©IDOSR PUBLICATIONS
International Digital Organization for Scientific Research ISSN: 2579-0811
IDOSR JOURNAL OF BIOCHEMISTRY, BIOTECHNOLOGY AND ALLIED FIELDS 4(2): 44-63, 2019.

The Glucose yield and increasing starch activity of the native amylase from *Bacillus subtilis* and cloned amylase from transformed *E.coli.*

Amasiorah V.I[1]., Oyeleke S. B[2]. Egwim E.C[2]. and Nna E[3].

[1]Department of Genetics and Genomics and Bioinformatics, National Biotechnology Development Agency (NABDA), Abuja, Nigeria.
[2]Department of Microbiology Federal University of Technology, Minna, Niger State, Nigeria.
[3]Safety Molecular and Pathology Laboratory (SMPL) Enugu, Nigeria.

ABSTRACT

This research paper involved the isolation of amylase gene from pure *Bacillus subtilis* isolated from a cassava waste dumpsite, ligation of the gene onto a plasmid and subsequent insertion of the recombinant plasmid in *Escherichia coli* (*E. coli*) to produce a cloned amylase enzyme. The biochemical tests showed that the isolate was Application Programming Interface (API 50CH) positive and oxidase negative. These results in conjunction with the polymerase chain reaction (PCR) and defined primer sets were used to identify the organism as *Bacillus subtilis*. Courtesy of three methods of DNA extraction, the average concentration of the organism (*Bacillus subtilis*) DNA was found to be 395.58µg while 1159 base pair was exhibited from the 2% gel electrophoresis of the amylase gene. The ligation and transformation processes were achieved using kits procured from Promega Corporation, U. S. A. which consists among other things pGEM®T Easy vector and JM109 High-Efficient competent *Escherichia coli* respectively. The enzyme analysis showed 0.63µg/ml/seconds and 0.66µg/ml/seconds activity for the native and cloned amylase respectively. The work concludes that the amylase gene from *Bacillus subtilis* isolated from a cassava waste dumpsite has been successfully transferred into *E. coli* which produced amylase more useful.

Keywords: Amylase, *Bacillus subtilis, Escherichia coli,* dumpsite and gene.

INTRODUCTION

Enzymes are biological substances found in living organisms in small quantities, able to hasten biochemical reactions, though remained unchanged at the end of the reaction. Enzymes increase the reaction rate without basically commencing it [1]. Enzymes can be sourced in three ways [2], [3], [4], from plants such as the enzyme pappain, from animal such as trypsin found in glands, and microorganisms (fungi and bacteria) via fermentation processes e.g. amylase [5]. The enzymes from microbes are favoured to those from both plants and animal sources since they are relatively inexpensive to manufacture, more convenient, dependable, predictable [6] and above all, they have short generation time. Genetic transformation (biotransformation) is a practice by which cells that are competent such e.g. *Escherichia coli* (*E. coli*) absorb fragments of DNA via their cell walls and getting changed or transformed in the process [7]. Ordinarily, a characteristic cloning exercise may involve the cutting of the plasmid DNA conferred with a specific marker gene e.g. an antibiotic resistance and the gDNA with a particular restriction enzyme called endonuclease at only one position [8]. Subsequently, the target gene is amplified using a

primer set in a process commonly referred to as polymerase chain reaction (PCR). Since the plasmid and the gene were cut with the same endonuclease, they possess opposite though sticky ends that permits them to fasten together [9].

However, modern technique regards the above method as not only wasteful in terms of fund and time but also more suitable for sequencing of the DNA fragment. Hence, the target gene such as amylase gene of *Bacillus subtilis* can be isolated from the gDNA with set of primers [10].

Virtually all amylase enzymes in use by our local industries are imported. In this regard, two hundred million naira is lost to foreign exchange annually via these industries. The use of recombinant DNA (rDNA) technology can generate more amylase than conventional methods yet of the same quality [11]. This research intends to address some of these prevailing challenges in the industrial sector by determining the glucose yield and increasing starch activity of the native amylase from *Bacillus subtilis* and cloned amylase from transformed *E. coli* [12], [13].

Aim and Objectives

To determine the glucose yield and increasing starch activity of the native amylase from *Bacillus subtilis* and cloned amylase from transformed *Escherichia coli.*

The objectives include:

i. To isolate amylase-coding gene from pure *Bacillus subtilis* isolated from the cassava waste dumpsite.
ii. To amplify the gene in vitro, ligate in a plasmid and insert in a competent *E. coli.*
iii. To clone (grow) the transformed *E. coli* with a view to generating amylase enzyme.
iv. To determine the glucose yield and increasing starch activity of the native amylase from *Bacillus subtilis* and cloned amylase from transformed *Escherichia coli.*

MATERIALS AND METHODS

Collection of Sample

The sample used for the isolation of *Bacillus subtilis* was soil. Five hundred grams (500g) of sample soil was collected from a cassava waste dumpsite in Minna, Niger State (Plate I) and taken to the Microbiology and Biotechnology Department of National Institute for Pharmaceutical Research and Development (NIPRD), Idu, Abuja for isolation and analysis. Subsequently, the isolated organism was taken to Safety Molecular and Pathology Laboratory, University of Nigeria Nsukka (UNN), Enugu Campus, Enugu State, for the biotransformation segment of the research work.

Preparation and Inoculation

The preparation of the media was carried out as described by [14]. Five grams (5g) of the soil sample was placed in a flask containing 45 ml of sterilised Ringer's solution and shaken to formulate a stock mix. The stock mix was placed onto a water bath maintained at 90°C for 1 hour to encourage the formation of spores as well as eliminating other unwanted microorganisms in the sample. The sample was serially diluted in six test tubes (from $10^{-1}$to 10^{-6}) in a Biohazard safety chamber (BSC) and the 10^{-6} dilution was used afterwards. The serial dilutions was achieved by adding one mililitre (1 ml) of the stock mix serially to 9 ml of Ringer's solution, placed in each of the six test tubes arranged on a test tube rack. The nutrient agar medium (100 ml) was prepared in agreement with the instructions of the manufacturers. The 100ml nutrient agar was supplemented with one gram of soluble starch in a conical flask to yield 1% starch nutrient agar. The medium was heated over an electric heater to enable the agar dissolve completely. With the aid of a syringe, 19 ml of the medium was dispensed in five slant bottles and autoclaved at 121°C for 15 minutes. Earlier on, the glass Petri dishes were washed, autoclaved at 121°C for 15 minutes and dried in a hot chamber oven at 180°C for one hour. The 19 ml agar-medium in the slant bottles was allowed to cool before inoculation with 1ml of

the diluted soil sample from the 10^{-6} dilution. The already cooled inoculated media (though not gelled) in the slant bottles were emptied into the sterile glass Petri dish and allowed to cool further in the BSC. The medium with the inoculated soil sample was incubated at 36.8°C in the Memmert incubator along with the control. The control consists of 1% starch nutrient agar only without the soil sample. Growth occurred after 24 hours.

Screening and Identification

The isolate was morphologically and biochemically characterized and identified up to genus level in accordance with the provisions of Determinative Manual of Bacteriology (Bergey's): identification flow chart (citation needed). The isolate was screened by sub culturing on fresh media (streak culture method); from 1% starch nutrient agar to another, 1% starch nutrient agar to 6.5% NaCl agar, 1% starch nutrient agar to Simmon citrate agar (SCA). Three plates were made for each medium including the control. From the Gram's stain result further inter media culture inoculation was made by streaking on the three media with the aid of a sterilised wire loop followed by incubation at 36.8°C. The morphology was reconciled by comparing the physical appearance with that of American Society of Microbiology (ASM) and subsequently a smear was made and viewed under a light microscope for Gram's stain. Further sub culture was made from the colony where positive result was obtained during the Gram's stain. Finally, the following biochemical tests; Gram stain, spore staining, starch hydrolysis, catalase, citrate utilization, 6.5% NaCl bacterial culture medium growth and incubation at 55°C were performed.

