ISOLATION AND SCREENING OF ENDOSULFAN-DEGRADING FUNGI FROM SOIL

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Abstract

Fungal colonies capable of tolerating and degrading endosulfan were enriched and isolated from soil with history of endosulfan application. The microorganisms were enriched using technical-grade endosulfan with gradual increase in concentration from 10 to 100 mgl^{-1}, each enrichment run lasting for 210 h. Four (4) fungal isolates, labeled as P701, P702, P601 and P801, were obtained by successive plating of enriched colonies. The isolates were screened based on α- and β-endosulfan degradation in nutrient media with endosulfan as sole-carbon source (NM-SCS) and as sole-sulfur source (NM-SSS). Results indicated that P601 grown in the NM-SSS exhibited the highest endosulfan degradation after 14 d (93.85% for α-endosulfan and 90.29% for β-endosulfan). Isolate P601 was identified as *Candida tropicalis* using the API C AUX yeast identification system.

Keywords: Bioremediation, Endosulfan, Enrichment process, Fungal isolate

Introduction

Endosulfan, a cyclodiene organochlorine pesticide, was the most widely used pesticide worldwide for the last three decades before it was banned or regulated due to its persistence and toxicity. Most of the endosulfan produced in the past decades now persists in soils where it was applied. Studies have proven that endosulfan applied in soils transports to waterways thru soil run-off [1,2]. This phenomenon endangers not only the aquatic ecosystem but also those that depend on aquatic life as well.

Several studies have been conducted from which the metabolic processes undergone by microbial communities in degrading endosulfan were understood. Different microbial isolates, whether bacteria or fungi were used to degrade endosulfan.

In the 1980s, considerable numbers of bacterial strains which possess the ability to degrade chloroaromatic compounds were isolated [3]. These are *Stenotrophomonas maltophilia* and *Rhodococcus erythropolis* [4], *Bacillus* sp. [3,5], *Mycobacterium* sp. [6], and *Klebsiella pneumoniae* KE-1 [7]. Likewise, a number of fungal species were also isolated: *Phanerochaete chrysosporium* [8], *Trichoderma harzianum* [9], *Chaetosartorya stromatoides*, *Aspergillus terricola*, *Aspergillus terreus* [10], and *Aspergillus niger* [11]. Most of the endosulfan-degrading microorganisms were isolated from endosulfan-contaminated soils. But Verma [12] was able to isolate apparently for the first time an endosulfan-degrading bacterium from the gut microflora of an Indian earthworm *Metaphire posthuma*. The microflora was enriched with endosulfan with a concentration of 80 μg ml^{-1}. The isolate, a *Rhodococcus* strain, was able to degrade endosulfan without the formation of endosulfan sulfate. An increase in free chloride ion concentration was observed in the course of degradation.

In this study, fungal colonies capable of tolerating and degrading endosulfan were enriched and isolated from soil with history of endosulfan application.

Experimental

Reagents and Media

Technical-grade endosulfan (73% α-endosulfan and 27% β-endosulfan) was used in this study. The chemicals and solvents used such as *n*-heptane, ethyl acetate and *n*-hexane were of analytical grade.

Two (2) nutrient media were used in this study, one with endosulfan as sole-carbon source (NM-SCS) and the other with endosulfan as the sole-sulfur source (NM-SSS). The compositions of the media adapted from Kumar et al. [4] and Gunam [13] were modified. NMCS is composed of (in gl^{-1}): KH_2PO_4, 2.44; Na_2HPO_4, 5.77; $(NH_4)_2SO_4$, 4.94; $MgSO_4$, 0.15 and 10 ml l^{-1} of a mineral solution. On the other hand, NM-SSS is composed of (in gl^{-1}): KH_2PO_4, 2.44; Na_2HPO_4, 5.77; $(NH_4)_2PO_4$, 5.57; glucose, 10 and 10 ml l^{-1} of a mineral solution. The mineral solution amended to both media is composed of 100 mM $MgCl_2 \cdot 6H_2O$, 50 mM $CaCl_2$, 10 mM $FeCl_3$, 100 uM $CuCl_2 \cdot 2H_2O$ and 500 uM $MnCl_2 \cdot 4H_2O$.

