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AMYLASE AND CELLULASE ENZYMES PRODUCTION FROM CORN COB USING *BACILLUS THURINGIENSIS* ISOLATED FROM THE SOIL

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Abstract

Amylases and cellulases are enzymes that hydrolyze complex carbohydrates into simple sugars. In this research work *Bacillus* species were isolated and identified to the genus level morphologically and using conventional biochemical tests. The isolates were then screened for amylase and cellulase enzymes production capabilities. The *Bacillus* spp isolates all showed varying degrees of amylase production that ranged from 11mm to 17mm which were evident by the production of clear zones of hydrolysis around the colonies when grown on nutrient agar supplemented with starch. The 10 isolates also showed varying degrees of cellulase production by the clear zones of hydrolysis as a result of cellulase enzyme production when grown on Caboxymethyl cellulase agar. The isolates were further used for amylase and cellulase enzymes production using pulverised corn cobs as substrate. The Isolates had amylase actvities that ranged from 1.18-2.98 U/ml and cellulase activities that ranged from 0.33-1.47FPU/ml. The DNS method of Zambere was used to determine amylase and cellulase activities. The filter paper assay was specifically used for cellulase determination. The three isolates with the highest amylase and cellulase enzyme production capacities were further characterized molecularly as *Bacillus thuringiensis* by sequencing their 16SrRNA genes. The sequences have been deposited in the gene bank data base. This research work has demonstrated the potential of corn cob as a cheap substrate for the production of amylase and cellulase enzymes. *Bacillus thuringiensis* isolates have demonstrated an exceptional ability to produce amylase and cellulase enzymes, these organisms have a potential for industrial production of amylase and cellulase enzymes.

KEYWORDS: Amylase, cellulase, corn cob, hydrolysis,Enzyme,Sequencing

INTRODUCTION

Enzymes are large biological molecules responsible for the thousands of chemical inter-conversions that sustain life (Grisham *et al*., 1999). Amylases are important enzymes employed in the starch industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents by degrading 1 – 4 linkage of starch. Beside their use in starch saccharification, they also find potential application in a number of industrial processes

such as in food, baking, brewing, detergent, textile and paper industries. With the advent of new frontiers in biotechnology, the spectrum of amylases application has expanded into many fields such as clinical, medical and analytical chemistry (Pandey *et al*., 2000). The main microbial sources for amylase production are *Bacillus* species (Nurmatov *et al*., 2001, Deutch, 2002, Dey *et al*. 2002) and *Asperigillus* species (Gigras *et al.,* 2002).

Cellulases are hydrolytic enzymes capable of hydrolyzing the most abundant organic polymer cellulose to small sugar components. Cellulases have potential in industries and are used in food, beverages, textile, laundry, paper and pulp industries etc. As lytic enzymes, they are also of major importance in the protoplast production for tissue culture and plant metabolites production (Bhat, 2000).

Amylases and cellulases are predominantly produced by fungi, studies about bacterial amylases and cellulases had been performed on *Bacillus subtilis, Paenibacillus, Clostridium cellulolyticum, Thermobifida fusca, and Clostridium thermocellum* (Gaurav *et al*., 2017)

A corncob, also called cob of corn, is the central core of an ear of corn (also known as maize in Spanish-speaking countries). It is the part of the ear on which the kernels grow. When harvesting corn, the corncob may be collected as part of the ear (necessary for corn on the cob), or instead may be left as part of the corn stover in the field for natural degradation. This substrate can however be channelled into production of enzymes.

Enzymes are relatively expensive reagents and enzyme production reveals that almost 50% of the cost of production is associated with capital investment, while the cost of raw materials accounts for almost one third of such costs. Substitute or complementation of feedstocks with lignocellulosic sources can result in an increased return on investment.

Methodology

Sample collection and preparation

Corn-cob samples were collected from the corn farm into polythene bags and were transported to the laboratory and pound using laboratory mortar and pestle to ensure homogenization of the sample. 20g of the corn-cob were further pulverizeded using a grinding machine..

Isolation of *Bacillus* species

Nutrient agar was used for the cultivation of bacteria; Nutrient agar at a concentration 24g/1 of distilled water. The media was autoclaved at 121^0C for 15mins after which it was dispensed into 90mm-diameter polystyrene sterile plastic petri-dishes. The freshly sieved soil was carefully mixed and pulverized with spatula on a large piece of paper. 1g of soil was weighed on a sterile aluminum foil and transferred immediately to the conical flask containing 150ml of normal then placed in a water bath that was preheated to 80^OC for 30min. It was allowed to cool while 0.1ml was innoculated onto plates of nutrient agar and spread evenly with a sterile bent glass rod. The inoculated media was then incubated at 37^OC for 24hr (Travers *et al*., 1987). Sub culturing was done on nutrient agar to isolate pure cultures.

