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Biodegradation potentials of edaphic bacterial isolates cultured on Haloxyfop R Methyl ester herbicide-mineral salt medium

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Abstract

The heterotrophic and haloxyfop-R methyl ester utilizing bacterial counts associated with top soil samples was evaluated. Three (3) bacterial isolates; *Bacillus* sp., *Micrococcus* sp. and *Staphylococcus* sp. were cultured and screened for their ability to utilize haloxyfop-R methyl ester as sole source of carbon and energy using the turbidimeteric procedure. The growth profiles of two axenic cultures; *Bacillus* sp. and *Micrococcus* sp. were determined using the shake flask test. Parameters which included pH, mean viable bacterial counts, optical density and dissolved CO_2 were determined during growth profiling using relevant procedures and equipment. The pH of the soil samples was 5.08 ± 0.02 for sample A and 4.62 ± 0.02 for sample B. The total heterotrophic bacterial count was 2.8 × 10^4 cfu/g ± 849 for A and 4.62 × 10^4 cfu/g ± 989 for B. The mean dissolved CO_2 data for *Micrococcus* sp. during the growth profile study ranged from 1.1 mg/l ± 0.1 to 6.8 mg/l ± 0.2. Axenic *Micrococcus sp.* was the most effective amongst the growth profile cultures in mineralizing the herbicide content of the culture medium.

Keywords: Axenic, Bacterial consortium, Growth profile, Haloxyfop-R methyl ester, Mineral salt medium, Mineralization, Shake flask.
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Introduction

Herbicides have been described as biologically active compounds utilized in the eradication of unwanted plants (Janaki *et al.*, 2015; Mesnage *et al.*, 2021). Commercial agricultural activities such as cash and tree crops cultivation are known to be reliant on the application of herbicides for weed control with the related objectives of maximization of crop yield and economic benefits to sustain a continually increasing global human population (Mesnage *et al.*, 2021). Herbicides are known to differ with respect to their persistence rates in soil and examples of persistent herbicides include; triazines, uracils, phenylureas, isoxazolidinones, imidazolinones coupled with several flora growth regulators which belong to the pyridine group (Jaboro *et al.*, 2019).

The fate of herbicides has become an important environmental issue, in view of the fact that targeted flora are only affected by low concentration of the applied chemicals (Sarkar, *et al.*, 2021), which has led to the greater likelihood of negative health effects on non-target fauna and flora arising from exposure to these applied chemicals (Mesnage *et al.*, 2021). Herbicides are essential chemicals for agriculture, but under some circumstances, they are also known to function as contaminants that can damage soils, groundwater, and surface water supplies (Mesnage *et al.*, 2021). While most herbicides are not intentionally sprayed on soil, there are a number of ways they can get into the edaphic environment. They include herbicide runoff from floral assemblage, leaching from dead plant material, and direct interception of spray by the edaphic surface in either the early planting season or during post-harvest applications (Zabaloy *et al.*, 2011).

Mineralization can be described as the process of pesticide degradation by microbiota with a metabolic capacity to utilize the recalcitrant

chemical moiety as a source of carbon and energy for growth and proliferation. This approach may cause the molecule to completely dissipate and transform into CO_2, H_2O and inorganic components (Mesnage *et al*., 2021). Parte *et al.* (2017) opined that various microorganisms ranging from bacteria to fungi were known to possess varying degrees of pesticide degradative abilities. The authors also stated that generally, microorganisms known to possess the ability to degrade pesticides have been cultured from an array of pesticide polluted soil habitats.

Haloxyfop-R methyl ester is a member of the aryloxyphenoxy-propionate herbicide (AOPPs) family based on 4-oxyphenoxypropanoic acid as their main skeletal form and on a global basis, the herbicide has been described as a widely utilized herbicide (Zhou *et al*., 2018). Haloxyfop-R methyl ester has a chemical formula of $C_{16}H_{13}ClF_3NO_4$ and a molecular weight of 375.7 g/mol respectively (Rathore *et al*., 2021). The herbicide is utilized for controlling the growth of annual and perennial weed plants in areas where economic plants such as sugar beet, fodder beet, potatoes, leafy vegetables, onions, sunflowers, soya beans, vines, strawberries and other broad leaf plants are cultivated (Rathore *et al*., 2021).