Enrichment of Microorganisms

Soil with previous endosulfan application was obtained from a low land rice field. Four (4) parallel enrichment runs were conducted with different final endosulfan concentrations as illustrated in Figure 1.
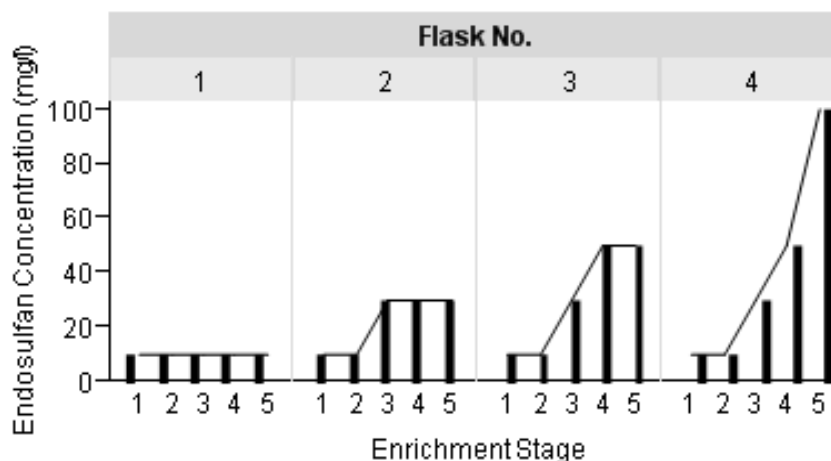

Figure 1. Endosulfan concentration during enrichment runs

For enrichment stage 1, twenty (20) grams of the soil sample was mixed in 250-ml conical flasks with 100 ml of sterilized potato dextrose broth containing 10 mgl^{-1} technical-grade endosulfan and shaken at 160 rpm for 18 h at ambient temperature. Five (5) milliliters of the supernatant liquid were re-suspended into 100 ml potato dextrose broth containing 10 mgl^{-1} technical-grade endosulfan and 0.5 gl^{-1} chloramphenicol for enrichment stage 2. The suspension was shaken at 160 rpm and at ambient temperature for 48 h. The inoculum was re-suspended 3 more times, each flask shaken for 48 h for the last three enrichment stages.

Isolation of Fungi

Eight (8) potato dextrose agar plates with 10 mgl^{-1} endosulfan and 0.5 gl^{-1} chloramphenicol were initially inoculated for the isolation procedure. Four (4) plates labeled P101, P102, P103 and P104 were plated with the inocula obtained from the 5th stage of enrichment after 24 h of suspension. The culture grown in the plates were successively re-plated to purify isolates (Figure 2). A total of 29 plates were used for the isolation from which 4 isolates were obtained. The isolates were constantly maintained by streak plating into fresh agar media.
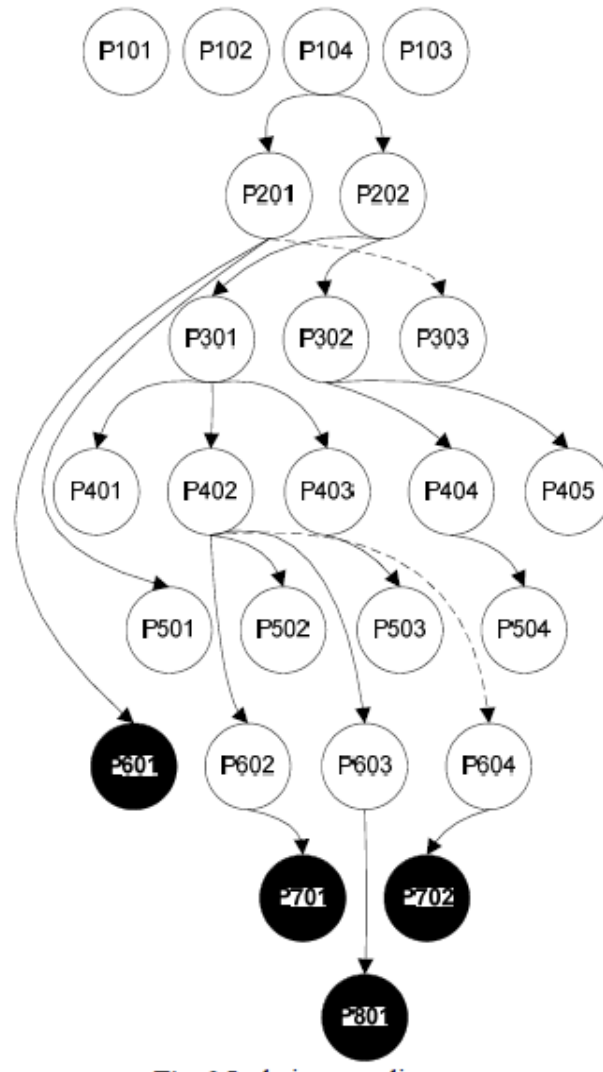
Figure 2. Isolation tree diagram

