



Atrazine degradation by *Bacillus safensis* strain BUK_BCH_BTE6 isolated from agricultural land in northwestern Nigeria

Faisal Muhammad¹, Hafeez Muhammad Yakasai^{1,*}, Mohd Yunus Shukor²

¹Department of Biochemistry, Faculty of Basic Medical Sciences, College of Health Sciences, Bayero University Kano, P.M.B. 3011, Kano State, Nigeria

²Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia

*Corresponding author: hmyakasai.bch@buk.edu.ng

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ABSTRACT Atrazine herbicide is known to disrupt the endocrine system and is potentially carcinogenic. Its continual application leads to high residue levels in soil, causing water pollution, which when consumed is associated with devastating health effects. This research reported the isolation and characterization of a new bacterial strains from active agricultural soil with the potential to biodegrade atrazine as a sole carbon source. An enrichment method was utilized to isolate the bacteria (A1, A2, B1, B2, C1 and C2) on mineral salt media (MSM) following serial dilution. Six isolates were screened for their tolerance to various concentrations of atrazine (500 to 1500 mg·L⁻¹), and only isolate B1 tolerated up to 1500 mg·L⁻¹ atrazine. The isolate was identified molecularly as *Bacillus safensis* strain BUK_BCH_BTE6 based on 16S rRNA gene sequencing and molecular phylogenetic analysis. Characterization of the isolate based on the effects of temperature, pH, substrate concentration, incubation time, inoculum size, and heavy metals revealed optimum growth and atrazine degradation at 35 °C, a pH of 7.5, 400 mg·L⁻¹, at 48 h, and inoculum size of 600 µL, respectively. The growth of the isolate was inhibited by 2 ppm Hg, Cd, Cr, Pb, Ar, and Ni, while Fe, Cu, and Zn stimulated it. GC-MS analysis revealed a degradation efficiency of 88.85% within 120 h, while metabolites such as desethyldeisopropylatrazine, deisopropylatrazine, N-ethylammelide, and cyanuric acid were also detected. This isolate is a highly atrazine-tolerant and efficient atrazine degrader that could be employed for bioremediation of atrazine-polluted sites.

KEYWORDS Bacteria; Bioremediation; GC-MS; Herbicide; Pollution

1. Introduction

The rise in population and the need for better agricultural production in addition to industrial advances and urbanization were the major sources of environmental contamination we are facing today (Ali et al. 2020). Increased industrialization, wars, use of intensive metals and organic substances have leveraged these problems (Benjamin et al. 2019). Modern agriculture depends on herbicides to eradicate weeds to maximize crop yield. Thus, these herbicides are of economic importance to the increasing world population (Singh and Singh 2016).

Atrazine (1-chloro-3-ethylamino-5-isopropylamino-2, 4, 6- triazine) is an herbicide of the triazine class with molecular formula C₈H₁₄ClN₅ (He et al. 2019). It is often present on surface and groundwater, with a half-life of 13–261 days (Li et al. 2019). It acts against broad-leafed weeds and some grass weeds that inhibit growth of crops, also restrain some perennial weeds (He et al. 2019). It is the most widely used herbicide worldwide on crops such

as sorghum (*Sorghum bicolor*), maize (*Zea mays*) and sugarcane (*Saccharum officinarum*) because of its low cost and effectiveness (Shamsedini et al. 2015).

Several tons of herbicides are applied every year in agriculture, causing harm to non-target organisms. They can reach water through runoff from treated plant and soil and are able to leach down the soil to contaminate groundwater or if immobile, they remain on the top soil where it accumulates as a toxin and affect microorganisms (Warsi et al. 2017). The application and continuous use of atrazine increases its residue in soil, causing water pollution. Atrazine monitoring in southwestern Nigeria, reveals higher concentrations of atrazine and its chloro-s-triazine metabolites in hand-dug wells than in borehole and stream water, with concentrations ranging between 0.01 mg·L⁻¹ and 0.08 mg·L⁻¹. Hazard index values associated with ingestion and dermal for both children and adults were below the acceptable limit of 2.0 µg·L⁻¹ in the US and above 0.1 µg·L⁻¹ in Europe. Atrazine concentration as low as 0.02 mg·L⁻¹ appears to trigger defense mechanisms capa-

ble of protecting the structural integrity of the brain. These concentrations may not pose any threat to brain function, but concern should be raised at $0.08 \text{ mg}\cdot\text{L}^{-1}$ (Owagbori-aye et al. 2022). Atrazine herbicide has negative effect on plant photosynthesis, microorganism and human health as it is considered an endocrine disruptor and carcinogen. This necessitates the need for its remediation. The conventional physicochemical approaches for remediating soil have some drawbacks such as high costs, formation of secondary pollutant and damage to soil fauna, all of which fail to completely remove atrazine (Olu-Arotiowa et al. 2019). Thus, innovative techniques are critically needed (Olu-Arotiowa et al. 2019). Bioremediation is considered a harmless, efficient, and economical biotechnological approach for the removal of atrazine in polluted areas. To date, report on the isolation and characterization of indigenous bacteria with atrazine-degrading capability in Nigeria is limited and perhaps lacking from the northwestern part of the country despite the extensive utilization of this herbicide in agricultural activity.

