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Isolation and Molecular Characterization of Pullulanase Producing *Bacillus* Strains

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ABSTRACT
Pullulanase is an extracellular carbohydrase responsible for the hydrolysis of pullulan and amylopectin toproduce maltotriose. The product maltotriose is used in detergent industry, bakery industry and in the production of biotechnological products. In the present investigation pullulanase producing bacillus species were isolated and characterized using different biochemical and molecular methodologies. The isolates were identified as Bacillus cereus and Bacillus thuringiensis respectively. The pullulanase acivity was higher in Bacillus cereus, 0.62U/ml than B. thuringiensis, 0.53U/ml. This research reveals that pullulanase enzyme production from these Bacillus species shows great promise for use in industrial processes.

KEYWORDS: *Pullulanase, Bacillus, Molecular, Characterization*

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INTRODUCTION
Pullulanase is an extracellular carbohydrase which debranches pullulan. They are also called debranching enzymes and have been widely used to hydrolyse 1,6-glucosidic linkages in starch, amylopectin, pullulan and other oligosaccharides to produce maltotriose [1].

Pullulanase is of great significance due to its wide area of potential application. It is a very potent enzyme for degradation of starch to glucose and maltose. It has been reported that Pullulanase enzyme is used on a large scale in glucose and maltose syrup industries. It is widely used in industries in the Saccharification of Starch. It converts starch into glucose and maltose which are used in the production of glucose syrup more efficiently [2]. The enzyme is used in detergent industry, baking industry and for the production of cyclodextrins which in turn is used in the production of Biotechnological products and low calorie beer [3].

A number of pullulanase have been produced and characterized from bacterial sources. Factors such as temperature, substrate concentration, agitation and time have been reported to greatly affect its production [4]. Pullulanase type I has been characterised from mesophilic bacteria such as *Aerobacter aerogenes* [5], *Bacillus acidopullulyticus, Klebsiella pneumonia* and *Streptomyces sp.* Moderate thermophilic gram positive bacteria such as *Bacillus flavocaldarius, Bacillus thermoleovorans, Clostridium sp,* and *Thermos caldophilus* also have ability to secrete pullulanase type I. Pullulanase type I from hyperthermophilic bacterium *Fervidobacterium pennavorans*, has also been reported.[6]. The aim of this study is the isolation and molecular characterization of pullulanase producing *Bacillus* strains.

MATERIALS AND METHODOLOGY
Screening, Isolation and identification of microorganism for the production of pullulanase enzyme
Collection of soil sample: Soil sample from different flour mills was collected and 1g of sample was weighed and a suspension was prepared using 10ml

of saline. It was allowed to settle down and the clear top layer in the tube was used as inoculums.

Isolation of organism by using specific media: The pullulanase media was sterilized and 500µl of the inoculum was inoculated through pour plate method. The plates were Incubated at 37ºC for two days and observed for the growth of microorganism in each plate. The colonies were marked; pure cultures of those isolated colonies were maintained.

Screening for the production of pullulanase

Fresh isolates that showed high pullulan degradation efficiency were inoculated in culture medium, which consisted of: {(g/l) cassava flour (10), NaCl (2), $MgSO_4.7H_2O$ (0.1), K_2HPO_4 (0.17), $KH_2PO_4.7H_2O$ (0.12) and $NaNO_3$ (5), pH 7} .The flask was loaded on a rotary shaker incubator at a speed of 200 rpm at 37°C for 48 h. The cells were removed from the culture medium by centrifugation at4,000 rpm for 20 min..Supernatant was collected and used for the pullulanase assay. Pullulanase assay was done as described by [9]. Pullulanase assay was determined by measuring the release of reducing sugar from pullulan. The reaction mixture containing 0.5 ml of crude enzyme and 0.5ml of (1% pullulan in 0.2M sodium acetate buffer- pH 5.0) was incubated at 40°C for 30 min. The reaction was stopped by the addition of 2 ml of 3, 5-dinitrosalicylic acid, followed by boiling for 10 min to develop color. The absorbance of the mixture was measured at 540 nm, and enzyme activity was calculated.

