

## Characterization of Butachlor Degradation by A Molybdenum-Reducing and Aniline-degrading *Pseudomonas* sp.

Sufyan, A.J.<sup>1</sup>, Ibrahim, S.<sup>1,2</sup>, Babandi, A.<sup>1</sup> and H.M. Yakasai<sup>1\*</sup>

<sup>1</sup>Department of Biochemistry, Faculty of Basic Medical Sciences, College of Health Science, Bayero University, Kano, P. M. B 3011, Nigeria.

<sup>2</sup>Centre for Biotechnology Research, Bayero University Kano P. M. B 3011, Nigeria.

\*Corresponding author:

Tel: +2348034966925

Email: [hmyakasai.bch@buk.edu.ng](mailto:hmyakasai.bch@buk.edu.ng)

### HISTORY

Received: 15<sup>th</sup> Oct 2021  
Received in revised form: 24<sup>th</sup> Nov 2021  
Accepted: 6<sup>th</sup> Dec 2021

### KEYWORDS

Bioremediation  
Butachlor-degrading  
Aniline-degrading  
Molybdenum-reducing  
*Pseudomonas* sp.

### ABSTRACT

Butachlor is a chloroacetanilide herbicide that is selective in action and commonly used for pre-emergence control of weeds. It is believed to have range of toxicity from acute to chronic and also can cause DNA strand breaks and chromosomal aberrations in humans. This study was aimed at characterizing the potential of previously isolated bacteria for butachlor degradation. The isolates from culture collection, labelled A-F were screened for butachlor degradation on Bushnell Hass agar media with butachlor as a sole carbon source. Isolate A (molybdenum-reducing and aniline-degrading *Pseudomonas* sp.) was observed to grow best and tolerated the highest concentration of butachlor supplemented in the media after 72 h of incubation at 37 °C. Characterization study revealed that the *Pseudomonas* sp. can utilize and grow with butachlor at optimum pH between 6.0 - 6.5, temperature between 30 – 37 °C and can tolerate up to 600 mg/L butachlor concentration with increase in growth as inoculum size increases. Additionally, this bacterial strain shows no lag phase regardless of the concentration of the herbicide used and reach its maximum growth after 24 h of incubation. The ability of this isolate to tolerate and utilize butachlor as sole carbon source makes it suitable for future bioremediation of this toxicant.

### INTRODUCTION

Herbicides are chemicals predominantly used in agricultural systems to control weeds. Although there exist other substances that can be used to achieve same result, herbicides are made solely for this purpose [1]. Butachlor (N'-(butoxymethyl)-2-chloro-2',6'-diethyl acetanilide) belongs to the chloroacetanilide compound of carbamate group which is selective in action and is widely used by farmers [2]. It is used in the pre-emergent control of weeds [3]. Butachlor can cause DNA strand breaks and chromosomal aberrations [4], it is cytotoxic and can cause apoptosis in humans [5], and can also cause dissipation of mitochondrial membrane potential [6]. It was reported to be toxic to certain aquatic lives [7]. In soil, butachlor is toxic to earthworms [8,9], while also inhibiting production and oxidation of methane in flooded tropical soils [10].

Looking at the vast number of threats that butachlor poses to the environment, finding a way to tackle these become necessary. This led to the exploration of various processes that can be applied to safely control this pollution in an eco-friendly manner. Nevertheless, biochemical and physical factors in the environment does affect the fate of these organic pollutants [11].

Bioremediation as a technique is a biological mechanism of recycling waste into form that can be used by microorganisms

[12]. However, microorganisms dwelling in these polluted environment are the key to the method of bioremediation/biodegradation [13]. Similarly, suitable physical factors like pH and temperature enhances their degrading ability [14]. In comparison to other methods of remediation, the low-cost feature of bioremediation made it the most adopted and suitable method for remediation. So far, bioremediation has been defined differently by different scholars, nevertheless, all the definitions emphasized on one process, degradation [15].

Perhaps, characterization works on butachlor-degrading isolates are grossly inadequate considering the utilization and toxicity caused by this herbicide. Thus, necessitate the need to isolate and characterize more microbes with potential to degrade this environmental contaminant. This work will therefore, screened previously isolated bacteria for their potential to degrade and utilized butachlor as sole carbon source.

