Scientia Africana, Vol. 23 (No. 1), February, 2024. Pp 73-84
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ISOLATION AND MOLECULAR CHARACTERIZATION OF CELLULASE-PRODUCING BACTERIA FROM WASTE DUMP SITE

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Received: 05-12-2023
Accepted: 04-01-2024

https://dx.doi.org/10.4314/sa.v23i1.6

Journal Homepage: http://www.scientia-african.uniportjournal.info
Publisher: *Faculty of Science, University of Port Harcourt.*

ABSTRACT

Cellulases are collections of extracellular enzymes and a complex mixture of enzyme proteins with various specificities. Cellulases work together to hydrolyze glycosidic bonds and generate monomers of glucose for fermentation. This investigation aims to isolate and molecularly characterize Bacillus species with cellulolytic ability. Bacillus species were isolated from soil samples retrieved from a dump site and screened for cellulolytic ability on carboxyl methylcellulose (CMC) agar. The percentage hydrolysis efficiency of isolates was determined and cellulase produced was quantified using CMC assay method. Biochemical identification was by Analytical Profile Index (API) Kit 50CHB/20E and API web software while molecular characterization employed 16S rRNA gene sequencing and blast search analysis. Bacillus megaterium (FSP_1) and Bacillus zanthoxyli (FSP_4) exhibited their cellulolytic potentials by presenting zones of clearance of about 21 ± 2.08 and 7 ± 1.00 mm on CMCA with hydrolysis efficiency of 250 and 600 % respectively. Quantification of crude cellulase revealed cellulase activity of 85 and 74µmol/ml for both bacteria species. Biochemically, the cellulolytic bacteria were identified as Bacillus megaterium (FSP_1) and Bacillus zanthoxyli (FSP_4) while molecularly, they were identified as Bacillus megaterium 14581 (FSP_1) and Bacillus zanthoxyli 1433 (FSP_4) with Reference Sequence (RefSeq) accession numbers NR_116873 and NR_164882, and showing maximum sequence similarity of 99 and 96 % respectively. Results obtained from this investigation, suggests that both bacteria species characterised, possesses good cellulolytic ability and hence can be utilized for the production of the enzyme cellulase which has a wide range of industrial application.

Keywords: Cellulolytic, Dumpsite, Hydrolysis efficiency, *Bacillus zanthoxyli.*

INTRODUCTION

Biochemical catalysts called enzymes accelerate the speed of chemical reactions within the cell. They are fundamentally in charge of all cellular reactions that result in the conversion of nutrients into energy. Enzymes have been discovered to be helpful in a variety of industrial processes for several decades, and numerous microorganisms, including bacteria, fungus, actinomycetes, yeast, etc., have been linked to the synthesis of enzymes with numerous industrial applications (Anoop Kumar *et al.*, 2019). When all industrial enzymes used today are considered, only 15% of commercial enzymes come from plants and animals, 35% originate from bacteria, and about 50% are from fungi origin (Saranraj and

Naidu, 2014). Recent advancements in biotechnology echo that numerous industrial enzymes including cellulases are finding new and varied uses industrially.

Cellulases, which include endocellulase or 1, 4-β-D-glucanglucanohydrolases (EC 3.2.1.4.), exocellulase or 1, 4-β-D-glucancellobiohydrolase (EC 3.2.1.91) and beta-glucosidase or β-D-glucosideglucohydrolases (EC 3.2.1.21), rank third among industrial enzyme classes globally in terms of dollar expenditure. Apart from protease, one of the most popular enzymes used in the industry is cellulase and it's been found to have several uses in industrial biotechnology (Anoop Kumar *et al*., 2019). The wide range of applications for the enzyme cellulase in different industrial processes, including extraction of juice from fruits and vegetables, modification of food tissues, production of ethanol from enzymatic hydrolysis of lignocellulosic biomass, removal of the soybean seed coat, paper recycling, extraction of green-tea components, detergent enzymes, cotton processing, starch processing, grain alcohol fermentation, malting and brewing, improving cattle feed quality and animal feed additives, reiterates their importance (Roussos, 1989; Zhou *etal*., 2004; Martins *et al*., 2008; Farinas *et al*., 2011; Agrawal, 2015). Several bacterial species of the *Bacillus* genus, including *B. subtilis* subsp. *subtilis* JJBS250 (Alokika *et al*., 2023), *B. subtilis* strain LFS3 (Rawat and Tewari, 2012), *B. subtilis* Strain CBS31 (Regmi *et al*., 2020), *Bacillus halodurans* IND18 (Vijayaraghavan *et al*., 2016), *B. subtilis* BC1 (Dehghanikhah *et al*., 2020), *B. subtilis* BY-4 (Ma *et al*., 2015), *B. subtilis* CD001 (Malik and Javed, 2021) and *B. subtilis* CD001 (Malik *et al*., 2022) have been linked to the synthesis of cellulase. Therefore, the isolation and molecular characterization of indigenous *Bacillus* species with cellulolytic ability is of utmost importance considering the vast application of the enzyme cellulase in numerous industrial processes.

