JEMAT 2025, Vol 13, No 1, 18-22
https://doi.org/10.54987/jemat.v13i1.1106

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JOURNAL OF ENVIRONMENTAL MICROBIOLOGY AND TOXICOLOGY

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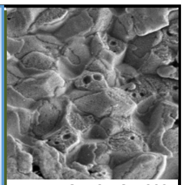

JEMAT VOL 13 NO 1 2025
SEM of Cocoa husk

Biodegradation of Butachlor by *Pseudomonas aeruginosa* Strain B2 Isolated From Agricultural Soil

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HISTORY

Received: 7th March 2025
Received in revised form: 15th May 2025
Accepted: 23rd July 2025

KEYWORDS

Gadar Kazaure
Pseudomonas aeruginosa
Butachlor
Herbicide
Bioremediation

ABSTRACT

The chloroacetamide herbicide butachlor (N-Butoxymethyl-2-chloro-2', 6'-diethyl acetanilide) is frequently used to eradicate undesirable weeds from paddy rice, corn, soybean, and other crop plants. Regular use of these pesticides has caused them to persist in soil and water, making them pollutants. As a result, the herbicide must be bio-remediated using an economical and environmentally responsible technique. This study examined the butachlor herbicide in a controlled laboratory setting. The soil was gathered from Gadar Kazaure's agricultural areas in Jigawa State, Nigeria. At 130 rpm and 30 ºC, the soil sample was enriched in Mineral Salt Medium (MSM) with 0.1 mL of butachlor as the only carbon source. After plating the enriched culture, the bacteria that grew the best were separated and designated as GKSS1, GKSS2, and GKSS3. The biodegradation of strains was evaluated at butachlor concentrations of 0.05 mL and 0.1 mL in 120 hours (pH 7.0, 30 ºC). The isolate (GKSS1) demonstrated a degradation efficiency of 75% and 90% at 0.05 mL and 0.1 mL of butachlor, respectively. *Pseudomonas aeruginosa strain B2* was determined to be the bacterial isolate GKSS1 by molecular and biochemical analysis. This work is the first to be published on the isolation of bacteria that break down butachlor from the sample collection location.

INTRODUCTION

According to [1], the biodegradation process cannot only break down living microorganisms but also immobilize unwanted chemicals such as hydrocarbons, POPs, chemical acids, and their derivatives for further degradation. There have been reports of other microorganisms, including bacteria, fungi, algae, and others, but Bacillus has been discovered to play a major part in the clean-up of harmful organic pollutants. According to the organic contamination that needs to be removed, a variety of methods have been employed, including biosparging, bioaugmentation, biostimulation, and bioventing. The chloroacetamide herbicide butachlor (N-Butoxymethyl-2-chloro-2', 6'-diethyl acetamide) is frequently used to eradicate undesirable weeds from paddy rice, corn, soybean, and other crop plants. Its widespread use and tenacity in soil and water have led to its existence as pa pollutant. In eastern nations, it is frequently applied as a pre-emergence or early post-emergence herbicide to cotton, wheat, barley, and beet fields [2]. The three primary goals of pesticides in agriculture are to increase crop output, improve crop quality, and lower labour and energy costs associated with crop production [3]. Notwithstanding the advantages, pesticides are substances that could be harmful to the environment [4]. According to recent investigations, the use of butachlor was found to be hazardous to aquatic organisms and to flow out with effluents, contaminating groundwater and rivers [5]. Direct application of butachlor to soil may cause it to seep into groundwater, which could be harmful to both human and animal health [6]. Butachlor is found in surface and groundwater as well as in the final drinking water of many nations due to its high water solubility (242 mg/L) and comparatively low soil adsorption coefficient [7].

According to [8], the effects of butachlor-induced toxicity on the cyanobacteria *Nostoc* sp. were investigated, observing notable alterations in growth rate, pigment synthesis, and photosynthetic system (II) activities. Soil containing butachlor can be hazardous to earthworms [9,10], alter microbial populations and activity [13], and occasionally negatively impact the growth and activity of beneficial microorganisms in soils [11]. *Rhodococcus* sp. was obtained from Chinese rice fields by [12], who also found that the strain was effective at breaking down 100 mg/L of butachlor after 5 days of incubation. It was discovered that butachlor biodegraded effectively and efficiently. Another study reported the isolation of a bacterium from agricultural soil, identified as *Bacillus* sp. The strain Hys-1 was isolated from China's active sludge and was able to degrade butachlor more than 90% (100 mg/L) in 7 days [13]. These studies provide evidence that the biodegradation of butachlor by bacterial strains isolated from agricultural soils could be an effective strategy for the remediation of contaminated soil. Utilizing these bacteria as a bioaugmentation agent in polluted areas may also hasten the elimination of harmful contaminants from the environment.