2. Materials and Methods

2.1. Sample collection

A random sampling technique was used to select three agricultural farmland from which soil samples were collected in Kura Local Government Area of Kano, Northwestern Nigeria, located at $11^{\circ}46'N$, $8^{\circ}49'E$ and $12^{\circ}01'N$, $8^{\circ}29'E$ (Umar et al. 2020). Soil samples were collected at the depth of 5–10 cm below the topsoil using a sterile scoop after removal of the top layer of the soil up to 1–5 cm. The samples in sterile containers were transported to the Microbiology laboratory at Bayero University Kano for analyses. The samples were dried, homogenized and passed through a 2 mm sieve. Analytical grade atrazine with 90% purity was procured from Emerald Scientific, USA. All experiments involving microorganisms were done in a class II biosafety cabinet.

2.2. Enrichment and isolation of atrazine-degrading bacteria

The bacteria were isolated using culture enrichment method in a mineral salt medium comprising $0.5 \text{ g}\cdot\text{L}^{-1}$ of KH_2PO_4 , $3.0 \text{ g}\cdot\text{L}^{-1}$ of K_2HPO_4 , $0.2 \text{ g}\cdot\text{L}^{-1}$ of $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, $0.5 \text{ g}\cdot\text{L}^{-1}$ of NaCl , and $1 \text{ m}\cdot\text{L}^{-1}$ and a solution of trace element concentrate was prepared according to Sawangjit (2016), containing $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, $1 \text{ g}\cdot\text{L}^{-1}$, $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, $5 \text{ g}\cdot\text{L}^{-1}$, $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, $0.4 \text{ g}\cdot\text{L}^{-1}$, $\text{MnSO}_4\cdot \text{H}_2\text{O}$, $1 \text{ g}\cdot\text{L}^{-1}$, EDTA , $2.5 \text{ g}\cdot\text{L}^{-1}$, $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$, $0.25 \text{ g}\cdot\text{L}^{-1}$, and $\text{Na}_2\text{B}_4\text{O}_7\cdot 10\text{H}_2\text{O}$, 0.2 g . The mineral salts were dissolved in 1 L deionized water and autoclaved at $121 \text{ }^{\circ}\text{C}$ for 15 min (Macwilliams and Liao 2016).

Into 250 mL Erlenmeyer flasks containing 100 mL of MSM which was supplemented with $100 \text{ mg}\cdot\text{L}^{-1}$ atrazine, 10 g of soil sample was inoculated and homogenized. The flasks were then incubated at $37 \text{ }^{\circ}\text{C}$ under orbital agitation at 150 rpm. After 7 d, 10 mL aliquot of each culture

was transferred to the new MSM broth (100 mL). Subsequently, each culture was serially diluted from 10^{-1} to 10^{-6} in sterile distilled water and proper dilutions of 1×10^{-6} were plated on MSM agar, supplemented with 0.1 mL of atrazine ($100 \text{ mg}\cdot\text{L}^{-1}$). The Petri dish was then incubated for 48 h at $37 \text{ }^{\circ}\text{C}$. Bacterial colonies grown were isolated according to their morphology, and the selected morphotypes were purified by repeated streaking on MSM agar (Muhammad et al. 2021). The isolates were then grown on the stock culture of nutrient agar and incubated at $37 \text{ }^{\circ}\text{C}$ for 24–48 h. Bacterial isolates were then stored refrigerated at $-20 \text{ }^{\circ}\text{C}$ for further studies.

2.3. Screening of atrazine-tolerant isolate

A total of six bacterial isolates (A1, A2, B1, B2, C1 and C2) were isolated based on their morphology after culture enrichment and tested for their ability to tolerate varying concentrations ($500 \text{ mg}\cdot\text{L}^{-1}$, $1000 \text{ mg}\cdot\text{L}^{-1}$ and $1500 \text{ mg}\cdot\text{L}^{-1}$) of atrazine in MSM as sole carbon source (Ahmad et al. 2022).

2.4. Molecular identification

Pure bacterial culture grown on Luria broth at $37 \text{ }^{\circ}\text{C}$ was used to extract genomic DNA and centrifuged for 2 min at 5000 rpm. The pellets were homogenized in 100 μL warmed Livak grind buffer (1.6 mL, 5M NaCl, 5.48 g sucrose, 1.57 g Tris, 10.16 mL 0.5M EDTA, 2.5 mL 20% SDS) and incubated at $65 \text{ }^{\circ}\text{C}$ for 30 min. About 14 μL of 8 M K-acetate was added and centrifuged for 20 min at $4 \text{ }^{\circ}\text{C}$. The pellet was rinsed in 100 μL ice-cold 70% ethanol dried and suspended in 100 μL dH_2O incubated for 10 min at $65 \text{ }^{\circ}\text{C}$ and the concentration of DNA was determined using a Nanodrop spectrophotometer (2000/2000c, Thermo Fisher Scientific, USA) (Brahmbhatt 2012).