MATERIALS AND METHODS

Collection of Soil Sample

Soil samples were aseptically retrieved from waste dump site of groundnut shell and spent mushroom substrate located in Federal Institute of Industrial Research (FIIR) Lagos State, Nigeria. The samples were collected from surface and at 5, 10 and 15 cm depth in sterile screw-capped containers from three distinct locations on the same site using sterile stainless-steel spatulas and thereafter conveyed to the laboratory for further analysis. To create the composite sample for isolation, all soil samples retrieved from the three locations were thoroughly and aseptically mixed together.

Isolation of Bacteria

Serial dilution of ten grams (10 g) of soil sample was carried out using 90 ml of sterile distilled water in a 100 ml screw-capped test tube and further dilutions made to 10^{-6}. One milliliter (1ml) of the diluent from 10^{-5} and 10^{-6} dilutions were cultured on nutrient agar (prepared according to manufacturer's instruction) using the pour-plating method and incubated for 20±4 hours at 37 ºC. Based on morphology, size, and color presented on the nutrient agar plate, different colonies that emerged after incubation were selected and series of sub-culturing done to obtain pure cultures which were maintained on Luria Bertani agar (LBA) slants and used in subsequent experiments.

Screening of Isolated Bacteria for Cellulase Producing Ability

A qualitative assay for cellulase production was done using the plate assay method. Twenty-four hour (24 h) pure cultures of bacterial isolates on nutrient agar were spot inoculated on Carboxyl Methyl Cellulose (CMC) agar screening medium (Composition (g/L): KH_2PO_4 1.0; CMC 26.0; KCL 0.2; Yeast extract 1.0; $MgSO_4.7H_2O$ 1.0; Agar 12.0) and incubated at 37 ºC for 20±4 h. The plates were flooded with 2 ml of Grams iodine solution and allowed to stand for 2 minutes

Scientia Africana, Vol. 23 (No. 1), February, 2024. Pp 73-84
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after which it was decanted. It was thereafter observed for cellulase activity indicated by a halo/clear zone around growing microbial colonies. Clear zones that appeared were indicative of the microorganism's ability to produce cellulase. The clear zones were measured and recorded accordingly (Osho *et al*., 2017).

Determination of Percentage Hydrolysis Efficiency of Isolated Bacteria

All isolated bacteria exhibiting cellulase ability were spot inoculated on CMC agar screening medium and after the prescribed incubation period of 20±4 h at 37 ºC, both their halo diameters (H) and colony diameters (C) were measured. Thereafter, the percentage hydrolysis efficiency was calculated for all isolated bacteria using the formula below (Sreedevi and Reddy, 2012).

$$\% \, Hydrolysis \, efficiency = \frac{H - C}{C} \times 100$$

Where:

H = Halo Diameter

C = Colony Diameter

Production of Crude Cellulase using *Bacillus megaterium* ATCC 14581 (FSP_1) And *Bacillus zanthoxyli* 1433 (FSP_4)

Inoculum was prepared by growing the bacteria species in an Erlenmeyer flask (250 ml) containing 100 ml of sterile tryptone soy broth. The inoculated broth was incubated for 24 h at 37 ºC. One (1) ml of inoculum was grown in 100 ml cellulase production medium (CPM) (composition g/L: $MgSO_4$ $7H_2O$ 0.5g; KCl 0.5g; K_2HPO_4 1g; Na_2NO_3 2g; peptone 2g and Cellulose 5g) and incubated at 37 ºC for 48 h at 150 rpm. The broth culture obtained from the submerged fermentation process was centrifuged for 20 mins at 4 ºC and at 4100 rpm after which the supernatant was utilized as an extracellular enzyme source (Mrudula and Murugammal, 2011)

