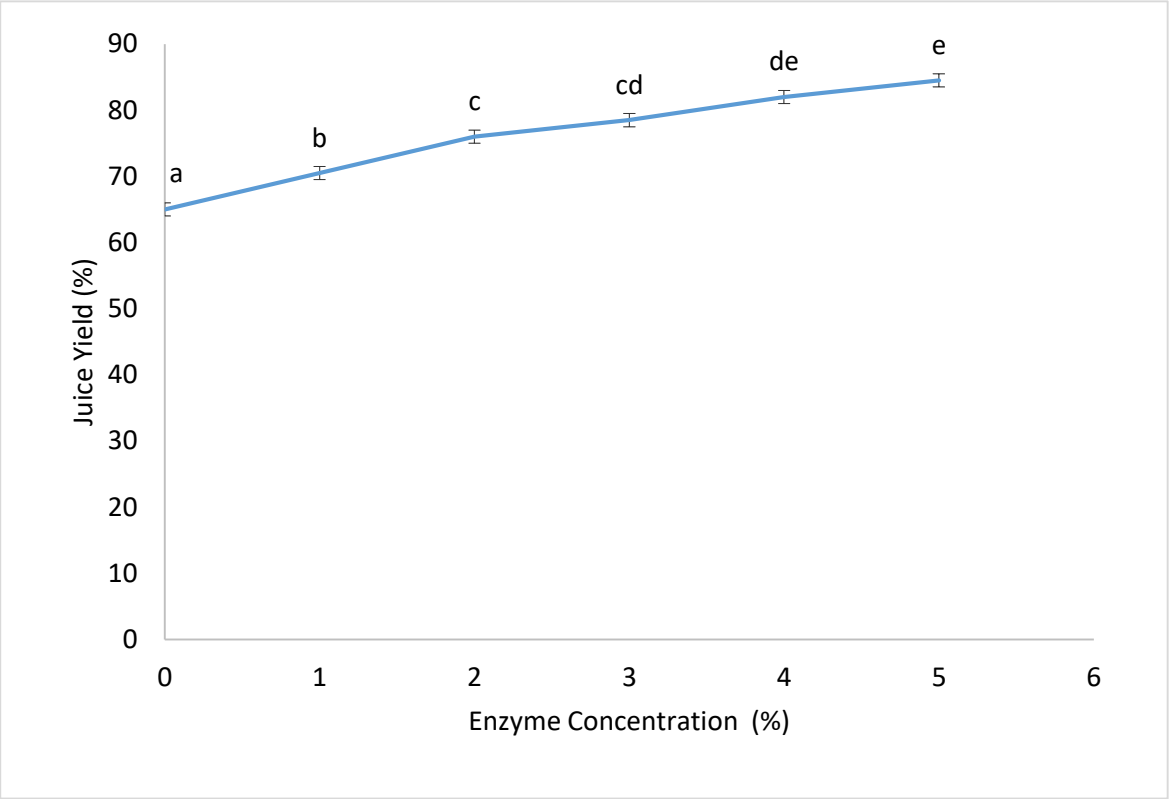


Figure 4.12: *Effect of Enzyme Concentration on Juice Yield. Data points with the same letter are not significantly different from the corresponding responses ($p<0.05$)*

The results from Figure 4.13 were obtained by the addition of different concentrations of produced pectinase enzymes to the apple juice. The absorbance readings indicated as clarity of the apple juice. As the enzyme concentrations were increased absorbance were decreased. The sharp slope of point a (apple juice without enzymes) and point b (apple juice with 1% concentration of produced pectinase enzymes) shows there was a significant difference in the absorbance readings based on apple juice treated with produced pectinase enzymes. The effect also was observed between 1% and 2% enzyme concentration, but there is no significant effect on absorbance readings at concentrations of 2% and 3%, 3% and 4%, 4% and 5%.

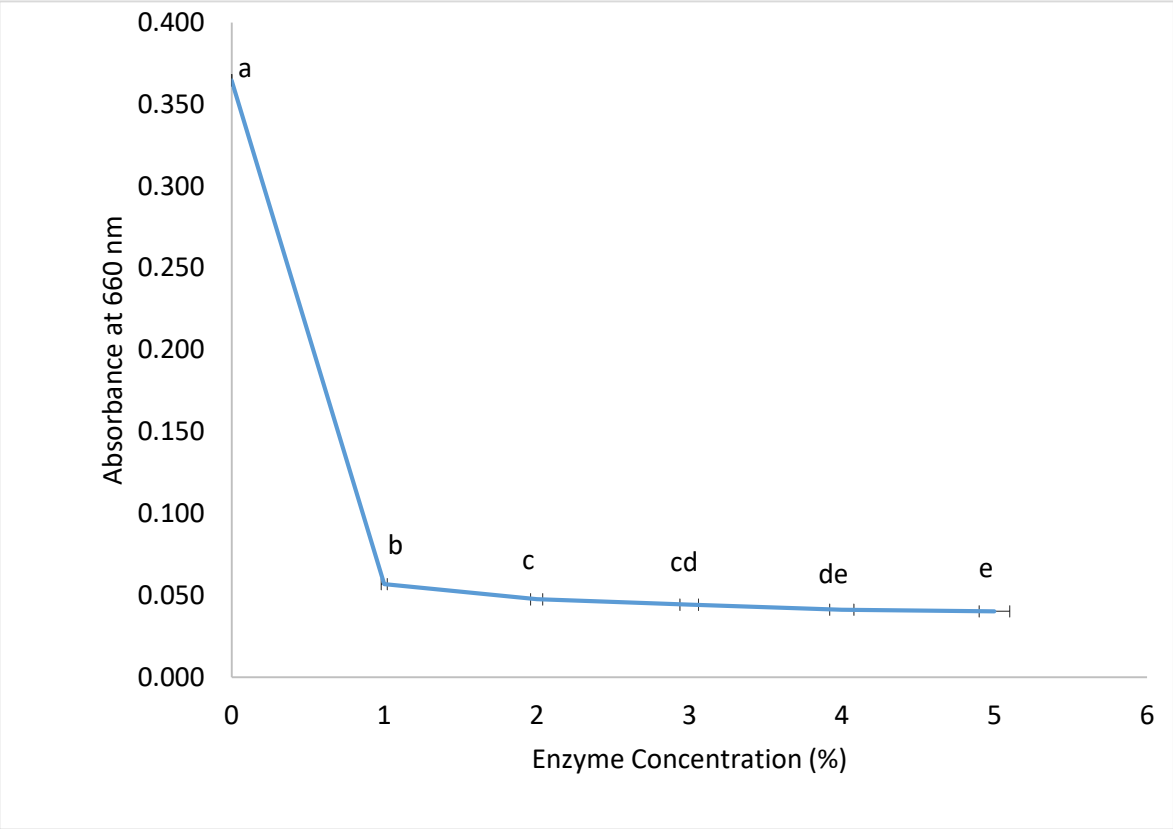


Figure 4. 13: *Effect of Enzyme Concentration on Absorbance (Clarity) of Juice. Data points with the same letter are not significantly different from the corresponding responses ($p<0.05$)*

The results obtained from Figure 4.14 shows the effect of pectinase enzyme concentration on the pH of the juice. From the results, there was a significant effect on pH when the pectinase enzymes increased from 0% to 5%. All pectinase enzymes concentration affect the pH of the juice except for 4% and 5%.

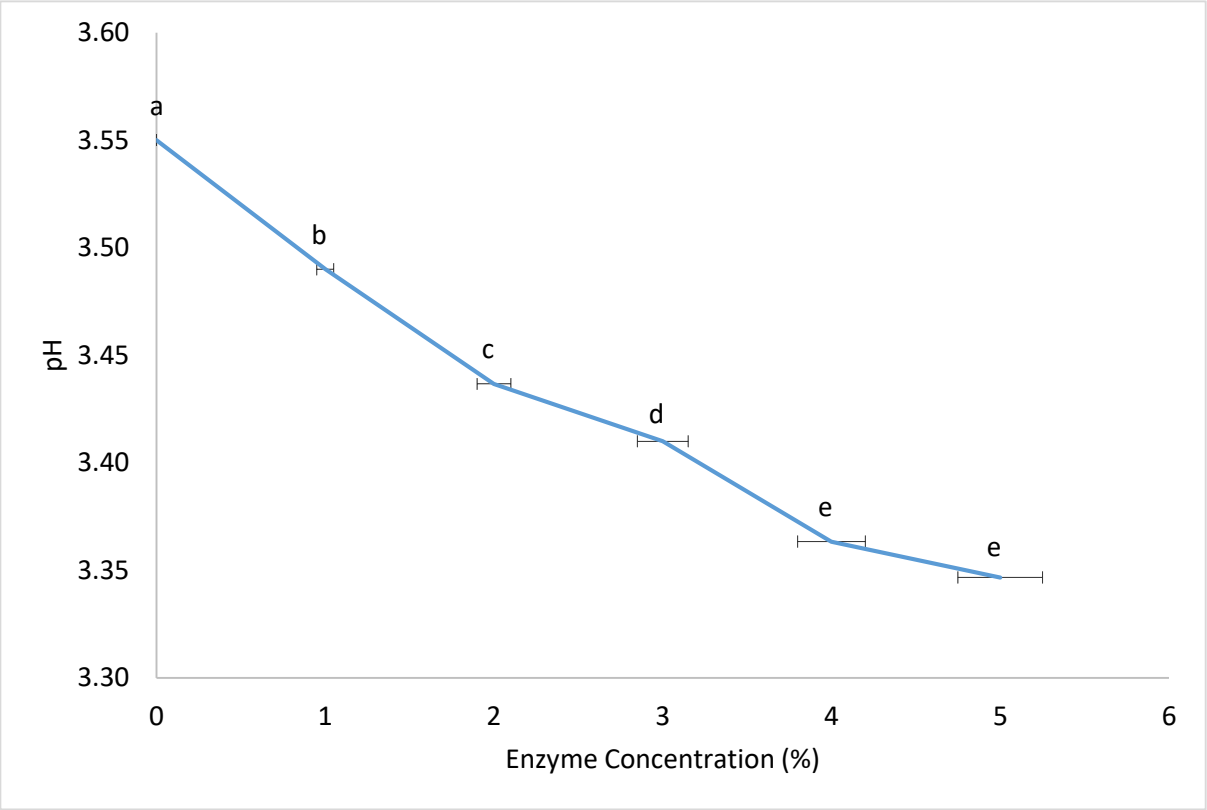


Figure 4. 14: *Effect of Enzyme Concentration on pH of juice. Data points with the same letter are not significantly different from the corresponding responses ($p<0.05$)*

Pectinase enzymes hydrolysis pectin, apple juice is one of the juices with a high amount of pectin. When juice is treated with pectinase the amount of pectin is expected to be reduced. From the results from Figure 4.15 the pectin content of the apple juice without being treated with pectinase enzymes was reduced from 0.45% to 0.01% of apple juice treated with 5% of produced pectinase enzymes. Pectin There was a significant effect on the percentage of pectin content at 0% and 1%, 3% and 4% pectinase enzyme concentration while there was no significant effect on pectin content at 1%, 2% and 3%, 4% and 5% pectinase enzymes concentration.