## MATERIALS AND METHODS

### Chemicals and equipment

Butachlor of high purity was procured from distributors in Sabon Gari market in Kano. Bushnell Hass media (g/L) containing CaCl<sub>2</sub>, 0.02; FeCl<sub>3</sub>, 0.05; MgSO<sub>4</sub>, 0.2; KH<sub>2</sub>PO<sub>4</sub>, 1.0; K<sub>2</sub>HPO<sub>4</sub>, 1.0; NH<sub>4</sub>NO<sub>3</sub>, 1.0 g/L was prepared in distilled water. Nutrient broth was used for growth of bacterial culture. All the chemicals used in this study were of analytical grade.

### Media and Media Preparation

#### Nutrient Agar

In a 250 ml conical flask, 3.4 g of nutrient agar was added to 120 ml of distilled water and autoclaved at 121 °C for 45 minutes. The medium was used to determine the viability of isolates.

#### Mineral salt medium

Media broth was prepared by adding (g/L); KH<sub>2</sub>PO<sub>4</sub>, K<sub>2</sub>HPO<sub>4</sub>, MgSO<sub>4</sub>, CaCl<sub>2</sub>, FeCl<sub>3</sub>, NH<sub>4</sub>NO<sub>3</sub> to 400 ml of distilled water in a 1000 ml beaker. Measured volume of the medium was then taken and transferred each into conical flasks, the flask containing the medium were then autoclaved at 121 °C for 45 minutes. 100 mg/L (unless otherwise stated) concentration of butachlor was used for degradation study. Agar (20 g/L) was added to solidify the media. 120 ml of solid medium was used in screening the six (6) isolates for butachlor degradation.

#### Screening for Butachlor-Degrading Isolates

Screening of isolates was carried out in petri dish. In each plate, 20 ml of prepared solid media was added under a lamina flow and allowed to solidify. To all the petri dish, bacterial isolates labelled (A-F) were inoculated and incubated for 72 h. Screening for maximum growth was done macroscopically.

#### Characterization of Butachlor-Degradation

Optimum condition for butachlor-degradation was determined using a method from [16] with some modifications. Liquid mineral salt media was used at a temperature of 37 °C, butachlor concentration of 100 mg/L and inoculum size of 100 µL unless otherwise mentioned. Each of the test was conducted in triplicates.

#### Effect of Incubation Time

The effect of incubation time was tested by inoculating *Pseudomonas* sp. into the liquid media in a 250 ml flask and incubated for 120 h. Optical density was measured at 24 h intervals using a spectrophotometer at a wavelength of 600 nm to observe the growth rate of the isolate. Control bottles were prepared without the inoculum and kept under the same condition as the inoculated test bottles.

#### Effect of Butachlor Concentration

The effect of butachlor concentration in the media was studied by adding *Pseudomonas* sp. inoculum of 100 µL volume into the liquid media containing different butachlor concentrations (50, 100, 200, 400, 600 and 800 mg/L) were used, tests were conducted in triplicates. Optical densities were measured at 24 h intervals using a spectrophotometer at a wavelength of 600 nm to observe the growth rate of the isolate. Control bottles were prepared without the inoculum and kept under the same condition as the inoculated test bottles.

#### Effect of pH

The effect of pH in the media was tested by putting *Pseudomonas* sp. inoculum of 100 µL volume into the liquid media of varying pH (5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) in a 250 ml bottles each and

incubated. The pH was adjusted using NaOH and HCl prior to the sterilization. Optical densities were measured at 24 h intervals using a spectrophotometer at a wavelength of 600 nm to observe the growth rate of the isolate. Control bottles were prepared without the inoculum and kept under the same condition as the inoculated test bottles.

#### Effect of Inoculum Size

The effect of inoculum size was tested by inoculating different volumes (50, 100, 200, 400, 600, 800 and 1000 µL) of *Pseudomonas* sp. into the liquid media in 250 ml bottles and incubated for 120 h. Optical densities were measured at 24 h intervals using a spectrophotometer at a wavelength of 600 nm to observe the growth rate of the isolate. Control bottles were prepared without the inoculum and kept under the same condition as the inoculated test bottles.