The PCR reaction was carried out using KAPATaq DNA polymerase. Total volume of 25 μL and comprise of 2 μL genomic DNA, 2.5 μL of 10 TaqA Buffer, 0.4 M (0.85 μL) of each of forward primer Bact1442-F (AGAGTTGATCCTGGCTCAG) and reverse primer Bact1492-R (GGTTACCTTGTTACGACTT) 1.25 mM (1.5 μL) of MgCl_2 , 0.25 mM (0.2 μL) of dNTPs mixes and 0.2 μL of Taq DNA polymerase, in ddH_2O (Sawangjit 2016). PCR amplification products were observed using agarose gel electrophoresis. A 14-well comb was placed, and the gel was allowed to set for 30 min. After the gel solidified, the comb was removed and the gel tray was placed in the buffer tank. The gel was submerged to a depth of 2 to 5 mm by pouring $0.5\times$ TBE into the tank (Li et al. 2020).

Purified PCR products were further amplified in one direction with 16S primer using Big DyeTM Terminator Ready Reaction Mix (ABI) and sequencing of the amplified product was done with an ABI 3130 Genetic Analyzer version (Applied Biosystem) (Singh and Singh 2016). The 16S rRNA gene sequence of the isolate was analyzed with nucleotide BLAST search in GenBank. Phylogenetic relationship was analyzed with other closely related in GenBank (Swissa et al. 2014). The sequences were obtained in FASTA format, checked for Multiple Sequence Align-

ment (MSA) using T-Coffee tool from EBI (EMBL), and a tree was created (Brahmbhatt 2012).

2.5. Characterization of atrazine-degrading bacteria

Various environmental factors affect the growth of microorganisms. A triplicate experimental run was conducted using one factor at a time (OFAT) to study the effect of growth parameters.

2.5.1 Effect of initial pH on atrazine degradation

The pH of the MSM (50 mL) supplemented with atrazine ($100 \text{ mg}\cdot\text{L}^{-1}$) was varied in a range between 5.5–8.5, bacterial culture ($100 \mu\text{L}$) was then inoculated and incubated at 37°C for 120 h. Bacterial aliquot (1 mL) was taken every 24 h to measure at OD_{600} (Shehu et al. 2021).

2.5.2 Effect of temperature on atrazine degradation

The incubation temperature of the inoculated MSM (50 mL) supplemented with atrazine ($100 \text{ mg}\cdot\text{L}^{-1}$) was varied from 25– 50°C , and incubated for 120 h. Bacterial aliquot (1 mL) was taken every 24 h to measure the OD_{600} (Muhammad et al. 2021).

2.5.3 Effect of substrate concentration on atrazine degradation

Into MSM (50 mL) supplemented with various concentrations of atrazine ($100\text{--}1000 \text{ mg}\cdot\text{L}^{-1}$) was inoculated with $100 \mu\text{L}$ bacterial culture and incubated at 37°C for 120 h. Bacterial aliquot (1 mL) was taken every 24 h to measure the OD_{600} .

2.5.4 Effect of incubation time on atrazine degradation

A culture media containing 50 mL of MSM supplemented with atrazine ($100 \text{ mg}\cdot\text{L}^{-1}$) was inoculated with $100 \mu\text{L}$ of bacterial culture and incubated at 37°C . Bacterial aliquot (1 mL) was taken every 24 h to measure the OD_{600} for 120 h.

2.5.5 Effect of inoculum size on atrazine degradation

Various inoculum sizes ranging between $100\text{--}1000 \mu\text{L}$ were inoculated into 50 mL each of MSM supplemented with atrazine ($100 \text{ mg}\cdot\text{L}^{-1}$) and incubated for 120 h at 37°C . An aliquot (1 mL) was taken every 24 h to measure the OD_{600} .

2.5.6 Effect of heavy metals on atrazine degradation

Heavy metals solution (2 ppm) was added into each MSM (50 mL) supplemented with atrazine ($100 \text{ mg}\cdot\text{L}^{-1}$) and inoculated with $100 \mu\text{L}$ of bacterial culture, then incubated at 37°C for 120 h. An aliquot (1 mL) was used at each 24 h to measure the OD_{600} .

2.5.7 Biodegradation study

An experiment with optimum conditions was set in 250 mL Erlenmeyer flasks with 100 mL of MSM, for 120 h. Microbial growth was assessed spectrophotometrically by measuring OD_{600} . Final atrazine residue at 120 h were extracted with 12 mL acetonitrile, and centrifuged at $9980 \times g$ for 6 min; extracts were concentrated to dryness by a rotary evaporator at 25°C . The dried residue was redissolved in 0.5 mL acetonitrile/water (20:80, v/v), and the final solutions were filtered through a $0.22 \mu\text{m}$ filter membrane before GC-MS analysis on an Agilent 19091S-433UI (HP-5MS Ultra Inert). The mobile phase consisted of acetonitrile and water (v/v, 80:20). Following injection of $2 \mu\text{L}$ of the sample onto the column, the analytes were eluted using an isocratic mode with a flow rate of $0.3 \text{ mL}\cdot\text{min}^{-1}$. The GC temperature program was 50°C for 1 min, ramped to 50°C at $10^\circ\text{C}\cdot\text{min}^{-1}$, ramped to 180°C at $5^\circ\text{C}\cdot\text{min}^{-1}$, and finally ramped to 230°C at $10^\circ\text{C}\cdot\text{min}^{-1}$ and held for 5 min. The injector temperature was held at 300°C for 5 min. Splitless injection was used. Helium was used as the carrier gas at a $1 \text{ mL}\cdot\text{min}^{-1}$ flow rate. The transfer line between the GC and mass spectrometer was held at 325°C , and the ion trap manifold was set to 330°C . A Varian 8200 Auto Sampler was used to perform sample injection and solvent flushing of the needle. Atrazine standard was $100 \mu\text{g}\cdot\text{L}^{-1}$ in 100% acetonitrile. After completion of the analysis, the GCMS post-run analysis was checked on the desktop. The data required for the report preparation was opened and integration was done. The mass spectrum of the atrazine standard was compared with that of the test sample to determine the metabolite present.

