RUGIPO Research & Technical Journal; Vol. 3, Issue 1: Issues in Science & Agricultural Technology; Sept, 2024 Edition
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ISSN: 2714-3643 E-mail: rugipotechjournal@rugipo.edu.ng, or rugiporetechjournal@gmail.com.
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# EFFECT OF SOME MICROORGANISMS WITH BIOREMEDIATION POTENTIALS AND THEIR CORRESPONDING CRUDE PROTEINS ON AQUACULTURE

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**Abstract**
The use of microorganisms for environmental clean-up has been invoke in recent times. The toxicity and pathogenicity of the isolates that expressed pollutant bioremediation potentials on an aquatic model have been a major concern in preventing the release of known microbes into polluted water bodies. Soil microbes were isolated and identified using the 16S RNA partial sequences molecular technique. The isolates identified to be homologous to *Metarhizium anisopliae*, *Penicillium ruben*, *Aspergillus terreus*, *Candidia tropicalis*, *Yarrowia lipolytica*, *Bacillus subtilis*, *Serratia marcescens Bacillus sphaericus* and *Pseudomonas aeruginosa* were all screened for cytochrome p450 protein production on SDS-PAGE. Lorke principle was used to evaluate the lethal dose 50 of the extracted crude protein from each experimental isolate. The cell and corresponding proteins were introduced into an aquaculture model containing *Clarias gariepinus*. All the isolates express the cytochrome p450 protein and, hence are potential bioreactors for environmental clean-up. *Bacillus sphaericus* and *S. merscecens* are most virulent at a concentration of 20% with a 14.14 ml lethal dose required to kill 50% of the cultivated fishes. However, *M. anisopliae* illustrated the least virulent potential with an 84.85 % dose required to kill 50% of the cultivated fishes which is quite high and hence may be regarded as a negligible dose. The cell and corresponding protein/exudates of *Serratia marcescens*, *B. sphaericus*, and *A. terreus* kill the cultivated *C. garipienus* in the aquifer model, hence are pathogenic to the fishes. However, the remaining isolates showed little or no negative effect on the growth rate of the cultivated fishes.

**Keywords:** Aquaculture, SDS-PAGE, *Clarias garipienus*, lethal dose 50, cytochrome p450

## Introduction

The development of aquaculture for fish has boosted and increased seafood in recent times. However, the contamination of aquifers due to oil spillages, dirt dumps and garbage has led to studies of the use of many techniques to bio-remediate the water bodies. These contaminations could lead to heavy metal accumulation and eventually poisoning of the water bodies and apex predators along the food chain. Among these techniques is the introduction of microorganisms that can clean up the environment. Fu and Wang (2011) and Deip *et al*. (2018) both reported the use of microbial cells and microbial proteins for the bioremediation and bioaccumulation process. This has been reported to work in a synergistic process where the microorganisms with the ability to secret some proteins which coat the surface of their cells to aid heavy metal uptakes and sequestration. Some of these microorganisms have been reported to express cytochrome p450 proteins, a protein that has been reported to show bioremediation potentials on heavy metals and detoxification of pollutants (Sakaki and Munetsuna, 2010). To harness the potential of microbial clean-up of aquifer pollutants, the concern of releasing pathogens into the water bodies is of great concern. This could be because some microbes with bioremediation potentials may also be a


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potential pathogen to aquatic animals (Tayabali *et al*., 2017). The introduction of the microbes into the water bodies may also interfere with the physicochemical characteristics of the water bodies hence upsetting the ecological balance of the aquifer. This work aims to isolate, identify and evaluate the toxicity of microbes reported to have bioremediation potentials.

**Materials and Methods**

**Isolation and identification of microbes from the soil sample.**

Isolation and identification of pathogens from soil samples at Orioke Iwa Mimo river bank, Ilaje, Ondo State, Nigeria using molecular techniques via partial sequences of the 16S rRNA gene. The collected soil was cultured and observed growth was isolated and enumerated using the standard procedure (Adamu *et al.*, 2015). The culture medium Nutrient Agar (NA) oxoid was used for the bacteria isolation while Saboraud Dextrose Agar (SDA) containing 5% chloramphenicol antibiotics was used for fungi isolation. The isolated microbes were lysed and the nucleotides were sequenced. The sequenced nucleotides were blasted against the National Center for Biotechnology Information (NCBI) gene bank and the percentage identity of each microbe was recorded.

