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*Full Length Research Paper*

# Microbial biodegradation of butachlor pollution (obsolete pesticide Machete 60% EC)

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**Accumulation of obsolete pesticides in the environment is a global problem. The scale of the problem in different countries varies depending on economic situation and social awareness. Only limited data are available on the microbial biodegradation of butachlor. Biodegradation of butachlor by different microorganisms was investigated. Six bacterial strains were isolated from an agricultural soil and found to be actively utilized butachlor, as a sole source of carbon and energy. Based on their morphological and biochemical categorization, the six bacterial and fungal isolates were identified as *Psedomonas alcaligens*, *Bacillus licheniformis*, *Bacillus megaterium*, *Trichoderma viride*, *Rhizobium huakuii* and *Bradyrhizobium japonicum*. Results show that the *Trichoderma viride* and *Psedomonas alcaligens* quickly degraded butachlor and reached nearly 98 and 75% in a medium containing 50 mg/kg of butachlor after 15 and 21 days, respectively. Our results can conclude that these two organisms can be used to degrade the obsolete butachlor formulation.**

**Key words:** Obsolete pesticides, herbicide, butachlor, biodegradation.

## INTRODUCTION

Herbicides are being increasingly used under intensive cultivations to control unwanted weeds, to minimize the cultivation cost as well as to sustain high yield. Butachlor (N-butoxymethyl-2-chloro- 2,6-diethyl acetanilide) is one of the most widely recommended herbicides. It is a pre-emergence herbicide belonging to chloroacetanilide group used widely in oriental countries for the control of annual grasses for rice cultivation (Jena, 1987; Yu et al., 2003). The recent studies, reported that the application of butachlor has adverse environmental impact; butachlor was found to flow out with effluents, causing contamination of river and ground water (Natarajan et al., 1993; Ohyama et al., 1986; Yamagishi and Akiyama, 1981), it has toxicity to aquatic organisms (Ateeq et al., 2002; Ateeq et al., 2006; Lin, 1997), and it has genotoxicity to the amphibian animals (Geng et al., 2005). Also, it could induce apoptosis in mammalian cells (Panneerselvam et al., 1999). Applying butachlor in soil can cause toxicity to earthworm (Muthukaruppan, 2005; Panda and Sahu, 2004), change the microbial populations and enzyme activities (Kole, 1989; Min et al., 2001), and sometimes adversely affects the growth and activities of beneficial microorganisms in soils (Kole, 1989). However, the data on butachlor biodegradation, is limited, and no systematic study to the best of our knowledge is reported in literature on butachlor degradation in soil by bacterial cultures. An earlier study reported enhanced butachlor degradation in wheat rhizosphere soil inoculated with the bacterial community HD (Yu et al., 2003). Other study by Madhaiyan (2006) was conducted to isolate and characterise butachlor-catabolizing bacteria and to assess their degradation kinetics and other auxiliary activities in soil. However, it has been reported that butachlor at higher concentrations exerts inhibitory effect on indole-3-acetic acid (IAA) synthesis. This result was supported by Dwivedi et al. (2010). They reported that

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the ability to degrade butachlor revealed the strain JS-1 as an effective bio-resource for application in bioremediation at the contaminated sites. Nowadays, with the progress of molecular biotechnology, evaluating the impact of pesticides application on ecological environment is more thorough and accurate using amplified (DNA) fragment length polymorphism (AFLP), amplified ribosomal DNA restriction analysis (ARDRA) (Vandamme et al., 1996; Vaneechoutte, 1996), and denaturing gradient gel electrophoresis (DGGE) (Muyzer, 1999; Muyzer et al., 1993).