The sprayed herbicide is usually taken in by the foliage and roots and hydrolyzed to haloxyfop which is then translocated to the meristematic tissues, hindering the proliferation of the weed plant (Rathore *et al*., 2021). Whilst AOPPs are regarded as non-toxic for economic plants, their residues in the edaphic niche can have deleterious effect on surrounding crops or non-target biota (Zhou *et al*., 2018).

Contamination of terrestrial niches arising from routine herbicide usage in agricultural (Pileggi et al., 2020; Tudi *et al*., 2021) and horticultural practices would warrant the need to develop sustainable approaches to remediate or mitigate the deleterious effects of herbicide applications on the agriculturally modified terrestrial environments. It has been revealed that the utilization of microorganisms for AOPPs removal in contaminated soils has some important benefits, which include cost-effectiveness and environmental friendliness (Hussain *et al*., 2015). In line with this revelation, several microorganisms with excellent degradation capacity for different AOPPs have been isolated in recent years (Zhou *et al*., 2018). However, despite the traditional usage of these herbicides in different parts of Nigeria for various activities such as agriculture (Kughur, 2012) with the attendant contamination of the recipient environment, there is a dearth of information with respect to the isolation of microorganisms that can be utilized in the degradation of herbicide residues. As such, this study aimed at the evaluation of the biodegradation potentials of phenotypically characterized soil edaphic bacterial cultures grown on Haloxyfop-R methyl ester liquid medium. To achieve this aim, several soils borne isolates were screened for their herbicide degradative abilities and cultures which showed appreciable degradative potentials were subjected to shake flask tests utilizing the herbicide as sole carbon and energy source.

Materials and Methods

Collection of soil samples and herbicide

A quantity of the top (0-15 cm) soil samples; 100 g were collected from two (2) locations at a fallow plant covered land within the botanical garden premises sited in the University of Benin, Ugbowo campus, Benin City. The coordinates of the sampled sites were A (N 06^0 23. 843′ E 005^0 36.978′) and B (N 06^0 23. 852′ E 005^0 36.967) and the samples were collected using a soil auger and the bored soils were placed in clean labeled polyethylene bags. The samples were taken to the laboratory for pH and bacteriological evaluation. The granular form of the herbicide; Haloxyfop-R methyl ester was purchased, and the trade name was Gallant®. The herbicide was also taken to the laboratory and utilized in relevant experiments.

Determination of pH and culturable bacteriological profile

A specific amount of collected soil sample; 10 g was weighed and transferred onto a 100 ml glass beaker. About 20 ml of a 0.01 M $CaCl_2$ solution was added and the resultant suspension was continuously stirred for 20 minutes. A calibrated electrode from a Suntex SP-2100 pH meter (Suntex Instruments Company. New Taipei City) was inserted into the suspension and steady pH readings were documented (Álvaro-Fuentes *et al.*, 2019).

The amount of the respective soil sample; 10 g were suspended in sterile conical flasks containing 90 ml of sterile peptone water diluent. The homogenate was serially diluted to 10^{-7} using tenfold dilution. Using the pour plate technique as described by Cappuccino and Welsh (2020), aliquots (1ml) from each dilution were plated in duplicates under aseptic conditions. Nutrient agar (NA) was utilized in the determination of the

heterotrophic bacterial counts (Bridson, 2006). For the culturing of Haloxyfop-R methyl ester degrading bacteria, modified mineral salt medium (MMSM) as described by Okpokwasili and Okorie (1988), modified by the addition of 1% Haloxyfop-R methyl ester which served as carbon source was used. An anti-fungus drug: nystatin was added to the labeled Petri dishes to inhibit fungal growth and the quantity added was 0.1 ml of 10 mg/l (Obiefuna and Onuorah, 2022). The labeled NA and MMSM agar plates were incubated aerobically at 35°C for 48hr and 7 days respectively. After incubation, counts obtained from culture plates were recorded and the cfu per one gram of the sample was derived in accordance with a formula stated by Yates *et al.* (2016).