#### Effect of Temperature

The effect of temperature was tested by inoculating *Pseudomonas* sp. into 100 ml of the liquid media in a 250 ml bottles and incubated for 120 h at different temperatures (25, 30, 37 and 40 °C). Optical density was measured at 24 h intervals using a spectrophotometer at a wavelength of 600 nm to observe the growth rate of the isolate. Control bottles were prepared without the inoculum and kept under the same condition as the inoculated test flasks.

## RESULTS AND DISCUSSION

### Screening for Butachlor-Biodegrading Isolates

A total of six (6) previously isolated molybdenum-reducing bacteria obtained from Agricultural soils in Kano state were screened for their potential to degrade butachlor and utilize it as sole source of carbon. Out of which isolate A (*Pseudomonas* sp.) was observed to tolerate and grow best on Bushnell Hass media containing butachlor as sole carbon source following 72 h of incubation at 37 °C, thus was chosen for further analysis.

### Characterization of Butachlor-Degradation by *Pseudomonas* sp.

#### Effect of Incubation Time

The result of effect of incubation time shows that *Pseudomonas* sp. grows exponentially from 0 to 24 h attaining optimum at 24 h, after which a significant ( $p < 0.05$ ) was observed (Fig. 1).

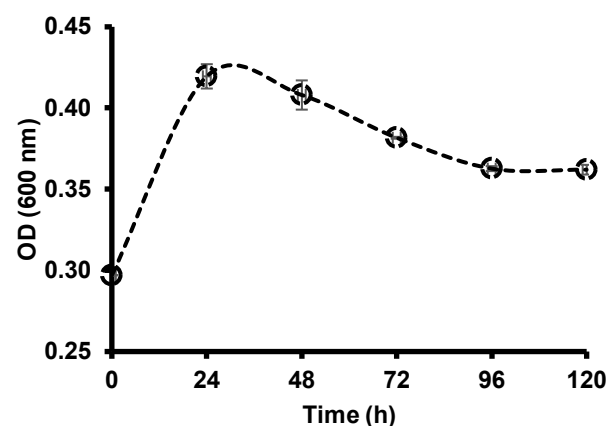


Fig. 1. Effect of incubation time on butachlor degradation by *Pseudomonas* sp. Data represent mean  $\pm$  standard deviation of the triplicates

### Effect of concentration

The effect of various concentrations of butachlor on its degradation was assessed between 50 – 800 mg/L. It was found that a concentration of 600 mg/L to be optimum for *Pseudomonas* sp. with a sharp decline in growth on increasing concentration to 800 mg/L (Fig. 2).

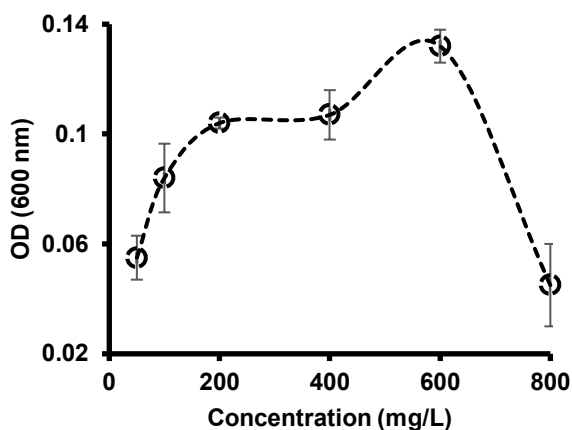


Fig. 2: Effect of various concentrations of butachlor (carbon source) on its degradation by *Pseudomonas* sp. after 24 h incubation at 37 °C. Data represent mean  $\pm$  standard deviation of triplicate measurements.

### Effect of inoculum size

An inoculum volume between 100 - 1000  $\mu$ L was used to determine the effect of inoculum size. It was found that the growth of this bacteria increases almost linearly with insignificant ( $p>0.05$ ) variation between 200 to 400  $\mu$ l (Fig. 3).