Assay for Crude Cellulase Activity

The enzyme assay was conducted using the CMC assay (Ghose, 1987), via measuring the amount of reducing sugar released. The experiment was carried out at 50 ºC using a reaction mixture comprising 0.5 ml of enzyme plus 0.5 ml of 2% substrate (CMC) dissolved in 0.1 M sodium acetate buffer (pH 4.0) and incubated at 37 ºC for 30 mins. Three (3) ml of DNS reagent was added to terminate the reaction and the mixture was heated for 10 minutes in a vigorously boiling water bath. The resulting solution's absorbance was measured at 540 nm using a UV 754 Spectrophotometer (Hospibrand, USA), and the glucose concentration was calculated using the glucose standard curve (El-Hadi *et al*., 2014; Osho *et al*., 2017). One unit of enzyme activity was defined as the amount of enzyme that releases 1 µ mole of glucose in a unit of time under assay conditions.

Biochemical Identification of Isolated Bacteria

The biochemical identification of the selected isolates was performed using API Kit and API online database identification software system (htpps://apiweb.biomerieux.com) following the manufacturer's instructions.

Molecular Characterization of Isolated Bacteria

Molecular characterization of the selected isolates was investigated using the 16S rRNA gene sequence and BLAST search analysis.

DNA was extracted using the Nigerian Institute of Medical Research (NIMR) Biotech DNA extraction kit 2, following the manufacturer's instructions. The polymerase chain reaction was carried out to amplify the 16S rRNA gene of the bacteria using the universal primer pair 799F (5′-AACMGGATTAGATACC-3′) and 1193R (3′-ACGTCATCCCCACCTTCC- 5′). The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µl of a reaction mixture, and the reaction concentration was brought down from 5X concentration to 1X

concentration containing 1X Blend Master mix Buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200µM of each deoxynucleoside triphosphates (dNTP)(Solis Biodyne), 25pMol of each primer (StabVida, Portugal), 2 unit of Hot FIREPol DNA polymerase (Solis Biodyne), Proofreading Enzyme, 5µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in a Techne^{3}Prime thermal cycler (24 x 0.2ml, DKSH Australia) for an initial denaturation of 95 °C for 15 minutes followed by 35 amplification cycles of 30 seconds at 95 °C; 1 minute at 61°C and 1 minute 30 seconds at 72 °C. This was followed by a final extension step of 10 minutes at 72 °C.

The amplification product was separated on a 1.5% agarose gel and electrophoresis was performed at 80V for 1 h 30 mins. DNA bands were visualized after electrophoresis using ethidium bromide staining and a 100bp DNA ladder (Solis Biodyne) was used as a DNA molecular weight marker. All PCR products were purified with a QIAquickPCR Purification kit based on the manufacturer's instructions and subsequently sent to Epoch Life science (USA) for Sanger sequencing. Using the online blast search at http://blast.ncbi.nlm.nih.gov/Blast.cgi, the corresponding sequences were identified.

Data Analysis

Results obtained were expressed as mean ± standard deviation and data analysis and graphs were constructed using SPSS and Microsoft Excel software version 2013.

RESULTS

Isolation and Screening of Bacteria for Cellulolytic Ability

The data represented in **Figure 1** shows the zone of hydrolysis exhibited by some of the isolated *Bacillus* species on CMC agar as revealed by Gram's iodine staining. Their ability to hydrolyze cellulose as a sole carbon source was depicted by the existence of a halo zone (zone of hydrolysis) surrounding the microbial colonies growing on CMC agar screening medium. Twenty (20) bacteria species were selected from the waste dump site based on different colonial morphology presented on nutrient agar plate, seventeen of them showed cellulase activity as a considerable difference in their abilities to break down cellulose was seen by their measured zone of hydrolysis exhibited. Isolate FSP_{20} had the highest zone of clearance of about 30±2.08 mm and FSP_4 had the least clearance zone of 7 mm±1.00 mm (**Table 1**).

