ISOLATION AND MOLECULAR IDENTIFICATION OF *LYSINIBACILLUS XYLANILYTICUS* SCREENED FOR AMYLASE PRODUCTION.

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Abstract
Amylases are enzymes that break down starch or glycogen .The production of economically important alpha-amylases essential for the conversion of starches in oligosaccharides. The optimum condition for growth of Lysinibacillus xylanilyticus was best observed in Nutrient broth. The optimum pH range was found to be 6-10 and optimum temperature for growth was Phylogenetic analysis based on 16S rRNA gene sequences indicated that Lysinibacillus xylanilyticus ILBB210 16S ribosomal RNA gene, with accession number KT340486.1 was isolated from the soil and used for the production of amylase enzyme, while Molecular characterization and identification of fungi by internal transcribing sequence (ITS) sequencing. Lysinibacillus xylanilyticus showed abilities to produce amylase enzyme using nutrient starch agar during primary screening. The results revealed the abilities of lysinibacillus xylanilyticus and Lactobacillus acidophilus to ferment all sugars. While Lactobacillus brevis and Staphylococcus aureus only fermented sucrose and glucose and finally Lactobacillus plantarum only fermented sucrose. the screening of amylase activities of Bacillus sp in which bacteria isolate S3 had the highest enzyme activity of 0.542 compared to S1, S2, S4, S5 and S6 which had enzyme activity of 0.244, 0.122, 0.134, 0.187 and 0.161 respectively.
Microorganisms of the Bacillus genus synthesise alpha amylase, and thus have the potential to dominate the enzyme industry. Bacillus sp are heterogeneous and are very versatile in their adaptability to environment. Various factors influence the nature of their metabolic process and the enzymes produced the result furthermore gives hope for a pharmaceutical composition wherein bonding amylase with another one auxillary material will be a good option to treat digestive disorders.

Keywords: amylase, Lysinibacillus Xylanilyticus

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1. INTRODUCTION

Amylases are enzymes that break down starch or glycogen (Vidyalakshmi *et al*., 2009) .The production of economically important alpha-amylases essential for the conversion of starches in oligosaccharides (Pederson and Nielsen, 2000). Amylases are extensively used for starch liquefaction and the paper, food, pharmaceutical and sugar industries (Bozic *et al*., 2011). Amylases with suitable properties are very useful in specific industries have been characterized by most available amylolytic microbial strains. Amylases stand out as a class of enzymes, which are of useful applications in the food, brewing, textile, detergent and pharmaceutical industries. (Paranthaman *et al*., 2009).

Microorganisms of the *Bacillus* genus synthesise alpha amylase, and thus have the potential to dominate the enzyme industry (Bozic *et al*., 2011). *Bacillus* sp are heterogeneous and are very versatile in their adaptability to environment. Various factors influence the nature of their metabolic process and the enzymes produced (Paranthaman *et al*., 2009).

The genus *Lysinibacillus* was proposed for organisms previously allocated to rRNA group 2 of the genus *Bacillus* as reported by Ahmed *et al*. (2007). A major chemotaxonomic feature for this genus is the presence of lysine and aspartate in the peptide subunit of the cell-wall peptidoglycan. Ahmed *et al* (2007) described the type species of the genus, *Lysinibacillus boronitolerans*, and also reclassified *Bacillus sphaericus* and *Bacillus fusiformis* to the genus *Lysinibacillus*. Recently, several other species have been added to this genus, *Lysinibacillus parviboronicapiens*, *Lysinibacillus*

xylanilyticus (Lee *et al.*, 2010), *Lysinibacillus macroides* (Coorevits *et al.*, 2012) and *Lysinibacillus magniferihumi* (Yang *et al.*, 2012).

