

Optimizing the Effect of pH and Temperature on Atrazine Degradation by *Bacillus safensis* strain BUK_BCH_BTE6 an Efficient Atrazine Tolerating Bacteria from an Agricultural Soil in Kura Local Government Area of Kano State, Nigeria

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Abstracts

The widespread and long term use of atrazine results in high residue levels of atrazine in soil, which further causes water contamination, it is considered as an endocrine disruptor and is potentially carcinogenic. Microbial degradation of herbicide represents a time cost effective way of eco-restoration. This research was aimed at isolating and characterizing bacteria capable of degrading and utilizing atrazine as a sole carbon source. An enrichment method was used to isolate the bacteria on mineral salt media (MSM) following serial dilution. The isolate was identified morphologically, biochemically and molecularly as *Bacillus safensis* strain BUK_BCH_BTE6 based on 16S rRNA gene sequence and molecular phylogenetic analysis. The effect of pH and temperature on the degradation of atrazine was studied in MSM medium supplemented with atrazine as sole carbon source. Growth and degradation of atrazine in this isolate was optimal at pH 7.5 and temperature of 35 °C. *Bacillus safensis* is highly efficient in atrazine degradation with an optimum range of pH and temperature. *Bacillus safensis* could be a suitable candidate for bioremediation of atrazine polluted sites.

Keywords: Atrazine, Degradation, pH, Temperature, Bacteria.

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Introduction

Worldwide, several anthropogenic activities have resulted in high increase of environmental pollution, and its effect has affect almost all components of the ecosystem (Izabel et al., 2020). Soil, as an important constituent of the terrestrial ecosystem is exposed to pollution from various sources, including industrialization and various agricultural practices. Different forms of anthropogenic pollutant entering the soil causing a very serious impact and risk to human health and terrestrial ecosystem (Sayara and Sánchez, 2020). High growth in demand and use of agricultural and industrial products have lead to rapid increases in polluted soils and waters (Zhao et al., 2018).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1, 3, 5- triazine) is a s-triazine herbicide with a molecular formula $C_8H_{14}ClN_5$ (He et al., 2019). It is a nonpolar, nonvolatile and low soluble compound (Olu-arotiowa et al., 2019). It is powdery white substance that is unstable at high temperature. It has a melting point of 173 – 175 °C, and a boiling point of 200 °C. it solubility in water is 33 mgL^{-1} at 20 °C, it is very soluble in organic solvents (Ariole and Abubakar, 2015). Atrazine is usually detected in the earth surface water and groundwater system because of it long half-life that ranges from 13 – 261 days (Li et al., 2019). Atrazine can restrain and remove broadleaf weeds and some grass weeds that affect crop growth, and also inhibit some perennial weeds (He et al., 2019). It is also

applied to soil as herbicide on crops such as sorghum, maize, sugarcane, ornamental plants and also applied to forests as well. It's considered among the top used herbicide in the world due to its low cost and effectiveness (Shamsedini, 2015). Atrazine is considered as a precedence pollutant that is toxic and carcinogenic. It is toxic when inhale, taken orally or excess exposure with the skin. likewise many studies on it toxicity reported atrazine to exhibit long term effect on reproductive and endocrine system, epidemiological effect to decrease sperm counts in men and is assume to be human carcinogen that lead to breast and ovarian cancer (Oluarotowa et al., 2019).

Previous research reported various bacteria capable of atrazine biodegradation. isolation of microorganisms with high atrazine tolerance and degradation is critically needed to reduce it adverse effects on human health and ecosystem (Shiri, 2016). This paper reports the efficiency of *Bacillus safensis* strain BUK_BCH_BTE6 in the degradation of atrazine in MSM media.