**Cell harvesting and production of clarified lysate**

A standard procedure described by Ge *et al.* (2018) with some modifications was embraced in the harvesting and production of clarified lysates of experimental microbial cells. The cell harvesting was done in two sets. Firstly, 25 ml was centrifuged at 5000 x g, for 10 min, the pellet was re-suspended by adding 40 ml of the spent medium and again centrifuged at 8000 x g for 10 min. Finally, the pelleted cells were re-suspended in 10 ml/g lysis buffer, disrupted by thermal shock at successive rotation at 43ºC for 3 min and cooled to the ambient temperature. Afterwards, 50 µl of benzamidine and phenylmethylsulfonyl fluoride (PMSF) were added to the final concentration and the samples were evaluated for total protein profile.

**Molecular weight estimation of CYP450 protein from isolates protein of interest (POI)**

The presence of CYP450 in the crude protein extract from all selected isolates was evaluated via their molecular weight on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Ge *et al*. (2018). Sterile glass plates were assembled, 20 ml of 15 % resolving gel was dispensed, and 2 ml of another resolving gel was then overlaid onto the gels, and allowed to polymerize. After which the overlay was poured off and the gel surface was rinsed with deionized water. To the gel, 8 ml of 5 % stacking gel was overlaid and fixed in an electrophoresis apparatus. To the electrophoresis wells, equal volumes of 20 µl of 1 x SDS and test sample preheated at 100 ºC in a test tube for 30 min and marker (17.671 – 103.142 kDa) respectively were loaded in the gel. The gel was then run at 100 V for 5 h, after which it was stained with silver stain for membrane-bound proteins for easy distinction when compared with the standard weight protein markers.

**Lethal dose 50 of isolated crude protein from isolates on the aquifer**

A lethal dose of 50 of each protein crude extract from each microbial isolate was evaluated using the Lorke principle as described by Erhirhie *et al*. (2018). An aquifer containing crude protein extract was prepared as 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% concentration and each was cultivated with 10 three days old Juvenile catfish (*C. gariepinus*) and was

inoculated with crude proteins from the nine microbial isolates proteins (Erhirhie *et al*. 2018).). The highest dose without mortality (a) and lowest dose with mortality (b) were used to calculate the lethal dose 50 as follows LD50 = (a x b)1/2

**Effect of Isolated Crude Protein on fish body weight in an aquifer**

A 100 ml of crude protein extracted from each isolate protein was inoculated into different aquifer models containing 10 three-day-old juvenile *C. gariepinus* cultures. The weight of fish in each model set-up was monitored and recorded weekly using an analytical weighing balance (BSA224S-CW model). The cultivated fishes were monitored for 42 days and the effect of each indigenous isolated microbial cell and corresponding extracted protein on the weight gain /loss was monitored and documented (Berlo *et al.*, 2022).