Bacteria of the *Bacillus* genus are omnipotent in nature, predominant in soil, and these bacteria have also been isolated frequently from water and air. Large amounts of bacilli (10 7-10 8 cfu) consistently enter the gastrointestinal and respiratory tracts of healthy people through air, water, and food and exist in the gut microflora. Some researches showed that *Bacillus* organisms are a normal component of human intestinal microflora (Anuraj *et al.*, 2012).

2. MATERIALS AND METHODS

2.1. Collection of Agro-allied Samples

Agro-allied wastes Such as wheat bran was bought from a local market within kaduna metropolis, while Rice bran was obtained from the institute for agricultural research Zaria (I.A.R), and banana peel and potatoe peels were obtaned from dumpsites within kaduna metropolis. Soil samples were collected from agro-allied dumpsites in central market, station market and kawo market by the means of sterilized spatula and these were collected in sterile polythene bags. The samples were then brought to the laboratory for microbiological Analysis.

2.2. Isolation of Bacillus Species from soil

From each Soil sample, 25g of sample was taken and with 225ml sterile saline (9 g/L NaCl). The samples was then serially diluted to 10-6. The 10^{-6} dilution factor was plated on starch- nutrient agar plate and skimed milk agar plate and incubated at 37^{o}C. Single colonies showing different morphological characteristics such as size, shape, colour, elevation and margin were identified from different plates streaked with diluted samples. Single colonies which formed clear halos with Gram's iodine were identified as starch utilizing strains. Selected single colonies were purified by repeated streaking and transferred to starch-nutrient agar slant (Vaseekaran *et al.*, 2010).

2.3. Characterization of Bacterial Isolate

Biochemical tests were carried out using standard methods. In accordance with Cheesbrough (2005).

2.4. Primary Screening of Bacillus sp for Amylase Production

Lysinibacillus xylanilyticus was screened primarily using starch agar medium by dissolving yeast extract of 1.5, peptone 0.5gm, sodium chloride 1.5gm, starch 10gm and agar 15 gm in one liter of distilled water, the pH of media was regulated to 5.6 using 0.1m Hydrochloric acid and was sterilized (Khan and Yadac,2011). Seventy five (75) micro litre of spore suspensions (75000 spores) of all isolates were transferred into holes bored with cork borer (radius 8 mm) in starch agar media and incubated at 28°C for 48 hours. After incubation period, the radius of zone of hydrolysis was determined by using weak iodine solution which was prepared by addition of 0.3% (w/v) of iodine I2 to 3% (w/v) of potassium iodide. The isolates with maximum zone of hydrolysis was selected for production of amylase (Dalal *et al.*, 2014).

2.5. Primary Screening of *Bacillus* Species For Amylase Production

The pure isolates of *Bacillus* sp were inoculated at the centre of the sterile starch agar media by pour plating and incubated at 37^{o}C for 24 hours. After incubation, 1% iodine solution was over layed on the agar plates and the observations was made to note the starch utilized zone around the colony. The pure bacterial isolate were screened for the production of extra cellular amylase production using starch agar medium. (Akansha and Varsha, 2013).

2.6. Molecular Characterization and Identification of Bacterial Isolates

Extraction of Bacteria DNA

Genomic DNA was extracted using phenol-chloroform extraction method. The PCR parameters for the amplification of 16S ribosomal DNA were optimized. fifty millimeter 50 ul of PCR master mix containing universal ribose primer set 27 F- (51 –GGA CTA CAG GGT ATC TAA T-31)/1492 R- (51

–AGA GTT TGA TCC TGG-31), 10 mM dNTPS, 10 PCR Buffer, 1 U Taq DNA polymerase, 2 mM Mg+ and (100-200 ng) template DNA. The PCR steps included initial denaturation at 94^{o}C for 5 minutes, with 25cycles of denaturation at 94^{o}C for 1 minutes, annealing at 52^{o}C for 1 minutes, elongation at 72^{o}C for 1 minutes and final extension at 72^{o}C for 7minutes. Approximately 1.5 kb amplicons were generated. The PCR product was purified using Gene JET PCR purification kit (Fermentas) using manufacturer's instructions. Purified PCR products were sequenced and sequence search similarities were conducted using basic local alignment search tool (BLAST). Phylogenetic analysis of sequence data of bacteria under study was aligned with reference sequence homology from the NCBI database using the multiple sequence alignment of MEGA 5.0 Program. (Ravindar and Namasivayam, 2013).