Purification and identification of isolates

The bacterial isolates were purified by sub-culturing the various cultures onto freshly prepared NA plates and Gram-stained (Brown and Smith, 2015). Phenotypic identification of both Gram positive and Gram-negative bacterial cultures was conducted using API 50CHB and API 20E strips (BioMerieux, Marseille) (Imarhiagbe *et al.*, 2016). Supplementary biochemical tests: endospore staining, and oxidase production were also conducted on the isolates.

Preparation of standard bacterial cultures

Standard suspensions of the bacterial isolates were prepared following the techniques previously described by Pepper *et al.* (2015) and Reddy *et al.* (2007). One hundred (100) ml of mineral salt broth was dispensed into flasks and seeded with isolate transferred from pure culture stock with the aid of a sterilized inoculating loop and incubated at 28°C for 24 hrs. After incubation, serial dilution and pour plating was done. The resultant plate counts were documented, and the values obtained were expressed as standard number of bacterial cells present in 0.1ml of the broth. This was utilized as the standardized bacterial culture.

Screening for the ability of bacterial isolates to utilize haloxyfop-R methyl ester as sole carbon and energy source.

The method as described by Okpokwasili and Okorie (1988) was adapted to screen the ability of the purified bacterial and fungal isolates to utilize Haloxyfop-R methyl ester as sole carbon and energy source using the modified mineral salts medium (Mills *et al.*, 1978). To a set of test tubes, 9 ml of mineral salt medium (MSM) was respectively added. One (1) gram of granular Haloxyfop-R methyl ester was weighed and dispensed to the respective tubes and capped just before autoclaving. Upon cooling, each of the first set of tubes was seeded with 0.1ml of standardized suspension of the respective prokaryotic cultures. A non-inoculated tube was used as control.

All the experimental tubes were incubated at 35°C for 10 days. The optical density of the respective tubes was taken at day 0 and day 10 respectively at a wavelength of 450 nm using a spectrophotometer: Biobase model BK-UV1800PC (Biobase bio-industry, Shandong). The difference between each OD reading was documented and the two cultured tubes which had the highest difference were adjudged to have the best screening potentials with respect to Haloxyfop-R methyl ester degradation.

Growth profile of axenic and mixed consortium of bacterial isolates in haloxyfop-R methyl ester medium

The growth profiles of the bacterial isolates which were selected from the screening test were determined using procedure previously described by Okpokwasili and Okorie (1988). Two (2) litres of MSM were prepared (pH 7.2). Two hundred-and forty-seven-point five (247.5) ml of the medium was dispensed onto nine (9) labeled 250 ml conical flasks and a measured quantity of granular herbicide (2.5g) was added to the respective flasks.

The flasks were sterilized and 2 ml of a 24 hr MSM broth culture of each of the bacterial culture's isolates were pipetted into each flask with the exception of the control flask in a sterile setting (Okpokwasili and Okorie, 1988). The flasks were incubated at 37°C for 13 days on an incubator shaker (Heidolph Unimax 2010, Heidolph Company, Wood Dale) at 120 rpm. Each flask was analyzed for Haloxyfop-R methyl ester utilization and mineralization at a 48 hr interval. The indicators of Haloxyfop-R methyl ester utilization and mineralization were Total viable count, pH, optical density (OD) and dissolved carbon dioxide values. Duplicate samples from the cultured and control flask were subjected to analysis and a mean value was derived from the duplicate values.

Viable mean bacterial count determination

The viable mean bacterial counts of each flask were ascertained using the pour plate technique (Yates *et al.*, 2016), with peptone water and

Nutrient agar (NA) utilized as diluent and general-purpose medium (Bridson, 2006). Plating was conducted in duplicates and 1 ml of an antifungal agent solution; Nystatin – 500mg in 50ml sterilized distilled water was dispensed onto each Petri dish prior to the addition of cool molten NA. The NA agar plates were incubated at 37°C for 24 hr and emergent discrete colonies were counted and recorded (Yates *et al*., 2016).

pH determination

The mean pH value of each culture flask was determined with the aid of a Suntex SP-2100 pH meter. The electrode was first calibrated with freshly prepared pH buffers 4.0, 7.0 and 14.0. The calibrated electrode was dipped into each beaker containing the samples and steady readings were recorded.

Evaluation of optical density

This was determined the aid of a Biobase Spectrophotometer model BK-UV1800PC. Ten (10) ml of the sample was dispensed into a clean cuvette under aseptic conditions and steady OD readings were recorded at 450 nm.

