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Original Article

α- and β-cyclodextrin production by cyclodextrin glucosyl transferase from *Bacillus* species using different carbon substrate

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ABSTRACT

α- and β-cyclodextrin (α-CD and β-CD) are compounds of great application in food, pharmaceutical, agricultural, and chemical industry. They are produced by action of starch substrates on CD glucosyl transferase (CGTase) which is an enzyme produced by *Bacillus* species. The objective of this study was to isolate three *Bacillus* species and determine the effect of different starch substrate of cassava, sweet potato, and cocoyam on the activity of CGTase and production of α-and β-CD. The three *Bacillus* species isolated were confirmed using polymerase chain reaction-16sr RNA sequencing and bio-informatics as *Bacillus cereus*, *Bacillus thurengiensis,* and *Bacillus licheniformis*. CGTase was successfully produced from the three *Bacillus* species identified. The results projected cassava starch as a better substrate for CGTase and CD production than sweet potato and cocoyam starches. NXS 003 (Nigerian *Xanthosoma* specie) was a better substrate than sweet potato and *edeuhie* (red cocoyam). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis result identified lower molecular weight CGTase in the three *Bacillus* species which ranged from 21.04 to 43.29kDa. CGTase from *B. licheniformis* produced highest level of α-CD and β-CD when cassava (30572 and 419) and sweet potato starch, respectively, were used as carbon source. CGTase from *B. cereus* produced highest level of α-CD and β-CD in *eheuhie,* while CGTase from *B. thurengiensis* showed highest α-CD and β-CD level in *edeuhie* and Um37.

Keywords: *Bacillus*, cyclodextrin glucosyl transferase, enzyme, starch, α-and β-cyclodextrin

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INTRODUCTION

Cyclodextrin glucosyl transferase (CGTase) is an enzyme that converts starch into cyclodextrins (CD) which are closed-ring structures having six or more glucose units joined by means of α-1, 4 glucosidic bonds. CGTase is assessed within the α-amylase family and is known to catalyze four different transferase reactions: Cyclization, coupling, disproportionation, and hydrolysis Three major sorts of CDs are produced by CGTase counting on number of glucose units, α-CD, β-CD, and γ-CD.[1] β- CD has the smallest amount solubility and may be easily obtained in pure form by selective precipitation and the most industrial applications are targeting β-CD. CD molecules have a singular structure of hydrophobic cavity and hydrophilic surface. Due to this feature, CDs form inclusion complex with a good sort of organic compounds[2] and increasing their water solubility and stability. These properties made CDs as important materials as molecular encapsulator for applications in food, pharmaceutical, dairy, and cosmetic industries.[3] A number of the physical and chemical changes caused by complexation with CD are solubilization of lipophilic compounds, stabilization of reactive compounds, removing bad smell and taste, fixation of volatile compounds, and controlled release of compounds.[4] Within the typical process for CD production by CGTase, the starch slurry is gelatinized by heat treatment and liquefied using CGTase or amylase and, therefore, the liquefied starch is cyclized by CGTase to produce CD.[5] CGTase produces α-, β-, and γ-CDs from starch in several ratios depending on the characteristics of CGTase and also the reaction conditions.

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CGTase producing bacteria is often found in various places such as soil, waste, plantation, hot springs, and even in deep sea mud. CGTase is produced by differing types of bacteria. Major CGTase producers belong to the genus *Bacillus*. However, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Thermococcus*, *Brevibacterium* sp., and hyperthermophilic archaea are reported as CGTase producers.[6] The consumption of CD is increasing worldwide at a far better rate. Use of CDs has increased annually around 20–30%, of which 80–90% is employed in food industries. CDs are utilized in produce low-cholesterol butter, utilized for flavor stabilization, and delivery in chewing gum, flavored tea, lemon, and grapes fruit candies.[7] CD is employed in fruit crush beverages to mask odors and to mask bitterness in beverages. It is used to convert ethanoic acid to powder. It also increases the solubility of sauce.[8] However, the cost of production is a limiting factor for the extensive industrial applications of CD.[9] Hence, many efforts are made to improve production of these cyclic oligosaccharides with a cost-effective method.

