



## Isolation and Characterization of *Photorhabdus Spp.* (Enterobacteriaceae) Isolated From *Heterorhabditis Bacteriophora* in Kashere, Nigeria

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**ABSTRACT:** *Photorhabdus* bacteria are symbiotically associated with the infective juveniles of entomopathogenic nematodes of the genus *Heterorhabditis*. These bacteria after infecting a susceptible host, produces a host of lethal toxins that kill the insect hosts within 24 to 72 hours. Consequently they have emerged and are trending as excellent biological control agents against insect pests of agricultural crops. Therefore, this study aim to isolate, identify and test the pathogenicity of *Photorhabdus* bacteria isolated from *Heterorhabditis bacteriophora*, entomopathogenic nematode from Kashere, Gombe State, Nigeria. To this end phenotypic and biochemical tests were conducted. The tests conducted showed that the isolate exhibited characteristics similar to those of *Photorhabdus* bacteria that have been identified. Last instar larvae of *G. mellonella* exposed to different concentrations of the bacterial isolate showed some level of susceptibility of the larvae to the bacterial isolate confirming its biological control potential.

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*Photorhabdus* species of bacteria are excellent candidates for the biological control of insect pests of agricultural crops. They have emerged as excellent alternatives to synthetic pesticides for the successful control of insect pests of agricultural crops worldwide Gaugler, 2002; Adams, 2006; Aliyu *et al.*, (2015). They have been characterized as non-spore producing, Gram-negative, facultative anaerobic motile rods Owuama, 2001; Saenz-Aponte *et al.*, (2014). These bacteria according to the report of Ferraira *et al.*, (2013), are oxidase positive. They live in the intestinal lumen of infective juveniles of *Heterorhabditis bacteriophora*. The relationship between the bacteria and the nematode is symbiotic. The nematode is dependent upon the bacteria for killing the host insect, creating a suitable environment for the development of the nematode, breaking down the hosts' tissues into utilizable nutrients and serving as a food source. The nematode protects the bacteria from the external environment since the bacterium is unable to survive living freely. The nematode acts as a vector, penetrates the host and regurgitates its symbiotic bacteria into the haemocoel of the host insect Hinchliffe *et al.*, (2010). The bacteria produce a spectrum of toxins and antibiotics Hinchliffe *et al.*, 2010; Lang *et al.*, (2014) which inhibit potential competitors Orozco *et al.*, (2013). The bacteria proliferate and provide nutrition

for the developing nematodes Webster *et al.*, (2002). The multiplication of the *Photorhabdus* bacteria leads to septicemia and toxemia and eventual death of the insect host within 48-72 hrs Saenz-Aponte *et al.*, (2014). About three species of *Photorhabdus* have been classified thus; *P. luminescens* Thomas and Poinar, *P. temperate* Fischer-Le Saux, *P. asymbiotica* Akhurst and seven subspecies have been described specifically for *P. luminescens* Saenz-Aponte *et al.*, (2014). The characterization and identification of these species and sub-species have been achieved by the use of morphological, biochemical as well as molecular characteristics as reported by Saenz-Aponte *et al.*, (2014). Though studies have been conducted on the exploitation of nematode-bacteria complex as potential biological pesticides against local pests of crops, similar study have not been reported in Kashere. Therefore, this study aim to isolate, identify and test the pathogenicity of *Photorhabdus* bacteria isolated from *H. bacteriophora* entomo-pathogenic nematode from Kashere in Nigeria

### MATERIALS AND METHODS

*Isolation and culture of symbiotic bacteria:* Entomopathogenic nematodes were isolated from soil samples collected in Kashere using an established *Galleria mellonella* L. (Lepidoptera: pyralidae) bait

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method and the white trap. The entomopathogenic nematodes were characterized and identified as *Heterorhabditis bacteriophora* Aliyu *et al.*, (2019). Dead larvae of *Galleria mellonella* infected 48h earlier with infective juveniles of *H. bacteriophora* were surface sterilized in 70% alcohol for 10 mins. The larvae were allowed to dry and a fore limb was removed with a sterile scissors. Using a sterile wireloop, a drop of oozing hemolymph from each larva was inoculated on to nutrient agar, nutrient agar bromothymol blue and MacConkey agar plates. The plates were incubated at 32°C for 48-72 hrs. Single colonies were picked with a sterile wire loop and sub-cultured twice to obtain a pure culture which was used for this study. The bacterial isolates were sub cultured on nutrient agar slants for storage Emeliahoff *et al.*, (2008).

*Phenotypic and biochemical characterization of bacterial symbionts:* Colony pigmentation, shape and size were examined on nutrient agar, MacConkey (Oxoid) and Nutrient agar bromothymol blue plates after 72h of incubation at 32°C. The light microscope was used to observe the isolates after Gram staining. The biochemical tests carried out included; production of acid from carbohydrates, citrate, indole production after 48 h, catalase and oxidase activities were also evaluated. Lecithinase, protease and lipolytic activity were carried out on egg yolk agar Jang *et al.*, (2011).