Determination of dissolved CO_2 values

Dissolved carbon (IV) oxide (CO_2) values were determined using the titrimetric method (Bastola *et al*., 2013).

Statistical evaluation of the growth profile data

All the mean data obtained for the axenic bacterial isolates and bacterial consortium were subjected to one way analysis of variance (ANOVA) with the aid of SPSS version 22. This was to assess if the observed differences in the mean values was significant at 95% probability level. Mean separation was also conducted using a post hoc test; Duncan's multiple range test at 95% probability level.

Results and Discussion

Soil pH, bacteriological counts and screening data

The pH of the soil samples was 5.08 ± 0.02 for A and 4.62 ± 0.02 for B (Table 1). The total heterotrophic bacterial count was 2.8×10^4 cfu/g ± 849 for A and 4.62×10^4 cfu/g ± 989 for B while the total Haloxyfop-R methyl ester utilizing bacterial count was 5.0×10^3 cfu/g ± 849 for A and 5.2×10^3 cfu/g ± 566 for B (Table 1).

The investigated top soil samples were acidic and this attribute might have had a direct effect on the microbial activity within the soil samples. Soil pH has been known to influence several factors which can directly affect soil microbial activity. Examples of these factors include; solubility and ionization of inorganic and organic soil solution components and these factors are known to consequently impact soil enzyme activity (Voroney, 2007).

Table 1: pH and bacteriological counts of the soil samples

Soil samples	pH	Total heterotrophic bacterial count $\times 10^4$ cfu/g	Total Haloxyfop-R methyl ester utilizing bacterial count $\times 10^3$ cfu/g
A	5.08 ± 0.02	2.8 ± 849	5.0 ± 849
B	4.62 ± 0.02	2.6 ± 989	5.2 ± 566

KEY: overall mean± Std. deviation

Three (3) bacterial isolates; *Bacillus sp. Micrococcus sp.* and *Staphylococcus sp.* were isolated from the soil samples (Table 2). These bacteria are present in either barren or plant covered soils and *Bacillus* spp. are known members of plant microbiomes (Voroney, 2007) (Goldman and Green 2015). Heterotrophic bacterial counts were detected for all the soil samples and despite the non – application of herbicides to the sampled area, varying counts of Haloxyfop-R methyl ester utilizing bacteria were recorded for the respective soils. These trends might be reflective of the abundance and intensity of microbial activities occurring within the sampled edaphic area and the expressed ability of the soil borne bacterial isolates to utilize the herbicide as a carbon and energy source despite the anthropogenic origin of the herbicide.

Two of the three bacterial isolates; *Micrococcus sp. and Bacillus sp.* exhibited the highest difference in optical density readings at the conclusion of the screening test (Table 2). These two isolates and a consortium of these cultures

were utilized in the subsequent growth profile study as they exhibited the best biodegradation potential when cultured on the herbicide for seven (7) days. This trend can be directly linked to the ability of the metabolic machinery within these bacteria to successful utilize the herbicide as sole carbon and energy source. The herbicide degrading attribute of *Bacillus* species has been also reported by Huang *et al.* (2018) and the authors gave examples of these herbicides degraded by the bacterium which included; endrin and glyphosate respectively.

Table 2: Haloxyfop-R methyl ester utilizing capabilities of the bacterial isolates

Bacterial isolate	Difference in absorbance reading	Decision
Micrococcus sp.	0.366	Selected
Bacillus sp.	0.810	Selected
Staphylococcus sp.	0.113	Not selected

Growth profile study

The mean counts recorded for *Micrococcus* sp. during the shake flask experiment ranged from 3.5 × 10^2 cfu/ml at Day 1 to 1.3 × 10^3 cfu/ml at Day 9 (Fig. 1). The mean counts recorded for Bacillus sp. during the growth profile study varied from 2.8 × 10^2 cfu/ml at Day 1 to 1.9 × 10^3 cfu/ml at Day 9 (Fig. 1). The mean counts recorded for the bacterial consortium during the growth profile study ranged from 3.4 × 10^2 cfu/ml at Day 1 to 1.2 × 10^3 cfu/ml at Day 9 (Fig. 1). The difference between the mean bacterial counts was significant ($p<0.05$).