Gram Stain

Procedure: A smear of the isolate was made by placing a drop of normal saline on the slide. Then, some of the sample was collected aseptically with the aid of the wire loop, mixed with the normal saline and fixed on the burner. It was kept for about 30minutes to cool and dry before staining with crystal violet solution (the primary dye) and kept for 60 seconds. It was washed with tap water making sure that all the excess water was drained off to avoid diluting the mordant. Subsequently, it was treated with Gram's iodine solution for 30 seconds and gently washed with tap water. It was decolourised with alcohol, which was added drop wise on the tilted slide until all free colours (blue) were removed. Finally, the stuff was washed by gently dropping tap water on the slide. Cautiously, the slide was flooded with safranin (counter stain) for 60 seconds. The smear was viewed under a light microscope in an oil immersion. The Gram stain was positive with purple colour and the cells rod-shaped.

Spore Stain

Procedure: The spore stain was carried out according to Wirtz's method (modified) as reported by [14]. One drop of normal saline was placed on a clean slide. The culture (over 24 hours) was picked aseptically from the petri dish with the aid of a wire loop, mixed with normal saline and fixed. The smear was flooded with Malachite green and brought to steaming. It was allowed to remain for 3 minutes, washed with tap water before applying safranin solution. The stain was kept on the bench for 30 seconds, washed with water, dried and examined under oil immersion. The stain was positive with light green colour.

Starch Hydrolysis

Procedure: Two plates of 1% starch nutrient agar medium were streaked with the isolated organism. After 24 hours, they were flooded with iodine and kept in the Biohazard safety chamber (BSC) for one hour. The iodine indicated a blue black colour with starch in the areas not streaked with the organism while the areas around the organism showed clear zones indicating that the starch may have been broken down by the organism.

Catalase Test

Procedure: One drop of three percent hydrogen peroxide (3% H_2O_2) was positioned on a glass slide. A little quantity of growth was collected from the culture medium with a sterilised wire

loop and mixed with the drop of H_2O_2. It was allowed to stand for a few seconds. Evolution of gas with burbling and frothing was observed showing catalase positive.

Citrate Utilization Test

Procedure: Simmon citrate agar (SCA) medium was aseptically prepared in a petri dish according to the manufacturer's instruction. Three (3) plates containing 19 ml of SCA were made with one serving as a control. The medium was streaked with the isolate and incubated at 36.8° for 24 hours. A colour change from green to blue was seen. The control was not inoculated with the isolate hence the colour remained green.

Biotransformation

Culture Revival/Sub Culture: With the aid of a wire loop, a little quantity of the isolate (*Bacillus subtilis*) was collected from the slant and streaked onto the nutrient agar medium. The culture was incubated at 36.6°C for 24 hours in a Kingjoe laboratory incubator model no KJ-9022A. Growth occurred after 24 hours.

Stock Culture: With the aid of a sterilised wire loop, some of the organism was collected from the slant, placed in a-2 ml Eppendorf tube, vortexed to emulsify and homogenize. 150 µl of glycerol and 850 µl of peptone were added. The stock was kept in the freezer at -20°C. It could stay in there for up to 20 years for use if there is no power failure.

Stock Resuscitation: Some culture of the *Bacillus subtilis* sample was collected from the stock culture with the aid of a sterilised wire loop, streaked on a nutrient agar and incubated for 24hrs. Growth was observed. Then the fresh culture was used for the confirmatory tests.

Application Programming Interface (API) 50CH B Test

Procedure: An API 50 CHB kit (BioMérieux, UK) was also used for the identification of this organism. Rapid growing *Bacillus subtilis* (twenty four hours) culture was harvested by collecting about ten colonies using a sterile wire loop. The sample was emulsified in 10 ml API 50 CHB/E medium to obtain a suspension and homogenised by vortexing for few seconds. The incubation box (consisting of a tray and a lid) was prepared by filling the honey comb wells with distilled water. Then the tubes (in five strips of ten each) were placed on the incubation box already filled with distilled water. Two hundred microlitre of the API emulsified suspension was inoculated into each of the fifty tubes. The tray was incubated at 24°C for 24 hours after covering with the lid. There was no colour changes in the first tube (control) and represented as negative (-). Others showed remarkable colour changes and marked as positive (+) if it tallies with the colour in the manufacturer's colour interpretation manual or (-) if it does not tally.

Extraction of Deoxyribonucleic Acid (DNA) of the *Bacillus subtilis*

Sample Preparation: Two hundred microlitres (200 µl) of the Tris-EDTA (TE) buffer was placed in an Eppendorf tube. Then a large quantity of *Bacillus subtilis* colonies (about 15 colonies) were collected and emulsified with the TE buffer. The mixture was incubated at 95°C in the Dri-block for 10 minutes and vortexed at 2,500 rpm for 15 seconds. Then the following methods were used for the DNA extraction on different occasion.

Genotek Method (modified) of Genomic DNA (gDNA) Extraction

Procedure: The DNA extraction kit used was procured from Genotek Incorporation Kanata, Ontario, Canada. Twenty microlitres (20 µl) of proteinase K was placed in a-2 ml Eppendorf tube. Two hundred microlitres (200 µl) of the *Bacillus subtilis* isolate was added as well as 200 µl of AL (ATL) buffer. The mixture was vortexed for about 10 seconds, emulsified and incubated in the heating block at 56°C for 10 minutes (mins). Twenty microlitres (20 µl) of PT-L2P was added and mixed by vortexing for about 15 seconds (secs). The resultant concoction was left on ice for ten minutes and spinned in the centrifuge at

room temperature for five minutes at 13, 000 revolutions per minute (rpm). With the aid of a 200 µl pipette, the upper liquid layer or supernatant was carefully moved into a clean 2 ml tube and the pellet containing the impurities was discarded. Six hundred microlitres (600 µl) of absolute ethanol (molecular grade) was added at room temperature to the supernatant and gently mixed by inverting 10 times. The mixture was allowed to stand for 10 minutes to allow the DNA to precipitate fully. With the centrifuge properly balanced, the mixture or concoction was spinned at room temperature for 3 minutes at 13,000 rpm. Then the upper layer or supernatant was discarded with a 200 µl pipette without tampering with the DNA. Two hundred and fifty microlitres (250 µl) of 70% ethanol was added and incubated for one min at room temperature. The mixture was spinned for 5 minutes at 13,000 rpm and the ethanol removed completely without meddling with the pellet. It was dried in the heating block at 56°C for 3 minutes, making sure that the pellet was dry before proceeding to the next step. Sixty microlitre (60 µl) of AE buffer (same as TE or BE buffer) was introduced in order to dissolve the DNA and allowed to stand at room temperature for 5 minutes with gentle pipette mixing or vortexing for at least 5 seconds. The mixture was spinned for 2 minutes at 8,000 rpm and the upper liquid layer (supernatant- or the DNA) placed in a 1.5 millilitre (1.5 ml) for proper storage and testing. The quality of the DNA was determined by adding 5 µl of the genomic DNA to 95 µl of AE or TE buffer to check the quantity and quality/ purity. The DNA suspension was read at A260/280 in the Eppendorf Biophotometer plus. The concentration and the ratio were recorded in the work sheet.

