

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI,

GHANA

COLLEGE OF SCIENCE

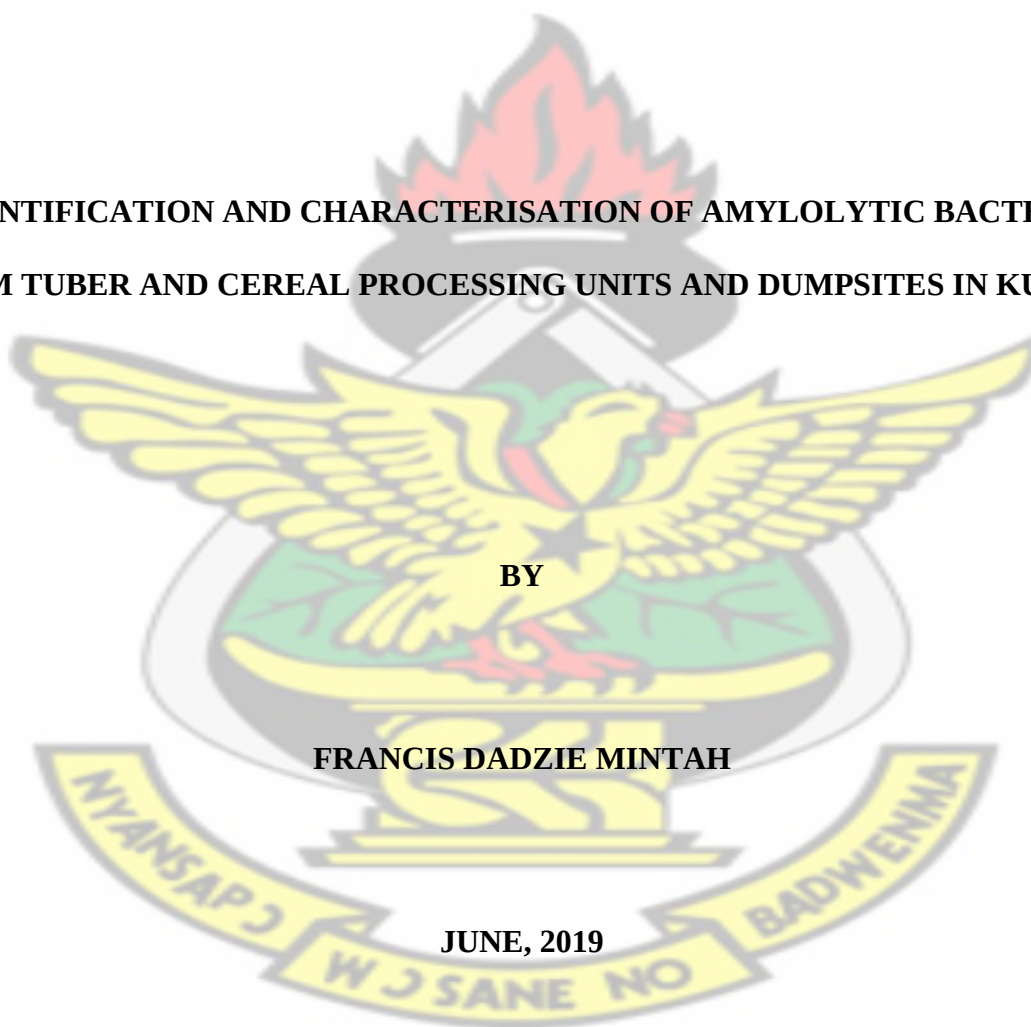
DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

**IDENTIFICATION AND CHARACTERISATION OF AMYLOLYTIC BACTERIA
FROM TUBER AND CEREAL PROCESSING UNITS AND DUMPSITES IN KUMASI**

BY

FRANCIS DADZIE MINTAH

JUNE, 2019



KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI,

GHANA

COLLEGE OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

**IDENTIFICATION AND CHARACTERISATION OF AMYLOLYTIC BACTERIA
FROM TUBER AND CEREAL PROCESSING UNITS AND DUMPSITES IN KUMASI**

**A THESIS SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY AND
BIOTECHNOLOGY, KWAME NKRUMAH UNIVERSITY OF SCIENCE AND
TECHNOLOGY, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE
AWARD OF MASTER OF PHILOSOPHY IN BIOTECHNOLOGY**

BY

FRANCIS DADZIE MINTAH (BSc. Agricultural Biotechnology)

JUNE, 2019

DECLARATION

I hereby declare that except for references cited in relation to other works, which have duly been acknowledged, this work is the outcome of my own research and that this thesis has neither in whole or part been submitted for any other degree at any other University.

Francis Dadzie Mintah
(PG6717816)

Signature

Date

We declare that we have supervised the above student to undertake the study submitted herein and confirm that she has our permission to submit.

Prof. (Mrs.) Antonia Yarbey Tetteh
(Supervisor)

Signature

Date

Dr. F. C. Mills-Robertson
(Co-Supervisor)

Signature

Date

Certified By:

Prof. (Mrs.) Antonia Yarbey Tetteh
(Head of Department)

Signature

Date

DEDICATION

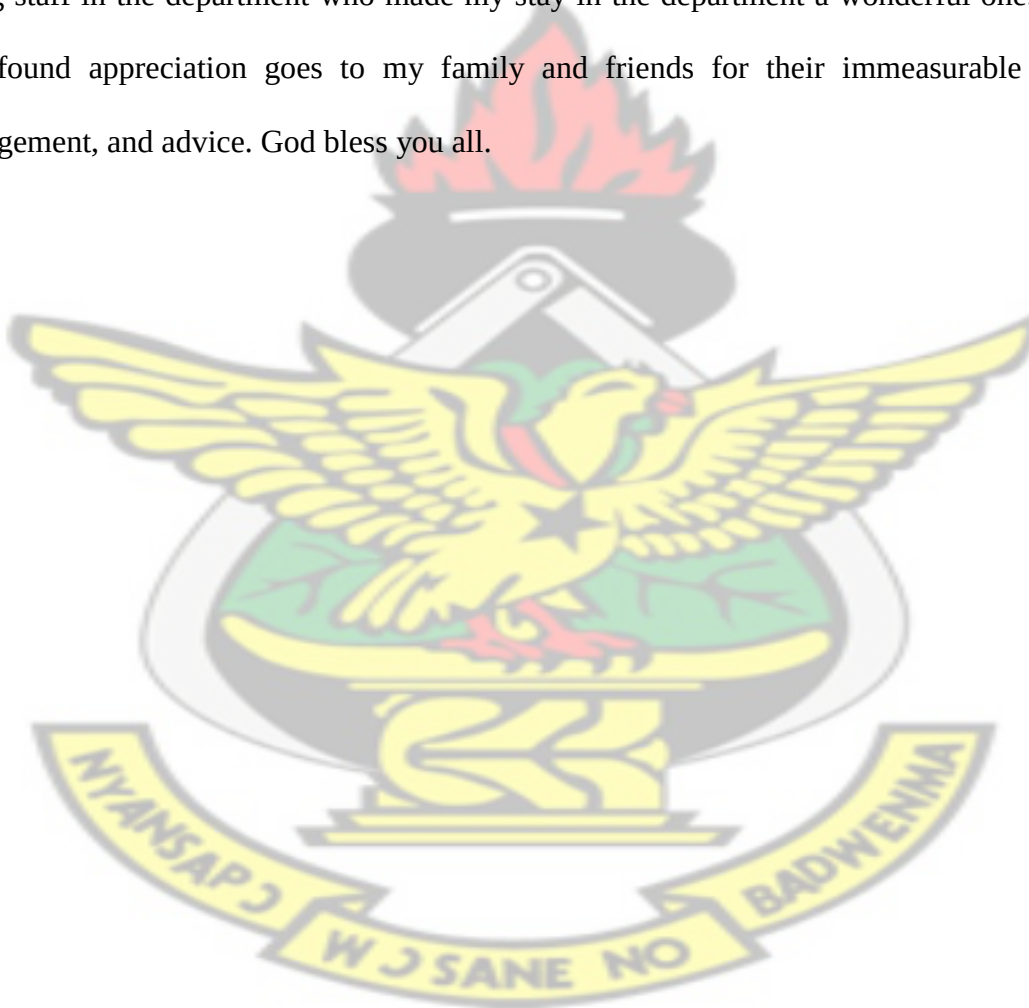
I dedicate this thesis to the Almighty God and my entire family.

KNUST



ACKNOWLEDGEMENT

I am most grateful to the Almighty God for his gift of life, grace, and strength throughout my academic pursuit. My heartfelt gratitude goes to my supervisor, Prof. (Mrs.) Antonia Yarbey Tetteh for her immense support, direction and guidance, constructive criticisms, time and financial assistance in making this study a success. I am also grateful to my co-supervisor, Dr. F. C. Mills-Robertson for his support, advice, and encouragement. Special thanks to all teaching and non-teaching staff in the department who made my stay in the department a wonderful one. Finally, my profound appreciation goes to my family and friends for their immeasurable support, encouragement, and advice. God bless you all.



ABSTRACT

Amylases are enzymes essential for commercial and industrial purposes in breaking down starch to soluble sugars. In Ghana, commercial amylases are absent though there is abundance of starchy materials in which amylase-producing bacteria inhabit. Therefore, this study was to identify and characterize amylolytic bacteria in cereal and tuber processing units and dumpsites in Kumasi using morphological and biochemical methods. Using a 3 x 3 Factorial experiment with three (3) replications using Completely Randomized Design (CRD), eighteen samples from three starchy sources, namely, cassava, maize, and rice were aseptically collected from three processing and waste dumpsites in Kumasi. Bacteria were morphologically and biochemically screened from the various substrates, rate of bacteria growth was determined and enzyme activities of the enzymes produced by the organisms were ascertained. Cluster analysis and multivariate analysis were conducted on the data obtained. Not all the substrates had the organisms, rice had highest while maize had the lowest making maize not suitable in producing amylase. A total of 50 bacteria isolates were identified which were grouped into 10 classes on the basis of the similarity in their qualitative characteristics where five were amylolytic and five were non-amylolytic. All bacteria were Gram positive and catalase positive. Across the three fermentation systems, only pH of substrate was significantly different ($P < 0.05$) whereas temperature and oxidation-reduction potential (ORP) were not. The pH of starch substrates ranged between 4.68 ± 0.58 and 6.45 ± 0.54 , temperature ranged between 26.83 ± 0.93 and 27.67 ± 1.25 °C, and ORP ranged between 72.5 ± 113.20 mV and 134.5 ± 24.50 mV. A cluster analysis based on Dice similarity index grouped the five amylolytic populations into two large clusters, cluster I and cluster II (with sub clusters A, B, and C). The clusters were putatively identified as *Staphylococcus* spp. for cluster I and IIC, *Bacillus* spp. for cluster IIA, and *Pseudomonas* spp. and *Staphylococcus* spp. for cluster IIB. Mean

zone of clearance across the four isolates was 5.39 ± 0.17 mm for 24-h incubation which increased to 7.44 ± 0.13 mm for 48 h of incubation. Significant growth of the bacteria cells were recorded for all isolates at 37 °C, pH 7.0, and 1.0% starch concentration with isolate IIA showing the highest growth across the various parameters. There were differences in the activities of the enzymes produced by the organisms, where *Bacillus* spp. had the highest followed by *Pseudomonas* spp. and *Staphylococcus* spp. Across the various isolates, optimum enzyme activity of 7.20 ± 0.30 to 12.60 ± 0.20 U/mL was exhibited at 75 °C whereas optimum activity of 5.55 ± 0.30 to 11.7 ± 0.20 U/mL was demonstrated at pH 7.0, 4.20 ± 0.30 to 9.30 ± 0.20 U/mL displayed at 1.0% substrate concentration, and 43.50 ± 4.00 to 88.50 ± 5.00 U/mL at 96 h of incubation. However, in an economic sense for industrial purposes, the 72 h incubation period can be used since enzyme activities at the said incubation time is not different from 96 h. These findings suggest that bacteria are good sources of amylase and good for industrial purposes. However, further studies are required to identify the species of the bacteria and its associated enzymatic properties such as thermostability. Moreover, the type of amylase produced needs to be ascertained.

TABLE OF CONTENTS

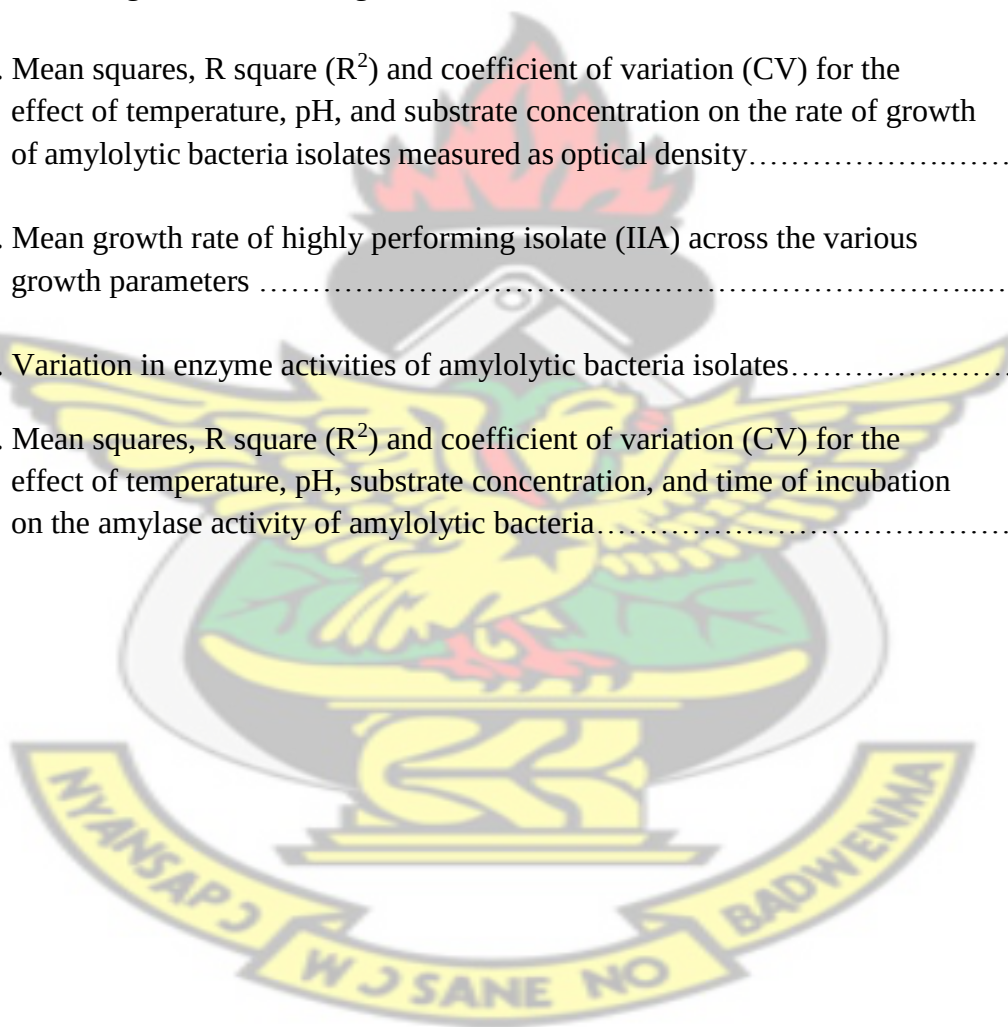
CONTENT	PAGE
DECLARATION	i
DEDICATION	ii
ACKNOWLEDGEMENT	iii
ABSTRACT.....	iv
TABLE OF CONTENTS.....	vi
LIST OF TABLES	ix
LIST OF FIGURES	x
LIST OF PLATE.....	xi
CHAPTER ONE	1
INTRODUCTION	1
1.0 Introduction	1
1.2 Justification	5
1.3 Research Hypothesis	6
CHAPTER TWO	7
LITERATURE REVIEW	7
2.1 Economic Importance of Natural Fermentations in Ghana.....	7
2.1.1 Types of Fermentation.....	9
2.2 Enzymes as an Industrial Workhorse.....	10
2.3 Enzyme Activity and its Measurement	11
2.3.1 Effects of Temperature on Enzyme Activity.....	11
2.3.2 Effects of pH on Enzyme Activity	13
2.3.3 Effects of Substrate Concentration on Enzyme Activity.....	14
2.3.4 Effect of Incubation Period on Enzyme Activity	15
2.4 Enzymatic Hydrolysis of Starch.....	16
2.5 Amylases	18
2.5.1 Purification of Amylase.....	19
2.5.2 Amylase-producing Bacteria	20
2.6 Isolation and Storage of Enzyme-Producing Organisms	21
2.7 Bacteria Characterisation	22
CHAPTER THREE	25

MATERIALS AND METHODS.....	25
3.1 Sample Collection	25
3.2 Physicochemical Properties of the Sources of Sample	25
3.3 Media and Culture Conditions for Amylolytic Bacteria Isolation	26
3.4 Isolation and Characterization of Starch- degrading Bacteria	26
3.5 Identification of Starch Utilizing Bacteria with Gram's iodine	26
3.6 Morphological Identification and Characterization of Starch-degrading Bacterial Strain .	27
3.6.2 Oxidase Test	27
3.6.3 Catalase Test.....	28
3.6.4 Gram Staining.....	28
3.7 Determination of Culture Conditions and Growth Kinetics of Isolates	28
3.7.1 Effect of Temperature on Culture Growth	28
3.7.2 Effect of pH on Culture Growth	29
3.7.3 Effect of Starch Concentration on Culture Growth	29
3.8 Determination of amylase activity	30
3.8.1 Glucose standard curve preparation	31
3.9 Kinetic Properties of Enzyme Activity	31
3.9.1 Effect of Temperature on Enzyme Activity	31
3.9.2 Effect of pH on Enzyme Activity	32
3.9.3 Effect of Starch Concentration on Enzyme Activity	32
3.9.4 Effect of Incubation Period on Enzyme Activity	32
3.10 Experimental Design	33
3.11 Data Analysis	33
CHAPTER FOUR.....	34
RESULTS	34
4.1 Bacteria Isolates in Substrates.....	34
4.2 Variations in Physicochemical Properties of Substrates at the Three Locations	35
4.3 Bacteria Populations in the Fermentation Systems	36
4.4 Clustering of Amylolytic Bacteria in Ghanaian Local Fermentation Systems	37
4.5 Amylolytic Activity of Isolates Based on Zone of Clearance.....	39
4.6 Rate of Bacteria Growth.....	42
4.6.1 Effects of Temperature, pH, and Substrate Concentration on Growth of Amylolytic Bacteria Isolates.....	42

4.7 Amylase Assay of Bacteria Isolates on Cassava Starch.....	50
4.8 Effects of Temperature, pH, Substrate Concentration, and Time of Incubation on Amylase Enzyme Activity	51
4.8.1 Temperature.....	51
4.8.2 pH	51
4.8.3 Substrate Concentration.....	52
4.8.4 Time of Incubation	52
CHAPTER FIVE	57
DISCUSSION	57
5.1 Bacteria Isolates in Substrates.....	57
5.2 Physicochemical Properties of the Fermentation Systems.....	58
5.3 Bacteria Populations in the Fermentation Systems	60
5.4 Amylolytic Activity of Isolates Based on Zone of Clearance.....	61
5.5 Effect of Temperature, pH, and Substrate Concentration on the Rate of Growth of Bacteria Isolates	61
5.6 Effects of Culture Conditions on Amylase Enzyme Activity	63
5.6.1 Temperature.....	63
5.6.2 pH	65
5.6.3 Substrate Concentration.....	66
5.6.4 Incubation Time.....	68
CHAPTER SIX.....	70
CONCLUSION AND RECOMMENDATIONS	70
6.1 Conclusion	70
6.2 Recommendations.....	71
REFERENCES	72
APPENDICES	91

LIST OF TABLES

	Page
Table 4.1. Mean Squares, R Squares and Coefficient of Variation for Physicochemical Properties for Processing of Maize, Cassava, Rice in Kumasi for Identification of Amylolytic Bacteria.....	35
Table 4.2. Bacteria population of fermentation systems and their morphologies.....	38
Table 4.3. Means, standard deviation and mean squares of zone of clearance of amylolytic Bacteria grown on starch agar.....	41
Table 4.4. Mean squares, R square (R^2) and coefficient of variation (CV) for the effect of temperature, pH, and substrate concentration on the rate of growth of amylolytic bacteria isolates measured as optical density.....	45
Table 4.5. Mean growth rate of highly performing isolate (IIA) across the various growth parameters	45
Table 4.6. Variation in enzyme activities of amylolytic bacteria isolates.....	51
Table 4.7. Mean squares, R square (R^2) and coefficient of variation (CV) for the effect of temperature, pH, substrate concentration, and time of incubation on the amylase activity of amylolytic bacteria.....	54



LIST OF FIGURES

	Page
Figure 4.1 Frequency of bacteria isolates from three starchy samples in three processing sites and farm in Kumasi.....	34
Figure 4.2 pH, Temperature, ORP of substrates from three different processing sites and farm in Kumasi.....	36
Figure 4.3 Cluster analysis of 23 colonies of amylolytic bacteria isolated from Ghanaian local fermentation systems.....	39
Figure 4.4. Zone of clearance of amylolytic bacteria isolates cultured on starch agar for 48 hours	40
Figure 4.5. Growth rate of amylolytic bacteria at 90 min of incubation period.....	46
Figure 4.6. Rate of growth of amylolytic bacteria isolate at varying temperatures.....	47
Figure 4.7 Rate of growth of amylolytic bacteria isolate at varying pH.....	48
Figure 4.8 Rate of growth of bacteria isolates at varying cassava starch concentrations.....	49
Figure 4.9 Glucose Standard Curve at 575nm.....	50
Figure 4.10 Effect of temperature on activity of amylase enzyme produced by the various bacteria isolates.....	54
Figure 4.11 Effect of pH on activity of amylase enzyme produced by the various bacteria isolates.....	55
Figure 4.12. Effect of substrate concentration on activity of amylase enzyme produced by the various bacteria isolates.....	55
Figure 4.13 Effect of time of incubation on activity of amylase enzyme produced by the various bacteria isolates.....	56

LIST OF PLATE

Page

Plate 4.1 Zone of clearance and Gram staining of amylolytic bacteria grown on starch agar....41

KNUST



CHAPTER ONE

INTRODUCTION

1.0 Introduction

Amylolytic bacteria are heterotrophic bacteria which live on starchy products for survival. In their feeding, they produce amylase to break down starch and release energy for their growth. Many bacteria species produce amylases, including *Bacillus* species, *Actinomyces*, *Escherichia coli*, *Micrococcus* species, *Pseudomonas* species, *Proteus* species, *Serratia* species, and *Cephalosporium* species. Of these, *Bacillus* species are the most important (Akansha and Varsha, 2013). Amylases are enzymes which break down starch into monosaccharides and oligosaccharides by targeting the α -1,4 and α -1,6 glycosidic bonds. Amylolytic bacteria are commonly found in traditional fermentation systems, soils, kitchen, and farm waste dumpsites.

Cassava (*Manihot esculenta*), and cereals such as maize (*Zea mays* L.), sorghum (*Sorghum bicolor*), and rice (*Oryza sativa* L.) are staple foods in West Africa. Traditionally, these staples are processed into varied products, of which fermentation is the predominant processing method employed. Some of the fermented products include *kenkey* and malt drink from maize in Ghana, *ogi* from maize in Nigeria, *pito* and porridge from sorghum and millet in Ghana, and cassava dough and *lafun* in Ghana, and Nigeria, respectively. The fermented foods, milling equipment, utensils, and waste dumpsites, a notable menace to solid waste management in Ghana, are reserve of microorganisms, particularly, amylolytic bacteria and fungi (Franz *et al.*, 2014; Fossi, *et al.*, 2005; Ali *et al.*, 1998; Hesseltine, 1979).

A five-year annual production of cereals from 2012 to 2016 in Ghana was estimated to be 1.8 million metric tonnes (mmt) for maize, 600,000 mt for rice, and 260,000 mt for sorghum (KNOEMA, 2018). Of these, 59 % of maize is processed by fermentation (Khan *et al.*, 2014) into various food products, while rice is consumed as such. Of the 18 mmt of cassava produced in Ghana annually from 2013 to 2017 (Statistics Research and Information Department – Ministry of Food and Agriculture, 2018), about 80 % is processed as fermented and unfermented food. A large amount of these foods and their wastes are therefore available on farms, in processing enterprises, and in homes. The high starch content of the produce, 73 % for maize (Khan *et al.*, 2014), 80 % for rice and 90 % for cassava (Moreno and Gourdj, 2015; Gu *et al.*, 2013) makes them ideal substrates for growth of microorganisms, hence, good source for amylolytic bacteria. The amylases produced by the amylolytic bacteria can be exploited for industrial purposes.

Commercial amylases are calcium-dependent metalloenzymes that hydrolyze starch into low molecular weight products such as dextrin, maltose, maltotriose, and glucose (Roy and Gupta, 2004) by the breakdown of glycosidic bonds. They are employed in industrial processes for production of ethanol, textiles, paper, brewage, detergents, adhesives, and diagnostics. Amylase trade accounts for approximately 25 % of the global market value of industrial enzymes (Rajagopalan and Krishnan, 2008) and was valued at \$US 6.4 billion in 2013, with a projection of \$US 7.1 billion in 2018, equivalent to a growth rate of 4.4 % (Business Communication Report, 2014). World enzymes are produced by about 12 major companies with top producers being Novozyme in Denmark, DuPont in U.S.A., and Roche in Switzerland (Shuang *et al.*, 2012). Commercial production of amylase in Ghana is absent, despite few research reports on amylase production from fungi (Johnson *et al.*, 2014) and lactic acid bacteria (Atter *et al.*, 2014). Ghana

spends more than US\$ 200 million annually on amylase import for ethanol production and brewery industries, and imports about 60 million litres of ethanol annually (Business Communication Report, 2014). Local production of amylases or amylolytic bacteria for seeding starch-based fermentation cultures should be explored as demand for amylases keeps rising.

Identification of amylolytic bacteria and characterization for commercial production of amylases should be possible owing to the wide variety of raw materials and diversity in processing techniques that have the potential to create variation in types of amylolytic bacteria and their performance in amylase production. Amylases with different catalytic activities are produced by plants, animals and microorganisms (Murakami *et al.*, 2008) in intracellular or extracellular form with bacteria and fungi amylases being the most widely used in industrial applications (Pandey *et al.*, 2000; Chi *et al.*, 2007; de Souza and de Oliveira Magalhaes, 2010). The most commonly used microbial amylases are alpha-amylase (α -amylase, E.C.3.2.1.1), beta-amylase (β -amylase, E.C.3.2.1.2), and gluco- or gamma-amylase (γ -amylase, E.C.3.2.1.3) (Fogarty and Kelly, 1980; Burhan *et al.*, 2003; Coutinho and Reilly, 1997). Alpha-amylase hydrolyzes the internal α -1,4-glycosidic bonds in starch to generate glucose, maltose and maltotriose units. Beta-amylase hydrolyzes the α -1,4-glycosidic bonds of starch and the β -anomeric maltose from the non-reducing ends, while, gluco-amylase hydrolyzes the α -1,6-glycosidic linkages and α -1,4-glycosidic linkages from the non-reducing ends of amylose and amylopectin into glucose (Baum and John-Hill, 1995; Nigam and Singh, 1995; Burhan *et al.*, 2003).