## RESULTS

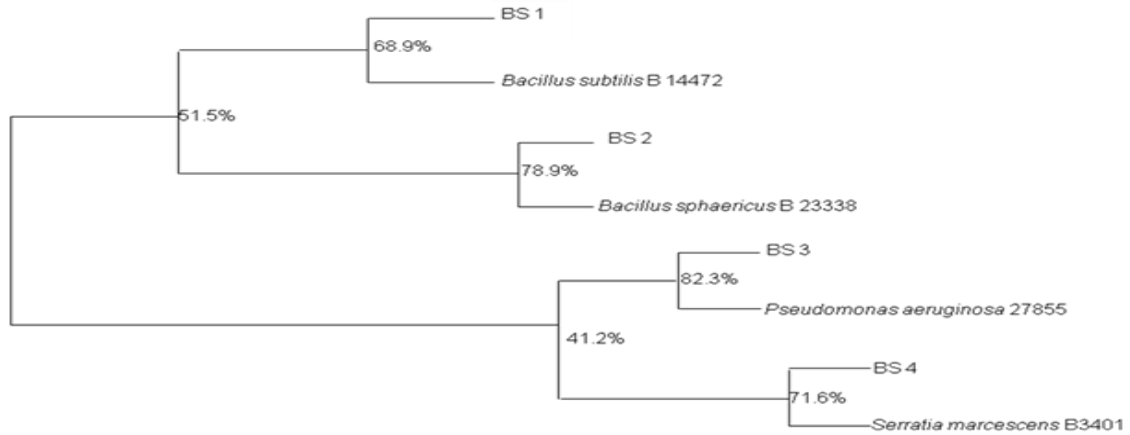


**Figure 1: Neighbour-joining tree of bacterial isolates from soil. The dendrogram was constructed by MEGA 6.0 software based on the partial sequences of the 16S rRNA gene. The scale on the bottom of the dendrogram shows the degree of dissimilarity.**

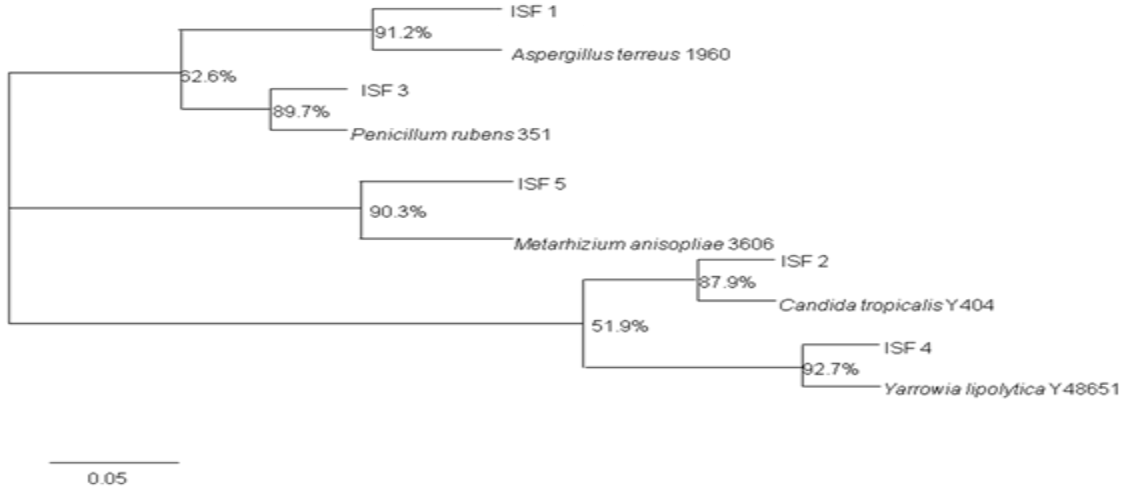


**Figure 2: Neighbour-joining tree of fungal isolates from soil. The dendrogram was constructed by MEGA 6.0 software based on the partial sequences of the 16S rRNA gene. The scale on the bottom of the dendrogram shows the degree of dissimilarity.**

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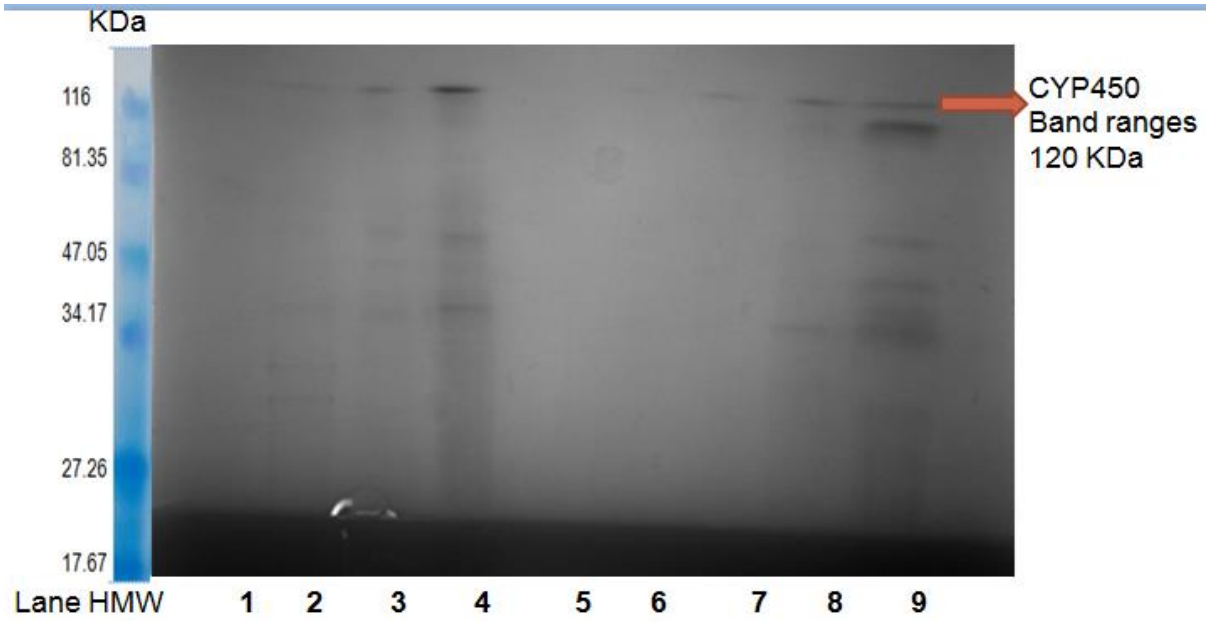