Comparative assessment of the mean bacterial counts for both the single bacterial cultures and the consortium of both bacterial cultures grown on the amended medium containing the herbicide as sole carbon source indicated that the axenic *Bacillus* sp. had maximal counts at Day 14 of the study. This observation might suggest that amongst the axenic growth profile isolates, *Bacillus* sp. cultured on the herbicide-MSM medium exhibited a greater tolerance and adaptation towards the changing micro-environmental conditions within the culture flasks as the shake flask study progressed. This observation was also supported by the screening results which indicated that the isolate displayed maximal herbicide biodegradation potential in comparison with *Micrococcus* sp. which came second in the screening test. Another possible reason for the comparative low *Micrococcus* counts was the likelihood that the prokaryote was less tolerant of the altered micro-environmental conditions within the culture flask occasioned by the increased concentrations of secondary metabolites such as organic acids arising from the metabolism of the herbicide content of the growth medium.

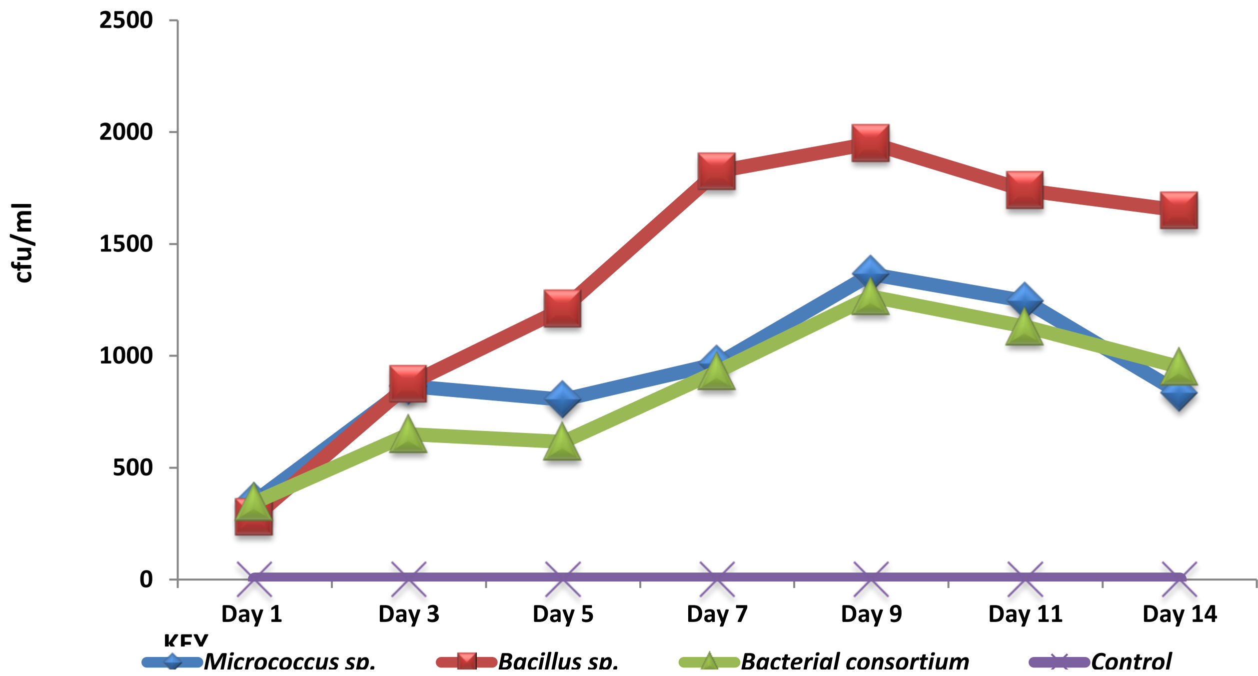

Fig. 1: Mean bacterial counts (cfu/ml) for the growth profile isolates cultured on Haloxyfop-R methyl ester mineral salt medium