MATERIALS AND METHODS

Study Area

The research area is Gadar Kazaure (Latitude, 12039' 2.16" N; longitude 80 24' 33.84" E) in Kazaure local government, Jigawa state, Nigeria where a lot of irrigation and farming activities are carried out.

Sample Collection

Using a personal interview, a soil sample was taken from agricultural fields (Site A and Site B) with a lengthy history of pesticide application at a depth of 5 to 10 cm. It was then taken to the Bayero University Kano Postgraduate Microbiology Laboratory. Local farmers and pesticide sellers were consulted to determine the best location for the sample collection.

Preparing the soil sample for processing

After being allowed to air dry at ambient temperature to eliminate any remaining moisture, the samples were sieved through a 2 mm screen to exclude any undesired material, which was stored in polyethylene bags and kept at 4°C for further analysis [17].

Source of Butachlor

Butachlor of high purity was procured from distributors in Sabon Gari market in Kano, Kano State, Nigeria.

Determination of Butachlor in Soil

A mixture of all the soil samples was prepared and checked for butachlor contamination using an HPLC instrument.

Pesticide extraction from soil

The soil samples were extracted using a modified version of [15], which is sonication extraction for low levels of pesticides and organics [15]. After being crushed with anhydrous $MgSO_4$ (1:1 w/w for sediment, 5:1 wt/wt for soil), three grams (3 g) of w/w soil sample was put into extraction tubes. Using a 10 ml solution of dichloromethane, acetone, ethyl acetate, and cyclohexane (2:1:1:1 v/v/v/v), the samples were extracted.

They were then sonicated for one minute with 3-s pulses using a sonic disruptor. To separate the extract from the pellet, samples were vortexed for five minutes and then centrifuged at 495 g for five minutes. Twice more, the same process was carried out without vortexing, using an extra 10 millilitres of extraction solvent each time. Following their combination, the extracts were evaporated to dryness under a nitrogen stream, re-dissolved in methanol, and analysed using an Agilent 1260 Infinity HPLC.

Enrichment, Serial Dilution and Isolation of Bacteria

To isolate the bacteria that break down butachlor, a traditional enrichment culture method was used. Inoculating 250 mL Erlenmeyer flasks with 50 mL of mineral salt medium (Bushnell Hass media (g/L) comprising $CaCl_2$, 0.0$_2$; $FeCl_3$, 0.05; $MgSO_4$, 0.2; KH_2PO_4, 1.0; KH_2HPO_4, 1.0; NH_4NO_3, 1.0 g/L was done using one gram (1g) of soil (mixture of all the soil sample) [15]. As a carbon source, 0.1 mL of butachlor was added to the media. The enrichment was carried out in triplicate, and the flask was incubated for five days at 30 °C on a rotary shaker at 130 rpm. Following five days of incubation, 3 mL of the enrichment culture was moved into 50 mL of new media and cultured for an additional five days.

One millilitre of each enrichment was serially diluted after six transfers, and 0.1 mL of the enriched culture 10^{-5} were then put onto nutritional agar medium plates. Each plate was incubated for 48 hours at 30 ºC. The colony exhibiting the highest rate of growth was isolated. After being purified through subculturing on nutrient agar, the isolates were designated as GKSS [14].

Extraction of the isolated bacteria with strong butachlor biodegradation potentials

The chosen bacterial isolate is cultured for 48 hours at 30 °C in a 250 mL Erlenmeyer flask filled with distilled water, 8 gL^{-1} of nutritional broth, and 8 gL^{-1} glucose. It is then supplemented with 0.1 mL butachlor. After centrifuging the bacterial cell or pellet for 20 minutes at 5000 rpm, it was repeatedly cleaned with phosphate buffer. The cell density was adjusted to OD 600nm = 1.00 and stored at 4 ºC for further studies [24].

Biodegradation studies

The experiment was carried out in a 250 mL Erlenmeyer flask that contained a biodegradation solution (100 mL MSM (Bushnell Hass media (g/L) containing $CaCl_2$, 0.02; $FeCl_3$, 0.05; $MgSO_4$, 0.2; KH_2PO_4, 1.0; KH2HPO4, 1.0; NH_4NO_3, 1.0 g/L was prepared in distilled water. 0.1 mL of the herbicides butachlor and 1 mL of pure culture GKSS1, GKSS2, and GKSS3 separately. The experiment was incubated for five days at optimal conditions (30 ºC, pH 7.0) at 130 rpm on a rotary shaker.

Every 24 hours, 1 and 2 mL of the sample were taken, and the bacterial growth was tracked by measuring the optical density (OD) of each isolate using a spectrophotometer set to 600 nm. The butachlor content in the sample was also measured at 24 and 120 hours. Using a Spectrophotometer set at 220 nm, GKSS1, a bacterial culture with a high potential for growth and degradation, was chosen for additional molecular identification and optimization testing. The following formula was used to calculate the pesticide's degradation:

$$\% \text{ Degradation} = 1 - \frac{A_{final}}{A_{initial}} \times 100$$

Where $A_{Initial}$ is the OD at the 24th hour of incubation and A_{final} is the OD at the 120th hour.