Identification of *Bacillus* species

Primary identification of the isolates was performed using morphological and cultural characteristic as described by Cheesebrough., 2010. Morphological identification of the isolates was done under the compound microscope to observe cell size, shape and arrangement after Gram staining of bacteria. The isolates were further characterized biochemically using conventional biochemical tests and sugar fermentation tests as described by Cheesebrough, 2010.

Screening of *Bacillus* isolates for cellulase production

Bacillus isolates were screened on selective carboxymethyl cellulose agar containing (g/l): $NaNO_3$ 2.0, KH_2 PO_4 1.0, $MgSO_4$.$7H_2O$ 0.5, KCl 0.5, carboxymethyl cellulose (1%), sodium salt 10.0, peptone 0.2, agar 17.0. Plates were inoculated with suspension of pure *Bacillus sp* culture and incubated at 30ºC. After 24h, plates were flooded with 1% Congo red solution for 15 min then de-stained with 1M NaCl solution for 15 min. The zones of decolorization around each colony were noted (Kanmani *et al*., 2011).

Screening of *Bacillus* for amylase production

A loop full of Pure *Bacillus* sp suspensions were streaked on starch agar plates and plates were incubated at 37^0C for 48hrs. After 48hrs, the plates will flood with lugols iodine, which is an indicator of starch. Excess of lugols iodine was drained off and plates were observed for clear zone around the colony against blue, black background. A clear halo zone around the

colony was indicative of amylase production. (Ashwini *et al*., 2011)

Cellulase production using corn-cob

4g pulverized corn cob was added to 50ml of Mandel and Sternberg's mineral media in different conical flasks. The initial PH of the media was adjusted to 5 before being autoclaved at 121^0C for 15mins. After cooling, the flasks were inoculated with 5ml of bacterial culture that was equivalent to 1.0 Mcfarland ($3.0x10^8$CFU/ml) and incubated at 30^0C on an orbital shacking bed at 120pm for 5 days. Samples were withdrawn after 5 days for cellulase activity using the endoglucanase activity method as described by Zakpaa *et al*., 2009.

Amylase production using corn-cob

10g of pulverized corn-cob was added to 1 litre of the fermentation medium which consisted of 6.0g bacteriological peptone, 0.5g SO4'7H20, 0.5g KC, 1.0g starch, PH7.0.5ml of the *Bacillus* species suspension was added and incubated at 35^0C 48hr, samples were collected after 48hr and tested for Amylases activity.

Molecular characterization of *Bacillus* isolates

Molecular characterization of the Bacillus isolates with the highest amylase and cellulase activities were done through PCR and sequencing of the 16SrRNA gene.

DNA Extraction

This was done by using DNA purification kit from Thermo Scientific Products. Single colonies of the isolates were separately inoculated into 10ml standard media broth and incubated overnight at 37ºC. The bacterial cells of 2×10^9 were harvested by centrifugation for 10 min at 500 × g and the supernatant was discarded. The pellet was re-suspended in 180 µL of digestion solution with addition of 20µL of proteinase K solution and mixed thoroughly by votexing to obtain a uniform solution. The sample was incubated at 56ºC by shaking in a water bath until the cells were completely lysed for 30min. 20uL of RNase A solution was added and mixed by votexing and incubated for 10 min at room temperature. 200ml of lysis solution was added and mixed by votexing for about 15 sec until a homogeneous mixture was obtained. To the mixture 400 µL of 50% ethanol was added and mixed by vortexing. The DNA was purified using GeneJET Genomic DNA Purification column with the aid of wash and elution buffers. The purified DNA was used immediately or stored at -20ºC.