The mean pH data recorded for *Micrococcus sp.* during the growth profile experiment ranged from 6.01 ± 0.05 at Day 14 to 7.30 ± 0.01 at Day 3 and 5 respectively (Fig. 2). The mean pH values recorded for *Bacillus* sp. during the growth profile study ranged from at 6.12 ± 0.002 at Day 11 to 7.31 ± 0.02 at Day 5 (Fig. 2). The mean pH data recorded for the bacterial consortium during the growth profile study varied from 5.75 ± 0.05 at Day 11 to 7.16 ± 0.005 at Day 3 (Fig. 2). The mean pH data recorded for the un-inoculated control during the growth profile study varied from 7.17 ± 0.01 at Day 5 to 7.26 ± 0.01 at Day 1 (Fig. 2). The variation between the mean pH readings was not statistically significant ($p>0.05$). In the course of the growth profile study, the pH of the agitated inoculated flasks decreased from neutral values at Day 1 to acidic values but the extent of the pH reduction was higher in the flask seeded with the bacterial consortium as this flask recorded the lowest mean pH amongst all the seeded flask. This observation might be the result of the combined intensity of microbial activities between both consortial members; *Bacillus* sp. and *Micrococcus* sp. which might have culminated in increased levels of several unidentified primary and secondary metabolites such as dissolved organic acids and gases which would accumulate in the surrounding medium leading to reduced pH readings.

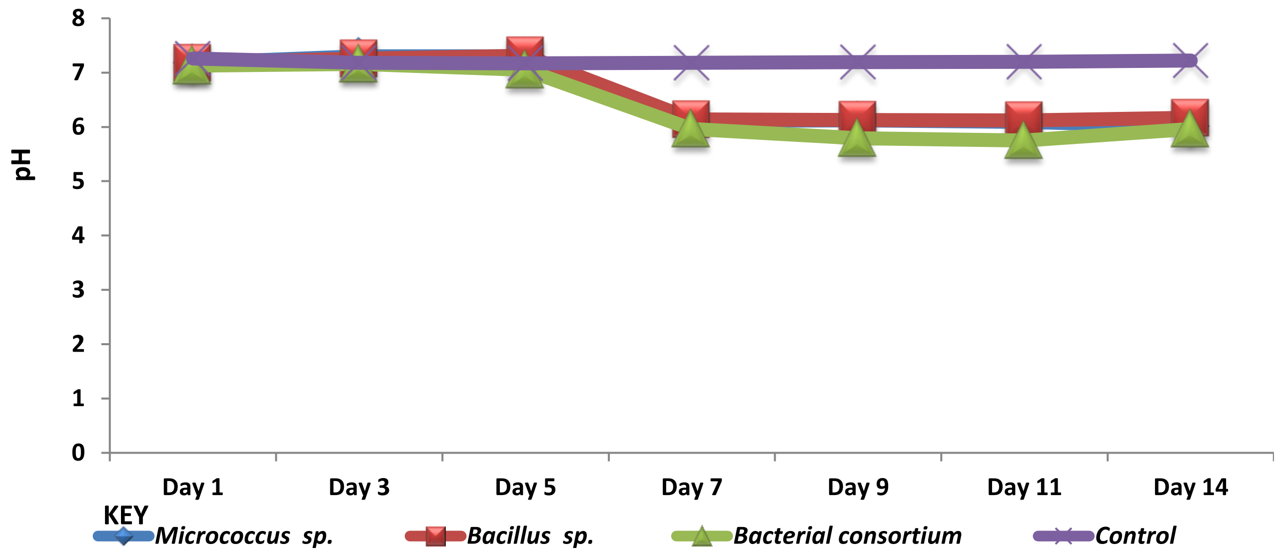

Fig. 2: Mean pH values for the growth profile isolates cultured on Haloxyfop-R methyl ester mineral salt medium