NucleoSpin Method of gDNA Extraction

Procedure: The DNA extraction kit used was procured from Clontech Laboratories, Incorporation, Mountain View, CA94043 USA. Lysing of the *Bacillus subtilis* cells: Some *Bacillus subtilis* (as much cells as possible) were collected in 1.5 Eppendorf tube and mixed or vortexed with 250 µl of TE buffer until it became turbid. The mixture was heated at 70°C for 10 minutes in the Dri block and subsequently DNA extraction process was performed. Then 25 µl of proteinase K and 200 µl buffer B3 were added to the sample and the concoction mixed thoroughly with vortex machine for 20 seconds so as to obtain a DNA of high yield and purity. The sample was incubated at 70°C for 15 minutes. Adjusting of DNA binding conditions: The sample was supplemented with two hundred and ten microlitre (210 µl) of absolute ethanol (96-100%) and vortexed again.

DNA binding: One nucleospin column was positioned in a 1.5 ml Eppendorf tube (collection tube). The sample mixture was loaded in it and then centrifuged for one minute at 11,000 rpm and the collection tube discarded together with the air flow.

Washing the silica membrane: First wash: The nucleospin column was positioned in a fresh 2 ml collection tube and 500 µl of Buffer BW (wash buffer) added. The mixture was centrifuged for one min at 11,000 rpm. The collection tube was discarded with the flow through. Second wash: The nucleospin column was positioned in a fresh 2-ml collection tube. Then six hundred microlitre Buffer B5 was added.

The mixture was spinned at 11,000 rpm for one min. The collection tube was re-used while the flow through discarded. Dry silica membrane: The nucleospin column was returned onto the collection tube and spinned at 11,000 rpm for 1 min eliminating the remaining ethanol.

Elute highly pure DNA: The nucleospin column was positioned in a 1.5 ml micro centrifuge tube. Then one hundred microlitre of 70°C BE Buffer (preheated) was introduced. The buffer was dispensed straight onto the silica membrane, allowed to stand at room temperature for one minute and spinned at 11,000 rpm for one minute. Finally, the absorbance was read with the aid of a Biophotometer: 5µl of sample plus 95 µl

of distilled water. The initialisation was done with Buffer AE.

TRIzol Method of gDNA Extraction

Procedure: The DNA extraction kit used was procured from Life Technologies – Invitrogen Grand Island, NY 14072, USA.

Phase Separation

Two hundred microlitre (200 µl) of the emulsified organism (as in sample preparation) was moved into a-2 ml tube. 750 microlitre of Trizol LS reagent was added and mixed by pipetting repeatedly. The mixture was allowed to stand for 5 minutes at room temperature (26°C). Then, two hundred microlitre of chloroform was introduced, the tube capped and vortexed for 5 seconds. It was allowed to stand for 15 minutes at room temperature and spinned at 12,000 rpm for 15 minutes thus obtaining 3 layers; aqueous layer RNA, interphase layer protein/DNA and organic DNA/protein.

DNA Precipitation: The upper most layers that is the aqueous phase containing the RNA was carefully removed with the aid of a micropipette, then the interphase/organic phase was supplemented with 300 µl of 100% ethanol and vortexed gently. Subsequently, the mixture was allowed to stand at 26°C for 3 minutes and spinned at 12,000 rpm for 5 minutes at room temperature.

DNA Wash: The ethanol supernatant which contains protein was removed as waste. The

DNA pellet was washed two times in 0.1M sodium citrate solution by addition of one millilitre of sodium citrate solution. The concoction was vortexed gently and incubated in the wash solution for 30 minutes at 26°C. It was spinned at 12000 rpm for 5 minutes at 26°C. The process was performed again. Then the DNA was re- suspended in 1 millilitre of 75% ethanol (molecular grade) after washes, incubated at 15-30°C for 20 minutes (periodic mixing) and spinned at 12,000 rpm for 5 minutes at room temperature (26°C). The supernatant was removed using two hundred microlitre pipette and discarded into the waste container. The pellet was allowed to dry by placing on the Dri block for 5 minutes.

Redissolving the DNA: The pellet was dissolved in 50 µl of TE buffer and placed on the Dri block at 55°C for 10 minutes to increase solubility. The sample solution was centrifuged at 12,000 rpm for 10 minutes to remove the insoluble materials/insoluble fragments such as membrane and protein it may contain. The supernatant containing the DNA was transferred into a new 1.5 ml Eppendorf tube, labelled and stored in the refrigerator at 4°C for testing.

Quick Preparation of *Bacillus subtilis* for PCR

Large number of colonies of *Bacillus subtilis* were placed in a tube (Eppendorf) containing two hundred microlitre of TE buffer and emulsified. The mixture was heated in a dry block (Dri block EB 3A) at 95°C and incubated at room temperature for 10 minutes. Finally, stuff was vortexed at 2,500 rpm for 5 minutes to obtain the genomic DNA.

PCR of *Bacillus subtilis* Genomic DNA (gDNA), Authentication and Identification of the Organism

Procedure: Ten microlitre (10 µ1) of Taq PCR master mix containing 100µM Taq polymerase and dNTP was placed in a PCR tube**.** Seven microlitre (7µ1) of diluted primer mix of concentration µM100 Bsub5f (Bsub5F; AAGTCGAGCGGACAGATGG) and µM100 Bsub3r (Bsub3R; CCAGTTTCCAATGACCCTCCCC)-diluted to 10µM, was added (from Integrated DNA Technologies, Belgium). Then 3 µl f the gDNA called the template DNA was introduced to make up a total volume of twenty microlitres. Earlier on, the master mix and primer mix were prepared as stated below:- The primer mix was prepared as follows: - 683 µl of µM100 Tris EDTA (TE) buffer was moved into a tube (Eppendorf) containing µM100 of the forward primer (Bsub5f). Also, 707 µl of µM100 TE was mixed with the reverse primer (Bsub3r) of molar µM100 in an Eppendorf tube. (These volumes 683 and 707µl can be obtained by

multiplying the primer yield by 10). The mixtures were vortexed for about 5 seconds to homogenize. 3.5 µl was collected from each of the Eppendorf tube to obtain 7 µl. At the same time the DNA polymerase enzyme was thawed by keeping in the heating block at 37 °C in Dri block machine for about 5 minutes. The DNA polymerase was added to dNTP as stated by the manufacturer to produce the master mix. The master mix, primer mix and template DNA were added as reaction mixture in one PCR tube as stated above, labelled and placed along with molecular water of the same quantity to act as control labelled as "1" and "2" respectively. Then the two tubes labelled as "1" and "2" were ran in the Thermocycler following the thermal profile below

The thermal profile was fine-tuned as follows-

i. Initial denaturation cycle at 94°C for 5 minutes
ii. Denaturation cycle at 94°C for 5 minutes
iii. Annealing cycle at 55°C for 5 minutes
iv. Initial extension (elongation) at 72°C for 1 minute or 30 seconds
v. Final extension/elongation of the DNA at 72°C for 5minutes
vi. Cooling for 4 minutes

PCR of *Bacillus subtilis* Amylase Gene, Authentication and Identification

Procedure: Ten microlitre (10µ1) of Taq PCR master mix containing the Taq polymerase and dNTP was placed in a PCR tube**.** Seven microlitre (7µ1) of diluted primer mix (BS168F, forward) of sequence; CTAATTCCATGGGGATGTTTGC AAAACGATTCA and BS168R (reverse) of sequence; GAACGCGGATCCTCAATGGGGAAGAGAA was added (from Integrated DNA Technologies, Belgium). Then 3 µl of the gDNA was introduced to make up a total volume of twenty microlitres. Master mix (supplied by the manufacturers) was thawed by keeping on the bench at room temperature. So also the primer mix which consists of the forward and reverse primers was mixed in the laboratory. The master mix, primer mix and template DNA were added as reaction mixture in one PCR tube as stated above, labelled and placed along with molecular water of the same quantity to act as control labelled as "1" and "2" respectively. Then the two tubes labelled as "1" and "2" were ran in the Thermocycler following the thermal profile below