2.4. DNA Amplification.

Fifty (50) millimeter of PCR master mix contained universal ribose primer set 27 F- (51 –GGA CTA CAG GGT ATC TAA T-31)/1492 R- (51 –AGA GTT TGA TCC TGG-31), 10 mM dNTPS, 10 PCR Buffer, 1 U Taq DNA polymerase, 2 mM Mg+ and (100-200 ng) template DNA. The PCR steps included initial denaturation at 94^{o}C for 5 minutes, with 25 cycles of denaturation at 94^{o}C for 1 minutes, annealing at 52^{o}C for 1 of MEGA 5.0 Program. (Ravindar and Namasivayam, 2013).

3. RESULTS AND DISCUSSION

Table 1 revealed the morphological and biochemical characteristics of the bacterial isolates. After some preliminary physiological tests a total of six (6) isolates were selected for further identification. The bacterial isolate (S3) was found to be positive in Gram staining, Spore formation, Cell shape, Motility, Citrate utilization Catalase, Lecithenase, Voges proskauer, Starch hydrolysis, Anaerobic growth, Growth at 42^{o}C, Casein hydrolysis except indole and methyl red, compared to S1, S2, S4, S5 and S6 which were positive in Gram staining, Spore formation, Cell shape, Motility, Catalase, Lecithenase, Voges proskauer, Starch hydrolysis, Anaerobic growth, Growth at 42^{o}C, Casein hydrolysis except citrate utilization, methyl red and indole.

Table 1: Biochemical Characterization of Bacteria Isolates from Agro-allied Waste

Bacterial Isolates	S1	S2	S3	S4	S5	S6
Grams Staining	+	+	+	+	+	+
Spore Formation	+	+	+	+	+	+
Cell Shape	Long rod shape	Cocci	Short Rod chain	Short Rod chain	Short Rod chain	Long rod shape
Motility	+	+	+	+	+	+
Citrate ultilization	-	-	+	-	-	+
Catalase	+	+	+	+	-	-
Lecithenase	+	-	+	-	+	+
Voges proskauer	+	+	+	+	+	+
Methly red	-	-	-	-	-	-
Starch hydrolysis	+	-	+	-	+	+
Indole	-	-	-	-	-	-
Anaerobic growth	+	+	+	+	+	+
Growth at 42^{o}C	+	+	+	+	+	+
Casein hydrolysis	+	+	+	-	-	+
Probable organism	*Lactobacillus brevis*	*Staphylococcus aureus*	*Lysinibacillus xylanilyticus*	*Lactobacillus acidophilus*	*Lactobacillus brevis*	*Lactobacillus plantarum*

Keys: + (positive), - (Negative), S1 – S6 – codes that represent each bacterial isolates before specific identification.

Table 2. Screening of Bacteria Isolates Using Triple Sugar Iron for Amylase Production

Organism	slant	butt	Gas	H2S	Probable Organisms
1	NC/ALK	NC/ALK	-	-	*Lactobacillus brevis*
2	AA	AA	-	-	*Staphylococcus aureus*
3	NC/ALK	NC/ALK	-	-	*Lysinibacillus xylanilyticus*
4	AA	AA	+	+	*Lactobacillus acidophilus*
5	NC/ALK	AA	-	-	*Lactobacillus brevis*
Control	NC/ALK	NC/ALK	-	-	

Keys: NC/ALK: No change/ Alkaline, AA: Acidic, + (positive), - (Negative)

Table 3: Sugar Fermentation Test for Characterization of Bacteria Isolates

Isolate code	Sucrose	Maltose	Manitol	Glucose	Probable organism
S1	+	-	-	+	Lactobacillus brevis
S2	+	-	-	+	*Staphylococcus aureus*
S3	+	+	+	+	*Lysinibacillus xylanilyticus*
S4	+	+	+	+	*Lactobacillus acidophilus*
S5	+	-	-	-	*Lactobacillus brevis*
Control	-	-	-	-	

Keys:+ (positive), - (Negative), S1 – S6 – Isolate codes.