Being enzymes, amylases are temperature-sensitive with a wide range of operative temperatures in industrial fermentation systems. Amylases occur in thermostable or temperature-labile forms,

with the thermostable form preferred for the high operative temperatures during starch liquefaction (Pandey *et al.*, 2000; Yasouri *et al.*, 2003). Bacterial amylases are preferred for their stability, low cost and ease of production with simple nutritional requirements in a wide variety of conditions (Uguru *et al.*, 1997), as well as their amenability to genetic modification (Kaur *et al.*, 2012; Nigam and Singh, 1995; Pandey, 2003; Aiyer, 2005; Ajayi and Fagade, 2007; Oyeleke and Oduwale, 2009). Some bacterial amylases can survive under wide range of temperatures of 25°C – 45°C; others can continue to grow slowly near 0°C and in hot springs above 80°C. Thermostable amylases have been produced industrially from several *Bacillus* species including *B. subtilis*, *B. coagulans*, *B. cereus*, *B. stearothermophilus*, *B. licheniformis*, and *B. amyloliquefaciens* (Fogarty and Kelly, 1980; Yasouri *et al.*, 2003; Prakash *et al.*, 2009; Deb *et al.*, 2013; Neelima and Reddy, 2013) having applications in starch processing industries (Pandey *et al.*, 2000).

Conventional culturing methods such as spread plate, streak plate, and pour plate are useful for identification, isolation, and determination of cell numbers of bacteria. Cells of amylolytic bacteria optimally grow at 37°C, pH 7.0, and 1.0% substrate concentration (Holt, 1994; Elmansy *et al.*, 2018). Characterization of microorganisms at various species levels may be carried out by morphological, biochemical, and molecular methods. Morphological characterization encompasses colony characterization and biochemical characterization, such as the catalase, oxidase, and starch hydrolysis tests (Kaur *et al.*, 2012). Morphological characterization is limited by its inability to distinguish differences among strains or races (Mohamed *et al.*, 2014), therefore requiring more discriminatory methods such as biochemical and molecular evaluations. Morphological characterization is cheaper than biochemical characterization and it gives a readily available information to guide selection of desired strains. Biochemical characterization further

provides information on metabolic activities of the microorganisms, which information, offers guide to the applicability of the culture. However, biochemical characterization is less accurate, less discriminatory, and less reproducible (Bessler *et al.*, 2009).

The only record in Ghana on identification of amylolytic microorganisms in fermentation systems was carried out on soil from cassava processing dumpsites from which three amylolytic fungal isolates were best performers (Johnson *et al.*, 2014). Their amylase activities ranged between 3.24 and 11.01 U/mL. There is lack of information on amylolytic bacteria, their identification and characterization from the natural fermentation systems and waste dumpsites in maize, cassava and rice in Ghana. The main objective of this study was to identify and characterize amylolytic bacteria in cereal and tuber processing units and dumpsites in Kumasi. The specific objectives were:

- i. To identify amylolytic bacteria from maize fermentation systems and dumpsites by morphological and biochemical methods.
- ii. To identify amylolytic bacteria from rice processing sites and farms by morphological and biochemical methods.
- iii. To identify amylolytic bacteria from cassava fermentations systems and dumpsites by morphological and biochemical methods.
- iv. To determine the growth conditions of the amylolytic bacteria isolates
- v. To determine the conditions for optimum amylase activity of the isolates

1.2 Justification

Agricultural wastes from cereal and cassava processing abound and constitute environmental nuisance in Ghana. Information on sources of amylolytic bacteria in Ghana will contribute to

commercial production of lyophilized bacterial culture and purified commercial amylases. Both products have the capacity to create jobs and grow the industrial ethanol production companies.

1.3 Research Hypothesis

Amylolytic bacterial strains are ubiquitous and can be found in starchy products. Different strains of bacteria can be found in a particular area due to the effect of evolutionary forces of mutation, genetic drift, recombination and gene flow. Hence, it is possible to identify variety of strains with different amylolytic activities.



CHAPTER TWO

LITERATURE REVIEW

2.1 Economic Importance of Natural Fermentations in Ghana

Fermentation is primarily considered as the conversion of sugar into organic acids or ethanol (Paulova *et al.*, 2013). There are two forms of fermentation, namely, natural and induced. Natural fermentation, which is often wild, occurs when the naturally occurring microflora in the substrate utilize nutrients for growth, thereby producing metabolites that may be useful to man. Naturally, fermentation is exploited in food preservation, for removal of antinutrients, and for enhancing the nutritive, organoleptic, and aesthetic value of food (Steinkraus, 2002; Simango, 1997) and enhances characteristics of food (Kasperski and Miskiewitz, 2008). In induced fermentation, pure cultures of microorganism are seeded into a medium for conversion of the raw materials into specific products such as enzymes, ethanol, and other metabolites (Borowiak *et al.*, 2012). The production of the beneficial products depends on several factors, including Fermented foods contribute to about one-third of the diets consumed across the entire world with Africa being the highest consumers (Campbell-Platt, 1994).

Fermentation offers a natural way of preserving foods. Further, fermentation reduces bulk density of food with direct reduction in energy for processing and time for cooking (Nout *et al.*, 1996). In Africa, raw materials such as sorghum, maize, cassava, and millet undergo fermentation to produce varying forms of foods including *kenkey*, *banku*, *gari*, *ogi*, *lafun*, *mahewu uji*, *injera* and *koko* in various ways that differ from one region to the other (Odunfa, 1985).

Several studies have been carried out on indigenous fermented foods in Africa (Amoa-Awua *et al.*, 2007; Atter *et al.*, 2014; Mugula *et al.*, 2003; Omafuvbe *et al.*, 2000; Sefa-Dedeh *et al.*, 2004). Amoa-Awua *et al.* (2007) investigated the microbiological and biochemical changes which occur in locally tapped palm wine of felled oil palm trees in Ghana. They identified that lactic acid bacteria were eminent at the preliminary stages of the tapping while acetic acid bacteria were identified when alcohol was substantial. Atter *et al.* (2014) investigated the microbiological and chemical changes which takes place in the souring and alcoholic fermentations of guinea corn (*Sorghum bicolor*) to make *burukutu* in Ghana. They identified that lactic acid bacteria and *Saccharomyces cerevisiae* were responsible for souring and alcoholic fermentations, respectively. The activities of the lactic acid bacteria were able to reduce the pH to 4.05, creating an uncondusive environment for the growth of organisms such as *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium*. Mugula *et al.* (2003) reported that the predominant organism in the fermentation of maize, sorghum mixtures to produce *togwa* in Tanzania was lactic acid bacteria and yeast.

Fermentation of soybean for traditional production of *daddawa* involves the activities of *Bacillus* species notably *B. subtilis*, *B. licheniformis* and *B. pumilus* (Omafuvbe *et al.*, 2000). These organisms reduce lipase and beta-fructofuranosidase activities in the fermenting seeds. The improvement of the nutritional quality of corn is enhanced with the fermentative activities of lactic acid bacillus such as *Lactobacillus plantarum*, *L. fermentum*, *L. cellobiosus*, *Pediococcus spp.* thereby producing a sour product (Sefa-Dedeh *et al.*, 2004). However, irrespective of the deficiency of essential amino acids in cereals, fermentation may be considered as the simplest but

economical means of enhancing their nutritional value, sensory properties, and functional qualities (Blandino *et al.*, 2002).

2.1.1 Types of Fermentation

Two methods of fermentation are in use, namely submerged and solid state fermentation. In submerged fermentations, microbes are inoculated into the substrates with moderately high water activity, approximately 95%. Submerged fermentation is often used for enzyme production owing to ease of manipulation and optimization of culture conditions such as temperature, humidity, carbon dioxide, dissolved oxygen, and pH (Cristobal *et al.*, 2008). Three methods of submerged fermentations include batch, fed-batch, and continuous cultivation systems (Paulova *et al.*, 2013). Batch culture is a closed system which involves the inoculation of constant number of viable cells into bioreactors containing appropriate culture medium and nutrients. The fed-batch cultivation is a semi-open system where the volume of the culture broth is gradually increased over time through the intermittent addition of one or more nutrients by aseptic means. Fed-batch cultivation has the possibility of enhancing the yield of products, product synthesis, and higher cell densities (Garcia-Garcia *et al.*, 2009). Continuous cultivation is an open system which involves aseptic continuous addition of nutrients and continuous removal of culture broth (containing cells and metabolites) at the same time. Continuous cultivation enables the possibility of optimizing broth conditions, and steady state and quality of product. Submerged fermentation is mostly applied in the food, alcohol and beverage industries.

In solid state fermentation, microbes are cultured on a solid substrate with relatively low water activity (Holker and Lenz, 2005). The low water activity results in poor heat and mass transfer

within the substrate which reduces the capacity to monitor and optimize the microbial growing conditions (Mitchel *et al.*, 1999; Paulova *et al.*, 2013; Bellon-Maurel *et al.*, 2003). Applications of solid state fermentations in Ghana include corn dough-making, *lafun* and *ogi*- making. Others include production of enriched animal feeds (Ugwuanyi *et al.*, 2009), fermented vegetables and ripening of cheeses, bio-beneficiation, bioremediation, bioleaching, and biopulping (Pandey *et al.*, 1999; Pandey, 2003 Medeiros *et al.*, 2000).

2.2 Enzymes as Industrial Workhorse

Enzymes are globular proteins that speed up the rates of chemical reactions. In speeding up a chemical reaction, enzymes break down complex products, such as polysaccharides, proteins, and lipids into sugars, amino acids, and fatty acids and glycerols, which are less complex substances. Enzymes are useful for many industrial applications. In the food industry, amylases are used for the conversion of starch into high fructose syrup; amylases are also used in the beverage industries for the conversion of starch into glucose. In industrial alcohol production, enzymes such as amylase and cellulase are used to break down starch and cellulose into sugars. Lipases and proteases are used in the detergent industry to remove stains from fabrics. In medical diagnostics, amylases find use in making of glucose strips for detection of diabetes.

Globally, over 500 industrial products are made using enzymes (Johannes and Zhao, 2006; Singh *et al.*, 2016). Global demand for industrial enzymes was US\$7.0 billion in 2017 and is projected to grow by 5.7 % per year amounting to US \$10.4.0 billion in 2024 (Srivastava and Srivastava, 2018). Current demand for enzymes in Sub-Saharan Africa is not available, though there are reports of increase in industrial processes that rely on enzymes. The demand for enzyme in Africa

is expected to rise by 7.6% over the same period. Enzymes that dominate the market include the carbohydrases, proteases, and lipases which are employed in the food and beverage industries, biofuel ethanol production, water treatment, and detergent industries. Companies in Ghana that employ enzymes are Caltech for production of ethanol (Caltech Ventures, 2017), and pharmaceutical companies. Regrettably, enzymes are not produced on commercial basis in Ghana.

2.3 Enzyme Activity and its Measurement

The ability of the enzyme to break down complex products into less complex ones is termed the activity of the enzyme. Enzyme activity is measured in enzyme units per millilitres (U/mL), meaning the amount of enzyme that converts 1 μ mole of substrate to product per minute. Enzyme activity is influenced by factors including temperature, time of incubation, pH, and concentration of substrate.

2.3.1 Effects of Temperature on Enzyme Activity

Increase in temperature increases the kinetic energy of both the substrate and enzyme molecules, thereby increasing enzymatic reaction (Singh *et al.*, 2015). The Q_{10} factor specifies that for every degree rise in temperature, the rate of reaction increases by two fold (Bai *et al.*, 2017). On the basis of optimum temperature activity of the enzyme, psychrophilic (0 – 20°C), mesophilic (20 – 40°C), and thermophilic (> 60°C) occur (Mehta and Satyanarayana, 2016). Amylases are considered to be stable at high temperatures. Thermostability of enzymes is functional within a narrow range. Amylase from *Bacillus* spp. are optimally produced and stable within 50-90°C (Bano *et al.*, 2011; Bozic *et al.*, 2011; Asoodeh *et al.*, 2010). For example, temperature optimum of α -amylase from *B. licheniformis* ATCC 6346 is 75°C. Increase in temperature to 85°C resulted in loss of 40% of

its activity (Vengadaramana *et al.*, 2014). According to Saito and Yamamoto (1975), α -amylase from *B. licheniformis* is easily lost above 76°C. Abdel-Fattah *et al.* (2013) reported a temperature optimum of alpha-amylase as 75°C in *Bacillus licheniformis* from Indonesia garden soil, which activity dropped beyond this temperature. However, a study carried out by Mohsen *et al.* (2005) on *B. subtilis* AX20, α -amylase demonstrated optimum activity of 22.8 U/mL at 40°C. In another vein, a study carried out on *Pseudomonas aeruginosa* from Indian garden soil showed optimum enzyme activity of 1.7 U/mL at 40°C (Raju and Divakar, 2013). Therefore, *Bacillus* seem considered to produce larger quantities of thermostable α -amylase.

On the contrary, fungi such as *Aspergillus* and *Penicillium* are also noted of producing high yields of amylase at varying temperatures (Hernandez *et al.*, 2006; Jin *et al.*, 1998; Kathiresan and Manivannan, 2006). However, high temperature usually distort fungal sporulation and germination, and metabolites formation (Bhargav *et al.*, 2008; Raghavaro *et al.*, 2003). A study carried out by Oyewole *et al.* (2011) reported that amylase produced by *Aspergillus niger* yielded an optimum activity of 3.5 U/mL at 70°C whereas another study on *Aspergillus niger* demonstrated an optimum activity of 55 U/mL at 30°C (Gupta *et al.*, 2003). Amylases from fungi are stable within 25 – 60°C due to the native structure of the enzyme (Jafari-Aghdam *et al.*, 2005).

Thermostable α -amylases have become a major requirement for most food and beverage industries such as distillery, baking, brewing, and cheese making (Gangadharan *et al.*, 2006). *Bacillus* spp. have become of enormous relevance in the production of thermostable α -amylase in starch processing industries (Schallmeyer *et al.*, 2004). Thermostable α -amylase producing *Bacillus* spp. include *B. amyloliquefaciens*, *B. coagulans*, *B. subtilis*, *B. licheniformis*, *B. stearothermophilus*,

and *B. brevis* (Ray *et al.*, 2010; Schallmeyer *et al.*, 2004). These species mostly require metal ions such as Ca^{2+} for their activity and stability during the starch hydrolysis into fructose and glucose syrups (Tonkova, 2006; Swain *et al.*, 2006).

2.3.2 Effects of pH on Enzyme Activity

pH plays a critical role in the activity of enzymes where changes in pH transforms the ionic charge of the acidic and basic groups associated to the shape of the enzyme. On the basis of enzyme activities, enzymes can be acidic (< 5.0), neutral (6-8), and alkaline (> 9.0) (Kikani and Singh, 2011; Pancha *et al.*, 2010). Under fixed temperature and pressure, limited pH range exist for every enzyme. This behavior is evident in amylase. A study carried out by Vengadaramana *et al.* (2014) on the production of α -amylase from *B. licheniformis* identified an optimum activity of 22.05 U/mL at pH 7.0. Moreover, studies on *Bacillus licheniformis* isolated from Indian soils waste mixture demonstrated optimum amylase activity of 3.23 U/mL at pH 7.0 (Alariya *et al.*, 2013; Vaseekaran *et al.*, 2010). Behal *et al.* (2006) identified a *Bacillus* sp. from soil which showed maximum enzyme activity of 5.12 U/mL at pH 8.0. Shanmugasundaram *et al.* (2015) also identified a *Bacillus subtilis* from a saltpan environment which expressed an amylase activity of 6.23 U/mL at pH 6.0. At similar conditions, *Pseudomonas aeruginosa* was identified from garden soil in India which exhibited an optimum enzyme activity of 4.7 U/ml at pH 7.0 (Raju and Divakar, 2013).

Moreover, a related study conducted by Alariya *et al.* (2013) identified *Pseudomonas flourescens* isolated from soil sample in India which showed an optimum amylase activity of 4.25 U/mL at pH 7.0. Therefore, enzymes from bacteria show optimum activity within the pH range of 6.0 - 8.0. On

the contrary, amylase from fungi shows optimum activities at relatively low pH (Bhargav *et al.*, 2008). A study carried out by Oyewole *et al.* (2011) reported that amylase produced by *Aspergillus niger* from a Nigerian abattoir yielded an optimum activity of 4.48 U/mL at pH 5.0 whereas another study on *Aspergillus niger* from Indian soil demonstrated an optimum activity of 27 U/mL at pH 5.0 (Gupta *et al.*, 2003). Although amylase activities of fungi are usually higher than that of bacteria, amylase from bacteria are good for industrial purposes than fungal amylase.

2.3.3 Effects of Substrate Concentration on Enzyme Activity

The production of enzymes always require a suitable substrate to support the growth of microorganisms in induced fermentation (Burhan *et al.*, 2003). Commonly used agro-industrial substrates for this fermentation process include wheat bran, cassava, maize, potato, rice, palm kernel, tamarind (Johnson *et al.*, 2014; Kaur *et al.*, 2012; Mitra and Chandra, 1994; Tengerdy, 1996). These agro-industrial substrates serve as a source of carbon source for the microbes to thrive on and have an effect on the yield and activity of the produced enzyme (Bhargav *et al.*, 2008). However, wheat bran has been extensively used and has the capacity of producing high levels of enzyme activities (Mitra and Chandra, 1994). For amylase production, starchy products or starch inhabiting environments is the suitable substrate for its activity.

A study conducted by Bharathiraja *et al.* (2016) on *Bacillus* spp. MTCC demonstrated an optimum activity of 1.28 U/mL at 4% starch concentration. Mishra and Behera (2008) identified *Bacillus* sp. from soil sample receiving kitchen waste in India which showed maximum amylase activity of 75.7 U/mL at 2% starch concentration. Moreover, Mamo *et al.* (1999) identified a *Bacillus* sp. WN11 which optimally produced amylase (1.46 U/mL) at 2% starch concentration. *Bacillus*

cereus from Cuddalore Fishing Harbour waters, India showed an optimum enzyme activity of 11.11 U/mL at 1% of starch concentration (Annamalai *et al.*, 2011). At similar conditions, *Pseudomonas aeruginosa* from garden soil in India shows maximum enzyme activity of 3.19 U/mL at 1% substrate concentration (Raju and Divakar, 2013). Moreover, a related study conducted by Alariya *et al.* (2013) identified *Pseudomonas fluorescens* from Indian soil which showed an optimum amylase activity of 3.25 U/mL at 1% substrate concentration. On the contrary, a study carried out by Gupta *et al.* (2003) reported that amylase produced by *Aspergillus niger* from an Indian soil shows an optimum activity of 48.8 U/mL at 0.5% starch concentration. Hence, bacterial amylases show maximum activity at higher starch concentrations than that of fungal amylases.

2.3.4 Effect of Incubation Period on Enzyme Activity

The longer an enzyme is incubated with its substrate, the greater the amount of product that will be formed. Subsequently, the rate of product formation slows down as the incubation proceeds, and if the incubation time is too long, then the measured activity of the enzyme is falsely low (Akansha and Varsha, 2013). Incubation is very essential in enzyme production and they can be done using several methods such as microwave drying, solar drying, and vacuum drying or combination of these methods. Incubation periods are hugely influenced by temperature; surface area, moisture content, and particle size of substrate (Pandey *et al.*, 1994; Pandey *et al.*, 1991). Very high and very low temperatures destroy the quality of product over time (Oberoi *et al.*, 2007). Incubation trays with larger surface areas have shorter incubation time than those with smaller areas (Pandey and Radhakrishnan, 1992). Depending on the source, enzymes are stable and optimally active at varying periods, thus fungal enzymes are active at long periods of incubation

whereas bacterial enzymes are not (Wadhwa *et al.*, 2005). These trends are evident in the activity of amylase. For instance, a study carried out by Mishra and Behera (2008) identified a *Bacillus* sp. from soil mixed with kitchen waste in India which showed a maximum activity of 78.0 U/mL at 96 h incubation. However, some *Bacillus* species are capable of exhibiting low enzyme activities at high incubation periods (Aiyer, 2005). At similar conditions, *Pseudomonas aeruginosa* was identified from garden soil in India which exhibited an optimum enzyme activity of 2.25 U/mL at 48 h (Raju and Divakar, 2013). A similar study carried out by Alariya *et al.* (2013) identified *Pseudomonas fluorescens* isolated from soil sample in India which showed an optimum amylase activity of 0.9 U/mL at 48 h. On the contrary, a study carried out by Oyewole *et al.* (2011) reported that amylase produced by *Aspergillus niger* from a Nigerian abattoir yielded an optimum activity of 4.48 U/mL at 96 h of incubation. *Aspergillus niger* from Indian soil showed optimum amylase activity of 43 U/mL at 120 h (Gupta *et al.*, 2003).

2.4 Enzymatic Hydrolysis of Starch

Most cereals and tubers such as maize, rice, and cassava are mainly composed of high starch content. Starch is a glucose polymer with a glycosidic bond linking them to one another. Two types of glucose polymers exist in starch, amylose and amylopectin. Amylose is a linear polymer consisting of up to 6,000 glucose units with α -1,4 glycosidic bonds. Amylopectin consists of short linear chains of about 10 - 60 glucose units in α -1,4 linkages joined by α -1,6 linkages to side chains of 15 - 45 glucose units. Maize starch has amylose content of 25 % and amylopectin of 75 % whereas rice starch has 10-20% amylose and 80-90%, and cassava starch has 20 % amylose and 80% amylopectin (Menzel, 2014). Gao *et al.* (2015) revealed that the proportion of amylose differ among varieties of a crop. The proportion of amylose in a starch granule has direct influence on

the physicochemical and functional properties of the starch. Moreover, the content of amylose helps in preventing leaching of the starch granules (Singh *et al.*, 2003) specifically due to variance in geographical origin and culture conditions.

Starch hydrolysis is possible through the use of acids or enzymes (Dziedzic and Kearsley, 1995; Azmi *et al.*, 2016). Due to the low yield of acid hydrolysis, formation of undesirable by-products (Ramprakash and Muthukumar, 2014), and poor flexibility of the method (Klinke *et al.*, 2004), hydrolysis of starch with enzymes has become more popular. Amylases, specifically, a combination of α -amylase and glucoamylase, can hydrolyze starch with 95% efficiency (Hua and Yang, 2016).

Enzymatic hydrolysis of starch has become a prominent and efficient method in providing a better understanding of the degradability of starch (Rocha *et al.*, 2010). According to Noda *et al.* (2004), enzyme hydrolysed starches have reduced molecular weight, paste viscidness, and swelling power. Starch enzymatic susceptibilities differ due to varying factors including starch source, granule size, content of amylose and amylopectin, crystallinity, type of enzyme, and hydrolysis condition, that is, temperature, pH, reaction duration and agitation rate (Li, 2004; Mussatto *et al.*, 2008; Tester *et al.*, 2006). For instance, corn starch is easily degraded than cassava starch due to its porous nature (Franco and Ciacco, 1992). Lower diameter starch granules are easily hydrolysed by enzymes than higher diameter starch granules due to the higher surface area of the former (Tester *et al.*, 2006; Yonemoto *et al.*, 2007). In enzymatic hydrolysis of cereals, less organised amorphous rings are easily degraded than the crystalline lamella (Oates, 1997). However, both crystalline lamella and amorphous rings of cereal starch granules can be hydrolysed by α -amylase at the same rate

(Colonna *et al.*, 1988). Starches with varying polymorphic structure types (i.e A, B, and C) have displayed diverse susceptibilities to enzyme hydrolysis (Lauro *et al.*, 1999). The work of Hoover and Zhou (2003) showed that α -amylase easily degrades legume granular starches (C-type) than potato or corn granular starches (B-type) and cereal or cassava granular starches (A-type). A study conducted by Amenaghawon *et al.* (2016) on enzymatic hydrolysis of cocoyam starch revealed that the higher the temperature the faster the hydrolysis reaction.

2.5 Amylases

Amylases contribute approximately 30% of the world enzyme market (Rajagopalan & Krishnan, 2008; Johnson *et al.*, 2014). They have become a significant industrial enzyme which is mostly used in the food, brewery, paper, pharmaceutical, detergent and textile industries (Pandey and Radhakrishnan 1992; Rameshkumar and Sivasudha, 2011; Fossi *et al.*, 2014). For instance, amylases are used in the brewing industry as a substitute for the expensive malt, enhancement of flour in the baking industry, removal of starch in the textile industries, and production of modified starch for the paper industries (Rameshkumar & Sivasudha, 2011). These enzymes are mostly used in the hydrolysis of starch to produce glucose and fructose polymers (Bessler *et al.*, 2009). Depending on the method of hydrolysis of polysaccharide bonds, enzymes can be classified into α -amylase, β -amylase, and γ -amylase.

The α -Amylase (E.C.3.2.1.1) is an endo-amylase that catalyses the hydrolysis of internal α -1,4-glycosidic linkages in low molecular weight starchy units such as maltose, and maltotriose (Gupta *et al.*, 2003; Rajagopalan & Krishnan, 2008, de Souza and de Oliveira Magalhaes, 2010). α -Amylase has a weak cleavage potential on terminal glucose residues or α -1,6-linkages (Whitcomb

and Lowe, 2007). The three dimensional structure of the enzyme enhance its specificity in binding to substrate, catalyzing, and breaking of glycoside links (Iulek *et al.*, 2000). The structure of an α -amylase is composed of three protein domains where the active site (substrate-binding) is situated in a long cleft located between the carboxyl end. Calcium (Ca^{2+}) ions are located between the A and B domains which may enhance the stabilization of the three-dimensional structure and also serve as an allosteric activator. In the catalytic center, the calcium ions and chloride ions are exposed at different regions of the three-dimensional structure of the enzyme (Payan, 2004). α -amylase or 1,4- α -D-glucan glucanohydrolase is considered as ubiquitous, most prominent and important form of industrial enzymes found in microbes, plants and higher organisms (Kandra, 2003). On the other hand, β -amylase or glycogenase or 1,4- α -D-glucan maltohydrolase (E.C 3.2.1.2) is a type of amylase synthesized by bacteria, fungi, and plants. This enzyme catalyses the hydrolysis of α -1,4-glycosidic linkages by cleaving off two glucose units specifically maltose at the same time. γ -amylase or glucoamylase or amyloglucosidase (E.C 3.2.1.3) is a type of amylase which cleaves α -1,6-glycosidic linkages. γ -amylase is most efficient in acidic environments, having an optimum pH of 3 (Saini *et al.*, 2017).

