OBAFEMI AWOLOWO UNIVERSITY

STUDIES ON AMYLASE SYNTHESIS BY THERMOPHILIC *Bacillus* sp. ISOLATED FROM REFUSE DUMP AND ITS ACTION ON STARCHY WASTE MATERIALS.

BY

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B. Sc. (MICROBIOLOGY) ADO-EKITI,

SCP09/10/R/0060

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, OBAFEMI AWOLOWO UNIVERSITY, ILE – IFE, NIGERIA, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN MICROBIOLOGY

2014

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POSTGRADUATE THESIS

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TITLE: Studies on Amylase Synthesis by Thermophilic *Bacillus* sp. Isolated from Refuse Dump and Its Action on Starchy Waste Materials

DEGREE: Master of Science (M. Sc.) Microbiology

YEAR: 2014

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CERTIFICATION

This research project was supervised by me and approved in accordance with the partial fulfilment for the award of Master of Science (M. Sc.) degree in Microbiology, Obafemi Awolowo University, Ile –Ife, Nigeria.

_____ _____

Dr. M. K. Bakare Date

(Supervisor)

_____ _____

Dr. I. O. Adewale Date

(Co-Supervisor)

DEDICATION

With humility and profound gratitude I dedicate this project to my supervisor; Revd. (Dr.) M. K. Bakare for both intellectual grooming and financial support during the course of this work.

I also dedicate this research to my mentor Dr. (Mrs.) A. O. Oluduro for her persistent provisions and guidance before, during and after this work. God will bless your glorious home and career. I gladly dedicate this thesis to Miss Yetunde Helen Abioye for your love, support and unfathomable hospitality throughout the course of my M. Sc. most especially during the practicals.

To my ineffable parents Mr. and Mrs. Samson Ayodele Omoboye and all my family members for prayers and support at all times, I heartily dedicate this thesis.

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ACKNOWLEDGEMENTS

I wish to express my profound gratitude to my supervisor, Revd. (Dr.) M. K. Bakare for his academic, financial and moral support during the course of this programme. Sir, I'm highly favoured to work under your able leadership and supervision. Also, to Dr. I. O. Adewale for his sacrifices and support for me in his laboratory.

My appreciation goes to Dr. (Mrs.) A. O. Oluduro for various provisions, encouragement and financial support during this research and M. Sc. at large. I wish to use this medium to thank Prof. Olu Odeyemi for all the provisions he made available for me in his laboratory throughout my M. Sc. programme, God will bless you Sir.

I thank Dr. D. A. Akinpelu for his fatherly love for me during this programme, always and for his purposeful leadership in the Department both in research and administration. Also, I am highly indebted to all my lecturers and the entire members of staff of the Department of Microbiology, Obafemi Awolowo University, Ile-Ife most especially Dr. B. O. Omafuvbe, Mr. O. Oyedeji and Mrs. T. O. Awotipe. I humbly acknowledge Prof. G. O. Babalola for his contribution to the thesis.

I am grateful to Dr. F. K. Agboola, Dr. (Mrs.) A. Kuku, Dr. R. Okonji and Dr. O. O. Odekanyin for their support during the course of this research.

I appreciate my fiancée Miss Helen Yetunde Abioye for assisting me to achieve success in this research. I am sincerely thankful to Mr. Biola Isawumi, Mr. Aregbesola Oladipupo, Mr. Ayodeji Ajayi, Mr. Osuji Chukwudi, Mrs. Oluwatoyin Aladejana, Mrs. Tope Ojo, Mr. Moses Ikechuckwu, Miss Ololade Ogunsade, Mr. Kazeem Alayande, Mr. Hamzat Oyelade, Mr. Adekunle Bamigbade, Mr. Dare Agunbiade, Miss. Yetunde Ojopagogo, Mr. Aruwajoye Olusanjo, Mr. Ogunkanbi Ademola, Mr. Abioye Oluwatayo, Mrs. Odun Awotorebo, Mr. Ayodele Omotoso, Miss Yewande Ajao, Mr. Tony Ejelonu,

Mr. Oluyemi Odeyemi, Mr. Taiwo Fadare, Mr. Osunde Michael, Abioye Babasanmi and Abioye Sola for their support during this research.

I am grateful to Mr. F. Oyelakin and all the members of Staff of the Biotechnology Centre, Federal University of Agriculture, Abeokuta for the molecular characterization of the bacterium used in this research.