The degradation percentage was calculated according to the formula:

$$\% \text{ Degradation} = \frac{(ACS - ACT)}{(ACS)} \times 100 \quad (1)$$

Note: ACS= Area under the curve of the standard and ACT= Area under the curve of the test sample.

3. Results and Discussion

3.1. Isolation, screening and identification atrazine-degrading bacteria

A total of six isolates (A1, A2, B1, B2, C1 and C2) were isolated and screened for atrazine degradation, of which isolates A1, A2, B2, C1 and C2 tolerated only concentrations between $500 \text{ mg}\cdot\text{L}^{-1}$ and $1000 \text{ mg}\cdot\text{L}^{-1}$ of atrazine. However, only isolate B1 tolerated up to $1500 \text{ mg}\cdot\text{L}^{-1}$ atrazine; hence, it was selected for the characterization work (Table 1).

The 16S rRNA gene sequences of the bacterium were compared with related sequences from the GenBank database using Blast Server at NCBI, and results show that the DNA sequences obtained were closely related to the partial sequences of several *Bacillus* sp. with almost 99% similarity. Molecular phylogenetic studies using the neighbor-joining method linked the iden-



FIGURE 1 Cladogram indicating genetic relationship between *B. safensis* strain BUK_BCH_BTE6 and 16S rRNA sequences of related microorganisms.

TABLE 1 Screening for atrazine tolerant bacterial isolate within 48 h of incubation.

Isolate	500 mg·L ⁻¹	1000 mg·L ⁻¹	1500 mg·L ⁻¹
A1	+	+	-
A2	+	-	-
B1	+	+	+
B2	+	+	-
C1	-	-	-
C2	+	-	-

Key: + = Growth, - = No growth

tity of the sequence to *Bacillus safensis*. Thus, this bacterium was tentatively assigned as *B. safensis* strain BUK_BCH_BTE6. Accession numbers are accompanied by the species name of the respective 16S rRNA gene sequence. Numbers at the nodes designate bootstrap values from the neighborhood-joining analysis of 1,000 resampled data sets. The sequence was deposited in the GenBank under the accession number OK180490.

The first pure bacterial culture to be isolated with potential for mineralization and utilization of atrazine as a sole carbon and nitrogen source for growth was *Pseudomonas* sp., which fully degrades it, making it a model of interest for analyzing the genetic and enzymatic basis of atrazine mineralization (Fernandes et al. 2020). To date, a number of *Bacillus* with atrazine degrading capability have been isolated from soils around the world. Interestingly, this work was the first to report the isolation of *B. safensis* from Nigerian soil that could tolerate up to 1500 mg·L⁻¹ atrazine and tolerated a number of toxic heavy metals, which is quite a novel feat.

3.2. Characterization of atrazine-degrading bacteria using one factor at a time (OFAT)

3.2.1 Effect of initial pH on atrazine degradation

The effect of pH on the growth of atrazine-degrading *B. safensis* in MSM is presented in Figure 2. The growth of this bacterium was shown to be optimum at pH 7.5 following 48 h incubation at 37 °C. A significant decrease in growth was observed at a pH higher than 7.5 ($p < 0.05$).

The optimum pH of 7.5 for atrazine degradation observed in this study (Figure 2), was in agreement with findings from literature reports (Abigail et al. 2013; Kolekar et al. 2014; Wang et al. 2014; Zhu et al. 2019; Li et al. 2019; Khatoon and Rai 2020). However, this finding contradicts the works of Debasmitta and Rajasimman (2013), who reported a pH of 6.7, while Shamsedini et al. (2015) reported a pH of 11, Mansee, Ayman and Bakry, Nabila and Doaa (2017) reported a pH of 5.0, while Zhao et al. (2017) and Zhao et al. (2018) reported a pH of 9.0. Research has indicated that an increase in soil pH may lead to an increase in soil microbial biomass and enzymatic process, it was also observed that atrazine degradation was higher at a pH

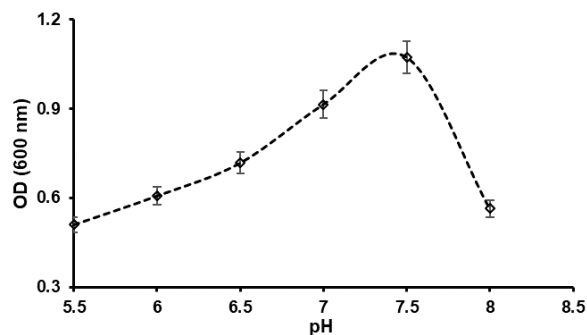


FIGURE 2 Effect of pH on the growth of strain BUK_BCH_BTE6 in MSM containing 100 mg·L⁻¹ atrazine, incubated at 37 °C for 48 h. Each value represents mean ± SD of three replicates.

range of 6.5–7.5 (Omotayo et al. 2016). Perhaps, pH is among the factors influencing the rate of some chemical and biological processes (Shamsedini et al. 2015). The changes in pH values affect both the structure and activity of protein, this also affects the activity of cellular enzymes. Drastic change in pH also damages the cell and membrane proteins, which in turn debilitate permeability barrier of cell (Cho et al. 2016).

