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Azotobacter spp. Bioremediation Chemosate

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Abstract: Bioremediation of pesticides is the best option available to date due to its eco-friendly, cost-effective and efficacious nature**.** The study aimed to evaluate the Azotobacter spp. bioremediation Chemosate in the different incubation period and concentrations (5, 10, 15, 20, 25 ppm). From local sites, different microbes were isolated and Azotobacter separated using selective methods for identification of characteristics. The best result for the growth of Azotobacter sp. was at 25 ppm/0.222-0.163, in 15 days; in addition, the great degradation rate % was 25 ppm / 54.16%, observed in 2 months, while the best degradation and residues of chemosate after its digestion through MSM and HPLC residues analyses were at 25 ppm, as seen in 1-2 months, respectively. The degradation ratio % reached 81-79 % for 1-2 months. This conclusion suggests that Azotobacter spp. degradation Chemosate principles applied via hydrolysis binds phosphorus bonds with oxygen and digests the pesticides to produce nitrogen and carbon as elements for its growth sequences, especially at 2 months/25ppm.

Key words: Azotobacter spp., chemosate, biodegradation.

Introduction

Chemosate is an organophosphate and heterogeneous compound, and works as a non-specific herbicide via killing plant leaves. It was used for the first time by Monsanto (Roundup) of the United States in the year 1917 (Valavanidis, 2018). Chemosate works towards the inhibition of a shikimic acid enzyme pathway, which is essential for some microorganisms and plants. In the case of its extensive use as a chemi-control for pests, they can cause potential hazards to humans and the environment besides the benefits of their application (Kaczyński and Łozowicka, 2015). In trace analysis, Chemosate is a difficult herbicide, has good water solubility that causes difficulty in determination of its physical and chemical properties (Kaczyński and Łozowicka 2015).

The microorganism's ability to eliminate pollutants is one of the bioremediation methods (Ibrahim et al., 2015). Bioremdiation is an effective supplementation method as it is eco-friendly, economically worthy, reduces the toxicity in the environment and mineralises the toxic pollutants. (Comeau et al., 1993; Finley et al., 2010; Ghassempour et al., 2002; Sørensenet al., 2008; Yasouri, 2006). Metabolic reaction is one of the microorganism degradation methods, applied across pesticides in soils, including catabolism strategies and the enzymes of co-metabolism (El-Sheikh and Ashour, 2010). The fate of organic-pesticides in the ecological system can be marked by applying biodegradation as a major agent. This study proposes to achieve and inspect the domestic bacterial separation when reacted with different glyphosate concentrations present in soil, and detect residues concentration from bacterial digestive via HPLC after extraction.

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Material and Methods

Materials

Chemosate is the trade name for Glyphosate, which was purchased from the local market. Materials were facilitated by the Remediation Pollutants Center. The Mineral Salt Media (MSM) was added to the growing Azotobacter spp. to investigate chemosate degradation, as shown in Table 1 (Sperber, 1957). As the only phosphorus and nitrogen source, also carbon resource Flasks (125 ml) were supplemented with Chemosate at concentrations of 5, 10, 15, 20 and 25 ppm.

Soil Samples Collection

Samples collected from the local Alkarkh region, 10g sample was taken from a depth of 0-15 cm, put in plastic packets, brought and kept in the laboratory until utilised (Mousa et al., 2019).

Isolation, Morphological and Biochemical Identification of Azotobacter

One gram of soil was taken from the stored plastic packets, diluted with 10 ml of distilled water (D.W.) as stock solution. The tubes were filled with 9 ml D.W., which means concentration stocks of up to 10^{-9} were prepared and kept under laminar airflow (Roychowdhury et al., 2017)

Bacterial, Morphological and Biochemical Identification

For the isolation of Azotobacter spp. from soil sample, Ashby media was prepared, dilutions stocked prepared (10^{-2}, 10^{-4}, 10^{-8}) were taken and 0.1 ml to the Ashby media were added together and kept on respective petri dishes, the dishes were kept under incubation conditions (37°C/7 days) (Aquilantia et al., 2004), and 50 ml/3 tubes Ashby medium were prepared, poured and allowed for solidification in slant positions, kept inside the incubator at 37°C for 48 hrs (Collee et al., 1989; Singh, 2011).