The mean OD data recorded for *Micrococcus* sp. during the growth profile study ranged from 0.76 ± 0.03 at Day 14 to 1.74 ± 0.01 at Day 3 (Fig. 3). The mean OD values recorded for *Bacillus* sp. during the growth profile study ranged from1.69 ± 0.003 at Day 1 to 1.99 ± 0.009 at Day 14 (Fig. 3). The mean OD data recorded for the bacterial consortium during the growth profile study ranged from1.44 ± 0.01 at Day 7 to1.91 ± 0.003 at Day 3 (Fig. 3). The mean OD data recorded for the un-inoculated control during the growth profile study varied from 1.51 ± 0.002 and 1.51 ± 0.006 at Day 11 and Day 14 to 1.53 ± 0.0001,1.53 ± 0.001 and 1.53 ± 0.001 at Day 3, 5 and 7 respectively (Fig. 3). The difference between the mean OD was significant (*p<0.05)*. OD values are an indirect measurement of bacterial growth and activity within a microenvironment (Mira *et al.*, 2022) and as such, directly corresponded with the bacterial counts reported for the bacterial isolates during the shakeflask experiment. The axenic *Bacillus* sp. cultured on the herbicide displayed the maximal mean OD reading which might suggest that the dissolved biomass of the bacterium was comparatively greater than the other test cultures grown on the same herbicide as sole carbon and energy source.

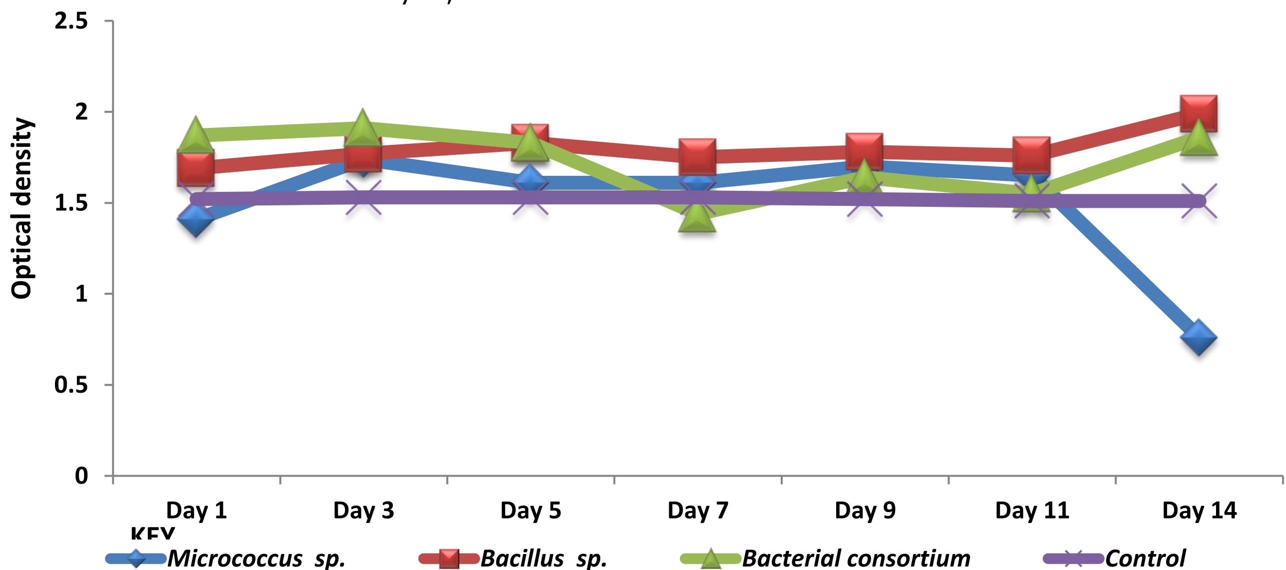

Fig. 3: Mean OD values for the growth profile isolates cultured on Haloxyfop-R methyl ester mineral salt medium

The mean dissolved CO_2 data recorded for *Micrococcus* sp. during the growth profile study ranged from 1.1 mg/l ± 0.1 at Day 14 to 6.8 mg/l ± 0.2 at Day 1 (Fig. 4). The mean dissolved CO_2 values recorded for *Bacillus* sp. during the growth profile study varied from 2.2 mg/l ± 0.3 at Day 14 to 7.3 mg/l ± 0.3 at Day 1 (Fig. 4*).* The mean dissolved CO_2 data recorded for the bacterial consortium during the growth profile study varied from 2.4mg/l ± 0.1 at Day 14 to 6.5 mg/l ± 0.2 at Day 3 (Fig. 4).