- Initial denaturation cycle at 94°C for five minutes
- Final denaturation cycle at 94°C for thirty seconds
- Annealing cycle at 55°C for thirty seconds
- Initial elongation (extension) at 72°C for thirty seconds
- Final elongation (extension) of the DNA at 72°C for five minutes
- Cooling for 4 minutes

The Cloning of the Competent *Escherichia coli* Ligation of the Amylase Gene onto the Vector; pGEM-T Easy

The plasmid vector was centrifuged at 24,000 rpm for 30 seconds to sediment the content. The ligation buffer (2X rapid) was vortexed briefly and kept at 37°C for one minute in order to thaw. The reaction set up was prepared in a tube (0.5ml) that has no affinity for DNA in the following way:- Five microlitre (5 µl) of the buffer was placed in the tube. One microlitre of the vector was added and 2 µl of the PCR product were added. One microlitre of the Ligase enzyme (T4 DNA) was added and one microlitre of the molecular water was added to make up a volume of 10 µl. It was emulsified properly with a bigger pipette (20 µl) and incubated overnight to produce the ligation reaction.

Enzyme assay Amylolysis of the Native and Cloned Amylase.

Procedure: One millilitre each of the *Bacillus subtilis* and the transformed *E. coli*, (15 colonies in 10 ml of molecular water) were aseptically inoculated in two separate plates containing 1% starch nutrient agar and 2 mm circular hole. The agar plates were kept to stand for 48 hours at 37°C. The culture was flooded with Lugol's iodine solution to determine the amylolysis.

Extraction and Recovery of the Native and Cloned Amylase (Crude Amylase)

Procedure: With the aid of a sterile wire loop, ten colonies each of the organisms (*Bacillus subtilis* and Transformed *E. coli*) were collected from the 24 hour culture, placed in two separate 250ml-Erlenmeyer flasks containing 1% starch broth and allowed to stand for 24 hours at 37°C. The flasks were positioned in a shaker incubator set at 120 rpm at 30°C. The extracellular enzyme solutions were obtained by centrifugation at 5000 rpm for 20 minutes using a high speed centrifuge. The supernatant obtained was collected and tested as protein and crude enzyme.

Partial Purification of the Native and Cloned Amylase

Procedure: The two organisms were propagated at 37°C for 24 hours in two separate 250ml-Erlenmeyer flasks containing 1% starch broth. The flasks were placed in a shaker incubator, set at 30°C and 120 rpm. Then, the mixture was spinned for 15 minutes at 10,000 rpm and the supernatant gathered as the foundation of crude amylase. Partial purification involves the addition of a solution of sixty five percent (w/v) sodium sulphate to the spelt out supernatant, spinning at 10,000 rpm and suspension of the pellet in 0.005 M Na_2HPO4 (pH 6.0). The partially purified amylase was used for further analysis of the enzyme.

Demonstration of Activity of the Native and Cloned Amylase using Glucose Oxidase Method

Procedure: One millilitre of one percent soluble starch in citrate phosphate buffer and pH of 6.4 was placed in a test tube. Zero point five millilitre of culture extract "enzyme" was pipetted into the test tubes. The mixture was kept for thirty minutes at 40°C in a water bath. A blank consisting of one millilitre of soluble starch in citrate phosphate buffer with 6.4 pH and 0.5 ml of molecular water was also incubated in the water bath at the same temperature and time. The glucose yield was by determined by means of 3, 5-Dinitrosalicyclic acid (DNSA) method of [1]. The reaction was terminated by introducing one millilitre of DNSA reagent in each test tube, immersed the tubes in a boiling water bath for 5 minutes and allowed to cool.

Then the absorbance was read as follows:- Five hundred microlitres (500 µl) of Glucose oxidase working reagent was placed in test tubes labelled reagent blank, sample blank, crude enzyme, partially purified enzyme and standard. Then 10 µl each of the substances was added accordingly to its labelled test tube. However, in the reagent blank 10 µl of molecular water was used. The test tubes containing the mixtures were kept to stand for twenty five minutes at room temperature or for ten minutes at 37°C in a heating block (Dri block). The reagent blank was used to initialize the machine before reading the absorbance starting from sample blank to the test samples.

The absorbance was measured at 500 nm wave length with Eppendorf Biophotometer plus (AG 22331) and DPU-414 Thermal printer. The optical density for the enzymatic activity was calculated from the glucose yield after incubation as shown:

$$\frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times \text{concentration of standard}$$

The enzyme activity is defined as glucose yield mg/ml divided by incubation period (in seconds).

Effect of Increasing Starch on the Activity of the Native and Cloned Amylase

Procedure: One millilitre (1 ml) of 1% soluble starch in citrate phosphate buffer having a pH of 6.4 was placed in a test tube. Zero point five millilitres (0.5 ml) of culture extract "enzyme" were pipetted into the test tubes. The reaction mixture was kept to stand for 30 minutes in a water bath at 40°C. A blank consisting of 1 ml of distilled water was also incubated in the water bath at the same temperature and time with the rest of the test tubes which were serially

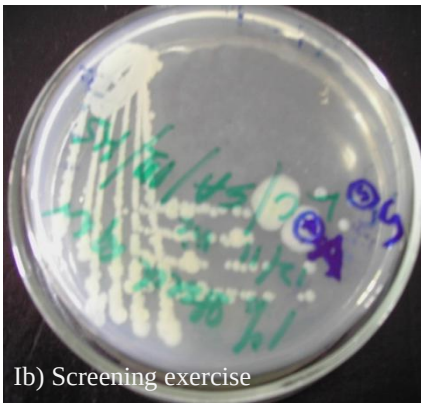
diluted with distilled water at 0.1 ml intervals to obtain equal volumes with one another.

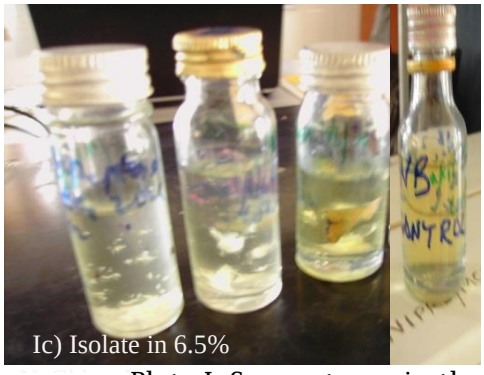
The glucose yield was determined by 3, 5-Dinitro salicyclic acid (DNSA) technique. The reaction was terminated by introducing one millilitre of DNSA reagent in each test tube. The test tubes were placed in a boiling water bath for 5 minutes and then incubated at room temperature to cool. The absorbance for all the test tubes was measured at 500 nm with Biophotometer as above. Subsequently the glucose yield was determined as described above.

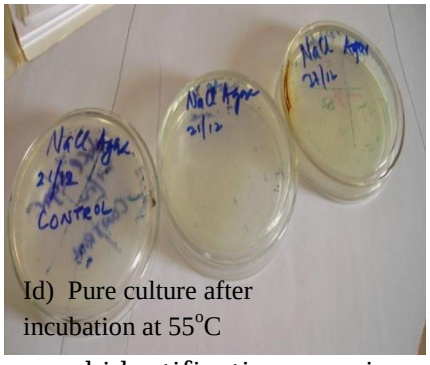
RESULTS AND DISCUSSIONS

After extensive screening and identification exercise (Plate I), the pure isolate obtained was subjected to various tests.