PCR Amplification of the 16SrRNA gene of the *B*acillus isolates

The target primers were for 16SrRNA. Bacterial universal primers 27F 5'(AGA GTT TGA TCM TGG CTC AG)3' and 1492R 5' (TAC GGT TAC CTT GTT ACG ACT T) 3' of about 1500bp size were used (Mojtaba and Hoda 2017). The PCR reaction mixture of 50µl contained 2.5 mM dNTPs, 5 µg genomic DNA template and 20 µmol of each primer. The PCR mixture was subjected to the following thermal conditions using DNA thermal cycler: The Products were isolated after 40 cycles of amplification with initial denaturation at 94 °C for 30 s, 30 sec at 58 °C and 2 minute at 72 °C, final extension for 3 minutes at 72 °C. Purification to obtain PCR products was done with the QIA quick PCR purification kit (Qiagen). All amplification products were separated on 1% agarose gel which was stained with ethidium bromide. The PCR products were observed under an ultraviolet (UV) light transilluminator (Sambrook *et al*., 1989).

Sequencing of PCR product

PCR products were sequenced by Macrogen biotech laboratory (Europe). The 16SrRNA sequences were analyzed against those available from the National centre for biotechnology information (NCBI) database using the BLAST search system to identify the most similar sequences

Phylogenetic analysis of the 16SrRNA gene sequences

The 16SrRNA sequences were analyzed for anomalies using Chromas lite (2.01) software package, then assembled using the CAP contig program in bioedit (7.1.3.0). FASTA and BLAST subroutine were used to determine the closest relatives in the Genbank database (Altschul *et al*., 1990). The sequences were compared with other closely related bacterial sequences from the Genbank using the FASTA Algorithm (Thompson *et al*., 1997). Sequences were aligned and analysed with CLUSTALX program (Thompson *et al*., 1997). The phylogenetic package MEGA6 was used to carry out the nucleotide substitution model of Jukes and Cantor in order to obtain distance matrices. Phylogenetic trees were then

constructed using the neighbour-joining method (Saitu and Nei, 1987).

16SrRNA gene sequence accession numbers

The 16SrRNA gene sequences of *Bacillus* species isolates were deposited in the Genbank with their accession numbers.

RESULTS

The morphological characteristics and biochemical characterization of the ten (10) presumptive *Bacillus* isolates (table 1) showed that most of the isolates had colonies that were rough, some were moist and creamy in colour. All the isolates were rod shaped, gram positive and motile. All the isolates had the ability to hydrolyze starch, they possessed endospores, they were catalase positive indicating that the all belong t the genus Bacillus.

The zones of hydrolysis of starch by the ten 10 isolates when grown on nutrient agar plates supplemented with starch (table 2) showed that all the isolates had zones of hydrolysis that ranged from 10 to 17mm, isolate B3 had the highest zone of hydrolysis while B9 had the lowest zone of hydrolysis.

The cellulase activities of all the *Bacillus* isolates before and after hydrolysis (Plates 1A,1B and 1C) shows clear zones of hydrolysis due to secretion of cellulase enzymes by some of the isolates . Plate 1 D showed nutrient agar plates supplemented with starch showing zone of hydrolysis produced by *Bacillus* species due to amylase production

The mean amylase and cellulase activities of all the isolates in different enzyme production media (table 3) showed that the amylase activities of the isolates ranged from 1.18-2.98 U/ml while the cellulase activities of isolates ranged from 0.33-1.47 FPU/ml

The sequencing codes of *Bacillus* isolates, the percentage nblast identity, the identity and accession numbers of the strains identified by 16SrRNA (Table 4) showed that the nblast identity of all the sequenced isolates ranged from 97.77-99.93 %. All the ten (10) *Bacillus* isolates were identified to the species level by sequencing of 16SrRNA genes, their various accession numbers were obtained and submitted to the Genbank.

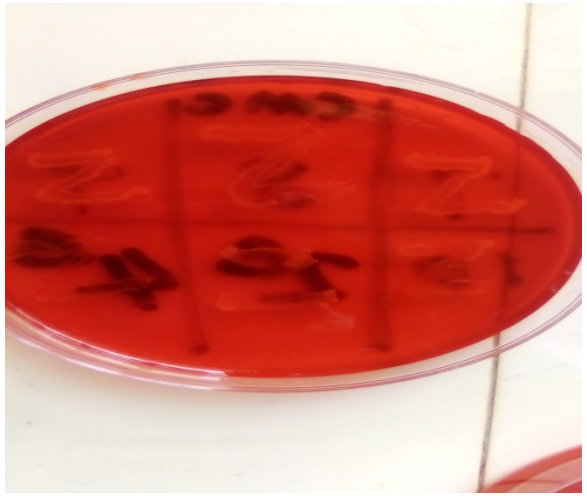
The Phylogenetic tree (Fig 1) showed evolutionary relationship of *Bacillus thuringiensis* obtained from soils in Jos, Nigeria (in red) with other strains available in the GenBank database. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Saitou *et al*., 1987). The tree with the highest log likelihood (-3072.6805) was shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 19 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1408 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. The tree was rooted using an *Escherichia coli* isolate from Nigeria. The tree showed that all three *Bacillus thuringiensis* isolates SXFB3, SXFB7 and SXFB8 clustered together with the strains gotten from the Genbank. This means that they share a similar evolutionary relationship that is different from the outdoor *E. coli* group as shown on the tree. The three *Bacillus thuringiensis* isolates were not closely clustered together which may be due to the fact that they may have evolved from different ancestors.

