ISOLATION AND CHARACTERIZATION OF GLYPHOSATE DEGRADING BACTERIA FROM KUBAU LOCAL GOVT. AREA, KADUNA STATE

ABSTRACT

Strains of bacteria with the ability of utilizing glyphosate as the sole source of carbon were isolated from soil exposed to glyphosate herbicide by culture enrichment method and identified through partial 16S rRNA gene sequence analysis. Sixteen different microbial strains were isolated from glyphosate-contaminated sites in Kubau local govt. area, Kaduna state. Two of the microbial strains: BBDD3 and BBD4 were found to be the best degraders and were able to withstand up to 16.4 mg/ml of the glyphosate concentration. On the basis of 16s rRNA and biochemical tests, BBDD3 and BBDD4 strains were identified as *Escherichia coli* and *Sporanaerobacter acetigenes* respectively. These strains demonstrate laboratory utilizing ability of 92% and 98% for 16.4 mg/ml of the glyphosate formulation, respectively. The best optimum conditions for degradation were observed at pH 6.0 for the strains and at the temperature of 35^{o}C for strain BBDD3 and BBDD4. Based on these results, the two strains displayed their potentials to be used in the bioremediation of glyphosate-contaminated environment.

Key words: *Escherichia coli; Sporanaerobacter acetigenes;*Isolation; Identification; Process optimization

1.0 INTRODUCTION

Glyphosate (N-phosphonomethylglycine) is a biocide effective against wide range of organisms that was introduced for weed control in agricultural production field in 1974 (Benbrook, 2016). Glyphosate is a biochemical that is applied to the leaves of plant to eliminate both broad leaf plant and grasses. The salts of sodium from glyphosate regulate plant growth and ripen fruit. Glyphosate is a non-selective herbicide that can kill most plants. It prevents the plants from making certain proteins that are needed for plant growth by stopping the shikimic acid pathway is necessary for plants and microorganisms (Malik *et al*.,1989).

The consistent use of glyphosate has caused increase in environmental and plant residues. Glyphosate is quite resistance to degradation due to the inert carbon-phosphate linkage in the molecule (Chekan *et al*., 2016). None the less, it is brakes down in dead plant material and soil by various microorganisms (Mamy*et al*., 2016). As a result of large scale and coupled with consistent use of glyphosate as well as its accumulation in the environment and edible products, several major concerns have arisen in recent years about harmful effects of glyphosate and Aminomethylphosphonic acid for soil and water quality, plants, animal and health (Battaglin *et al*., 2014; Seralini *et al*., 2014). In 2015, the international agency for research on cancer (IARC, Lyon in France), a research arm of the world health organization, classified glyphosate as "probably carcinogenic" (IARC, 2015).

The purpose of this study was to isolate and characterize glyphosate degrading bacteria from Glyphosate contaminated soil..

2.0 MATERIALS AND METHODS

2.1: materials

Study site

Kubau local government area was chosen for this study after numerous consultations of maize farmers in Pambegua indicate their extensive use of glyphosate herbicide for farming activities. Kubau is a local government area in Kaduna State, Nigeria. Kubau was chosen for this study because it is a popular local government in terms of farming activities, its headquarters are in the town of Anchau. It has an area of 2,505 km^2 and a population of 282,045at the 2006 population census.

The chemicals and reagents used for this study are of good quality with high percentage purity. These are k_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$, NaCl, $FeSO_4.7H_2O$, NH_4NO_3 and $CaCl_2 2H_2O$**,** Crystal Violet, Gram's iodine, Alcohol and Safranin**,** hydrogen peroxide, tetramethyl-phenylene diamine dihydrochloride, and N-phosphonomethylglycine.