Materials and Methods

Samples Collection

Soil profiles of 1 – 30 cm under the surface were sampled using a sterile scoop after removal of the top layer of the soil up to 1 – 5 cm. Three soil samples were collected from agricultural soil in Kura Local Government Area of Kano state located between latitudes 10°03' and 12°37' North and longitudes 7°03' and 9°5' East of the Greenwich meridian (Ayodele, 2016). All samples were transferred to sterile polyethene bags, transported to the Microbiology laboratory in Bayero University Kano for analyses. The samples were dried, homogenized and passed through a 2 mm sieve. Atrazine used was of analytical grade with 90% purity.

Enrichment and Isolation of Atrazine Tolerating and Degrading Bacteria

The atrazine tolerating and degrading bacteria were isolated using culture enrichment method. The mineral salt medium contained (g/L of distilled water): 0.5 gL⁻¹ of KH₂PO₄, 3.0 gL⁻¹ of K₂HPO₄, 0.2 gL⁻¹ of MgSO₄·7H₂O, 0.5 gL⁻¹ of NaCl, and 1 mL⁻¹ of trace element concentrate solution according to Sawangjit (2016). Trace element concentrate solution had the following composition: FeSO₄·7H₂O, 1 gL⁻¹, ZnSO₄·7H₂O, 5

gL⁻¹ CuSO₄·5H₂O, 0.4 gL⁻¹, MnSO₄·H₂O, 1 gL⁻¹, EDTA, 2.5 gL⁻¹, Na₂MoO₄·2H₂O, 0.25 gL⁻¹, and Na₂B₄O₇·10H₂O, 0.2 g. All contents were dissolved in 1L distilled water, heated to dissolve and sterilize by autoclaving for 15 minutes at 121 °C (Macwilliams, 2016).

A total of 10 g of each sample was initially inoculated and homogenized in 250 ml Erlenmeyer flasks containing 100 ml of mineral salt media and was supplemented with 10 ml of atrazine as sole carbon source at a final concentration of 100 mg L⁻¹. The flasks were then incubated under orbital agitation (150 rpm) at 37 °C. After 7 days, a 10 ml aliquot of each culture was transferred to new MSM broth (100 mL) with same atrazine volume. Subsequently, each culture was serially diluted from 10⁻¹ to 10⁻⁶ in sterile distilled water and proper dilutions 10⁻⁶ was plated on MSM agar, supplemented with 0.1 mL of atrazine (100 mgL⁻¹). The Petri dish was then incubated at 37 °C for 48 hours (Wang et al., 2015).

Effect of Initial pH on Atrazine Degradation

The effect of pH ranges: 5.5, 6.5, 7.0, 7.5, 8.0, and 8.5 was assessed. Mineral salt medium (50 ml) was introduced into conical flask, 100 µL of bacterial culture was used and supplemented with 100 µL of atrazine with initial concentration of 100 mgL⁻¹ and incubated at 37 °C for 120 hours. Bacterial aliquot (1 ml) was taken after every 24 hours to measure the OD₆₀₀ for bacterial growth (Mansee et al., 2017).

Effect of Temperature on Atrazine Degradation

The effect of temperature ranges was assessed. 50 ml of Mineral salt medium was introduced into conical flask, 100 µL of bacterial culture was used and supplemented with 100 µL of atrazine with initial concentration of 100 mgL⁻¹ and incubated at different temperature ranges: 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, and 50 °C for 120 hours. Bacterial aliquot (1 ml) was taken after every 24 hours to measure the OD₆₀₀ for bacterial growth (Ariole and Abubakar, 2015).

Results

Isolation, Identification and Screening of Atrazine-Degrading Bacteria

After inoculation of the culture with MSM agar and incubating at 37 °C for 48 hours and further

sub culturing for 24 hours at 37 °C resulted in the appearance of white, round, slimy shape colonies with raised elevation and rough edge which look like fried egg.

Based on Morphological identification (Table 1), it was found that after visualization under light microscope, the isolates were all gram positive and rod shape with one cocci.