3.2.2 Effect of temperature on atrazine degradation

The temperature effect on the growth of atrazine-degrading *Bacillus safensis* in MSM is presented in Figure 3. The result shows that a temperature between 35 and 40 °C was optimum for growth following 48 h incubation. A significant decline in growth ($p < 0.05$) was observed at temperatures below 30 °C higher than 40 °C.

The optimum temperature between 35 and 40 °C for the growth of atrazine degrading isolate reported in this study (Figure 3) was similarly reported (Ariole and Abubakar 2015; Mansee, Ayman and Bakry, Nabila and Doaa 2017; Zhao et al. 2018). However, lower optimum temperature of 30 °C was equally reported (Zhang et al. 2009; Abigail et al. 2013; Kolekar et al. 2014; Wang et al. 2014; Andleeb et al. 2016; Zhu et al. 2019; Li et al. 2019; Zhang et al. 2019). Whereas, Swissa et al. (2014) and Debasmita and Rajasimman (2013) reported an optimum temperature of 28 °C and 29.3 °C, respectively for atrazine biodegradation. The variation in optimum temperature could be due to the differences in the species of isolates, geographical location from which the isolate was isolated, targeted concentration of atrazine, as well as other conditions prevailed. This study shows that growth and degradation abilities of the bacteria were both inhibited at temperatures higher than 40 °C or lower than 35 °C, indicating that the isolate is a mesophile (growth in moderate temperature) and that temperature plays an active role in bacterial metabolism and atrazine degradation. All life activities leading to growth are catalyzed by the enzymes found in the cell, the activity of which is affected by temperature, which also influences growth (Charpe et al. 2019). Thus, the effect of accelerated degradation in trop-

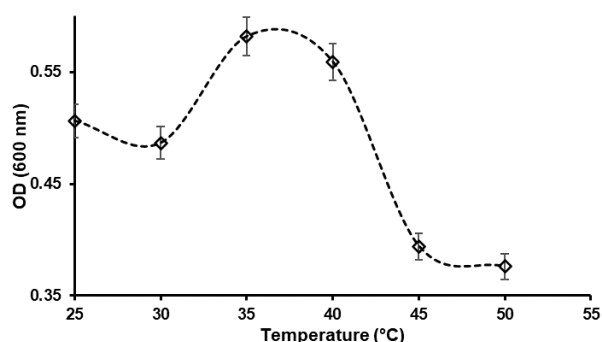


FIGURE 3 Effect of temperature on growth of strain BUK_BCH_BTE6 in MSM containing 100 mg·L⁻¹ atrazine, incubated for 48 h. Each value represents mean ± SD of three replicates.

ical zones might be stronger than in temperate regions (Omotayo et al. 2016).

3.2.3 Effect of atrazine (carbon source) concentration on its degradation

The effect of atrazine (substrate) concentration on the growth of *B. safensis* in MSM is presented in Figure 4. A concentration of 400 mg·L⁻¹ atrazine was seen to produce optimum growth for this bacterium following incubation at 37 °C for 48 h. A significant decrease in growth ($p < 0.05$) was observed at concentrations higher than 400 mg·L⁻¹, though the difference was between 600 mg·L⁻¹ and 1000 mg·L⁻¹.

The linear increase in growth observed at atrazine concentrations between 50–400 mg·L⁻¹ (Figure 4), shows that lower concentrations of this herbicide did not produce toxicity to this bacterial isolate. However, the decline in OD₆₀₀ as concentration increased beyond 400 mg·L⁻¹, reveals that concentrations above the optimum are toxic to the bacterial cell, slowing down the growth of the isolate. The optimum concentration found in this study was similar to that reported by Abigail et al. (2013), but deviated from 50 mg·L⁻¹ reported (Shiri et al. 2016; Zhao et al. 2017, 2018; Zhang et al. 2019). Similarly, an optimum concentration of 100 mg·L⁻¹ atrazine was reported (Zhang et al. 2009; Kolekar et al. 2014; Ye et al. 2016; Ariole and Abubakar 2015; Li et al. 2019). Other studies (Wang et al. 2014; Zhu et al. 2019) reported an optimum concentration of 200 mg·L⁻¹, Khatoon and Rai (2020) reported 200 ppm, Debasmita and Rajasimman (2013) reported 12 mg·L⁻¹ and 35 ppm was reported by Swissa et al. (2014), Shamsedini et al. (2015) reported 0.1 mg·L⁻¹, Mansee, Ayman and Bakry, Nabila and Doaa (2017) reported 0.06 mM and Li et al. (2020) reported 80 mg·L⁻¹. The ability of *B. safensis* strain BUK_BCH_BTE6 to tolerate and degrade up to 1000 mg·L⁻¹ with optimum atrazine concentration at 400 mg·L⁻¹ makes it superior to other *B. species* and most other atrazine-degrading microbes reported to date. Microbial growth is influenced by concentration of substrate. Higher concentrations of substrate affect growth and may