CDs are used as drug carriers and tableting vehicles. They are applied to reduce the bitter or aggravating taste and bad odor of medicine. Works within the past with β-CD within the area of flavor binding have checked out selectivity, separation retention, and stability of aroma compounds.[9] Binding of aroma (guest) compound to the β-CD molecules (host) causes the formation of an inclusion complex. Investigations into the discharge of compound from β-CD have been mainly supported formal (and informal) sensory observation.[10] CDs are declared to be "Generally Recognized as Safe" and have not any adverse effects on the absorption of certain nutrients.[11] CGTase enzyme is industrially a very important enzyme because of its application in production of CD. However, there are two limitations associated with solvent method of CGTase production. The yield of CD is low, thus cost is high and solvents that are used to precipitate CDs are highly toxic.[12]

CGTase from alkaliphilic *Bacillus* species was the first bacteria that lead to mass production of α-CD, β-CD, and γ-CD by overcoming these problems.[12] Since then, it is known that alkaliphiles are candidate organisms for CGTase production. Hence, this will reduce cost of production, increase the yield and produce nontoxic CD for food applications. The bacterial strain *Bacillus macerans* is the foremost often used source of the CGTase enzyme. Various sorts of starch are often used as a substrate for CGTase including corn and potato starches. Starch consists of amylopectin and amylose, but amylopectin gives higher yields than amylose, because the reaction with CGTase begins at the non-reducing end of the starch molecule.[13] This work reports isolation and identification of latest *Bacillus* species from umudike soil using different starch source: Three cassava varieties (30572, 419, and umu 37), one sweet potato root (x-igbariam) and two cocoyam varieties (edeuhie and NXS003), and its activity during production of α- and β- CD.

MATERIALS AND METHODS

Isolation of the CGTase Producing *Bacillus* Species

The method described in[14] was used for the isolation of CGTase producing bacillus species. Soil sediment and water sample were collected from different locations at Michael Okpara University of Agriculture, Umudike. The samples were collected in sterile test tubes, transferred to laboratories and kept in refrigerator at 4°C. Both dilution plate and enrichment methods were used. For the enrichment method, exactly 1 g of sample was subjected to heat treatment in water for 30 min at 90°C in a water bath to kill most of the vegetative cells and thus to eliminate non-spore forming bacteria. After heat treatment, the soil sample was serially diluted, spread plated on nutrient agar, and incubated at 30°C for 48 h. The colonies that came up on agar plates were purified by picking single colonies with different morphologies and purified using streak plate method and stored at −80°C in nutrient broth containing glycerol. The culture was maintained on nutrient agar pH10.

Screening for CGTase – Producing Bacteria

The isolated organisms were screened for aerobic alkaliphilic CGtase- producing bacteria using direct and indirect methods. The direct method for screening for CGTtase activity was carried out using Horikoshi II agar medium containing 0.02% (w/v) phenolphthalein. This method depends on the fact that the CGTase released to the surrounding alkaline medium converts starch to CD that forms inclusion complex with phenolphthalein, resulting in a color change that is detected as a halo zone around the colonies of the CGTase-producing strains.[15] The Horikoshi II agar medium (pH10.5) contained soluble starch (10 g/l), yeast extract (5 g/l) polypetron 95 g/l), $MgSO_4 \times 7H_2O$ (0.2 g/l), k_2HP0_4 (g/l), Nacl (5 g/l), Na_2CO_3 (10 g/l), and agar (15 g/l).

The isolated organism was serially diluted up to 10^{-5} and 0.5 ml of each dilution was spread on the surface of Horikoshi II agar medium containing 0.02% w/v phenolphthalein. The plates were incubated for 5 days at 37°C and the colonies that were surrounded by a halo zone resulting from the cyclodextrine – dye complex were selected for further examination.