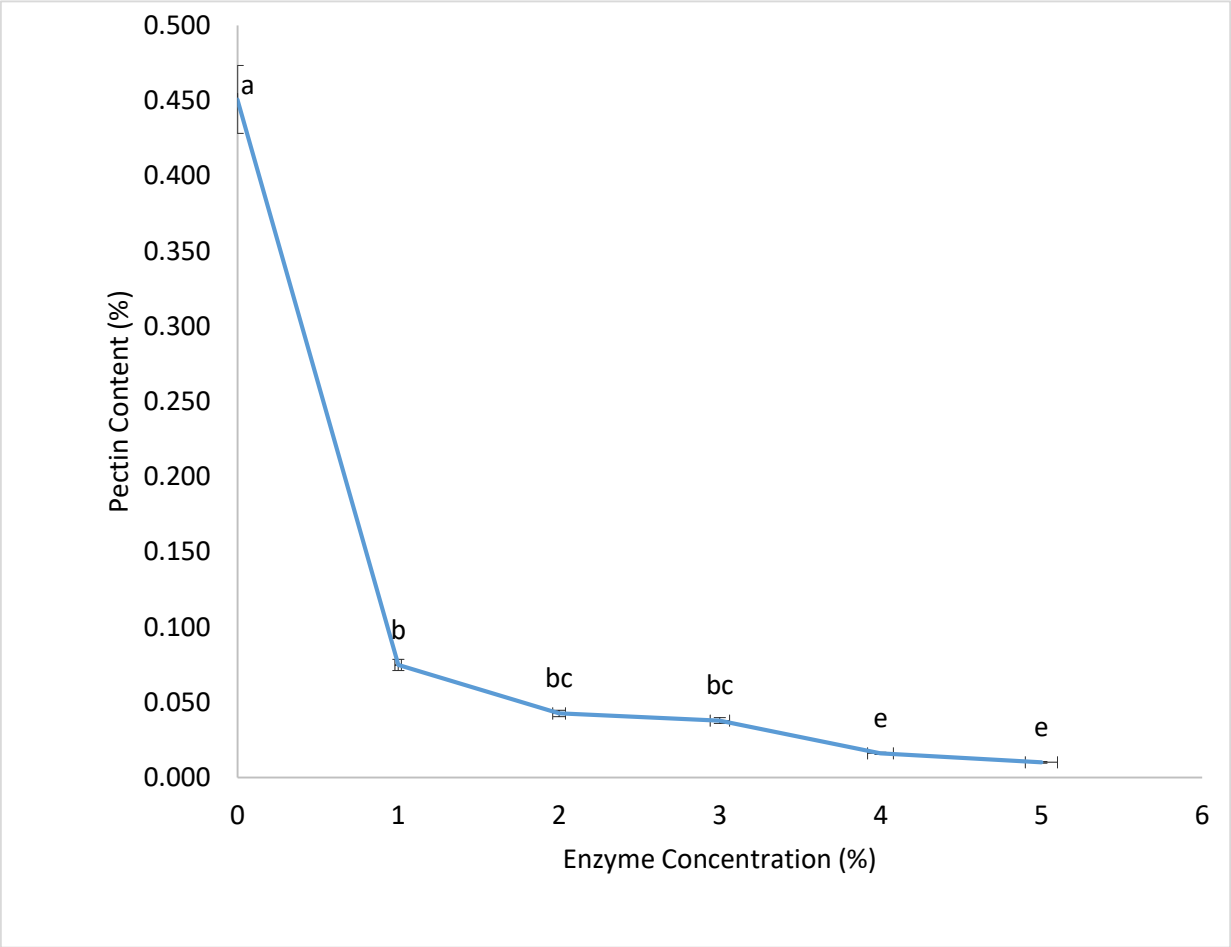


Figure 4.15: *Effect of Enzyme Concentration on Pectin Content in the Juice. Data points with the same letter are not significantly different from the corresponding responses ($p<0.05$)*

4.9 Correlation Coefficients of Absorbance, Pectin Content and pH of Juice after treated with produced pectinase enzymes

Correlation Coefficients of Absorbance, Pectin Content and pH of juice after being treated with produced pectinase enzymes are shown in Table 4.5 there is a strongly significantly positive correlation between absorbance and pectin content (r= 0.99) as absorbance of the juice treated with different concentration of produced pectinase enzymes decreased, the pectin content also was decreased. This positive correlation was observed in the pH of the juice as absorbance decreased the pH decreased (r= 0.77). There is a strong positive correlation between pectin content and pH (r=0.82) as pectin content decreased the pH of the juice also decreased.

Table 4. 5: Correlation Coefficients of Absorbance, Pectin Content and pH of juice after treated with produced pectinase enzymes

	Absorbance	Pectin content	pH
Absorbance	1.000	0.993***	0.769***
Pectin content		1.000	0.823***
pH			1.000

Key: ***= Significant at P<0.001

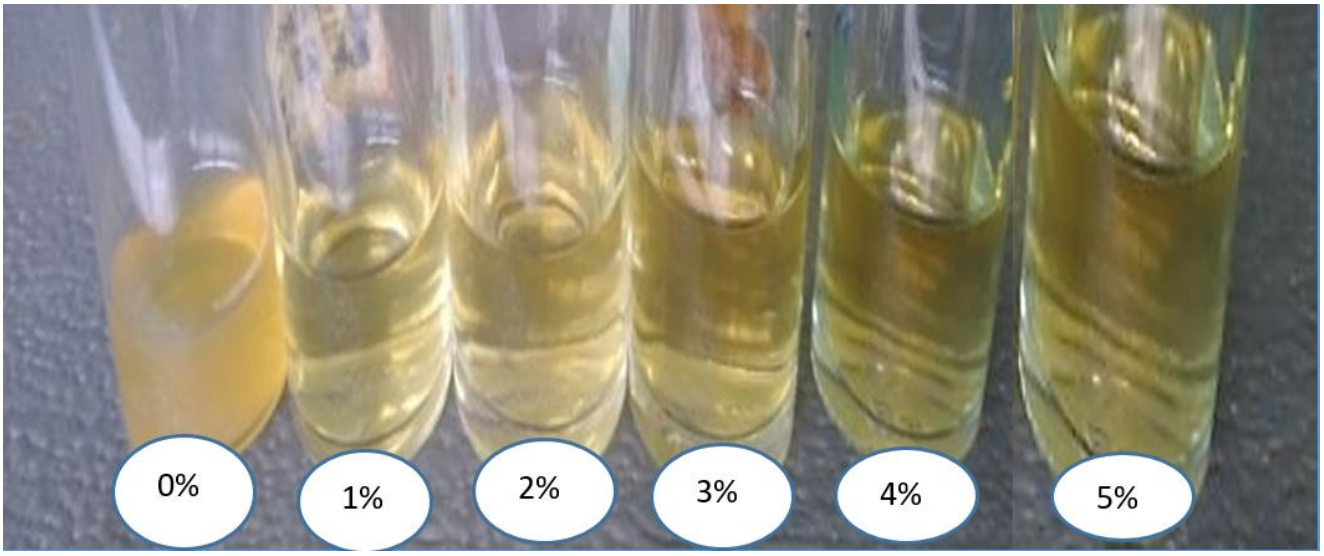


Figure 4. 16: *A- Juice without Pectinase Enzyme (Control), Juice Treated with Produced Pectinase Enzyme*

CHAPTER FIVE

DISCUSSION

5.1 Macroscopic and microscopic features for Aspergillus brasiliensis identification

Aspergillus brasiliensis is among the most essential microorganisms in biotechnology. Mostly, researchers isolated this fungal from soil and decaying organic matter for different uses. In this study, the fungal was isolated from decaying orange peels and used for pectinase enzyme production. The characteristics of the isolate were consistent with reports by several scientists. *Aspergillus brasiliensis* isolated from soil samples using a PDA medium showed the same characteristics of initial growth of white colonies then after the production of spores with time causes the black colouration (Toma *et al*., 2021). This fungal was also isolated by Bellaouchi *et al*. (2021) from date by-products and showed similar morphological characteristics on a PDA medium. Other authors isolated *A.niger* using different mediums as reported by Bandh *et al*. (2012). The medium used were Czapek yeast agar and Malt extract agar and both of the colonies on the media were observed green and the reverse was white to cream. This means the type of media for fungal isolation can give different macroscopic characteristics. In addition to that the size of the colony also were measured and an average diameter was 55 to 70 mm after 7 days of incubation at 30℃. Yin *et al*. (2015) reported a diameter of 15mm on PDA after 4 days of incubation at 26℃ which differ according to Oyebamiji *et al*. (2018) 40 up to 50mm diameter on PDA after 7 days of incubation at 27℃. The number of days of incubation and temperature can affect the growth of the colonies. The diameter of the colony on the PDA medium was found to reach up to 100 mm 7 days after inoculation (Jaidev & Narasimha, 2010).

Microscopic characteristics also were significantly crucial for the identification of fungi. Microscopic observation of the isolate was conducted to identify the fungal. The size, shape, colour, hyphae, conidiophores and arrangement of spores were used for *Aspergillus brasiliensis* identification. The diameter of conidia was approximately 4 - 5 μm this also was reported by Thorati *et al*. (2016) 3.6 to 4.4 μm. Conidiophores length was 550 μm and vesicle diameter was 68 μm.