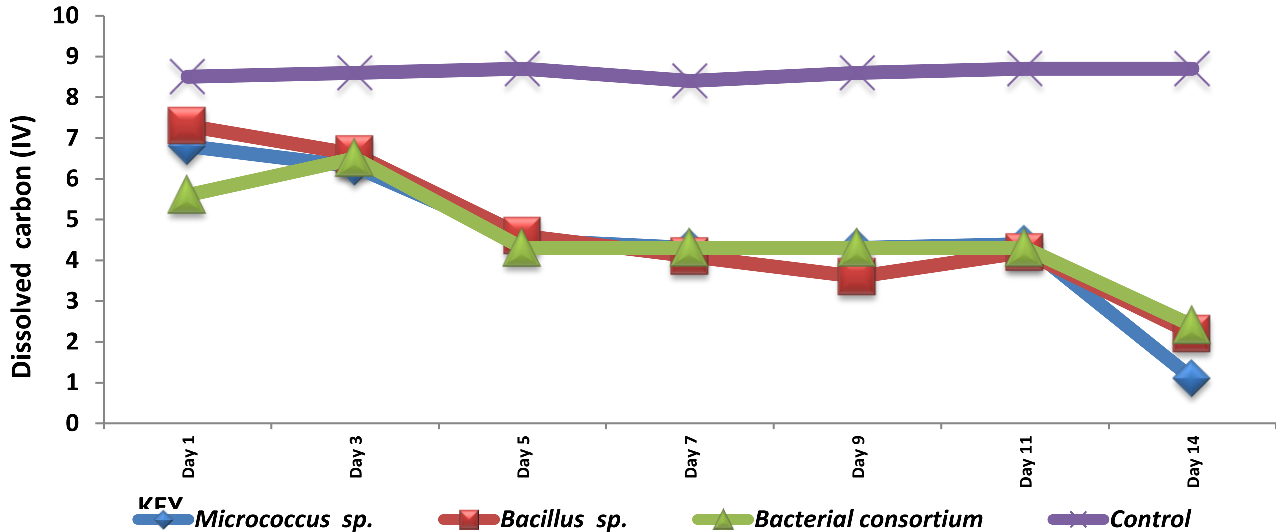

Fig. 4: Mean dissolved CO_2 (mg/l) values for the growth profile isolates cultured on Haloxyfop-R methyl ester mineral salt medium.

The mean dissolved CO_2 values recorded for the un-inoculated control during the growth profile study ranged from 8.4 mg/l ± 0.1 at Day 7 to 8.7mg/l ± 0.1, 8.7 mg/l ± 0.2 and 8.7mg/l ± 0.01 at Day 5, 11 and 14 respectively (Fig. 4). The variation between the mean CO_2 values was significant (*$p<0.05$).* The dissolved CO_2 content of the seeded flasks was used as a direct evaluation of mineralization rate/complete biodegradation of the herbicide constituent of the medium by axenic or bacterial consortia. The progressive reduction in the dissolved CO_2 content might have been indicative of increased herbicide mineralization activity by the seeded culture. Expectedly, a consistent mean dissolved CO_2 value was observed for the un-inoculated control and this trend could have signified that no mineralization or complete biodegradation took place in the control flask during the shake flask study.

Comparatively, the flask seeded with axenic *Micrococcus* sp. had the least mean dissolved CO_2 reading as at Day 14 of the incubation period. This observation would indicate that the bacterium might have mineralized or completely degraded higher amounts of dissolved herbicide moieties in comparison to the other growth profile isolates. The greater mineralizing ability of this bacterium grown on the herbicide might have been directly responsible for the very low counts recovered in respect of this bacterium at Day 14 as the herbicide content which served as both the energy and carbon source might have been severely reduced culminating in the reduction of colony counts associated with the bacterium.

Conclusion

Several heterotrophic and Haloxyfop-R methyl ester utilizing bacterial counts were documented for acidic soils collected from a fallow plant covered land. Two (2) screened bacterial isolates; *Bacillus* sp. and *Micrococcus sp.* exhibited varying degradative potentials when cultured on Haloxyfop-R methyl ester modified medium. With reference to mineralization of the herbicide, the axenic *Micrococcus sp.* was the most effective amongst the growth profile cultures. It is recommended that further studies aimed at evaluating the effect of extra carbon source exemplified by glucose on bacterial biodegradation of Haloxyfop-R methyl ester should be conducted.

Disclosure statement

The authors declare that there are no conflicts of interest.

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