Table 1: Morphological Identification of the Isolate

Isolate	Gram Reaction	Shape	Colour
A ₁	Positive	Rod	Purple
A ₂	Positive	Rod	Purple
B ₁	Positive	Rod	Purple
B ₂	Positive	Cocci	Purple
C ₁	Positive	Rod	Purple
C ₂	Positive	Rod	Purple

A total of six isolates were isolated from the atrazine contaminated soil samples, five were able to partially tolerate the varying concentrations (500 mgL⁻¹, 1000 mgL⁻¹ and 1500 mgL⁻¹) of atrazine (Table 2). Only one isolate

tolerates all the concentrations of atrazine after 120 hours of incubation and therefore was used for the main study. The bacterium was identified to its generic level according to its characteristics, that may include morphological and physiological.

Table 2: Screening/Tolerance Test for Atrazine-Degrading Bacteria Grown in MSM after 48 Hours of Incubation at 37 °C

Isolate	500 mgL ⁻¹	1000 mgL ⁻¹	1500 mgL ⁻¹
A1	+	+	-
A2	+	-	-
B1	+	+	+
B2	+	+	-
C1	-	-	-
C2	+	-	-

Biochemical test for the isolate (Table 3), indicated that the isolate is positive for catalase test, oxidase test, Voges-Proskauer test and nitrate reduction test, while negative for citrate

utilization test, starch hydrolysis test, methyl red test, H₂S production test, indole production test and urease production test.

Table 3: Biochemical tests for the candidate isolate (B₁) used for characterization and degradation study

Biochemical Test	Result
Methyl Red	+
H ₂ S Production	+
Indole Production	+
Citrate Utilization	+
Starch Hydrolysis	+
Urea Production	+
Catalase	-
Oxidase	-
Voges-Proskauer	-
Nitrate Reduction	-

Keys: + =Positive, =Negative

Molecular identification of the isolate was presented in Figure 1. It was found that after subjecting the PCR product to sequencing using cycle sequencing kit genetic analyzer from both forward and reverse directions. The 16S rRNA gene sequences of the bacterium obtained was compared with GenBank database using Blast Server at NCBI. The analysis show that the DNA sequences obtained were closely related to the partial sequence of several *Bacillus* sp. with over

99.91 % similarity. Molecular phylogenetic studies using the neighbor joining method linked the identity of the obtained bacterium sequence to *Bacillus safensis*. Thus, this bacterium is tentatively assigned as *Bacillus safensis* Strain BUK_BCH_BTE6. The bacterial sequence was deposited in the GenBank with the accession number OK180490.1