Growth Percentage and Degradation %

The hydrolysis ability was measured (2 days to 2 months) via spectrophotometer OD_{600}. The degradation % for (1-2) months was measured, from the extracts prepared (equal volumes of ethyl acetate and Mineral Salt Media) and utilised as a reagent, for two times, centrifuged at 3000 rpm/10 minutes, filtered and then anhydrous sodium sulphate utilized for drying the extract by keeping it in a glass-fiber paper (Roychowdhury et al., 2017). Using equation (1), the degradation % was measured (Tang and You, 2012):

$$P = (1\text{-}C_1/C_0) \times 100\% \quad (1)$$

P = Chemosate degradation %

C_1= Chemosate concentration via test sample

C_0= the control

HPLC Analysis

Each prepared ethyl acetate extract was analysed using HPLC conditions, as shown in Table 2 (Islas et al., 2014). The final concentration (Alehagen, 2011) of Chemosate was calculated using equation 2:

$$\text{Pest. con. sp (mg/L)} = \text{Asa} \times \text{Cs}/\text{As} \times \text{Csa} \quad (2)$$

where Cs = Concentrate of standard (mg/L);
As = Standard peak area

Table 1: The Mineral Salt Media (MSM)

Weight (g)	*Compounds*	*Note*
0.2	Potassium dihydrogen phosphate	Section A sterilised separately at 125 ºC /25 min
0.5	Dipotassium hydrogen phosphate	
1	Ammonium sulphate	Section B -B: All mixed and added to part A of 1 litre flask, and adjusted at - (pH 7.0 ± 0.3)
0.2	Magnesium sulphate heptahydrate	
0.2	Sodium chloride	
0.05	Calcium chloride dihydrate	
0.025	Ferrous sulphate heptahydrate	
0.005	Molybdic acid sodium salt dihydrate	
0.005	Manganese sulphate	

Csa = Sample concentration mg/L;
Asa = Sample peak area

Table 2: HPLC analysis

HPLC Condition analysis	
UV-Vis Detector	254 nm
Manual Injector Equipped	20-µL loop
*Column	C-18 *
Mobile phase	Acetic acid (1%) with CH_3OH (6:4)
Rate Flow (ml/min)	1.0
Temperature	24 ±1°C

* ZORBAX (5µm; 150 mm × 4.6 mmi.d.)

Results and Discussion

Morphological and Biochemical tests

The Azotobacter spp. identification tests are shown in Table 3 (Aquilantia et al., 2004; Karunya and Saranraj, 2014).

Hydrolysis and Bacterial Growth

Growth of Azotobacter spp.

The best growth of Azotobacter sp. was observed as 25 ppm /0.222,0.163 in 1-2 months, Figure 1.

Degradation Rate %

The best Azotobacter sp. degradation rate % results were 25 ppm/54.16%, in 2 months, as shown in Figure 2.

HPLC Test Chemosate Residues

The Azotobacter sp. showed greatest digestive and biodegradation in Chemosate with 25 ppm

MSM, which was 2.83/3.1 mg/L/(1-2) months, Figure 3.

Microbial degradation of phosphorus, and organic pesticides and the bioremediation strategies progress for contaminated agricultural sites depends upon the beginning of microbes degrading, which is also a growing area of research worldwide (Karunya and Saranraj, 2014; Upadhyay et al., 2015). *Bacillus megaterium* presented the greatest growth and degradation % at two months/5 and 25 ppm. The

Table 3: The Azotobacter identification tests

Morphological tests		*Biochemical tests*	
Spore shape	Spherical	Catalase	+
Colonies	Yellowish Brown; Dark	Starch Hydrolysis	+
Motility	+	Indol/ Methyl Red	+
Gram stain	-	Nitrate reduction test	-
Aerobic	+	Caesin test	+
Temperature	26±3 C, optimum 28 C	Voges-Proskauer test	-
pH	7	Catalase	+

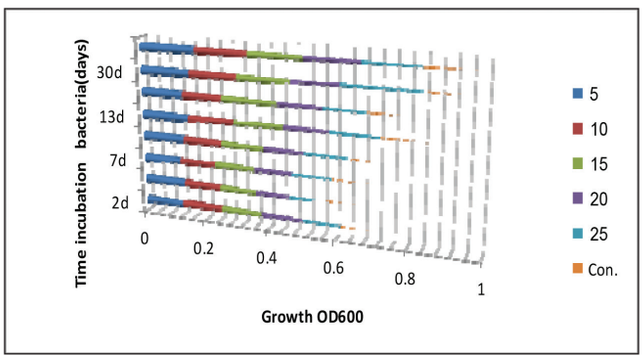

Figure 1: Chemosate and Azotobacter growth % on mineral salt media.