Identification of microorganisms by morphological and biochemical test The colony characteristics of the test organism was observed and recorded.

Gram staining: A thin smear of the pure culture was made on a clean, grease free glass slide, heat fixed. Crystal violet was added, left for 1min followed by water wash, added grams iodine for 1min and washed, 70% alcohol for 30 sec, washed and added a counter stain saffrainin for 30 sec and observed under oil immersion.

Methyl red – Voges proskauer: MRVP broth was autoclaved and about 10ml of broth was taken in four test tubes. Labelled as MR control, MR test, VP control, VP test. The test organism was inoculated into the MR test and VP test tubes, all the tubes were incubated at 37ºC for 24 – 48 hrs. After the incubation time methyl red solution was added to the MR test and control tubes. For VP, Barrets reagent I (40% KOH) and Barrets reagent II (α-naphthol in 95ml of 95% alcohol) was added and observed for the results.

Catalase test: The container containing Hydrogen peroxide solution was shaken to expel the dissolved oxygen. One drop of the solution was dropped on a clean glass slide followed by the addition of a loop-full 24-hour old inoculum on the slide. The presence of gas bubbles indicated a positive test while the absence of gas bubbles indicated negative reaction.

Citrate utilization: The media (Simons citrate media) was prepared, autoclaved and two slants were prepared out of which one was labelled as control and another as test. The tube (test) was inoculated with the test organism and incubated for 2 days to observe the results.

Spore stain

A drop of distilled water was placed on a clean grease free glass slide and a colony from the isolate was picked with a sterilized wire loop and emulsified. The glass slide was passed over the flame three times to heat fix. The smear was flooded with malachite green and allowed to heat for 3-5 minutes, the stain was rinsed off with tap water. Safranin was used to counter stain the smear and allowed to stand for 60 seconds. The slide was then rinsed to remove the secondary stain and allowed to air dry. It was observed under the microscope using x 100 objective (oil immersion). The vegetative cells appeared pink, while the spores appeared green in colour.

Starch hydrolysis

A starch agar was prepared, containing : g/500ml: peptone (5.0), sodium chloride: (5.0), yeast extract (1.5), beef extract (1.5), soluble starch (1.5) and agar-agar (15). A single was streak inoculation of the organisms was made at the centre of the labeled plates. The plates were incubated for 48 hours at 37°C. Following incubation, the plates were flooded with iodine solution using a dropper and allowed to stand for 30 seconds. Excess iodine was poured off and the plates were examined for clear zones around the bacterial growth. a positive test was indicated by clear zones around the line of growth showing that the organism hydrolyzed the starch.

Motility Test

A semisolid agar was prepared in a test tube and sterilized by autoclaving. It was allowed to cool and then it was inoculated with the test organism using a straight wire by making a stab down the centre of the tube to about half the depth of the medium. It was incubated at 37°C and examined at time intervals: 12 hours, 24, 36 and 48 hours . the non motile bacteria had growth confined to the stab line, having sharply defined margins and leaving the surrounding medium

very transparent. The motile bacteria didn't grow on a confined stab line but had hazy growth that spread throughout the medium, rendering it slightly opaque.

Molecular identification of the pullulanase producing isolates

Extraction of DNA

Hundred (100) mg of the bacterial cell was added to 200µl of isotonic buffer. The solution was mixed in a bashing lysis tube. Seven hundred and fifty (750) µl of lysis solution was added to the tube. The resulting solution was filled with bashing beads and mixed using a vortex mixer for five minutes. The solution contained in the tube was then centrifuged for 1 minute at 10000 x g using a microcentrifuge.400 µl of the supernatant was transferred to a spin filter in a collection tube and centrifuged at 7,000 x g for 1 minute. One thousand, two hundred (1200) µl of bacterial DNA binding buffer was added to the filtrate in the collection tube. 800µl of the mixture was transferred to a spin column in a collection tube and centrifuged at 10,000 x g for 1 minute, this procedure was repeated then, 200 µl of DNA pre-wash buffer was added to the zymo spin in a new collection tube and centrifuged at 10,000 x g for 1 minute. Then, 500 µl of the bacterial DNA wash buffer was added to the Zymo spin column and centrifuged again at 10,000 x g for 1 minute. The zymo spin column was transferred to a clean 1.5ml microcentrifuge tube and100 µl of DNA elution buffer was added directly to the column matrix. It was centrifuged at 10,000 x g for 30 seconds to elute the DNA. The DNA was then suitable for PCR and other downstream applications.

