

Isolation and identification of phytase-producing *Bacillus* and *Enterobacter* species from Nigerian soils

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Abstract

The bioavailability of phosphorus and other nutrients in feed for monogastrics is restricted by the presence of phytate. Exogenous supply of phytase produced by bacteria and other microbes can enhance the bioavailability of these nutrients and reduce phosphorus pollution. The objective of the study was to isolate bacteria with phytase-producing potentials from soil; which may be employed for the bioavailability of phosphorus and other nutrients in feed for monogastrics in Nigeria. Top soil samples were collected from two dumpsites in Lagos, Nigeria and bacteria were isolated and screened for potentials to produce phytase. The isolates with phytase potential were primarily identified by their cultural and biochemical characteristics and then confirmed using the 16S rRNA sequencing, after which their expressed phytases were quantified. A total of six isolates belonging to three species were identified as phytase producers. Sequence data analyses revealed these to be *Bacillus subtilis* (2), *Bacillus amyloliquefaciens* (3) and *Enterobacter cloacae* (1) with accession numbers MH879827 and MH879832; MH879828, MH879830 and MH879831; and MH879829 respectively. Phenotypic phytase activity was highest in *E. cloacae* ODS 29 (9.69 ± 0.04 U/ml) and least in *B. subtilis* ODS 10 (8.83 ± 0.02 U/ml). In conclusion, phytase-producing *Bacillus* and *Enterobacter* species were isolated and characterized from Nigerian soils. These bacteria species could be used in biotechnological applications.

Keywords: *Bacillus*, *Enterobacter*, Monogastrics, Phylogenetic analysis, Phytase

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Introduction

Legumes, oil seed crops and cereals serve as major sources of nutrients for humans and animals but they contain phytic acid; an anti-nutrient, which is the major storage form of phosphorous and other minerals in plants tissues, accounting for approximately 70 - 80% of the total phosphorous (Blaabjerg et. al., 2011). Phytate chelates phosphorus and other minerals, and also binds starch and proteins making them non bioavailable. Monogastric animals are unable to produce the requisite enzyme, phytase needed for the hydrolysis of phytate and as such cannot utilize these nutrients. This leads to nutrient deficiencies and can interfere with growth performance of

animals. To alleviate nutrient deficiencies, animal feed is supplemented with phosphorus sources often in excess and this together with undigested phytate in feed can cause a high fecal phosphorus load in areas of intensive livestock production, resulting into environmental phosphorus pollution. As such, the enzymatic hydrolysis of phytate into inorganic phosphates and divalent cations in the intestine of monogastric animals is most desirable. Phytases catalyse the breakdown of phytic acid complexes to improve the nutritional value of feed and decrease the amount of phosphorous excreted. Naves et. al. (2014), Kumar et. al. (2015) and Abdel-Megeed and Tahir (2015) amongst others have reported the effectiveness of supplemental microbial

phytases in improving utilization of phosphate from phytate. Phytases can be derived from a host of sources including plants, animals and microorganisms; however, the production of phytase from microbial origin is of great potential in developments. Currently, phytase-producing microorganisms include yeasts such as *Pichia anomala* (Vohra et. al., 2010), molds such as *Aspergillus ficcum* (Zhang et. al., 2010), members of the genus *Bacillus* obtained from different soil sources viz; *B. subtilis* from Syrian soil (Abd-Alhadi et. al., 2015) and *Bacillus* species from an abattoir soil (Onawola et. al., 2016a) as well as from a host of other bacteria. Towards improving the nutritional value of plant-based feed for monogastric animals, reduction of environmental phosphorus pollution in areas of intensive livestock farming and reduction of cost incurred by farmers in Nigeria due to use of imported phytase, this study was aimed to isolate and characterize phytase-producing bacteria which could be employed in the production of phytase for use in animal feed in Nigeria.