Table 1: Morphological Characteristics and Biochemical characterization of *Bacillus* spp.

Isolate	Colonial morphology	Vegetative morphology	Gram rxn	Starch hydrolysis	Presence of spore	Catalase test	Xylose fermentation	Identity
B1	Spherical,Rough,dry,creamy	Rod shape						*Bacillus spp*
			+	+	+	+	+	
B2	Moist, creamy	Rod shape	+	+	+	+	+	*Bacillus spp*
B3	Rough, dry, creamy	Rod shape	+	+	+	+	+	*Bacillus spp*
B4	Spherical,Rough,dry,creamy	Rod shape	+	+	+	+	+	*Bacillus spp*
B5	Moist, creamy	Rod shape	+	+	+	+	+	*Bacillus spp*
B6	Rough, dry, creamy	Rod shape						*Bacillus spp*
			+	+	+	+	+	
B7	Spherical,Rough,dry,creamy	Rod shape	+	+	+	+	+	*Bacillus spp*
B8	Moist, creamy	Rod shape	+	+	+	+	+	*Bacillus spp*
B9	Rough, dry, creamy	Rod shape	+	+	+	+	+	*Bacillus spp*
B10	Rough,dry,creamy	Rod shape	+	+	+	+	+	*Bacillus spp*

Table 2: *Bacillus* spp isolates and their zone of hydrolysis on nutrient agar plates supplemented with starch (screening for amylase production)

Isolates	Zone of Hydrolysis (mm)
B1	13
B2	13
B3	17
B4	15
B5	15
B6	13
B7	15
B8	16
B9	10
B10	15



A

B

D

Plate 1 A: *Bacillus* species inoculated CMC Plates without zone of hydrolysis (before hydrolysis)

Plate 1 B and Plate 1 C: *Bacillus* species inoculated CMC plates showing clear zones of hydrolysis due to cellulase production (after hydrolysis)

Plate 1 D: Nutrient agar plates supplemented with starch showing zone of hydrolysis produced by *Bacillus* species due to amylase production

Table 3: Mean Amylase and cellulase Activities in Enzyme production media using different *Bacillus* species

Isolate	Amylase activity (U/ml)	Cellulase activity (FPU/ml)
B1	1.18	0.34
B2	2.00	0.34
B3	2.62	1.47
B4	1.32	1.32
B5	1.66	0.99
B6	2.11	0.45
B7	2.98	1.45
B8	2.34	1.46
B9	1.56	0.56
B10	1.44	0.33

Table 4: The sequencing codes of *Bacillus* isolates, percentage nblast identity and identity and of the strains characterised by sequencing of 16SrRNA gene.

Isolate Code	Highest nBLAST identity (%)	E-Value	Highest query coverage (%)	Isolate identity
SBX3	99.79	0.0	99	*Bacillus thuringiensis*
SBX7	99.93	0.0	100	*Bacillus thuringiensis*
SBX8	99.79	0.0	100	*Bacillus thuringiensis*