Amplification of DNA by polymerase chain reaction (PCR)

The PCR cocktail mix consisted of: 2.5µl of 10x PCR buffer, 1µl if 25mM $MgCl_2$,1µl each of forward primer (27F TCCTCCGCTTATTGATATGS) and reverse primer(1535R GGAAGTAAAAGTCGTAACAAGG), 1µl of DMSO, 2µl of 2.5m MDNTPs, 0.1µl of 5 µ/µl Taq DNA polymerase, and 3µl of 10ng/µl DNA. The total reaction volume was made up to 25µl using 13.4µl nuclease free water. Initial denaturation of the DNA was done at 94°C for 5minutes, followed by 36 cycles of denaturation for 94°C for 30 seconds, annealing was done at 54°C for 30 seconds and elongation was done at 72°C for 45 seconds. This was followed immediately by a final elongation at 72°C for 7 minutes and its hold-temperature was at 10°C. Amplified fragments were visualized on safe-view stained 1.5% agarose electrophoresis gels. The size of the amplicon is about 650bp and the DNA ladder from NEB.(IITA, Ibadan).

Sanger Sequencing of Amplified DNA

A GeneAmp PCR system 9700 was used for the sequencing. The tubes, which contained the products from the PCR amplification were placed in a thermal cycler and set to the correct volume. An initial denaturation was performed at 96°C for 1 minute. Then, the following was repeated for 25 cycles: a rapid thermal ramp at 96°C for 10 seconds, a rapid thermal ramp of 50°C for 5 seconds, a rapid thermal ramp of 60°C for 10 minutes.

In order to purify, the 96-well reaction plate was removed from the thermal cycler and briefly spun**.** Five**,** 5µl of 125mM EDTA was added to each well. 60µl of 100% ethanol was added to each well. The plate was sealed with aluminium tape and mixed by inverting it four times. It was then incubated at room temperature for 15 minutes. A Beckman Allegra 6A centrifuge was used to spin the plate at 1650 x g for 45 minutes at 4°C. The plate was inverted and spinned up to 185 x g, then removed from the centrifuge and 60µl of 70% ethanol was added to each well. The mixture was spun at 1650 x g for 15 minutes at 4°C. The plate was inverted and spun up to 185 x g for 1 minute, then it was removed from the centrifuge and stored at 4°C.

RESULTS AND DISCUSSION

Isolation, morphological and biochemical identification of pullulanase producing bacteria:

Forty bacterial isolates were obtained from the different cassava processing sites. The identification of the isolates obtained from different cassava processing sites which was done through primary screening on pullulan media showed five of the isolates to possess pullulan degradation potential by forming zones of clearing on the pullulan media plates. These isolates were identified by employing morphological and biochemical identification test procedures, which revealed the presumptive organism to be *Bacillus* sp (Table 1). Similar reports have been made [10], where different Bacillus species showed varying degrees of pullulan degradation. Cassava wastes from processing sites are rich sources of different microorganisms with many industrial advantages.

Table 1: Biochemical Identification of pullulanase producing isolates

Isolate No	Pul Degrd(mm)	Starch hydrolysis (mm)	Catalase	VP	Motility	Citrate
Bp1	9.00	10.00	+	-	-	+
Bp2	15.00	15.00	+	+	+	-
Bp3	13.00	11.00	+	+	+	+
Bp4	10.00	13.00	+	-	-	+
Bp5	6.00	9.00	+	+	-	-

Pullulanase Production and Assay

The production and assay of pullulanase for the isolates (Table 2) which showed pullulan degradation potential revealed isolate BP2 and BP3 as the isolates with the highest pullulanase activity (0.62U/ml and 0.53U/ml). These two isolates were selected for molecular identification. [2] reported a similar findings in which Bacillus sp was found to produce pullulanase enzyme in large quantity.