Obsolete pesticides can shortly be defined as stocked pesticides that can no longer be used for their original purpose or any other purposes and therefore require disposal. Such pesticides can no longer be used because their use has been banned, because they have been deteriorated, or because they are not suitable for the use originally intended and cannot be used for another purpose, nor can they easily be modified to become usable (FAO, 1995). Obsolete pesticides, as well as their preceding utilitarian versions, consist of active substances, that is, chemical compounds or substances which, while penetrating natural environment (waters, soil, food chain), modify and harm it, often becoming an ecological time bomb (Egenhofer, 2009). Spreading obsolete pesticides in the environment is a global problem. The scale of the problem varies depending on economic situation and social awareness. Many countries enacted strictly laws to pesticide trade and its waste management, . the The efforts to resolve the pesticide problem legally and effectively seem to bring first results (Egenhofer, 2009). There are yet some places in developing countries where agricultural crops are considerably contaminated with pesticide waste as Ethiopia, Tanzania, Botswana, Mali, and Madagascar (Aqiel Dalvie et al., 2006; Martínez, 2004; Naidoo 2003).If microorganisms are used to biodegrade sustained, toxic pollutants such as persistent organic pollutants (POPs) to live organisms (Alexander, 1999; Kulkarni, 2006;Nishino and Spain, 1993; Oh et al., 2003), it is also possible to exploit them to neutralize organic obsolete pesticides. Nowadays, the use of microorganisms to biodegrade this kind of waste is almost imperceptible, as the most popular way of managing with them is thermal utilization (Martínez, 2004). Bacteria and fungi show the biggest capability of degrading pesticides. Bacteria, actinomycetales (special group of bacteria), and fungi show the biggest capability of degrading pesticides. The species of bacteria and soil fungi distinguishing the strongest activity in degrading pesticides are: bacteria of the genera arthobacter, bacillus, corynebacterium, flavobacterium, and pseudomonas; actinomycetales of the genera nocardia and streptomyces; and fungi penicillium, aspergillus, fusarium, and trichoderma (Feakin, 1995; Kucharski, 2009; Omar, 2001; Rousseaux et al., 2001). Kolić (2007), Topp (2001), and Vibber et al. (2007) pointed out in their research, the biggest biodegradation potential concerning bacteria *Pseudomonas* sp. and *Nocardioides* sp.

The aims of this study were the determination of the ability of six different microorganisms isolated from soil to transform obsolete butachlor formulation (Machete 60% EC), to investigate biodegradation rate of Machete 60% EC, and to provide basic information for developing regulations regarding the safe disposal of butachlor to protect the environment and public health.

## MATERIALS AND METHODS

The 127 soil samples were collected from the surface 10 cm layer at the agricultural ground of Alkharj, Saudi Arabia. 25 g were collected for every soil sample in plastic bags and transported at once in cold storage containers to the laboratory for further investigation. The mass soil was a little air-dried, thoroughly mixed, and then sieved through 0.20 mm mesh sieve for incubation experiment and chemical analysis, respectively.

### Search for biotopes consisting of butachlor-degrading microorganisms

Bacteria capable of degrading butachlor were isolated from soil samples by the enrichment culture technique. The soil homogenate was inoculated in 50-ml nutrient broth (Sigma, USA) and Sabouraud dextrose broth (Difco, USA). All purified microorganisms were tested for their abilities to grow in the presence of pesticides using butachlor in nutrient agar medium: Beef extract, 3.0 g, peptone, 5.0 g; agar-agar 20.0 g and distilled water to make 1 L ($H_2O$) 1.0 L at pH 7.2 (Jacobs, 1960) then incubated for at 37°C for 24 h. For fungal isolates, Sabouraud dextrose agar (Difco, USA) with butachlor were used and incubated for 5-7 days at 25 °C. The resulting colonies were repeatedly subculture in M9 medium containing 10 ppm butachlor to confirm their butachlor-catabolising ability. Inoculated plates were incubated at 37 °C for 24h and 25 °C for 7 days for bacterial and fungal growth, respectively. The growth of microorganisms used for standing the toxicity of pesticide was determined and recorded as growth or inhibition. Identification and characterization of the isolates were carried out on the basis of the colony morphology, biochemical characteristics following Bergey's manual of systematic bacteriology (Bergey's 1984) and polymerase chain reaction used as a supporting tool in the identification of the bacterial isolates (Singh, 2009).