Ia) Initial culture at sixth dilution


Ib) Screening exercise


Ic) Isolate in 6.5%



Id) Pure culture after incubation at 55^{o}C

- Plate I: Some stages in the screening and identification exercise

The isolate was morphologically and biochemically characterised. The result of the biochemical tests revealed that the isolate was catalase positive, starch hydrolyser, citrate utiliser and spore formers (Plates II, III and IV respectively below).

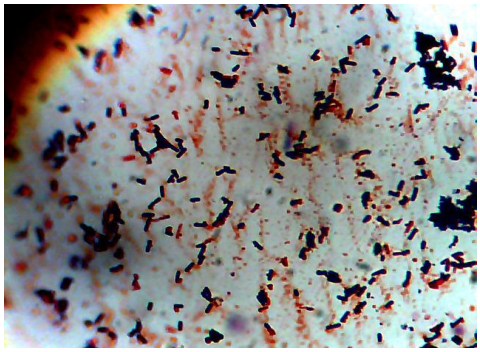


Plate II: Gram positive

Plate III: Catalase positive

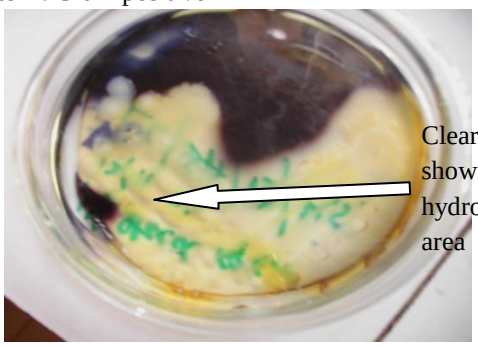
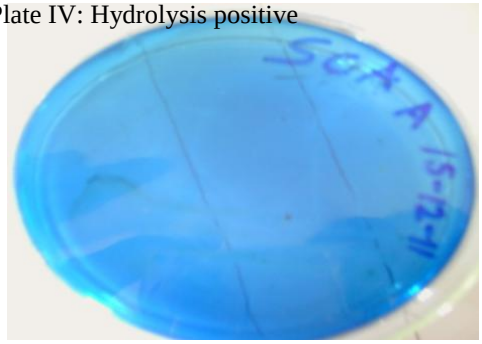


Plate IV: Hydrolysis positive



Equally, [12] in their work made the same observation that the isolate was Gram-positive, spore former and grow in the presence of oxygen hence, aerobic spore formers. The catalase result agrees with that of Vidhya1 & Thatheyus (2013) as published in their research work. The amylolytic test for the native enzyme in *Bacillus subtilis* revealed remarkable halo zones showing the presence of amylase; 1.74cm diameters (24hrs), 2.58cm diameter (48hrs) and 3.02cm diameter (72hrs) with a mean value of 2.45cm (Plate VI). Similar amylolytic result was obtained by [2] who recorded 2.3cm zone of inhibition for similar test.

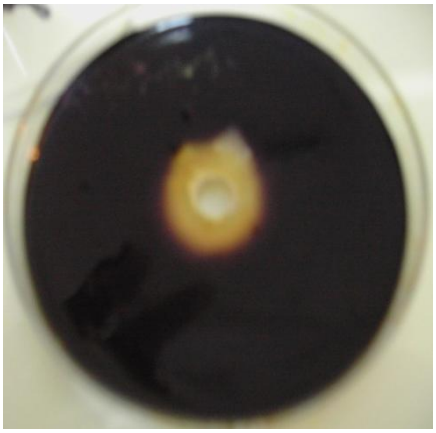


Plate V: The amylolysis of the native amylase in *Bacillus subtilis*

Confirmatory results showed that the isolate was oxidase negative thus concurring with the observation of Vidhya1 & Thatheyus (2013) who reported the following results: Gram's stain, positive; Simmon citrate, positive; oxidase, negative and catalase, positive. The Application Programming Interface (API) 50 CH result-a biochemistry/bioinformatics test specifically designed for *Bacillus subtilis* showed positive for the organism. A similar finding on API 50CH was reported by [7] whose result showed positive for *Bacillus subtilis.* Besides, the API 50CH results indicated series of positive and negative outcomes due to colour changes prompted by the various fermenting sugars in the tubes (Table 1 and Plate VII).

Table 1: Application Programming Interface (API) 50 CH Test Showing Various Active Ingredients in the Various Tubes and their Corresponding Results after Fermentation by *Bacillus subtilis*

Tube	Test	Active ingredients	Result
0		Control (0)	-
1	GLY	Glycerol	+
2	ERY	Erythritol	-
3	DARA	D-Arabinose	-
4	LARA	L-Arabinose	-
5	RIB	D-Ribose	-
6	DXYL	D-Xylose	-
7	LXYL	L-Xylose	-
8	ADO	D-Adonitol	-
9	MDX	Methyl-βD-Xylopyranoside	-
10	GAL	D-Galactose	-
11	GLU	D-Glucose	+
12	FRU	D-Fructose	+
13	MNE	D-Mannose	+
14	SBE	L-Sorbose	-
15	RHA	L-Rhamnose	-
16	DUL	Dulcitol	-
17	INO	Inositol	+
18	MAN	D-Mannitol	+
19	SOR	D-Sorbitol	+
20	MDM	Methyl-αD-Mannopyranoside	-
21	MDG	Methyl-αD-Glucopyranoside	+
22	NAG	N-AcetylGlucosamine	-
23	AMY	Amygdalin	+
24	ARB	Arbutin	+
25	ESC	Esculin ferric citrate	+
26	SAL	Salicin	+
27	CEL	D-Celiobose	+
28	MAL	D-Maltose	+
29	LAC	D-Lactose (bovine origin)	-
30	MEL	D-Melibiose	+
31	SAC	D-Saccharose (sucrose)	+
32	TRE	D-Trehalose	+
33	INU	Inulin	+
34	MLZ	D-Melezitose	-
35	RAF	D-Rafinose	+
36	AMD	Amidon (starch)	+
37	GLYG	Glycogen	+
38	XLT	Xylitol	-
39	GEN	Gentiobiose	-
40	TUR	D-Turanose	+
41	LYX	D-Lyxose	-
42	TAG	D-Tagatose	-
43	DFUC	D-Fucose	-
44	LFUC	L-Fucose	-
45	DARL	D-Arabitol	-
46	LARL	L-Arabitol	-
47	GNT	Potassium gluconate	-
48	2KG	Potassium-2-ketogluconate	-
49	5KG	Potassium-5-ketogluconate	-

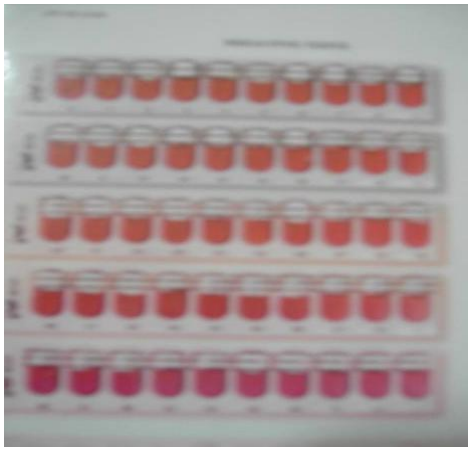


Plate VI: Original colour before API 50CH test

Plate VII: Colour change after API 50CH test

The gDNA for the *Bacillus subtilis* was extracted using 3 different methods of DNA extraction; Genotek (527.85µg), Nucleospin (370.50µg) and TRIzol (288.40µg) thus recording an average concentration of 395.58µg. Further confirmatory results were obtained during this molecular analysis of the isolate with specific primer sets and polymerase chain reaction (PCR) and electrophoresis. The primers consist of forward (Bsub5F) and reverse (Bsub5R) in the form: Bsub5F; AAGTCGAGCGGACAGATGG and Bsub3R; CCAGTTTCCAATGACCCTCCCC. The PCR product was authenticated by running on 2% agarose gel electrophoresis (Plates VIII and IX).