In screening for CGTase producers using the indirect method, alkaliphilic bacteria with starch degrading enzymes were first isolated using Horikoshi II agar medium (without phenolphthalein) after 5 days of incubation at 37°C, the plates were stained with an iodine solution to detect starch hydrolysis, which appears as a clear zone around growing bacteria.[15] Next, the starch – degrading enzyme – producing stain was further screened for CGTase activity using Horikoshi II agar medium containing phenolphthalein (0.2%w/v) as described above.

Bacteria Identification

DNA isolation and identification

Pure genomic DNA was isolated from CGTase – producing bacteria following the method of.[16] Briefly, the culture was grown overnight in 3 ml nutrient broth with shaking at 30°C. A 1.5 ml of the culture was centrifuged at 12000 rpm for 10 min and the result and pellet were resuspended in 567 µl × TE butter (10mM tris pH 8.0, l mM EDTA). Proteinase K and sodium dodecyl sulfate (SDS) were added to final central of 100 µg/ml and 0.5%, respectively, and incubated at 37°C for 1 h after incubation, Nacl (5 m) and CTAB/Nacl (10%w/v ethyl trimethyl ammonium bromide in 0.7 m Nacl were added and incubated at 65°C for 10 min. The mixture was extracted once again with an equal volume of chloroform-isoamyl alcohol (24:1) and phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated from the aqueous phase using 0.6 volumes of isopropanol and washed once with 70% ethanol. The DNA pellet obtained after final centrifugation was vacuum dried and dissolved in 50 µl 1 × TE buffer. DNA quantification was done using a UV-1601 spectrophotometer (Shimadzu Corporation, Japan).

Molecular identification of three Bacillus species

For identification of the selected CGTase-producing alkaliphilic bacteria, polymerase chain reaction (PCR) amplification of genomic DNA of three strain (B1, B2, and B3) was done using universal bacteria forward primers 16F27 (5-AGAGTTTGATCMTGGCTCAG G-3) and a reverse primer,16r1525(5-AAGGAGGTGWTCCARCCGCA-3).[17] The 10 µl reaction mixture contained 10 mg of genomic DNA, 0.5 µM each primer, and PCR super mix high fidelity (Taq and Go, Qbio gene, Iilkirch, France) Table 1. The amplification reactions were performed in a peltier PTC-2000 thermal cycler with the following conditions: Table 2. As molecular marker, the GeneRular™100 bp DNA Ladder (MBI Fermentas) was used. The primer pair covered V4 region (approx. 600 base pair) of the DNA fragment.

Table 1: PCR-blast identification of *Bacillus cereus*

Scientific name of organism	Nucleotide Assembly contig	Reference Coding	NCBI Accession number	Taxanomy ID	Lineage
Bacillus cereus VD102	AHEW01000010	EJR49530	IIK_02385	1053228	-Cellular organism-*Bacteria-Firmicutes-Bacilli-Bacillaceae-Bacillus-Bacillus cereus group-Bacillus cereus*
Closest relatives *Bacillus pumilus*	A0A0S3NX16_ BACPU	TUAT1	AKO65_00605	1408	Cellular organisms›*Bacteria*›Terrabacteria group›*Firmicutes*›*Bacilli*›*Bacillales*›*Bacillaceae*›*Bacillus*
Bacillus sp Bacillus simplex	A0A120GN05-1	B14905	BB14905_05738 AS888_10360	388400 1478	Cellular Organisms›*Bacteria*›Terrabacteria group›*Firmicutes*›*Bacilli*›*Bacillales*›*Bacillaceae*›*Bacillus* Cellular Organisms›*Bacteria*›Terrabacteria group›*Firmicutes*›*Bacilli*›*Bacillales*›*Bacillaceae*›*Bacillus*›*Bacillus cereus group*›

PCR: Polymerase chain reaction, NCBI: National Centre for Biotechnology Information