Materials and methods

Collection of Soil Samples

A total of six (6) top soil samples were each obtained from the dumpsites of Olusosun, Ojota and the Federal Institute of Industrial Research Oshodi, (FIIRO) Lagos, Nigeria respectively. A sample of top soil was aseptically taken from each sampling point on the two locations using sterile spatulas and transferred into sterile screw cap bottles. These were capped tightly and transferred into a clean and dry plastic bucket that was sterilized on site using 70% ethanol and conveyed to the laboratory. All samples from each location were aseptically pooled together and mixed to obtain the composite sample for each dumpsite.

Isolation and Screening for Phytase-Producing Bacteria

This was carried out as described by Demirkan et. al. (2014) and Onawola et. al. (2016a) on 1g each of composite soil sample obtained from each dumpsite. One gram each of composite samples was aseptically weighed and transferred into 9 ml of sterile distilled water in screw cap test tubes and were regarded as the stock suspensions. These were capped tightly and contents mixed by gently turning the bottles up and down. One milliliter of each of the stock suspension was serially diluted in

sterile distilled water up to the 4th dilution. One hundred microlitre of the 4th dilutions were each spread-plated on nutrient agar plates and the plates incubated at 35 to 37 °C for 24 hours (h). Based on morphological differences, bacteria colonies were aseptically picked out and sub cultured repeatedly on nutrient agar plates until pure and thereafter preserved on Luria Bertani agar slants. A loopful of each isolate was cultured overnight in 10ml of tryptone soya broth and 100µl each were aseptically inoculated into correspondingly labeled agar wells on sterile phytase specific medium (PSM) comprising (g/L): Glucose 15.0; NH₄NO₃ 5.0; KCl 0.5; MgSO₄.7H₂O 0.5; FeSO₄.7H₂O 0.01; MnSO₄.4H₂O 0.01; calcium phytate (Sigma-Aldrich) 0.5; Agar 15.0; pH 5.5 and incubated at 37°C for 72 h without inversion. The development of a colony surrounded by a clear zone was considered a potential to produce phytase and the zone diameters were measured using a ruler and reported in millimeters. Bacterial isolates from Olusosun dumpsite were coded ODS with arabic numerals while isolates from FIIRO dumpsite were coded FDS with arabic numerals.

Phenotypic Identification and Biochemical Characterization of Phytase-Producing Isolates

Phenotypic identification was carried out as described by Holt et. al. (1994) while determination of the carbohydrate fermentation profile was carried out using the Analytical Profile Index (API) 50 CHB/20E (for *Bacillus*) and API 20 E (for *Enterobacter*) kits of Biomereux according to manufacturer's instruction.

Amplification and Sequence Determinations of the 16S rRNA Genes of the Bacterial Isolates

Genomic DNA was extracted from each isolate and purified using the DNA isolation kit Quick - DNA™ (Zymo Research, USA). Isolation of the 16S rRNA genes was carried out using the primers: 27F: 5'- AGAGTT TGATCC TGG CTC AG -3', T^M 58°C and 1492R: 5'- GGTTAC CTT GTT ACG ACT TT -3', T^M 53°C and the One Taq® Quick load SM0486S master mix as source of all dNTPs and polymerizing enzyme. PCR mixtures contained per assay: 12.5µl (1X) of master mix, 0.5µl of each primer (0.24 mM), 5µl of DNA (template), 6.5µl of sterile DNase-free water. PCR profile of 30 minutes consisted of 30 cycles, each of 30 s (initial and final) denaturation at 94°C, 45 s annealing at 51°C and 1 and 5 min (initial and final) primer

extension at 68°C respectively. A negative control was included and PCR products were analyzed on 1.5% agarose gel and purified. Nucleotide sequences were determined by the Sanger's method on the ABI 3130XL genetic analyzer system.