Screening of Isolates

The inocula for the screening experiments were prepared by aseptically cutting 1 cm x 1 cm agar blocks per isolate where luxurious growth was observed and placing the agar blocks in flasks containing 50 ml potato dextrose broth with 50 mgl^{-1} technical-grade endosulfan and 0.5 gl^{-1} chloramphenicol.

The cultures were allowed to sporulate for 28 h in an orbital shaker oscillating at 160 rpm under ambient temperature. Approximately 10 ml of the suspensions were collected from each of

the flasks. The collected samples were centrifuged. The supernatant liquid was discarded and the residues were washed twice with sterilized distilled and deionized water. The mycelia were re-suspended again in NM-SCS and NM-SSS. Sixty (60) milligrams of the wet mycelia of each isolate were suspended in 100 ml of the two media. Technical-grade endosulfan was added to both media to make a concentration of 50 mgl^{-1}. The suspensions were shaken at 160 rpm for 14 d at ambient temperature. The shake-flasks were shielded from light to remove the effect of light on endosulfan degradation and fungal growth.

Gas Chromatographic Analyses

Five (5) milliliters of the culture broth in the screening experiments were collected and the samples were placed in 15-ml vials to which 20 ul of 250 ppm trans-chlordane were added as surrogate standard. Anhydrous sodium sulfate and Tween 80 were added to prevent formation of emulsion and increase the recovery of the analyte. Five (5) milliliters of ethyl acetate/*n*-heptane (1:1, v/v) were added to the mixtures. The contents of the vials were mixed in a vortex mixer and then sonicated for 30 minutes at 30-35°C. The sonicated mixtures were then centrifuged and the ethyl acetate/*n*-heptane layers were collected. The solvent was evaporated and the residue was re-dissolved in 1 ml n-hexane.

The concentrations of α-endosulfan, β-endosulfan and endosulfan sulfate in the samples were determined using Gas Chromatography equipped with Electron Capture Detector (Hewlett-Packard 5890 Dual ECD) with columns RTx-CL Pesticides (30 m, 0.32 mm ID, 0.5 um) and Stx-CL Pesticides (30 m, 0.32 mm ID, 0.25 um).

Results and Discussion

Fungal Isolates

Four (4) isolates were obtained from the enrichment and isolation processes. Two (2) of the isolates are molds labeled as isolates P701 and P702. The other 2 isolates are yeasts labeled as P601 and P801.