I wish to also use this medium to appreciate my pastors Pastor (Dr.) R. O. Salawu, Pastor (Dr.) Victor Bakare and Rev. (Canon) M. A. Enimola for their prayers and precious encouragements during my study and research.

To crown it all, I acknowledge my parents Mr. and Mrs. Samson Ayodele Omoboye; Mrs. Funmilayo Fajemila, Mr. Olugbenga Omoboye and my other siblings for their unfathomable support.

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ABSTRACT

This study isolated and characterized α-amylase-producing thermophilic bacteria from a refuse dumpsite as well as characterized the partially purified α-amylase produced by the isolated bacteria. This was with a view to obtaining a thermostable α-amylase-producing bacteria capable of digesting raw starches.

Four soil samples were collected at different dumpsites in Ile-Ife, Osun State, Nigeria. Serially diluted inocula were plated on nutrient agar in order to isolate bacteria for their α-amylase activity. The isolated bacteria were identified by morphological and biochemical characterization while the bacteria of interest was identified by molecular analysis using 16S rRNA gene sequencing. The bacterium with the highest α-amylase activity was selected for enzyme production, purification and characterization. The optimal conditions for α-amylase secretion by the bacterium were determined by varying the pH, temperature, percentage soluble starch, nitrogen sources and carbon sources. Sources of raw starches were also varied. The enzyme was partially purified by ion exchange chromatography on CM Sepharose CL-6B. The molecular weight of the enzyme was determined using gel filtration on Sephadex G-100. Kinetic parameters (K_m and V_{max}) of the purified enzyme, effects of temperature, pH, metal ions and ethylenediaminetetra acetic acid (EDTA) were studied.

The isolated and identified bacteria were *Bacillus alvei* (40%) ***Bacillus licheniformis*** (40%) and *Bacillus brevis* (20%) while ***Bacillus*** *licheniformis* RD24 was identified by 16S rRNA gene sequencing. The peak of amylase productivity was at 20 h of incubation (925 μg/ml/min). The optimum pH and temperature for the production of *Bacillus licheniformis* RD24 α-amylase were 7 (with 150 ± 1.33 μg/ml/min) and 45 ^{o}C (with 58 ± 1.66 μg/ml/min enzyme activity) respectively. One percent (1%) starch composition of the enzyme production medium gave highest enzyme activity of 102 ± 5.3 μg/ml/min.

Peptone gave an enzyme activity of 165 ± 8.97 μg/ml/min and yeast extract gave 52.26 ± 2.86 μg/ml/min. Starch gave the highest activity of 33 ± 4.98 Units/ml followed by lactose (32 ± 2.99 Units/ml) and melibiose (27 ± 2.99 μg/ml/min). Of the raw starches, cassava flour gave the highest specific activity of 72 ± 0.07 Units/mg protein, while sorghum starch gave the lowest specific activity 5 ± 1.52 Units/mg protein. The specific activity of the partially purified *Bacillus licheniformis* RD24 α-amylase for starch was 1.634 Units/mg protein with a purification fold of 4.76. The partially purified *Bacillus licheniformis* RD24 α-amylase had a molecular weight of 50 kDa. The V_{max} and K_m of the partially purified *Bacillus licheniformis* RD24 α-amylase with soluble starch as substrate were 4654 ± 108 Units/mg protein and 79.11 ± 1.84 mg/ml respectively. The optimum pH and temperature of partially purified *Bacillus licheniformis* RD24 α-amylase were 8.0 and 70 ^{o}C respectively. Sodium ion had stimulatory effect on the enzyme while Mg^{2+}, Ca^{2+} and Al^{3+} inhibited the enzyme. EDTA inhibited the enzyme at all concentrations.

The study concluded that α-amylase synthesized by ***Bacillus licheniformis*** RD24 had a unique characteristic of thermostability and ability to withstand alkaline pH.

CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

Starch is the most abundant material reservoir in superior vegetable beings destined to nourishment. It is a basic source of chemical energy for life sustenance, which is continuously renewed by sun. Starch production in the earth was estimated to be in the order of 2.0 x 10^{10} tonnes/year, which corresponds to about 80 % of total food production worldwide (Sarikaya *et al.*, 2000). In the most developed tropical countries, where agricultural products like tubers and cereals are abundant, a valuable fraction of starch is yearly lost because of different causes, among which waste and inadequate storage account for (Okolo *et al.*, 1995). Therefore, the conversion of agricultural raw materials into higher added-value products, by enzymatic saccharification, can represent an effective strategy of resources conservation (Okolo *et al.*, 1995). Starch is an abundant carbon source in nature, and α-amylase (1, 4-α-D-glucanohydrolase; EC 3.2.1.1), which hydrolyzes α-1,4-glucosidic linkage in starch-related molecules, is one of several enzymes involved in starch degradation (Verma *et al.*, 2011).