The molecular identity of the most potent bacterial isolate (GKSS1)

Following the extraction and clean-up of the isolate's genomic DNA, the 16S rRNA gene was amplified using templates from the polymerase chain reaction (PCR) to describe the isolated strain from the experiment that can break down butachlor. Targeting the 16S rRNA gene, PCR amplification was performed using the Taq Mix (2x) (Master Mx) and universal primers 27F (forward) and 1426R (reverse).

The following parameters were used to set up each PCR reaction separately for the sample: Five minutes of initial denaturation at 95 °C, 30 seconds of denaturation at 95 °C, 30 seconds of annealing at 58 °C, and 30 seconds of extension at 72 °C. Completed the last extension at 72 °C for five minutes after repeating steps 2-4 for thirty to thirty-five cycles [19,22].

RESULTS

The initial butachlor concentration in the soil sample, according to **Table** 1, was 4.743 ug/mL, which was calculated using the butachlor concentration standard curve.

Butachlor-biodegradation

The bacterial isolates GKSS1 and GKSS2 were chosen for secondary screening due to their high butachlor-degrading ability (75% and 56%) after primary screening of three (3) isolates on 50 mL of mineral salt medium supplemented with butachlor (50 mgL^{-1}), supplemented with MSM (**Table 2**). Following secondary screening, the most effective isolate, GKSS1, was selected for additional research because of its excellent utilization as a sole source of carbon and energy in MSM and its tolerance to up to 100 mgL-1 of butachlor. After five days of incubation, 90% of the butachlor was degraded by the strain, leaving 10% butachlor remaining in 0.1 mL at the minimum cell density (OD600 = 1.00) under ideal conditions (30 $^{°C,\ pH}$ 7.0) with 120-150 rpm on a rotary shaker.

Molecular Identification of the Most Potent Isolate

The query sequence was 97.59% similar to the *Pseudomonas aeruginosa strain B2* strains in the database, according to the molecular test results of the most powerful butachlor-degrading organism isolated from agricultural lands (**Table 3**). The gel electrophoresis findings for the PCR products derived from the 16S rRNA gene amplification are shown in **Fig. 1**. The ladder and the bacterial isolate's amplified product are shown in lanes 1 and 4. The existence of a DNA band in lane 4 confirms the success of the PCR amplification.

Table 1. Initial concentration of the pesticide from the sol sample by HPLC.

RetTime (Min)	Type	Area (mAU·s)	Amt/Area	Amount (µg/mL)	Grp	Name
4.332		20.49497	0.231449	4.74354		Butachlor

Table 2. Degradation Capacity of the Screened Butachlor Degrading Bacteria.

Type of Bacterial Screening	Butachlor Degradation (%)		
	GKSS1	GKSS2	GKSS3
Primary screening	75	56	48
Secondary screening	90	68	-

Keys: (-) Not selected for secondary screening.

Table 3. Summary of 16S rRNA sequencing results of the isolate to identify the closest homologs.

Sample ID	Sequence (bp)	Closest Homolog	% Identity	Accession No.
GKSS1	1245	*Pseudomonas aeruginosa* strain B2	97.59%	JQ900536.1

DISCUSSION

Gram's Bacterial Isolate Reaction

The nutrition agar plate's colony appeared milky and mucoid under a microscope. The rod-shaped bacterial cell was Gram-negative. Gram-negative *Pseudomonas sp.* bacteria have been found to be able to eliminate butachlor [22].

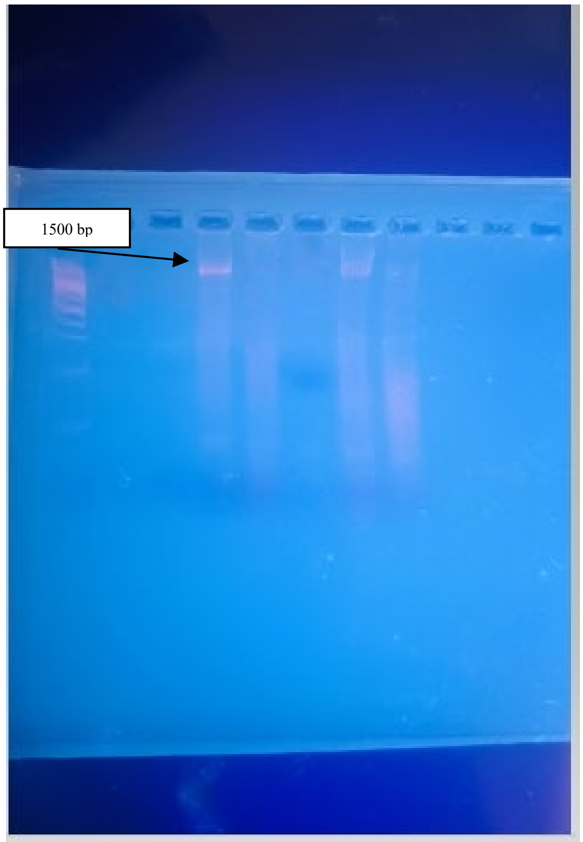

Fig. 1. Gel Electrophoresis of the amplified 16S rRNA genes.