Gas – production of gas by organism by upward shift of the medium , Slant – TSI prepared in slant, Butt – change in colour at the buttom of the medium, H^2S – production of H^2S gas at the surface of the medium, S1 – S6 – codes that represent each bacterial isolates before specific identification.

Table 2 revealed the screening of bacterial isolate using triple sugar iron (TSI).The Bacterial isolates with code S2 and S4 were positive for acid formation at the slant and butt medium, but S2 was negative for both gas and H^2S production while S4 was positive for gas and H^2S production. Furthermore S1, S3 and S5 showed no change or Alkaline condition of the medium and were all negative for gas and H^2S production.

Table 3 showed sugar fermentation test for bacterial isolates. The results revealed the abilities of *lysinibacillus xylanilyticus* and *Lactobacillus acidophilus* to ferment all sugars. While *Lactobacillus brevis* and *Staphylococcus aureus* only fermented sucrose and glucose and finally *Lactobacillus plantarum* only fermented sucrose.

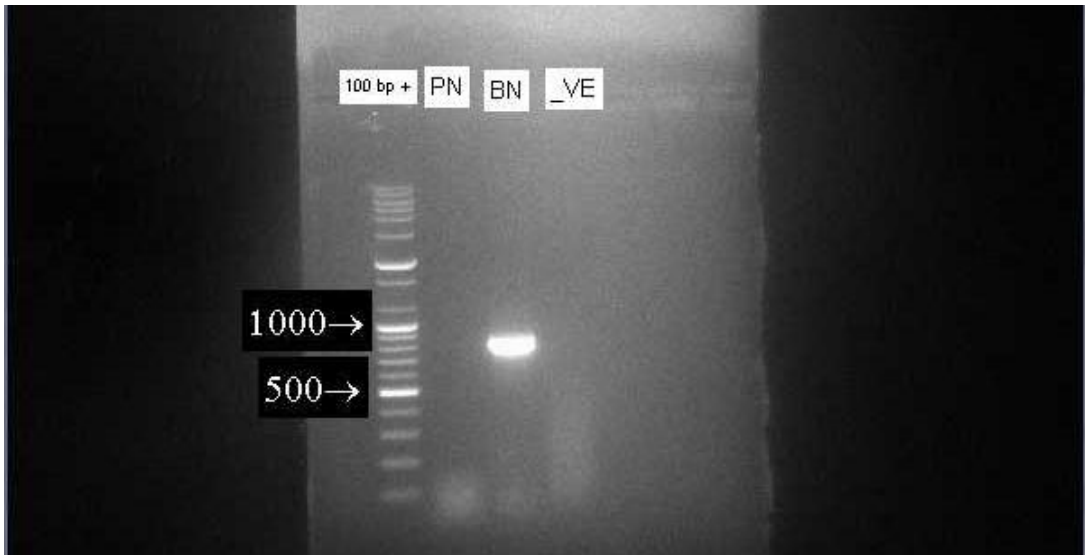

Figure 1: Agarose Gel image for *Lysinibacillus xylanilyticus*

Key: UV transiluminator (1-302nm) of Agarose gel electrophoresis of DNA profiles of *lysinibacillus xylanilyticus;* lane BN; Marker: 900 bp^+

3.1. Molecular Identification of *Bacillus* Specie

Genomic DNA of the studied strains were extracted and the yield of genomic DNA varied from 60 to 140 ng/µl. The PCR amplification produced a fragment of approximately 800-1000 bp (plate. 1). A BLAST search performed on the 16S rRNA primary sequences of the bacterial specie resulted in the highest similarity score. The maximum identity score after BLAST with 16S rRNA gene sequence of S3 showed 99% identity with *Lysinibacillus xylanilyticus* ILBB210 16S ribosomal RNA gene, with accession number KT340486.1. (Table 4).