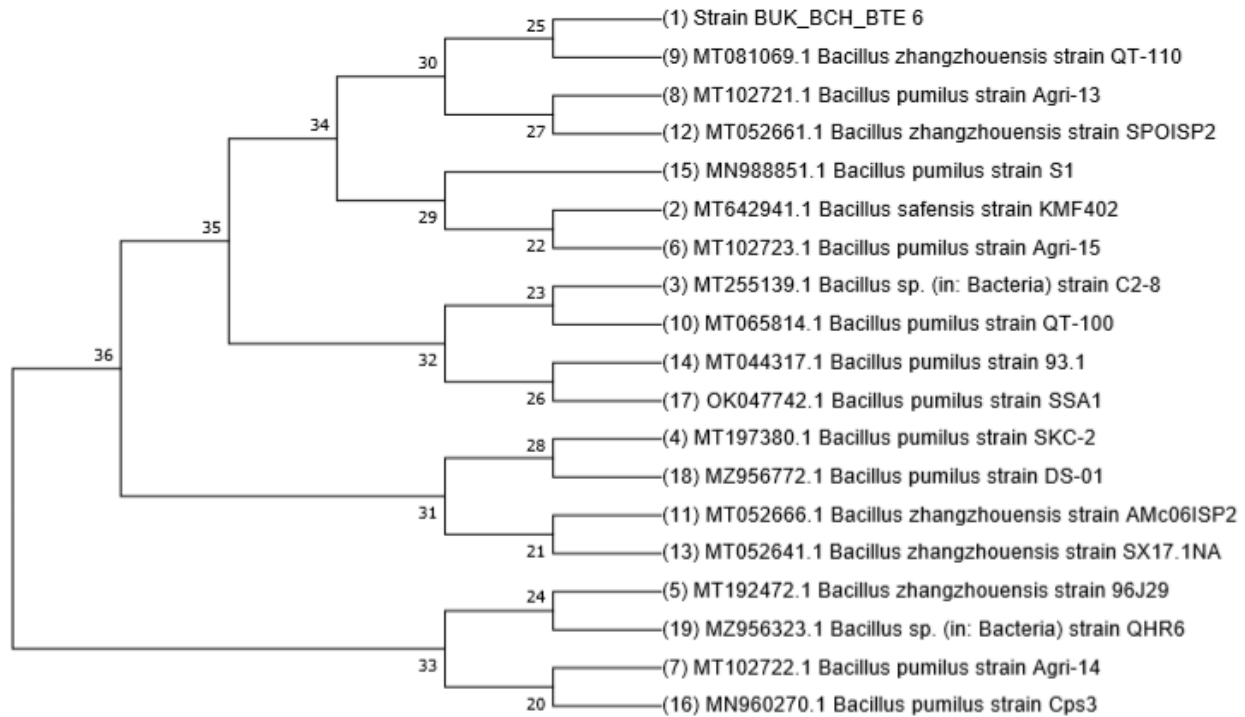


Figure 1: Cladogram (neighbor-joining method) indicating the genetic relationship between *Bacillus safensis* and reference related microorganisms based on 16S rRNA gene sequence. Species name are accompanied by the accession numbers of their 16s rRNA gene sequence. Numbers at the nodes indicate bootstrap values from the neighborhood-joining analysis of 1,000 resampled data sets.

Characterization of Atrazine-degrading Bacteria using One Factor at a Time (OFAT)

Several factors arising from the environment are known to affect the growth of microorganism. A triplicate experimental run was conducted using one factor at a time (OFAT) to study the effect of pH and Temperature for maximum atrazine growth using the bacterial strain.

Effect of initial pH on the growth of atrazine-degrading bacteria

The effect of various pH on the growth of atrazine-degrading *Bacillus safensis* on MSM media was presented in figure 2. It was found that growth of this bacterium tends to be optimum at pH 7.5 following 48 hours' incubation at 37 °C. A significant ($p < 0.05$) decrease in growth was observed at pH higher than 7.5.

Statistical analysis showed a significant difference ($p < 0.05$) between the pH values of 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0.

Effect of temperature on the growth of atrazine-degrading bacteria

The effect of various temperature on the growth of atrazine-degrading *Bacillus safensis* strain BUK_BCH_BTE6 on MSM media was presented in figure 3. It was found that growth of this bacterium tend to be optimum at 35 °C following 48 hours' incubation. A significant ($p < 0.05$) decrease in growth was observed at temperature higher than 40 °C. Statistical analysis showed a significant difference ($p < 0.05$) between the temperature of 25 °C, 30 °C, 35 °C, 40 °C, 45 °C and 50 °C.