2.2. Methods

2.1Culture and Growth Condition

The different media viz. mineral salts medium and Nutrient Agar were used during the study. The pH of media is between 7.0-7.2 for both media. A mineral salt medium (modified) of Dworkin and Foster (1958), was used, the medium was prepared using k_2HPO_4,KH_2PO_4, $MgSO_4.7H_2O$,NaCl, $FeSO_4.7H_2O$, NH_4NO_3 and $CaCl_2 2H_2O$. HCL was used to wash all glass wares thoroughly and rinsed with deionized water to avoid contamination. The medium was autoclaved at 121^0c for 15mins prior to the addition of the filter sterilized glyphosate (Bush killer) containing 41g/100ml. The soil sample was air dried and sieved using a 2mm mesh. Five grams of each soil sample was suspended in 250ml flask containing a mixture of 50ml of mineral salt medium and 1ml of the glyphosate herbicide (Bush killer) as the sole carbon source. This concentration is equivalent to the field application rate. The flask was incubated on a rotary shaker at 120rpm for 7days at 30^0C under agitation. Then 5mL of the fermented broth was transferred to 50mL fresh minimum salt media containing 1 g/L glyphosate. Four subcultures were conducted and followed by serial dilutions (10^{-2} to 10^{-6}) of final enrichment cultures were plated on Luria broth agar plates containing 0.5 g/L glyphosate. Colonies were picked on the basis of distinct colony morphology and followed by inoculation into a liquid medium to test the use of glyphosate (Akhter and Laz, 2013).

2.2 Identification of the isolates

Bacterial colonies with distinct morphology were isolated and repeatedly sub-cultured using Nutrient agar. Identification of the isolates was affirmed by standard bacteriological methods after characterization (Holt *et al*., 1994). The probable identities for the pure cultures of bacteria isolates were determined when subjected to various biochemical tests. The result of each test was recorded and the probable identity of the isolates was deduced (Buchanan and Gibbons, 1974). Meanwhile, Gene fragments specific for the highly variable region of the bacterial 16s rRNA gene was amplified by PCR as described by Penaloza-Vazquez *et al.,* 1995 This reaction was carried out by the universal primer 16s forward and 16s reverse (Sigma USA). The primer sequences were as follows: 5'- GAGTTTGATCCTGGCTCAG -3' forward primer and 5'-GAAAGGAGGTGATCCAGCC -3' reverse primer (Penaloza-Vazquez *et al.,* 1995). PCR reagents were all used from the Invitrogen. PCR analysis was conducted in a 50µl reaction mixture containing 5µl of 10X PCR buffer, 4µl of 2.5mM dNTPs, 2µl of 50mM MgCl2, 2.5µl of each primer (10µlM), 0.2µl of 5U/µl tag DNA polymerase, 3µl genomic DNA and rest of the PCR water. As a PCR program, an initial denaturation step at 95^{o}C for 5min. followed by 30 cycles of denaturation at 94^{o}C for 1min. primer annealing at 55^{o}C for 1 min, 1min of primer extension at 72^{o}C, and 10min of final extension at 72^{o}C for one cycle. Amplicons were separated by agarose gel electrophoresis (1%) in 0.5X Tris/borate/EDTA buffer. Ethidium bromide was stained with the products, destined with distilled water, visualized under UV light and photographed on a gel-documentation system (Akhter and Laz, 2013).

Phylogenetic analysis

The phylogenetic analysis for the isolates was carried out using Molecular Evolutionary Genetics Analysis (MEGA) software (Kumar *et al., 2018*).

2.3 Identification of Glyphosate Metabolites

Using centrifugation at 8000 rpm and 4°C for 15 min coupled with washing twice with a phosphate buffer (0.05 M; pH 7.0), followed by re-suspension in the same buffer and disintegrated using an ultrasonic probe (45cycles of 5s on and 5s off at 280W), cells were harvested during exponential growth. Centrifugation of the homogenate was conducted at 10000rpm for 15min at 4°C and the supernatant was filtered through 0.22µm membrane filters. Subsequently, 0.3mL of the filtrates was added to 2.7mL of the phosphate buffer (0.05M; pH 7.0) with 0.5g/L glyphosate. The mixtures were incubated at 33°C for 2 h. 0.5mL HCl (1 M) was added, and the reaction mixtures were used as samples for the analysis of glyphosate metabolites. HPLC (LC-20A Shimadzu, Japan) with UV detection at 240 nm was employed in detecting glycine in the culture liquid and soil extracts using an Intersil ODS-SP C18 reversed-phase column (5µm × 4.6mm × 150mm) and 10mM phosphonic acid as the mobile phase at a flow rate of 0.8mL/min (Haoyu*et al*., 2015).

3 Results and discussion

3.1 Isolation of glyphosate degrading bacteria

In the present study, sixteen strains of different bacteria were isolated through bacterial morphology differences from the sites contaminated with glyphosate in Kubau local govt. area, Kaduna state, Nigeria. Two of the microbial strains named; BBDD3 and BBD4 were found to be the best degraders as they were able to withstand 16.4 mg/ml of the glyphosate concentration. These two strains were selected for further studies.