2.5.1 Purification of Amylase

The purification of the enzyme is a crucial step in every enzyme production since the enzymes are present with several proteins and ions that will affect the efficiency of the enzyme. Therefore, amylase can be purified by several methods such as ammonium sulphate precipitation, acetone precipitation, ethanol precipitation, and activated charcoal. With respect to ammonium sulphate precipitation, the culture filtrate is treated with ammonium sulphate fractionation (usually 30 – 100 %). The precipitated protein is washed using acetone buffer (usually 0.1 M, pH 4.4) and later

washed with water (Madiah *et al.*, 2000). Ammonium Sulphate precipitation was used by Raul *et al.* (2014) to purify amylase produced from *Bacillus* spp. on wheat bran and the specific amylase activity was 13.14 U/mL. Acetone precipitation is similar to ammonium sulphate precipitation unless that the protein pellet is vacuum or air dried (Wessel and Flugge, 1984). A study carried out by Bole *et al.* (2013) used both ammonium sulphate precipitation and acetone precipitation on amylase produced from airborne-bacteria isolates on starch and the optimum enzyme activity was 0.533 U/mL.

Ethanol precipitation deals with the washing of the protein pellet with ethanol and later dried in dry air. Activated charcoal involves the mixing of activated charcoal and protein pellet in solution in order to wash the protein through centrifugation for the clear supernatant to be collected as the enzyme. Kareem *et al.* (2011) used activated charcoal to purify glucoamylase and the enzyme yield was 3.5 U/mL. Conclusively, in purifying enzymes, several factors such as pH, temperature, preservation of SH group, and prevention of proteases should be considered in order to minimize their action on the purification process (Ashwini *et al.*, 2011).

2.5.2 Amylase-producing Bacteria

Amylase can be obtained from different sources such as plant, animals, and microbes (Jain *et al.*, 2012). Comparatively, microbial sources of amylase are more productive due to fastness and ease in enzyme production, and cost effectiveness of the product (Leveque *et al.*, 2000). Microbial sources are predominantly bacteria and fungi. However, bacterial amylases are preferred for their stability, low cost and ease of production with simple nutritional requirements in a wide variety of conditions (Uguru *et al.*, 1997), as well as their amenability to genetic modification (Kaur *et al.*,

2012; Nigam and Singh, 1995; Pandey, 2003; Aiyer, 2005; Ajayi and Fagade, 2007; Oyeleke and Oduwole, 2009). Some bacteria including *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens* have been identified as efficient producers of amylase (Alariya *et al.*, 2013; Raju and Divakar, 2013; Ashwini *et al.*, 2011, Kar and Ray, 2008).

2.6 Isolation and Storage of Enzyme-Producing Organisms

Mostly, the methods used for microbial isolation are serial dilution (Gopinath *et al.*, 2005) and substrate selection (Anbu *et al.*, 2004). These methods have been used in several studies in isolating efficient microbes from their rich source environment (Kaur *et al.*, 2012; Atter *et al.*, 2014; Johnson *et al.*, 2014). Production or secretion of amylase can be screened by varying forms of methods, including solid-based or solution-based techniques. The solid-based method is carried out on nutrient agar plates containing starch as the substrate, whereas solution-based methods include the dinitro salicylic acid (DNS) and Nelson-Somogyi (NS) techniques. Gopinath *et al.* (2003) applied the solid agar method to determine the amylase activity of *Aspergillus versicolor*, and *Penicillium* sp. in their study. Contrarily, Gusakov *et al.* (2011) applied the solution-based method to detect the release of reducing sugars from substrate hydrolysis by *Bacillus* spp. amylase. They found that the amylase activity could reach up to 0.75 U/mL after 24 h of incubation. For future purposes, enzyme producing bacteria are usually kept on nutrient slants at -20°C for long term storage and 4°C for short term storage (Grisham *et al.*, 1999; Pandey *et al.*, 2000; Hernandez *et al.*, 2006; Jain *et al.*, 2012).

2.7 Bacteria Characterisation

Bacteria classification can be carried out based on morphological, biochemical, and molecular characterization (Axelsson, 2004). According to Bergey (1938), morphological classification of bacteria is done based on colony shape, size, texture, margin, opacity, colour, consistency, and elevation. Morphological and biochemical methods are carried out to ascertain the genus of bacteria under surveillance. Biochemical classification of bacteria involves the chemical processes in the bacteria such as include oxidase, catalase, indole, voges proskauer, starch hydrolysis, gram staining, urease test, simmons citrate test, H₂S production (Sharma, 2009). Several studies have characterised organisms using both morphological and biochemical tests (Bhaskara Rao *et al.*, 2011; Kaur *et al.*, 2012; Johnson *et al.*, 2014). Due to limitation of genus identification associated with morphological and biochemical characterization, a well-established phylogeny with respect to 16S rRNA gene provides an enhancement on the previous methods of bacteria classification (Garrity *et al.*, 2004). This phenomenon is notably regarded as molecular characterization.

Molecular characterization encompasses DNA extraction, PCR amplification, sequencing, and bioinformatics analysis. The PCR amplification can be carried out either using the 16S rRNA sequence or 16S rDNA sequence depending on the interest of sequencing (Chatellier, 2014). According to Altschul *et al.* (1990) and Stackebrandt and Goebel (1994), most microbiologist prefer the use of 16S rRNA to 16S rDNA since 16S rRNA have most proprietary databases. Kaur *et al.* (2012) characterised *B. amyloliquefaciens* from a potato field as an amylolytic producing bacteria using morphological tests, biochemical tests, and 16S rRNA sequence. Identification of bacterial species on the basis of 16S rDNA sequencing predominantly remains to be extensively used (Clarridge, 2004; Woo *et al.*, 2008; Schlager *et al.*, 2012). Jain *et al.* (2012) characterised

Bacillus sp. from arid and semi-arid regions as an amylolytic producing bacteria using morphological tests, biochemical tests, and 16S rDNA sequence.

2.8 Growth Kinetics of Bacteria

Kinetics of growth is considered as the rate of changes in the quantity of cells in a defined system (Egli, 2009). It has indispensable tool in areas such as microbial genetics, selection, evolution, and industrial and environmental biotechnology. In the area of microbial growth kinetics, it is focused on the use of parameters in constructing models to predict the growth processes of the microbes. Classic models assume that growth kinetics is governed by the extracellular concentration of a single nutrient, though under environmental and biotechnological conditions, microbial cells utilize mostly mixtures of substrates (Egli *et al.*, 1993). The major step ahead in microbial growth kinetics came with the work of Monod (1949). He refined and calibrated the method for quantifying microbial growth using turbidimetry and demonstrated how growth can be mathematically described in terms of growth yield, specific growth rate, and substrate concentration (Monod, 1949).

In the growth cycle of cells, exponential increase is observed in protein and RNA whereas other cellular components such as cell wall components most likely do not (Panikov, 1995). However, since protein and RNA are the major cellular components, they dominate the overall growth rate. Hence, the growth during the division cycle of a cell is probably so close to exponential that it will be experimentally indistinguishable and difficult to show the contrary. The cultivation of cells involves four classical phases; which are: lag, exponential or log, stationary, and declining phases (Monod, 1949). The lag phase is the initial stage of bacterial cells where no cell division is

observed but there is cellular activity such as increase in cell size. Subsequently, the cells enter into the exponential or log phase where cell division by binary fission occurs and metabolic activity is high. At the stationary phase, the growth of bacterial cells reaches a plateau where the number of dividing cells equals the number of dying cells. Finally, the death phase is reached when waste products increase due to less availability of nutrients for cell growth.



CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample Collection

Eighteen samples from three starchy waste sources, namely, cassava (*Manihot esculenta* Crantz), maize (*Zea mays* L.), and rice (*Oryza sativa* L.), were aseptically collected into sterile plastic bags from three processing and waste dumpsites in Aboabo, Oforikrom, and Ejisu in the Ashanti region of Ghana. Sampling was carried out from 15th November to 20th December 2017. At each site, 300-gram samples were collected from three locations representing replicates, from the interior of the milling machine, soil around milling machine, and soil from the waste dumpsite of the milling factory. On the rice farm, collection was made from three different sites where harvesting and winnowing are carried out. The plastic bags were labelled as Maize Oforikrom (MOF 1, 2 and 3), Maize Aboabo (MAB 1, 2 and 3), Cassava Oforikrom (COF 1, 2 and 3), Cassava Aboabo (CAB 1, 2 and 3), Rice Aboabo (RAB 1, 2 and 3) and Rice Ejisu (REJ 1, 2 and 3).

3.2 Physicochemical Properties of the Sources of Sample

Temperature, pH, and oxidation reduction potential (ORP) of the cereal, tuber, and soil samples at their point of collection were read with a digital pH/Temperature/ORP meter (Milwaukee Calibration and Quality Control Instrument FH58). For pH and oxidation-reduction potential, the probe was inserted into a solution of five gram sample in an equal volume of sterile distilled water whereas the temperature was measured in the fermentation systems. ORP was measured in order to ascertain the level of oxidation and turbidity in the fermentation system that is impeding bacteria metabolism.

3.3 Media and Culture Conditions for Amylolytic Bacteria Isolation

Starch nutrient (SN) agar, composed of nutrient agar (Sharma, 2009) and cassava starch purchased from a local enterprise in Kumasi was used to grow, select, and maintain bacterial cultures. The SN agar was prepared from 14 g nutrient agar (containing 0.5% peptone, and 0.3% beef extract) and 5 g starch powder suspended in 500 mL distilled water. The mixture was held at 100°C in a water bath for one hour to ensure complete dissolution followed by sterilization in an autoclave (Shanmugasundaram *et al.*, 2015) at 121°C and 15 psi for 15 min. About 22 mL of media was poured into sterile petri plates.

3.4 Isolation and Characterization of Starch- degrading Bacteria

To 1.0 g of sample in a test tube were added 9 mL of saline (0.9% NaCl), mixed thoroughly, and the supernatant serially diluted to 10^{-3} with the saline. A 10 μ L of the diluted sample was plated on the starch nutrient agar plate by spreading with an inoculating loop and incubated at 37°C for 24 h. Identified colonies were sub-cultured at the same conditions to obtain pure isolates. Pure isolates were maintained on starch-agar slants at 4°C for further analysis.

3.5 Identification of Starch Utilizing Bacteria with Gram's iodine

A 10 μ l of the pure bacteria culture was inoculated onto a starch nutrient agar plate and incubated for 24 h at 37°C. The plates were flooded with Gram's iodine solution and allowed to sit for 15 min. Plates were examined for development of halo, that is, the clear, non-blue and transparent surrounding zone arising from hydrolysis of starch. The diameter of the halo were measured and bacterial samples which showed highest level of starch degradation were kept for further studies (Thippeswamy *et al.*, 2006).

3.6 Morphological Identification and Characterization of Starch-degrading Bacterial

Strain

Single bacterial colonies on the plates were examined for morphological characterization. The characteristics and their scores included colony sizes (CS: mean diameter of a colony from four measurements, small (0), large (1)); colony shape (CSH: principal shape of the colony, circular (0), irregular (1), punctiform (2), filamentous (3), spindle (4), rhizoid (5)); colony texture (CT: appearance of surface of colony: smooth (0), rough (1), glistening (2), wrinkled (3), dull (4), veined (5)); colony margin (CM: shape of margin of colony, entire (0), undulate (1), irregular (2), filamentous (3), lobate (4), curved (5)); colony elevation (CE: rise of the colony above the agar, raised (0), flat (1), convex (2), crateriform (3)), colony opacity (COP: ability for light to pass through colony, opaque (0), translucent (1), transparent (2), iridescent (3)), colony consistency (COC: retaining ability of colony shape, moist (0), dry (1), viscid (2), brittle (3), mucoid (4)), and colony colour (CCL: pigmentation of colony, white (0), cream (1), red (2), pink (3)) were recorded after sub-culturing (Bergey *et al.*, 1938). Biochemical tests, including Gram staining, oxidase and catalase tests, were carried out.

3.6.2 Oxidase Test

This test sought to ascertain whether the isolated bacteria produce cytochrome C oxidase. One loop of bacterial culture was smeared on a filter paper soaked with 1% oxidase reagent for 10 sec and the change in colour was observed and recorded. Positive reaction (1) turned purple while negative reaction (0) gave no change in colour.

3.6.3 Catalase Test

One loop of bacterial isolate was smeared on a glass slide with a drop of 3% hydrogen peroxide and the bubbling reaction was observed. Positive catalase test (1) gave immediate bubbling while negative catalase test (0) showed no bubbling.

3.6.4 Gram Staining

Gram staining was done by smearing 1 μL of the bacterial isolate on a glass slide and crystal violet, gram's iodine, 95% ethanol, and safarin used to flood the glass slide in a respective manner in order to differentiate the organisms based on their cell wall constituents. Smeared organisms were observed under a light microscope ($\times 100$) with oil immersion to determine the shape (GRArr - bacillus (0), coccus (1), spiral (2)), and classification (GRStain) of either Gram negative (-) (0) or Gram positive (+) (1). Gram positive bacteria is stained purple by the crystal violet while the Gram negative bacteria does not retain the purple stain and are counter-stained pink.

3.7 Determination of Culture Conditions and Growth Kinetics of Isolates

The optimum conditions of growth for each isolate, namely, temperature, pH, and starch concentration, were determined. These parameters were ascertained based on the time in which the isolates degrade the starch and also grow or multiply in numbers (Monod, 1949).

3.7.1 Effect of Temperature on Culture Growth

1.0 mL of each of the selected bacteria culture was added to 2.0 mL of 1% soluble starch in a test tube. Vortex was done to ensure complete dissolution of the isolates in the starch solution. Each

mixture was incubated at varying temperatures of 28°C, 31°C, 34°C, 37°C, 40°C and 42°C each for 2 h in a rotary shaker (Kaur *et al.*, 2012) at 200 revolutions per minute. All experiments were done in duplicates to ensure accuracy in measurements. The absorbance of each sample at their respective temperatures were measured at 600 nm at 0 min, 5 min, 30 min, 60 min, and 90 min. Results were recorded and plotted on a graph and the temperature reporting high degradation of starch was selected as the optimum temperature for maximum bacterial growth. The selected temperature was used for further studies in determining the pH and starch concentration.

3.7.2 Effect of pH on Culture Growth

1.0 ml of each bacterial isolate was added to a test tube containing 2 mL of 1% soluble starch. Vortex was done to ensure complete dissolution of the mixture. Each bacteria isolate was incubated at the optimum temperature with varying pH range of 6.8 – 7.2 for 2 h. pH was adjusted by adding few drops of 2M HCl and 2M NaOH. The absorbance of each sample at their respective pH were measured at 600 nm at 0 min, 5 min, 30 min, 60 min, and 90 min. All experiments were done in duplicates to ensure accuracy in measurements. The optimum pH parameter was used to determine the effect of starch concentration on the growth kinetics of the isolates.

3.7.3 Effect of Starch Concentration on Culture Growth

1.0 ml of each bacterial isolate was added to different test tubes containing 2 mL of 0.5%, 1%, 1.5%, 2.0%, and 2.5% soluble starch. Each mixture was vortexed to ensure complete dissolution of the mixture. Each mixture was adjusted to the optimum pH and incubated at the optimum temperature for 2 h. Absorbance were measured at 600 nm at 0 min, 5 min, 30 min, 60 min, and 90 min. All experiments were done in duplicates to ensure accuracy in measurements.

3.8 Determination of amylase activity

Amylase activity is defined as the amount of enzyme that releases one μmol reducing sugar equivalent to glucose over a specific time, pH and temperature. In determining the amylase activity of the selected strains, the selected strains were activated using the same culture medium [starch (1.5 g/L) and nutrient agar (12.5 g/L)] at pH 7. One loop of bacterial strains grown on starch-nutrient agar slants were transferred to activation medium (5 mL) and incubated in a shaker water bath at 42°C at 120 rpm for 12 h. The fermentation medium was inoculated with the activated culture and incubated at 42°C at 120 rpm for 24 h in a shaker water bath. Fermentation medium contained soluble starch (2g); peptone (2g); $(\text{NH}_4)_2\text{SO}_4$ (2g); NaCl (2g); KH_2PO_4 (1g); K_2HPO_4 (2.5g); FeCl_3 (1g); MgCl_2 (0.01g); CaCl_2 (0.01g) at pH 7 (Hamilton *et al.*, 1999). The fermentation medium was topped up to a 20 mL.

The spent medium was centrifuged at 3000 rpm for 20 min and filtered using a filter paper. Cell-free filtrate was used as enzyme source for the assay of α -amylase activity. 0.5 mL of diluted enzyme solution was added to 0.5 mL of 20 g/L starch at pH 7 in 0.01 M phosphate buffer and incubated at 37°C for 15 mins. 2 mL of dinitrosalicylic acid (DNS) reagent was added to the solution and the mixture was boiled for 15 min and cooled to room temperature (Miller, 1959). The direct contact of glucose with DNS results in the reduction of the DNS to 3-amino-5-dinitrosalicylic acid which is characterised with a red complex colour. The red complex colour is formed when the reduced DNS is heated in boiling water. The heat then denatures the enzyme and ensures that the enzymatic reaction is completely stopped. Absorbance was read at 540 nm for each isolate to estimate reducing sugars released (Fisher and Stein, 1961; Kaur *et al.*, 2012). Using a standard glucose curve, concentration of the glucose was obtained.

The activity of amylase enzyme was determined by:

Enzyme activity IU/mL =

$$\frac{\text{Concentration of reducing sugar} \times 1000 \times \text{dilution factor}}{\text{Molecular weight of glucose} \times \text{time} \times \text{enzyme volume}}$$

3.8.1 Glucose standard curve preparation

A mixture of 3 mL of DNS reagent and 3 mL each of graded concentrations of soluble glucose was prepared in a partly-covered test tube. Glucose solution was prepared at different concentrations of 0 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL, and 1.0 mg/mL. The mixture was heated at 90°C for 15 min. The red-brown colour formed was stabilized by the addition of 1 mL 40% Rochelle salt ($\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$) solution. The mixture was cooled and absorbance was read at 575 nm (Wang, 2015).

3.9 Kinetic Properties of Enzyme Activity

Enzyme activity is highly influenced by several factors, namely, temperature, pH, substrate concentration, and incubation period. Therefore, further studies were conducted on the enzyme activity of the extracted amylase enzyme in order to ascertain the thermo stability of the enzyme and its optimum production parameter.

3.9.1 Effect of Temperature on Enzyme Activity

0.5 mL of amylase enzyme was added to 0.5 mL of 1% soluble starch. The mixture was incubated in a rotary shaker at varying temperatures of 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C at 20

minutes each. The DNS method was conducted and absorbance were measured at 540 nm (Bharathiraja *et al.*, 2016).

3.9.2 Effect of pH on Enzyme Activity

A mixture of 0.5 mL of amylase enzyme and 0.5 mL of 1% soluble starch was prepared. Few drops of 2M HCl and 2M NaOH was added to adjust the pH to the various pH ranges of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 in different test tubes. The mixture was incubated for 20 minutes at 37°C in a rotary shaker. DNS method was conducted and absorbance measured at 540 nm (Bharathiraja *et al.*, 2016).

3.9.3 Effect of Starch Concentration on Enzyme Activity

A mixture of 0.5 mL of amylase enzyme and 0.5 mL of soluble starch was prepared. Varying concentrations of 0.5%, 1.0%, 1.5%, 2.0%, and 2.5% of the soluble starch were prepared. The mixture was incubated 37°C for 20 minutes and the DNS method was conducted. Absorbance were measured at 540 nm (Bharathiraja *et al.*, 2016).

3.9.4 Effect of Incubation Period on Enzyme Activity

A mixture of 0.5 mL of amylase enzyme and 0.5 mL of 1% soluble starch was prepared. The mixture was incubated at 37°C on a rotary shaker at different incubation periods. The incubation periods were 24 h, 48 h, 72 h, 96 h, 120 h. DNS method was conducted and absorbance were measured at 540 nm (Bharathiraja *et al.*, 2016).

3.10 Experimental Design

The experimental design used for this research was a 3 x 3 Factorial experiment with three (3) replications using Completely Randomized Design (CRD). The researcher used CRD because it is simple and easy to work with, the experimental material is homogenous and also gives equal probability for every experimental unit to receive a treatment. However, due to lack of restrictions it is relatively low in accuracy causing experimental error (Saunders *et al.*, 2012).

3.11 Data Analysis

Statistical analysis such as frequencies, means, standard deviation (SD), R squared, ANOVA, and coefficient of variation (CV) were conducted on the various data using the SAS 9.3 (SAS Institute, Cary, 2011). The NTSYS software version 2.21q was used to conduct a cluster analysis based on the dice similarity index to construct a dendrogram for the identified amyolytic bacteria populations. Cluster analysis was conducted in order to group or link organisms together based on the similarities in their morphological and biochemical characteristics. Data was entered in Microsoft Excel 2013 before imported into the NTSYS 2.21q and SAS 9.3 for their respective analysis. All data were statistically tested at 95% and 99% significance level. Results were shown in tables, graphs, and phylogenetic tree.

CHAPTER FOUR

RESULTS

4.1 Bacteria Isolates in Substrates

Cassava, maize, and rice from three processing communities in Kumasi were examined to identify amylolytic bacteria. Fermented Cassava and maize were sampled each from two mills in Aboabo and Oforikrom. Rice was sampled from a mill in Aboabo and rice farm in Ejisu. A spread plate culture on starch agar was carried out. A total of 50 bacteria isolates were identified of which 18 were from cassava, 12 from maize, and 20 from rice substrates. Variation in the number of isolates obtained in the various processing sites were observed. For cassava, an equal number of bacteria isolates, nine in each case were identified in the two processing sites. For maize, four and eight bacteria isolates were identified in Aboabo and Oforikrom, respectively. Nine and eleven bacteria isolates were identified in rice processing sites in Aboabo and Ejisu farm, respectively. In general, rice samples produced the least number of isolates except the farm sample which gave the highest number of isolates among the three substrates (Figure 4.1).

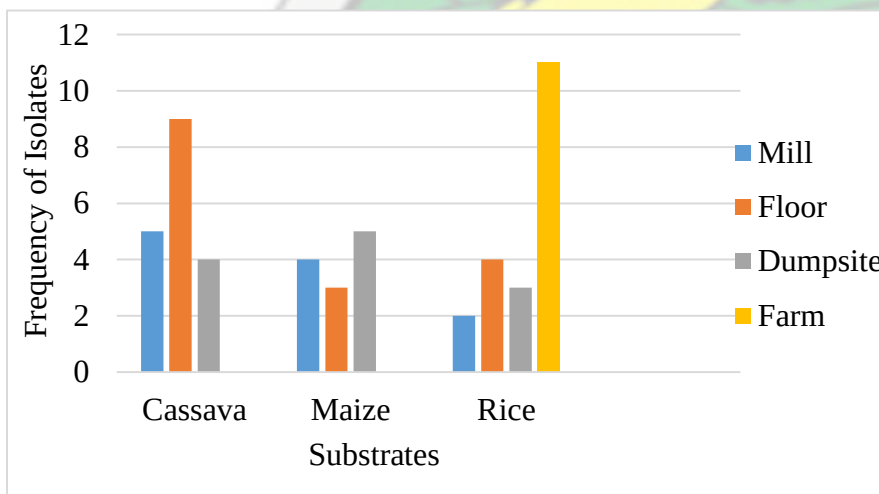


Figure 4.1 Frequency of bacteria isolates from three starchy samples in three processing sites and farm in Kumasi

4.2 Variations in Physicochemical Properties of Substrates at the Three Locations

Variation in the physicochemical properties of the three fermentation systems was observed (Table 4.1). Main effect of substrate was important whereas main effect of location and interaction of substrates and location were not ($P < 0.05$).

Table 4.1 Mean Squares, R Squares and Coefficient of Variation for Physicochemical Properties for Processing of Maize, Cassava, Rice in Kumasi for Identification of Amylolytic Bacteria

	Degree of freedom	Mean Square		
		pH	Temp.	ORP
Substrate	2	3.80*	1.16	5770.69
Location	2	0.60	3.38	3339.42
Substrate x Location	1	0.25	4.08	14283.00
		pH	Temp.	ORP
R ²		0.49	0.50	0.43
CV		17.96	3.88	55.05

*($P < 0.05$)

The pH of substrate was significant ($P < 0.05$) across the three fermentation systems whereas temperature and Oxidation-reduction Potential (ORP) were not. The pH value of maize fermentation system, 4.68 ± 0.58 , was lower than cassava 6.15 ± 1.49 , and rice 6.45 ± 0.54 (Figure 4.2). The temperature of all three fermentations systems across all locations ranged between 26.83 ± 0.93 and $27.67 \pm 1.25^\circ\text{C}$. Across the various locations, ORP value of cassava fermentation system, 72.5 ± 11.56 mV was lower than maize (134.5 ± 24.50 mV), and rice (128.5 ± 37.55 mV) (Figure 4.2) but not significant ($P > 0.05$). Overall mean and coefficient of variation for pH, temperature, and ORP for all the substrates were 5.7 ± 1.21 and 21.07%, $27 \pm 1.25^\circ\text{C}$ and 4.59%, and 111.83 ± 68.81 mV and 61.52%, respectively.