Degradation of Endosulfan Isomers

The degradation of α-endosulfan was found to be greater than that of β-endosulfan for all isolates and media (Figure 3). Isolate P601 exhibited the highest total endosulfan degradation of 84.56% (10349.70 ugl^{-1}) in 14 d while isolate P702 exhibited the lowest total endosulfan degradation at 43.18% (2299.52 ugl^{-1}) in 14 d. On the other hand, the screening flasks with nutrient medium containing endosulfan as the sole-sulfur source (NM-SSS) showed higher total endosulfan degradation at 73.40% (6077.23 ugl^{-1}) in 14 d than the nutrient medium with endosulfan as the sole-carbon source (NM-SCS) at 45.21% (2365.36 ugl^{-1}) in 14 d.
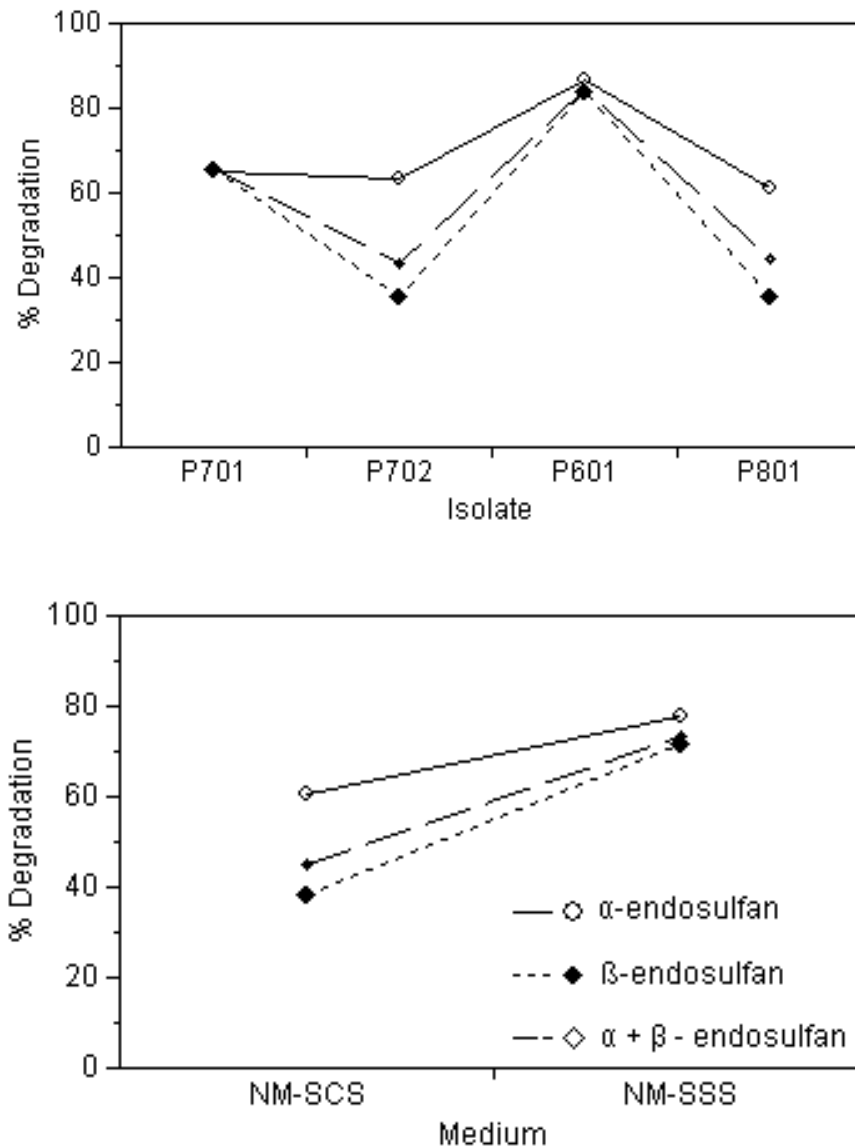
Figure 3. Main effects plots for endosulfan degradation

The responses were also analyzed in a prediction profiler using JMP (version 8, SAS Institute Inc.) The desirabilities were set to vary linearly with α-endosulfan and β-endosulfan degradation. The profiler gives the optimum setting as P601 for isolate and NM-SSS for medium when the desirabilities were set to maximum (obtained as 0.95). The optimum responses were given as 93.85 % for α-endosulfan degradation, 90.29 % for β-endosulfan degradation and 91.35 % for total endosulfan degradation. The interaction effects plot for endosulfan degradation illustrates the interactions between the isolates and the media used (Figure 4). The top right box shows the variation of total endosulfan degradation with medium using different isolates. It was observed that isolates P702, P601 and P801 prefers NM-SSS while isolate P701 prefers NM-SCS. It was also observed that isolate P601 exhibited the highest endosulfan degradation for both media (the lines for P701, P702 and P801 are below the line for P601).