Generic Identification of Phytase-Producing Isolates and Evolutionary Relationship

Comparison of the six partial 16S rRNA gene sequences to the Genbank database was done using the BlastN program available at <http://www.ncbi.nlm.nih.gov>. All organisms used for the analyses were phytase-producing bacteria and their sequences were of reference sequence (RefSeq) on the database. Cluster analysis was performed and model for the analysis was computed as described by Tamura et. al. (2013). Evolutionary history was inferred as described by Kimura (1980) and Felsenstein (1985). The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed and initial tree(s) for the heuristic search were obtained by applying the Neighbor-Join and BioNJ algorithms. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 1.1455)]. The analysis involved 22 nucleotide sequences and evolutionary analyses were conducted in MEGA6.

Phytase Production and Estimation of Phytase Activities

This was carried out as described by Saribuga et. al. (2014) and Onawola et al. (2016b) but with slight modification. One hundred microlitre of a washed suspension of an overnight culture of each phytase-producing bacteria in tryptone soya broth were aseptically inoculated into 50ml of correspondingly labeled sterile phytase production medium (PPM) broths comprising (g/L): Glucose 15.0; NH₄NO₃ 5.0; KCl 0.5; MgSO₄.7H₂O 0.5; FeSO₄.7H₂O 0.01; MnSO₄.4H₂O 0.01; sodium phytate (Sigma-Aldrich) 5.0 for phytase production; pH 5.5 and thereafter incubated at 37°C for 72 h with shaking at 180 rpm. The activities of the expressed phytases were quantified by reacting 100µl of clear filtrate (the enzyme solution) with 200µl of buffered substrate (1.5mM sodium phytate in 0.2M sodium acetate, pH

5.5) and incubated at 37°C for 15 min. Reaction was terminated with 300µl of trichloro acetic acid (TCA) and 400µl of ammonium molybdate reagent. The absorbance of the clear solutions were determined at 700nm against a reference blank which contained distilled water in place of enzyme solution and amounts of inorganic phosphate liberated were calculated from a standard curve of 1µmol/ml to 10 µmol/ml orthophosphate from dipotassium hydrogen phosphate. One unit of phytase activity (U/ml) was defined as the amount of phytase which liberated 1 µmol of orthophosphate from phytate per minute under the assay conditions. Isolates which had significantly higher phytase activities over the others were considered to be phytase hyper-producers.

Statistical Data Processing

Data obtained were analyzed by the One-way Analysis of Variance using the statistical package for the social sciences (SPSS) of IBM Corporation. Results were expressed as Mean ± SEM and significant differences were evaluated using the Tukey HSD at $p \leq 0.05$.

Results

A total of fifty three bacterial isolates were obtained with four (4) and two (2) strains having potential to produce phytase from the Olusosun and FIIRO dumpsites, translating to about 12% and 10.5% respectively. Comparison of the partial 16S rRNA gene sequences of the isolates to the Genbank database revealed all isolates except ODS 29 to show high similarities to the RefSeq for the genus *Bacillus* with ODS 25, ODS 33 and FDS 10 having 99% identity to *B. amyloliquefaciens* and ODS 10 and FDS 16 having 99% identity to *B. subtilis*. Phylogenetic relations of ODS 25, ODS 33, FDS 10 and FDS 16 showed closest homology with *B. amyloliquefaciens* DSM 7 (NC 014551.1) while that of ODS 10 revealed closest homology to *B. subtilis* subsp. *subtilis*. The comparison of the partial 16S rRNA gene sequence of ODS 29 showed 99% similarity to the RefSeq for *E. cloacae* and closest phylogenetic homology with *E. cloacae* subsp. *cloacae*. Cluster and Neighbor-joining phylogenetic analyses revealed all six isolates to be grouped in three clusters.

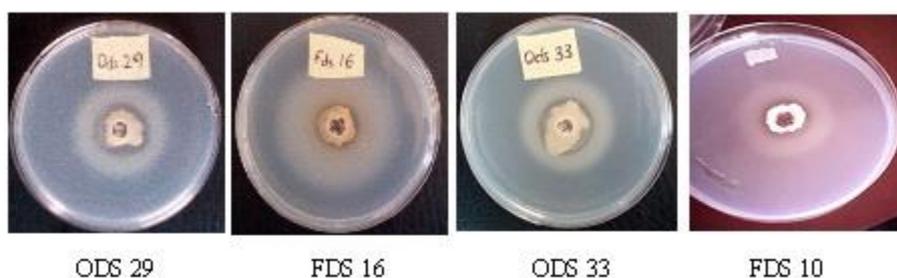


Figure 1. Clear zone formation by some phytase-producing bacteria.