Table 2: PCR-blast identification of *Bacillus thuringiensis*

Scientific name of organism	Nucleotide Assembly contig	Reference Coding	NCBI Accession number	Taxanomy ID	Lineage
Bacillus thuringensis	A0A0N7JLM0	DUF4183	BTXL6_21680	1428	Cellular organism-*Bacteria-Firmicutes-Bacilli-Bacillaceae-Bacillus-Bacillus cereus* group-*Bacillus thuringensis*
Closest relatives *Bacillus cereus*	A0A0S3NX16_ BACPU	*MSX-D12*	II9_03080	1053222	Cellular Organisms›*Bacteria*›Terrabacteria group›*Firmicutes*›*Bacilli*›*Bacillales*›*Bacillaceae*›*Bacillus*›*Bacillus cereus group*›*Bacillus cereus*
Bacillus mycoides *Bacillus anthracis*	A0A0B5S7A6 A0A120GN05-1	B14905 ABW01_ 28365	BG05_1517 A0A0J1HK80	1405 1392	Cellular organisms›*Bacteria*› *Terrabacteria group*›*Firmicutes*›*Bacilli*›*Bacillales*›*Bacillaceae*›*Bacillus*›*Bacillus cereus group*›*Bacillus cereus* Cellular Organisms›*Bacteria*›Terrabacteria group›*Firmicutes*›*Bacilli*›*Bacillales*›*Bacillaceae*›*Bacillus*›*Bacillus cereus group*›*Bacillus cereus*

PCR: Polymerase chain reaction, NCBI: National Centre for Biotechnology Information

The obtained PCR products were analyzed through 0.8% w/v agarose gel electrophoresis. The amplified 16 s products where sliced out of the agarose gel with a sterile razor blade, and the DNA was purified from the agarose using the QIA quick Gel extraction Kit (Qiagen, Valencia, CA), following the manufacturers instruction. The purified DNA was sequenced in AB1377 automated sequencer using the PRISM ready reaction kit (Applied Biosystem, Foster City, CA). The sequence data were compared with the BLAST program available at Gene Bank of the National Centre for Biotechnology Information (http//www.ebi.ac.uk/tools/sss/psisearch). The amino acid and nucleotide sequence we obtained were compared with other sequence using the BioEdit 7.01 program.

Production of CGTASE

Three *Bacillus* species (B_1, B_2, and B_3,) identified in plate 2, 3, and 4 were inoculated into nutrient medium containing (g/l) soluble potato starch 10, peptone 10, yeast extract 5, and sodium chloride 5, for production of CGTase. The pH was maintained at 10 by addition of sterile 10% sodium carbonate after autoclaving. The organism was cultivated in 250 ml flasks containing 50 ml. medium incubated in an orbital shaker at 15 rpm at 30°C for 24 h. After 24 h, the broth was centrifuged at 10,000 g at 4°C for 20 min. The cell free supernatant was used as crude enzyme.[18]

Enzyme assay

The assay for CGTase was performed using phenolphthalein reagent as described by.[18] Exactly 100 ml of crude enzyme extract was added to 1 ml of 1% soluble potato starch in 0.005 M Tris-HCL butter pH 8.5 and incubated at 60°C for 20 min.

After incubation, the reaction mixture was cooled in ice. 4 ml of 1 mm phenolphthalein reagent was added to the tubes and the absorbance measured immediately at 550 nm. One unit of CGTase activity was defined as the amount of enzyme released by 1 µg β-CD per min under the defined assay condition.

Electrophoresis analysis of the isolated CGTase enzyme

Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dedecyl SDS–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of.[19] The experiment was carried out in reductive and non-reductive condition in parallel.

CD Production

This was done according to the method of.[20] The starch produced from cassava varieties (30572, 419, and umu 37), sweet potato (*x-igbariam*), and cocoyam (*edeuhie* and NXS003) containing mainly amylose and amylopectin was used as source of carbon for the production of CD. Substrate solution was prepared in phosphate buffer pH7.0, in a manner allowing the desired concentration to be reached after addition of the dissolved enzyme (CGTase). Different isolated starch samples were dissolved in a steam water bath. The resultant substrate solution was cooled to 45°C and the necessary CGTese was added. The enzyme reaction was conducted in 100 ml Erlenmeyer flasks containing 50 ml of the reaction medium at 45°C on a reciprocal shaker for 20 h. Samples were taken and the α and β-CD analyzed.