3.2 Effects of pH, temperature, inoculum size and Glyphosate concentration on BBD3 and BBD4 growth.

The result in (Fig. 1) displayed the bacterial growth evolution at different temperatures (30, 35, 40 and 45^{o}C) for the isolated bacterial strains. An increase significantly in the growth rate of strain BBDD3 and BBDD4 were noted at 35^{o}C. Using U.V spectrophotometer, strain BBDD3 and BBDD4 however showed better growth or degradation rate at 35^{o}C. Moreover, the optical density obtained for BBDD3 and BBDD4 were 0.242 and 0.119 respectively. Report showed that *E. coli* has a wide range of growth temperature between 18-47^{o}C and 17-46^{o}C respectively (Akiyoshi *et al.,* 1981).

The glyphosate concentration effect on the growth of BBDD3 and BBDD4 strains were shown in Fig 2. Generally, the growth of BBDD3 strain was greater at 12.3mg/ml concentration of glyphosate with absorbance of 0.182. Whereas BBDD4 strain growth was favorable in 8.2mg/ml concentration of glyphosate in the culture medium with an absorbance of 0.207. Generally, the isolates w survive and grow at the designed variation of glyphosate concentration in the culture medium. A report by (Motharasan *et al.,* 2017) showed the ability of glyphosate degrading bacteria to degrade up to about 3ml/L of glyphosate formulation.

The effect of pH on the growth of BBDD3 and BBDD4 is shown in Fig 3. Generally, the growth of both strains was greater in the medium at pH 6 over 60 h. When the initial pH was higher than pH 6, there is an increase in the degradation rate of strain BBDD3 and BBDD4. Uniquely, slightly acidic pH is favorable for glyphosate degradation by the strains. This can be also explained during the acclimation process via the introduction of

glyphosate into the culture media which can result in an acidic condition, thus making the microbes to be able to acclimatize to this state, leading to a better degradation process in the acidic environment. However, limited literature is available regarding glyphosate-degrading bacteria favoring acidic condition as most glyphosate degraders prefer neutral-alkaline pH for optimum degradation rate (Singh and Walker, 2006).

The effect of inoculum size on the growth of BBDD3 and BBDD4 bacterial strains were shown in Fig 4. Generally, BBDD3 and BBDD4 strains grow better at 800ul and 100ul concentration of the inoculum with absorbance of 0.097 and 0.110 respectively. All the isolates were generally able to survive and grow at the designed variation of inoculum size concentration in the glyphosate containing culture medium and this suggest the extensive use of glyphosate. Inhibition of growth was not observed in the isolates which are in conformity with report published by (Motharasan *et al.*, 2017).