Table 1: Zone diameter by phytase-producing bacteria after 72 hours of incubation

Isolate code	Zone diameter (mm)
ODS 10	25.0
ODS 25	26.0
ODS 29	32.0
ODS 33	31.1
FDS 10	29.0
FDS 16	28.0

Table 2: Phenotypic and structural characteristics of the phytase-producing bacteria species.

Ic	Cs	Col	Margin	Elevation	Shape	Tx	Cm	Mot	Gr	Ti
ODS 10	Sm-all	Off-white	Entire	Raised	Circular	Ns	Rods	Motile	+	<i>Bacillus</i>
ODS 25	Big	Cream	Undulate	Umbonate	Irregular	S	Rods	Motile	+	<i>Bacillus</i>
ODS 29	Sm-all	Cream/Iridescent	Serrated	Flat	Circular	Ns	Rods	Motile	-	<i>Enterobacter</i>
ODS 33	Big	Cream	Entire	Umbonate	Irregular	S	Rods	Motile	+	<i>Bacillus</i>
FDS 10	Big	Cream	Undulate	Umbonate	Irregular	S	Rods	Motile	+	<i>Bacillus</i>
FDS 16	Sm-all	Off-white	Undulate	Raised	Irregular	Ns	Rods	Motile	+	<i>Bacillus</i>

Ic, Isolate code; **Cs**, Colony size; **Col**, Colour; **Tx**, Texture; **Cm**, Cellular morphology; **Mot**, Motility; **Gr**, Gram reaction; **Ti**, Tentative identity; **Ns**, non-slimy; **S**, slimy; **+**, positive; **-**, negative

Table 3: Biochemical characteristics of the phytase-producing bacteria species

Isolate code	ODS 10	ODS 25	ODS 29	ODS 33	FDS 10	FDS 16
Biochemical						
Tests						
Oxidase	+	+	-	+	+	+
Catalase	+	+	+	+	+	+
ONPG	+	+	+	+	+	+
ADH	-	-	+	-	-	-
LDC	-	-	-	-	-	-
ODC	-	-	+	-	-	-
CIT	+	+	+	+	+	+
H ₂ S	-	-	-	-	-	-
URE	-	-	-	-	-	-
TDA	-	-	-	-	-	-
IND	-	-	-	-	-	-
VP	+	+	+	+	+	+
GEL	+	+	-	+	+	+
GLU	-	+	+	+	+	+
NIT	+	+	+	+	+	+
N ₂			-			
MOB			+			
McC			+			
OF-O			+			
OF-F			+			

+, positive reaction; -, negative reaction

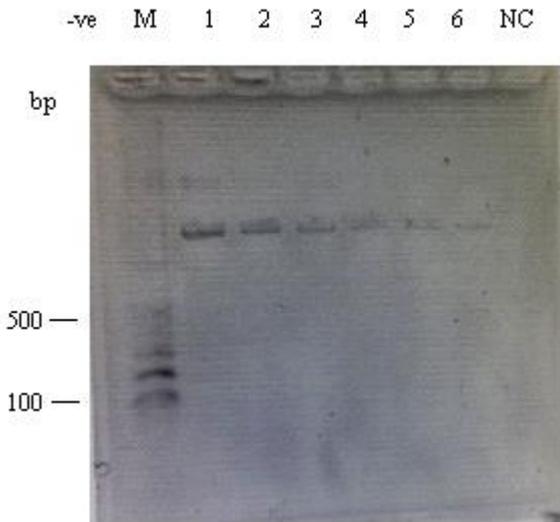
Table 4: Carbohydrate fermentation profile of the phytase-producing bacteria species