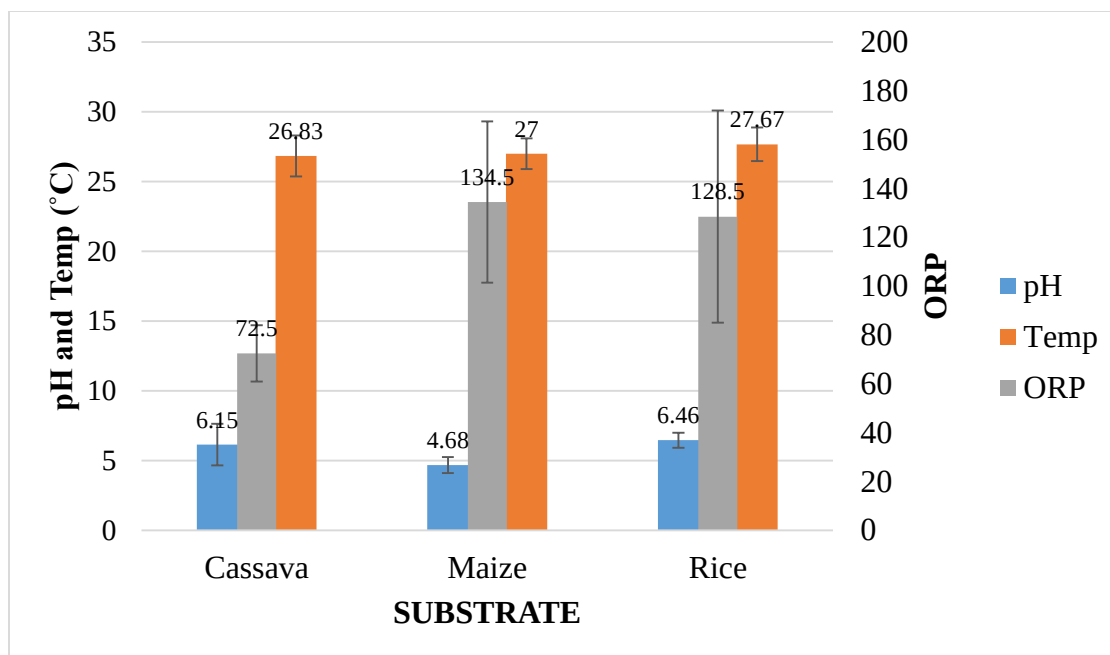


Figure 4.2 pH, Temperature, ORP of substrates from three different processing sites and farm in Kumasi

4.3 Bacteria Populations in the Fermentation Systems

Of the total of 50 colonies obtained from the three fermentation systems at the three locations, bacteria were grouped into 10 classes on the basis of the similarity in their qualitative characteristics (Table 4.2). All bacteria had raised and moist colonies which were Gram positive and catalase positive. There were five amylolytic and five non-amylolytic groups. The non-amylolytic group included Group 1, which were smooth, white, small, translucent, and circular colonies with entire margin and oxidase-negative cocci; Group 3 was made up of red, smooth, small, opaque and circular colonies with entire margin and oxidase-negative bacilli. The others were Group 4 which was made up of red, smooth, small, opaque and circular colonies with entire margin and oxidase-negative cocci; Group 5 was white, rough, small, translucent, and irregular colonies with undulate margin and oxidase-negative cocci; and Group 6 was cream, rough, large, opaque, and irregular colonies with undulate margin and oxidase-negative cocci.

The amylolytic groups included: Group 2 consisted of cream, smooth, small, opaque, and circular colonies with entire margin and oxidase-negative cocci; Group 7 was made up of cream, rough, small, opaque, and irregular colonies with undulate margin and oxidase-negative cocci. Group 8 colonies were white, smooth, large, translucent, and irregular with entire margin and oxidase-positive bacilli. The others were Group 9, having white, rough, large, translucent, and irregular colonies with undulate margin and oxidase-negative bacilli, while Group 10 was white, rough, large, translucent, and irregular colonies with undulate margin and oxidase-positive cocci.

All amylolytic colonies were universally present in rice fermentation systems. Rice had the largest number of amylolytic organisms followed by cassava where three isolates were identified and maize where only two amylolytic organisms were found. In contrast, cassava fermentation systems had some amylolytic organisms but the non-amylolytic organisms were more. The non-amylolytic colonies for cassava outnumbered the amylolytic organism. Rice and maize had the same number of non-amylolytic colonies.

4.4 Clustering of Amylolytic Bacteria in Ghanaian Local Fermentation Systems

A cluster analysis based on Dice similarity index grouped the five amylolytic populations of the 23 colonies into two large clusters, cluster I and cluster II (Figure 4.3). Cluster I was made up of 11 distinctive colonies that were common to all substrates, namely, rice, cassava, and maize and all locations, namely, Aboabo, Oforikrom, and Ejisu. The putative organism in cluster I was *Staphylococcus aureus*. The 12 colonies of Cluster II were grouped into three sub-clusters, A, B and C which were confined to specific substrates and locations. Cluster IIA was predominantly in cassava fermentation systems in all locations. The putative organisms were *Bacillus subtilis*, *B. licheniformis*, *B. amyloliquefaciens*. Cluster IIB was mostly found in rice fermentation systems in all locations having *Pseudomonas aeruginosa* and *Staphylococcus aureus* as putative organisms.

Table 4.2 Bacteria population of fermentation systems and their morphologies

	Group	Characteristics	Substrate	Putative Organisms	Isolate	Locations	Cluster
Non-amylyolytic	1	White, smooth, small, translucent, and circular colonies with entire margin and oxidase-negative cocci	Cassava, maize, rice	<i>Lactococcus lactis</i>	-	-	-
	3	Red, smooth, small, opaque and circular colonies with entire margin and oxidase-negative bacilli	Cassava		-	-	-
	4	Red, smooth, small, opaque and circular colonies with entire margin and oxidase-negative cocci;	Cassava, rice	<i>Lactococcus lactis</i>	-	-	-
	5	White, rough, small, translucent, and irregular colonies with undulate margin and oxidase-negative cocci	Cassava, maize, rice	<i>Lactococcus lactis</i>	-	-	-
	6	Cream, rough, large, opaque, and irregular colonies with undulate margin and oxidase-negative cocci	Cassava, maize	<i>Lactococcus lactis</i>	-	-	-
Amylyolytic	2	Cream, smooth, small, opaque, and circular colonies with entire margin and oxidase-negative cocci	Cassava, maize, rice	<i>Staphylococcus aureus</i>	CYSS, MYSS, RYSS	Oforikrom, Aboabo, Ejisu	I
	7	Cream, rough, small, opaque, and irregular colonies with undulate margin and oxidase-negative cocci	Maize, rice	<i>Staphylococcus aureus</i>	MCRS, RCRS	Oforikrom, Aboabo	IIC
	8	White, smooth, large, translucent, and irregular with entire margin and oxidase-positive bacilli	Cassava, rice	<i>Bacillus subtilis</i> , <i>B. licheniformis</i> , <i>B. amyloliquifaciens</i>	CWSL, RWSL	Aboabo, Oforikrom	IIA
	9	White, rough, large, translucent, and irregular colonies with undulate margin and oxidase-negative bacilli	Rice	<i>Pseudomonas aeruginosa</i> ,	RWRL	Ejisu	IIB
	10	White, rough, large, translucent, and irregular colonies with undulate margin and oxidase-positive cocci	Cassava, rice	<i>Staphylococcus aureus</i>	CWRL, RWRL	Aboabo, Ejisu	IIB

Cluster IIC was common to maize fermentation systems in all locations having *Staphylococcus aureus* as a putative organism. All three bacteria types, namely, *Bacillus*, *Staphylococcus*, and *Pseudomonas* were present in rice fermentation systems whereas two bacteria types, *Staphylococcus* and *Bacillus* were present in cassava fermentation systems. Only one bacteria organism, *Staphylococcus* was present in maize. The type of amylolytic organism present is dependent on the substrate.

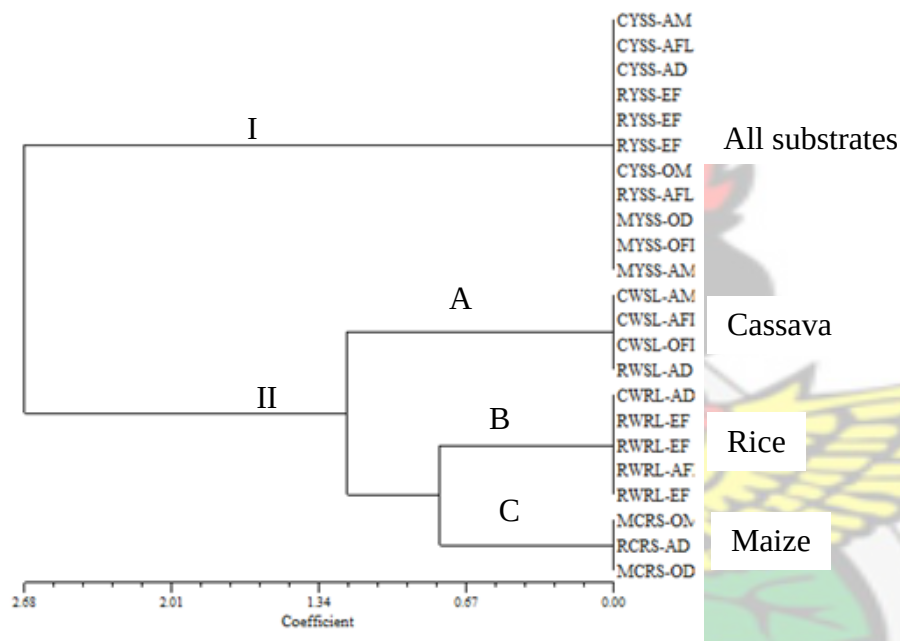


Figure 4.3 Cluster analysis of 23 colonies of amylolytic bacteria isolated from Ghanaian local fermentation systems

4.5 Amylolytic Activity of Isolates Based on Zone of Clearance

Amylolytic activity was estimated as zone of clearance upon culture of the isolates on starch agar for 24 to 48 hours (Figure 4.4). All amylolytic isolates exhibited significant ($P < 0.01$) increase in zone of clearance with time of culture. Moreover, zone of clearance of clusters were significantly different ($P < 0.01$). An R^2 of 0.99 accounted for variance in the data, and a coefficient of variation of 2.50% signifies large precision of data. Mean zone of clearance across the four isolates was 5.39

± 0.17 mm for the 24-h incubation, and increased to 7.44 ± 0.13 mm in 48 h of incubation (Table 4.3).

At the 24-hour period of incubation, cluster IIA exhibited the largest zone of clearance (6.73 ± 0.15 mm), then cluster IIB (6.03 ± 0.15 mm), cluster I (5.53 ± 0.28 mm), while cluster IIC (3.30 ± 0.12 mm) showed the least. At 48 hours of incubation, the zone of clearance increased for all clusters, specifically, to 9.58 ± 0.13 mm in cluster IIA (41.79% increase), to 8.10 ± 0.12 mm (35.00% increase) in cluster IIB, to 7.90 ± 0.12 mm in cluster I (30.42% increase), to 4.18 ± 0.17 mm in cluster IIC (27.30% increase). With respect to mean zone of clearance over the entire incubation period, cluster IIA was the largest (8.15 ± 0.14 mm), followed by cluster IIB (7.06 ± 0.13 mm), then cluster I (6.71 ± 0.20 mm), and the least was cluster IIC (3.74 ± 0.14 mm). (Table 4.3).

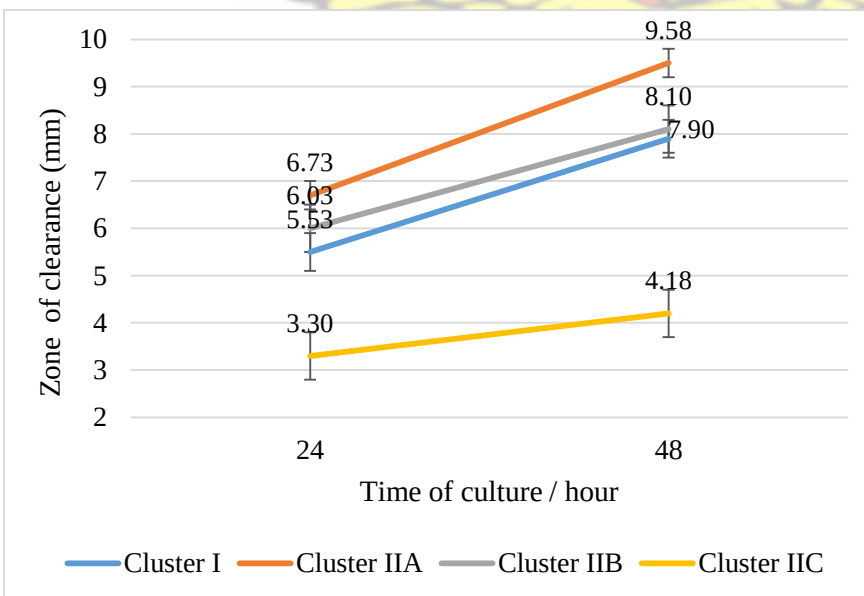


Figure 4.4 Zone of clearance of amylytic bacteria isolates cultured on starch agar for 48 hours

Table 4.3 Means, standard deviation and mean squares of zone of clearance of amylolytic bacteria grown on starch agar

Variable		Mean	Degree of freedom	Mean Squares
Time (h)	24	5.39±0.17 ^b	1	33.42**
	48	7.44±0.13 ^a		
Cluster	CI	6.71±0.20 ^c	3	28.50**
	CIIA	8.15±0.14 ^a		
	CIIB	7.06±0.13 ^b		
	CIIC	3.74±0.14 ^d		

** (P < 0.01); Means of categories with different superscripts are significantly different at P < 0.05

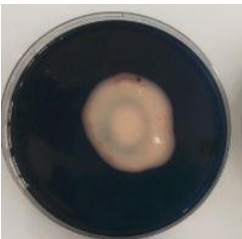
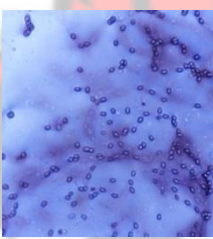
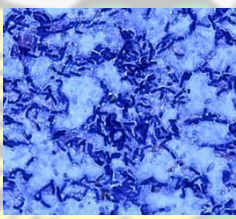
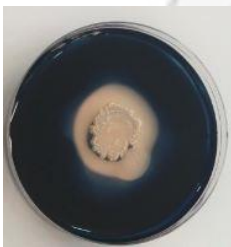
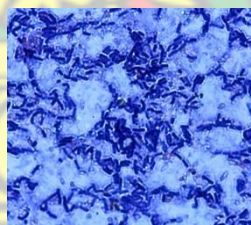
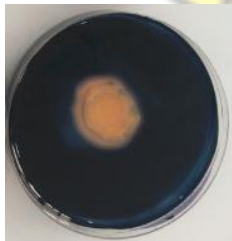
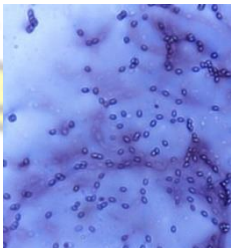
Zone of clearance	Gram Staining	Isolate
		<i>Staphylococcus</i> species
		<i>Bacillus</i> species
		<i>Pseudomonas</i> species
		<i>Staphylococcus</i> species

Plate 4.1 Zone of clearance and Gram staining of amylolytic bacteria grown on starch agar

4.6 Rate of Bacteria Growth

Optical densities of bacteria grown in starch at varying temperature (Figure 4.6), pH (Figure 4.7), and substrate concentration (Figure 4.8) were estimated as rate of bacteria growth after being incubated within 0 mins to 90 mins.

4.6.1 Effects of Temperature, pH, and Substrate Concentration on Growth of Amylolytic Bacteria Isolates

4.6.1.1 Temperature

Effects of temperature, isolate, time of incubation on growth of amylolytic bacteria isolates were significantly different ($P < 0.01$) (Table 4.4). Moreover, both two-way and three-way interaction effects were significantly different ($P < 0.01$). The variance in the data was accounted for with an R^2 value of 0.99, with the data showing a high precision of 3.76%. At each temperature, a lag period of 0 - 5 min was observed for each isolate, after which a sharp rise in growth rate occurred until 90 min used in this study. Incubation at 34°C exhibited a fluctuating growth rate for all isolates (Figure 4.6). Mean growth of isolates at all temperatures were significantly different ($P < 0.05$) except for 28°C and 31°C. Highest growth rate occurred at 37°C for all isolates. At this temperature, growth rate was highest in isolate IIA ($OD = 0.22 \pm 0.09$) followed by isolate IIB ($OD = 0.209 \pm 0.09$), then isolate I ($OD = 0.204 \pm 0.07$) and isolate IIC ($OD = 0.183 \pm 0.05$). The incubation of isolate IIA at 37°C showed a logarithmic growth throughout the entire culture period, where maximum growth was observed at 90 min of culture. At 42 °C, no growth was observed for all the isolates across the entire time culture (Figure 4.6).

4.6.1.2 pH

Main effect of pH, isolate, time, and two-way and three-way interactions were significantly different ($P < 0.01$) (Table 4.4). A coefficient variation of 6.02% signifies that the data was highly precise and an R^2 value of 0.99 accounted for the variation in the data. A lag period between 0 – 5 min was observed for all isolates at all pH levels then an exponential increase in growth was observed between 5 – 90 min for all isolates at all pH levels except for pH 6.7 (Figure 4.7). Mean growth of isolates across the various pH levels were significantly different ($P < 0.05$) with highest growth rate occurring at pH 7.0. At this pH, isolate IIA showed the highest growth rate ($OD = 0.23 \pm 0.04$) followed by isolate IIB ($OD = 0.20 \pm 0.02$), then isolate I ($OD = 0.14 \pm 0.03$), and isolate IIC ($OD = 0.09 \pm 0.02$). At a pH of 7.0, an increase in time of culture caused a continual rise in growth of the highly performing isolate, IIA. At pH 6.7, 6.8, and 7.1 virtually no growth was observed for all the isolates across the entire culture period (Figure 4.7).

4.6.1.3 Substrate Concentration

Effects of isolate, starch concentration, and time of culture on bacteria growth rate were significantly different ($P < 0.01$). Moreover, the two-way and three-way interactions effects on bacteria growth rate were significantly different ($P < 0.01$) (Table 4.4). An R^2 of 0.98 accounted for variance in the data with a low precision of 30.29%. For all isolates, a sharp rise in growth rate was observed throughout the entire culture period. Among the entire starch concentrations, 1.0% showed the maximum growth rate ($OD = 0.106$) followed by 0.5% ($OD = 0.062$) then 2.0% ($OD = 0.042$), and the least ($P > 0.05$) growth rate at 1.5% ($OD = 0.014$) and 2.5% ($OD = 0.011$) (Figure

4.8). At the 1.0% starch concentration that produced the optimum growth rate, the mean growth rate across the clusters were: isolate IIA (0.14 ± 0.01) > isolate IIB (0.12 ± 0.04) > isolate I (0.09 ± 0.01) > isolate IIC (0.06 ± 0.01). At the optimum temperature of 37°C, pH of 7.0, and time of culture of 90 min, a bimodal growth pattern in response to substrate concentration was observed between 0% to 1.5% on one hand and between 1.5% to 2.5% on the other hand (Figure 4.5). The highest growth in the first mode occurred at 1.0% for all isolate whereas the highest growth in the second mode occurred at 2.0% for all isolate. In the first mode, the highest growth rate ($OD = 0.391 \pm 0.008$) was observed in isolate IIB, then isolate IIA ($OD = 0.362 \pm 0.078$), isolate I ($OD = 0.283 \pm 0.004$), and isolate IIC ($OD = 0.104 \pm 0.01$). In the second mode, a similar trend was observed where isolate IIA gave the highest growth rate ($OD = 0.175 \pm 0.008$) as compared to isolate IIB ($OD = 0.168 \pm 0.008$), isolate I ($OD = 0.082 \pm 0.008$), and isolate IIC ($OD = 0.032 \pm 0.008$). The least growth of up to $OD = 0.1$ was observed for all isolates at 0.5%, 1.5%, and 2.5%. At starch concentration of 1.5% and 2.5%, virtually no growth was observed for all isolates across the entire culture period (Figure 4.8).

Table 4.4 Mean squares, R square (R^2) and coefficient of variation (CV) for the effect of temperature, pH, and substrate concentration on the rate of growth of amylolytic bacteria isolates measured as optical density

Effect of temperature on growth rate					
	Degree of freedom	Mean Squares	R ²	CV (%)	
Time (min)	4	0.329**	0.99	3.76	
Isolate	3	0.002**			
Temperature (°C)	4	0.181**			
Temp (°C) x Isolate	12	0.000**			
Time (min) x Isolate	12	0.001**			
Temp (°C) x Time (min) x Isolate	48	0.001**			
Effect of pH on growth rate					
	Degree of freedom	Mean Squares	R ²	CV (%)	
Time (min)	4	0.093**	0.99	6.09	
Isolate	3	0.018**			
pH	4	0.151**			
pH x Isolate	12	0.007**			
Time (min) x Isolate	12	0.004**			
pH x Time (min) x Isolate	48	0.002**			
Effect of substrate concentration on growth rate					
	Degree of freedom	Mean Squares	R ²	CV (%)	
Time (min)	4	0.064**	0.98	30.29	
Isolate	3	0.019**			
Starch concentration (%)	4	0.062**			
Isolate x Starch concentration (%)	12	0.005**			
Time (min) x Isolate	12	0.007**			
Starch conc. (%) x Time (min) x Isolate	48	0.006**			

**($P < 0.01$)

Table 4.5 Mean growth rate of highly performing isolate (IIA) across the various growth parameters

Factor	Time (min)				
	0	5	30	60	90
Temperature at 37 $^{\circ}\text{C}$	0.011 $\pm$ 0.000	0.066 $\pm$ 0.000	0.178 $\pm$ 0.001	0.339 $\pm$ 0.000	0.518 $\pm$ 0.001
pH (7.0)	0.000 $\pm$ 0.000	0.040 $\pm$ 0.003	0.142 $\pm$ 0.001	0.382 $\pm$ 0.003	0.604 $\pm$ 0.004
Starch concentration at 1%	0.000 $\pm$ 0.000	0.066 $\pm$ 0.002	0.078 $\pm$ 0.028	0.211 $\pm$ 0.001	0.363 $\pm$ 0.078

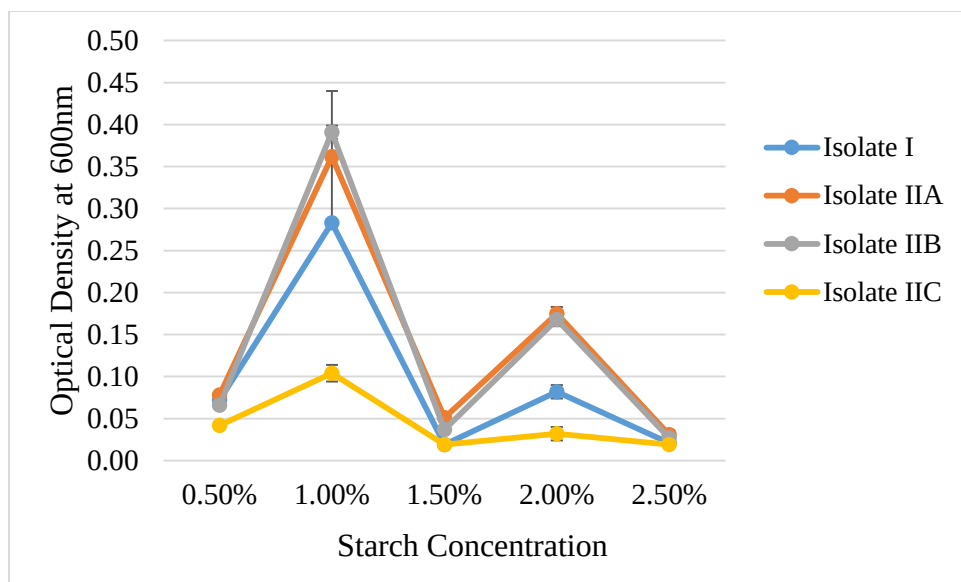


Figure 4.5 Growth rate of amylolytic bacteria at 90 min of incubation period



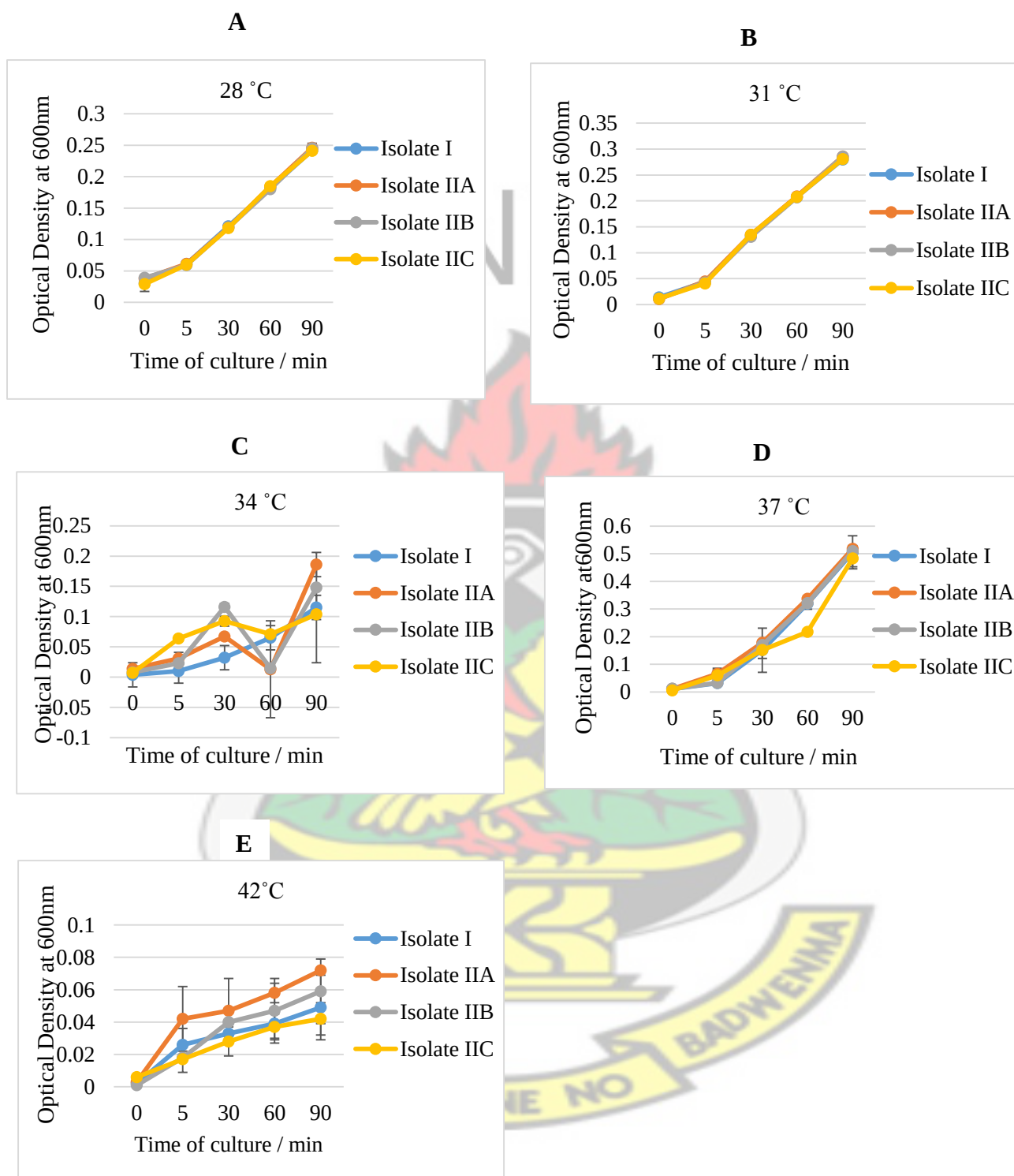


Figure 4.6 Rate of growth of amylolytic bacteria isolate at varying temperatures. (A) 28 °C (B) 31 °C (C) 34 °C (D) 37 °C (E) 42 °C