Microorganisms, which were able to utilize butachlor as a sole carbon and/or nitrogen source, were tested for their abilities to degrade these pesticides. Tested microorganism was incubated into liquid aqueous (basal medium): $KH_2PO_4$, 1.0 g; $K_2HPO_4$ 1.0 g; $NH_4NO_3$ 1.0, g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2$, 0.02 g, $Fe(SO_4)_3$ 0.01gm and distilled water to make 1 L ($H_2O$)1.0 L at pH 7. 0 (Miles, 1996), with pesticide used for 21 day at (30 ±2°C). Samples were taken from treatments and control at intervals of 0, 1, 3, 6, 9, 12 and 15 days for determination. Each culture was filtered through whatman No.1 filter paper. Pesticide residues were extracted from filtrate using the method adopted by Letizia (1992).

Analyses of butachlor pesticide were performed using its formulation Machete 60 EC containing Butachlor in quantity of 600 g $L^{-1}$ of pesticide (it was confirmed with quantity examination with pure 98% butachlor analytical standard). The material was taken from the stock of pesticides of Plant Breeding Station, Faculty of Agriculture, King Saud University. Herbicide in the form of aqueous emulation was put into sterile medium. The sterility of herbicide-contaminated medium was checked before each test. Recovery

**Table 1.** Pesticide-tolerance of microorganisms.

| Microorganism | Growth inhibition by Butachlor |
|---|---|
| *Psedomonas alcaligens* | - |
| *Bacillus licheniformis* | + |
| *Bacillus megatherium* | - |
| *Trichoderma viride* | - |
| *Rhizobium huakuii* | + |
| *Bradyrhizobium japonicum* | - |

+ = growth was inhibited; - = no inhibition.

studies were carried out by spiking there replicates of control samples (untreated medium) with 10, 50, and 100 $\mu gkg^{-1}$ of butachlor. Samples were analyzed as previously described and mean values of three replicates were calculated. Recovery percent was 96.5% (92 to 101). The detection limit and limit of quantification of butachlor were 0.2 and 1.0 $\mu gkg^{-1}$, respectively. Stock solution of butachlor was prepared by dissolving 5 mg dissolved in 50 mL n-hexane to obtain concentration 0.1 mg $mL^{-1}$. A working standard solution of 1 µg $mL^{-1}$ was prepared by appropriately diluting the stock solution with n-hexane. Stock solution was stored at -20 ± 2°C, and working standard solutions were stored in the dark ≤s 4°C when not in use. Butachlor residue analysis was performed with Agilent technologies Agilent 7890 gas chromatography with $^{63}Ni$ electron capture detector (GC-ECD), into a J&W DB-1701 capillary column 30m x 0.35 mm x 0.25 µm film. Data analysis was performed using Chemistation software.

## RESULTS

The experiment scheme was to determine which of the studied microorganisms, *Psedomonas alcaligens*, *Bacillus licheniformis*, *Bacillus megatherium*, *Trichoderma viride*, *Rhizobium huakuii*. and Bradyr*hizobium* sp. is butachlor resistant, and then the materials consisting of such microorganisms were analyzed on microorganism's biodegrading butachlor content to determine if bacteria and fungi strains are responsible for biodegradation. This is the first study that examined the use of microorganisms in the biodegradation of obsolete herbicide butachlor. Results in Table 1 show the inhibition of different microorganisms growth by butachlor. These results reflect that there are different resistant levels in the tested microorganisms to Butachlor. Two strains of bacteria had growth inhibition *Bacillus licheniformis* and *Rhizobium huakuii*, while the other four strains *Psedomonas alcaligens, B. megatherium, T. viride* and *Bradyrhizobium sp.* showed a great growth without any inhibition.