Isolate code	⇒	ODS 10	ODS 25	ODS 29	ODS 33	FDS 10	FDS 16
Acid production from carbohydrates:							
Control		-	-	-	-	-	-
Glycerol		+	+	-	+	+	+
Erythritol		-	-	-	-	-	-
D-Arabinose		-	-	-	-	-	-
L-Arabinose		+	+	+	+	+	+
D-Ribose		+	+	-	+	+	+
D-Xylose		+	+	-	+	+	+
L-Xylose		-	-	-	-	-	-
D-Adonitol		-	-	-	-	-	-
MDX		-	-	-	-	-	-
D-Galactose		-	-	-	-	-	-
D-Glucose		+	+	-	+	+	+
D-Fructose		+	+	+	+	+	+
D-Manose		+	+	-	+	+	+
L-Sorbose		-	-	-	-	-	-
L-Rhamnose		-	-	+	-	-	-
Dulcitol		-	-	-	-	-	-
Inositol		+	+	-	+	+	+
D-Manitol		+	+	+	+	+	+
D-Sorbitol		+	+	+	+	+	+
MDM		-	-	-	-	-	-
MDG		+	+	-	+	+	+
NAG		-	+	-	+	+	+
Amygdalim		+	+	+	+	+	+
Arbutin		+	+	-	+	+	+
Esculin Ferric Citrate		+	+	-	+	+	+
Salicin		+	+	-	+	+	+
D-Celibiose		+	+	-	+	+	+
D-Maltose		+	+	-	+	+	+

D-Lactose	-	-	-	-	-	-
D-Melibiose	w	-	+	-	-	w
D-Saccharose (sucrose)	+	+	+	+	+	+
D-Trehalose	+	+	-	+	+	+
Inulin	+	+	-	+	+	+
D-Melezitose	-	-	-	-	-	-
D-Raffinose	+	+	-	+	+	+
Amicon (starch)	+	+	-	+	+	+
Glycogen	+	+	-	+	+	+
Xylitol	-	-	-	-	-	-
Gentiobiose	+	+	-	+	+	+
D-Turanose	+	+	-	+	+	+
D – Lyxose	-	-	-	-	-	-
D – Tagatose	-	-	-	-	-	-
D – Fucose	-	-	-	-	-	-
L – Fucose	-	-	-	-	-	-
D – Arabitol	-	-	-	-	-	-
L – Arabitol	-	-	-	-	-	-
Potassium gluconate	-	-	-	-	-	-
Potassium - 2 - ketogluconate	-	-	-	-	-	-
Potassium - 5 - ketogluconate	-	-	-	-	-	-

+, positive reaction; -, negative reaction; w, weakly positive

Presumptive isolates: ODS 10 and FDS 16, *Bacillus subtilis*; ODS 25, ODS 33 and FDS 10, *Bacillus amyloliquefaciens*; ODS 29, *Enterobacter cloacae*



M, DNA ladder; **NC**, negative control; **-ve**, negative terminal. **Lanes 1 to 6** = ODS 29, ODS 33, FDS 16, ODS 25, ODS 10 and FDS 10 respectively

Figure 2: Gel analyses of the amplified 16S rRNA genes of the six phytase-producing bacteria.