**Plate 1**: The SDS-PAGE of crude membrane protein obtained from selected microbes. Some showing the band of CYP450 ≓116 kDa

**Key**: 1= *A. terreus* 2= *P. ruben*, 3= *M. anisopliae*, 4= *Y. lipolytica*, 5= *C. tropicalis*, 6= *P. aeruginosa*, 7= *S. marcescens*, 8 = *B. subtilis*, 9 =*B. sphaericus*, HMW =High range Molecular Weights size markers

Plate 1 above showed the various molecular bands representing various proteins based on molecular weight separation. Cytochrome P450 protein corresponding to 120 KDA is present in all the well-containing crude extracts from the various selected isolates.

**Table 1: Lethal dose 50 of microbial isolates cell crude protein extract**

| Orgs | 10% | 20% | 30% | 40% | 50% | 60% | 70% | 80% | 90% | 100% | $LD_{50}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **BSP** | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 4 | 4 | 64.81 |
| **PAP** | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 4 | 4 | 64.81 |
| **CTP** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 4 | 74.83 |
| **ATP** | 0 | 0 | 0 | 0 | 1 | 2 | 2 | 4 | 4 | 4 | 44.72 |
| **BSPH** | 0 | 1 | 2 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 14.14 |
| **PRP** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 2 | 74.83 |
| **YLP** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 2 | 74.83 |
| **SMP** | 0 | 2 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 14.14 |
| **MAP** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 2 | 74.83 |

Key: BSP= *Bacillus subtilis* protein, PAP= *Pseudomonas aeruginosa* protein, CTP= *Candida tropicalis* protein, ATP= *Aspergillus terreus* protein, BSPH= *Bacillus sphaericus* protein, PRP= *Penicillium ruben* protein, YLP= *Yarrowia lipolytica* protein, SMP=*Serratia marcescens* protein, MAP= *Metarhizium anisopliae* protein

Table 1 shows the lethal dose 50 of protein extracts from all the isolated microbial samples with *M. anisopliae* protein, *Y. lipolytica* protein, *P. ruben* protein, and *C. tropicalis* protein showing the highest lethal dose of 50 concentrations needed to kill half of the cultivated fishes while *B. sphaericus* proteins and *S. marcescens* proteins showed little concentration of 14.14% enough to kill half of the cultivated fishes.

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**Table 2: Average weight gain/loss in grams of juvenile *C. gariepinus* cultured in an aquarium inoculated with microbial isolates and their corresponding crude membrane proteins concentration of (100 ml/ 3000 ml).**