5.2 Molecular characterization

According to Demirci *et al*. (2021), there are more than 300 *Aspergillus* species. Some of the species have similar morphological characteristics. The molecular technique is the only method that can be used for the identification of similar fungi species even for morphologically indistinguishable species. In this study, the isolate was identified as *A. niger* after sequences were blasted against the fungal sequences in the MEGA version 6.0 database for identification.

5.3 Orange waste nutritional profile

From the results of proximate analysis, orange waste contains a lot of nutrients which can support the growth of microorganisms for enzyme production. The result of crude protein was 15.5% more than the amount of protein 7.15% reported by Olabinjo *et al*. (2017) and 5.17% reported by Zaker *et al*.(2016). The crude fat result was 3.56% less than the amount of fat presented by Naqvi *et al*. (2021) which was 7.9%**.** Ash was 5.6% less than presented by Mondal *et al*. (2012) which was 3.55% and the value was near the amount of ash 5.25% reported by Pathak *et al*. (2017). The crude fibre was 11.62% near the value of 12% reported by Olabinjo *et al*. (2017). The amount of carbohydrates obtained by subtraction was 54.35%. This means more than half of the orange waste is composed of carbohydrates which can be the source of energy for fungal growth such as *Aspergillus brasiliensis* for enzyme production. This was supported by Ghimire (2019) who studied citrus waste as the alternative media for the cultivation of fungi. One of the wastes was the orange waste and the result obtained showed that the waste supported the growth of *Aspergillus brasiliensis.*

5.4 Optimization of fermentation condition for maximum pectinase enzymes production based on clear zone diameter

Enzymes activity can be qualitatively determined using the clear zone diameter. The solidified pectin-containing media with holes can be filled with pectinase enzymes and the clear zone diameter can give the preliminary results of the enzyme activity. The pectinase enzymes produced from different combined conditions were tested based on their activity using the clear zone diameter. The results obtained showed that there was a significant difference in the clear zone diameter of the pectinase enzymes produced under different combined conditions at p values < 0.05. The optimum conditions for the largest clear zone diameter (12mm) were a temperature of 31℃, pH 4 and 7 days of fermentation. Clear zone diameters were a preliminary qualitative analysis of testing pectinase enzyme activity in which the enzymes degrade the pectin from the solidified pectin agar to form the clear zone after being flooded with coloured solution e.g. iodine. The results obtained were not far away from that of other

researchers who used quantitative procedures for pectinase enzyme activity determination. A temperature of 30 ℃ and pH 3-4 were reported for optimum pectinase enzyme production using coffee pulp residues and *ficus religiosa* leaves by *Aspergillus* species (Dasari, 2020; Hidayah *et al*., 2020). Other researchers use the clear zone diameter to identify microorganisms producing pectinase enzymes. The same procedures were used but instead of filling the holes with enzymes the isolates were streaked on the solidified medium (Pectin – Agar medium) and the plates were incubated for 24 hours at 30°C. At the end of the incubation period, iodine solution were flooded on the plate and occurrence of a clear halo zone around the microorganisms indicates the ability of the microorganisms to produce pectinase enzymes (Abate & Oumer, 2018). The pectinolytic activity of a 20 mm clear zone diameter was obtained around *Tetracoccosporium* species during the screening of different fungal isolates from vegetable wastes (Haile & Ayele, 2022).

5.5 Optimization of fermentation condition for maximum pectinase enzymes production based on polygalacturonase, pectin lyase and pectin esterase enzyme activity

The optimum condition giving the maximum polygalacturonase, lyase and esterase enzyme activities were at a temperature of 35℃, pH 4 and 6 days of fermentation (figure 4.9). The specific enzymes activities were 44.58 U/g, 16.53 U/g and 74 U/g respectively. The results were within the range of *A. niger* optimum growth condition in which most filamentous fungi grow in acidic conditions with pH between 4.0 to 5.0 (Sathiyaraj *et al*., 2011). Other researchers used *Aspergillus sojae* for polygalacturonase enzyme production. The maximum polygalacturonase enzyme activity obtained was 29 U/g after 5.5 days of fermentation using crushed maize as a solid substrate (Ustok *et al*., 2007). Results revealed by da Câmara Rocha *et al*. (2020) stated that the highest polygalacturonase activity produced at 38 U/g was obtained on the third day of fermentation using yellow mombin pulp residues as the substrate. Most of the researchers used different types of substrates for pectinase enzyme production. The optimum temperature for maximum pectinase enzyme activity reported was between 25℃ to 35℃ using *Aspergillus* species (Núñez Pérez *et al*., 2022). Diversification of the conditions can alter the results of enzymes production, depending on the type of microorganism used for enzyme production as the temperature and pH exceeding the optimum can reduce production of enzymes as the microorganisms might acquire stress. Time is very crucial as microorganisms utilize the materials for their growth at the end they rich optimum growth with high enzyme production. Exceeding at that point microorganisms start to die due to lack of food due to higher microbial load. At the optimum condition microorganisms (*Aspergillus brasiliensis)* digest orange waste materials into hydrolysate for their growth. During the digestion process, *Aspergillus brasiliensis produce* enzymes which can be recovered

by filtration and water purification. In this study the wastes used was orange wastes as contain higher amount of pectin *Aspergillus brasiliensis* produced more pectinase enzyme to convert pectin into simple form sugars e.g. monosaccharide and oligosaccharide. Utilization of pectin containing waste for pectinase enzyme production was done by Doan *et al*. (2021) using *Bacillus amyloliquefaciens* strain, orange waste was one of the wastes used for the study and was the third in pectinase enzyme production before wheat bran and banana wastes.

5.6 Comparison of commercial and optimized pectinase enzymes activity

The crude pectinase enzyme produced by isolated *Aspergillus brasiliensis was* compared with commercial pectinase enzymes in terms of their activity. From the results in Table 4.5, the optimized pectinase enzyme was more active nearby twice than the commercial pectinase enzyme produced by *Aspergillus oryza.* The pectin esterase activity was not detected in the commercial pectinase enzyme, this depends on the type of application of the enzyme produced as pectinase enzyme with the addition of pectin esterase stated to be more useful in juice processing because pectin esterase causes aggregation of the cloud particles within a short time and works well at the natural acidic pH of the juice (Garg *et al*., 2016). Among all pectinolytic enzymes, polygalacturonase is the most used for industrial applications, this is due to its specificity during hydrolysis as it breaks the glycosidic bond and reduces the viscosity of the solution to a greater extent (Satapathy *et al*., 2020). The comparative study conducted by another researcher on the potential use of crude pectinase enzyme produced by *Bacillus subtilis* isolated from hazelnut shell in clarification of carrot juice. Under the same conditions, the crude bacterial pectinases provided a good clarity result compared to commercial fungal pectinase enzymes. The result was 95% clarity of crude pectinase enzyme while commercial pectinase enzyme was 78% (Uzuner & Cekmecelioglu, 2015).

5.7 Characterization of polygalacturonase, pectin esterase and pectin lyase

Temperature and pH are some of the factors that can affect the activity of enzymes. Increasing temperatures generally speeds up a reaction up to the point where extremely high temperatures can cause enzymes to be denatured as the enzymes are protein in nature. From the results obtained the polygalacturonase enzyme activity was 41.13 U/g which was the highest at the temperature of 30℃. Exceeding the temperature of 30 ℃ polygalacturonase enzyme activity started to decline to 7.87 U/g at a temperature of 70 ℃, this means when a temperature increased more the polygalacturonase enzyme was losing its activity and it will reach a point where the enzyme will be denatured and stop working. Normally the polygalacturonase enzyme works at a temperature between 30℃ to 50℃ as reported by

Nirmaladevi *et al*. (2014) and Oat (2020). The pH of optimum polygalacturonase enzyme activity was 5. At that pH, the polygalacturonase enzyme activity was 44.73 U/g. Most of the researchers reported a pH of 4 up to 6 for optimum polygalacturonase enzyme activity (Ahmed & Sohail, 2020; Patel *et al*., 2022).

A temperature of 40 ℃ was the optimum for pectin lyase and pectin esterase enzyme activities. The enzyme activities at that temperature were 13.17 U/g and 74.69 U/g respectively. A review done by Patel *et al*. (2022) showed that most of the pectin lyase and pectin esterase work well at temperatures around 40℃ to 60℃. On the other side the variation of pH on testing the optimum working pH for pectin lyase and pectin esterase showed that at the pH of 4, both of the enzymes showed the highest activity. The enzyme activities value were 12.74 and 71.45 respectively. The results were also reported by Dal Magro *et al*. (2019). Based on the optimum pH of all groups of pectinase enzymes produced the results show that the enzymes work well under acidic medium pH of 4 to 5 that gave a good performance for apple juice extraction and clarification as the acidic pectinase enzymes used in juice and wine processing to degrade pectin structure.

5.8 Application of pectinase enzymes produced by isolated *Aspergillus brasiliensis* in apple juice extraction and clarification

In this study, the effect of crude enzymes on apple juice extraction was found that an increase in the amount of enzymes in apple pulp increased the percentage of juice yield. Juice yield was increased from 65% to 84.5%, this is due to enzymes hydrolysed pectin in apple pulp might have loosened the cell wall and released the cell content thus, increased the volume of the juice. The percentage of juice yield of 4% and 5% concentration of enzymes are the same therefore 4% concentration can be used for maximum percent of juice yield as the small percentage is more preferred to reduce the cost of production.

Apple juice is one of the juices which consumers prefer to drink when it is clear. In juice clarity, as the enzyme concentration increased the absorbance at 660 nm was decreased. The lower the absorbance means the higher the clarity of the juice and the cloudiness reduced. But there is no significant difference between 3% and 4% and between 4% and 5% enzyme concentration on juice clarity based on absorbance readings at 660nm. At the highest pectinase enzyme concentration treated in different varieties of grapes juice, the lowest absorbance was observed by Aponso *et al*. (2017) and this was explained by the degree of juice clarity.