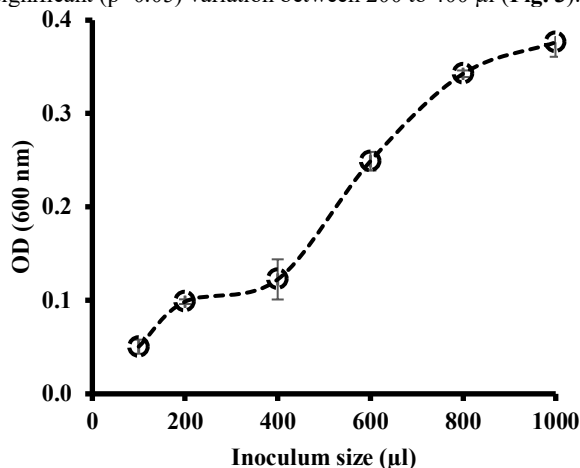


Fig. 3. Effect of inoculum sizes on butachlor-degradation by *Pseudomonas* sp. after 24 h incubation at 37 °C. Data represent mean  $\pm$  standard deviation of triplicate measurements.

### Effect of initial pH

The effect of pH on butachlor-degradation by *Pseudomonas* sp. was determined over a pH range of 5.5 - 8.0. The result obtained showed that the growth was optimum at pH between 6.0 and 6.5 with the latter having slightly higher support which was insignificant ( $p>0.05$ ). Although, the growth decreases as the pH increased from 6.5 to 8.0 over 24 h incubation, there was an insignificant ( $p>0.05$ ) difference in growth at pH between 7.0 and 7.5 (Fig. 4).

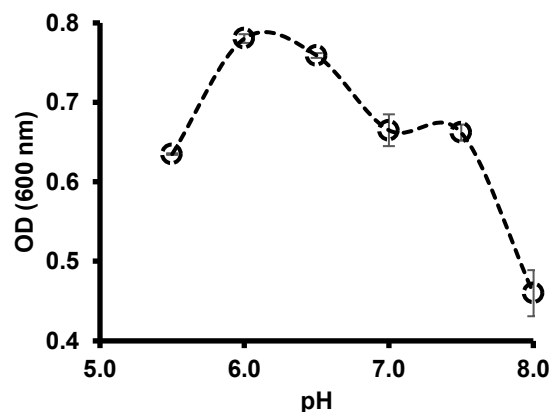


Fig. 4. Effect of various initial pH on butachlor-degradation by *Pseudomonas* sp. after 24 h incubation. Data represent mean  $\pm$  standard deviation of triplicate measurements.

### Effect of Temperature

The effect of temperature on the growth and butachlor-degrading potentials of *Pseudomonas* sp. was tested within a temperature range of 25 - 45 °C. The result obtained showed that the growth of this bacterium was optimum at temperature between 30 and 37 °C with insignificant difference ( $p>0.05$ ) between this temperature range. Additionally, growth of this bacterium was lowest at 25 and 45 °C after 24 h incubation (Fig. 5).

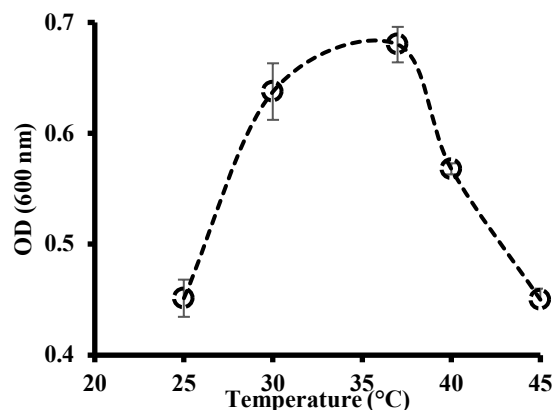


Fig. 5. Effect of temperatures on butachlor degradation by *Pseudomonas* sp. after 24 h incubation. Data represent mean  $\pm$  standard deviation of triplicate measurements.

## DISCUSSION

The present study showed that the bacteria quickly adapt in the media showing a very small lag phase (or none at all), growing exponentially for up to 24 h where the maximum growth was observed in all cases. Growth declined after 24 h due to exhaustion of carbon source (butachlor) and accumulation of toxic substances. Another study of *Pseudomonas* sp. potential to degrade butachlor showed that the bacteria can grow in liquid salt media containing the herbicide no matter the concentration of the chemical added to the media, hence considered effective for remediation of this chemical [17]. Additionally, in another study of butachlor degradation where *Mycobacterium* sp. J7A and *Sphingobium* sp. J7B isolated from rice paddy soil were used to biodegrade butachlor in a syntrophic process, they were capable of degrading this pollutant completely in 24 h [3].