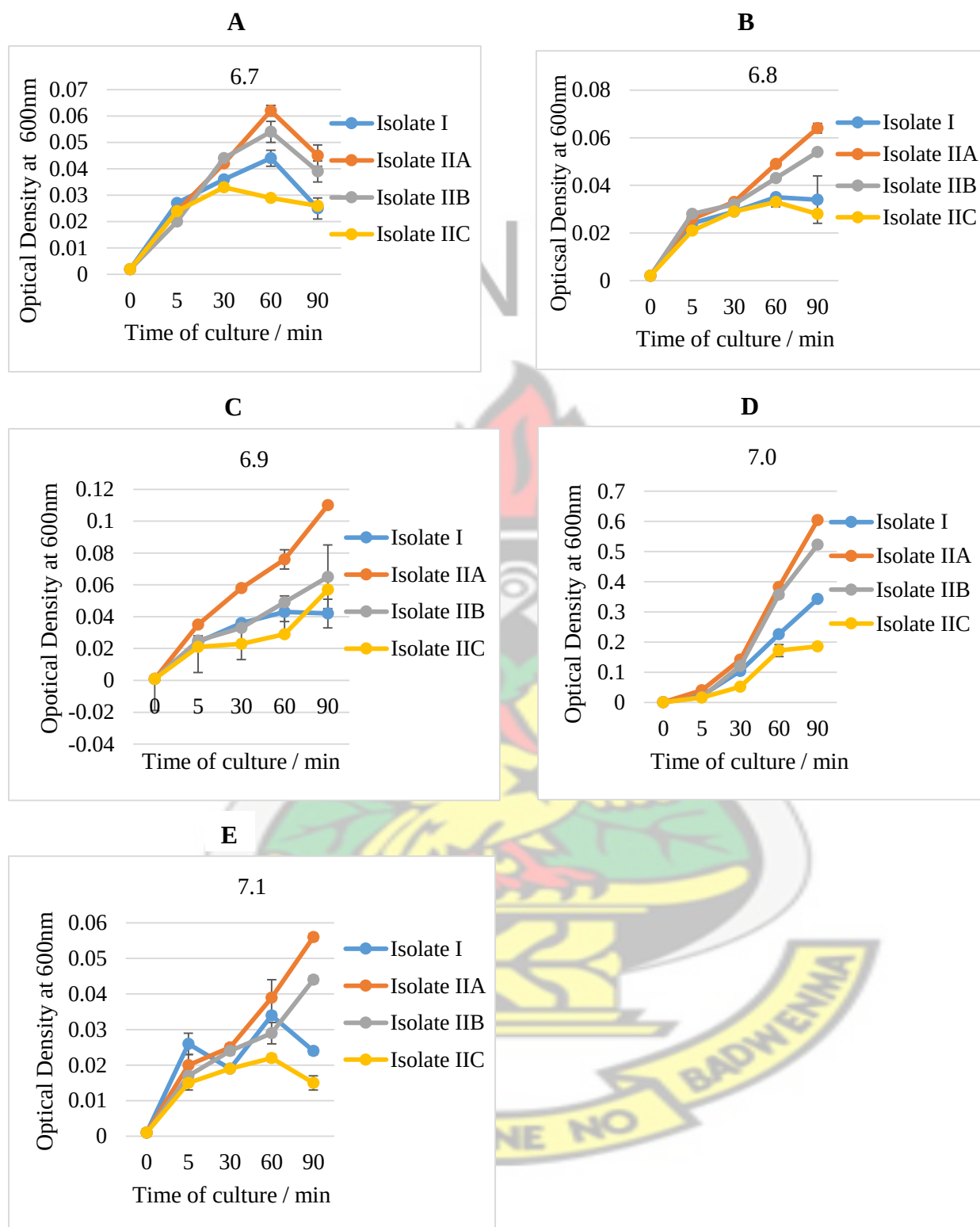


Figure 4.7 Rate of growth of amylolytic bacteria isolate at varying pH (A) 6.7 (B) 6.8 (C) 6.9 (D) 7.0 (E) 7.1

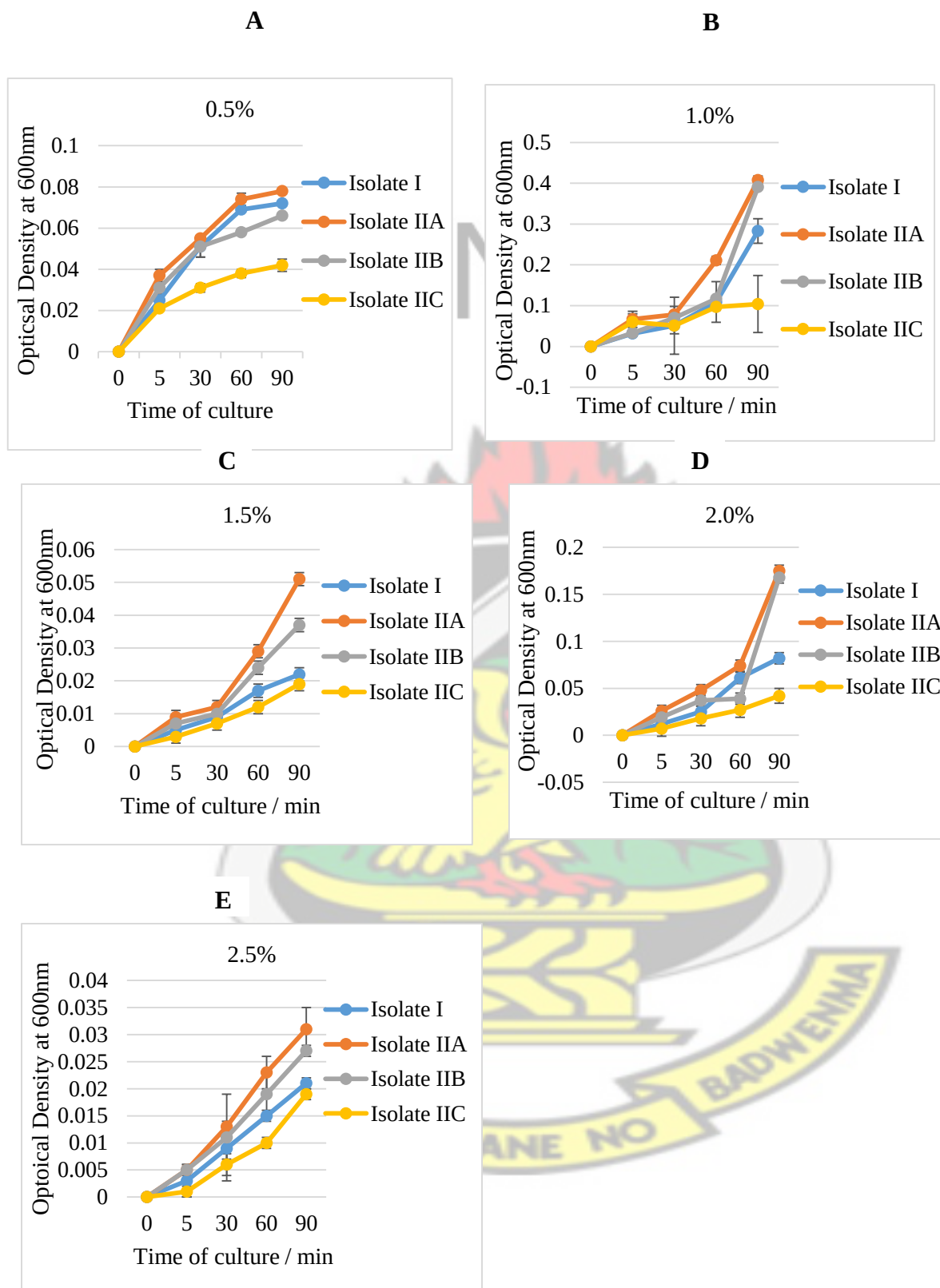


Figure 4.8 Rate of growth of bacteria isolates at varying cassava starch concentrations (A) 0.5% (B) 1.0% (C) 1.5% (D) 2.0% (E) 2.5%

4.7 Amylase Assay of Bacteria Isolates on Cassava Starch

The four isolates were evaluated for their amylase production properties. Amylase assay from bacteria isolates on cassava starch were validated based on the optimum condition for growth and DNS method for quantitative and qualitative assays. Production of the red coloration by the addition DNS to the culture indicated the presence of sugars arising from the breakdown of starch by amylase. The intensity of the red color which is measured as absorbance provided an estimate of the amylase activity. All four isolates produced amylase but at different ($P < 0.01$) capacities (Table 4.6). Amylase activity ranged from 1.20 ± 0.04 U/ml (isolate IIC), 1.65 ± 0.01 U/ml (isolate I), 2.55 ± 0.04 U/ml (isolate IIB) to highest value at 3.75 ± 0.03 U/ml which is isolate IIA at 37 °C at 1.0% starch concentration.

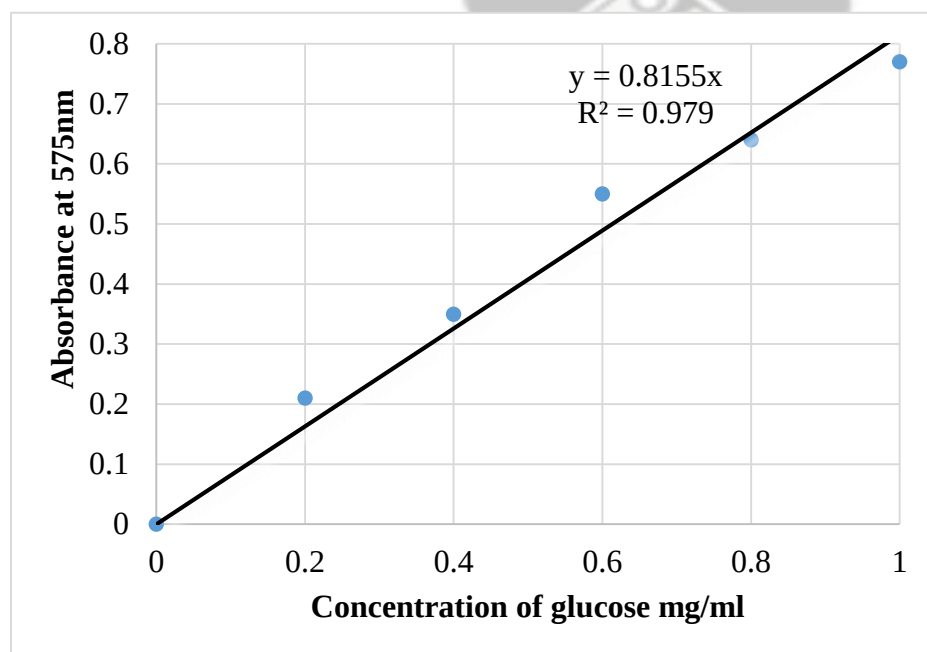


Figure 4.9 Glucose Standard Curve at 575nm

Table 4.6 Variation in enzyme activities of amylolytic bacteria isolates

Variable		Mean $\pm$ Standard Deviation	Mean Square	R ²	CV (%)
Isolate	I	1.65 $\pm$ 0.01	2.531**	0.99	1.17
	IIA	3.75 $\pm$ 0.03			
	IIB	2.55 $\pm$ 0.04			
	IIC	1.20 $\pm$ 0.04			

**P < 0.01

4.8 Effects of Temperature, pH, Substrate Concentration, and Time of Incubation on Amylase Enzyme Activity

4.8.1 Temperature

Effects of temperature, isolate and two-way interaction between temperature and isolate on enzyme activity were significantly different (P < 0.01) (Table 4.7). An R² value of 0.99 accounted for the variance in the data with a high precision of 0.68%. At low temperature of 25°C to 55°C incubation, low enzyme activity (mean values of 1.05 $\pm$ 0.01 – 3.73 $\pm$ 0.04 U/ml) was observed across the isolates. Above 55°C, enzyme activity exhibited a three to sevenfold increase up to mean value of 7.40 $\pm$ 0.03 – 9.64 $\pm$ 0.04 U/ml with maximum activity occurring at 75°C for each of the isolates. At the optimal temperature of 75°C, isolate IIA produced the highest amylase activity of 12.60 $\pm$ 0.20 U/ml followed by isolate IIB (10.65 $\pm$ 0.20 U/ml), then isolate I (8.10 $\pm$ 0.30 U/ml), and isolate IIC (7.20 $\pm$ 0.30 U/ml) (Figure 4.10).

4.8.2 pH

Effect of pH, isolate, and two way interaction between pH and isolate on enzyme activity of the isolates were significantly different (P < 0.01) (Table 4.7). Wide variations in enzyme activity from pH 3 to 10 was observed. Enzyme activity increased in a linear fashion beginning from 1.58 $\pm$ 0.01 U/ml at pH 3.0 to a highest value of 8.70 $\pm$ 0.04 U/ml at pH 7.0 and thereafter dropped

rapidly to 4.43 ± 0.04 U/ml at pH 10.0 across the isolates. At the pH optima of 7.0, the amylase activity across the various isolates were 11.7 ± 0.20 U/ml for isolate IIA, 9.45 ± 0.20 U/ml for isolate IIB, 8.10 ± 0.30 U/ml for isolate I, and 5.55 ± 0.30 U/ml for isolate IIC (Figure 4.11).

4.8.3 Substrate Concentration

Main effects of substrate concentration, isolate and two-way interaction (between substrate concentration and isolate) effects on enzyme activity were significant ($P < 0.01$) (Table 4.7). Majority of the variance in the data ($R^2 = 0.99$) was explained with a high precision of 0.69 %. Across the isolates, enzyme activity increased gradually from a mean value of 4.69 ± 0.04 U/ml at 0.5 % substrate concentration to a mean value of 7.06 ± 0.04 U/ml at an optimal substrate concentration of 1 %. Beyond 1% substrate concentration, enzyme activity declined to a minimum value of 3.34 ± 0.04 U/ml at 2.5 % substrate concentration. Except for isolate IIA which sustained a high enzyme activity of 9.30 ± 0.04 to 9.18 ± 0.03 U/ml at a wide concentration range of 1 % to 2%, all other isolates demonstrated a rapid linear drop in enzyme activity after 1% from a maximum mean value of 9.18 ± 0.03 U/ml to the lowest mean value of 1.65 ± 0.04 U/ml at 2.5% substrate concentration. At the optimum starch concentration of 1.0%, isolate IIA produced the highest amylase activity of 9.30 ± 0.20 U/ml, then isolate IIB (9.18 ± 0.20 U/ml), isolate I (5.55 ± 0.30 U/ml), and the lowest enzyme activity in isolate IIC (4.20 ± 0.30 U/ml) (Figure 4.12).

4.8.4 Time of Incubation

Enzyme activities of the isolates were ascertained at optimum conditions of 75 °C, pH of 7.0, and 1.0 % substrate concentration over an incubation period of 120 hours at 24 hour intervals. Main effect of time of incubation on enzyme activity was significant ($P < 0.01$) while the two-way

interaction effect between time of incubation and isolate on enzyme activity was not ($P > 0.01$) (Table 4.7). A high precision of 14.93% in the variance of the data was explained by an R^2 value of 0.97. Enzyme activities of the isolates showed a linear increase from 0 h to 96 h after which it dropped. Enzyme activity in isolate I increased from 0.00 ± 0.00 to 49.50 ± 4.00 U/ml at 96 h and dropped to 46.50 ± 4.00 U/ml at 120 h. Similarly, enzyme activity increased from 0.00 ± 0.00 to 43.50 ± 4.00 U/ml at 96 h and dropped to 34.50 ± 4.00 U/ml at 120 h in isolate IIC, then from 0.00 ± 0.00 to 72.00 ± 5.00 U/ml at 96 h and dropped to 61.50 ± 5.00 U/ml at 120 h in isolate IIB. Finally, enzyme activity in isolate IIA increased from 0.00 ± 0.00 to 88.50 ± 5.00 U/ml at 96 h and dropped to 78.00 ± 5.00 U/ml at 120 h. There were variations in the pattern of increase in enzyme activities across the isolates. Between 24 h and 96 h of incubation where maximum enzyme activities were exhibited, all isolates showed a little over twofold increase. Isolate I showed the highest (from 18.00 ± 4.00 to 49.50 ± 5.00 U/ml), then isolate IIC (from 16.35 ± 4.00 to 43.50 ± 4.00 U/ml), cluster IIA (from 37.50 ± 5.00 to 88.50 ± 5.00 U/ml), and cluster IIB (from 34.50 ± 5.00 to 72.00 ± 5.00 U/ml). Isolate IIA and IIB exhibited identical enzyme activities in the first few hours of fermentation but thereafter segregated, with isolate IIA outperforming isolate IIB. This trend was also evident between isolate I and IIC (Figure 4.13).

Table 4.7: Mean squares, R square (R^2) and coefficient of variation (CV) for the effect of temperature, pH, substrate concentration, and time of incubation on the amylase activity of amylolytic bacteria

Effect of temperature on amylase activity				
	Degree of freedom	Mean Squares	R ²	CV (%)
Temperature (°C)	6	86.757**	0.99	0.68
Isolate	3	31.747**		
Temperature (°C) x Isolate	18	1.679**		
Effect of pH on amylase activity				
	Degree of freedom	Mean Squares	R ²	CV (%)
pH	7	46.881**	0.99	0.64
Isolate	3	41.888**		
pH x Isolate	21	0.888**		
Effect of substrate concentration on amylase activity				
	Degree of freedom	Mean Squares	R ²	CV (%)
Starch concentration (%)	4	16.024**	0.99	0.69
Isolate	3	48.646**		
Starch concentration (%) x Isolate	12	0.942**		
Effect of time of incubation on amylase activity				
	Degree of freedom	Mean Squares	R ²	CV (%)
Time of incubation (h)	5	4435.586**	0.97	14.93
Isolate	3	2437.328**		
Time of incubation (h) x Isolate	15	133.102		

**($P < 0.01$)

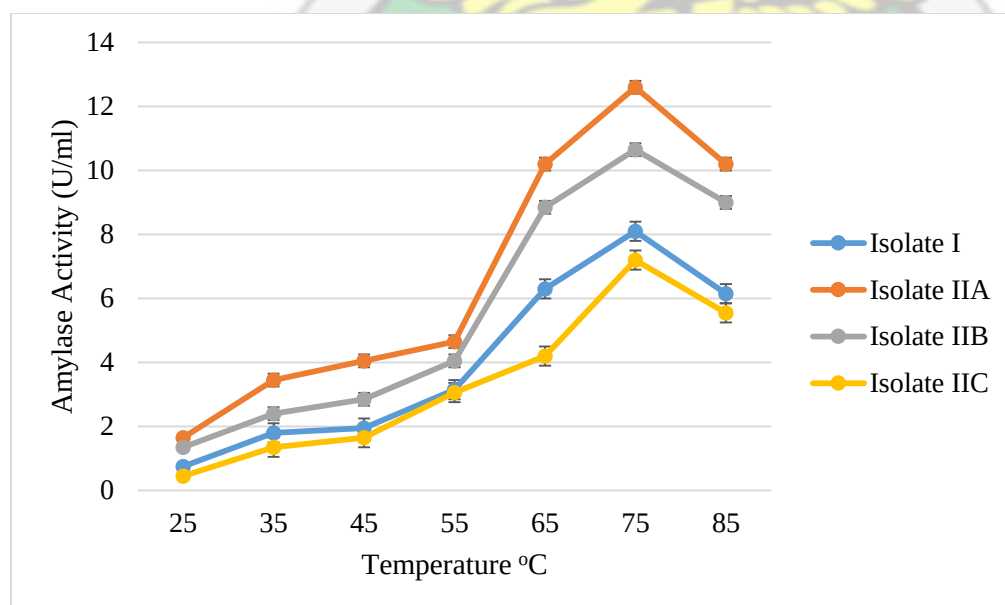


Figure 4.10 Effect of temperature on activity of cell free filtrate of amylase enzyme produced by the various bacteria isolates at pH 7.0 and starch concentration of 1.0%

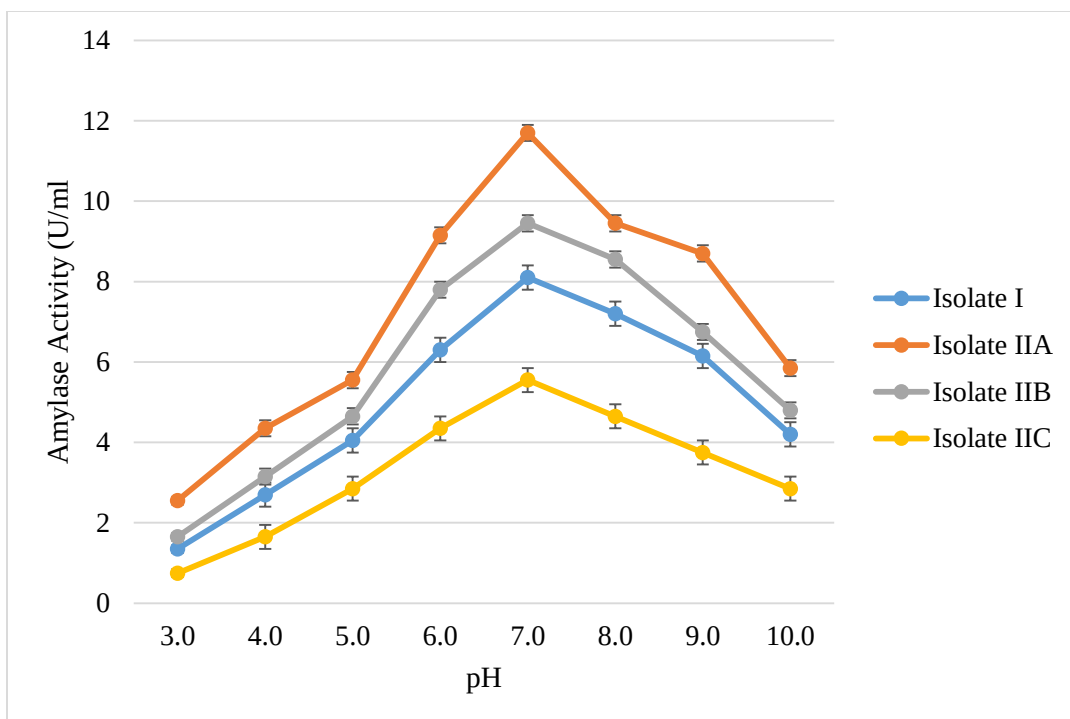


Figure 4.11 Effect of pH on activity of amylase enzyme produced by the various bacteria isolates

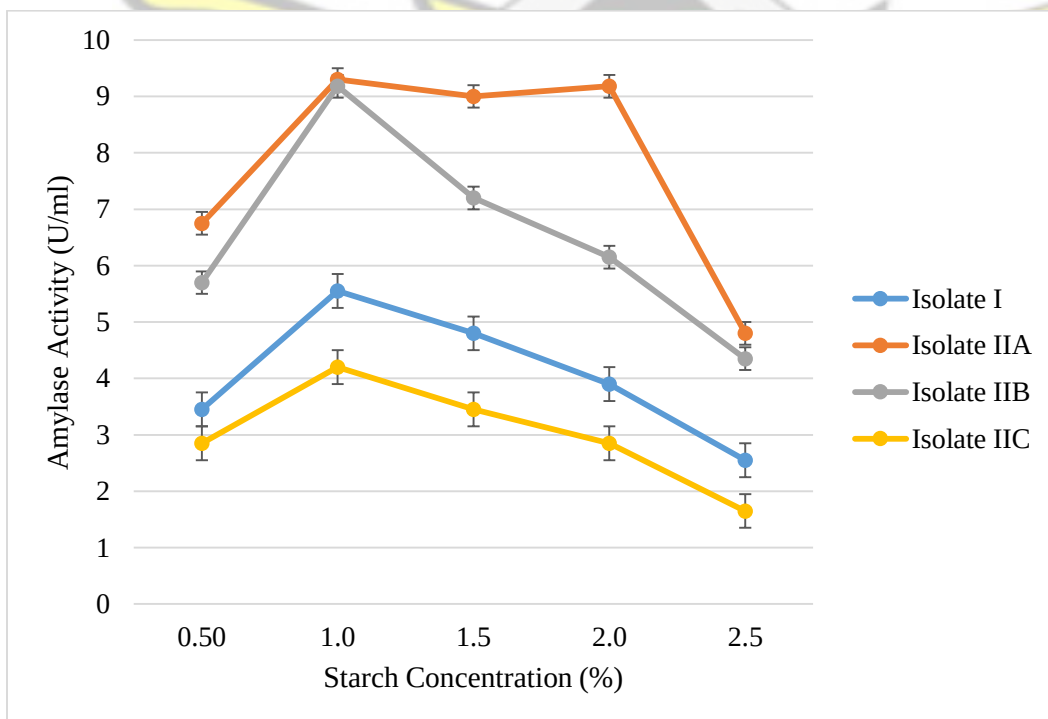


Figure 4.12. Effect of substrate concentration on activity of amylase enzyme produced by the various bacteria isolates at pH 7.0 and starch concentration of 1.0%