This study report that different butachlor biodegradation level was obtained with *Psedomonas alcaligens*, *B. megatherium*, *T. viride*, and *Bradyrhizobium japonicum.* When butachlor was the only source of nitrogen and carbon, the reduction percent were 94.7, 51.5, 97.6 and 58.5 % after 21, 30, 15 and 30 days for *Psedomonas sp*., *B. megatherium*, *T. viride*, *Bradyrhizobium japonicum*, respectively. This result proved that *T. viride* and *Psedomonas alcaligens* can use butachlor as a source for both carbon and nitrogen (Table 2).

## DISCUSSION

During periodic cultivation, representative samples were taken approximately 1, 3, 5, 7, 9, 12, 15, 21 and 30 days. Results show that the strains, *T. viride* and Psedomonas *alcaligens* quickly degraded butachlor reached nearly 98 and 75%, respectively. Figure 1 shows the degradation rate of butachlor by all tested microorganisms.

Results obtained from this work can be compared to the results of many other works concerning pesticides biodegradation using microorganisms as well as with the results of works concerning mechanisms of active pesticides degradation. However, the authors reported that there is only very few data available in the research database regarding the use of biodegradation to utilize obsolete pesticides.

Butachlor residue analysis proved that the strain *T. viride* as a fungus of the *Trichoderma* genus was frequently recognized as good at butachlor herbicide biodegradation. Although the microbial degradation of butachlor is too weak but Trichoderma showed good ability to degrade butachlor which might be due to the mycelium of *Trichoderma* which can produce a wide variety of enzymes, including cellulases (degrading cellulose) and chitinases (degrading chitin), so it can grow directly on wood and can be a parasite of other fungi because the cell walls of fungi are primarily composed of chitin, a polymer of n-acetyl-glucosamine.

The mineralization rate of the herbicides depends on environmental factors, which was proved by Kodama (2001).Changing the substrate reaction and incubation temperature, he determined optimal parameters of simazine biodegradation for *Penicillium steckii* and *Moraxellaovis*. In the case of fungi, the best were: pH 7 to 8, 30°C, and traces of glucose and yeast extract insubstrate (over 50% subsidence of simazine in 5 days); in the case of bacteria, it was pH 5 and 35°C. Nearly 100% of simazine was degraded after 40 days of incubation of mixed bacteria culture (therein Arthrobacter

**Table 2.** Biodegradation rate and reduction percentage of butachlor (n=3).

| Time (days) | *Pseudomonas* sp. | | *Bacillus megaterium* | | *Trichoderma viride* | | *Bradyrhizobium* sp. | |
|---|---|---|---|---|---|---|---|---|
| | Concentration (µg/kg) | Reduction % | Concentration (µg/kg) | Reduction % | Concentration (µg/kg) | Reduction % | Concentration (µg/kg) | Reduction % |
| 0 | 100 ± 0.1 | - | 100 ± 0.12 | - | 100± 0.08 | - | 100± 0.11 | - |
| 3 | 84.4 ± 0.06 | 15.6 | 95.4± 0.08 | 4.6 | 80.1± 0.09 | 19.9 | 95.2± 0.09 | 4.8 |
| 5 | 72.1 ± 0.11 | 27.9 | 91.1± 0.05 | 8.9 | 64.2± 0.1 | 35.8 | 89.4± 0.07 | 10.6 |
| 7 | 66 ± 0.12 | 34 | 86.3± 0.13 | 13.7 | 53.6± 0.05 | 46.4 | 85.2± 0.11 | 14.8 |
| 9 | 53 ± 0.07 | 47 | 79.8± 0.2 | 20.2 | 35.8± 0.09 | 64.2 | 81.4± 0.09 | 18.6 |
| 12 | 39 ± 0.13 | 61 | 71.3± 0.07 | 28.7 | 20.4± 0.07 | 79.6 | 74.8± 0.09 | 25.2 |
| 15 | 25.4 ± 0.09 | 74.6 | 64.2± 0.11 | 35.8 | 2.4± 0.06 | 97.6 | 68.5± 0.08 | 31.5 |
| 21 | 5.3 ± 0.08 | 94.7 | 57.1± 0.11 | 42.9 | nd | | 57.3± 0.12 | 42.7 |
| 30 | nd | | 48.5± 0.13 | 51.5 | | | 41.5± 0.1 | 58.5 |

Values are Mean± SD; nd, not detected.