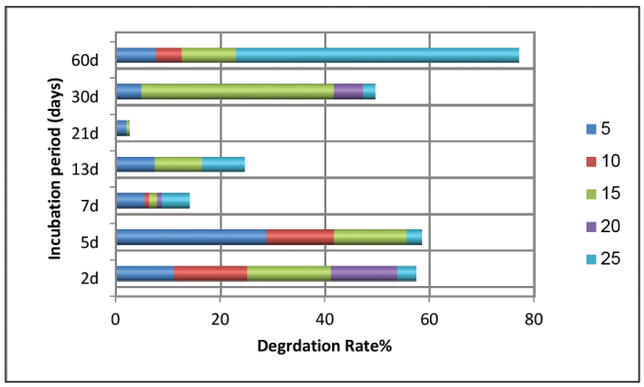

Figure 2: Chemosate degradation % in mineral salt media.

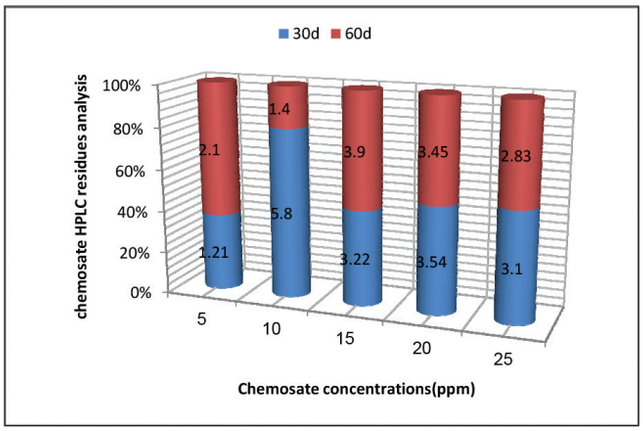

Figure 3: Chemosate residues concentrations % in HPLC.

capacity of degradation organophosphate pesticide like Chlorpyrifos via *Bacillus megaterium* (600 mg/L) reached 80.9%/10 days (Karunya and Saranraj, 2014), while in 20 ppm/21days, it reached 72.3%, also improving significantly, degraded atrazine (50 mg/kg) significantly (99.0% in a week) (Shweta et al., 2017; Zhu et al., 2017). Bacteria such as *Bacillus megaterium*, *Pseudomonas fluorescens* showed increased degradation in organic phosphorus pesticides, while *Bacillus subtilis* showed an opposite reaction towards the increasing concentrations (Mkpuma et al., 2015); also *B. megaterium* showed positive degradation ability towards Chlorpyrifos in 1-2 weeks (Chandrashekar et al., 2017), and 83% degradation rate towards Monocrotophos (MCP), which is an analysis towards CO_2, NH_4 and HPO_3 during metabolic degradation (Borji et al., 2014). The glyphosate degradation rate % via *Bacillus megaterium* reached 71% for 25 ppm /2 months (Mousa et al., 2019). With agreement to this, Azotobacter spp. utilises Chemosate as a nitrogen and carbon source. Also using an increased concentration of digestive phosphate media was observed to show a highly significant increase in the biodegradation of the species, under the conditions: incubation period of 1-2 months, media concentration of 25 ppm, the biodegradation reached 81-79%.

It was observed that the concentration of glyphosate generally reduces along with bacterial growth. Phosphate ester and phosphonate are the analytical hydrolysis results of phosphorus. The esters groups are vulnerable to hydrolysis sites. Besides hydrolysis, oxidation is also one of the major reactions, including alkylation and dealkylation (Singh, 2011). Detoxification is one of the microbial degradation principles via hydrolysis of phosphorus bonds with oxygen (P–O-alkyl, P–O-aryl) (Mousa et al., 2019).

Conclusion

From the Alkarkh region, Azotobacter spp. was isolated and collected in soil samples, and utilised for morphological tests to identify Azotobacter spp. and biochemical tests. The greatest growth rate of the species was at 25 ppm/0.163 in 1-2 months, the highest degradation rate % was 25 ppm/54.16%, in 2 months, while the best Azotobacter sp. degradation and residues results of chemosate after the digestion with Mineral Salt Media at 25 ppm concentration were (2.83 and 3.1) mg/L / (1-2) months, respectively. From all the conclusions, it is noted that using Azotobacter sp. with glyphosate (as the source for carbon and phosphorus could be well exploited for bioremediation of contaminated sites with good results after 1-2 months with 25 ml/L percentage of degradation (81-79%).

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