Pathogenicity test of the bacterial isolate on *G. mellonella*: Pasteur pipette was used to transfer 0.5ml of 0, 10<sup>-2</sup>, 10<sup>-4</sup> and 10<sup>-6</sup> cells/ml of the bacterial isolate to the feed of larvae of *G. mellonella* which was thoroughly mixed. Three replicates of the experimental design were used and five larvae each were introduced into each concentration per unit. The containers were kept in a dark cupboard at room temperature and observed daily for mortality during 5 days.

Similarly a drop of oozing hemolymph from dead *G. mellonella* larvae was extracted and cultured on MacConKey, NBTA and Nutrient agar to verify that the bacteria isolate was responsible for the death of the larvae. Statistical analysis was done using ANOVA to determine the effect of the concentrations of the bacterial isolates on the larvae of *G. mellonella* Jang *et al.*, (2011).

## RESULTS AND DISCUSSION

*Phenotypic and Biochemical characterization of bacterial endosymbionts:* The colonies absorbed dye on MacConkey and NBTA plates. On MacConkey agar colonies absorbed neutral red dye and appeared pink and mucoid, (a) on nutrient agar they appeared milky

white and shiny (b) while on nutrient bromothymol blue NBTA plates colonies absorbed the blue colour of the dye and appeared dark bluish-green (c). At 72 h of incubation, distinct colonies on NBTA appeared convex, small and 1mm in size.



Plate 1a: Pink colonies of bacteria on MacConkey agar

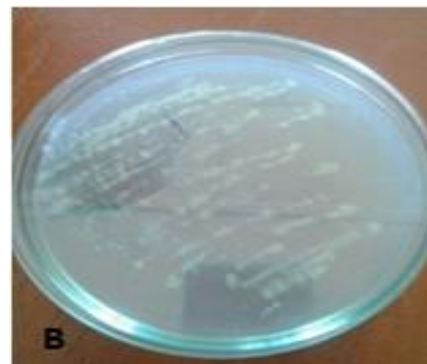


Plate 1b: Milky white colonies on nutrient agar

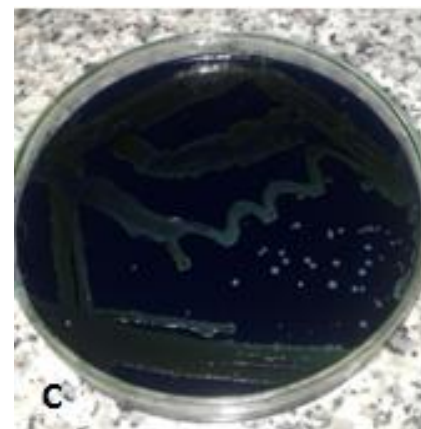


Plate 1c: Bluish-green colonies on nutrient agar bromothymol blue (NBTA)

After Gram staining, cells of the bacterial isolate appeared as Gram-negative rods. From results of the biochemical tests, the bacterial isolate was able to ferment xylose, glucose, mannose and fructose as sources of carbohydrates, it was citrate positive, indole positive, oxidase positive as well as catalase positive. Lecithinase, protease and lipolytic activities were all negative.

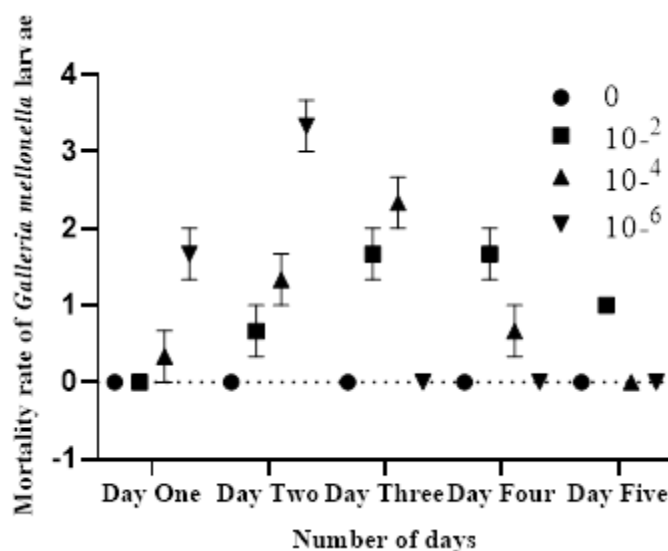
*Pathogenicity test of the bacterial isolate on G. mellonella*: Pathogenicity tests showed that the bacterial isolate was effective against the larvae of *G. mellonella* at various concentrations, however, variations were observed Table 1 and Figure 1. The effect of  $10^2$  CFU/ml of the isolate commenced 48 h after exposure to *G. mellonella* and was slower at killing the larvae whereas concentrations of  $10^4$  and

$10^6$  CFU/ml of the isolate was effective at 24 h with  $10^6$  CFU/ml killing all exposed larvae within 48 h. The control had no effect on the larvae of *G. mellonella*. Results of ANOVA showed that there was a significant difference between the means of the concentrations of the isolate against the larvae of *G. mellonella*.