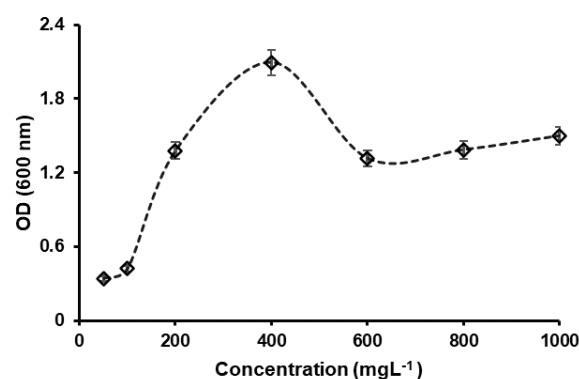


FIGURE 4 Effect of atrazine (substrate) concentration on growth of strain BUK_BCH_BTE6 in MSM incubated at 37 °C. Each value represents mean ± SD of three replicates.

inhibit the metabolism of microorganisms (Khatoon and Rai 2020). This result shows that this isolate is a good atrazine degrader that could be used for remediation in a broader range of atrazine concentrations.

3.2.4 Effect of inoculum size on atrazine degradation

The effect of inoculum size on the growth of atrazine-degrading *B. safensis* in MSM is presented in Figure 5. The growth of the bacterium was found to be optimum when the inoculum was 600 µL following 48 h incubation at 37 °C. A significant decrease in growth ($p < 0.05$) was observed at inoculum sizes below 400 µL and higher than 600 µL.

The optimum inoculum size from this study was found to be 600 µL (Figure 5). The insignificant effect observed after increasing the inoculum from 600–800 µL could probably be due to the attainment of equilibrium of microbial load. Similar studies on atrazine degradation reported different inoculum sizes; Zhang et al. (2009) and Abigail et al. (2013) reported 3%, Debasmita and Rajasimman (2013) reported 5%, Zhao et al. (2017) reported 100 µL and 10% was reported by Zhao et al. (2018). According to Zhang et al. (2009), a lower inoculum size prevents sufficient contact of the strain with the atrazine in the medium, resulting in a lower degradation rate, while a higher inoculum size results in carbon source competition at the initial stage of the strain growth causing malnutrition in strains and a significant influence on biodegradation rate. A high inoculum size results in an increase in atrazine-degrading capability and may play a simulative role in the degradation of atrazine by the isolate and increase the pollutant removal capacity. Hence, different inoculum size levels result in different degradation rates (Zhao et al. 2017).

3.2.5 Effect of incubation time on atrazine degradation

The effect of incubation time on the growth of atrazine-degrading *B. safensis* in MSM is presented in Figure 6.

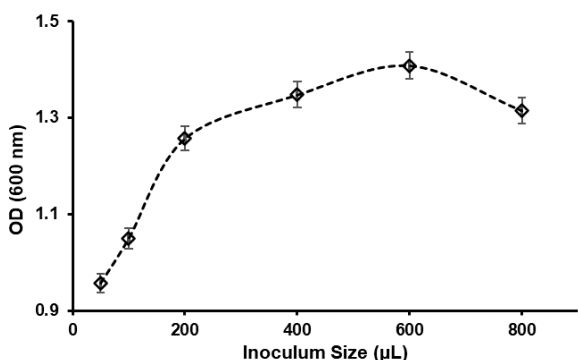


FIGURE 5 Effect of inoculum sizes on growth of strain BUK_BCH_BTE6 in MSM containing 100 mg·L⁻¹ atrazine, incubated at 37 °C for 48 h. Each value represents mean ± SD of three replicates.

The growth of this isolate reveals a linear pattern within the first 24 h, reaching optimum after 48 h of incubation at 37 °C. A significant decrease in growth was observed at incubation times above 48 h ($p < 0.05$).

In this study, the optimum growth was observed after 48 h of incubation (Figure 6), which could be due to the lag phase of the isolate. Atrazine degradation was inefficient at low incubation time, but increases with higher incubation period, indicating that time might positively affect atrazine degradation. Similar findings were reported (Abigail et al. 2013; Zhao et al. 2018; Li et al. 2019; Zhang et al. 2019; Li et al. 2020), and deviated from 72 h reported (Wang et al. 2014; Andleeb et al. 2016; Omotayo et al. 2016). Moreover, Ye et al. (2016) reported 36 h, Kolekar et al. (2014) and Shiri et al. (2016) reported 120 h, Zhao et al. (2017) reported 14 h, Swissa et al. (2014) reported 6 h, Dehghani et al. (2013) reported 24 h, while Ariole and Abubakar (2015) and Mansee, Ayman and Bakry, Nabila and Doaa (2017) reported an optimum incubation time of 20 days for atrazine degradation. It is well established that to ensure an optimal reproducible physiological state of bacterial cells during growth incubation time needs to be strictly respected (Chikere and Ugueri 2014).