3.2. Screening of Amylase Activities of Bacillus Species

Table 5 shows the screening of amylase activities of *Bacillus* sp in which bacteria isolate S3 had the highest enzyme activity of 0.542 compared to S1, S2, S4, S5 and S6 which had enzyme activity of 0.244, 0.122, 0.134, 0.187 and 0.161 respectively.

Table 4 Gene Sequence For Identification Of *Lysinibacillus Xylanilyticus*

TAC AGA CCA GAA AGT CGC CTT CGC CAC TGG TGT TCC TCC AAA TCT CTA CGC ATT TCA CCG CTA CAC TTG GAA TTC CAC TTT CCT CTT CTG CAC TCA AGT CCC CCA GTT TCC AAT GAC CCT CCA CGG TTG AGC CGT GGG CTT TCA CAT CAG ACT TAA AGG ACC GCC TGC GCG CGC TTT ACG CCC AAT AAT TCC GGA CAA CGC TTG CCA CCT ACG TAT TAC CGC GGC TGC TGG CAC GTA GTT AGC CGT GGC TTT CTA ATA AGG TAC CGT CAA GGT ACA GCC AGT TAC TAC TGT ACT TGT TCT TCC CTT ACA ACA GAG TTT TAC GAT CCG AAA ACC TTC TTC ACT CAC GCG GCG TTG CTC CAT CAG GCT TTC GCC CAT TGT GGA AGA TTC CCT ACT GCT GCC TCC CGT AGG AGT CTG GGC GGT GCC TTG TCC CAG GTG CGA GGA ACC TCC CCT TGA CGA GCT CTC AAA ACT TAG GGC CAC GTC A

A - ADENINE; T - THYMINE; G - GUANINE; C – CYTOSINE

key: Identification of *Lysinibacillus xylanilyticus* at 99% with Accession number KT340486.

Table 5: Screening of Amylase Activities of Bacterial Isolates

Isolate code	Absorbance (540nm)	Enzyme Activity (iu/ml)	Bacterial
S1	1.322	0.244	*Lactobacillus brevis*
S2	0.922	0.122	*Staphylococcus aureus*
S3	2.933	0.542	*Lysinibacillus xylanilyticus*
S4	1.112	0.134	*Lactobacillus acidophilus*
S5	1.009	0.187	*Lactobacillus brevis*
S6	0.870	0.161	*Lactobacillus plantarum*

Figure 2: Starch utilization of *Lysinibacillus xylanilyticus* after iodine staining on nutrient starch medium

4. CONCLUSION

The optimum condition for growth of *Lysinibacillus xylanilyticus* was best observed in Nutrient broth. The optimum pH range was found to be 6-10 and optimum temperature for growth was observed to be at 30-40oC.
Phylogenetic analysis based on 16S rRNA gene sequences indicated that *Lysinibacillus xylanilyticus* ILBB210 16S ribosomal RNA gene, with accession number KT340486.1 was isolated from the soil and used for the production of amylase enzyme, while Molecular characterization and identification of fungi by internal transcribing sequence (ITS) sequencing. Identifying *Lysinibacillus xylanilyticus* showed abilities to produce amylase enzyme using nutrient starch agar during primary screening.

Recommendation

1. The result furthermore gives hope for a pharmaceutical composition wherein bonding amylase with another one auxillary material will be a good option to treat digestive disorders.
2. Alpha amylase produced after solid state fermentation from microorganisms appears to have potential in industries due to its thermal, pH and detergent stability.
3. Data originated from this study will help to design experimental set up for large scale production of amylase.

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