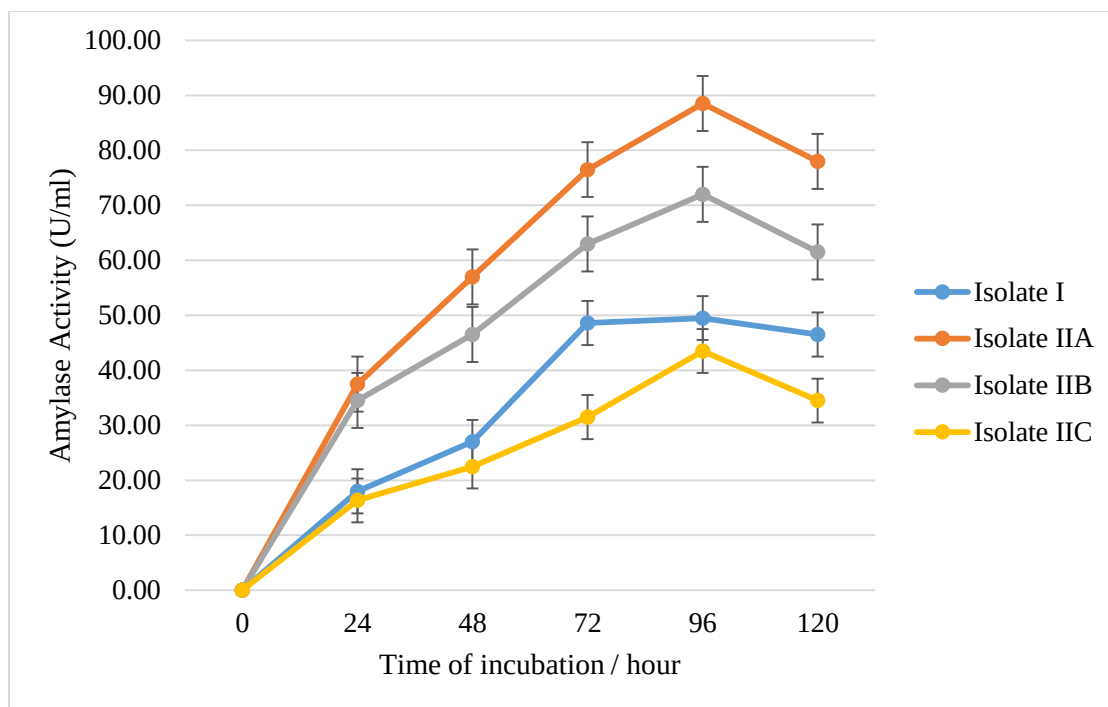
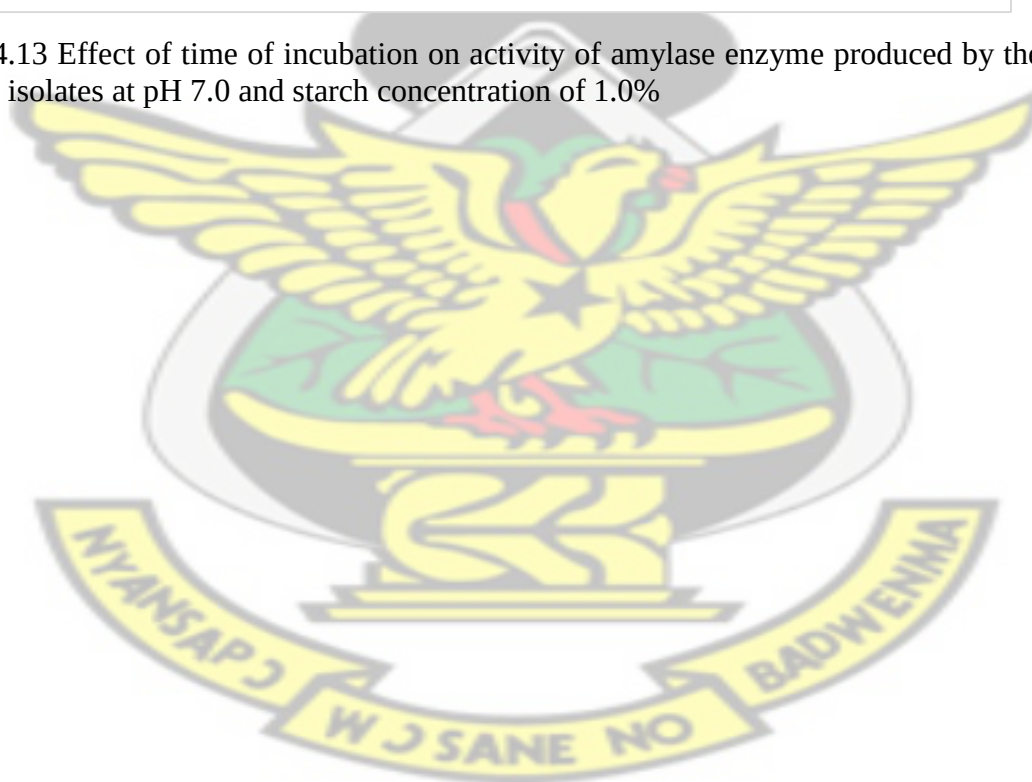


Figure 4.13 Effect of time of incubation on activity of amylase enzyme produced by the various bacteria isolates at pH 7.0 and starch concentration of 1.0%



CHAPTER FIVE

DISCUSSION

5.1 Bacteria Isolates in Substrates

Bacteria colonies were present in all substrates and in all the processing sites and farm. Variations in the number of isolates observed in the different substrates and processing sites were remarkable. The least number of bacteria isolates identified in the rice mill and dumpsite samples was not unexpected since the milling and the dumpsite conditions were devoid of water. Bacteria thrive well under moist condition. In contrast, the highest number of the bacteria isolates from the farm rice sample, though dry as in the mill and dumpsite may have been caused by its mixture with soil, which often has high bacteria populations (Akpan and Adelaja, 2004). Bacteria isolates from maize and cassava substrates were high because the substrates are fermented for several days before milling. The higher water content of cassava mash than the maize meal may have contributed to the much higher bacteria number of isolates in the cassava sample. Bacteria types which were expected in the maize and cassava substrates were likely to be both aerobes and anaerobes. At the beginning of fermentation which involves the steeping of maize and cassava in water, aerobes are expected to grow. The exhaustion of oxygen by the aerobes will lead to the multiplication of spore forming anaerobes.

Exposure of material during milling which incorporate air would favor the growth of aerobes. The known amylase-producing bacteria include both aerobes such as *B. subtilis*, *B. megaterium*, *B. pumilus* and anaerobes such as *B. licheniformis* and *B. amyloquifaciens*, and *E. coli* (El-Fallal *et al.*, 2012; Franz *et al.*, 2014; Gangadharan *et al.*, 2006; Kaur *et al.*, 2012). The higher starch content

of both maize and cassava combined with the process of fermentation make the substrates ideal sources of amylolytic bacteria. To the best of my knowledge, no record of amylolytic bacteria in maize and cassava traditional fermentation systems is documented in Ghana. Fermentations cannot become a reality without the use of microorganisms. Information on fermentation presupposes that the bacteria isolates identified may have the capacity to hydrolyse starch. According to Ross *et al.* (2002), fermentation uses microorganisms to produce metabolites capable of producing desirable products at the detriment of the development and survival of undesirable microflora in the food.

5.2 Physicochemical Properties of the Fermentation Systems

Physicochemical properties of the various fermentation systems were similar except pH. The low pH of the maize fermentation system of 4.68 was similar to values 4.77 to 4.83 reported on other maize fermentation systems (Nout *et al.*, 1996). Indeed the maize fermentation is a starting material for *kenkey*, an indigenous food in Ghana where maize kernels are soaked in water for 48 hours and milled. Akharaiya and Omoya (2008) reported a drop in pH of steeped maize kernels from 5.10 to 4.51 within 48 hours. Their result concurred with the pH of 4.68 observed in the 48-hour old maize fermentation system used in current work. In their work, the drop in pH was attributed to microbiological activity which begins with yeasts and mold and succeeded by bacteria, specifically lactic acid bacteria which produces lactic acid in such fermentation systems (Nout *et al.*, 1996; Akharaiya and Omoya, 2008).

The pH of the cassava fermentation system of 6.15 was similar to the pH value of 6.2 as reported in a cassava fermentation system after 36-hour of fermentation (Padonou *et al.*, 2009). However, Oyewole (1992) reported a decrease of pH from 6.3 to 4.0 after a 96-hour fermentation period of

cassava. Cassava fermentation is an important indigenous system for making *gari* and *agbelima* in Ghana and *lafun* in Nigeria where cassava is peeled, washed, mashed, and fermented before drying, sieving and roasting. Fermentation of cassava for the making of *gari* begins during the soaking of the cassava in water for about 36 hours before mashing (Quaye *et al.*, 2009). At this pH, the microbial activity of lactic acid bacteria increases and enhance the detoxification of the cassava from linamarin and cyanide. Therefore, the cassava fermentation systems used for this study have enough lactic acid bacteria and are likely to reduce when the pH drops over a period of time and microbial activities (Ajayi and Fagade, 2007; Azmi *et al.*, 2016).

The pH of the rice fermentations system of 6.46 reported in this study does not corroborate with the pH values of 4.56 to 3.53 after 48-hour of fermentation period as reported by Phattrat and Meawang (2015). Rice fermentation is characterised by soaking the rice in water and wet milling. However, dry milling of rice is practiced in Ghana. The expressed high value of pH in the fermentation systems for this study can be characterised by the low temperature associated with the fermentation systems adopted for this study.

ORP does not only indicate the reduction and oxidation capacity of the environment but also reflects the metabolic activity of microorganisms (Liu *et al.*, 2017). This can be attributed to the fact that the electron donor NAD(P)H resulting from glycolysis and biomass formation, and electron acceptor oxygen dissolved from sparging and/or agitation (Danakar *et al.*, 2016; Gao *et al.*, 2015). Therefore, maize and rice fermentation systems were expected to have an increasing number of bacteria than cassava fermentation systems. However, across the various locations, with the exception of cassava from dumpsite, cassava from floor and mill produced increasing number

of bacteria than rice and maize from floor and mill. Environmental factors such as temperature, ecological zone, mill composition and other physiological conditions have a direct influence of shifting the redox potential of substrates (Zhu *et al.*, 2015). However, the varying ORP of the different fermentation systems can be controlled by energy input, gas sparging, and redox reagents (such as FeCl₃, Na₂S, potassium ferricyanide, dithiothreitol, cysteine, methyl viologen, neutral red, H₂O₂, and even directly NADH and NAD⁺ as additives) (Thrash and Coates, 2008). These have the potential of enhancing the cultivation of bacteria and its metabolic production.

5.3 Bacteria Populations in the Fermentation Systems

The three amylolytic bacteria namely, *Staphylococcus* spp., *Pseudomonas* spp., and *Bacillus* spp. identified in the fermentation systems were expected since the carbon source and growth parameters used support the growth of these organisms (Swain *et al.*, 2006; Tonkova, 2006). Bhaskara *et al.* (2011) and Kaur *et al.* (2012) identified amylolytic *Bacillus* species from soil samples from a marine environment and potato field in India, respectively. Amylolytic *Pseudomonas aeruginosa* was identified by Pathak *et al.* (2015) from agricultural waste which was similar to the findings of this study but the organism identified by them was Gram negative. Amylase producing *Staphylococcus aureus* was identified by Bala *et al.* (2013) from a soil sample in Nigeria. To the best of my knowledge, this is the first report about amylolytic organisms in maize fermentation systems in Ghana.

5.4 Amylolytic Activity of Isolates Based on Zone of Clearance

The bacteria isolates demonstrated sustained amylolytic activity over the 48 hour incubation period and differences in rate of increase were observed across the clusters. Amylolytic activity indicates the production of amylase. Amylase production decreased in the order cluster IIA > cluster IIB > cluster I > cluster IIC. The putative organisms of cluster IIA are *B. subtilis*, *B. licheniformis*, *B. amyloquificiens* in rice and cassava fermentation systems. These organisms were absent from the maize fermentation system (Table 4.2). Similar findings were reported by Awais *et al.* (2008) and Atanasova-Pancevska *et al.* (2016) on the same cassava starch substrate. *Bacillus* species usually predominate over other microorganisms in wild fermentation systems (Moshafi *et al.*, 2011). The highest value of zone of clearance, 9.5 mm, for the bacteria isolate IIA was three times higher than the 3.1 mm recorded by Oyeleke and Oduwole (2009) from Nigerian soil but one and half times lower than the 13.1 mm recorded by Shanmugasundaram *et al.* (2015) from saltpan environment in India. The high zone of clearance for bacteria isolates from saltpan environment arises from their extreme growth as compared to fermentation systems (Selvamani *et al.*, 2005). There is a clear indication that cluster IIA, which is noted to be a *Bacillus* species degrades starch faster hence its rate of bacteria growth and amylolytic activity is expected to be higher than the other isolates. A confirmatory test for the actual *Bacillus* species based on molecular methods is required.

5.5 Effect of Temperature, pH, and Substrate Concentration on the Rate of Growth of Bacteria Isolates

The growth of bacteria isolates was estimated using the optical densities of the culture. Temperature, pH, substrate concentration, and time had strong effect on the rate of growth of

bacterial cells. Highest growth rate of all bacteria isolates occurred at 37°C, pH of 7.0, and 1.0% substrate concentration. However, virtually no growth was observed at temperature of 42°C, pH 6.7, 6.8, and 7.1, and substrate concentration of 1.5% and 2.5%. This is due to the fact that the reliability of the reading fall within the error margins range for the operating temperature of 37°C. Nevertheless, the readings are likely to change at different operating temperature and use of a much sensitive spectrophotometer. Results were consistent with reports of Kaur *et al.* (2012) and Bhaskara *et al.* (2011) who identified similar growth rate for *Bacillus* species obtained from Indian soil samples. *Bacillus* species thrive well at temperatures between 25 – 35°C (Cole *et al.*, 1990). Metabolic processes of bacteria cells are high at optimum temperatures. Temperatures below or above the optimum temperatures for bacteria growth lead to denaturation of proteins, loss of fluidity, and decreased chemical reactions and diffusion of bacteria cells (Huang *et al.*, 2011). However, at high temperatures, the spores in some *Bacillus* species provide dormancy for them to survive and their enzymatic proteins change shape as spore dehydrates. An effect of temperature on the growth of bacteria ensures enzymatic reactions as governed by the Arrhenius law (Ratkowsky *et al.*, 1983). For endothermic reactions, the reaction rate approaches to zero in the Arrhenius equation as temperature decreases. Therefore, growth of bacteria are high at 37°C due to increased metabolic processes and enzymatic reactions.

Bacteria cells have an ideal buffer system which capacitates them to adjust and grow in the pH which is optimal for them (Atanasova-Pancevska *et al.*, 2016). Low growth rate of bacteria cells at low pH levels could be as a result of the direct effect of the H⁺ ions on cellular components. Glucose concentration in the culture can inhibit the growth of bacteria cells but after adjusting to the optimal pH, they exploit the sugars and increase in growth. The identified growth parameters

are mostly characterised with bacteria isolated from soils. Conclusively, bacteria isolate IIA was identified as the one showing high growth rates at the various growth parameters. There is a clear indication that this isolate was highly sensitive to temperature and pH than substrate concentration due to the high growth rates exhibited at these growth parameters. Growth properties of bacteria cells are usually unstable due to the influence from environmental conditions such as temperature, pH, and substrate concentration (Rosello-Mora and Aman, 2001). Therefore, the growth parameters of bacteria isolates can change when the strains undergo genetic modifications.

5.6 Effects of Culture Conditions on Amylase Enzyme Activity

5.6.1 Temperature

Effect of temperature on enzyme activity reaching an optimum at 75°C was also reported by Abdel-Fattah *et al.* (2013). At this temperature, the isolates had optimum enzyme activities of 7 – 12 U/mL. Abdel-Fattah *et al.* (2013) reported a relatively lower enzyme activity of 2.5 U/mL in *Bacillus licheniformis* from Indonesia garden soil studied under identical growth conditions. Optimum amylase activities of 7.0 U/mL and 3.0 U/mL at lower temperatures of 45°C and 55°C have also been reported for *B. subtilis* and other *Bacillus* spp., respectively, from saltpan environment and garden soil (Shanmugasundaram *et al.*, 2015; Asad *et al.*, 2011). Similarly, the works carried out on *Bacillus coagulans*, *B. thermooleovorans* and *B. licheniformis*, *B. brevis* MTCC 7521 reported amylase activities of 15 – 40 U/mL at a temperature of 50°C (Babu and Satyanarayana, 1993; Malhotra *et al.*, 2000; Das *et al.*, 2004; Najafi *et al.*, 2005). At similar conditions, *Pseudomonas aeruginosa* was identified from garden soil in India which optimally exhibited an enzyme activity of 1.7 U/mL at 40°C (Raju and Divakar, 2013). Moreover, a related

study conducted by Alariya *et al.* (2013) identified *Pseudomonas flourescens* isolated from soil sample in India which showed an optimum amylase activity of 2.25 U/mL at 35°C.

Optimal amylase activities at low temperatures do not fit the general behavior of bacteria amylases which are often thermostable (Fossi *et al.*, 2005) unless genetically modified. Although the enzyme activities in this study is consistent with the behavior of most *Bacillus* species the amylase activity is low. As temperature increases, kinetic energy increases which enhances substrate-enzyme association for reactivity. However, high temperatures may affect the genes that code for protein production, including the enzyme (Aiba *et al.*, 1983) and also directly denature the enzymes. Amylases are heat-labile enzymes where β -amylase denatures at 70°C and α -amylase retains its potency beyond 80°C (Reed, 1975; Bernfeld, 1951; Ajayi and Fagade, 2007). At these denaturing temperatures, the hydrogen and ionic bond interactions of the enzyme are broken which leads to a change in the active sites (Bertoldo and Antranikian, 2002). This may reduce activity of some α -amylase. However, the amylase activity of the enzyme identified in this study increased until 75°C making it an α -amylase which is thermostable in nature. The optimum amylase activities of bacterial α -amylases are recorded within 25 – 100°C (Rao and Satyanarayana, 2007). Amylases produced by thermophilic bacteria have an inherent stability and longer shelf-life making them useful for many commercial applications (Bertoldo and Antranikian, 2002; Fitter and Heberle, 2000; Rasooli *et al.*, 2008) such as the saccharification and enzymatic liquefaction stages in ethanol production where temperatures rise to above 50°C and 30°C, respectively (Neves *et al.*, 2016).

5.6.2 pH

Increasing levels of pH resulted in increased amylase enzyme activity up to pH 7.0 after which the activity dropped for all isolates. Highest enzyme activities from all isolates ranged between 5.55 – 11.7 U/mL at pH optimum of 7.0. The enzyme activities of the isolates of the present study exceeded those of *Bacillus subtilis* isolated from Indian soil (0.35 U/mL) and *Bacillus licheniformis* isolated from Indian soils waste mixture (3.23 U/mL) at pH 7.0 (Alariya *et al.*, 2013; Vaseekaran *et al.*, 2010). Several species of *Bacillus* including *B. thermooleovorans*, *B. coagulans*, and *B. brevis* also exhibited optimum enzyme activities of 2.33 – 4.12 U/mL at pH of 7.0 (Medda and Candra, 1989; Tsvetkov and Emanyilova, 1989). On the contrary, a study carried out by Behal *et al.* (2006) identified a *Bacillus* sp. which showed maximum enzyme activity of 5.12 U/mL at pH 8.0. Moreover, Shanmugasundaram *et al.* (2015) identified a *Bacillus subtilis* from a saltpan environment which expressed an amylase activity of 6.23 U/mL at pH 6.0. At similar conditions, *Pseudomonas aeruginosa* was identified from garden soil in India which exhibited an optimum enzyme activity of 4.7 U/mL at pH 7.0 (Raju and Divakar, 2013). Moreover, a related study conducted by Alariya *et al.* (2013) identified *Pseudomonas fluorescens* isolated from soil sample in India which showed an optimum amylase activity of 4.25 U/mL at pH 7.0. However, no work has been identified with *Staphylococcus* species unless genetically modified species.

Most bacterial α -amylase exhibit optimum activity at slightly acidic to neutral pH (Hamilton, 1999). Contrarily, fungal species optimally produce amylase at pH below 6.0 (Fossi *et al.*, 2005; Johnson *et al.*, 2014; Oguntimehin, 1998). Different bacterial amylases exhibit varying optimum activities at different pH levels depending on the environmental conditions. The active site of the tertiary structure of an enzyme is held intact by several ionic and hydrogen bonds in order to bind

to the substrate. The occurrence of these bonds is as a result of attraction existing between oppositely charged groups on the amino acids making up the enzyme protein. Hence, at the pH of 7.0 where optimum activity are exhibited there is a complete exposure of the active sites to the substrates. On the other hand, at extremely high and low pH, the hydrogen and ionic bonds within the enzyme are interfered with charged H^+ and OH^- ions causing a change in shape of the active sites of the enzyme, thereby, decreasing enzyme activities. The loss of activities at extremes pH of 3.0 and 10.0 suggest that the protein has lost its function, probably arising from a high proton concentration that distorts the tertiary structure of the enzyme and high OH^- that distorts the ionic bonding (Annamalai *et al.*, 2011). The activity of enzymes persists within 10% deviation from the optimum pH since at these levels there can be reformation of bonds to enhance enzyme-substrate interactions. The high amylase activities exhibited by the bacteria isolates identified in this study can be attributed to the genetic mutation of the species, and time of incubation or fermentation. The optimum pH of 7.0 identified for the amylolytic bacteria identified in this study will be very useful for commercial applications such as ethanol and detergent production (Neves *et al.*, 2016; Saini *et al.*, 2017). To the best of my knowledge, this is the first report on amylolytic activity of *Bacillus* species isolated from maize, cassava, and rice fermentation systems in Ghana.

5.6.3 Substrate Concentration

A linear increase of enzyme activities was exhibited up to 1.0% substrate concentration after which enzyme activities decreased for all isolates. Optimum enzyme activities of 4.20 – 9.30 U/mL were demonstrated at 1% starch concentration for all isolates with isolate IIA which is noted to be a *Bacillus* species exhibiting the highest enzyme activity. The finding of this study is similar to one carried out on *Bacillus cereus* from Cuddalore Fishing Harbour waters, India where optimum

enzyme activity of 11.11 U/mL was exhibited at 1% of starch concentration (Annamalai *et al.*, 2011). On the contrary, Mishra and Behera (2008) reported an optimum amylase activity of 75.7 U/mL at 2% starch concentration for a *Bacillus* sp. identified from soil mixed with kitchen waste in India. Moreover, Mamo *et al.* (1999) identified a *Bacillus* sp. WN11 which optimally produced amylase (1.46 U/mL) at 2% starch concentration. At similar conditions, *Pseudomonas aeruginosa* was identified from garden soil in India which optimally exhibited an enzyme activity of 3.19 U/mL at 1% substrate concentration (Raju and Divakar, 2013). Moreover, a related study conducted by Alariya *et al.* (2013) identified *Pseudomonas flourescens* isolated from soil sample in India which showed an optimum amylase activity of 3.25 U/mL at 1% substrate concentration. However, no work has been identified with *Staphylococcus* species unless genetically modified species. At low substrate concentrations, high enzyme activities are exhibited since the catalytic sites of the enzymes are emptied and waiting to bind to the substrate.

On the other hand, high substrate concentrations saturate the enzyme thereby decreasing the enzyme activities of the enzyme. Increasing substrate concentration increases diffusion within substrate molecules, thereby, decreasing rate of enzyme reaction and activity. Nevertheless, no effect on the rate of reaction will be observed after a certain concentration where it will no longer be the limiting factor. At this point, the enzyme will become saturated with the substrate and enzyme activities will be expressed at maximum possible rate. However, a 2% starch concentration is mostly suitable for optimum fungal amylase activity (Fossi *et al.*, 2005; Johnson *et al.*, 2014). Commercial applications such as desizing of textiles and sizing and coating of paper are effective at optimum substrate concentration of 1.0% (Allan and Henrik, 1997; Okolo *et al.*, 1996). The

disparities in the amylase activities identified in this study as compared to other studies can be as a result of the cassava starch substrate used and environmental factors.

5.6.4 Incubation Time

Amylase activities of isolates increased with increasing period of incubation up to 96 h after which a decline was exhibited. Optimum enzyme activities of 43.50 - 88.50 U/mL were demonstrated at 96 h incubation period for all isolates where isolate IIA showed the highest amylase activity. Finding of this study is similar to the enzyme activity of 78.0 U/mL at 96 h incubation period as demonstrated by a *Bacillus* sp. identified from soil mixed with kitchen waste in India (Mishra and Behera, 2008). However, some *Bacillus* species are capable of exhibiting low enzyme activities at high incubation periods (Aiyer, 2005). At similar conditions, *Pseudomonas aeruginosa* was identified from garden soil in India which exhibited an optimum enzyme activity of 2.25 U/mL at 48 hours (Raju and Divakar, 2013). A similar study carried out by Alariya *et al.* (2013) identified *Pseudomonas fluorescens* isolated from soil sample in India which showed an optimum amylase activity of 0.9 U/mL at 48 hours. The longer an enzyme is incubated with its substrate, the higher the enzyme activity due to increased diffusion among the substrate and enzyme molecules. However, with unstable enzymes, denaturation of proteins occurs at prolonged time of culture causing a loss or reduction in enzyme activities (Robinson, 2015).

A decrease in the amylase activity signifies that the cells have reached the declining phase hence low synthesis of amylase. The optimum time of incubation of 96 h identified in this study can be relevant in commercial applications such as saccharification in ethanol production, desizing of textiles, and sizing of paper (Allan and Henrik, 1997; Neves *et al.*, 2016; Okolo *et al.*, 1996; Siani

et al., 2017). However, in an economic sense for industrial purposes, the 72 h incubation period can be used since enzyme activities at the said incubation time is not different from 96 h. This will aid to reduce cost in terms of resources to the industry for an additional 24 h to reach 96 h.

KNUST



CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

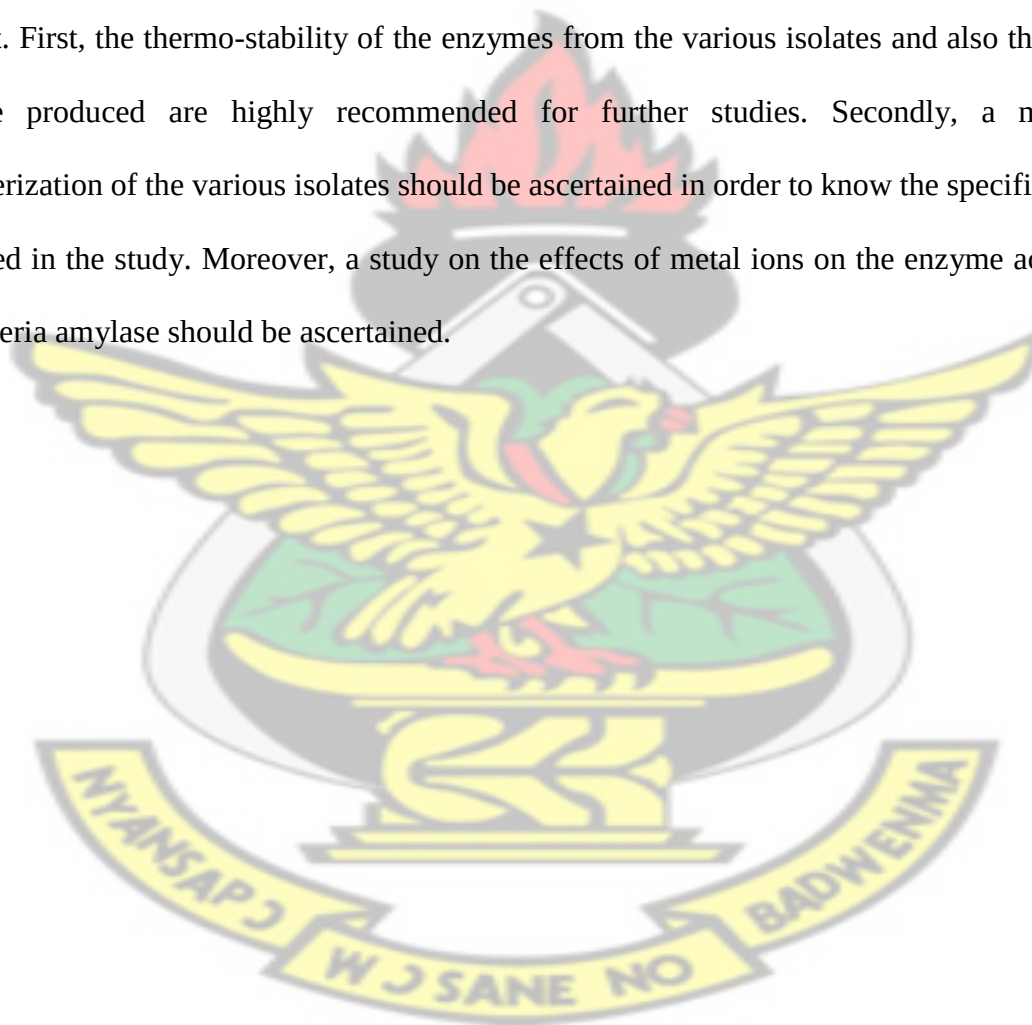
Amylase is essential for various commercial applications in industries such as detergent, food, paper, textile, beverage industries. The source of amylase include plant, animal, and microbial sources. Bacteria are noted of been good producers of amylase as compared to other microorganisms. Using various microbiological identification methods, amylolytic bacteria were identified from maize, cassava, and rice fermentation systems. These amylolytic bacteria exhibited different growth parameters. Amylolytic bacteria identified showed morphological and biochemical characteristics of *Bacillus* species, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. All the bacteria isolates showed various levels of zone of clearance at different incubation periods suggesting that all isolates were amylolytic in nature by degrading starch into soluble sugars. However, growth parameters such as temperature, pH, and substrate concentration had effects on the growth of the bacteria cells. Using the DNS method, all bacteria isolates showed significant enzyme production whose enzyme activity was affected by temperature, pH, substrate concentration, and time of incubation.