| SN | Day 1 | Day 7 | DAY 21 | DAY 28 | DAY 35 | DAY 42 |
|---|---|---|---|---|---|---|
| **BSP** | 76.50±0.50[b] | 81.67±1.53*[d] | 90.19±2.25**[c] | 89.20±0.80**[b] | 99.06±0.34***[b] | 116.26±0.74****[c] |
| **BS** | 74.61±0.44[b] | 77.43±0.53[d] | 88.49±0.32*[c] | 86.56±0.88*[b] | 94.48±0.48**[b] | 110.44±0.83***[b] |
| **PAP** | 66.56±0.47[a] | 116.40±1.51*[h] | 111.44±0.51*[e] | 111.62±0.36*[d] | 114.67±0.61*[c] | 128.19±1.49**[d] |
| **CPA** | 62.26±0.57[a] | 85.28±0.43*[e] | 90.84±0.45**[c] | 95.12±0.09***[c] | 99.59±0.67***[b] | 115.87±0.57****[c] |
| **CTP** | 76.43±0.61[b] | 77.16±0.94[d] | 105.49±0.47**[d] | 117.35±0.51****[d] | 100.40±0.72*[b] | 110.73±1.27***[b] |
| **CCT** | 76.21±0.03[b] | 71.44±0.50[c] | 111.34±0.59*[e] | 119.47±0.52**[d] | 127.48±0.50**[d] | 139.62±1.85****[f] |
| **ATP** | 86.26±0.16[c] | 123.62±0.43**[i] | 134.16±0.31***[f] | 134.40±0.53***[e] | 122.46±0.57**[d] | 110.70±2.04*[b] |
| **CAT** | 80.11±0.89**[c] | 63.27±1.10*[b] | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] |
| **BSPH** | 82.71±0.32*[c] | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] |
| **BSPHP** | 68.21±0.18**[a] | 62.77±0.65*[b] | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] |
| **PRP** | 90.64±0.36[d] | 113.70±0.56*[h] | 114.24±0.70*[e] | 114.70±0.46*[d] | 116.43±1.27*[c] | 125.10±1.18**[d] |
| **CPR** | 88.51±0.45[d] | 86.35±1.54[e] | 139.57±0.85**[f] | 142.38±0.45**[f] | 154.10±0.46***[e] | 131.24±0.94*[e] |
| **YLP** | 97.62±0.51[e] | 105.60±0.50*[g] | 100.18±0.28[d] | 98.61±0.55[c] | 101.95±0.63[b] | 117.73±0.32**[c] |
| **CYL** | 77.80±0.27[b] | 85.71±0.44*[e] | 85.23±0.38*[c] | 91.51±0.52**[b] | 100.78±1.16***[b] | 106.17±0.57****[b] |
| **SMP** | 80.81±1.09*[c] | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] |
| **CSM** | 88.71±0.52***[c] | 83.92±0.94**[d] | 66.65±1.00*[b] | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] |
| **MAP** | 64.39±0.72[a] | 193.62±0.78*[j] | 202.89±0.76**[g] | 203.47±0.62**[g] | 207.00±1.64**[f] | 238.80±1.86***[h] |
| **CMA** | 68.65±0.70*[a] | 60.36±0.56[b] | 115.24±0.98**[e] | 112.16±0.65**[d] | 116.27±0.91**[c] | 140.40±0.53***[f] |
| **Control** | 78.93±0.89[b] | 92.47±0.52*[f] | 118.03±0.95**[e] | 134.35±0.59***[e] | 153.03±1.06****[e] | 185.33±0.58****[g] |

Values with different alphabetical along columns and * across rows are significantly different at $p<0.05$
Key: BSP=*Bacillus subtilis* protein, BS=Cell of *Bacillus subtilis*, PAP= *Pseudomonas aeruginosa* protein, CPA= Cell of *Pseudomonas aeruginosa*, CTP= *Candida tropicalis* protein, CCT= Cell of *Candida tropicalis*, ATP= *Aspergillus terreus* protein, CAT= Cell of *Aspergillus terreus*, BSPHP= *Bacillus sphaericus* protein, BSPH= Cell of *Bacillus sphaericus*, PRP= *Penicillium ruben* protein, CPR= Cell of *Penicillium ruben*, YLP= *Yarrowia lipolytica* protein, CYL= Cell of *Yarrowia lipolytica*, SMP=*Serratia marcescens* protein, CSM= Cell of *Serratia marcescens*, MAP=*Metarhizium anisopliae* protein, CMA=Cell of *Metarhizium anisopliae* ,

The result presented in the Table showed the effect of the cell culture and its extracted proteins on the growth rate and death rate of cultivated fishes for 42 days.