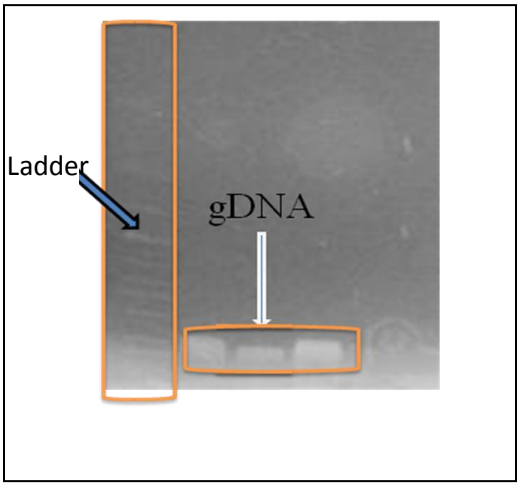


Plate VIII: The 2% agarose gel electrophoretic result showing the gDNA of the *Bacillus subtilis*

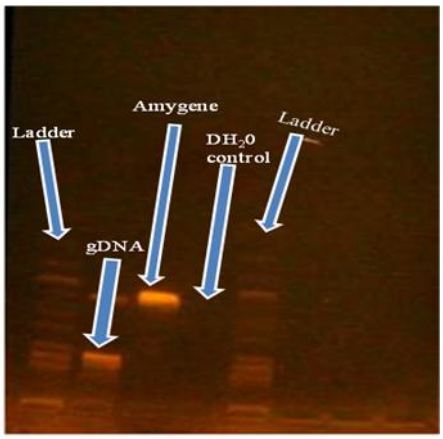


Plate IX: The 2% agarose gel electrophoretic result of the gDNA and amylase gene

In addition to that, further molecular analysis and confirmatory studies was performed on the target gene (the amylase gene) courtesy of PCR with the following set of primers from the 16S rRNA genes; BS168F (forward): CTAATTCCATGGGGATGTTTGC AAAACGATTCA and BS168R (reverse): GAACGCGGATCCTCAATGGGGAAGAGAA. Consequently, the result of the electrophoretic analysis was 1159 base pair and was used to authenticate the gene. Each of the above PCR consists of 35 cycles of initial denaturation, final denaturation, annealing, initial elongation, final elongation. The cycles and the standard conditions (thermal profiles) declared above were attained after several repeats and changes in annealing temperature. The profiles yielded satisfactory PCR products suggesting that accurate thermal profiles have been employed. The ligation process was attained by carefully following the laid down guidelines provided by the kit manufacturers, Promega Corporation, U.S.A. The plasmid vector used was pGEM®-T Easy and the PCR product (the gene of amylase) was successfully inserted and ligated into it to generate a recombinant plasmid carrying an amylase gene. The successful recombination of amylase gene into the plasmid DNA prompted the transformation of *E.coli* by inserting the recombinant plasmid into it. The *E.coli* strain used for this transformation was JM109 High- Efficient competent *E. coli* cell that came with the kit. After 24 hours of incubation period, growth occurred and white colonies were seen (Plate X).

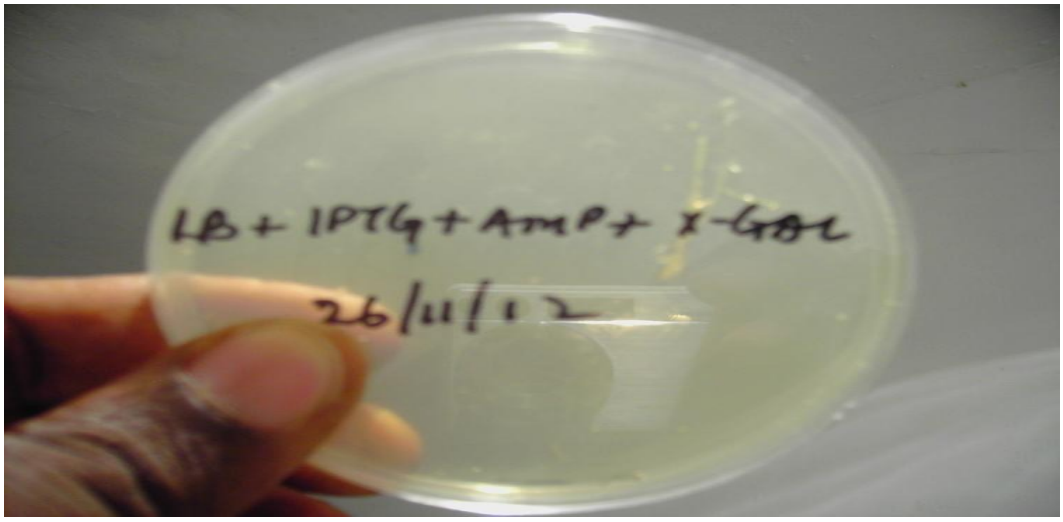


Plate X: Transformed *E. coli* shown in white colonies

The growth was not so luxuriant and could be due to high level of salt during the preparation of the media such as super optimal broth catabolic (SOC) medium and lysogenic broth (LB). However, the growth in 1% starch nutrient broth was very rich judging from the observed turbidity of the medium. The introduction of amylase gene into the *E.coli* strain thereafter rendered it capable of amylase activity which was earliest demonstrated by Bennett *et al.*, 1985. The transformed colonies expressed the amylase gene and produced amylase enzyme. The amylolytic result showed halo zones on the 1% starch nutrient agar. The cloned amylase from the transformed *E.coli* had halo zones of 1.8cm in 24hrs and 3.2 in 48hrs from the amylolysis showing an average of 2.5cm as shown below (Plate XI).