Measurement of β-CD

The concentration of β-CD was analyzed by decrease in absorbance at 550 nm due to phenolphthalein β–CD complex formation[18] with modification. To 0.2 ml of β-CD solution, 0.05 mmol/L Tris-HCL buffer, and pH 8.0, 1.0 ml of the phenolphthalein working solution were added.

The tube was mixed by vortexing and absorbance was measured immediately. The working solution was prepared by diluting 2 ml of 3 mmol/L phenolphthalein in ethanol (stock solution) to 100 ml with 125 mmol/l $NaCO_3$/$NaHCO_3$ buffer, pH 10.5.

Measurement of α-CD

The concentration of α-CD was assayed by the[21] method. Exactly, 1.0 ml amount of 1% soluble starch prepared in 50 mol/l phosphate buffer pH7 was added with 0.1 ml of properly diluted CD and incubated at 40°C for 10 min. The reaction was stopped by immediately cooling the tube with water followed by addition of 0.1 ml of 1.2 mol/L Hcl then 2 ml of methyl orange solution with concentration of 0.035 mol/L was added to the mixture and the tubes were maintained at 115°C for 30 min. The absorbance was measured at 507 nm and its decrease in absorbance (with respect to a control tube without enzyme) was correlated with the amount of α-CD. One unit of enzyme was defined as the amount of enzyme that produced 1 µmol of α–CD/min under standard conditions. The colorimetric determination of α- and β-CD of dye that undergoes spontaneous decolorization. The absorbance difference α-CD was analyzed to obtain β-CD.

α- and β - CD were calculation as follows:

Dye (D) + CD ---------- complex (1)

Kd is given by [D] [CD]/[C], where/[ ]denotes concentration of the indicated species. Since

[DE] = [D] + [C], and [CDt] = [CD]+[EC] (2)

Kd can be written as:

kd = [Dt]–[C] [CD]/[C] (3)

Assuming that the δA is directly proportional to [C] and Amax as the limiting δA under [CDt]>>[C], we can obtain the appropriate equation:

$$\delta A = (\delta Amax)\ [CDt]/(kd+[CDt]) \quad (4)$$

The fit of δA versus [CDt] at the appropriate wavelength gives Kd and δAmax, this was extrapolated from the standard graph.

α- and β-CD standard curve

α- and β- CD standard curve was plotted using different concentration of α- and β- CD (0.25, 0.5….2.0 and 0.5, 0.1, 0.15….0.4, respectively) from Sigma Aldrich company with cat no: C4642-1G and W4032826-25G, respectively. Absorbance at 507 and 550 nm for α- and β-CD was used to plot a graph of concentration against absorbance. The intercept of the graph fitted the standard equation.

RESULTS AND DISCUSSION

Isolation of Three *Bacillus* Species

The soil samples produced a yellow hollow different types of colonies on the agar plates with different morphologies. Five isolates achieved growth in pH range 7–11 and in temperature range 10–45°C. Three *Bacillus* species showed largest zone of hydrolysis and designated B1, B2, and B3. These isolates were confirmed by 16S rDNA sequence and bio-informatics analysis as *Bacillus cereus*-B1, *Bacillus thurengiensis* -B2, and *Bacillus licheniformis*-B3.

16sr RNA analysis result for three *Bacillus* spp.

PCR amplification of genomic DNA with universal primers specific for 16sr RNA amplification was successful and the product when bidirectionally sequenced with 16s specific primers, the data aligned to the closest homology of each samples. Based on Bioedit ABI chromatogram, nucleotide homology and blast alignment for B1 with accession number 11k_02385 and taxonomy ID 1053228 were detected from Gene bank to be *B. cereus* as shown in Table 1. B2 was detected to be *B. thurengiensis* with accession number BT1158_13250 and taxonomy number 529122, as shown in Table 2 while B3 was detected to be *B. licheniformis* with accession number Bali_ C0930 and taxonomy ID 766760 as shown in Table 3.