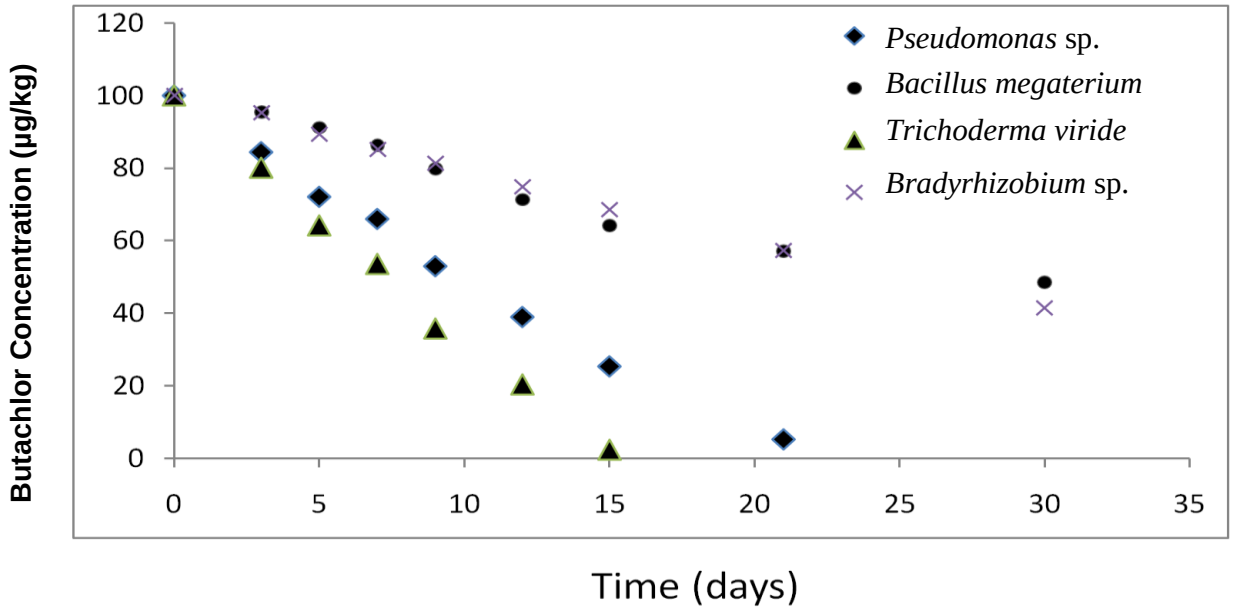


**Figure 1.** Butachlor degradation rate by different microorganisms strains.

sp.) in bioreactor. It was found that simazine herbicides were used by microorganisms both as a carbon and nitrogen source (Mondragon-Parada et al., 2008). All of these experiments together with our tests results as well as many others show the possibility of using different microorganism's populations to the process of contaminated systems biodegradation. In the present work, bacterial and fungal strains having fundamental impact on biodegradation of herbicides were added individually to the culture media. Butachlor residue analysis by GC-ECD gas chromatography showed essential herbicide residual behavior in the media with all the tested microorganisms which shows that there were significant differences in the degradation rate of butachlor with the tested microorganisms.

## Conclusions

Six different microorganisms' strains isolated from soil were tested to degrade butachlor. Results show that growth inhibition of the two microorganisms and four of these tested microorganisms had different degradation rate for butachlor. According to the data, *T. viride* had the highest degradation rate followed by *Psedomonas alcaligens*; 97.6 and 94.7% butachlor reduction in 15 and 21 days, respectively. These results show that both of these two organisms can be used to degrade the obsolete butachlor formulation. These results provide basic information for developing regulations regarding the safe disposal of butachlor using eco-friendly method. We suggest that further studies should use different microorganisms in the degradation of different obsolete pesticides, to protect the environment and public health.

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