The box at the lower left of Figure 4 shows the variation of total endosulfan degradation with isolate at different media. It clearly shows that NM-SSS significantly enhances the degradation capability of P601 among the rest of the isolates.
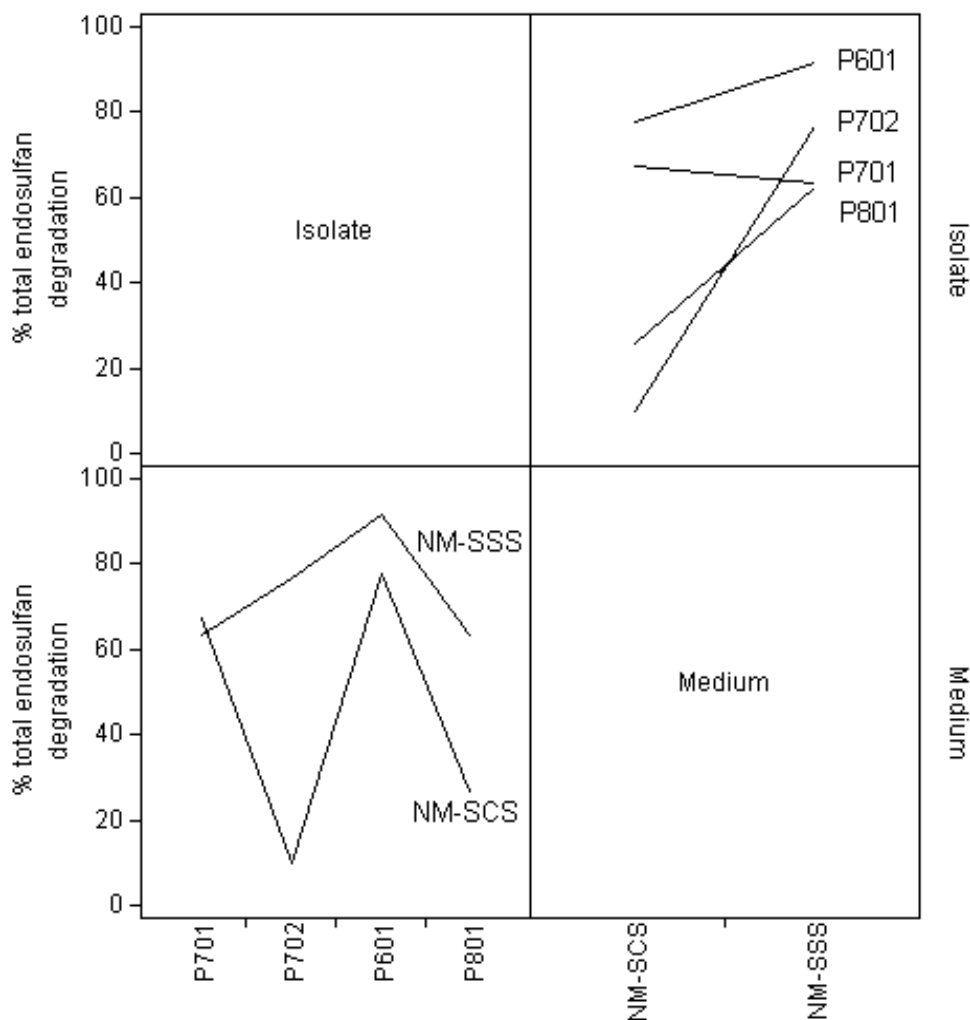
Figure 4. Interaction effects plot for endosulfan degradation

Degradation of Endosulfan Sulfate

The variation of endosulfan sulfate degradation with nutrient medium for each isolate shows that only isolate P601 favors endosulfan sulfate degradation with endosulfan as the sole sulfur source more than as the sole-carbon source as shown in Figure 5. However, isolate P601 exhibited minimal variation in endosulfan sulfate degradation with the change in nutrient medium. On the other hand, isolates P702 and P801 utilizing endosulfan as the sole-sulfur source exhibited net endosulfan sulfate formation rather than degradation at 49.79% and 62.80% increase in endosulfan sulfate concentration respectively. The isolates utilizing endosulfan as the sole sulfur source show wider variation in endosulfan sulfate degradation than that with endosulfan as the sole-carbon source.

Increase in Biomass

The main effects plots for increase in biomass show that the increase in biomass with respect to the isolates and medium has a similar profile with endosulfan degradation (Figure 6). Isolate P601 exhibited the highest increase in biomass at 1956.40 mg l^{-1} (dry weight) while isolate P801 exhibited the lowest increase in biomass at 83.35 mg l^{-1} (dry weight). The similarity in the main

effects profiles for endosulfan degradation and increase in biomass hints possible correlation between the two measured parameters.
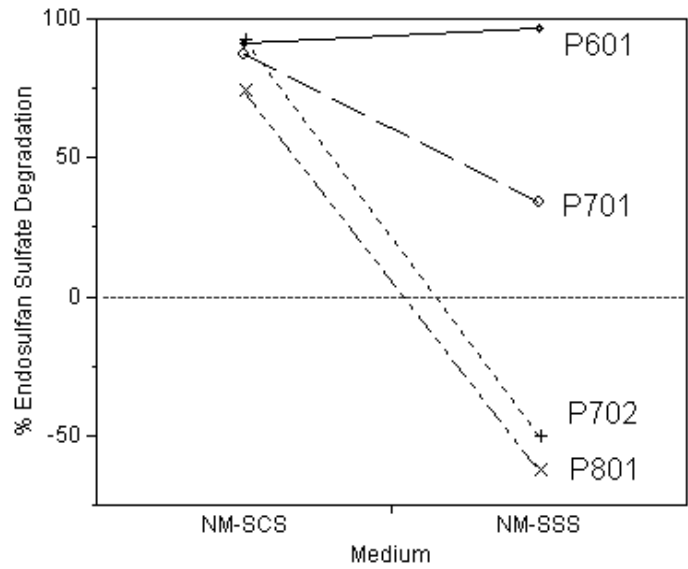