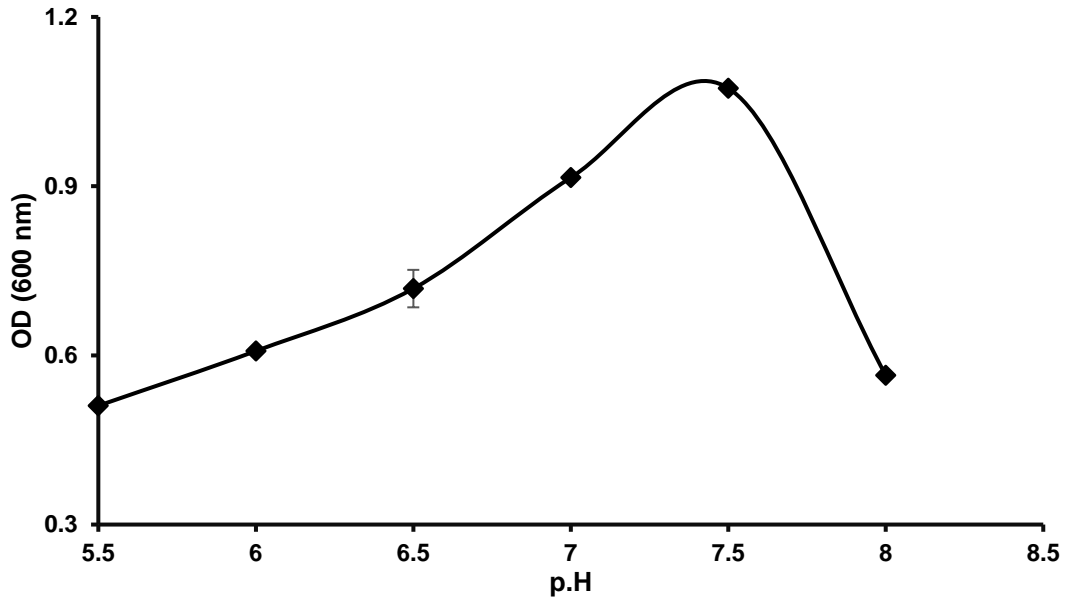


Figure 2: Effect of pH on the growth of atrazine-degrading *Bacillus safensis* strain BUK_BCH_BTE 6 in MSM media supplemented with 100 mgL⁻¹ initial atrazine, incubated for 48 hours at 37 °C. Data are mean ± SD of triplicate determinations.

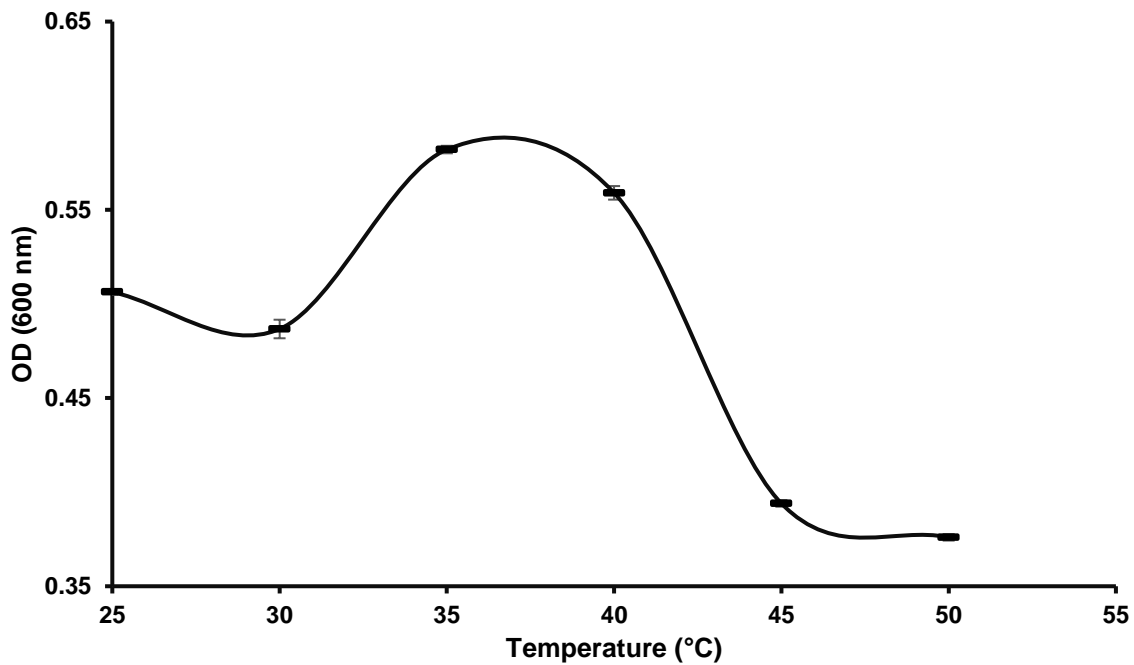


Figure 3: Effect temperature on the growth of atrazine-degrading *Bacillus safensis* strain BUK_BCH_BTE 6. in MSM media supplemented with 100 mgL⁻¹ initial atrazine, incubated for 48 hours at 37 °C. Data are mean ± SD of triplicate determination.