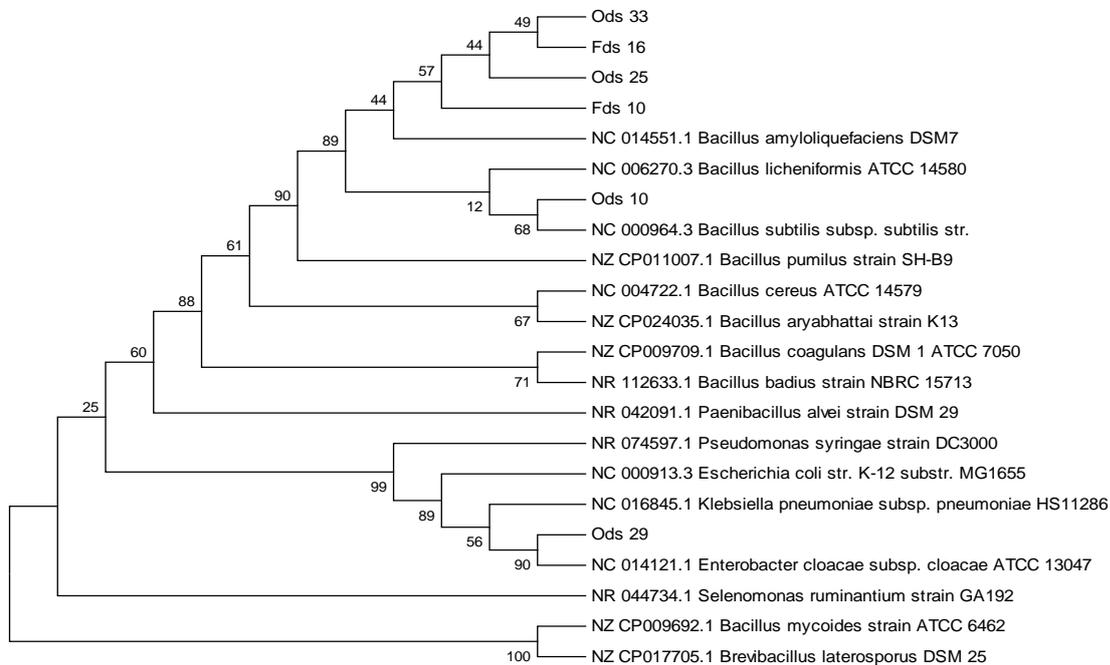


Figure 3: Phylogenetic relations of the phytase-producing bacteria with closely related RefSeq phytase-producing bacteria strains. ODS 25, ODS 33, FDS 10 and FDS 16 are more closely related to *B. amyloliquefaciens* DSM 7, while ODS 10 and ODS 29 are more closely related to *B. subtilis* subsp. *subtilis* and *E. cloacae* subsp. *cloacae* respectively. Similarity and homology of the neighboring sequences are shown by bootstrap values greater than 50%.