Considering the findings of this study, it can be asserted that bacteria isolates from fermentation systems exhibit optimum enzyme activities at thermophilic temperature range (75°C) , neutral pH (7.0), low substrate concentration (1.0%), and increased period of incubation (96 h). To the best of my knowledge, this is the first report on amylolytic activity of *Bacillus* species, *Pseudomonas* species, and *Staphylococcus* species isolated from maize, cassava, and rice fermentation systems in Ghana. However, there is a clear indication that their application for commercial purposes such

as enzymatic liquefaction and saccharification of ethanol production, slurry making in detergent production, desizing of textiles, and sizing and coating of paper are possible.

6.2 Recommendations

Although a milestone has been achieved in the characterisation of bacteria amylase from fermentation systems but to fully understand the behavior of such amylase several studies will be relevant. First, the thermo-stability of the enzymes from the various isolates and also the type of amylase produced are highly recommended for further studies. Secondly, a molecular characterization of the various isolates should be ascertained in order to know the specific species identified in the study. Moreover, a study on the effects of metal ions on the enzyme activity of the bacteria amylase should be ascertained.



REFERENCES

- Abdel-Fattah, Y. R., Soliman, N. A., El-Toukhy, N. M., El-Gendi, H. and Ahmed, R. S. (2013). Production, purification, and characterization of thermostable α -amylase produced by *Bacillus licheniformis* isolate AI20. *Journal of Chemistry* 1:1-11.
- Aiba, S., Kitai K. and Imanaka, T. (1983). Cloning and expression of thermostable α -amylase gene from *Bacillus stearothermophilus* in *Bacillus stearothermophilus* and *Bacillus subtilis*. *Applied Environmental Microbiology* 46:1059-1065.
- Aiyer, P. V. (2005). Amylases and their applications. *African Journal of Biotechnology* 4:23-29.
- Ajayi A. O. and Fagade O. E. (2007). Growth pattern and structural nature of amylases produced by some *Bacillus* species in starchy substrates. *African Journal of Biotechnology* 5:440-444.
- Akansha, K. and Varsha, N. (2013). Production of amylase enzyme by isolated microorganisms and its application. *International Journal of Pharmacy and Biological Science* 3:354-360.
- Akharaiyi, F. C. and Omoya, F. O. (2008). Effect of processing methods on the microbiological quality of liquid pap ogi prepared from maize. *Trends in Applied Sciences Research* 3:330-334.
- Akpan, I. and Adelaja, F.A. (2004). Production and stabilization of amylase preparations from rice bran solid medium. *World Journal for Microbiology and Biotechnology* 20:47-50.
- Alariya, S. S., Sethi, S., Gupta, S., and B. Gupta, L. (2013). Amylase activity of a starch degrading bacteria isolated from soil. *Archives of Applied Science Research* 5:15-24.
- Ali, S., Mahmood, S., Alan, R. and Hossain, Z. (1998). Culture conditions for production of glucoamylase from rice bran by *Aspergillus terreus*. *MIRCEN Journal of Applied Microbiology and Biotechnology* 5:525-532.
- Allan, S. and Henrik, B. F. (1997). Recombinant alpha amylase mutants and their use in textile desizing, starch liquification and washing. *International Applied Science* 12:205-210.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* 215:403-410.

- Amenaghawon, N., Osagie, E. and Ogbeide, S. (2016). Optimisation of Combined Acid and Enzymatic Hydrolysis of Cocoyam Starch to Produce Fermentable Hydrolysate. *Pertanika Journal of Science and Technology* 24(1):123-136.
- Amoa-Awua, W. K., Sampson, E. and Tano-Debrah, K. (2007). Growth of yeast, lactic acid and acetic acid bacteria in palm wine during tapping and fermentation from felled oil palm (*Elaeis guineensis*) in Ghana. *Journal of Applied Microbiology* 102:599-606.
- Anbu, P., Hilda, A. and Gopinath, S. C. B. (2004). Keratinophilic fungi of poultry farm and feather dumping soil in Tamil Nadu, India. *Mycopathologia* 158:303–309.
- Annamalai, N., Thavasi, R., Vijayalakshmi, S. and T. Balasubramanian (2011). Extraction, purification and characterization of thermostable, alkaline tolerant α -amylase from *Bacillus cereus*. *Indian Journal of Microbiology* 51:424–429.
- Asad, W., Asif, M. and Rasool, S. A. (2011). Extracellular enzyme production by indigenous thermophilic bacteria: partial purification and characterization of α -amylase by *Bacillus* sp. wa21. *Pakistan Journal of Botany* 43(2):1045-1052.
- Ashwini, K., Gaurav, K., Karthik, L. and Bhaskara Rao, K. V. (2011). Optimization, production and partial purification of extracellular α -amylase from *Bacillus* sp. *marini*. *Archives of Applied Science Research* 3(1):33-42.
- Asoodeh, A., Chamanic, J. and Lagzian, M. (2010). A novel thermostable, acidophilic α -amylase from a new thermophilic “*Bacillus* sp. *Ferdowsicus*” isolated from Ferdows hot mineral spring in Iran: Purification and biochemical characterization. *International Journal of Biological Macromolecules* 46:289–297.
- Atanasova-Pancevska, N., Popovska, I., Davalieva, K. and Kungulovski, D. (2016). Screening for antimicrobial activity of *Bacillus subtilis* and *Paenibacillus alvei* isolated from rotten apples compost. *Bulgarian Society of Microbiology* 1:56-64.
- Atter, A., Obiri-Danso, K. and Amoa-Awua, W. K. (2014). Microbiological and chemical processes associated with the production of *burukutu*, a traditional beer in Ghana. *International Food Research Journal* 21:1769-1776.
- Awais, M., Pervez, A., Qayyum, S. and Saleem, M. (2008). Effects of glucose, incubation period and pH on the production of peptide antibiotics by *Bacillus pumilus*. *African Journal of Microbiology Research* 2:114-119.

- Axelsson, L. (2004). Lactic acid bacteria: classification and physiology. In: Salminen S, von Wright A, Ouwehand AC (eds.) Lactic acid bacteria. Microbiological and functional aspects, 3rd ed. Marcel Dekker, New York, Pp 1–66.
- Azmi, A. S., Yusuf, N., Jimat, D. N. and Puad, N. I. M. (2016). Co-production of lactic acid and ethanol using *Rhizopus* spp. from hydrolyzed inedible cassava starch and leaves. IIUM Engineering Journal 17:1-10.
- Babu, K. R. and Satyanarayana, T. (1993). Parametric optimization of extracellular α -amylase production by thermophilic *Bacillus coagulans*. Folia Microbiology 38:77–80.
- Bai, Z. Lin, J., Wu, J., Shi, R., Li, X. and Chen, B. (2017). Q_{10} values vary with different kinetic properties of C mineralization. Journal of Soil Ecology 63:8-13.
- Bala, J. D., Abioye, O. P., Auta, H. S., Damisa, D., Adabara, N. U. and Tuggen, T. O. (2013). Screening of soil microorganisms for amylase production. Indian Journal of Biotechnology 7:22-27.
- Bano, S., Qader, S. A. U., Aman, A., Syed, M. N. and Azhar, A. (2011). Purification and characterization of Novel α -Amylase from *Bacillus subtilis*. AAPS PharmSci Tech 12:2–22.
- Baum, J.S., and Jonh-Hill, W. (1995). Polysaccharides. In: Introduction to organic and biological chemistry, MacMillan Publishing Company, New York.
- Behal, A., Singh, J., Sharma, M. K., Puri, P. and Batra, N. (2006). Characterisation of alkaline α -amylase from *Bacillus* sp. AB 04. International Journal of Agriculture and Biology 8:80–83.
- Bellon-Maurel, V., Orliac, O. and Christen, P. (2003). Sensors and measurements in solid state fermentation: A review. Process Biochemistry 38: 881–896.
- Bergey, D. H. (1938). Manual of determinative bacteriology.
- Bernfeld, P. (1951). Enzymes of starch degradation and synthesis. In: “Advances in Enzymology” (ed. Nord FF). Interscience Publications Inc. New York 379-424.
- Bertoldo, C., and Antranikian, G. (2002). Starch-hydrolyzing enzymes from thermophilic Archaea and bacteria. Current Opinion in Chemical Biology 6:151-160.

- Bessler, C., Schmitt, J., Maurer, K-H. and Schmid, R. D. (2009). Directed evolution of a bacterial α -amylase: Toward enhanced pH-performance and higher specific activity. *Protein Science*, 12:2141-2149.
- Bharathiraja, B., Jayakumar, M., Nithyananthi, M. T. J., Muthukumar, P. V. and Saravanaraj, A. (2016). Production and kinetics of amylase from starch using mutant strain of *Bacillus* sp MTCC 1434. *Journal of Chemical and Pharmaceutical Sciences* 9:281-289.
- Bhargav, S., Panda, B. P., Ali, M. and Javed, S. (2008). Solid-state fermentation: An Overview. *Chemical Biochemical Engineering* 22(1):49–70.
- Bhaskara, R. K. V., Ashwini, K., Gaurav, K. and Karthik, L. (2011). Optimization, production and partial purification of extracellular α -amylase from *Bacillus* sp. *marini*. *Archives of Applied Science Research* 3:33-42.
- Blandino, A., Al-Aseeri, M. E., Pandiella, S. S., Cantero, D. and Webb, C. (2002). Cereal-based fermented foods and beverages. *Food Research International* 36:527-543.
- Bole, S., Maji, A., Dey, A., Acharya, A., Dubey, S. and Rakeshlal, P. (2013). Isolation, purification and characterization of amylase from airborne-bacteria. *World Journal of Pharmacy and Pharmaceutical Sciences* 2:41-52.
- Borowiak, D., Miskiewitz, T., Miszczak, W., Cibis, E. and Krzywonos, M. (2012). A straightforward logistic method for feeding a fed-batch baker's yeast culture. *Biochemical Engineering Journal* 60:36–43.
- Bozic, N., Ruiz, J., Lopez-Santin, J. and Vujcic, V. (2011). Production and properties of the highly efficient raw starch digesting α -amylase from a *Bacillus licheniformis* ATCC9945a. *Biochemical Engineering Journal* 53:203–209.
- Burhan, A., Nisa, U., Gokhan, C., Omer, C., Ashabil, A. and Osman, G. (2003). Enzymatic properties of a novel thermophilic, alkaline and chelator resistant amylase from an alkalophilic *Bacillus* sp. isolate ANT-6. *Process Biochemistry* 38:1397–1403.
- Business Communication (2014). Annual report on enzyme production and demand. <https://thebusinesscommunication.com/what-is-annual-business-report/>
- Caltech Ventures (2017). Starch processing in Ghana. <https://www.google.com/search?q=Caltech+Ventures&oq=Caltech+Ventures&aqs=chrome..69i57.507j0j4&sourceid=chrome&ie=UTF-8>

- Campbell-Platt, G. (1994). Fermented Foods-a world perspective. *Food Research International* 27:253-257.
- Chatellier, S., Mugnier, N., Allard, F., Bonnaud, B., Collin, V., Belkum, A., Veyrieras, J-B. and Emler, S. (2014). Comparison of two approaches for the classification of 16S rRNA gene sequences. *Journal of Medical Microbiology* 63:1311-1315.
- Chi, H.L.Z., Wang, X., Duan, X., Ma, L. and Gao, L. (2007). Purification and characterization of extracellular amylase from the marine yeast *Aureobasidium pullulans* N13d and its raw potato starch digestion. *Enzyme Microbial Technology* 40:1006-1012.
- Clarridge, J. E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Review* 17:840–862.
- Colonna, P., Buleon, A. and Lemane, F. (1988). Action of *Bacillus subtilis* α -amylase on native wheat starch. *Biotechnology and Bioengineering* 31:895-904.
- Cole, M. B., Jones, M. V. and Holyoak, C. (1990). The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. *Journal of Applied Microbiology* 69:63-72.
- Coutinho, P. M. and Reilly, P. J. (1997). Glucoamylase structural, functional and evolutionary relationships. *Functional Genetics* 29:334-347.
- Cristobal, N. A., Gutiérrez-Sánchez, G., rado-Barragán, P. A., Rodríguez-Herrera, R., Martínez-Hernandez, J. L. and Contreras-Esquivel, J. C. (2008). Perspectives of solid state fermentation for production of food enzymes. *American Journal of Biochemistry and Biotechnology* 4(4):354-366.
- Danakar, C., Vishwakarma, A. and Raghayendra, A. S. (2016). Alternative oxidase pathway optimizes photosynthesis during osmotic and temperature stress by regulating cellular ROS, malate valve and antioxidative systems. *Frontiers in Plant Science* 7:68.
- Das, K., Doley, R. and Mukherjee, A. K. (2004). Purification and biochemical characterization of thermostable, alkalophilic, extracellular α -amylase from *Bacillus subtilis* DM-03, a strain isolated from the traditional food of India. *Biotechnology and Applied Biochemistry* 40: 291–298.
- de Souza, P. M. and de Oliveira Magalhaes, P. (2010). Application of microbial amylase in industry. *Brazilian Journal of Microbiology* 41:850-861.

- Deb, P., Talukdar, S. A., Mohinsa, K., Sarker, P. K. and Sayem, S. A. (2013). Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001. SpringerPlus 2:154.
- Dziedzic, S. Z. and Kearsley, M. W. (1995). Handbook of starch hydrolysis products and their derivatives. Boston, USA: Springer.
- Egli, T., Lendenmann, U. and Snozzi, M. (1993). Kinetics of microbial growth with mixtures of carbon sources. Advances in Microbial Ecology 63: 289–298.
- Egli, T. (2009). Growth Kinetics, Bacteria. Encyclopedia of Microbiology. (Moselio Schaechter, Editor) Oxford: Elsevier. Pp. 180-193.
- El-Fallal, E., El-Sayed, A. K., Abou-Dobara, M. S. and Omar, N. F. (2012). Starch and microbial α -amylase: From concepts to biotechnological applications. In book: Carbohydrates—comprehensive studies on glycobiology and glycotechnology, Publisher: Intech, Croatia.
- Elmansy, E. A., Asker, M. S., El-kady, E. M., Hassanein, S. M., El-Beih, F. M. (2018). Production and optimization of α -amylase from thermo-halophilic bacteria isolated from different local marine environments. Bulletin of the National Research Centre 42:31-37.
- Fisher, E. H. and Stein E. A. (1961). DNS Colorimetric Determination of Available Carbohydrates in Foods. Biochemical Preparations 8:30-37.
- Fitter, J. and Heberle, J. (2000). Structural equilibrium fluctuations in mesophilic and thermophilic α -amylase. Biophysical Journal 79:1629-1636.
- Fogarty, W. M. and Kelly, C. T. (1980). Amylases, Amyloglucosidases and Related Glucanases. In: Rose A. H. (Ed.). Microbial Enzymes and Bioconversions: Economic Microbiology. Academic Press, London Pp. 115–170.
- Fossi, B. T., Tavea, F. and Ndjonenkeu, R. (2005). Production and partial characterization of a thermostable amylase from Ascomycetes yeast strain isolated from starchy soils. African Journal of Biotechnology 4:14-18.
- Fossi, B. T., Tavea, F., Jiwoua, C. and Ndjouenkeu, R. (2014). Screening and phenotypic characterization of thermostable amylases producing yeasts and bacteria strains from some Cameroonian soils. African Journal of Microbiology Research 3:504-514.

- Franco, C. M. L. and Ciacco, C. F. (1992). Factors that affect the enzymatic degradation of natural starch granules: effect of size of the granules. *Starch*, 44:422-426.
- Franz, C. M. A. P., Huch, M., Mathara, J. M., Abriouel, H., Benomar, N. and Reid, G. (2014). African fermented foods and probiotics. *International Journal of Food Microbiology* 190:84–96.
- Gao, H., Cai, J., Han, W., Huai, H., Chen, Y. and Wei, C. (2015). Comparison of starches isolated from three different *Trapa* species. *Food Hydrocolloids* 37:174-181.
- Gangadharan, D., Sivaramakrishnan, S., Nampoothiri, K. M. and Pandey, A. (2006). Solid culturing of *Bacillus amyloliquifaciens* for alpha amylase production. *Food Technology and Biotechnology* 44:269–274.
- Garcia-Garcia, I., Santos-Duenas, I. M., Jimenez-Ot, C., Jimenez-Hornero, J.E. and Bonilla-Venceslada, J. L. (2009). Vinegar engineering. In *Vinegars of the World*, eds. L. Solieri,, and P. Giudici, 97–120. Milan, Italy: Springer-Verlag.
- Garrity, G. M., Bell, J. A. and Liburn, T. G. (2004). Taxonomic Outline of the Prokaryotes. *Bergey's Manual of Systematic Bacteriology*, Second Edition, Springer-Verlag, New York.
- Gopinath, S. C. B., Hilda, A., Lakshmi Priya, T., Annadurai, G. and Anbu, P. (2003). Purification of lipase from *Geotrichum candidum*: conditions optimized for enzyme production using Box-Behnken design. *World Journal of Microbiology and Biotechnology* 19:681–689.
- Gopinath, S. C. B., Anbu, P. and Hilda, A. (2005). Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments. *Mycoscience* 46:119–126.
- Grisham, C., Reginald, M. and Garrett, H. (1999). *Biochemistry: Handbook of Amylases and Related Enzymes* (2nd Ed.). Saunders College Publications. Pergamon Press, Oxford, Pp. 117–120.
- Gu, R., Day, L. and Li, R. (2013). Characterisation of AMT-mediated high-affinity ammonium uptake in roots of maize (*Zea mays* L.). *Plant Cell Physiology* 54:1515-1521.
- Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K. (2003). Microbial α -amylase: a biotechnological perspective. *Process Biochemistry* 38:1599-1616.

- Gusakov, A. V., Kondratyeva, E. G. and Sinitsyn, A. P. (2011). Comparison of two methods for assaying reducing sugars in the determination of carbohydrase activities. *International Journal of Analytical Chemistry* 6:1-4.
- Hamilton, L. M., Kelly, C. T. and Fogarty, W. M. (1999). Production and properties of raw starch digesting amylase of *Bacillus* sp. *Process Biochemistry* 35: 27-31.
- Hernandez, M. S., Rodriquez, M. R., Perez-Guerra, N. and Perez-Roses, R. (2006). Amylase production by *Aspergillus niger* in submerged cultivation on two wastes from food industries. *Journal of Food Engineering* 73:93-100.
- Hesseltine, C. W. (1979). Some important fermented foods of Mid-Asia, the Middle East, and Africa. *Journal of American Oil Chemists Society* 56:367–374.
- Holker, U. and Lenz, J. (2005). Solid-state fermentation—Are there any biotechnological advantages? *Current Opinion of Microbiology* 8:301–306.
- Holt, J. H. (1994). *Bergey's manual of Determinative Bacteriology* 9th edition the williams & Wilkins 428 East preston street Baltimore, Maryland 21202, U.S.A.
- Hoover, R. and Zhou, Y. (2003). *In vitro* and *In vivo* hydrolysis of starches by α -amylase and resistant starch formation in legumes – a review. *Carbohydrate Polymers* 54:401-417.
- Hua, X. and Yang, R. (2016). Enzymes in Starch Processing. In Chandrasekaran, M. (Ed.) *Enzymes in Food and Beverage Processing*. Boca Raton: CRC Press. Pp. 139-170.
- Huang, L., Hwang, A. and Philips, J. (2011). Effect of temperature on microbial growth rate – mathematical analysis: the Arrhenius and Eyring-Polanyi connctions. *Journal of Food Science* 76:553-560.
- Iulek, J., Franco, O. L., Silva, M. and Grossi-de-Sa, M. F. (2000). Purification, biochemical characterization and partial primary structure of a new α -amylase inhibitor from Secale cereal (Rye). *The International Journal of Biochemistry and Cell Biology* 32:11-12.
- Jafari-Aghdam, J., Khajek, K., Ranjbar, B. and Nemat-Gorgani, M. (2005). Deglycosylation of glucoamylase from *Aspergillus niger*: effects on structure, activity and stability. *Biochemistry and Biophysics* 1750: 61-68.
- Jain, K. P., Gaur, D. and Bajpai, V. (2012). Production of extracellular α -amylase by thermophilic *Bacillus* spp. isolated from arid and semi-arid region of Rajasthan, India. *Journal of Microbiology and Biotechnology Research* 2(5):675-684.

- Jin, L. Z., Ho, Y. W., Abdullah, N. and Jalaludin, S. (1998). Growth performance, intestinal microflora populations and serum cholesterol of broilers fed diets containing *Lactobacillus* cultures. *Poultry Science* 77:1259-1263.
- Johannes, T. W. and Zhao, H. (2006). Directed evolution of enzymes and biosynthetic pathways. *Current Opinion in Microbiology* 9:261-267.
- Johnson, F. S., Obeng, A. K. and Asirifi, I. (2014). Amylase production by fungi from cassava processing site. *Journal of Microbiology and Biotechnology Research* 4:23-30.
- Kandra, L. (2003). α -Amylases of medical and industrial importance. *Journal of Molecular Structure* 666-667.
- Kar, S. and Ray, R. C. (2008). Partial characterization and optimization of extracellular thermostable Ca^{2+} inhibited α -amylase production by *Streptomyces erumpens* MTCC 7317. *Journal of Science and Industrial Research* 67:58-64.
- Kareem, S. O., Akpan, I., Popoola, T. O. S. and Sanni, L. O. (2011). Activated charcoal - a potential material in glucoamylase recovery. *Enzyme Research* 4:1-12.
- Kasperski, A. and Miskiewitz, T. (2008). Optimization of pulsed feeding in a baker's yeast process with dissolved oxygen concentration as a control parameter. *Biochemical Engineering Journal* 40:321-327.
- Katiresan, K. and Manivannan, S. (2006). α -Amylase production by *Penicillium fellutanum* isolated from mangrove rhizosphere soil. *African Journal of Biotechnology* 5:829-832.
- Kaur, A., Kaur, M., Lal Samyal, M. and Ahmed, Z. (2012). Isolation, characterization and identification of bacterial strain producing amylase. *Journal of Microbiology and Biotechnology Research* 2:573-579.
- Khan, A. H., Minhas, N. M., Asad, M. J., Iqbal, A., Ilyas, M. and Mahmood, R. T. (2014). Estimation of protein, carbohydrate, starch and oil contents of indigenous maize (*Zea mays* L.) germplasm. *European Academic Research* 2:5230-5240.
- Kikani, B. A. and Singh, S. P. (2011). Single step purification and characterization of a thermostable and calcium independent α -amylase from *Bacillus amyloliquifaciens* TSWK1-1 isolated from Tulsi Shyam hot spring reservoir, Gujarat (India). *International Journal of Biological Macromolecules* 48:676-681.