The pH of apple juice was decreased from 3.55 to 3.35, pH of the juice was decreased this might be due to the release of carboxylic groups and galacturonic acids due to the enzyme action on pectin in juice. The pH was stable at the concentration of 4% and 5%. pH of guava juice decreased from 4.5 to 3.05 as reported by Kant *et al*.,(2013) as the number of enzymes increased.

To conclude that the percentage of pectin content was determined by using the gravimetric method based on the chelation reaction of pectin with metal ions Ca^{2+}, Firstly the clarified apple juice was added with NaOH to speed up hydrolysis of pectin from clarified apple juice. Pectin hydrolyses very faster in the presence of excess alkaline such as NaOH. Under acidic conditions, Calcium chloride is added and boiled to produce Calcium pectate. The amount of precipitated Calcium pectate was estimated using equation 3.11. Pectin content decreased from 0.45% to 0.040% as the enzyme concentration increased and there was a large difference in pectin content between apple juices without pectinase enzymes and those treated with pectinase enzymes. This means that produced pectinase enzymes were active in the pectin polymer that the pectin content was reduced. Although, some of the pectinase enzyme concentrations showed no significantly different in pectin content as shown in Figure 4.15.

5.9 Correlation Coefficients of Absorbance, Pectin Content and pH of Juice after treated with produced pectinase enzymes

The amount of pectin in the juice is the one which causes cloudiness and when the light passes the absorbance is expected to be high. The positive correlation between Absorbance and pectin content of the juice after being treated with produced pectinase enzymes was due to the reduced pectin content that cause the reduction of absorbance readings. Hydrolysis of the pectin from the apple juice release the carboxylic group of the pectin which causes the pH of the juice to be reduced. This means there was a positive correlation between pectin content and pH, as more hydrolysis of pectin occurred the amount of pectin reduced to form a galacturonic acid which cause the pH of the juice to be reduced. The same to the correlation coefficients between Absorbance and pectin content. There were positive correlation coefficients between Absorbance and pH. Absorbance is affected by the amount of pectin, as pectin hydrolysed the absorbance decreased and pH decreased. Reduction in the pH was observed by Amobonye *et al*. (2022) due to the release of galacturonic acid as the product of pectin after the hydrolysis action of the pectinase enzyme. Instead of absorbance, a researcher uses a change in per cent transmittance at 660 nm with a UV-VIS spectrophotometer to explain the clarity improvement of pear juice. The transmittance percentage was increased as the amount of pectinase enzyme increased.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

The use of waste materials is a cost-effective approach for enzyme production, which can yield active products for industrial applications. In this study, orange processing waste was identified as a suitable source for pectinase enzyme production using solid-state fermentation with isolated *A. brasiliensis* under optimized conditions. Pectinase enzyme activity was significantly affected by temperature, pH, and incubation time. The enzyme was successfully applied to improve the yield and clarify apple juice, as demonstrated by the reduction in pH and pectin content. Therefore, the isolated *A. brasiliensis* strain produced active enzymes that could be used in juice extraction and clarification. Further studies are required to characterize the enzyme for potential heterologous expression and its application in upcycling waste into valuable products using indigenous microorganisms through biotechnology.

6.2 Recommendations

i. Isolation of the different microorganisms from the environment can be performed to determine their uses in agro waste conversion to develop value added products

ii. Growth parameters should be determined for maximum production of the value-added products.

iii. Uses of the value-added products produced should be tested for its efficiency

REFERENCES

Abdel-Moneim, M. A., Hamady, G. A. A., & Motawe, H. F. A. (2014). The use of orange waste with and without enzymes in broilers' diets and its effect on their performance, carcass traits and some blood parameters. *Res. J. Anim. Vet. Fish. Sci*, *2*(12), 14-19.

Adeyefa, O. M., & Ebuehi, O. A. T. (2020). Isolation, identification and characterization of pectinase producers from agro wastes (*Citrus sinensis* L. and *Ananas comosus* L.). *World J Agric Soil Sci*, *4*(3), 1-6. https://doi.org/10.33552/WJASS.2020.04.000589

Aggarwal, R., Dutta, T., & Sheikh, J. (2020). Extraction of pectinase from Candida isolated from textile mill effluent and its application in bio-scouring of cotton. *Sustainable Chemistry and Pharmacy*, *17*, 100291. https://doi.org/10.1016/j.scp.2020.100291

Ahmed, A., & Sohail, M. (2020). Characterization of pectinase from *Geotrichum candidum* AA15 and its potential application in orange juice clarification. *Journal of King Saud University-Science*, *32*(1), 955-961. https://doi.org/10.1016/j.jksus.2019.07.002

Ahmed, I., Zia, M. A., Hussain, M. A., Akram, Z., Naveed, M. T., & Nowrouzi, A. (2016). Bioprocessing of citrus waste peel for induced pectinase production by *Aspergillus niger*; its purification and characterization. *Journal of Radiation Research and Applied Sciences*, *9*(2), 148-154. https://doi.org/10.1016/j.jrras.2015.11.003

Ajayi, A. A., Osunkoya, F. A., Peter-Albert, C. F., & Olasehinde, G. I. (2014). Clarification of apple juice with laboratory- produced-pectinase obtained from the deterioration of apple (*Malus domestica*) fruits by *Aspergillus niger*. *International Journal of Advanced Biotechnology and Research*, *5*(2), 134-140. https://doi.org/10.4314/ijbcs.v5i3.72206

Al-Maqtari, Q. A., Waleed, A. A., & Mahdi, A. A. (2019). Microbial enzymes produced by fermentation and their applications in the food industry-A review. *International Journal of Agriculture Innovations and Research*, *8*(1), 2319-1473.

Alsohaili, S., & Bani-Hasan, B. M. (2018). Morphological and Molecular Identification of Fungi Isolated from Different Environmental Sources in Northern Eastern Jordan Deseret. *Jordan Journal of Biological Sciences, 11*(3), 329–337.

Alsudani, A. (2021). Production of Polysaccharides and Single-Cell Protein by Some Local Isolates of *Trichoderma* spp. *Pakistan Journal of Biological Sciences, 24*(9), 971–977. https://doi.org/10.3923/pjbs.2021.971.977

Amanat, F., Yaqoob, A., Ali, A., & Sajjad, M. (2019). Extracellular Production of Pectinase from Bacteria Isolated from Rotten Apples from Lahore, Pakistan. *BioScientific Review*, *1*(3), 37-45. https://doi.org/10.32350/BSR.0103.05

Amilia, K. R., Sari, S. L. A., & Setyaningsih, R. (2017). *Isolation and screening of pectinolytic fungi from orange (Citrus nobilis Tan.) and banana (Musa acuminata L.) fruit peel*. In *IOP Conference Series: Materials Science and Engineering* (Vol. 193, No. 1, p. 012015). IOP Publishing. https://doi.org/10.1088/1757-899X/193/1/012015

Amin, F., Bhatti, H. N., & Bilal, M. (2019). Recent advances in the production strategies of microbial pectinases: A review. *International Journal of Biological Macromolecules*, *122*, 1017-1026. https://doi.org/10.1016/j.ijbiomac.2018.09.048

Amobonye, A., Bhagwat, P., Ruzengwe, F. M., Singh, S., & Pillai, S. (2022). Pear juice clarification using polygalacturonase from *Beauveria bassiana*: Effects on rheological, antioxidant and quality properties. *Polish Journal of Food and Nutrition Sciences*, *72*(1), 57-67.

Anbu, P., Gopinath, S. C., Chaulagain, B. P., Tang, T. H., & Citartan, M. (2015). Microbial enzymes and their applications in industries and medicine 2014. *BioMed Research International*, *122*, 1-3 https://doi.org/10.1155/2015/816419

Angebault, C., Payen, M., Woerther, P. L., Rodriguez, C., & Botterel, F. (2020). Combined bacterial and fungal targeted amplicon sequencing of respiratory samples: Does the DNA extraction method matter? *PLoS One*, *15*(4), e0232215. https://doi.org/10.1371/journal.pone.0232215

Ani, P. N., & Abel, H. C. (2018). Nutrient, phytochemical, and antinutrient composition of *Citrus maxima* fruit juice and peel extract. *Food Science & Nutrition*, *6*(3), 653-658. https://doi.org/10.1002/fsn3.604

AOAC (2000). *Official Methods of Analysis of Analytical Chemists*. (17th ed.). AOAC International, Gaithersburg.

AOAC (2005). *Official Methods of Analysis of Analytical Chemists*. (18th ed.). AOAC International, Washington D.C., USA.

Aponso, M. M. W., Marapana, R. A. U. J., & Manawaduge, R. (2017). Physicochemical analysis of grape juice from Israel blue (*Vitis vinifera* L.) grape cultivar under different processing conditions and a comparison with Red Globe and Michele Palieri grape varieties. *Journal of Parmacognosy & Phytochemistry*, *6* (3), 381-385.

Babagil, A., & Nadaroglu, H. (2022). Purification of pectin lyase enzyme from *Bacillus pumilus* bacteria by three-phase partitioning method (TPP), nanoflower preparation and investigation of fruit

juice clarification. *Biointerface Research in Applied Chemistry*, *12*(3), 3938-3955. https://doi.org/10.33263/BRIAC123.39383955

Babigumira, W. (2017). *A Sector Guide for Processed Fruits Juice in the East African Community.* http://ateas.co.ke/downloads/EAC-Market-Place-A-Sector-ide-for-Processed-Fruit-Juice-in-the-EAC.pdf

Bandh, S. A., Kamili, A. N., & Ganai, B. A. (2012). Identification of some Aspergillus species isolated from Dal Lake, Kashmir by traditional approach of morphological observation and culture. *Afr. J. Microbiol. Res*, *6*(29), 5824-5827. https://doi.org/10.5897/AJMR12.039

Behailu, A., & Abebe, G. (2018). Isolation, production and characterization of amylase enzyme using the isolate Aspergillus niger FAB-211. *International Journal of Biotechnology and Molecular Biology Research*, *9*(2), 7-14. https://doi.org/10.5897/IJBMBR2018.0289

Bellaouchi, R., Abouloifa, H., Rokni, Y., Hasnaoui, A., Ghabbour, N., Hakkou, A., ... & Asehraou, A. (2021). Characterization and optimization of extracellular enzymes production by *Aspergillus niger* strains isolated from date by-products. *Journal of Genetic Engineering and Biotechnology*, *19*(1), 50. https://doi.org/10.1186/s43141-021-00145-y

Cardoso, L., Karen, Y., & Karp, S. (2017). Microbial production of carotenoids: A review. *African Journal of Biotechnology*, *16*, 139–146. https://doi.org/10.5897/AJB2016.15763

Chandrasekaran, M. (Ed.). (2012). *Valorization of food processing by-products*. CRC press.