## Discussion

The expression of molecular band weight homologous to cytochrome p450, in all the selected microbes gives incites to one of the models operated by the organism in


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environmental clean-up mechanisms. This protein has been studied to exhibit bioremediation and detoxification potentials of heavy metals (Giwa *et al*., 2022). Hence any microbes with the ability to secrete it may be relevant in environmental clean-up.

The lethal dose of 50 of each experimental protein illustrated that *B. sphaericus* and *S. merscecens* are most virulent at a concentration of 20% with a 14.14 ml lethal dose required to kill 50% of the cultivated fishes. However, *M. anisopliae* illustrated the least virulent potential with an 84.85 % dose required to kill 50% of the cultivated fishes which is quite high and hence may be regarded as a negligible dose. The average weight response of juvenile *C. gariepinus* cultured in an aquarium inoculated with isolates and their corresponding crude membrane proteins concentration of (100 ml/3000 ml) showed that both the exogenous and endogenous extracted proteins and culture cell membrane proteins respectively had a significant effect on the growth and death rate of the cultured fishes at probability level $P < 0.05$. All the microbes and the corresponding crude extract illustrated little or no effect on the growth rate of the fishes except *S. marcescens*, *B. sphaericus* and *A. terreus* microbial cells and corresponding proteins. Extracted crude protein from *A. terreus* cell (ATP) treatment of the cultivated fishes illustrated no decline in the fish's growth rate measured in terms of weight gain however, there was a stationary phase recorded from day 21 to day 28. This was followed by the succession of weight decline from day 28 to day 35 and eventually day 42. However, the *A. terreus* cell culture (CAT) treatment of the cultivated fishes illustrated a gradual decline in the fish's weight from day 1 to day 7 of cultivation. All the fishes in this group died before day 21 of the inoculated cell. Hence, the virulence of the microbial cell culture of *A. terreus* was higher than the extracted corresponding crude protein. Also, the extracted crude protein from *B. sphaericus* appeared to be more virulent compared with the cultured cell. The cell culture reduced the weight of the fish from day 1 till day 7 after which every cultivated fish died. However, the crude protein extract was observed to kill all cultivated fishes before day 7. Therefore, the virulence of the crude protein extract appears to be more potent compared with the cultured cell of *B. sphaericus. Serratia marcescens* followed a similar trend after the order of *B. sphaericus* cell-cultured and extracted protein.

Similar trends have been reported by Sheehan *et al*. (2022). He noted that exotoxins are more potent in intensive care medicine by acting as super-antigens. Day 28 to day 42 was noted to illustrate a decrease in the weight of the fishes in this treatment group as some fishes died in this period of weight stationary and decline phase. Therefore, the death of the fishes exposed to the cell-free extracts and the bacterium cells suggests that there is a need for more research about the genome of our isolate. Probably, it may have taken up some virulent genes for pathogenicity from the environment. Earlier, Park *et al.* (2010) had reported that *B. sphaericus* produced two types of proteins namely; crystal toxins and Mtx toxins which produce a larvicidal effect on certain insects by acting on specific receptors in the midgut of culicid larvae, causing a lethal cytopathological effect. Park *et al*. (2010) confirmed the insecticidal activity of *B. sphaericus* against mosquito larvae showing a high toxicity on these insects, with very specific targets, affecting only a small number of susceptible species. Park *et al*. (2010) confirmed the insecticidal activity of *B. sphaericus* against mosquito larvae showing a high toxicity on these insects,

with very specific targets, affecting only a small number of susceptible species.

## Conclusion and Recommendation

The toxicity and pathogenicity of the soil isolates and their corresponding crude protein exudate on an aquatic model were evaluated. Among the selected soil microorganisms, cells and corresponding protein/exudates of *S. marcescens, B. sphaericus,* and *A. terreus* kill the cultivated *C. garipienus* in the aquifer model and, hence are pathogenic to the fishes. The lethal dose 50 of protein from each experimental microorganism illustrated that *B. sphaericus* and *Serratia merscecens* are the most virulent while, *M. anisopliae* illustrated the least virulent potential. Even though many of these isolated microorganisms showed high bioremediation potentials due to the expression of cytochrome p450 proteins, some of them are equally pathogenic and should be avoided as bioremediation agents in water bodies intended for fish cultivation.

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