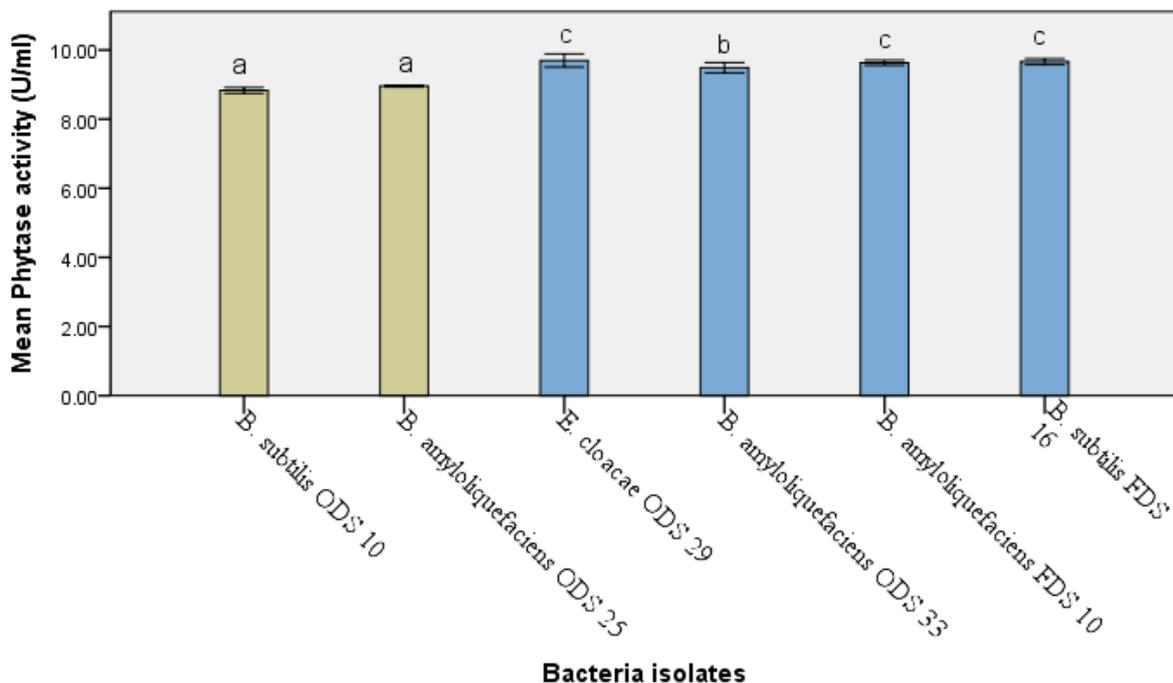


Figure 4. Phytase activities of bacteria and selection of hyper-producing strains. Result shows *E. cloacae* ODS 29, *B. amyloliquefaciens* ODS 33 and FDS 10, and *B. subtilis* FDS 16 to be hyper-producers of phytase. Any two means not followed by the same letter are significantly different.

Discussion

Clear zone or halo formation by bacteria isolates on phytase specific medium is an indication of a potential to produce phytase, the enzyme required for the solubilization of phytate. The observation of clear zones surrounding the colonies of some of the isolates (Figure 1) agrees with the findings of Demirkan et. al. (2014), Abd-Alhadi et. al. (2015) and Onawola et. al. (2016b) who had isolated phytase-producing bacteria strains from the soil respectively. Phytase production by these isolates could be attributed to the possession of phytase-encoding genes in the DNA of the organisms, without which the enzyme cannot be synthesized. The observed phenotypic and structural characteristics of the isolates (Table 2) collectively satisfied the criteria for the genus *Bacillus* and *E. cloacae* as given by Bergey's manual of determinative bacteriology (Holt et. al. 1994) and agree with the findings of Logan and Vos (2009) for *B. amyloliquefaciens* and *B. subtilis*; and Brenner and Farmer (2001) for *E. cloacae* respectively. The differences observed in the phenotypic and structural characteristics as well as in the carbohydrate fermentation profile (Table 4) among the *Bacillus* species could be attributed to differences in their DNA

sequences and possibly in their 16S rRNA genes (sequences available at NCBI data base) thus characterizing each species and differentiating them as strains. However, similarities exist between similar strains.

The observed similarity of the phenotypic, biochemical characteristics of isolate ODS 29 to the *Enterobacter* species (Tables 2, 3 and 4) and the high similarity of its partial 16S rRNA gene sequence to that of RefSeq for *E. cloacae* suggests it to be *Enterobacter cloacae*. As such, in consideration of the phenotypic, biochemical and carbohydrate fermentation profile, sequence identity and phylogenetic analysis (Figure 3), the isolate ODS 29 was preliminary identified as *E. cloacae* and based on these data, we assigned our strain as *E. cloacae* strain ODS 29 with the accession number **MH879829**. Previously, few phytase-producing *Enterobacter* species from different soil portions and origins had been isolated and characterized; but until now, information reporting isolation of phytase-producing *E. cloacae* from Nigerian soil is scarce and seems to have been made available for the first time by the authors.

The observation of similarities in the phenotypic and biochemical characteristics of isolates ODS

25, ODS 33 and FDS 10 to the *Bacillus* species (Tables 2, 3 and 4) and the high similarity of their partial 16S rRNA gene sequences to that of RefSeq for *Bacillus amyloliquefaciens* suggests these isolates to be *Bacillus amyloliquefaciens*. Although these isolates exhibited similar phenotypic and biochemical characteristics, yet there exist differences in the order of their nucleotide bases in the gene studied, causing them to be distinct from each other. This is evident in the phylogenetic relation (Figure 3), revealing a strain variation of the species. Therefore in consideration of their phenotypic, biochemical and carbohydrate fermentation profile, sequence identities and phylogenetic analyses, they were preliminary identified as *B. amyloliquefaciens* and based on these data; they were assigned *B. amyloliquefaciens* strains ODS 25, ODS 33 and FDS 10 with the accession numbers **MH879828**, **MH879830** and **MH879831** respectively.