Test set to determine the tolerance of this bacterium to the effect of the chemical showed a remarkable resistance capability. Although the bacterium is believed to be highly tolerant to the harmful effect of this herbicide, a concentration of 800 mg/L was found to be very toxic to the current microorganism and therefore inhibits its growth, the growth curve showed a steep fall signifying a steady decline phase. This finding goes somewhat in line with the findings of another study that used *Pseudomonas putida* G3 to biodegrade butachlor, the bacteria was found to survive up to 1000 mg/L concentration [18] thus more tolerant than the present bacterium. The high tolerance exerted by this strain is probably due to its ability to utilize the chemical more effectively and have better enzymes that can easily degrade it into simpler metabolites that can be consumed compared to the strain used in this study.

Another experiment set to determine the optimum inoculum size revealed that after 24 h of incubation, the organism showed the highest growth with 1000 µL of the inoculum compared to all to any other tested volume, this can be explained looking at the fact that the bacterium has no lag phase as none is observed in any case, adaptation was therefore fast and so was achieving exponential phase. The nature of the growth curve also showed that the growth rate of the bacterium increases with increasing volume of inoculum after 24 h incubation period. Similar findings were reported by [18,19] that highest growth and degradation of butachlor is achieved at high inoculum volume.

In the present study, pH of 5.5 - 8.0 range was also tested. pH being an important physical parameter affecting the growth of microorganisms in any environment by influencing its metabolic processes while also affecting the transport of molecules in and out of the cell, almost any microbe has a certain range of pH in which it grows and conduct its activities best. This study showed that the isolate grows best at a pH range between 6.0 - 6.5 when exposed to this pollutant signifying the strong possibility of the strain being acidophile in nature. Other studies conducted using different other microorganisms showed different optimal pH range, of which *Pseudomonas alcaligenes*, *Bradyrhizobium* sp. *Bacillus megaterium*, with pH 5.0 as optimal, a pH that is more acidic than the optimum of the strain used in this study and also *Catellibacterium caeni* sp. nov DCA-1T with optimal pH between 6.0 – 9.0 [20,21].

Finally, test aimed at determining the optimum temperature that supports the best growth of this bacteria was also carried out within a range of 25 - 45 °C, of the various set temperature values, the optimum temperature for this *Pseudomonas* sp. to degrade butachlor was found to be between 30 - 37 °C after 24 h incubation. This correspond to the findings of [16] in a study aim at using *Bacillus altitudinis* to biodegrade butachlor, the optimum temperature was also found to be 37 °C. An optimum temperature of 35 °C was also reported in the butachlor biodegradation of three bacteria (*Pseudomonas alcaligenes*, *Bradyrhizobium* sp. *Bacillus megaterium*) [20], a value that lies within the optimum range of this study. Since temperature has a great impact on the metabolic processes occurring in the organism as it affects the rate of enzyme activity and deviation from the optimum temperature may lead to the loss of activity of enzymes as a result of denaturation or inactivation, this temperature is therefore necessary to insure the best tolerance and activity of the bacterium for effective bioremediation.

## CONCLUSION

The *Pseudomonas* sp. used in this study was found to best degrade butachlor at pH between 6.0 and 6.5, temperature between 30 - 37 °C and can tolerate the pollutant up to 600 mg/L concentration with continuous increase in growth as inoculum size increases. Additionally, this study also revealed that the bacterium has no lag phase regardless of the concentration of the butachlor used. Furthermore, 24 h of incubation was found to be the time period over which the bacterium achieves its maximum growth.