Starch is the most common carbohydrate in the human diet and is contained in many staple foods. The major sources of starch intake worldwide are the cereals (rice, wheat, and maize) and the root vegetables (sweet potatoes and cassava) (Eliasson, 2004). Many other starchy foods are grown, some only in specific climates, including acorns, bananas, barley, breadfruit, colacasia, katakuri, millet, oats, polynesian arrowroot, sago, sorghum, sweet potatoes,

rye, chestnuts and yams, and many kinds of beans, such as favas, lentils, mung beans, peas, and chickpeas (Eliasson, 2004).

1.2 Enzymes and Alpha Amylase

Enzymes are among the most important products acquired for human needs in the areas of industrial, environmental and food biotechnology through microbial sources. Alpha amylase is a hydrolytic enzyme and in recent years, interest in its microbial production has increased dramatically due to its wide spread use in food, textile, baking and detergent industries (Asgher *et al*., 2007). Starch-degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefits (Buzzini and Martini, 2002; Oyeleke and Oduwole, 2009). Amylases are hydrolases that function by the breakdown or hydrolysis of starch into reducing fermentable sugars, mainly maltose and reducing non fermentable or slowly fermentable dextrins (Oyeleke *et al*., 2010). Amylases are hydrolyzing enzyme in function which causes hydrolysis of starch molecules. In biotechnology amylases are of the most important enzymes used (Aneja, 2003; Burhan *et al*., 2003).

Alpha amylases (endo - 1, 4 – α – D - glucan glucanohydrolase, E.C. 3.2.1.1) are extracellular endo-enzymes that randomly cleave the 1, 4 α-linkage between adjacent glucose units in the linear amylose chain and ultimately generate glucose, maltose and maltotriose units. Among various extracellular enzymes, α-amylase ranks first in terms of commercial exploitation (Babu and Satyanarayana, 1993) and accounts for 12 % of the sales volume of the world enzyme market (Baysal *et al.*, 2003). Spectrum of applications of α-amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, they also find applications in bakery, brewery, detergent, textile, paper and distilling industry

(Ramachandran *et al.*, 2004). The enzyme is extensively used in many industries including starch liquefaction, brewing, food, paper, textile and pharmaceuticals (Arikan, 2008).

Alpha amylases (1, 4 α -D-glucan glucohydrolase, E.C. 3.2.1.1) are extra cellular enzymes that break down the internal α-1, 4 linkages in starch to form glucose, maltodextrins and maltose. Industrially, α-amylase plays a vital role in starch liquefaction, brewing and food industries (Akpan *et al.*, 2004; Thippeswamy *et al.*, 2006; Gangadharan *et al.*, 2008; Rajagopalan and Krishnan, 2008; Rasiah and Rehm, 2009). *Bacillus* species such as, *B. subtilis, B. amyloliquefaciens, B. stearothermophilus* and *B. licheniformis* are well known potential producers of α-amylase (Gangadharan *et al.*, 2006; Rasiah and Rehm, 2009).

Alpha-amylase has been obtained from several fungi, yeast, bacteria and actinomycetes; however enzymes from fungi and bacteria sources have dominated applications in industrial sectors (Pandey *et al.*, 2000). Evidences of amylase in yeast, moulds and bacteria have been reported and their properties documented (Buzzini and Martini, 2002; Oyeleke and Oduwole, 2009).

1.3 Industrial Application of Amylases

Food and beverage industries employ β-amylase [EC. 3.2.1.2] to convert starch into maltose solutions (Fogarty and Kelly, 1990). Amylases from various fungal and bacterial species have been studied in a great detail and they have been found to be a very good source for amylases production (Khan and Briscoe, 2011).

Bacteria belonging to the genus *Bacillus* have been widely used for the commercial production of thermostable α-amylase (Kubrak *et al.,* 2010). Studies on bacteria amylase especially in the developing countries have concentrated mainly on *Bacillus* spp probably because of the simple nature and nutritional requirements of this organism (Omemu *et al.*, 2005; Ajayi and Fagade, 2006; Oyeleke and Oduwole, 2009). *Bacillus* species are heterogeneous forms

of organisms and they are very versatile in the adaptability to the environment (Aqeel and Umar, 2010).