The observation of similarities in the phenotypic and biochemical characteristics of isolates ODS 10 and FDS 16 to the *Bacillus* species (Tables 2, 3 and 4) and the high similarity of their partial 16S rRNA gene sequences to that of RefSeq for *Bacillus subtilis* suggests these isolates to be *Bacillus subtilis*. However, the phylogenetic relation of FDS 16 showed it to be closely related to *B. amyloliquefaciens* DSM 7 (NC 014551.1). This disparity in sequence identity and phylogenetic homology of isolate FDS 16 may be attributed to the slight differences occurring in the genotype of the members of the *B. subtilis* group, possibly due to the presence of particular alleles at specified loci in each organism. The observed disparity agree with the findings of Rooney et. al. (2009) and Kanghae et. al. (2016) who had reported that the *B. subtilis* group is complex, comprising several closely related members which include: *B. amyloliquefaciens*, *B. subtilis* and *B. licheniformis*.

The slight differences in the *B. subtilis* group might thus account for the presence of isolate FDS 16 in the cluster group of *B. amyloliquefaciens*, whereas FDS 16 is genotypically *B. subtilis*. Ash et. al. (1991) and Kanghae et. al. (2016) had reported that phylogenetic analysis of the 16S rRNA genes may fail to distinguish the *B. subtilis* species. Therefore in consideration of the phenotypic, biochemical and carbohydrate fermentation profile, sequence identity together with the phylogenetic analysis, isolates ODS 10 and FDS 16 were preliminary identified as *B. subtilis* and

based on these data, they were assigned *B. subtilis* strains ODS 10 and FDS 16 with accession numbers **MH879827** and **MH879832** respectively.

The observation of significant differences in quantitatively determined activities of phytase (Figure 4) may be a reflection of the differing abilities of the bacteria to produce phytase. The observed differences in activities of the phytases in comparison with what has been obtained for some strains of *Bacillus* and *Enterobacter* species reported in literature such as *B. subtilis* DR6, 129 - 378 mU/ml (Singh et. al., 2013), *B. cereus* 300.8 - 323.9 mU/ml (Sajidan et. al., 2015), *E. cloacae* PSB-45, 0.305U/ml (Kalsi et. al., 2016) and *B. subtilis* 55 - 88 U/ml (Shamna et. al., 2012) may possibly be due to strain difference, the application of optimized culture conditions or an engineered state of strains. The higher activities of extracellular phytase produced by the bacteria under study may possibly be attributed to the soil source from where they were obtained. Soil from sources such as dumpsites which accommodate a wide range of microorganisms and sources which accommodate substrates of an enzyme may likely house microorganisms possessing an efficient ability for the production of the enzyme. As such, the observed higher phytase activities of the isolates under study in the native state over strains earlier reported with lower phytase activities may suggest a potential for their use in biotechnological applications.

Conclusion

Few members of the phytase-producing *E. cloacae* strains with molecular data have been reported and of these, information reporting isolation and identification of phytase-producing *E. cloacae* strains from Nigeria soil is scarce and seems to have been made available for the first time by the authors. In addition, *E. cloacae* ODS 29 and the *Bacillus* isolates showed higher phytase activities than has been observed for some similar strains from other origins. As such, they may be considered as having potential for biotechnological applications, possibly in feed, for the reduction of environmental phosphorus pollution or in the solubilization of organic phosphorus in soil. However, further investigation into the development of the strains for economic considerations both from the environment and feed-conversion point of view is being considered for future study.

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