Chilakamarry, C. R., Sakinah, A. M., Zularisam, A. W., Sirohi, R., Khilji, I. A., Ahmad, N., & Pandey, A. (2022). Advances in solid-state fermentation for bioconversion of agricultural wastes to value-added products: Opportunities and challenges. *Bio Resource Technology*, *343*, 126065. https://doi.org/10.1016/j.biortech.2021.126065

Collados, A., Conversa, V., Fombellida, M., Rozas, S., Kim, J. H., Arboleya, J. C., ... & Perezábad, L. (2020). Applying food enzymes in the kitchen. *International Journal of Gastronomy and Food Science*, *21*, 100212. https://doi.org/10.1016/j.ijgfs.2020.100212

da Câmara Rocha, J., da Silva Araújo, J., de Paiva, W. K. V., Ribeiro, E. S. S., de Araújo Padilha, C. E., de Assis, C. F., dos Santos, E. S., de Macêdo, G. R., & de Sousa Junior, F. C. (2020). Yellow mombin pulp residue valorization for pectinases production by *Aspergillus niger* IOC 4003 and its application in juice clarification. *Biocatalysis and Agricultural Biotechnology*, *30*, 101876. https://doi.org/10.1016/j.bcab.2020.101876

Dal Magro, L., Kornecki, J. F., Klein, M. P., Rodrigues, R. C., & Fernandez-Lafuente, R. (2019). Stability/activity features of the main enzyme components of rohapect 10L. *Biotechnology Progress, 35*(6), e2877. https://doi.org/10.1002/btpr.2877

Dasari, P. K. (2020). Parametric optimizations for pectinase production by *Aspergillus awamori*. *GSC Biological and Pharmaceutical Sciences, 12*(2), 093-098 https://doi.org/10.30574/gscbps.2020.12.2.0247

Daskaya-Dikmen, C., Karbancioglu-Guler, F., & Ozcelik, B. (2018). Cold active pectinase, amylase and protease production by yeast isolates obtained from environmental samples. *Extremophiles*, *22*, 599-606. https://doi.org/10.1007/s00792-018-1020-0

de Castro, R. J. S., & Sato, H. H. (2015). Enzyme production by solid state fermentation: general aspects and an analysis of the physicochemical characteristics of substrates for agro-industrial wastes valorization. *Waste and biomass valorization*, *6*, 1085-1093. https://doi.org/10.1007/s12649-015-9396-x

de la Torre, I., Martin-Dominguez, V., Acedos, M. G., Esteban, J., Santos, V. E., & Ladero, M. (2019). Utilisation/upgrading of orange peel waste from a biological biorefinery perspective. *Applied Microbiology and Biotechnology, 103*(15), 5975–5991. https://doi.org/10.1007/s00253-019-09929-2

Demirci, E., Arentshorst, M., Yilmaz, B., Swinkels, A., Reid, I. D., Visser, J., ... & Ram, A. F. (2021). Genetic characterization of mutations related to conidiophore stalk length development in *Aspergillus niger* laboratory strain N402. *Frontiers in Genetics*, *12*(3), 1-11. https://doi.org/10.3389/fgene.2021.666684

Doan, C. T., Chen, C. L., Nguyen, V. B., Tran, T. N., Nguyen, A. D., & Wang, S. L. (2021). Conversion of pectin-containing by-products to pectinases by *Bacillus amyloliquefaciens* and its applications on hydrolyzing banana peels for prebiotics production. *Polymers*, *13*(9), 1483. https://doi.org/10.3390/polym13091483

Dou, Z., Toth, J. D., & Westendorf, M. L. (2018). Food waste for livestock feeding: Feasibility, safety, and sustainability implications. *Global food security*, *17*(6), 154-161. https://doi.org/10.1016/j.gfs.2017.12.003

El-Bakry, M., Abraham, J., Cerda, A., Barrena, R., Ponsá, S., Gea, T., & Sanchez, A. (2015). From wastes to high value-added products: novel aspects of SSF in the production of enzymes. *Critical Reviews in Environmental Science and Technology*, *45*(18), 1999-2042.

http://www.tandfonline.com.egertonelibrary.remotexs.co/doi/abs/10.1080/10643389.2015.1010423

FAO. (2019). *The state of food and agriculture 2019. Moving forward on food loss and waste reduction.* Rome. http://www.fao.org/3/ca6030en/ca6030en.pdf

FAO. (2020). *Statistical database of the Food and Agriculture Organization of the United Nations, Rome, Italy*. FAOSTAT. http://www.fao.org/faostat/en/#data/QC

Frisvad, J. C., Møller, L. L., Larsen, T. O., Kumar, R., & Arnau, J. (2018). Safety of the fungal workhorses of industrial biotechnology: update on the mycotoxin and secondary metabolite potential of *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*. *Applied Microbiology and Biotechnology*, *102*, 9481-9515. https://doi.org/10.1007/s00253-018-9354-1

Gani, G., Naik, H. R., Jan, N., Bashir, O., Hussain, S. Z., Rather, A. H., ... & Amin, T. (2021). Physicochemical and antioxidant properties of pear juice prepared through pectinase enzyme-assisted extraction from William Bartlett variety. *Journal of Food Measurement and Characterization*, *15*, 743-757. https://doi.org/10.1007/s11694-020-00676-x

Garg, G., Singh, A., Kaur, A., Singh, R., Kaur, J., & Mahajan, R. (2016). Microbial pectinases: an ecofriendly tool of nature for industries. *3 Biotech*, *6*, 1-13. https://doi.org/10.1007/s13205-016-0371-4

Ghimire, T. (2019). *FRUITS PEEL WASTE AS A FUNGAL GROWTH MEDIUM* (Doctoral dissertation, Tribhuvan University). http://202.45.146.37:8080/jspui/handle/123456789/21

Giavasis, I. (2013*). Production of microbial polysaccharides for use in food*. *Microbial Production of Food Ingredients, Enzymes and Nutraceuticals.* Elsevier https://doi.org/10.1533/9780857093547.2.413

Guerrand, D. (2018). *Economics of food and feed enzymes. In Enzymes in Human and Animal Nutrition*, 487–514. Academic press. https://doi.org/10.1016/B978-0-12-805419-2.00026-5

Haile, S., & Ayele, A. (2022). Pectinase from microorganisms and its industrial applications. *The Scientific World Journal*, *2022*(1), 1881305. https://doi.org/10.1155/2022/1881305

Heerd, D., Diercks-Horn, S., & Fernández-Lahore, M. (2014). Efficient polygalacturonase production from agricultural and agro-industrial residues by solid-state culture of *Aspergillus sojae* under optimized conditions. *SpringerPlus*, *3*, 1-14. https://doi.org/10.1186/2193-1801-3-742

Hidayah, A. A., Azizah, A., Winarsa, R., & Muzakhar, K. (2020, November). Utilization of coffee pulp as a substrate for pectinase production by *Aspergillus* sp. VTMS through solid state

fermentation. *In AIP Conference Proceedings* (Vol. 2296, No. 1). AIP Publishing. https://doi.org/10.1063/5.0030474

Iwuagwu, J. O., & Ugwuanyi, J. O. (2014). Treatment and valorization of palm oil mill effluent through production of food grade yeast biomass. *Journal of Waste Management*, *2014*(1), 439071. https://doi.org/10.1155/2014/439071

Jahan, N., Shahid, F., Aman, A., Mujahid, T. Y., & Qader, S. A. U. (2017). Utilization of agro waste pectin for the production of industrially important polygalacturonase. *Heliyon*, *3*(6). https://doi.org/10.1016/j.heliyon.2017.e00330

Jaidev, L. R., & Narasimha, G. (2010). Fungal mediated biosynthesis of silver nanoparticles, characterization and antimicrobial activity. *Colloids and surfaces B: Biointerfaces*, *81*(2), 430-433. https://doi.org/10.1016/j.colsurfb.2010.07.033

John, F., & Soloman, P. (2020). Optimization of cellulase enzyme production by co-cultures of fungi isolated from lignocellulosic waste. *International Journal for Modern Trends in Science and Technology*, *6*(5), 1–8. https://doi.org/10.46501/IJMTST060504

Joshi, V. K., Parmar, M., & Rana, N. (2011). Purification and characterization of pectinase produced from apple pomace and evaluation of its efficacy in fruit juice extraction and clarification. *Indian Journal of Natural Products and Resource, 2*(2), 189-197 http://nopr.niscair.res.in/handle/123456789/12142

Kant, S., Vohra, A., & Gupta, R. (2013). Purification and physicochemical properties of polygalacturonase from *Aspergillus niger* MTCC 3323. *Protein Expression and Purification*, *87*(1), 11-16. https://doi.org/10.1016/j.pep.2012.09.014

Kapoor, M., Panwar, D., & Kaira, G. S. (2016). *Bioprocesses for enzyme production using agro-industrial wastes: technical challenges and commercialization potential. In Agro-industrial wastes as feedstock for enzyme production,* 61-93. Academic Press. https://doi.org/10.1016/B978-0-12-802392-1.00003-4

KC, S., Upadhyaya, J., Joshi, D. R., Lekhak, B., Kumar Chaudhary, D., Raj Pant, B., ... & Raghavan, V. (2020). Production, characterization, and industrial application of pectinase enzyme isolated from fungal strains. *Fermentation*, *6*(2), 59. https://doi.org/10.3390/fermentation6020059

Khedkar, R., & Singh, K. (2018). Food industry waste: A panacea or pollution hazard?. *Paradigms in Pollution Prevention*, 35–47. https://doi.org/10.1007/978-3-319-58415-7_3