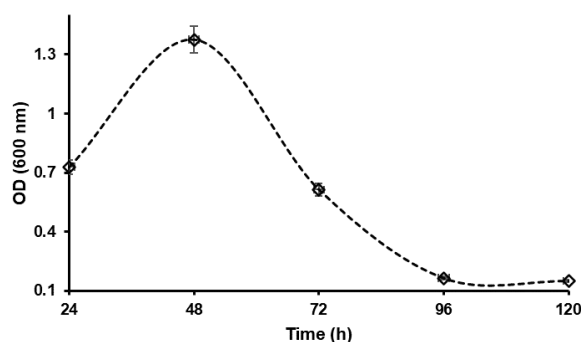


FIGURE 6 Effect of incubation time on growth of strain BUK_BCH_BTE6 in MSM containing 100 mg·L⁻¹ atrazine, incubated at 37 °C. Each value represents mean ± SD of three replicates.

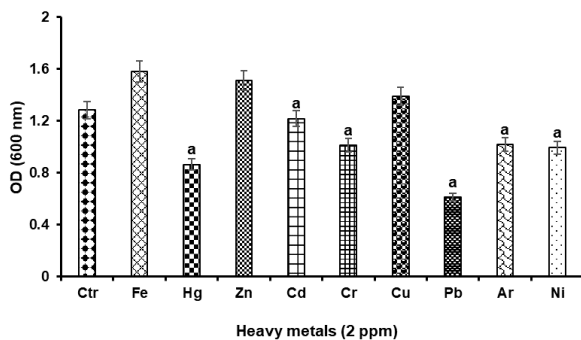


FIGURE 7 Effect of heavy metals on growth of strain BUK_BCH_BTE6 in MSM containing 100 mg·L⁻¹ atrazine. Each culture was separately supplemented with various metals, incubated at 37 °C for 72 h. Each value represents mean ± SD of three replicates. a Value indicated significant ($p < 0.05$) inhibition relative to control.

3.2.6 Effect of heavy metals on atrazine degradation

Assessing the effect of heavy metals on the growth of atrazine-degrading *B. safensis* in MSM showed that the metals affect the growth of this bacterium differently. At 2 ppm concentration, Fe, Zn and Cu were found to promote the growth of this bacterium as indicated by their increased OD₆₀₀ relative to control. Whereas Cd, Ar, Cr, Ni, Hg and Pb were found to inhibit the growth of this isolate relative to control. The order of inhibition was Pb>Hg>Ni>Cr>Ar>Cd following incubation at 37 °C for 72 h (Figure 7). Statistical analysis also showed significant differences (*p* < 0.05) between control, Fe, Hg, Zn, Cd, Cr, Cu, Pb, Ar and Ni.

The effect of various heavy metals on the growth of this isolate (Figure 7) showed that the growth and maximum tolerance level of the isolate is between 2–4 ppm. At concentrations higher than 4 ppm, the growth and metabolic activities of the isolate were inhibited. This result is in agreement with that of Alvarez et al. (2017) and Zhang et al. (2019). The need to assess the effect of various heavy metals on the growth of atrazine-degrading isolate arises from the fact that these metals often occur as co-contaminants with other herbicides in the polluted environment. Heavy metals like Cd, Ni, Cr, Pb, As, Hg are discharged by industries, which in trace amounts can cause toxicity to flora and fauna (Meenambigai et al. 2016). While other heavy metals are essential elements to all known life forms as they have a number of functions in biological systems. The toxicity depends on factors like the absorbed dose, the route of exposure, and the period of exposure. A number of homeostatic mechanisms are possessed by the cells to deal with the level of heavy metals and decrease the toxicity produced by excessive level

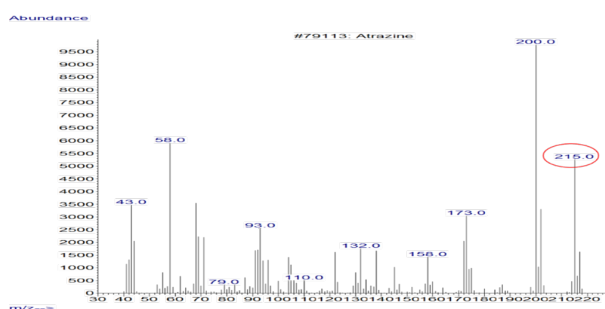


FIGURE 8 GC-MS mass spectrum, peak with MW of 215.0, Rt at 18.029 min indicates atrazine standard.

TABLE 2 Percentage degradation of residual atrazine (400 mg·L⁻¹).

Treatment area	Area under curve	Retention time	Degradation (%)
Atrazine standard	649.735	18.029	
Atrazine test sample	72.469	18.030	88.85

% Degradation = (ACS-ACT)/(ACS) x 100
 ACS= Area under the curve of the standard
 ACT= Area under the curve of the test sample