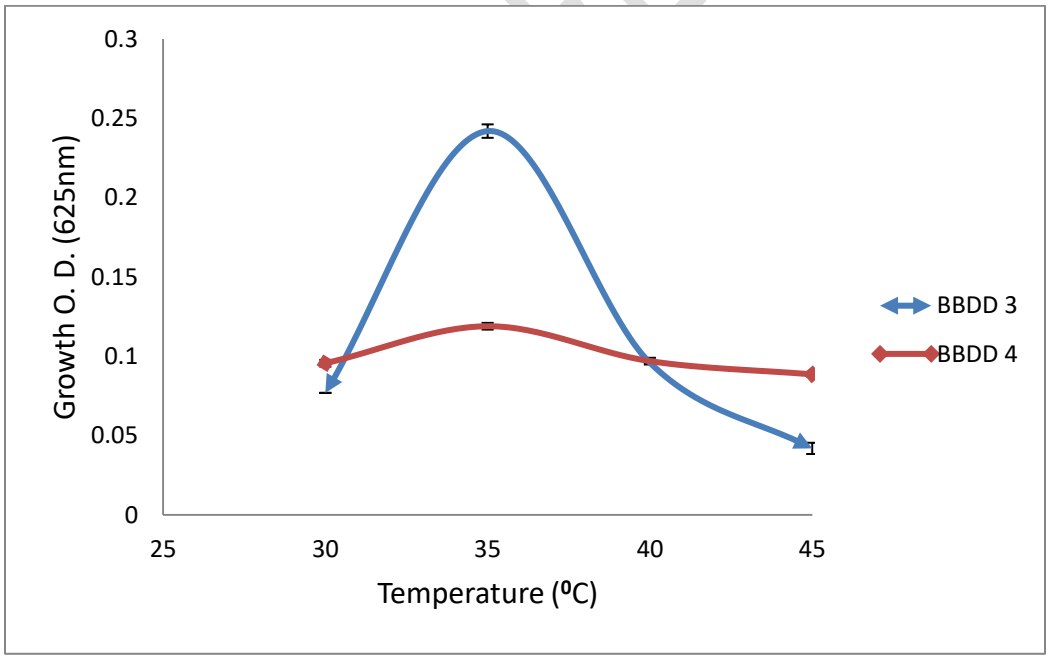


Fig 1 showed the growth response of isolate BBDD3 and BBDD4 at different temperature where "T" indicates Temperature

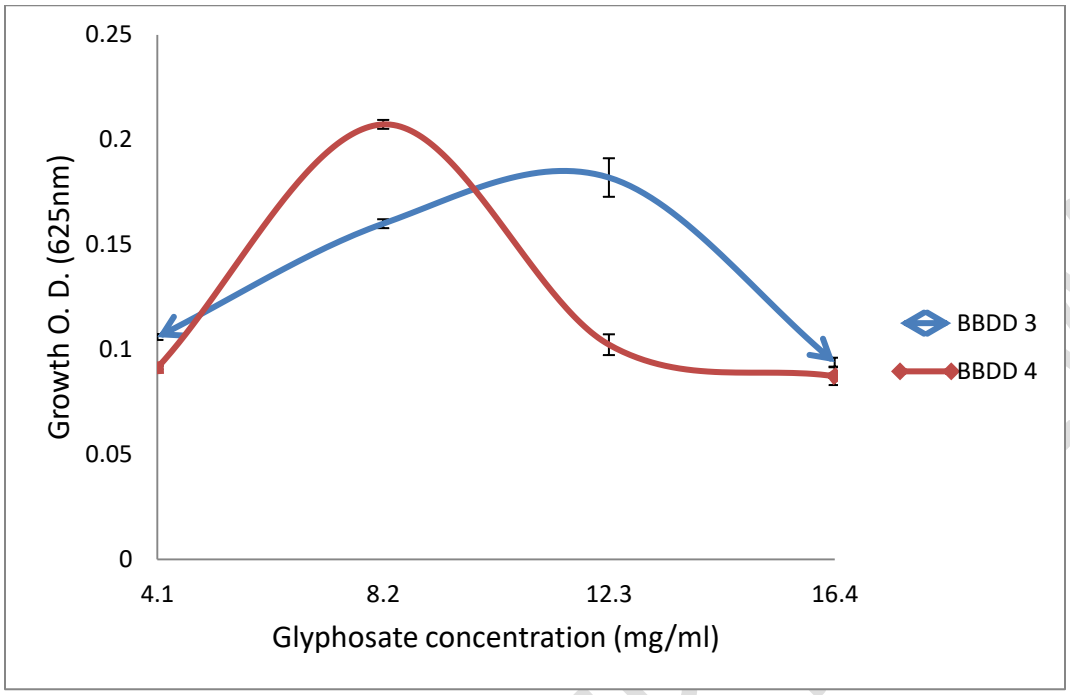


Fig 2 showed the Growth response of isolate BBDD3 and BBDD4 at different glyphosate concentrations where "C" indicates concentration of glyphosate