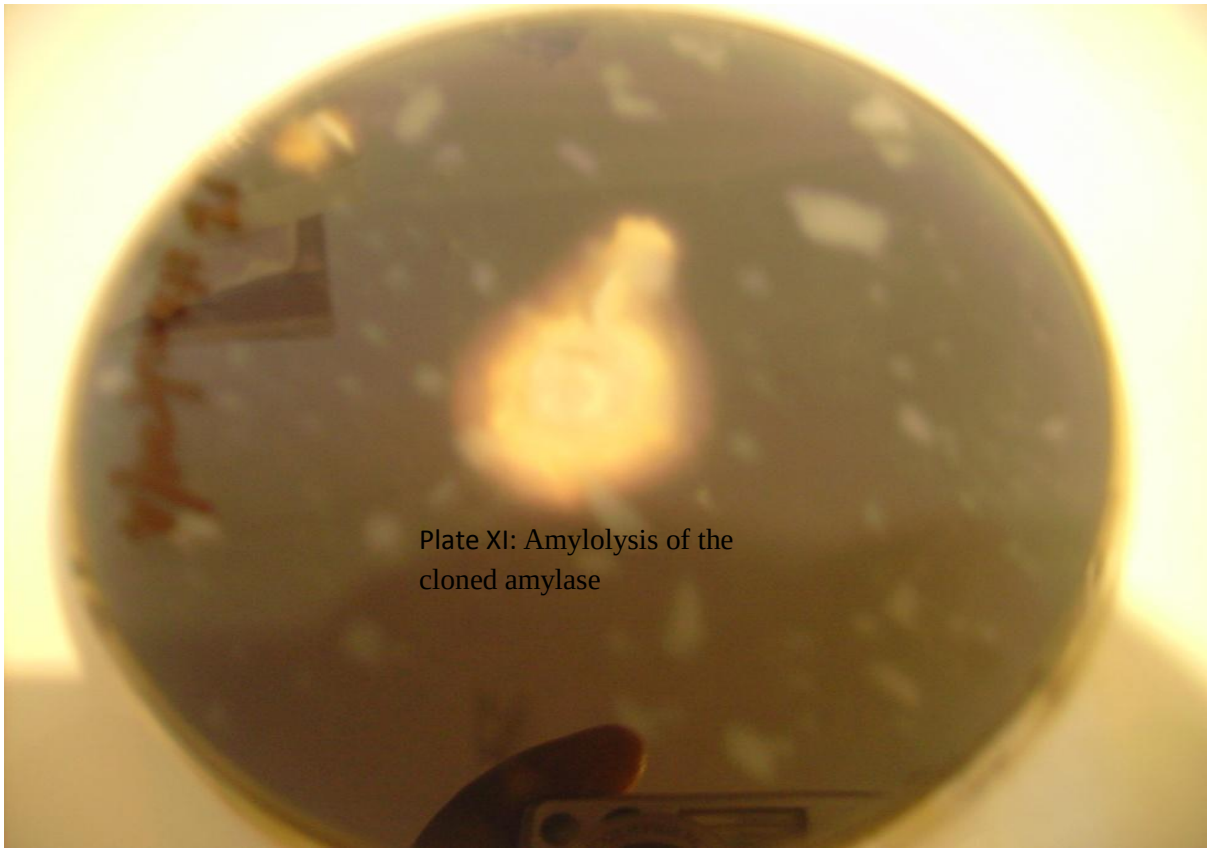


Plate XI: Amylolysis of the cloned amylase

Table 2: Glucose Yield of the Native Amylase from *Bacillus subtilis* using Glucose Oxidase Method

Test tube No	Sample blank (T_0)	Reagent blank (T_r)	Crude (C)	Partially Purified (P)	Standard (T_s)
T_s (O.D)	-	-	-	-	0.71
T_s (O.D)	-	-	-	-	0.69
T_r (O.D)	-	0	-	-	-
T_r (O.D)	-	0	-	-	-
T_0 (O.D)	0.04	-	-	-	-
T_0 (O.D)	0.041	-	-	-	-
P_1 (O.D)	-	-	-	0.857	-
P_2 (O.D)	-	-	-	0.897	-
C_1 (O.D)	-	-	0.601	-	-
C_2 (O.D)	-	-	0.685	-	-
Total (O.D)	0.081	0	1.286	1.754	1.40
Average (O.D)	0.041	0	0.643	0.877	0.70
Yield (mg/dl)	5.59	0	87.79	119.69	95.5
Yield difference	-	-	82.2	114.1	-
Yield (mg/ml)	-	-	0.82	1.14	-

Yield difference = yield for crude or purified – yield (blank)
Glucose yield for purified = 1.14 mg/ml
Activity (i.e. glucose yield/30x60 seconds) = 0.63 µg/ml/second
Specific activity (enzyme activity/ total protein) =0.63/7.17mg/ml
= 0.088 µg/ml/second/mg protein

Table 3: Glucose Yield of the Cloned Amylase from Transformed *E.coli* by Glucose Oxidase Method

Test tube No	Sample blank	Reagent blank)	Crude	Partially Purified	Standard
T_s (Op.D)	-	-	-	-	0.714
T_s (O.D)	-	-	-	-	0.71
T_r (O.D)	-	0	-	-	-
T_r (O.D)	-	0	-	-	-
T_0 (O.D)	0.05	-	-	-	-
T_0 (O.D)	0.04	-	-	-	-
P_1 (O.D)	-	-	-	0.891	-
P_2 (O.D)	-	-	-	0.94	-
C_1 (O.D)	-	-	0.652	-	-
C_2 (O.D)	-	-	0.71	-	-
Total (O.D)	0.09	0	1.362	1.831	1.42
Average (O.D)	0.045	0	0.681	0.916	0.71
Yield (mg/dl)	5.91	0	92.91	124.97	95.5
Yield difference	-	-	87	119.06	-
Yield (mg/ml)	-	-	0.87	1.19	-

- Yield difference = yield (for crude or purified) – yield for blank
- Glucose yield for purified = 1.19 mg/ml
- Enzyme activity = 0.66 µg/ml/second
- Specific activity (purified) = 0.66/6.46mg = 0.102 µg/ml/second/mg protein

Consequently, the enzyme activity was found to be higher in the clone than the native thereby collaborating with the results of [5], who stated a higher activity in the cloned enzyme. Also, the specific activity was higher in the cloned amylase than in the native organism. This observation was in line with the work of [3] who recounted a similar experience of higher specific activity. Therefore, it can be inferred that the cloned amylase may be more useful in industries where both quality and quantity are fundamental.

The effect of increasing starch substrate showed that the native amylase had the ability to digest starch at varying quantities with a falling sugar peak of 1.12mg/ml and an activity of 0.62µg/ml/sec. The activity of the enzyme increased sharply with increase in starch (substrate) level until the catalytic sites got saturated then the activity decreased. It follows therefore, the enzyme catalysed reaction mechanism: initially the enzyme catalytic site was vacant; ready to receive the substrate for binding, most of the time, and the rate at which the product was formed depended on the substrate availability.

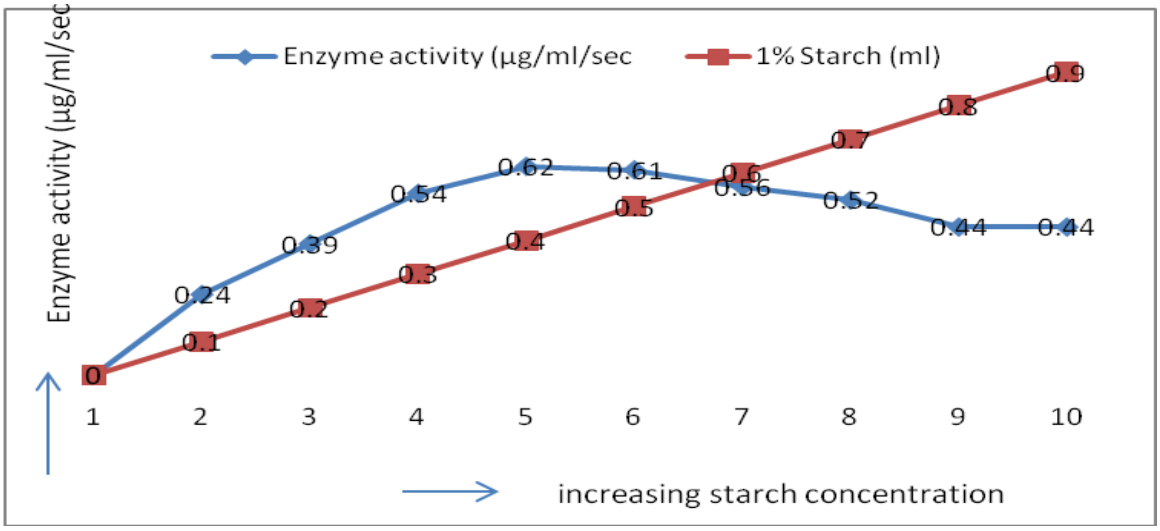


Figure 1. Effect of increasing starch on the activity of the native amylase

[6], his finding suggests that enzyme may be helpful in industries where enzyme-substrate association is very vital. The cloned amylase enzyme showed the same pattern with the native enzyme.

