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# Bioremediation of Trichlorpyr Butoxyethyl Ester (TBEE) in bioreactor using adapted *Pseudomonas aeruginosa* in scale up process technique

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**Abstract**
*Pseudomonas aeruginosa* has been adapted at varying concentrations of pesticide - Trichlorpyr Butoxy Ethyl Ester (TBEE) viz. 10,25 50, 75 and 100 mg/l in MSM using incubator shaker at 37 ºC and 150 rpm by scale up process technique. In the beginning 10 mg/l concentration of TBEE was supplied to microorganism in minimal salt medium (MSM) under controlled conditions for 14 days. The culture was subsequently scaled up to higher concentration of TBEE by transferring one milliliter MSM containing 10 mg/l to 25 mg/l concentration in MSM and incubated for continuous 14 days. The Pseudomonas aeruginosa (NCIM 2074) was adapted in increasing concentration of TBEE at 50 mg/l, 75 mg/l and 100 mg/l; after every 14 days using a total period of 70 days. During adaptation period, the *pseudomonas aeruginosa* degraded the TBEE completely at concentrations of 10 mg/l and 25 mg/l; while 49% and 23% degradation in 75 mg/l and 100 mg/l of TBEE was observed. Hence bioremediation studies has been carried out at concentrations 10, 25 50 mg/l of TBEE using flask shake method. GCMS data showed that TBEE degraded 100% within 3 days at 10 mg/l concentration and 5 days at 25 mg/l initial concentrations. While in case of 50 mg/l initial concentration of TBEE the compound was found persisting till 8 days period. The major intermediated, during the bioremediation of TBEE, were found to be 3,5,6-trichloro-2-pyridinol and 2,4,6-trichloro benzene amine. These intermediates are less toxic than parent compounds which on long term acclimatization would convert into environment friendly compounds.

**Keywords:** Bioremediation, TBEE, *Pseudomonas aeruginosa*.

## Introduction

In India, pesticides like TBEE is most commonly manufactured in industries and used in agriculture for preventing, controlling and mitigating the pest. The waste generated during the manufacturing and formulation processes/operations are treated by physico- chemical and biological methods. In spite of the present treatment technologies, the residual waste is still found persisting in the soil-water environment (Fulekar 2005a, b). The WHO data indicate that 2-3 % of applied pesticides are effectively used in mitigating the pest in agriculture environment while rest of the pesticide remains in the soil (World Health Organization 1990). The residual pesticide comes in contact with water causing surface / ground water pollution leading to toxicity to biotic environment. Therefore, bioremediation technique for treatment of pesticide is of paramount importance (Fulekar and Geetha. 2007).

In the present study, bioremediation potential of *Pseudomonas aeruginosa* has been assessed by increasing its adaptability to increasing concentration of pesticide such as TBEE using scale-up process technique (Fulekar 2008). Adapted *Pseudomonas aeruginosa* was used in bioremediation of TBEE in flask shaker method at 10 mg/l, 25 mg/l, 50 mg/l. pH and bacterial growth were observed during biodegradation of parent compound. The biodegradation of TBEE and its intermediates were studied using GC-MS. The intermediates were found less toxic than parent compound, which on long run acclimatization could convert into environmental friendly compounds under controlled environmental conditions. The biodegradation and conversion of parent compound into its less toxic intermediates were assessed during the study.

## Materials and Methods

*Microorganisms:* Pure culture of *Pseudomonas aeruginosa*-(NCIM 2074),

strain was procured for the bioremediation work from national Council of Industrial Meteorologists (NCIM), Pune, India. The culture was maintained on nutrient agar slants. Technical grade TBEE was adapted in Minimal Salt Medium using scale up process followed by bioremediation in Flask shaker method.