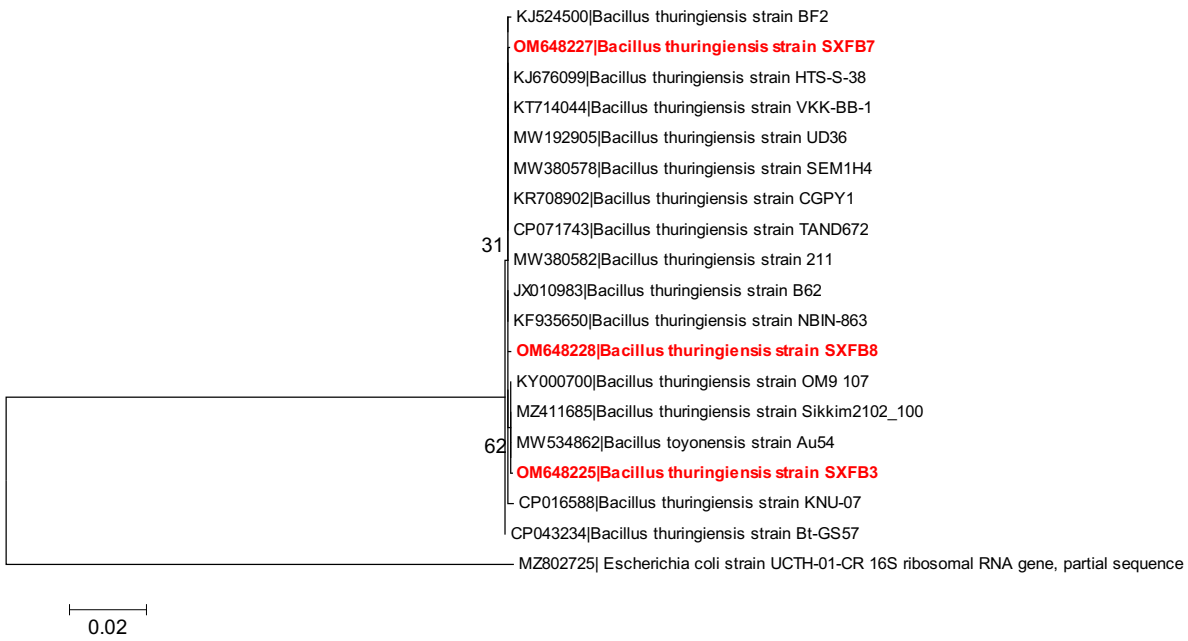


Fig 1: Phylogenetic tree showing evolutionary relationship of *Bacillus thuringiensis* obtained from soils in Jos, Nigeria (in red) with others stains available in the GenBank database.

DISCUSSION

A total of ten (10) *Bacillus* species were identified morphologically and biochemically and they all showed exceptional abilities to produce amylase enzyme when grown on nutrient agar plates supplemented with starch which was evident by the appearance of clear zones of hydrolysis around all the *Bacillus* specie isolates. Isolate B3 had the highest amylase enzyme activity as compared to the other nine (9) isolates. This could be as a result of the difference in the genetic makeup of the isolates. The isolates also produced cellulase enzyme which was evident by the clear zones of halos around the colonies due to hydrolysis of cellulose.

Isolate B3, B7 and B8 produced amylase enzymes with activities that were comparable to the amylase activities reported by other workers (Koel *et al*., 2014 and Mendu *et al*., 2005). The isolates also produced cellulase with activities that coincided with the enzyme activities reported by other workers (English *et al*., 1967, Guoxi *et al*., 2020 and Poorna *et al*.,2007)

This research work has demonstrated the potential of corn cob as cheap substrate for the production of amylase and cellulase enzymes.

A total of three *Bacillus* isolates that had very high amylase and cellulase activities were identified to the species level by sequencing the 16SrRNA gene. The isolates sequences and their various accession numbers were obtained and deposited at the Genbank. A Phylogenetic tree was constructed for the three *Bacillus* isolates to show evolutionary relationship of the three *Bacillus* species obtained from soils in Jos, Nigeria with others strains available in the GenBank database and the tree was rooted using an *Escherichia coli* isolate from Nigeria. The tree showed that all three *Bacillus thuringiensis* isolates SXFB3, SXFB7 and SXFB8 clustered together with the strains gotten from the Genbank. This means that they share a similar evolutionary relationship that is different from the outdoor *E. coli* group. The three *Bacillus thuringiensis* isolates were not closely clustered together which could be so if they have evolved from different ancestors. In the present research work, amylase and cellulase producing bacteria have demonstrated relatively high abilities to secrete those enzymes and therefore have potential for industrial production of those enzymes using cheap substrates.

CONCLUSION

A total number of ten *Bacillus* species with abilities to secrete amylase and cellulase enzymes were isolated and identified morphologically and biochemically. Three *Bacillus* species with high amylase and cellulase activities were characterized molecularly as *Bacillus thuringiensis* by

sequencing the16srRNA genes. The three isolates codes and accession numbers have been deposited in the gene bank.

AUTHUR'S CONTRIBUTION

Raplong H.H. participated in sample collection, analysis and wrote the manuscript. Jurbe F.D. and Musa O. carried out part of the analysis and recording of data, Adamu R.R., Dalhatu A.H. and Uzoma S.S. contributed to sample collection, analysis and revised the manuscript.

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