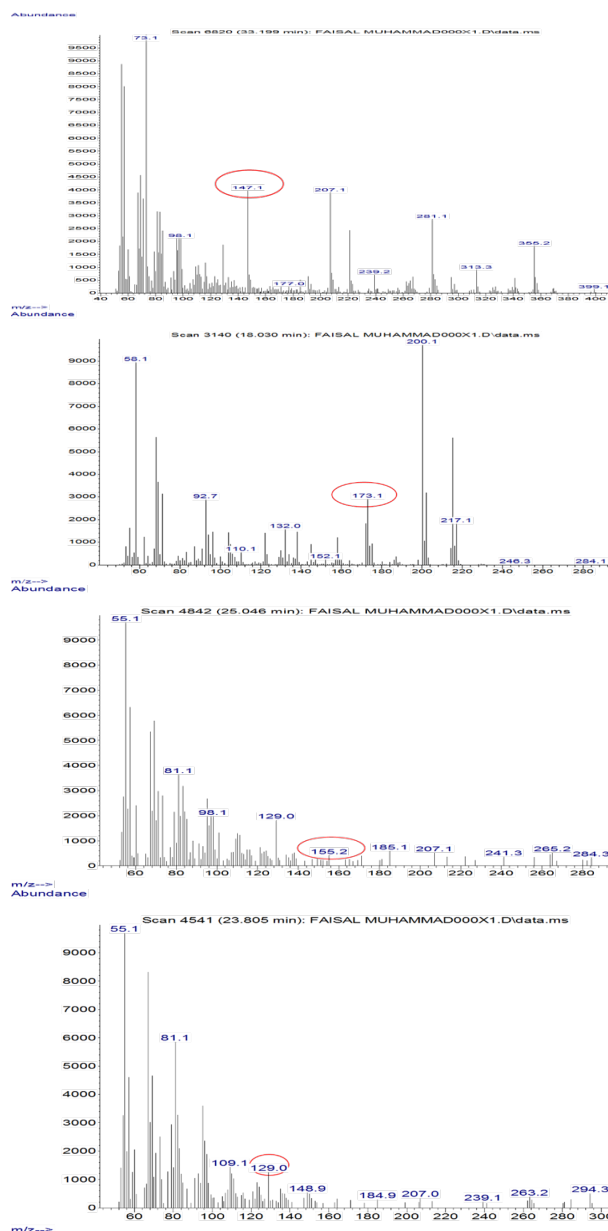


FIGURE 9 (a) GC-MS mass spectrum of 400 mg·L⁻¹ residual atrazine, peak with MW of 147.1 indicates desethyldeisopropylatrazine. The culture (600 µL) of strain BUK_BCH_BTE6 was inoculated and incubated at 35 °C for 120 h. (b) GC-MS mass spectrum of 400 mg·L⁻¹ residual atrazine, peak with MW of 173.1 indicates deisopropylatrazine. The culture (600 µL) of strain BUK_BCH_BTE6 was inoculated and incubated at 35 °C for 120 h. (c) GC-MS mass spectrum of 400 mg·L⁻¹ residual atrazine, peak with MW of 155.2 indicates N-ethylammelide. The culture (600 µL) of strain BUK_BCH_BTE6 was inoculated and incubated for 120 h at 35 °C. (d) GC-MS mass spectrum of 400 mg·L⁻¹ residual atrazine, peak with MW 129.0 indicates cyanuric acid. The culture (600 µL) of strain BUK_BCH_BTE6 was inoculated and incubated for 120 h at 35 °C.

(Alvarez et al. 2017).

3.2.7 Atrazine biodegradation study

The gas chromatography mass spectroscopy (GC-MS) analysis of the atrazine standard is presented in Figure 8. Atrazine, with a molecular weight of 215.0 was identified at a retention time (Rt) of 18.029.

3.2.8 Mass spectrum of atrazine metabolites

The result of GC-MS analysis of atrazine degraded residue was presented in Figures 9a – 9d. It was observed that an inoculum of 600 μ L *B. safensis* strain BUK_BCH_BTE6 degraded 88.85% (Table 2) atrazine at an optimum concentration of 400 $\text{mg}\cdot\text{L}^{-1}$, pH of 7.5 for 120 h of incubation at 35 °C in MSM. The GC-MS analysis also identified 4 major intermediates that are associated atrazine degradation pathway viz. Desethyldeisopropylatrazine (6-chloro-2,4-diamino-1,3,5-triazine) (C₃H₄CIN₅) (Figure 9a), deisopropylatrazine (2,4-diamino-1,3,5-triazine) (C₅H₈CIN₅) (Figure 9b), N-ethylammelide (2,4-dihydroxy-1,3,5-triazine) (C₅H₈N₄O₂) (Figure 9c), and cyanuric acid (1,3,5-triazine-2,4,6-triol) (C₃H₃N₃O₃) (Figure 9d).

4. Conclusions

A bacterium with potential to tolerate up to 1500 $\text{mg}\cdot\text{L}^{-1}$ of atrazine herbicide (as sole carbon source) was isolated from agricultural soil in Kano, Northwestern Nigeria and identified as *Bacillus safensis* strain BUK_BCH_BTE6 based on 16S rRNA sequencing and molecular phylogenetic analysis. The isolate tolerated 2 ppm heavy metals and degraded 88.85% of 400 $\text{mg}\cdot\text{L}^{-1}$ atrazine to cyanuric acid at an optimum pH of 7.5, temperature 35 °C, and inoculum size of 600 μ L after 120 h. With the characteristics of this isolate, this finding has provided basic information about indigenous isolates that could be exploited for bioremediation of atrazine-polluted sites. This isolate is a high atrazine tolerant and efficient atrazine degrader that could be employed for bioremediation of atrazine-polluted sites.

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Authors' contributions

HMY, MYS designed the study. FM, HMY carried out the laboratory work. FM, HMY, MYS, analyzed the data. FM, HMY, wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that there is no conflict of interest concerning the publication of this manuscript.

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