Effect of Various Carbon Sources Starch from Cassava: 419, UM37, 30572, Sweet Potato (X-Igbariam), and Cocoyam (NXS003, Edeuhie) on CGTASE Activity of Three *Bacillus* Species

The effect of various carbon sources (starch from cassava: 419, um37, 30572, sweet potato (*x-igariam*), and cocoyam: nxs003, *edeuhie*) on CGTase activity of three *Bacillus* species is shown in Table 4 that shows cassava posed a far better carbon source for the three *Bacillus* species for production of CGTase than sweet potato and cocoyam starch source. Cassava sample um37 and 419 recorded the very best enzyme activity 1.033u/ml and 1.039μ/ml, respectively, for *B. thurengiensis* and

Table 3: PCR-blast identification of *Bacillus licheniformis*

Scientific name of organism	Nucleotide Assembly contig	Reference Coding	NCBI Accession number	Taxanomy ID	Lineage
Bacillus licheniformis	Q65MH8	*ATCC 14580*	BL03073	279010	-Cellular organism-Bacteria -*Firmicutes-Bacilli -Bacillaceae-Bacillus -Bacillus subtilis group-Bacillus licheniformis*
Closest relatives *Bacillus licheniformis*	A0A0H4X901	*WX-02*	MUY_000843	1126218	-Cellular organism-Bacteria-*Firmicutes -Bacilli-Bacillaceae-Bacillus-Bacillus subtilis group-Bacillus licheniformis*
Bacillus paralicheniformis	R9TQT8	*ATCC 9945a*	BaLi_c09040	766760	Cellular organisms›*Bacteria*›Terrabacteria group›*Firmicutes*›*Bacilli*›*Bacillales*›*Bacillaceae*›*Bacillus*›*Bacillus subtilis group*›*Bacillus paralicheniformis*

PCR: Polymerase chain reaction, NCBI: National Centre for Biotechnology Information

Table 4: Effect of different carbon sources from cassava: 419, um 37, 30572, sweet potato (X-igariam) and cocoyam (nxs003, *edeuhie*) on CGTASE activity of three *Bacillus* species

Bacillus spp	419	30572	Um37	Pot.	ede	Nxs
Bacillus cereus	0.715^{c}	0.406^{c}	0.850^{a}	0.621^{a}	0.482^{b}	0.703^{a}
Bacillus thuringiensis	0.860^{b}	0.704^{b}	1.033^{a}	0.418^{b}	0.319^{c}	0.658^{b}
Bacillus licheniformis	1.039^{a}	0.777^{a}	0.874^{b}	0.343^{c}	0.711^{a}	0.553^{c}

419, um37, and 30572 are cassava starch, ede (*edeuhie*) and Nxs (*xanthosoma*) are cocoyam starch, pot (*x-igbariam*) is white skinned sweet potato.

B. licheniformis. *B. licheniformis* has highest CGTase activity (1.039 μ/ml) in cassava sample 419 and lowest activity in sweet potato starch (0.343 μ/ml).