Klinchongkon, K., Khuwijitjaru, P., & Adachi, S. (2017). Degradation kinetics of passion fruit pectin in subcritical water. *Bioscience, Biotechnology, and Biochemistry, 81*(4), 712–717. https://doi.org/10.1080/09168451.2016.1277941

Kot, A. M., Pobiega, K., Piwowarek, K., Kieliszek, M., Błażejak, S., Gniewosz, M., & Lipińska, E. (2020). Biotechnological methods of management and utilization of potato industry waste. A review. *Potato Research*, *63*, 431-447. https://doi.org/10.1007/s11540-019-09449-6

Kumar, V. N., Rebello, S., Raveendran, S., Parameswaran, B., Pandey, A., Aneesh, E. M., & Prabhakumari, C. (2022). Enzymes Production from Food Waste and Their Application. *Research Anthology on Emerging Techniques in Environmental Remediation*, 293-307. https://doi.org/10.4018/978-1-5225-7706-5.ch001

Kurnar, N. K., & Reddy, D. S. R. (2008). Optimization of pectinase production from *Manihot utilissima* by *Aspergillus niger* NCIM 548 using statistical experimental design. *Res J Microbiol*, *3*(1), 9-16. https://doi.org/10.3923/jm.2008.9.16

Lee, J. (2017). *Sustainable production of microbial lipids from renewable biomass: evaluation of oleaginous yeast cultures for high yield and productivity* (Doctoral dissertation, Kansas State University). https://krex.k-state.edu/dspace/handle/2097/35300

Li, C., Zhou, J., Du, G., Chen, J., Takahashi, S., & Liu, S. (2020). Developing *Aspergillus niger* as a cell factory for food enzyme production. *Biotechnology Advances*, *44*, 107630. https://doi.org/10.1016/j.biotechadv.2020.107630

Li, J., Ma, D., Tian, J., Sun, T., Meng, Q., Li, J., & Shan, A. (2023). The responses of organic acid production and microbial community to different carbon source additions during the anaerobic fermentation of Chinese cabbage waste. *Bioresource Technology, 371*, 128624. https://doi.org/10.1016/j.biortech.2023.128624

Li, P. J., Xia, J. L., Shan, Y., Nie, Z. Y., Su, D. L., Gao, Q. R., ... & Ma, Y. L. (2015). Optimizing production of pectinase from orange peel by *Penicillium oxalicum* PJ02 using response surface methodology. *Waste and Biomass Valorization*, *6*, 13-22. https://doi.org/10.1007/s12649-014-9317-4

Mahan, K. M., Le, R. K., Wells Jr, T., Anderson, S., Yuan, J. S., Stoklosa, R. J., ... & Ragauskas, A. J. (2018). Production of single cell protein from agro-waste using *Rhodococcus opacus*. *Journal of Industrial Microbiology and Biotechnology*, *45*(9), 795-801. https://doi.org/10.1007/s10295-018-2043-3

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31*(3), 426-428. https://doi.org/10.1021/ac60147a030

Mondal, A. K., Sengupta, S., Bhowal, J., & Bhattacharya, D. K. (2012). Utilization of fruit wastes in producing single cell protein. *International Journal of Science, Environment and Technology*, *1*(5), 430-438. https://doi.org/10.1002/appl.202100007

Muriithi, J., Matofari, J. W., & Nduko, J. M. (2022). Amylolytic microorganisms from diverse tropical environments: Isolation, identification, and amylase production. *Applied Research, 1*(1–2), e202100007. https://doi.org/10.1002/appl.202100007

Naqvi, S. A. Z., Irfan, A., Zaheer, S., Sultan, A., Shajahan, S., Rubab, S. L., ... & Acevedo, R. (2021). Proximate composition of orange peel, pea peel and rice husk wastes and their potential use as antimicrobial agents and antioxidants. *Vegetos*, *34*, 470-476. https://doi.org/10.1007/s42535-021-00213-1

Nielsen, S. (2017). *Food analysis*. Springer. https://doi.org/10.1007/978-3-319-45776-5

Nighojkar, A., Patidar, M. K., & Nighojkar, S. (2019). Pectinases: production and applications for fruit juice beverages. *Processing and Sustainability of Beverages*, *2*, 235-273. https://doi.org/10.1016/B978-0-12-815259-1.00008-2

Nirmaladevi, D., Anilkumar, M., & Srinivas, C. (2014). Production and characterization of exopolygalacturonase from *Fusarium oxysporum* F. sp. *Lycopersici*. *Int J Pharma Bio Sci*, *5*, 666-75.

Núñez Pérez, J., Chávez Arias, B. S., de la Vega Quintero, J. C., Zárate Baca, S., & Pais-Chanfrau, J. M. (2022). Multi-objective statistical optimization of pectinolytic enzymes production by an *Aspergillus* sp. on dehydrated coffee residues in solid-state fermentation. *Fermentation*, *8*(4), 170. https://doi.org/10.3390/fermentation8040170

Olabinjo, O. O., Ogunlowo, A. S., Ajayi, O. O., & Olalusi, A. P. (2017). Analysis of physical and chemical composition of sweet orange (*Citrus sinensis* L.) peels. *Int. J. Environ. Agric. Biotechnol*, *2*(4), 2201-2206.https://doi.org/10.22161/ijeab/2.4.80

Ortiz, D. L., Batuecas, E., Orrego, C. E., Rodríguez, L. J., Camelin, E., & Fino, D. (2020). Sustainable management of peel waste in the small-scale orange juice industries: A Colombian Case Study. *Journal of Cleaner Production, 265,* 121587. https://doi.org/10.1016/j.jclepro.2020.121587

Ortiz, G. E., Ponce-Mora, M. C., Noseda, D. G., Cazabat, G., Saravalli, C., López, M. C., ... & Albertó, E. O. (2017). Pectinase production by *Aspergillus giganteus* in solid-state fermentation: optimization, scale-up, biochemical characterization and its application in olive-oil

extraction. *Journal of Industrial Microbiology and Biotechnology*, *44*(2), 197-211. https://doi.org/10.1007/s10295-016-1873-0

Otles, S., & Kartal, C. (2018). *Food waste valorization. In Sustainable food systems from agriculture to industry*, 371-399. Academic Press. https://doi.org/10.1016/B978-0-12-811935-8.00011-1

Oumer, O. J., & Abate, D. (2018). Screening and Molecular Identification of Pectinase Producing Microbes from Coffee Pulp. *BioMed Research International, article* e2961767. https://doi.org/10.1155/2018/2961767

Oyebamiji, G. H., Jonathan, G. S., Akinyemi, D. S., & Popoola, K. K. (2018). Fungal and insect pests of the edible mushroom *Pleurotus ostreatus*. *Notulae Scientia Biologicae*, *10*(3), 379-386. https://doi.org/10.15835/nsb10310284

Panwar, D., Panesar, P. S., & Chopra, H. K. (2019). Recent trends on the valorization strategies for the management of citrus by-products. *Food Reviews International*, *37*(1), 91-120 https://www.tandfonline.com/doi/abs/10.1080/87559129.2019.1695834

Patel, A. K., Dong, C. D., Chen, C. W., Pandey, A., & Singhania, R. R. (2023). *Production, purification, and application of microbial enzymes. In Biotechnology of microbial enzymes*, 25-57. Academic Press. https://doi.org/10.1016/B978-0-12-803725-6.00002-9

Patel, V. B., Chatterjee, S., & Dhoble, A. S. (2022). A review on pectinase properties, application in juice clarification, and membranes as immobilization support. *Journal of Food Science*, *87*(8), 3338-3354. https://doi.org/10.1111/1750-3841.16233

Pathak, P. D., Mandavgane, S. A., & Kulkarni, B. D. (2017). Fruit peel waste: characterization and its potential uses. *Current Science*, *113*(3), 444-454.

Patidar, M. K., Nighojkar, S., Kumar, A., & Nighojkar, A. (2018). Pectinolytic enzymes-solid state fermentation, assay methods and applications in fruit juice industries: A review. *3 Biotech*, *8*(4), 199. https://doi.org/10.1007/s13205-018-1220-4

Picot-Allain, M. C. N., Ramasawmy, B., & Emmambux, M. N. (2022). Extraction, characterisation, and application of pectin from tropical and sub-tropical fruits: A review. *Food Reviews International*, *38*(3), 282-312. https://doi.org/10.1080/87559129.2020.1733008

Purnachandra Reddy, M., & Saritha, K. V. (2015). Bio-catalysis of mango industrial waste by newly isolated *Fusarium* sp. (PSTF1) for pectinase production. *3 Biotech*, *5*, 893-900. https://doi.org/10.1007/s13205-015-0288-3

Rana, N., Verma, N., Vaidya, D., & Dipta, B. (2017). Application of bacterial amylase in clarification of juices and bun making. *Journal of Pharmacognosy and Phytochemistry*, *6*(5), 859-864.

Rathinavel, S., & Saravanakumar, S. S. (2021). Development and analysis of silver nano particle influenced PVA/natural particulate hybrid composites with thermo-mechanical properties. *Journal of Polymers and the Environment*, *29*(6), 1894-1907. https://doi.org/10.1007/s10924-020-01999-y

Ravindran, R., & Jaiswal, A. K. (2016). Microbial enzyme production using lignocellulosic food industry wastes as feedstock: A review. *Bioengineering*, *3*(4), 30.https://doi.org/10.3390/bioengineering3040030

Ricci, A., Diaz, A. B., Caro, I., Bernini, V., Galaverna, G., Lazzi, C., & Blandino, A. (2019). Orange peels: from by-product to resource through lactic acid fermentation. *Journal of the Science of Food and Agriculture*, *99*(15), 6761-6767. https://doi.org/10.1002/jsfa.9958

Robinson, P. K. (2015). Enzymes: Principles and biotechnological applications. *Essays in Biochemistry*, *59*, 1–41. https://doi.org/10.1042/bse0590001

Rouse, A. H., & Atkins, C. D. (1955). *Pectinesterase and pectin in commercial citrus juices as determined by methods used at the Citrus Experiment Station*. https://agris.fao.org/agris-search/search.do?recordID=US201300455909

Ruiz, H. A., Rodríguez-Jasso, R. M., Rodríguez, R., Contreras-Esquivel, J. C., & Aguilar, C. N. (2012). Pectinase production from lemon peel pomace as support and carbon source in solid-state fermentation column-tray bioreactor. *Biochemical Engineering Journal*, *65*, 90–95. https://doi.org/10.1016/j.bej.2012.03.007

Sadh, P. K., Duhan, S., & Duhan, J. S. (2018). Agro-industrial wastes and their utilization using solid state fermentation: A review. *Bioresources and bioprocessing*, *5*(1), 1-15. https://doi.org/10.1186/s40643-017-0187-z

Salim, A. A., Grbavčić, S., Šekuljica, N., Stefanović, A., Tanasković, S. J., Luković, N., & Knežević-Jugović, Z. (2017). Production of enzymes by a newly isolated *Bacillus* sp. TMF-1 in solid state fermentation on agricultural by-products: the evaluation of substrate pre-treatment methods. *Bioresource technology*, *228*, 193-200. https://doi.org/10.1016/j.biortech.2016.12.081

Saranraj, P., & Naidu, M. A. (2014). Microbial pectinases: A review. *Global J Trad Med Syst*, *3*(1), 1-9.