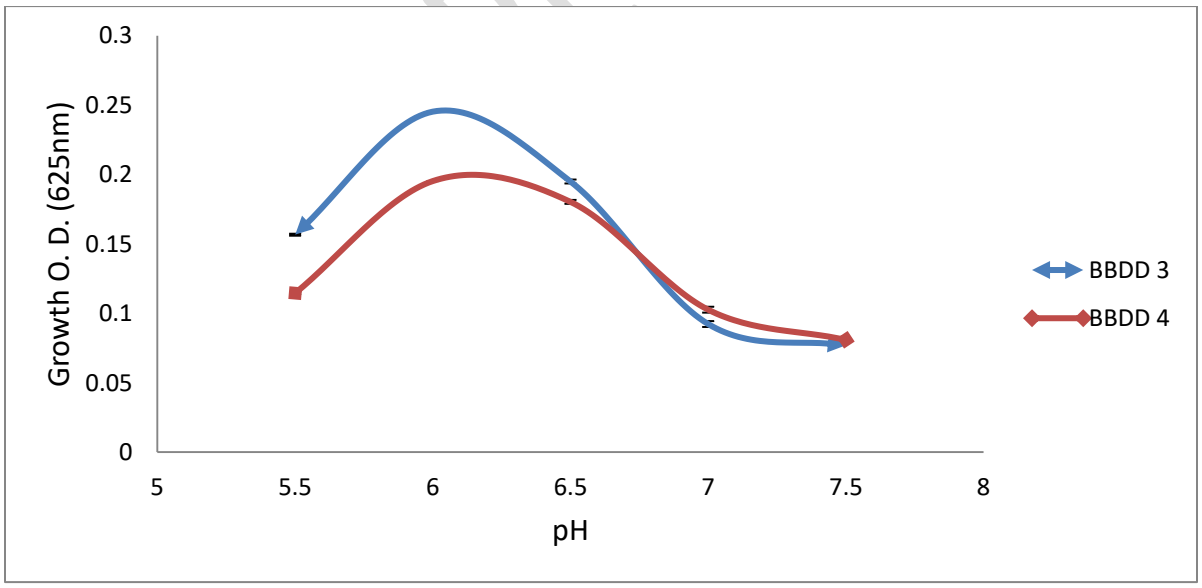


Fig 3 showed the growth response of isolate BBDD3 and BBDD4 at different pH concentrations

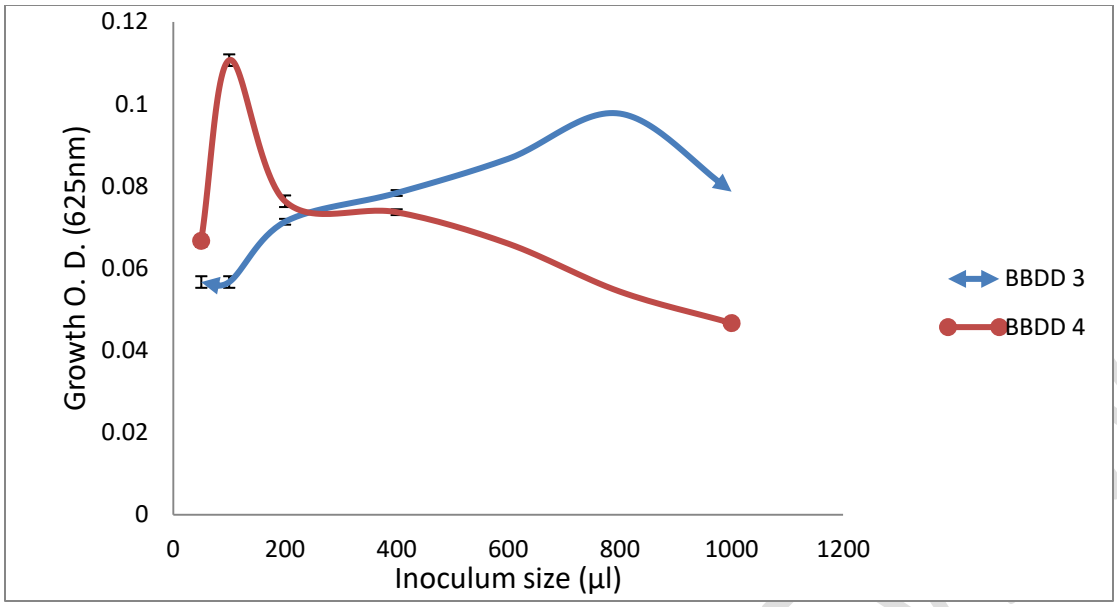


Fig 4 showed the growth response of isolate BBDD3 and BBDD4 at different inoculum size concentrations.