B. thurengiensis recorded its highest CGTase activity in cassava sample 37 um (1.033 μ ml) and lowest in sweet potato starch (0.418 μ/ml). *B. cereus* had its highest activity in cassava sample um37 (0.850 μ/ml) and lowest in cassava sample 3072. Cassava has proved to be a far better carbon source for production of CGTase enzyme. Nxs 0003 (Nigerian *Xanthosoma* specie) showed a far better carbon source than sweet potato starch and *edeuhie* (red cocoyam). The rationale might be due to the lower amylopectin content of sweet potato starch in comparison with cassava and NXS003. Amylopectin gives higher CGTase activity than amylose because the reaction with CGTase starts from the non-reducing end of starch molecules.[13] Enzyme activity of 2.2 u/mL was reported for *Bacillus* species from soluble starch in.[22] *Bacillus* spp. has been previously reported for CD and CGTase production by.[23] This result is in line with the work of[24] who observed highest CGTase activity of *Bacillus megaterium* in cassava medium followed by soluble starch medium. Differing types of starch are often used as substrate for CGTase production. Potato starch has reportedly been used for CGTase production. Maize and wheat starches gives low yield of CGTase due to its high amylose content.[6] The results of this work projected cassava as a far better substrate. This might be as a result of its high amylopectin and low liquefaction temperature in cassava starch.[25]

SDS-PAGE Result for Relative Molecular Mass (MW) of CGTASE from Three *Bacillus* Species

SDS-PAGE proved to be a reliable method for determining the relative MW of an unknown protein. Three samples of CGTase from three *Bacillus* species (*B. cereus*, *B. thurengiensis,* and *B. licheniformis*) were produced using cassava. The CGTase produced was successfully purified by separating its protein on an equivalent gel with a group of molecular standards. Next, a graph of log MW versus relative migration distance RF was plotted which supports the values obtained for the bands within the MW standard. The MW of the unknown protein band was then calculated by extrapolation using these graphs. The key to work out MW accurately is chosen separation conditions which will produce a linear relationship between log MW and migration within the likely MW region of the unknown protein.

The result SDS-PAGE in Table 5 recorded highest relative MW in *B. thurengensis* (43.29kDa) at $r^2 = 0.998$ while *B. cereus* had lowest relative MW (21.04kDa) at $r^2 = 0.998$.[26] Estimated the MW of purified CGTase from *Amphibacillus* spp. NRC-wn as 36kDa through SDS-PAGE which makes it one among the least CGTtase reported in the literature. This work achieved a way lower relative MW in *B. cereus* (21.04kDa)[27] described a CGTase from *Amphibacillus* spp NPST-10 strain with relative MW of 92kDa. Most CGTase display a high MW between 60 and 110kDa.[28,29] However, a couple of CGTase with a lower MW is reported like 56kDa CGTase from *Bacillus sphaericus* strain 41.[30]

α- and β-CD Standard Curve

1. The experimental data in fitted the equation of β-CD standard curve

$$\delta A = (0.800 \times [\beta\text{-CD}])/(0.428 + [\beta\text{-CD}]) \quad (8)$$

2. The experimental data fitted the equation of α-CD standard curve

$$\delta A = (0.177 \times [\alpha\text{-CD}])/(0.130 + [\alpha\text{-CD}]) \quad (9)$$

Effect of Carbon and CGTASE Source on Levels of α- and β-CD

Figures 1 and 2 shows the effect of carbon and CGTase source on the levels of α- and β-CD. α-CD was highest in Ede 2 (0.1497 δA) and lowest Nxs 1 (0.1380 δA). CGTase from B.cereus produced highest α-CD in Ede 1(0.1486 δA), CGTase from *B. thurengiensis* produced highest α-CD in Ede 2 (0.1497 δA) while CGTase from *B. licheniformis* produced highest α-CD in 30572-3 (0.1460 δA) and 419-3 (0.1459 δA). This report shows that CGTase from *B. licheniformis* produces higher level of α-CD when cassava is employed as carbon source.

β-CD showed its highest level in pot 3 (0.5776 δA) and lowest in um37-1 (0.4354 δA). CGTase from *B.cereus* produced highest β-CD in Ede 1 (0.4999 δA), CGTase from *B. thurengiensis* produced highest β-CD in um37-2 (0.5216 δA) while CGTase from *B. licheniformis* produced highest β-CD in pot 3 (0.5776 δA). β-CD was predominant of the three *Bacillus* species analyzed.