**Table 1.** Mean Mortality/day of *Galleria mellonella* larvae  
Mortality/day of *G. mellonella* larvae

| Concentration (CFU/ml) | 1                        | 2                        | 3                        | 4                        | 5                        |
|------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| 0                      | 0.00 ± 0.00 <sup>a</sup> | 0.00 ± 0.00 <sup>a</sup> | 0.00 ± 0.00 <sup>a</sup> | 0.00 ± 0.00 <sup>a</sup> | 0.00 ± 0.00 <sup>a</sup> |
| $10^2$                 | 0.00 ± 0.00 <sup>a</sup> | 0.67 ± 0.58 <sup>a</sup> | 1.67 ± 0.58 <sup>b</sup> | 1.67 ± 0.58 <sup>b</sup> | 1.00 ± 0.58 <sup>b</sup> |
| $10^4$                 | 0.33 ± 0.58 <sup>a</sup> | 1.67 ± 0.58 <sup>b</sup> | 2.33 ± 0.58 <sup>b</sup> | 0.67 ± 0.58 <sup>a</sup> | 0.00 ± 0.00 <sup>a</sup> |
| $10^6$                 | 1.67 ± 0.58 <sup>b</sup> | 3.33 ± 0.58 <sup>c</sup> | 0.00 ± 0.58 <sup>a</sup> | 0.00 ± 0.00 <sup>a</sup> | 0.00 ± 0.00 <sup>a</sup> |

Mean ± Standard Deviation (SD). Means with different superscript across the column differ significantly.



**Fig. 1.** Mortality rate of *G. mellonella* larvae exposed to different concentrations of bacteria

Pigmentation of colonies is attributed to the ability of *Photorhabdus* bacteria to produce diffusible pigments, a characteristic similar to all described species of the genus *Photorhabdus* Saenz-Aponte *et al.*, (2014). They absorbed neutral red on MacConkey and bromothymol blue on NBTA respectively as shown in plates (a) and (c) above. These properties are similar to those of *Photorhabdus* bacteria described by Eun-Kung *et al.*, 2012; Orozco *et al.*, (2013). Bacterial isolates obtained from *Heterorhabditis bacteriophora* nematode presented a Gram-negative cell morphology which is characteristic of *Photorhabdus* sp. this observation is similar to that observed by Boemare and Akhurst 2006; Saenz-Aponte *et al.*, (2014). Furthermore, the isolate like all species of described *Photorhabdus* bacteria are shiny after 48 h of incubation, a characteristic also observed by Sing *et al.*, (2012). The luminescence and morphological characters exhibited by the isolate

confirms that the isolate is part of the genus *Photorhabdus*. Like described species, the isolate was able to ferment xylose, glucose, mannose and fructose as sources of carbohydrates which shows that they are facultative anaerobes, an observation similar to that of Eun-Kung *et al.*, 2012; Saenz-Aponte *et al.*, (2014). Also formation of gas bubble upon addition of hydrogen peroxide was an indication of a positive test for catalase. The biochemical tests revealed that the isolate from *Heterorhabditis bacteriophora* had properties similar to described species for *Photorhabdus* bacteria. The isolate exhibited weak lecithinase and lipase activity and there was no production of proteases due to the absence of protein hydrolysis on the egg yolk agar. The weak activity is an indication of presence of phase II cells of *Photorhabdus* sp. whose activities are usually weak or completely negative for these enzymes when compared to phase I cells of *Photorhabdus* bacteria an

observation shared with Burnell and Stock (2000). The isolate shares similar phenotypic and biochemical properties with that of described species of *Photorhabdus luminescens*. Pathogenicity test confirmed susceptibility of *G. mellonella* larvae to various concentrations of the bacterial isolate with 10<sup>6</sup> CFU/ml having the highest activity against the larvae within 48 h. an observation similar to that of Saenz-Aponte *et al.*, (2014). The pathogenicity test also confirms the biological control potential of the isolate against larvae of *G. mellonella*.

**Conclusion:** The bacterial endosymbionts were successfully isolated and characterized as *Photorhabdus luminescens*, however, there is a need for molecular characterization of the isolate in order to classify it to its sub-species level. The pathogenicity of the isolate against *G. mellonella* larvae was established; hence it's biological control potential against insect pests of agricultural produce can further be exploited. This report also serves as a baseline data for further research in this area.

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