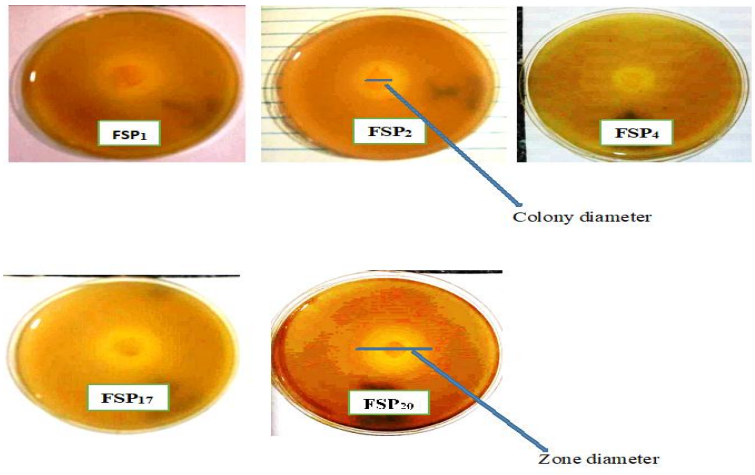

Figure 1: Carboxyl methyl cellulose (CMC) hydrolysis zones of some isolated bacteria as revealed by Gram's iodine staining.

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Table 1: Zone of clearance of cellulase enzyme as revealed by *Bacillus* species obtained from waste dump site on CMC agar screening medium.

Isolate Code	Diameter of Clear Zone(mm)*
FSP_1	21 ± 2.08
FSP_2	20 ± 1.53
FSP_3	20 ± 3.61
FSP_4	7 ± 1.00
FSP_5	28 ± 1.52
FSP_6	22 ± 1.53
FSP_7	10 ± 1.52
FSP_8	21 ± 3.05
FSP_9	11 ± 2.08
FSP_{10}	-
FSP_{11}	-
FSP_{12}	8 ± 2.08
FSP_{13}	22 ± 2.52
FSP_{14}	19 ± 2.65
FSP_{15}	-
FSP_{16}	19 ± 1.73
FSP_{17}	28 ± 2.08
FSP_{18}	10 ± 2.08
FSP_{19}	26 ±3.06
FSP_{20}	30 ± 2.08

***(**Mean of three replicates ± standard deviation),CMC (carboxyl methyl cellulose) – (no clear zone)

Determination of Percentage Hydrolysis Efficiency of Isolated Bacteria

The percentage hydrolysis efficiency for all isolated bacteria species ranged between 11 and 600% as can be seen in **Figure 2.** Isolate FSP_4 exhibited the highest efficiency of 600%, followed by FSP_1 with 250%, while the least of about 11% was observed in FSP_{18}.This further reiterates the high cellulose degrading capabilities of isolating FSP_4 and FSP_1.