*Nutrient Culture Medium:* The nutrient culture medium was prepared to assess pesticide as a carbon source for microorganism in the enrichment study (Siddique et al. 2003). The medium FTW media (Herman and Frankerberger 1999) comprised of (in gm/l): $K_2HPO_4$- 0.255, $KH_2PO_4$-0.255, $(NH_4)_2SO_4$-0.255, $MgSO_4.7H_2O$-0.05, $CaCO_3$- 0.005 and $FeCl_2.4H_2O$-0.005 was blended with 1 ml of trace elements solution (Focht, 1994). The Focht trace element solution contained (in mg/l): $MgSO_4.H_2O$-169, $ZnSO_4.7H_2O$-288, $CuSO_4.5H_2O$-250, $NiSO_4.6H_2O$-26, $CoSO_4$-28 and $Na_2.MoO_4.2H_2O$-24.

*Pesticide spiking:* Erlenmeyer flasks (250 ml) and nutrient culture media were autoclaved for 20 minutes at 121ºC. A 500µl acetone containing pesticide at varying concentrations separately was aseptically added to autoclaved dried Erlenmeyer flasks allowing the acetone to evaporate (Siddique et al. 2003).After complete evaporation of acetone from the Erlenmeyer flasks, 100ml culture media was added under laminar flow hood so as to reach the desired pesticide concentration (Brinch 2002).

*Scale-up technique:* 1ml subcultured *pseudomonas aeruginosa* (nutrient broth) was inoculated into Erlenmeyer flasks (250ml) containing nutrient culture media with a TBEE concentration of 10mg/l. the inoculated flasks were kept in orbital shaker incubator at 160 rpm, 30 º C. for 14 days (Collins and Lyne 1985). After 14 days, 1 milliliter of this culture media was added with a TBEE concentration of 25mg/l, the flasks were again kept on orbital shaker incubator at 160 rpm, 30 ºC for another 14 days. Likewise, the microbial culture was subcultured into nutrient culture media with a pesticide concentration of 50mg/l and 75mg/l and was kept on orbital shaker incubator at 160 rpm, 30 ºC for increasing a total period of 70 days at the frequency of 14 days for interaction of microorganisms with the compound. (Goudar and Strevett 2000).At this stage, the bacteria were found adapted to TBEE and by assessing the pesticide as sole source of carbon for growth and maintenance.

**Results and Discussion**

Bioremediation of pesticide-TBEE was carried out by *Pseudomonas aeruginosa* (NCIM 2074) using a scale up process technique followed by shake flask bioreactor methods under controlled environmental conditions. In the present investigation, the strain *pseudomonas aeruginosa* has been enriched and adapted in mineral salt medium (MSM) containing TBEE as the sole source of carbon at varying concentrations viz 10,25, 50, 75 and 100 mg/l; respectively. The scale up process has been carried out with successive frequent sub-cultures from lower concentration of TBEE to higher concentration after a period of every 14 days under continuous incubation (30ºC) and shaking at 160 rpm in an incubator shaker. The GC-MS data of the scale up process, illustrates that, TBEE has degraded upto 49% in 75 mg/l TBEE containing MSM, and 23% in 100 mg/l TBEE containing MSM; respectively. In rest of the concentrations, i.e. 10mg/l and 25mg/l TBEE was completely degraded during a period of their incubation for 14 days. Thus it was observed that the microorganism *Pseudomonas aeruginosa* was able to degrade TBEE at concentration ranging from 50-75 mg/l while it was partially active against higher concentrations of TBEE.

The adapted *Pseudomonas aeruginosa* in scale up technique has been used to study the bioremediation of TBEE in a bioreactor under controlled environmental conditions. A TBEE concentration of 10, 25, 50 mg/l separately in triplicate setup of experiment has been taken for the bioremediation study. (Maloney et al., 1988).The *Pseudomonas aeruginosa* inoculated and uninoculated control samples were incubated under continuous shaking to provide aerobic conditions. At periodic intervals, the samples were drawn aseptically from triplicate flasks and analyzed for TBEE degradation and its intermediates by GCMS after its extraction in hexane (Grant et al 2002).

The concentration of TBEE and its intermediates during bioremediation of 10, 25, 50 mg/l TBEE in MSM are estimated and presented in figure 1. The GC-MS data illustrates that TBEE was completely degraded within a period of 3 days in the case of 10 mg/l MSM, while in the case of 25 mg/l; traces of TBEE were detected till 5$^{th}$ day of the study. In the case of 50 mg/l TBEE containing MSM, the pesticide was persisting for a period of 8 days. The compound trichlopyr acid was found to be major metabolite of TBEE degradation in MSM. The presence of secondary intermediates like 3,5,6-trichloro-2-pyridinol and 2,4,6-trichloro benzene amine were also detected during the bioremediation study.