Figure 5. Interaction effects Plot for endosulfan sulfate degradation
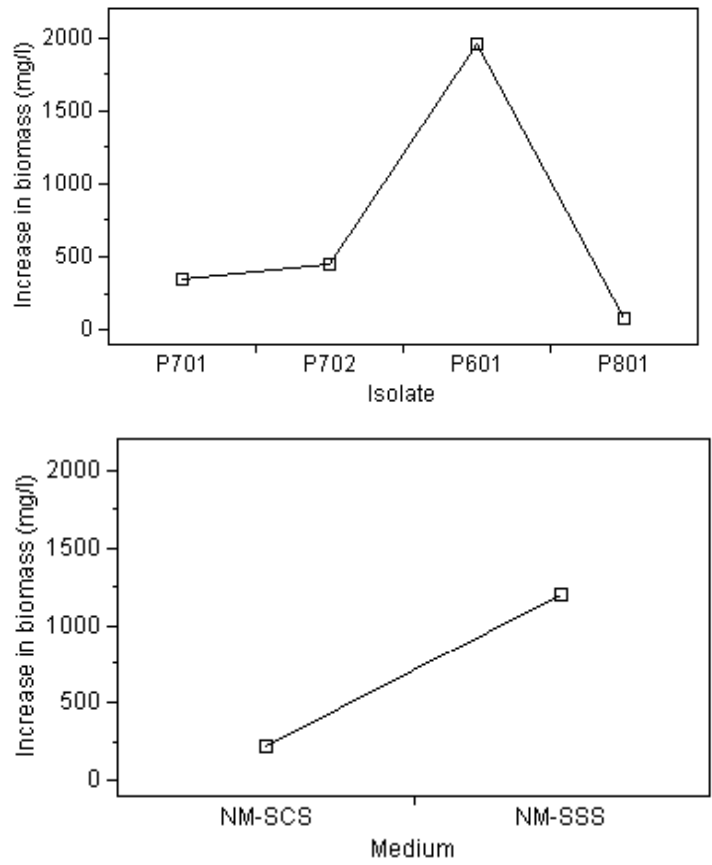

Figure 6. Increase in biomass per run

Identification of Yeast Isolate

Isolate P601 was identified as *Candida tropicalis* using the API C AUX Identification System (Good identification at 95.8%) by the Philippine National Collection of Microorganisms (PNCM) of the University of the Philippines National Institute of Molecular Biology and Biotechnology (U.P. BIOTECH).

Conclusions

This study investigated the biodegradation of α- and β-endosulfan in soil using a fungal isolate identified as *Candida tropicalis*. Indigenous microorganisms present in a soil sample with history of endosulfan application were enriched using Potato Dextrose broth amended with technical-grade endosulfan with concentrations gradually increased from 10 to 100 mgl^{-1}. Luxurious growth was observed on all flasks after 210 h of enrichment. The colonies from the flask with the highest concentration of endosulfan (100 mgl^{-1}) were inoculated into plates for fungal isolation. Four (4) fungal isolates (2 mold species, labeled as P701 and P702, and 2 yeast species labeled as P601 and P801) were obtained after a total of 29 plates were used for isolation and purification.

The isolates and the nutrient medium whether endosulfan is the sole-carbon source or the sole-sulfur source was screened based on α- and β-endosulfan degradation. Isolate P601 showed the highest endosulfan degradation at 84.56% total endosulfan degradation along with the nutrient medium with endosulfan as the sole-sulfur source (NM-SSS) at 73.40% total endosulfan degradation. Combination of the two yields 91.35% total endosulfan degradation (93.85% for alpha-endosulfan and 90.29% for beta-endosulfan). The results obtained from this study may be applied to future soil biodegradation studies.

Acknowledgement

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