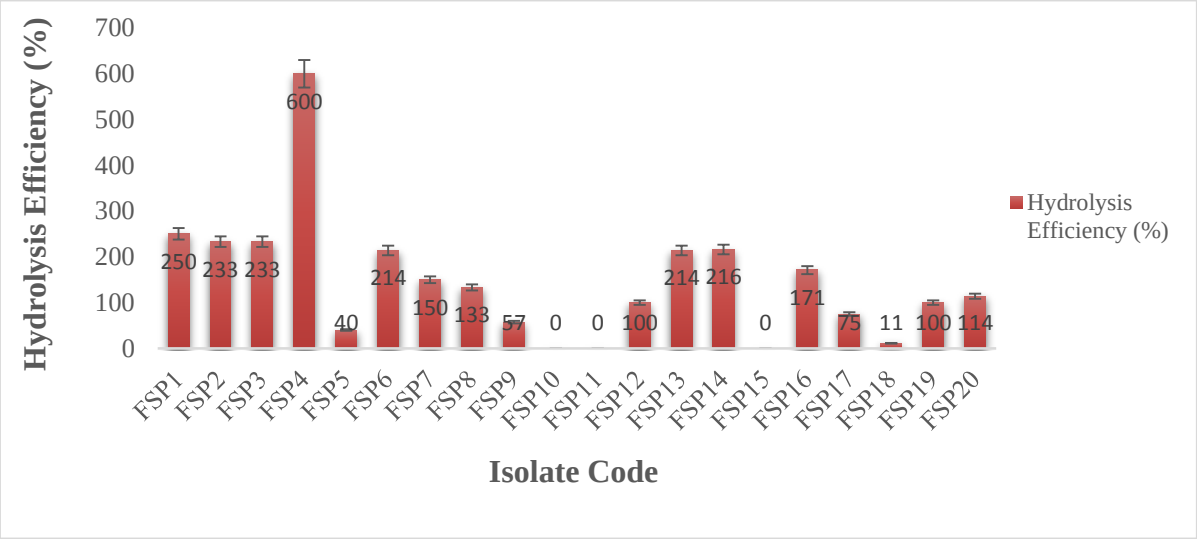

Figure 2: Hydrolysis efficiency (%) of isolated bacteria showcasing their potential to breakdown cellulose as the sole carbon source.

Assay for Activity of Crude Cellulase Produced by *Bacillus megaterium* ATCC 14581 (FSP1) and *Bacillus zanthoxyli* 1433 (FSP4)

Figure 3 shows the quantitative analysis of crude cellulase produced by *Bacillus megaterium* 14581 (FSP_1) and *Bacillus zanthoxyli*1433 (FSP_4) using CMC assay method. Both species of *Bacillus* showed appreciable cellulase activities of 85µmol/ml and 74µmol/ml respectively.

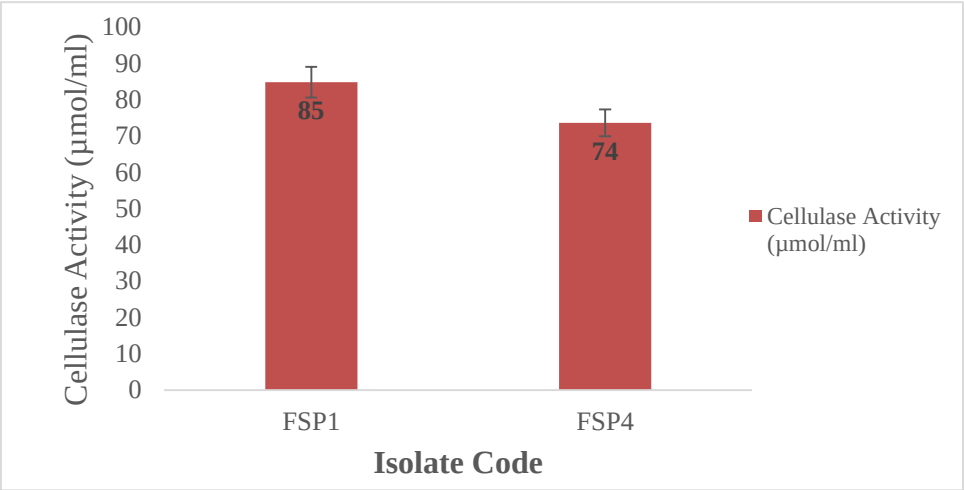

Figure 3: Crude cellulase activities of *Bacillus megaterium*14581 (FSP_1) and *Bacillus zanthoxyli* 1433 (FSP_4) at 540 nm.

Biochemical Identification Isolated Bacteria

In **Table 2, t**he investigation of carbohydrate metabolism using an analytical profile index kit (API Kit) 50CHB/20E and API web that aids in the identification of selected *Bacillus* species using biochemical assays was displayed. FSP_1 was identified as *Bacillus megaterium* and FSP_4 as *Bacillus zanthoxyli* respectively based on the way they metabolized different carbohydrates.

Table 2: Biochemical profile of cellulolytic *Bacillus* species using API Kit 50CHB/20E and APIweb (software).