The Initial concentration of the butachlor in the soil sample

The WHO has set a limit of quantification (LOQ) of 0.001 to 0.015 mg/kg for butachlor in soil samples [21]. This range indicates that the amount of butachlor in the agricultural soil sample (0.005 mg/kg) can be trusted for additional research on the pesticides' (butachlor) capacity to be biodegraded by isolated bacteria from the same agricultural soil sample. This is important for determining the risks that pesticide residues in agricultural areas pose to humans and the environment.

Pesticide biodegradation (Butachlor)

The development of a bacterial strain in 100 mg/L and the breakdown of butachlor were examined concurrently. However, there was no butachlor bio-removal in the control sample. This study demonstrates that degradation is caused by biotic activity and that high butachlor elimination takes place within a certain time frame.

https://doi.org/10.54987/jemat.v13i1.1106

Occurrence of Butachlor-Degrading Bacteria

The main and secondary phases comprised the two stages of the bacterial screening process. This enables a more thorough selection of the optimal microorganisms for butachlor biodegradation. In the first stage, the three bacterial isolates that were shown to be able to biodegrade butachlor were the primary focus. The biodegradation capacity of isolates GKSS1 and GKSS2 is over 50% in **Table 1**, but that of isolate GKSS3 is less than 50%.

Even while they all rose at almost the same pace, certain isolates showed a higher ability for biodegradation than others because they were all above 40% degradation. Thus, isolates GKSS1 and GKSS2 were chosen for further research to identify the best bacterium for decomposing butachlor. Other researchers have identified novel isolates such as *Stenotrophomonas acidaminiphila* JS-1 that grew in M9 medium with butachlor, degrading it in 20 days. It followed first-order kinetics (k = 0.17 day^{-1}, t½ = 4 days).

The strain also produced 21 µg/ml of IAA in tryptophan-supplemented medium, with lower butachlor concentrations enhancing IAA production while higher levels inhibited it [25]. In another study, butachlor degradation was found to be faster in rhizosphere and inoculated rhizosphere soils compared to non-rhizosphere soils across all concentrations. Inoculated rhizosphere showed the highest degradation rate constants (up to 0.2355 day^{-1}) and shortest half-lives (as low as 2.9 days), highlighting the efficiency of bacterial community HD in enhancing butachlor breakdown [26]. Furthermore, a soil isolate called isolate A, a molybdenum-reducing and aniline-degrading *Pseudomonas* sp. isolated from Nigerian soil, exhibited optimal growth at 30–37 °C and pH 6.0–6.5, tolerating up to 600 mg/L of butachlor. It showed no lag phase and reached peak growth within 24 hours. Its ability to utilize butachlor as a sole carbon source suggests strong bioremediation potential [27].

Screening

The butachlor biodegradation capacity of the two bacterial isolates utilized in the secondary screening phase was found to be adequate. As seen by the reduction in the biodegradation period from 4 to 3 days, resting cells are more active than the inoculum used in the initial screening stage. Given that their ability to withstand environmental stress during that phase may be greater than that of bacterial cells grown at the exponential phase of growth, the preparation of the resting cells, which were harvested at the early stationary phase after 48 hours of incubation, may serve as the rationale [8]. These cells are thought to have a higher biodegradation capability. Therefore, isolate GKSS1 was selected for additional examination since it showed a greater ability to degrade butachlor within the shortest time.

CONCLUSION

In summary, a native strain of *Pseudomonas aeruginosa* strain B2 that can break down butachlor was isolated from agricultural fields that had a documented history of using butachlor for more than ten years. After a rigorous screening process, one of the bacterial isolates was selected as the best for butachlor degradation at an ideal pH of 6.5, an incubation temperature of 37 °C, and a shaking speed of 120–150 rpm. This isolate demonstrated tolerance up to 0.6 mL of butachlor concentration with an inoculum size of 1 mL. As far as we are aware, this is the first study to use *Pseudomonas aeruginosa* strain B2 from the sample collection region to degrade butachlor. The only carbon source the isolates used for growth and energy requirements was butachlor. Given its environmentally benign and economical approach to bio-remediating the contaminant, further investigation is warranted for its full utilization in butachlor degradation at large-scale experiments.

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