Figure 2 depicts mild reduction in pH of the inoculated growth culture liquid medium was observed during the bioremediation study in flask bioreactors (fig 2). In the case of 10 mg/l TBEE containing MSM, the pH has been found varying from initial pH 7 to 6.58. The flask bioreactor with 25 mg/l TBEE containing MSM, the initial pH decreased from 7 to 6.5 on the 10$^{th}$ day of the experiment. Similar reductions in pH were recorded in the case of 50 mg/l TBEE containing MSM varying from initial pH 7 to 6.5. in the control uninoculated flask the pH was found to remain constant.

During the bioremediation study, fluctuations in bacterial growth in flask bioreactor were recorded as absorbance at 550 nm (fig. 3). The strain *P. aeruginosa* was found to attain exponential growth phase on the 1$^{st}$ day of the experiment at all the concentrations of TBEE in MSM. The bacteria were found to attain maximum growth between 4$^{th}$-5$^{th}$ day of the experiment in all the concentrations of TBEE in MSM and thereafter slow reduction in bacterial growth was recorded. In the control uninoculated flask bioreactor, sudden fall in the growth was observed during the experiment. The bioremediation of TBEE could be effectively used for remediation of pesticides contaminated soil-water environment.

## Conclusion

The bacterial strain *P. aeruginosa* was found adapted to TBEE ranging from 10-50mg/l in MSM while in the higher concentrations ranging from 25-100 mg/l TBEE, the growth of bacteria and degradation rate was found decreased. During bioremediation, TBEE was found converted into trichlopyr acid, 3,5,6-trichloro-2-pyridinol and 2,4,6-trichloro benzene amine . Ester cleavage was the principle reaction involved in the fenvalerate degradation leading to loss of insecticidal activity leading to formation of secondary metabolite 3-phenoxy benzoic acid. These intermediates are less toxic than TBEE which on long term acclimatization might be converted into biomass and nutrients.

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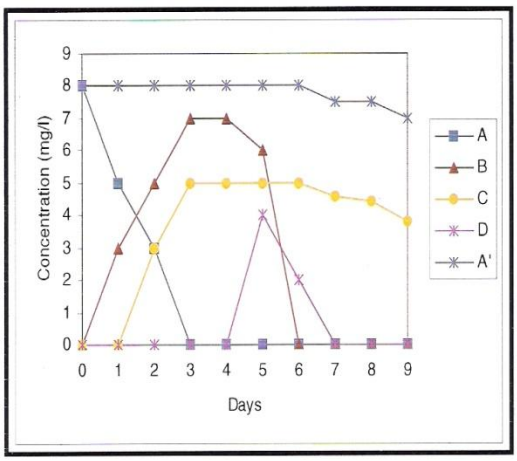
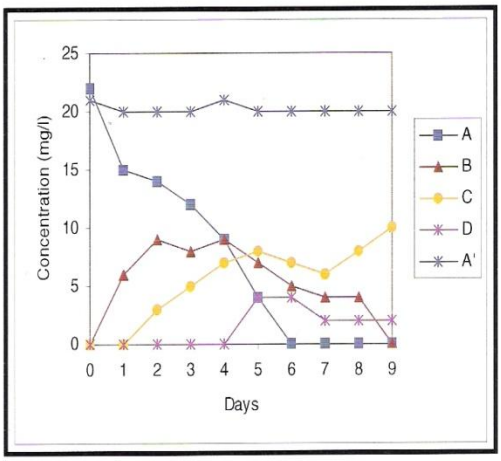
**Figures follow…..**




(a)