S/ NO	Biochemical Test	Results Obtained			Biochemical Test	Results Obtained	
		Bacillus megaterium	*Bacillus zanthoxyli*			*Bacillus megaterium*	*Bacillus zanthoxyli*
	AP1 50CHB						
1	Control	-	-	34	Inulin	+	-
2	Glycerol	+	+	35	D-Melizitose	+	-
3	Erythritol	-	-	36	D-Raffinose	+	-
4	D-Arabinose	-	-	37	Amidon(starch)	+	-
5	L-Arabinose	+	-	38	Glycogen	+	-
6	D-Ribose	+	+	39	Xylitol	-	-
7	D-Xylose	+	-	40	Gentiobiose	+	-
8	L-Xylose	-	-	41	D-Turanose	+	+
9	D-Adonitol	-	-	42	D-Lyxose	-	-
10	Methyl –BD Xylopyranoside	-	-	43	D-Tagatose	-	-
11	D-Galactose	+	+	44	D-Fucose	-	-
12	D-Glucose	+	+	45	L-Fucose	-	-
13	D-Fructose	+	+	46	D-Arabitol	-	-
14	D-Manose	+	+	47	L-Arabitol	-	-

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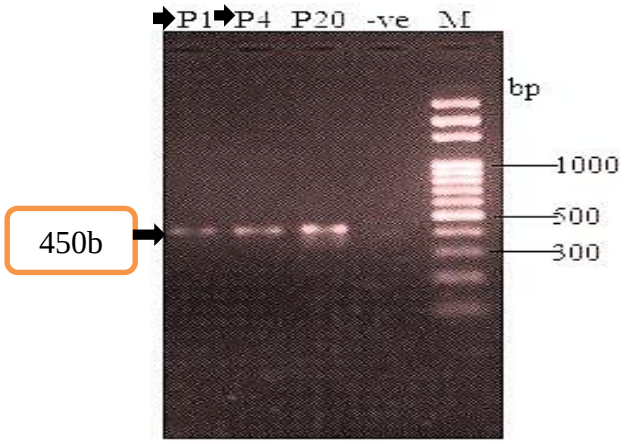
15	D-Sorbose	-	-	48	Potassium Gluconate	-	-
16	D-Rhamnose	-	-	49	Potassium 2-KetoGluconat	-	-
17	Dulcitol	-	-	50	Potassium 5-KetoGluconate	-	-
18	Inositol	+	-				
19	D-Manitol	+	-		**API 20E**		
20	D-Sorbitol	+	-	1	Ortho-Nitrophenyl-βD.Galactopyromosidase	+	-
21	Methyl-αD-Mannopyranoside	-	-	2	Arginine Dihydrolase	-	-
22	Methyl-αD-Glucopyranoside	+	+	3	Lysine Decarboxylase	-	-
23	N-AcetylGlucosamine	+	-	4	Orninthine Decarboxylase	-	-
24	Amygdalin	+	-	5	Citrate	-	-
25	Arbutin	+	-	6	Hydrogensulphide production	-	-
26	Esculin ferric citrate	+	-	7	Urease	-	-
27	Salicin	+	-	8	TryptophaneDeaninase	-	-
28	D-Cellobiose	+	-	9	Indole production	-	-
29	D-Maltose	+	+	10	Vogesproskauer	+	-
30	D-Lactose	+	-	11	Gelatinase	+	-
31	D-Melibiose	+	-	12	Nitrate reduction	-	+
32	D-Saccharose	+	+				
33	D-Trehalose	+	+		**ISOLATE CODE**	**FSP_1**	**FSP_4**

KEY: + (positive), - (negative)

Molecular Characterization of Isolated Bacteria

Figure 4 shows the analysis of PCR products of *Bacillus megaterium* (FSP_1) and *Bacillus zanthoxyli* (FSP_4) on a 1.5 % agarose gel electrophoresis using 100 bp DNA ladder. The presence and size of the expressed genes of both *Bacillus* species was seen to be approximately 450 bp. The isolates showed blast homology values of 99 and 96%, and were assigned Reference Sequence (RefSeq) Accession number and name NR_116873 (*Bacillus megaterium* ATCC 14581) and NR_164882 (*Bacillus zanthoxyli* 1433) respectively as seen in **Table 3**.



Key: M: DNA Ladder, -Ve: Negative Terminal, P4: Lane 2 (*Bacillus zanthoxyli*), P1: Lane3 (*Bacillus megaterium*), bp; Base pair.

Figure 4: PCR products of cellulolytic *Bacillus* species on 1.5 % agarose gel electrophoresis using 100bp DNA ladder showing the presence and the size of the expressed genes to be approximately 450bp.