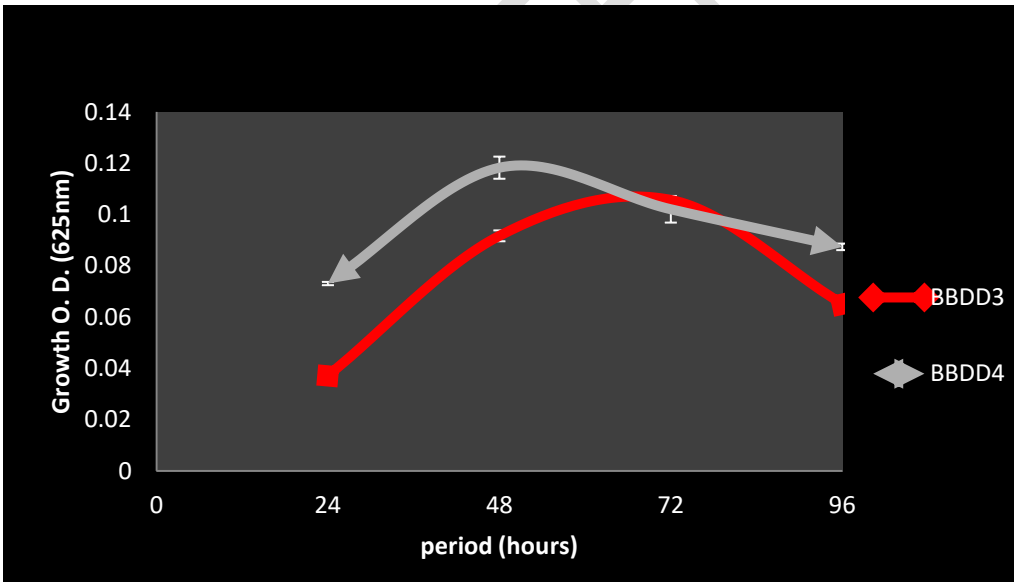


Fig 5 showed the biodegradation potential of the“BBDD” isolates

Table 1.A display of biochemical test results for BBDD bacterial isolates

TEST	BBDD3	BBDD4
Gram staining	-	+
Motility	+	+
Catalase	+	-
Oxidase	-	-
Indole	+	-
Methyl red	+	+
Starch hydrolysis	+	+
Sugar fermentation	+	+
Citrate utilization	-	+
Hemolytic	-	-
Voges-proskauer	-	-
Suspected bacteria	*Bacillus Sp.*	*Acetobacter Sp.* .

Phylogenetic analysis:

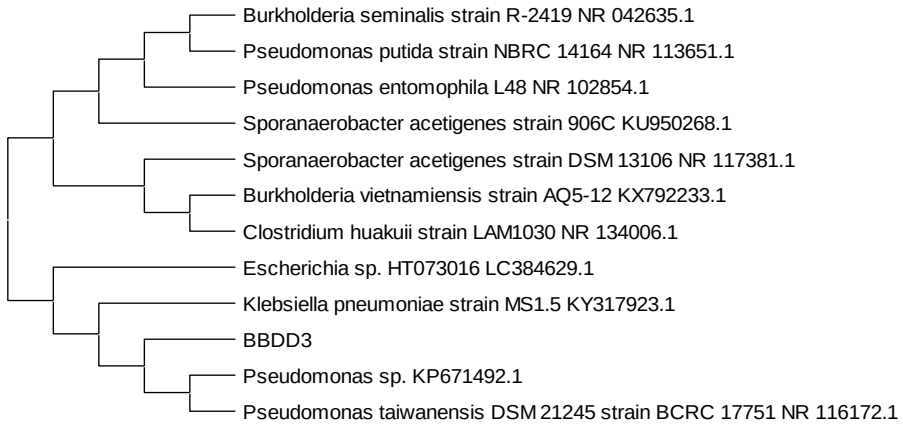


Fig 6 phylogenetic analysis of isolate BBDD3 and some glyphosate degrading species by neighbor joining method