Sarfraz, M., Iqbal, K., Wang, Y., & Bhutta, M. S. (2023). Role of agricultural resource sector in environmental emissions and its explicit relationship with sustainable development: Evidence from agri-food system in China. *Resources Policy*, *80*, 103191.https://doi.org/10.1016/j.resourpol.2022.103191

Satapathy, S., Rout, J. R., Kerry, R. G., Thatoi, H., & Sahoo, S. L. (2020b). Biochemical prospects of various microbial pectinase and pectin: An approachable concept in pharmaceutical bioprocessing. *Frontiers in Nutrition, 7*, 1-17. https://doi.org/10.3389/fnut.2020.00117

Satari, B., & Karimi, K. (2018). Citrus processing wastes: Environmental impacts, recent advances, and future perspectives in total valorization. *Resources, Conservation and Recycling*, *129*, 153-167. https://doi.org/10.1016/j.resconrec.2017.10.032

Sathiyaraj, G., Srinivasan, S., Kim, H. B., Subramaniyam, S., Lee, O. R., Kim, Y. J., & Yang, D. C. (2011). Screening and optimization of pectin lyase and polygalacturonase activity from ginseng pathogen *Cylindrocarpon destructans*. *Brazilian Journal of Microbiology*, *42*, 794-806.

Sewalt, V., Shanahan, D., Gregg, L., La Marta, J., & Carrillo, R. (2016). The generally recognized as safe (GRAS) process for industrial microbial enzymes. *Industrial Biotechnology*, *12*(5), 295-302. https://doi.org/10.1089/ind.2016.0011

Sharma, H. P., Patel, H., & Sugandha. (2017). Enzymatic added extraction and clarification of fruit juices: A review. *Critical Reviews in Food Science and Nutrition*, *57*(6), 1215-1227.http://www.tandfonline.com.egertonelibrary.remotexs.co/doi/abs/10.1080/10408398.2014.977434

Sharma, K. M., Kumar, R., Panwar, S., & Kumar, A. (2017). Microbial alkaline proteases: Optimization of production parameters and their properties. *Journal of Genetic Engineering and Biotechnology*, *15*(1), 115-126. https://doi.org/10.1016/j.jgeb.2017.02.001

Shiv Kumar, S. K. (2015). Role of enzymes in fruit juice processing and its quality enhancement. *Advance in Applied Science Research*, *6*(6), 114-124.

Siddeeg, S. M., Tahoon, M. A., & Rebah, F. B. (2019). Agro-industrial waste materials and waste water as growth media for microbial bioflocculants production: A review. *Materials Research Express*, *7*(1), 012001. https://doi.org/10.1088/2053-1591/ab5980

Singh, R. S., Singh, T., & Pandey, A. (2019). Microbial enzymes—an overview. *Advances in Enzyme Technology*, 1–40. https://doi.org/10.1016/B978-0-444-64114-4.00001-7

Singh, R., Kumar, M., Mittal, A., & Mehta, P. K. (2016). Microbial enzymes: Industrial progress in 21st century. *3 Biotech*, *6*, 1-15. https://doi.org/10.1007/s13205-016-0485-8

Socas-Rodríguez, B., Álvarez-Rivera, G., Valdés, A., Ibáñez, E., & Cifuentes, A. (2021). Food by-products and food wastes: Are they safe enough for their valorization? *Trends in Food Science & Technology*, *114*, 133-147. https://doi.org/10.1016/j.tifs.2021.05.002

Takagi, S., Kojima, K., & Ohashi, S. (2020). Proteomic analysis on *Aspergillus* strains that are useful for industrial enzyme production. *Bioscience, Biotechnology, and Biochemistry*, *84*(11), 2241-2252. https://doi.org/10.1080/09168451.2020.1794784

Thorati, M., Mishra, J. K., & Kumar, S. (2016). Isolation, identification of endophytic Fungi from mangrove roots along the coast of South Andaman Sea, Andaman and Nicobar Islands. *India. J Mar Biol Oceanogr*, *5*, 2. https://doi.org/10.4172/2324-8661.1000157

Tolkacheva, A. A., Cherenkov, D. A., Korneeva, O. S., & Ponomarev, P. G. (2018). Enzymes of industrial purpose-review of the market of enzyme preparations and prospects for its development. *Proceedings of the Voronezh State University of Engineering Technologies*, *79*(4), 197-203. https://doi.org/10.20914/2310-1202-2017-4-197-203

Toma, M. A., Nazir, K. N. H., Mahmud, M. M., Mishra, P., Ali, M. K., Kabir, A., ... & Alim, M. A. (2021). Isolation and identification of natural colorant producing soil-borne *Aspergillus niger* from Bangladesh and extraction of the pigment. *Foods*, *10*(6), 1280. https://doi.org/10.3390/foods10061280

Torres-León, C., Ramírez-Guzman, N., Londoño-Hernandez, L., Martinez-Medina, G. A., Díaz-Herrera, R., Navarro-Macias, V., ... & Aguilar, C. N. (2018). Food waste and byproducts: An opportunity to minimize malnutrition and hunger in developing countries. *Frontiers in Sustainable Food Systems*, *2*, 1-17. https://doi.org/10.3389/fsufs.2018.00052

Ustok, F. I., Tari, C., & Gogus, N. (2007). Solid-state production of polygalacturonase by *Aspergillus sojae* ATCC 20235. *Journal of Biotechnology*, *127*(2), 322-334. https://doi.org/10.1016/j.jbiotec.2006.07.010

Uzuner, S., & Cekmecelioglu, D. (2015). Optimising clarification of carrot juice by bacterial crude pectinase. *International Journal of Food Science & Technology*, *50*(12), 2707-2712. https://doi.org/10.1111/ijfs.12961

Voběrková, S., Maxianová, A., Schlosserová, N., Adamcová, D., Vršanská, M., Richtera, L., ... & Vaverková, M. D. (2020). Food waste composting-Is it really so simple as stated in scientific literature? –A case study. *Science of the Total Environment*, *723*, 138202. https://doi.org/10.1016/j.scitotenv.2020.138202

Wang, F., Du, C., Chen, J., Shi, L., & Li, H. (2021). A new method for determination of pectin content using spectrophotometry. *Polymers*, *13*(17), 2847. https://doi.org/10.3390/polym13172847

Yafetto, L. (2022). Application of solid-state fermentation by microbial biotechnology for bioprocessing of agro-industrial wastes from 1970 to 2020: A review and bibliometric analysis. *Heliyon*, *8*(3). https://doi.org/10.1016/j.heliyon.2022.e09173

Yin, G., Padhi, S., Lee, S., Hung, R., Zhao, G., & Bennett, J. W. (2015). Effects of three volatile oxylipins on colony development in two species of fungi and on Drosophila larval metamorphosis. *Current Microbiology*, *71*(3), 347-356. https://doi.org/10.1007/s00284-015-0864-0

Zaker, M. A., Sawate, A. R., Patil, B. M., & Sadawarte, S. K. (2016). Studies on Effect of Orange Peel Powder Incorporation on Physical, Nutritional and Sensorial Quality of Cookies. *International Journal of Engineering Research & Technology*, *5*(9), 2278-0181. https://doi.org/10.17577/IJERTV5IS090125

Zema, D. A., Calabrò, P. S., Folino, A., Tamburino, V. I. N. C. E. N. Z. O., Zappia, G., & Zimbone, S. M. (2018). Valorisation of citrus processing waste: A review. *Waste management*, *80*, 252-273. https://doi.org/10.1016/j.wasman.2018.09.024

Zeng, J., Zeng, H., & Wang, Z. (2022). Review on technology of making biofuel from food waste. *International Journal of Energy Research*, *46*(8), 10301-10319. https://doi.org/10.1002/er.7868

APPENDICES

Appendix A: Design of experiment

StdOrder	RunOrder	PtType	Blocks	temperature	pH	Time (Days)	Yield (Clear zone diameter)
44	1	0	1	30	5	6	
26	2	2	1	30	6	4	
11	3	2	1	30	6	4	
34	4	2	1	35	5	8	
13	5	0	1	30	5	6	
18	6	2	1	25	5	8	
16	7	2	1	25	5	4	
32	8	2	1	35	5	4	
24	9	2	1	30	4	4	
10	10	2	1	30	4	8	
7	11	2	1	25	6	6	
15	12	0	1	30	5	6	
42	13	2	1	30	6	8	
5	14	2	1	25	4	6	
12	15	2	1	30	6	8	
45	16	0	1	30	5	6	
22	17	2	1	25	6	6	
20	18	2	1	25	4	6	
23	19	2	1	35	6	6	
6	20	2	1	35	4	6	
3	21	2	1	25	5	8	
19	22	2	1	35	5	8	
29	23	0	1	30	5	6	
33	24	2	1	25	5	8	
30	25	0	1	30	5	6	
36	26	2	1	35	4	6	