## REFERENCES

1. Fishel F, Ferrell J, Macdonald G, Sellers B. Herbicides: How Toxic Are They? 2016;1–2.
2. Jaiswal A, Kumar A, Mishra S. Detection of Butachlor in Viscera by Thin Layer Chromatography. J Indian Soc Toxicol. 2011;7(1):38–40.
3. Kim NH, Kim DU, Kim I, Ka JO. Syntrophic biodegradation of butachlor by Mycobacterium sp. J7A and *Shingobium* sp. J7B isolated from rice paddy soil. FEMS Microbiol Lett. 2013;344(2):114–20.
4. Abigail MEA, Samuel SM, Ramalingam C. Addressing the environmental impacts of butachlor and the available remediation strategies: a systematic review. Int J Environ Sci Technol. 2015;12(12):4025–36.
5. Panneerselvam N, Sinha S, Shanmugam G. Butachlor is cytotoxic and clastogenic and induces apoptosis in mammalian cells. Indian J Exp Biol. 1999;37(9):888–92.
6. Dwivedi S, Saquib Q, Al-Khedhairi AA, Musarrat J. Butachlor induced dissipation of mitochondrial membrane potential, oxidative DNA damage and necrosis in human peripheral blood mononuclear cells. Toxicology. 2012 Dec 8;302(1):77–87.
7. Tilak KS, Veeraiah K, Thathaji PB, Butchiram MS. Toxicity studies of butachlor to the freshwater fish *Channa punctata* (Bloch). J Environ Biol. 2007; 28(2 SUPPL.):485–7.
8. Gobi M, Gunasekaran P. Effect of Butachlor Herbicide on Earthworm *Eisenia fetida* —Its Histological Perspicuity. Appl Environ Soil Sci. 2010; 2010: 1–4.
9. Muthukaruppan G, Janardhanan S, Vijayalakshmi G. Sublethal Toxicity of the Herbicide Butachlor on the Earthworm *Perionyx sansibaricus* and its Histological Changes (5 pp). J Soils Sediments [Internet]. 2004;5(2):82–6. Available from: <https://doi.org/10.1065/jss2004.09.111>
10. Mohanty SR, Nayak DR, Babu YJ, Adhya TK. Butachlor inhibits production and oxidation of methane in tropical rice soils under flooded condition. Microbiol Res. 2004;159(3):193–201.
11. Boyd SA, Shelton DR, Berry D, Tiedje JM. Anaerobic Biodegradation of Phenolic Compounds in Digested Sludged. 1983;46(1):50–4.
12. Abatenh E, Gizaw B, Tsegaya Z, Wassie M. Application of microorganisms in bioremediation-review. J Environ Microbiol. 2017;1(1):2–9.
13. Verma JP, Jaiswal DK. Book Review: Advances in Biodegradation and Bioremediation of Industrial Waste. Front Microbiol. 2016;6(January):2.
14. Khan SA, Hamayun M, Khan AL, Ahmad B, Lee I. Influence of pH, temperature and glucose on biodegradation of 4-aminophenol by a novel bacterial. 2009; 8(16): 3827–31.
15. Azubuike CC, Chikere CB, Okpokwasili GC. Bioremediation techniques—classification based on site of application: principles, advantages, limitations and prospects. World J Microbiol Biotechnol. 2016;32(11).
16. Kaur R, Goyal D. Biodegradation of Butachlor by *Bacillus altitudinis* and Identification of Metabolites. Curr Microbiol [Internet]. 2020;(0123456789). Available from: <https://doi.org/10.1007/s00284-020-02031-1>
17. Duc HD, Thi N, Thuy D, Thi H, Truc T, Nhu TH, et al. Degradation of butachlor and propanil by *Pseudomonas* sp. strain But2 and *Acinetobacter baumannii* strain DT. 2020;(June):1–8.

18. Mohanty SS, Jena HM. Degradation kinetics and mechanistic study on herbicide bioremediation using hyper butachlor-tolerant *Pseudomonas putida* G3. *Process Saf Environ Prot* [Internet]. 2019; 125: 172–81. Available from: <https://doi.org/10.1016/j.psep.2019.03.014>
19. Jena HM, Mohanty SS. Evaluation of butachlor biodegradation efficacy of *Serratia ureilytica* strain AS1: a statistical optimization approach. *Int J Environ Sci Technol* [Internet]. 2018;(0123456789). Available from: <https://doi.org/10.1007/s13762-018-1958-6>
20. Sherif HAA, Mounir MSB. Microbial biodegradation of butachlor pollution (obsolete pesticide Machete 60% EC). *African J Microbiol Res*. 2013;7(4):330–5.
21. Zheng J, Li R, Zhu J, Zhang J, He J, Li S, et al. Degradation of the chloroacetamide herbicide butachlor by *Catellibacterium caeni* sp. nov DCA-1T. *Int Biodeterior Biodegradation* [Internet]. 2012 Sep 1 [cited 2020 Mar 8]; 73: 16–22. Available from: <https://www.sciencedirect.com/science/article/pii/S0964830512001369>.