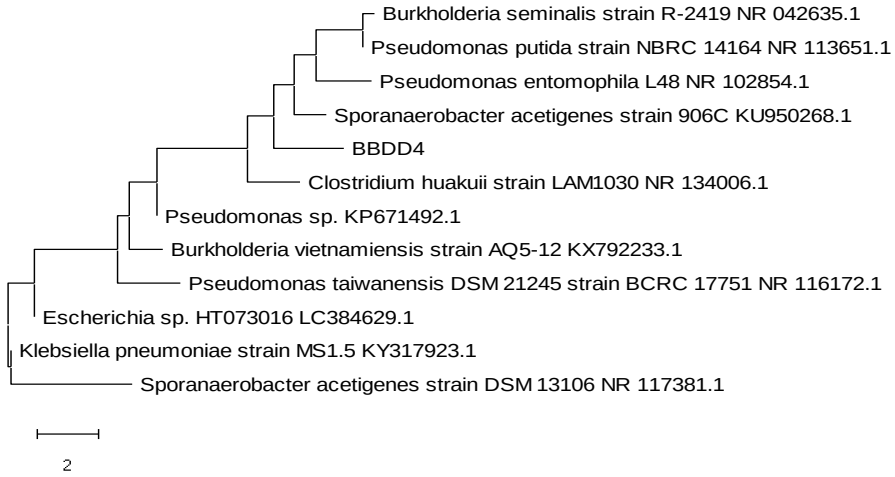
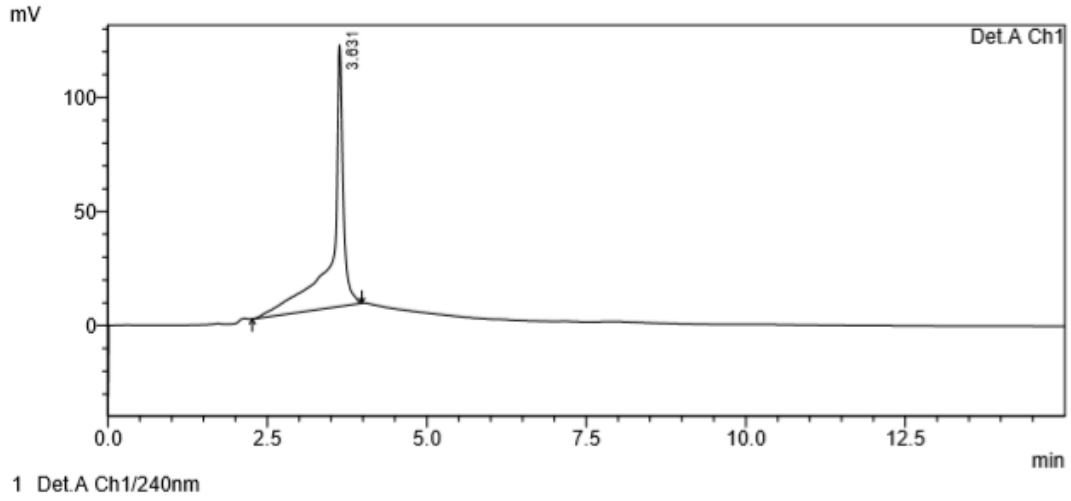


Fig 7 phylogenetic analysis of isolate BBDD4 and some glyphosate degrading species by neighbor joining method

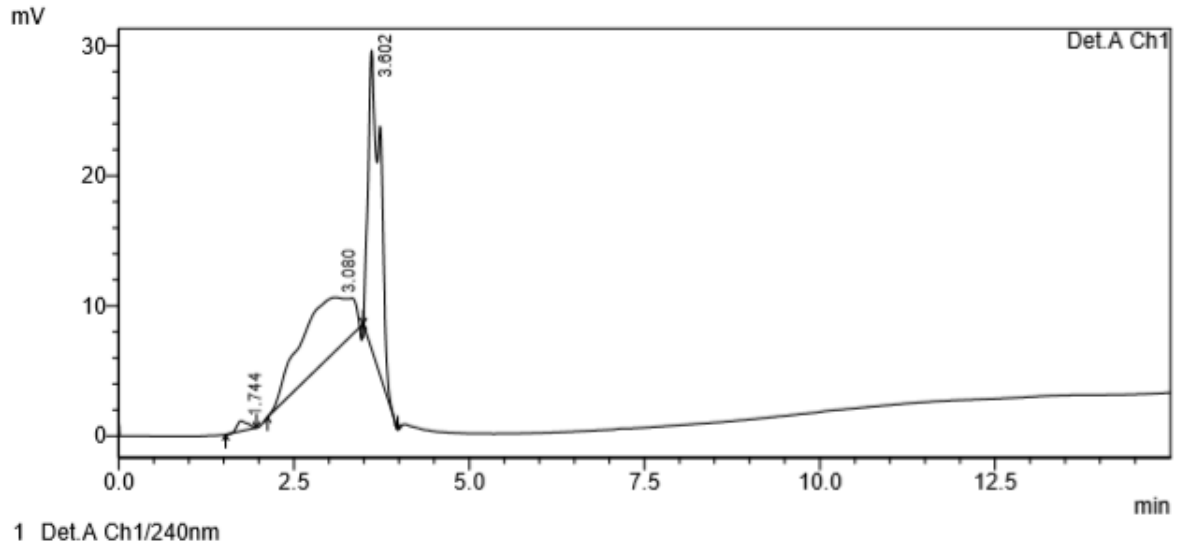


PeakTable

Detector A Ch1 240nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.631	1374052	114595	100.000	100.000
Total		1374052	114595	100.000	100.000