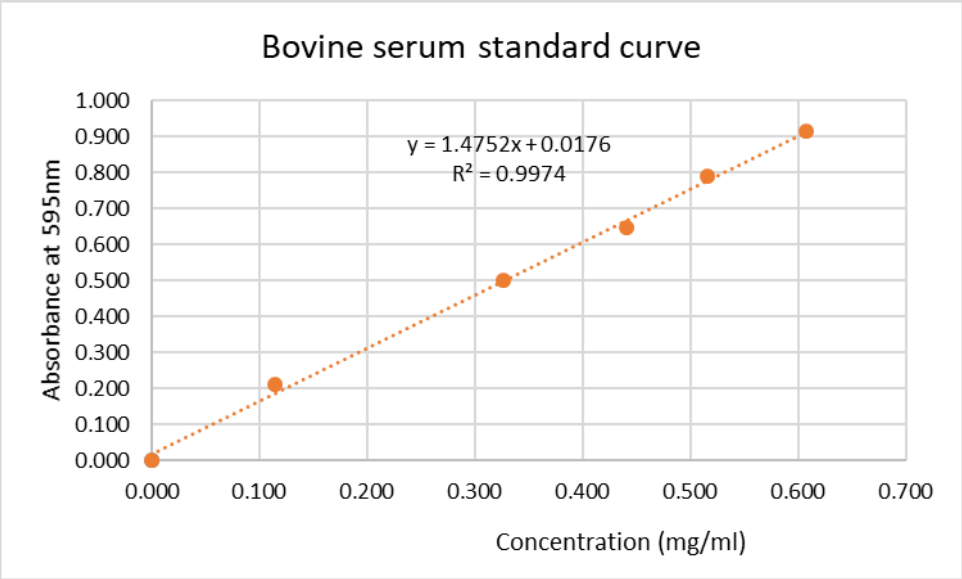
43	27	0	1	30	5	6	
1	28	2	1	25	5	4	
14	29	0	1	30	5	6	
25	30	2	1	30	4	8	
4	31	2	1	35	5	8	
28	32	0	1	30	5	6	
9	33	2	1	30	4	4	
39	34	2	1	30	4	4	
27	35	2	1	30	6	8	
17	36	2	1	35	5	4	
41	37	2	1	30	6	4	
21	38	2	1	35	4	6	
2	39	2	1	35	5	4	
40	40	2	1	30	4	8	
37	41	2	1	25	6	6	
38	42	2	1	35	6	6	
35	43	2	1	25	4	6	
8	44	2	1	35	6	6	
31	45	2	1	25	5	4	

Appendix B: Design of experiment

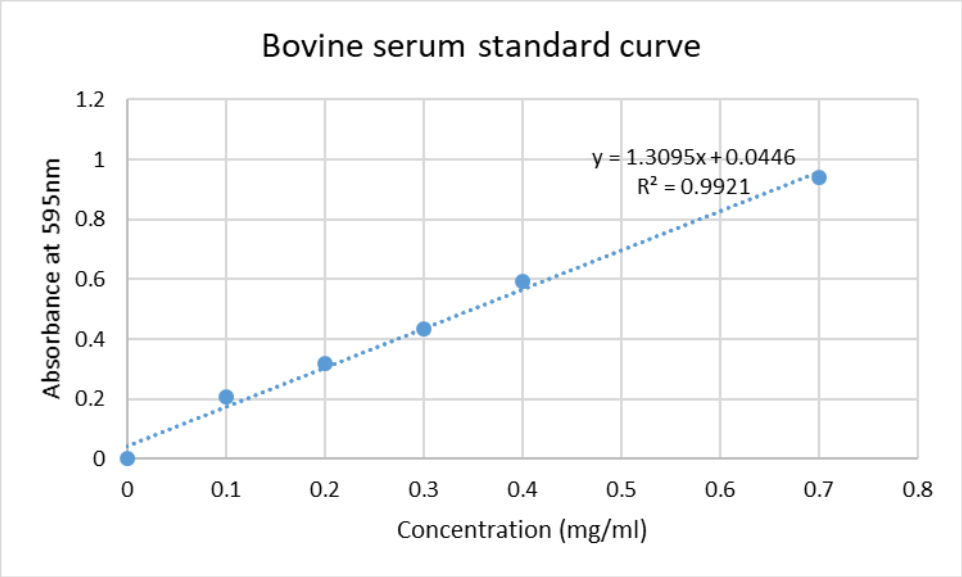
StdOrder	RunOrder	PtType	Blocks	temperature	pH	Time (Days)	Enzyme activity		
							Polygalactu ronase	Pectin lyase	Pectin esterase
44	1	0	1	30	5	6			
26	2	2	1	30	6	4			
11	3	2	1	30	6	4			
34	4	2	1	35	5	8			
13	5	0	1	30	5	6			
18	6	2	1	25	5	8			
16	7	2	1	25	5	4			
32	8	2	1	35	5	4			
24	9	2	1	30	4	4			
10	10	2	1	30	4	8			
7	11	2	1	25	6	6			
15	12	0	1	30	5	6			
42	13	2	1	30	6	8			
5	14	2	1	25	4	6			
12	15	2	1	30	6	8			
45	16	0	1	30	5	6			
22	17	2	1	25	6	6			
20	18	2	1	25	4	6			
23	19	2	1	35	6	6			
6	20	2	1	35	4	6			
3	21	2	1	25	5	8			
19	22	2	1	35	5	8			
29	23	0	1	30	5	6			
33	24	2	1	25	5	8			
30	25	0	1	30	5	6			
36	26	2	1	35	4	6			
43	27	0	1	30	5	6			

1	28	2	1	25	5	4			
14	29	0	1	30	5	6			
25	30	2	1	30	4	8			
4	31	2	1	35	5	8			
28	32	0	1	30	5	6			
9	33	2	1	30	4	4			
39	34	2	1	30	4	4			
27	35	2	1	30	6	8			
17	36	2	1	35	5	4			
41	37	2	1	30	6	4			
21	38	2	1	35	4	6			
2	39	2	1	35	5	4			
40	40	2	1	30	4	8			
37	41	2	1	25	6	6			
38	42	2	1	35	6	6			
35	43	2	1	25	4	6			
8	44	2	1	35	6	6			
31	45	2	1	25	5	4			

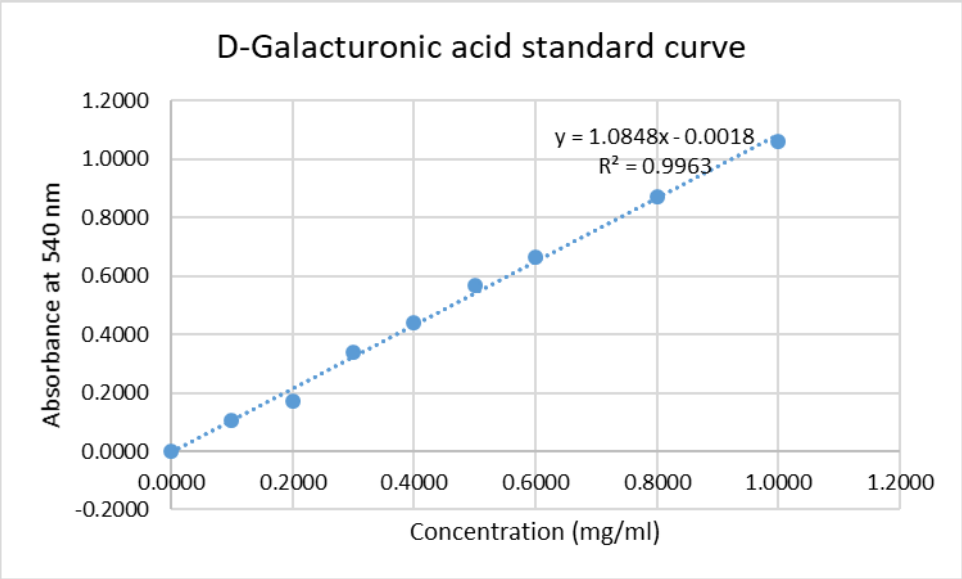
Appendix C: Standard graph for protein quantification (For produced pectinase enzyme)



Appendix D: Standard graph for protein quantification (For commercial pectinase enzyme)



Appendix E: Standard graph for polygalacturonase activity



Appendix F: Publication in a peer reviewed journal

Biomass Conversion and Biorefinery
https://doi.org/10.1007/s13399-023-04603-0

ORIGINAL ARTICLE

Pectinolytic Enzyme Production from Orange Processing Waste Using *Aspergillus brasiliensis* Strain

Flomena Cyprian Laswai[1] · Joseph Wafula Matofari[1] · John Masani Nduko[1]

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Abstract

This study explored the potential of utilizing orange processing waste as a cost-effective substrate for the production of pectinolytic enzymes using an isolated strain of *Aspergillus brasiliensis*. The study focused on optimizing various process parameters, such as temperature, pH, and incubation time, for the production of enzymes through solid-state fermentation using a Box-Behnken design (BBD). The maximum pectinase enzyme activities of polygalacturonase, lyase, and esterase were 44.58 U/g, 16.53 U/g, and 74 U/g, respectively. These optimal enzyme activities were observed for enzyme extracts from cells cultivated at 35 °C and pH 4.0 for 6 days. The crude enzyme extract was characterized, and its potential application in apple juice extraction and clarification was evaluated. The results showed that the enzyme extract was effective in improving juice yield from 65% (for control) to 84.5% at 4% enzyme loading. The enzyme was also effective in clarifying the juice as demonstrated by reduced pectin content (from 0.45% to 0.04%) and pH (from 3.55 to 3.35) of the juice, leading to reduction in absorbance at 620 nm. This study highlights the potential of orange processing waste as a sustainable and cost-effective substrate for pectinolytic enzyme production and demonstrates the effectiveness of using *Aspergillus brasiliensis* strain and BBD in optimal enzyme production. The findings suggest that this approach could be useful for the industrial-scale production of pectinolytic enzymes for various applications.

Appendix G: NACOSTI Research authorization

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **343552**

Date of Issue: **28/January/2022**

RESEARCH LICENSE

This is to Certify that Ms.. Flomena Cyprian Laswai of Egerton University, has been licensed to conduct research in Nakuru on the topic: VALORISATION OF ORANGE (Citrus sinensis) PROCESSING WASTES FOR PECTINASE ENZYMES PRODUCTION USING Aspergillus niger for the period ending : 28/January/2023.

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