- Klinke, H. B., Thomsen, A. and Ahring, B. K. (2004). Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Applied Microbiology and Biotechnology* 66(1):10-26.
- KNOEMA (2018). World annual production of cereals. Available on <https://knoema.com/WESP2019/world-economic-situation-and-prospects-2019> [Accessed on 12-07-18].
- Lauro, M., Forsell, P. M. and Linko, M. T. (1999). Alpha-amylolysis of large barley starch granules. *Cereal Chemistry* 76:925-930.
- Leveque, E., Janecek, S., Haye, B. and Belarbi, A. (2000). Enzymes. *Enzyme and Microbial Technology* 26:3-14.
- Li, J. H. (2004). Starch from hull-less barley: *in-vitro* susceptibility of waxy, normal, and high-amylose starches towards hydrolysis by alpha-amylases and amyloglucosidase. *Food Chemistry* 84:621-632.
- Liu, C-G., Qin, J-C. and Lin, Y-H. (2017). Fermentation and Redox Potential. *Fermentation Processes* 3:12-32.
- Madiah, M. S., Ariff, A. B., Khalil, M. S., Suraini, A. A. and Karim, M. I. A. (2000). Partial purification and some properties of α -amylase and glucoamylase obtained as by-product from direct fermentation of sago starch to solvent by *Clostridium acetobutylicum*. *Pakistan Journal of Biological Sciences* 3:744-749.
- Malhotra, R., Noorwez, S. M. and Satyanarayana, T. (2000). Production and partial characterization of thermostable and calcium independent α -amylase of extreme thermophile *Bacillus thermooleovorans* NP54. *Letters of Applied Microbiology* 31:378-384.
- Mamo, G., Gashe, B. A. and Gessese, A. (1999). Highly thermostable amylase from a newly isolated thermophilic *Bacillus* sp. WN 11. *Journal of Applied Microbiology* 86:557-560.
- Medda, S. and Chandra, A. K. (1989). New strains of *Bacillus licheniformis* and *Bacillus coagulans* producing thermostable α -amylase active at alkaline pH. *Journal of Genetic Applied Bacteriology* 48:47-58.
- Medeiros, A. B. P. Pandey, A., Freitas, R. J. S., Christen, P. and Soccol, C. R. (2000). Optimization of production of aroma compounds by *Kluyveromyces marxianus* in solid-state

- fermentation using factorial design and response surface methodology. *Biochemical Engineering Journal* 6:33–39.
- Mehta, D., and Satyanarayana, T. (2016). Bacterial and archeal α -amylases: diversity and amelioration of the desirable characteristics for industrial applications. *Frontiers of Microbiology* 28:1129-1145.
- Menzel, C. (2014). Starch structures and their usefulness in the production of packaging materials. Swedish University of Agricultural Sciences, Doctoral Thesis.
- Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry* 31:426-428.
- Mishra, S. and Behera, N. (2008). Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes. *African Journal of Biotechnology* 7:3326-3331.
- Mitra, P. and Chandra, A. L. (1994). *Solid State Fermentation* (ed. Pandey, A.), Wiley Eastern Publishers, New Delhi, Pp. 134–136.
- Mitchell, D. A., Stuart, D. M. and Tanner, R. D. (1999). Solid State Fermentation, Microbial Growth Kinetics. In *Encyclopedia of Bioprocess Technology—Fermentation, Biocatalysis and Bioseparation*. M.C. Flickinger and S.W. Drew, eds. Wiley & Sons, U.S.A Pp. 2408–2428.
- Mohamed, M. H., Gaber, A. and El-Hallous, E, I. (2014). Molecular and morphological characterization of *Trichoderma harzianum* from different Egyptian Soils. *Wulfenia Journal* 4:12-70.
- Mohsen, F. J., Dileep D. and Deepti D. (2005). Purification and characterization of an extracellular α -amylase from *Bacillus subtilis* AX20. *Protein Expression and Purification* 41:349-354.
- Monod, J. (1949). The growth of bacterial cultures. *Annual Review Microbiology* 3:371–394.
- Moreno, P. and Gourdj, S. (2015). Cassava starch content and its relationship with rainfall. CCAFS Info Note. Copenhagen, Denmark.
- Moshafi, H. M., Forootanfar, H., Ameri, A., Shakibaie, M., Dehghan-Noudeh, G. and Razavi, M. (2011). Antimicrobial activity of *Bacillus* sp. strain FAS 1 isolated from soil. *Pakistan Journal of Pharmaceutical Science* 24:269-275.

- Mugula, J. K., Nnko, S. A. M., Narvhus, J. A. and Sorhaug, T. (2003). Microbiological and fermentation characteristics of *togwa*, a Tanzanian fermented food. *International Journal of Food Microbiology* 80:187-199.
- Murakami, Y., Tatebayashi, K. and Saito, H. (2008). Two adjacent docking sites in the yeast *Hog1* mitogen-activated protein (MAP) kinase differentially interact with the Pbs2 MAP kinase kinase and the Ptp2 protein tyrosine phosphatase. *Molecular and Cellular Biology* 28:2481-94.
- Mussatto, S. I., Dragone, G., Fernandes, M., Milagres, A. M. and Roberto, I. C. (2008). The effect of agitation speed, enzyme loading and substrate concentration on enzymatic hydrolysis of cellulose from brewer's spent grain. *Cellulose* 15:711.
- Najafi, M. F., Deobagkar, D. and Deobagkar, D. (2005). Purification and characterization of an extracellular α -amylase from *Bacillus subtilis* AX20. *Protein Experimental Purification* 41: 349–354.
- Neves, P. V., Pitarelo, A. P. and Ramos, L. P. (2016). Production of cellulosic ethanol from sugarcane bagasse by steam explosion: effect of extractives content, acid catalysis and different fermentation technologies *Bio-resource Technology* 208:184-194.
- Neelima, D. and Reddy, B. I. (2013). Isolation and characterization of amylase producing *Bacillus subtilis* strain SS7 flour mill waste dump soil and optimization for amylase production. *International Journal of Recent Scientific Research* 4:2149-2154.
- Nigam, P. and Singh, D. (1995). Enzymes and microbial systems involved in starch Processing. *Enzyme and Microbial Technology* 17:770–778.
- Noda, T., Tsuda, S., Mori, M. and Takigawa, S. (2004). The effect of harvest dates on the starch properties of various potato cultivars. *Food Chemistry* 86:119-125.
- Nout, M. J. R., Kok, B., Vela, E. Ncheb P. F. and Romhouts, F. M. (1996). Acceleration of the fermentation of kenkey, an indigenous fermented maize food of Ghana. *Food Research International* 28:599-604.
- Oates, C. G. (1997). Towards an understanding of starch granule structure and hydrolysis. *Trends in Food Science and Technology* 8:375-382.
- Oberoi, H., Singh, K., Kalra, L., Dewinder, S., Uppal, S. and Kumar, T. (2007). Effects of Different Drying Methods of Cauliflower Waste on Drying time, Colour Retention and

- Glucoamylase Production by *Aspergillus niger* NCIM 1054. International Journal of Food Science and Technology, 42:228–234.
- Odunfa, S. A. (1985). African fermented foods. In Microbiology of Fermented Foods Woods B.J.B. ed. Elsevier Applied Science Publishers, London Pp. 167-195.
- Oguntimehin, G. B. (1998). Growth of and amylase production by *Bacillus licheniformis* isolated from cassava processing waste. Nigerian Food Journal 11:58-67.
- Okolo, B. N., Ezeogu, L. I. and Ebisike, C. O. (1996). Raw starch digesting amylase from *Thermoactinomyces thalophilus*. Journal of Microbiology and Biotechnology 12:637-638.
- Omafuvbe, B. O., Shonukan, O. O. and Abiose, S. H. (2000). Microbiological and biochemical changes in traditional fermentation of soybean for soy-daddawa- Nigerian food condiment. Food Microbiology 17:469-474.
- Oyeleke S. B. and Oduwole, A. A. (2009). Production of amylase by bacteria isolated from a cassava waste dumpsite in Minna, Niger State, Nigeria. African Journal of Microbiology Research 3:143-146.
- Oyewole, O. B. (1992). Cassava Processing In Africa. Applications of Biotechnology to Fermented Foods: Report of an Ad Hoc Panel of the Board on Science and Technology for International Development. Washington (DC): National Academies Press (US); National Research Council (US) Panel on the Applications of Biotechnology to Traditional Fermented Foods.
- Oyewole, O. A., Oyeleke, S. B., Dauda, B. E. N. and Emiade, S. (2011). Production of amylase and protease enzymes by *Aspergillus niger* and *Penicillium frequestans* isolated from abattoir effluent. Microbiology Journal 1:174-180.
- Padonou, S. W., Hounhouigan, J. D. and Nago, M. C. (2009). Physical, chemical and microbiological characteristics of lafun produced in Benin. African Journal of Biotechnology 8:3320-3325.
- Pancha, I., Jain, D., Shrivastav, A., Mishra, S. K. and Shethia, B. (2010). A thermoactive α -amylase from a *Bacillus* sp. isolated from CSMCRI salt farm. International Journal of Biological Macromolecules. 47:288–291.
- Pandey, A., Selvakumar, P., Carlos, P., Soccol, R. and Poonam, N. (1991). Solid State Fermentation for the Production of Industrial Enzymes. Bioresearch Technology 37:169–172.

- Pandey, A. and Radhakrishnan, S. (1992). Packed-bed column bioreactor for production of enzyme. *Enzyme Microbiology Technology* 14:486 - 488.
- Pandey, A., Selvakumar, P. and Ashakumary, L. (1994). Glucoamylase production by *Aspergillus niger* on rice bran improved by adding nitrogen sources. *World Journal of Microbiology and Biotechnology*, 10(3):348-349.
- Pandey, A., Selvakumar, P., Soccol, C. R. and Nigam, P. (1999). Solid-state fermentation for the production of industrial enzymes. *Current Science* 77:149–162.
- Pandey, A., Nigam, P., Soccol, C. R., Soccol, V. T., Singh, D. and Mohan, R. (2000). Advances in microbial amylases. *Biotechnology Application in Biochemistry* 31:135-152.
- Panikov, N. S. (1995). Microbial Growth Kinetics. *Journal of Basic Microbiology* 36:120.
- Pathak, A. P., Jethaliya, C. S., Sarsar, M. S. and Jadhav, S. R. (2015). Isolation and characterisation of potential amylase producing strain from the agriculture waste. *International Journal of Advances In Pharmacy, Biology And Chemistry* 4:829-832.
- Paulova, L., Patakova, P. and Branyik, T. (2013). General fermentation with enhanced technology. *Engineering Aspects of Food Biotechnology* 1:89-105.
- Payan, F. (2004). Structural basis for the inhibition of mammalian and insect alpha-amylases by plant protein inhibitors. *Biochemical Biophysics Acta* 1696:171-80.
- Phattra, B. and Maweang, M. (2015). Effects of natural fermentation on the rice slurry properties related to rice paper production. *Journal of Food Science and Agricultural Technology* 1:22-25.
- Prakash, B., Vidyasagar, M., Madhukumar, M. S., Muralikrishna, G. and Sreeramulu, K. (2009). Production, Purification and Characterization of Two Extremely Halotolerant, Thermostable, and Alkali-stable α -amylases from *Chromohalobacter* sp. Research Society of Japan (Ed.), Pergamon Press, Oxford. Pp. 40-45.
- Quaye, W., Gayin, J., Yawson, I. and Plahar, W. A. (2009). Characteristics of various cassava processing methods and the adoption requirement in Ghana. *Journal of Root Crops* 35:59-68.

- Raghavarao, K. S. M. S., Ranganathan, T. V. and Karanth, N. G. (2003). Some engineering aspects of solid state fermentation. *Journal of Biochemistry Engineering* 13:127–35.
- Rajagopalan, G. and Krishnan, C. (2008). Alpha-amylase production from catabolite depressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysates. *Bioresources Technology* 99:3044-50.
- Raju, N. V. E. and Divakar, G. (2013). Production of amylase by using *Pseudomonas aeruginosa* isolated from garden soil. *International Journal of Advances in Pharmacy, Biology and Chemistry* 2:50-56.
- Rameshkumar, A. and Sivasudha, T. (2011). Optimization of nutritional constitute for enhanced alpha amylase production using by solid state fermentation technology. *International Journal of Microbiological Research* 2:143-148.
- Ramprakash, B. and Muthukumar, K. (2014). Comparative study on the production of biohydrogen from rice mill wastewater. *International Journal of Hydrogen Energy* 39:14613-14621.
- Rao, J. L. U. M. and Satyanarayana, T. (2007). Purification and characterization of a hyperthermostable and high maltogenic alpha-amylase of an extreme thermophile *Geobacillus thermoleovorans*. *Applied Biochemistry and Biotechnology* 142:179-193.
- Rasooli, I., Astaneh, S. D. A., Borna, H. and Barchini, K. A. (2008). A thermostable α -amylase producing natural variant of *Bacillus* spp. isolated from soil in Iran. *American Journal of Agricultural and Biological Sciences* 3:591-596.
- Ratkowsky, D. A., Lowry, R. K., Mcmeekin, T. A. and Stokes, A. N. (1983). Model for bacterial culture growth rate throughout the entire biokinetic temperature range. *Journal of Bacteriology* 154:1222-1226.
- Raul, D., Biswas, T., Mukhopadhyay, S., Das, K. S. and Gupta, S. (2014). Production and partial purification of alpha amylase from *Bacillus subtilis* (MTCC 121) using solid state fermentation. *Biochemistry Research International* 1:1-5.
- Ray, A. K., Roy, T., Mondal, S. and Ringø, E. (2010). Identification of gut-associated amylase, cellulase, and protease-producing bacteria in three species of Indian major carps. *Aquaculture Research* 41:1462–1469.
- Reed, G. (1975). *Enzymes in Food Proceeding* (2nd Edition). Academic press New York 10005 U.S.A.

- Robinson, P. K. (2015). Enzymes: principles and biotechnological applications. *Essays in Biochemistry* 59:1-41.
- Rocha, T. D., Carneiro, A. P. D. and Franco, C. M. L. (2010). Effect of enzymatic hydrolysis on some physiochemical properties of root and tuber granular starches. *Food Science Technology* 30:544-551.
- Ross, P., Morgan, S. and Hill, C. (2002). Preservation and fermentation: past, present and future. *International Journal of Food Microbiology* 79:3-16.
- Rosselló-Mora, R. and R. Amann. (2001). The species concept for prokaryotes. *FEMS Microbiology Letters* 25:39-67.
- Roy, I. and Gupta, M. N. (2004). Hydrolysis of starch by a mixture of glucoamylase and pullulanase entrapped individually in calcium alginate beads. *Enzyme Microbiology and Technology* 34:26-32.
- Saito, N. and Yamamoto K. (1975). Regulatory factors affecting α -amylase production in *B. licheniformis*. *Journal of Bacteriology* 121:848-856.
- Saunders, M., Lewis, P. and Thorn hill, A. (2012). *Research Methods for Business Students*, Pearson Education Limited.
- Schallmey, M., Singh, A. and Ward, O. P. (2004). Developments in the use of *Bacillus* species for industrial production. *Canada Journal of Microbiology* 50:1-7.
- Schlager, R., Simmon, K. E. and Fisher, M. A. (2012). A systematic approach for discovering novel, clinically relevant bacteria. *Emergency Infectious Disease* 18:422-430.
- Sefa-Dedeh, S., Cornelius, B., Amoa-Awua, W., Sakyi-Dawson, E. and Afoakwa, E. O. (2004). The microflora of fermented nixtamalized corn. *International Journal of Food Microbiology* 96:97-102.
- Selvamani, P., Latha, S., Sen, D. J. and Gupta, J. K. (2005). Antibacterial activity of *Commiphora caudate* and *Commiphora berryi* leaves. *Indian Drugs* 42:696-698.
- Shanmugasundaram, S., Eswar, A., Mayavu, P., Surya, M. and Anbarasu, R. (2015). Screening and identification of amylase producing bacteria from marakkanam saltpan environment, Tamil Nadu, India. *Asian Journal of Biomedical and Pharmaceutical Sciences* 5:35-37.

- Sharma, A. K. (2009). Manual of Microbiology: Tools & Techniques, Ane Books, Delhi, India, **1**, 141-171.
- Shuang, L., Xiaofeng, Y., Shuai, Y., Muzi, Z. and Xiaoning, W. (2012). Technology prospecting on enzymes: application, marketing and engineering. Computational and Structural Biotechnology Journal 2:9-17.
- Siani, R., Saini, H. S. and Dahiya, A. (2017). Amylases: characteristics and industrial applications. Journal of Pharmacognosy and Phytochemistry 6:1865-1871.
- Simango, C. (1997). Potential use of traditional fermented foods for weaning in Zimbabwe. Journal of Social Science and Medicine 44:1065–1068.
- Singh, N., Singh, J., Kaur, L., Sodhi, N. S. and Gill, B. S. (2003). Morphological, thermal and rheological properties of starches from different botanical sources. Food Chemistry 81: 219-231.
- Singh, R., Kumar, M., Mittal, A., and Mehta, P. K. (2016). Microbial enzymes: industrial progress in 21st century. Biotechnology 6:174-191.
- Singh, K., Shandilya, M., Kundu, S. and Kayastha, A. M. (2015). Heat, Acid and Chemically Induced Unfolding Pathways, Conformational Stability and Structure-Function Relationship in Wheat α -Amylase. PlosOne 10:1-18.
- Srivastava, A. and Srivastava, M. (2018). Allied Market Research. Enzymes market share and trends. Global industry demand 2024. <https://www.alliedmarketresearch.com/enzymes-market>.
- Stackebrandt, E. and Goebel, B. M. (1994). Taxonomic Note: A Place for DNA-DNA Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology. International Journal of Systematic and Evolutionary Microbiology 44: 846-849.
- Statistics Research and Information Department – Ministry of Food and Agriculture (2018). Annual production of crops in Ghana. http://mofa.gov.gh/site/?page_id=79
- Steinkraus, K. H. (2002). Comprehensive reviews in food science and food safety. Institute of Food Technologists 1:26.
- Swain, M. R., Kar, S., Padmaja, G. and Ray, R. C. (2006). Partial characterization and optimization of production of extracellular α -amylase from *Bacillus subtilis* isolated from culturable cowdung microflora. Polish Journal of Microbiology 55:289–296.

- Tengerdy, R. P. (1996). Bioreactors for Solid State Fermentation of Lignocellulosics. *Journal of Scientific and Industrial Research* 55: 313-316.
- Tester, R. F., Qi, X. and Karkalas, J. (2006). Hydrolysis of native starches with amylases. *Animal Feed Science and Technology* 130:39-54.
- Thippeswamy, S., Girigowda, K. and Mulimani, H. V. (2006). Isolation and identification of α -amylase producing *Bacillus* spp. From dhal industry waste. *Indian Journal of Biochemistry and Biophysics* 43:295-298.
- Thrash, J. C. and Coates, J. D. (2008). Review: Direct and indirect electrical stimulation of microbial metabolism. *Environmental Science Technology* 42:3921-3931.
- Tonkova, A. (2006). Microbial starch converting enzymes of the α -amylase family. In: Ray, R.C., Wards, O.P., ed. *Microbial Biotechnology in Horticulture*, Volume 1. Enfield, NH: Science Publishers, pp. 421– 472.
- Trevan, M.D. (1980) *Immobilized Enzymes: An Introduction and Applications in Biotechnology*, John Wiley & Sons, Chichester.
- Tsvetkov, V. T. and Emanyilova, E. I. (1989). Purification and properties of heat stable α -amylase from *Bacillus brevis*. *Applied Microbiology and Biotechnology* 31:246-248.
- Uguru, G. C., Akinyanju, J. A. and Sani, A. (1997). The use of sorghum for thermostable amylase production from *Thermoactinomyces thalophilus*. *Letters in Applied Microbiology* 25: 13-16.
- Ugwuanyi, J. O., McNeil, B. and Harvey, L. M. (2009). Production of protein-enriched feed using agro-industrial residues as substrates. In: *Biotechnology for Agro-Industrial Residues Utilization*, eds., P. Singh Nigam and A. Pandey Springer, Berlin, Germany. Pp. 77–104.
- Vaseekaran, S., Balakumar, S. and Arasaratnam, V. (2010). Isolation and identification of a bacterial strain producing thermostable α -amylase. *Tropical Agricultural Research* 22:1-11.
- Vengadaramana A., Balakumar S. and Vasanthi A. (2014). Effect of temperature, pH, substrate (starch) and glucose on stability of α -amylase from *Bacillus licheniformis* ATCC 6346. *Scholars Academic Journal of Pharmacy* 3:492-495.
- Wadhwa, M., Kaushal, S. S. and Bakshi, M. P. S. (2005). Nutritive evaluation of vegetable wastes as complete feed for goat buck. *Small Ruminant Research* 64:197-310.

Wang, N. S. (2015) Starch Hydrolysis by Amylase; Experiment 5

Wessel, D. and Flugge, U. I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Analytical Biochemistry* 138:141 – 143.

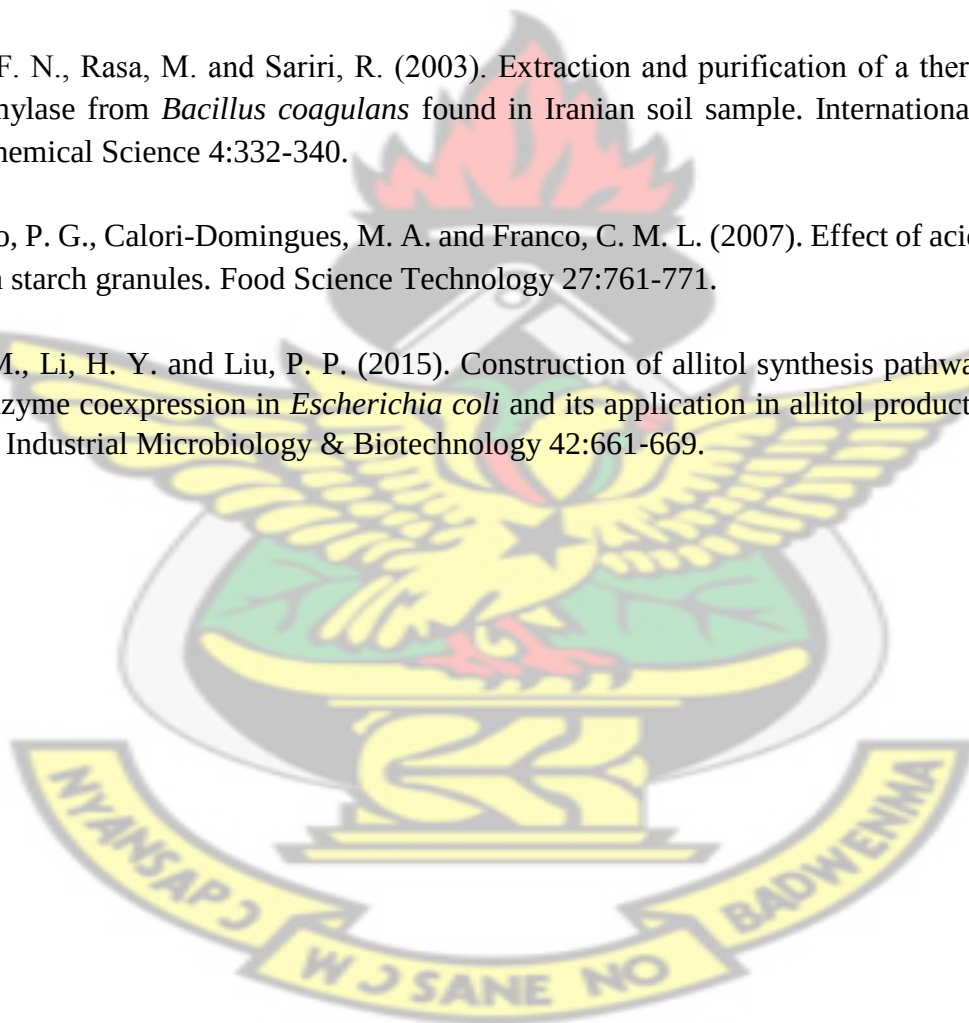
Whitcomb, D. C. and Lowe, M. E. (2007). Human pancreatic digestive enzymes. *Digestive Disease Science* 52:1-17.

Woo, P. C., Lau, S. K., Teng, J. L., Tse, H. and Yuen, K. Y. (2008). Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clinical Microbiology Infections* 14:908–934.

Yasouri, F. N., Rasa, M. and Sariri, R. (2003). Extraction and purification of a thermostable α -amylase from *Bacillus coagulans* found in Iranian soil sample. *International Journal of Chemical Science* 4:332-340.

Yonemoto, P. G., Calori-Domingues, M. A. and Franco, C. M. L. (2007). Effect of acid hydrolysis on starch granules. *Food Science Technology* 27:761-771.

Zhu, Y. M., Li, H. Y. and Liu, P. P. (2015). Construction of allitol synthesis pathway by multi-enzyme coexpression in *Escherichia coli* and its application in allitol production. *Journal of Industrial Microbiology & Biotechnology* 42:661-669.



APPENDICES

APPENDIX A

SERIAL DILUTION (10-fold)

Purpose: To achieve an appropriate concentration for a specific test method or a countable agar plate.

EQUIPMENT

Test tubes, Pipette, sterilized distilled water, vortex and soil sample

METHOD

1. Add 20 grams of soil sample into a test tube and add 100 ml of sterilized distilled water (Stock solution).
2. Vortex the stock solution
3. Pipette 1 ml of stock solution suspension into 9 ml of sterilized distilled water in a different test tube.
4. Repeat 2 & 3 to the degree of concentration you desire.

APPENDIX B

PREPARATION OF STARCH NUTRIENT AGAR (500 ml)

Purpose: To support the colonies formation or growth of microorganisms (bacteria).

EQUIPMENT

Nutrient Agar, peptone, beef extract, yeast extract, sodium chloride (NaCl), cassava starch, distilled water, autoclave, sterilized petri plate, cotton wool, conical or volumetric flask, water bath

METHOD

1. Prepare your beef extract by removing all fatty parts and ligaments on the lean part of the heart of a cow.
2. Mince the lean meat and blend with 700 ml of distilled water. Blended meat will be steamed in a water bath for 4 hours during which it will be stirred occasionally to avoid over-heating and boiling.
3. Cool after steaming and filter with a cotton wool. Weigh 250 g of the beef extract into a 1 litre beaker.
4. Weigh and add 5 g peptone, 2.5 g NaCl, 0.75 g yeast extract, 5 g starch, and 10 g of nutrient agar to the beaker and add distilled water to reach the 500ml mark of the beaker.

5. Adjust the pH to 7.0 by adding few drops of 0.1 N NaOH.
6. Autoclave for 20 mins at 121°C and 20 Psi.
7. Pour starch nutrient agar into sterilized petri plates and incubate at room temperature to solidify.

APPENDIX C

GRAM STAINING

Purpose: To know whether a bacteria species is either gram positive (+) or gram negative (-).

EQUIPMENT

Distilled water, glass slide, pipette, sterilized loop, crystal violet, gram's iodine, ethanol, safarin

METHOD

1. Put a drop of ddH₂O onto the glass slide.
2. Pick a sample of the bacterial growth from your petri plate using a loop and add it to the drop of water on the glass slide to form a thin layer.
3. Allow it to air dry.
4. Flood slide with Crystal violet and allow it to stain for 1 minute.
5. Gently rinse with water.
6. Flood slide with Gram's iodine and allow it to stain for 1 minute and gently rinse with water.
7. Flood slide with ethanol and allow it to stain for 30 seconds and gently rinse with water.
8. Flood slide with Safarin for 1 minute and rinse with water.
9. Air dry the slide.

APPENDIX D

BIOCHEMICAL TESTS

Starch Hydrolysis Test

Purpose: To identify the bacteria which produce the enzyme amylase.

EQUIPMENT

Iodine solution, cultured plate.

METHOD

1. Add iodine onto the cultured plate and allow it to stay for 1 minute.

2. Dispose off the excess iodine solution.

Oxidase test

Purpose: To differentiate between enteric (oxidase +ve) and non-enteric (oxidase –ve) organisms

EQUIPMENT

Oxidase reagent, distilled water, sterile loop, filter paper and petri dish.

METHOD

1. Add water to oxidase reagent.
2. Add a few drops to the filter paper placed in a petri dish.
3. Take a sample of the bacterial growth using a sterile loop and smear on the filter paper.
4. Leave for 5 to 10 seconds.

Catalase test

Purpose: To differentiate between *Staphylococcus* (catalase positive) and *Streptococcus* (catalase negative) species.

EQUIPMENT

Glass slide, 3% Hydrogen peroxide

METHOD

1. Place a drop of 3% hydrogen peroxide on a slide
2. Add a sample of the bacterial culture onto it.
3. Check the bubbling reaction