Fig 8 displayed the HPLC analytical result of isolate BBDD3



PeakTable

Detector A Ch1 240nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.744	8382	782	1.618	2.813
2	3.080	235053	4181	45.370	15.037
3	3.602	274641	22844	53.012	82.150
Total		518075	27807	100.000	100.000

Fig 9 displayed the HPLC analytical result of isolate BBDD4

The results obtained from the chromatogram shown in Fig 8 and Fig 9 displayed HPLC chromatogram for isolate BBDD3 and BBDD4 samples respectively. Using the peak height and area under the standard, the efficiency of the chromatographic separation and also the response factor (R.F) were made known (https://chromeacademy.com). The R.F was used to determine the amount of metabolic byproduct glycine detected in the chromatogram by HPLC quantitation (https://chromeacademy.com). The amount of glycine detected in strains BBDD4 and BBDD3 were 0.711mg and 3.56mg respectively. According to published reports, a GP degradation pathway has been proposed (Liu *et al*., 1991; Sviridov *et al*., 2011). Glyphosate degradation is catalyzed by Carbon-Phosphate lyase thereby forming sarcosine, which eventually transforms into formaldehyde and glycine, a reaction catalyzed by sarcosine oxidase. In order to elucidate the pathway for Glyphosate degradation in BBDD3 and BBDD4, the degradation product

(glycine) of GP was analyzed. Meanwhile, trace amounts of glycine was detected in the strains, indicating the involvement of this pathway in the strains.

3.3 Phylogenetic tree and BLAST

Before sequencing, the 16S rRNA DNA for the four isolates was run on gel for separation which is subsequently followed by PCR amplification using universal primer and the isolates' rRNA DNA. Molecular phylogenetic analysis for glyphosate degrader each of isolate BBDD3 and BBDD4 reveal cluster after a successful BLAST. The partial sequences for 6S rRNA gene of the strains were submitted to Gen Bank and comparison was conducted with sequences of previously deposited strains. Strains BBDD4 cluster strongly (93.92% bootstrap support) with *Sporanaerobacter acetigenes* DSM13106 having accession number of NZ_FQXR01000034.1. Of the named species, BBDD3 was related most closely to *Escherichia coli* 0157:H7 str. Sakai DNA (80.31% bootstrap support and accession number of NC_002695.2).

Bacteria from genus Pseudomonas and Bacilli (*E. coli*) are known to be very active metabolically and can utilize a broad-spectrum of xenobiotic compounds. *E. coli* and *Pseudomonas pseudomallei* (Penaloza-Vazquez *et al.,* 1995) were reported to have glyphosate degrading ability. Pseudomonas sp. strain LBr was isolated by Jacob *et al*. (1988) which completely utilized via metabolism 3.21 g/L GP with a degrading efficiency of about 2g GP/g dry biomass. On comparison with these previously Pseudomonas strains which were reported, the 2 isolated bacteria in the present study exhibit a record high Glyphosate-degrading abilities.

Obtained results from the bootstrap analysis of the partial 16S rRNA was consistent with the morphological results and the biochemical properties (see Table 1). And as such, the isolated

strain BBDD3 and BBDD4 were identified as *Escherichia coli* 0157:H7 str. Sakai DNA and *Sporanaerobacter acetigenes* DSM13106.

Sporanerobacter acetigens DM13106 has been reported to possess glyphosate degradation ability (www.mmtb.tu-bs.de/pathway).

Conclusion

The results presented in this work revealed that the four isolates, characterized with remarkable glyphosate degrading ability could be potential agents in developing bio-surfactant for bioremediation of soil contaminated with glyphosate herbicide. More so, new glyphosate degrading strains were added to the list of glyphosate-degraders. The genetic capacity of the isolates can be exploited for the remediation of glyphosate polluted sites.

DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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