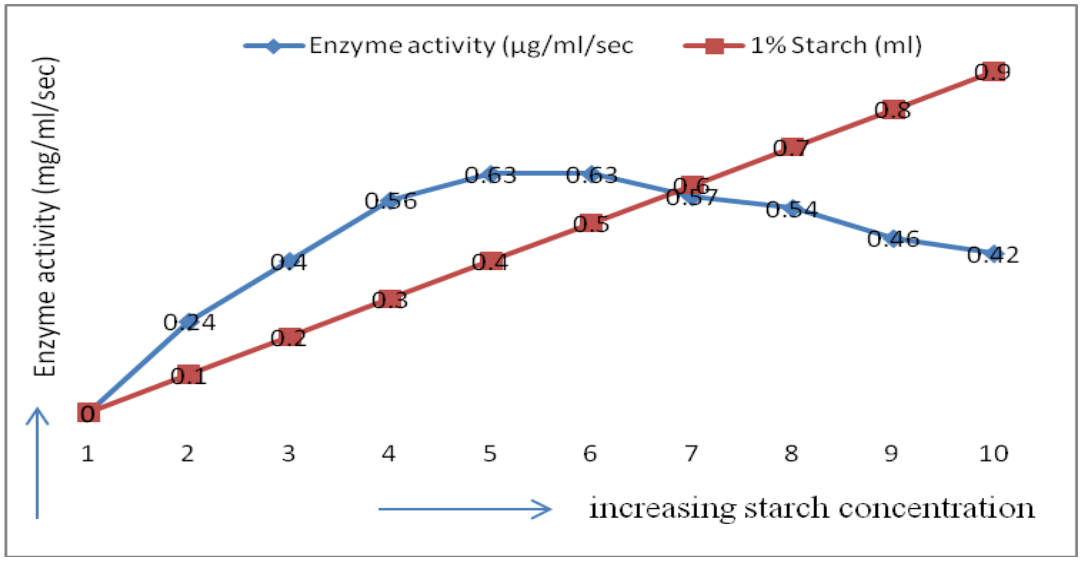


Figure 2 Effect of increasing starch on the activity of the cloned amylase

The activity of the enzyme rose sharply with rise in the starch (substrate) concentration until the catalytic sites were saturated then the activity decreased. It therefore, followed the enzyme catalysed reaction mechanism. Initially the enzyme catalytic site was vacant; ready to receive the substrate for binding, most of the time, and the rate at which the product was formed depended on the substrate availability. However, it exhibited a higher peak of 1.17mg/ml with an activity of 0.65µg/ml/sec. The enzyme and specific activities of the cloned amylase were better than that of the native amylase. These views bear witness to the findings of [7] [8] respectively. The finding suggests that the cloned amylase may as well be helpful in industries where enzyme-substrate association is very critical and above all, may be more efficient than native.

CONCLUSION

This study of amylase gene cloning from the *Bacillus subtilis* isolated from a cassava waste dumpsite successfully conferred amylolytic ability onto the *E. coli* since the transgenic *E. coli* demonstrated amylolytic activity soon after. The cloned enzyme compared favourably with the native enzyme following the usual catalysed reactions and in fact showing more returns. This is because the enzyme and specific activities of the cloned amylase were found to be higher than that of the native amylase. Also, the average generation time for *Bacillus subtilis* is 120 minutes as reported by [8] while that of *E. coli* is 20 minutes. It therefore means that biotransformation method of amylase production may yield six times as more enzymes as the conventional method even with better activity and efficiency as stated above. A combination of these properties makes the cloned amylase very competitive for industrial and economic growth. Considering the foregoing, the rDNA method may be a better means of amylase production in industries for commercial purposes.

RECOMMENDATIONS

The study hereby makes the following recommendations:-

i. Industries engaged in commercial amylase production should embrace rDNA technology.
ii. Industries involved in amylase by-products should produce the enzyme using rDNA technology because the technology has been simplified over the years.
iii. Training and retraining should be given to researchers and an awareness be created for the general public on the importance of rDNA technology.
iv. Relevant facilities and conducive atmosphere should be provided for such researchers.
v. Financial aids and fellowships should be granted for such research work

REFERENCES

1. Adejumo, A. L., Agboola, F. K., & Layokun, S.K (2012). Production and partial characterization of a thermostable extracellular α-amylase by a bacterium isolated from Cow dung microflora. *Fermentation Technology and Bioengineering, 2*(3), 14-22.
2. Adrianne, M. & Helen, K. (2001). Recombinant DNA and biotechnology: A Guide for Students. Washington, D.C: ASM Press.
3. Aiyer, D. P. V. (2004). Effect of C: N ratio on α-amylase production by *Bacillus licheniformis* SPT 27. *African Journal of Biotechnology, 3, 519-522.*
4. Ajayi, A. O. & Fagade, O. E. (2006). Growth pattern and structural nature of amylases produced by some *Bacillus* species in starchy substrates. *African Journal Biotechnology*, *5* (5), 440-444.
5. Alberts, B., Alexander, J., Julian, L., Martin, C., Keith, R. & Peter, W. (2002). Molecular biology of the cell. New York: Garland Science. Pp. G: 35.
6. Ali, S., Mahmood, S., Alan, R. & 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.
7. Ara, K., Ozaki, K., Nakamura, K., Yamane, K., Sekiguchi, J. & Ogasawara, N. (2007). *Bacillus* minimum genome factory: Effective utilization of microbial genome information. *Biotechnology and Applied Biochemistry, 46* (3), 169-78.
8. Atiyeh, M., Reza, H. S., Mehdi, R. & Vahab, J. (2010). Characterization of an α-amylase with broad temperature activity from an acid-neutralizing *Bacillus cereus* strain. *Iranian Journal of Biotechnology, 8* (2), 103-111.
9. Ayogu, T. E. & Amadi, E. S. (2010). Amylase production by *Rhizopus*

nigricans using mashed Maize. *The Internet Journal of Microbiology, 8*, 1. DOI: 10.5580/f95.

10. Bandow, J. E., Brutz, H. & Hecker, M. (2002). *Bacillus subtilis* Tolerance of moderate concentrations of Rifampin Involves the B-dependent general and multiple stress response. *Journal of Bacteriology*, *184* (2), 459-467.
11. Barnett, M. J., Fisher, R. F., Jones, T., Komp, C., Abola, A. P., Barloy-Hubler, F., ... Long, S. R. (2001). Nucleotide sequence and predicted functions of the entire Sinorhizobium meliloti pSymA megaplasmid. *Proceedings of the National Academy of Sciences*, *98* (17), 9883-9888.
12. Bennett, B.M., Brien, J.F., Nakatsu, K., & Marks, G.S. (1985) Role of hemoglobin in the differential biotransformation of glyceryl trinitrate and isosorbide dinitrate by human erythrocytes. *Journal of Pharmacology and Experimental Therapeutics 234*, 228-232.
13. Cheesbrough, M. (2003). Medical laboratory manual: Tropical health technology, low priced edition (pp. 20-35). United Kingdom: Doddington, Cambridgeshire.
14. Oyeleke, S. B. & Manga, B. S. (2008). Essential of laboratory practicals in microbiology (1st edn).(pp. 36-58) Niger State: Tobest Publisher.