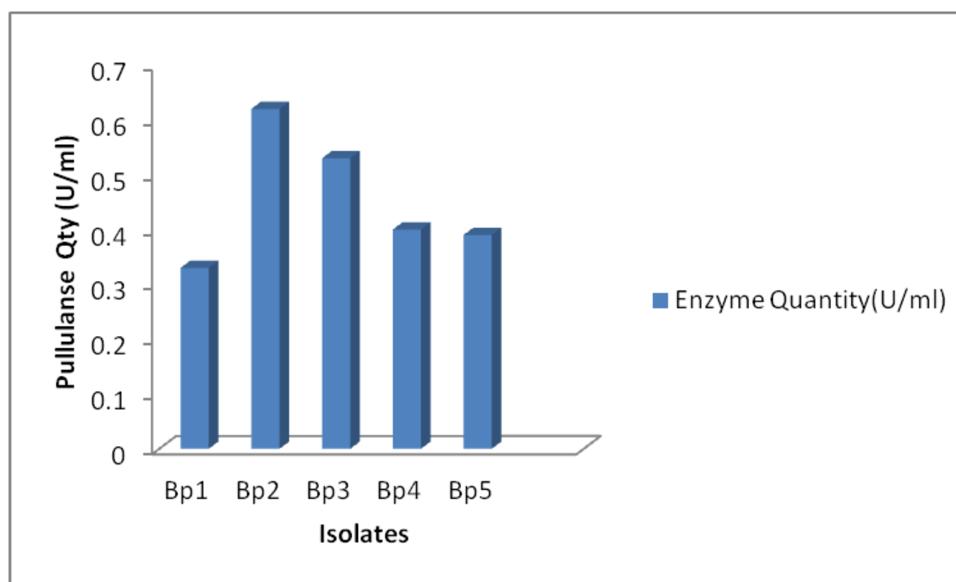

Fig 1: pullulanse Quantity in choice isolates

Molecular identification of pullulanase producing bacteria

In 1% agarose gel electrophoresis, 1500bp band was obtained by PCR amplification (Plate 1). Also partial sequencing of 16SrRNA was done to identify the isolate. BLAST analysis of the sequence data revealed that the isolates showed closest similarity (99%) with *Bacillus thuringiensis* and *Bacillus cereus*.

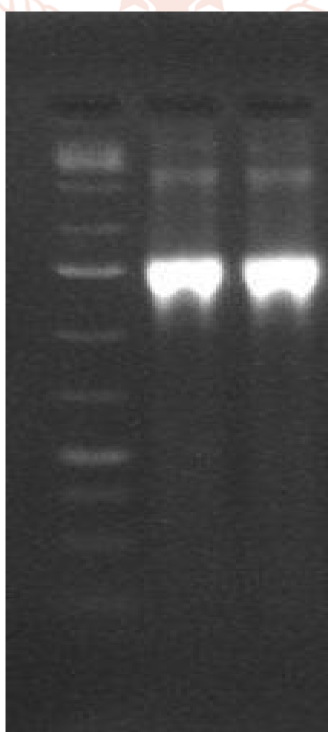

Plate 1: PCR on gel electrophoresis for *B. cereus* and *B. thuringiensis*

Phylogenetic analysis: the phyletic relationship between the Bacillus species. *Bacillus cereus* was clustered towards *B. mycoides*, while *B. cereus* clustered towards *B. weidmanni.* This shows great similarity between the species (Fig 1). The Bacillus species possessed separate branches, representing different species of different strains within a group of Bacillus genus, therefore indicating a level of singularity in the different identities.