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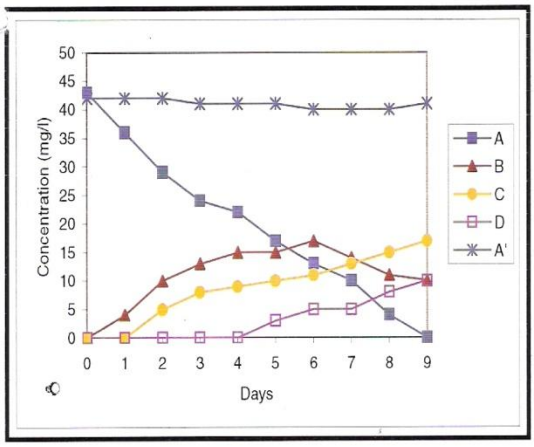


(c)

**TBEE Containing MSM** : **a- 10mg/l; b-25mg/l; c-50mg/l**

**Bioremediation Of TBEE- A- TBEE; B- Trichlorpyr acid;**
**C-3,5,6-trichloro-2-pyridinol;**
**D-2,4,6-trichlorobanzeneamine; A'- TBEE control**

**Fig. 1**

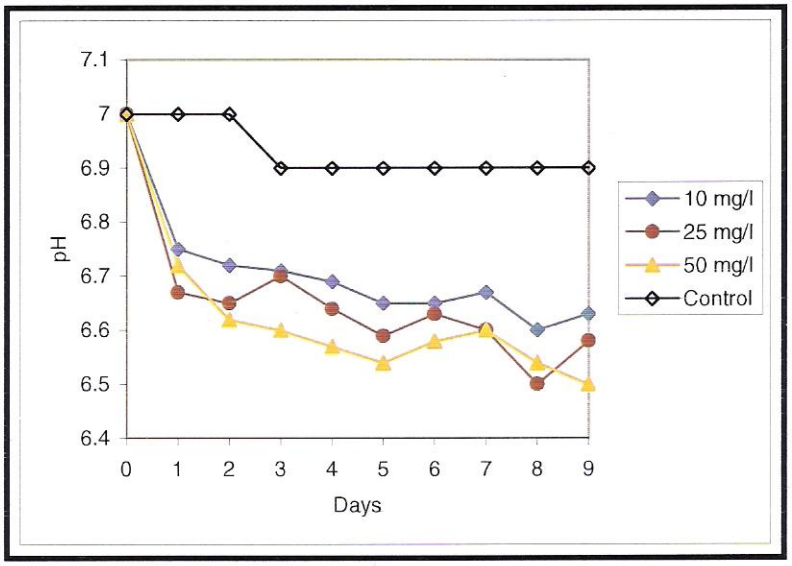


Fig. 2. Variation in pH during bioremediation of TBEE in MSM

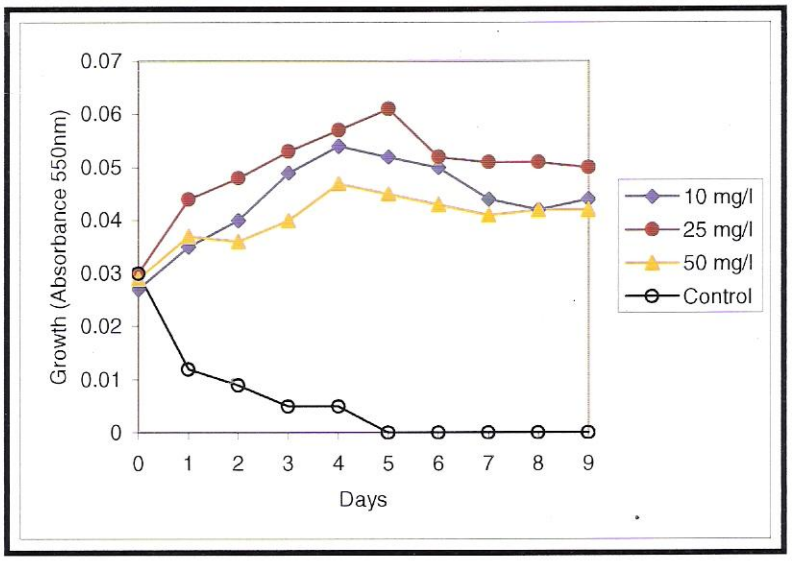


Fig. 3. Variation in bacterial growth during bioremediation of TBEE in MSM