Discussion

There is a significant diversity in the genera of bacteria capable of degrading atrazine (Li et al., 2019). Based on this research, a bacterium with potential of atrazine degradation was isolated from agricultural soil and identified as *Bacillus safensis* strain BUK_BCH_BTE6 according to 16S rRNA gene sequencing and molecular phylogeny. The bacterial sequence was deposited in the GenBank with the accession number OK180490.1

pH is among the most important parameters influencing the effectiveness of many chemical and biological activities (Shamsedini et al., 2016). The changes in values of pH affect both the structure and activity of protein, this also affects the activity of cellular enzymes. Drastic change in pH also damages the cell membrane and membrane proteins, which in turn debilitate permeability barrier of cell. The pH of the environment of the organism is frequently altered by metabolic activity of organism itself or by other activities in nature. This is implied when culture media are incorporated with buffers that can resist the change in pH (Cho et al., 2016). The effect of various pH on the growth of atrazine-degrading *Bacillus safensis* on MSM media was presented in **Figure 2**. It was found that growth of this bacterium tends to be optimum at pH 7.5 following 48 hours' incubation at 37 °C. A significant ($p < 0.05$) decrease in growth was observed at pH higher than 7.5. Statistical analysis showed a significant difference ($p < 0.05$) between the pH values of 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0.

This rate is consistent with findings from similar studies Abigail et al (2013); Kolekar et al (2014); Wang et al (2014); Zhu *et al* (2019); Li *et al* (2019) and Khatoon and Rai (2020). This implies that pH plays a significant role in microbial metabolism and atrazine degradation is pH dependent (Andleeb et al., 2016).

All life processes leading to growth are catalyzed by the enzymes present in the cell. The activity of enzymes is influenced by temperature and therefore temperature is able to influence growth (Charpe et al., 2019). The effect of various temperature on the growth of atrazine-degrading *Bacillus safensis* on MSM media was presented in Figure 3. It was found that growth of this bacterium tend to be optimum at 37 °C following 48 hours' incubation. A significant ($p < 0.05$)

decrease in growth was observed at temperature higher than 40 °C. Statistical analysis showed a significant difference ($p < 0.05$) between the temperature ranges of 25 °C, 30 °C, 35 °C, 40 °C, 45 °C and 50 °C.

Similar results were obtained by Ariole and Abubakar (2015); Mansee et al (2017) and Zhao et al (2018). Growth and degradation abilities were both inhibited when the temperature was higher than 40 °C or lower than 35 °C. This result indicate that the bacterium is mesophilic and that temperature have significant effect in bacterial physiological process and biodegradation of atrazine herbicide. The results also revealed that the optimal temperature of 35 °C for atrazine degradation is close to the optimal growth temperature for *Bacillus sp.*, which is 37 °C, this result is in agreement with that of Omotayo et al (2016). Results of this research prove the statement that the rate of microbial and chemical degradation are influenced by higher temperature and the rate at which pesticides dissipates is significantly faster under tropical conditions. As such, the overall degradation in tropical regions might be favorable than in temperate regions (Omotayo et al., 2016).

Conclusion

A bacterium with the ability to grow on and tolerate up to 1500 mgL⁻¹ of atrazine herbicide (as sole carbon source) was isolated from agricultural soil in Kura Local Government Area of Kano State, Nigeria. Morphological and biochemical identifications revealed the isolate as gram positive, rod shaped, which was identified as *Bacillus safensis* strain BUK_BCH_BTE6 based on 16S rRNA gene sequencing and subsequent molecular phylogenetic analysis. *Bacillus safensis* has an optimum pH range of 7.5 and temperature of 35 °C. As such *Bacillus safensis* have great potential of atrazine-degradation and can be used as a suitable candidate for bioremediation of atrazine polluted sites.

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