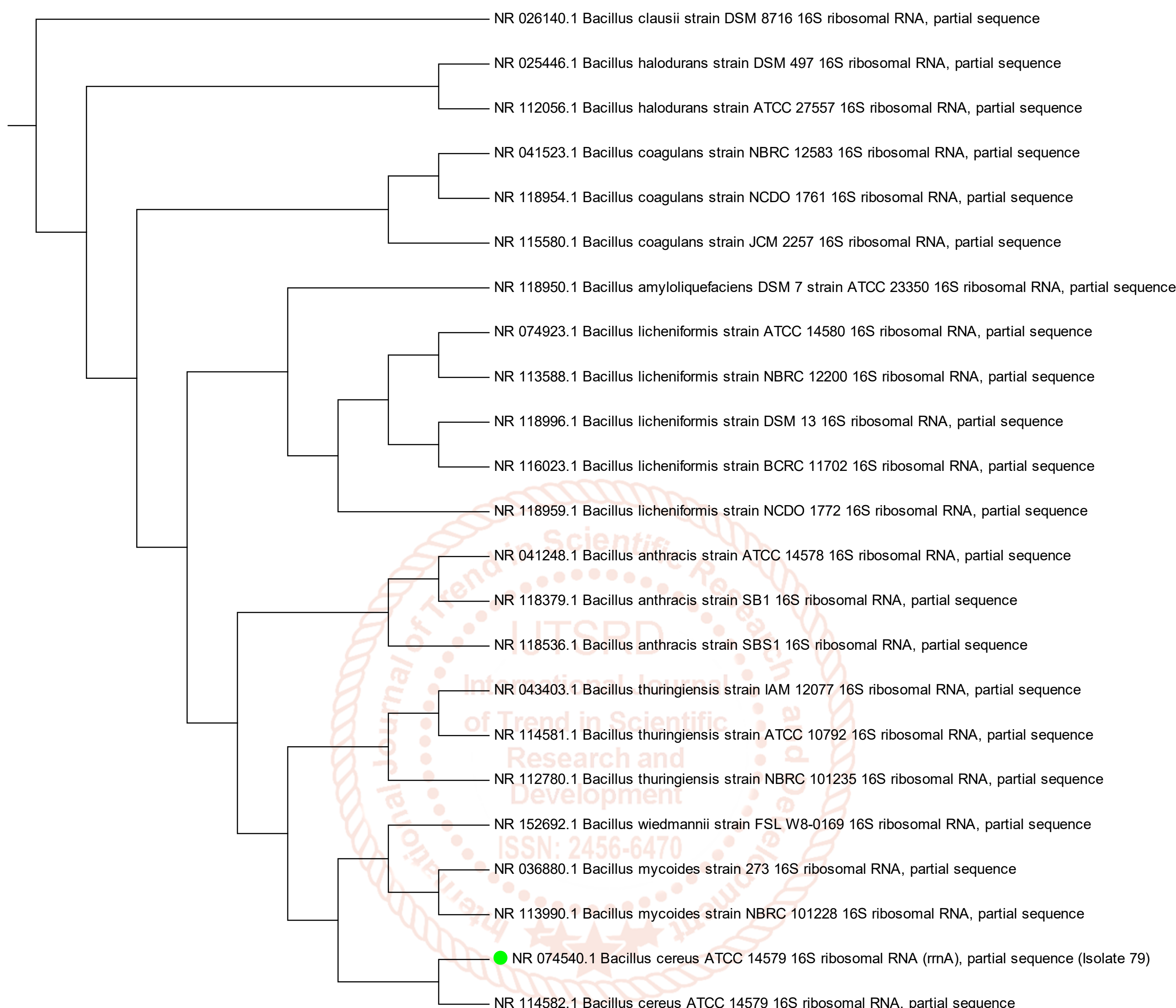

Fig 2: Phylogenetic tree of *Bacillus cereus*

CONCLUSION

Biochemical and Molecular identification tests confirmed the presence of pullulanase-producing Bacillus species: *B. thuringiensis* and *B.cereus* from cassava processing sites. Enzyme assay confirmed these organisms as producers of the pullulanase enzyme in high quantites. This research has therefore shown that the production of extracellular enzyme, pullulanase by these *Bacillus* species is of great value for many industrial processes.

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