This result is in line with the report of[25] which indicated that β-CD was the predominant product of the *Amphibacillus* sp. NRC-WN CGTase, followed by α- and γ-CD. Because the

Table 5: SDS-PAGE result showing molecular weight (mw) of CGTase from three *Bacillus* species

CGTase source	Equation	Molecular weight (kDa)	r^2
Bacillus cereus	−0.774×+1.721… (Equation 5)	21.040	0.998
Bacillus thurengensis	−1.334×+2.234… (Equation 6)	43.29	0.998
Bacillus licheniformis	−1.258×+2.175… (Equation 7)	42.36	0.995

SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, CGTase: Cyclodextrin glucosyl transferase

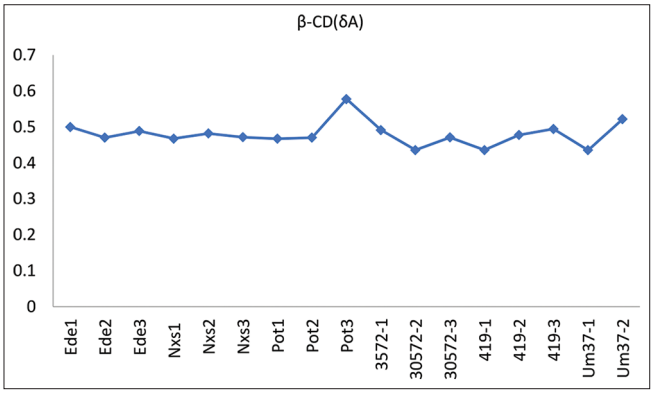

Figure 1: Effect of carbon and cyclodextrin glucosyl transferase source on levels of β-cyclodextrin

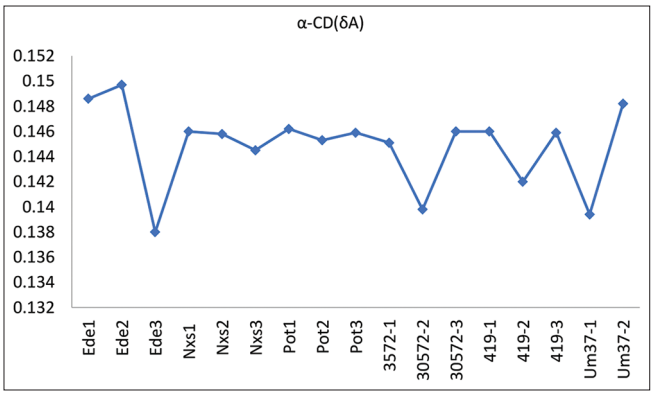

Figure 2: Effect of carbon and cyclodextrin glucosyl transferase source on levels of α- cyclodextrin

separation of various CDs is expensive and time consuming, a CGTase that predominantly synthesizes one sort of CD is of interest. While most CGTases produce mixtures of all kinds of CDs, CGTases from alkaliphilic bacteria convert starch into β-CD as the major product, though still in a mixture with the other CDs in several ratios.[28]

CONCLUSION

Bacillus species isolated was confirmed using PCR-16sr RNA sequencing and bio-informatics as *B. cereus, B. thurengiensis,* and *B. licheniformis*. CGTase was successfully produced from the three *Bacillus* species identified. This study has projected cassava starch as a better substrate for CGTase and CD production than sweet potato and cocoyam starches. NXS 003 (Nigerian *xanthosoma* specie) was a better substrate than sweet potato and *edeuhie* (red cocoyam). This was as a result of the higher amylopectin content of cassava when compared with other starches analyzed. SDS-PAGE result identified lower molecular weight CGTase in the three *Bacillus* species identified which ranged from 21.04 to 43.29kDa. Few CGTase with the lower molecular weight has been reported in the literature. CGTase from *B. licheniformis* produced highest level of α-CD and β-CD when cassava (30572 and 419) and sweet potato starch, respectively, were used as carbon source. CGTase from *B. cereus* produced highest level of α-CD and β-CD in *eheuhie,* while CGTase from *B. thurengiensis* showed highest α-CD and β-CD level in *edeuhie* and Um37.

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