Table 3: NCBI BLAST results of 16S rRNA gene sequences of *cellulolytic Bacillus species*

Isolate code	Strain	16S rRNA identity	Blast homology value	RefSeq accession number
FSP_1	14581	*Bacillus megaterium*	99 %	NR_116873
FSP4	1433	*Bacillus zanthoxyli*	96%	NR_164882

DISCUSSION

Waste dump sites are known to harbor vast array of *Bacillus* species with the ability to produce numerous enzymes including cellulases. According to Kiio *et al*., (2016), the utilization of CMC agar screening medium depicts the organism's ability to hydrolyze carboxyl methyl cellulose as a sole carbon source usually indicated by clear zone formation around growing microbial colonies (**Figure 1**). About 85% of the total number of bacterial species isolated showed varying degrees of hydrolysis depicted by their measured zones of hydrolysis on CMC agar after 24h of incubation (**Table 1**). The range of zones of clearance (7 mm-30 mm) obtained in the current study, is comparable with those reported by previous researchers including; Liang *et al*. (2014), 20 mm – 43 mm, Rasul *et al*. (2015), 7 mm – 9.5 mm, and Osho *et al*. (2017), 24 mm -28 mm for cellulolytic bacteria species. Islam and Roy (2018), stated that microbial isolates with cellulase activity on soluble cellulose such as CMC, represent mainly endoglucanase and beta-glucosidase activities. The current study isolates can thus be said to possess endoglucanase and beta-glucosidase activities respectively.

When microorganisms are screened for their enzyme-producing capabilities, percentage hydrolysis efficiency is usually employed to select bacteria species with the best degrading perspective. Osho *et al*. (2017), reported hydrolysis efficiency of between 20 and 100% for cellulase-producing *Bacillus* species and linked the best activity to the highest hydrolysis efficiency. In the current study, the percentage hydrolysis efficiency obtained ranged between 11% and 600% with highest efficiency of 600% and 250% observed in *Bacillus megaterium* (FSP_1) and *Bacillus zanthoxyli* (FSP_4) respectively (**Figure 2**). The clear disparity observed in the values obtained for percentage hydrolysis efficiency between the previous and current study, clearly signifies that the current study isolates possess better cellulose-degrading perspective.

Figure 3 shows the quantitative assay for the crude cellulase produced by *Bacillus megaterium* (FSP_1) and *Bacillus zanthoxyli* (FSP_4) in the submerged fermentation process. Although the former exhibited a better cellulase activity than the latter with a difference of about 11µmol/ml, results obtained further prove the cellulase-producing proficiency of these study isolates. This study was similar to those carried out by Debnath *et al*. (2020), Debnath *et al*. (2018), and Ilyas *et al*. (2011).

The investigation of carbohydrate metabolism that aids in the identification of *Bacillus* species using biochemical assays, employing API Kit 50CHB/20E and an identification software system called the API web, identified the study isolates as *Bacillus megaterium* (FSP_1) and *Bacillus zanthoxyli* (FSP_4)(**Table 2**). The use of API kit for preliminary identification of bacteria species, is in agreement with work done previously by various researchers which includes; the identification of *Bacillus* sp. isolated from marine water (Alnahdi, 2012), and the identification of *Bacillus* sp. isolated from municipal waste water (Sonune and Garode, 2018).

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Among all bacterial species, *Bacillus* species are a varied group. Janda and Abbott, (2007) and Kai, (2019) stated that 16s rRNA gene sequencing approach has always been used to identify bacteria molecularly and that this method is more reliable for identifying microorganisms. Additionally, the 16s rRNA gene sequencing method has also been applied to genetic research and diversity of bacteria, including *Streptococcus* (Sasaki *et al*., 2004). Therefore, the findings of this study (**Figure 4 and Table 3**) are consistent with those of other researchers who used the NCBI BLAST tool to identify bacteria based on their 16S rRNA gene sequences (Lele and Deshmukh, 2016; Mohammad, 2017).

Conclusively, results obtained from the current investigation reiterate the potentiality of microbial species such as *Bacillus megaterium* ATCC 14581 (FSP_1) and *Bacillus zanthoxyli* 1433 (FSP_4) in producing the enzyme cellulase which is of great industrial importance due to its numerous applications in diverse sectors.

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