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Removal of Aclonifen with Some Soil Microorganism as Chemical Oxygen Demand and Investigation Population-Time Relationship

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Abstract – **The study is about the removal of Aclonifen as COD parameter and monitoring the population dynamic of isolated bacteria and fungi. Some bacteria and fungi were isolated from soil samples for their identifications before the use of herbicide. Removal rates of Aclonifen was determined by microbiological degradation with these enriched bacteria and fungi in liquid media. With these microbial cultures, removal rate by bioremediation method studies were carried out. In addition to microbial degradation, with the measurements of the turbidity and the numerical value (colony number) is monitored with isolated bacteria and fungi species. The removal efficiency for *Micrococcus yuannesis* was 93% and *Micrococcus luteus* was 70%, by the fungi *Penicillium talaromyces*, and *Metacordcyceps chlamydosporia* species, were 53% and 91% respectively in five days. Bioremediation of Aclonifen results have shown differences with respect to species in liquid media. Degradation time of Aclonifen in liquid culture media with isolated bacteria and fungi varied between 10 min. and 120 min.**

Keywords – **Aclonifen, Bioremediation, Colony Number, Herbicide, Population Dynamic.**

I. INTRODUCTION

Inadequate mineral nutrient, especially nitrogen, and phosphorus, often limits the growth of hydrocarbon utilizing bacteria in water and soil. Addition of nitrogen and phosphorus to a soil has been shown to accelerate the biodegradation of the pesticides [1]. Addition of a carbon source as a nutrient in contaminated soil is known to enhance the rate of pollutant degradation by stimulating the growth of microorganisms responsible for biodegradation of the pollutant. It has been suggested that the addition of carbon in the form of pyruvate stimulates the microbial growth and enhances the rate of degradation [2]. It is observed that utilization of organic waste in the bioremediation of soil seems a highly potential area.

Pesticide biodegradation is a ubiquitous process. It has been documented in a wide range of habitats, including soils, ground water and sewage sludge, surface, sediments, etc. The ubiquity of pesticide degradation suggests that bioremediation strategies can play a sigificiant role in the treatment of pesticide wastes. The microbial degradation of pesticides offers a promising strategy by which toxic chemicals may safely to untoxic chemicals. For that reason there is a need to isolate and identify the microorganisms that subsist interact in contaminated fields [3].

The aerobic degradation of nitrobenzene by *M. luteus* Z3. Strain Z3 was able to utilize nitrobenzene as a sole source of carbon, nitrogen, and energy. Z3 tolerated nitrobenzene up to the concentration of 250mgl−1 [4]. The biochemical and molecular modes of pesticide degradation by microorganisms have been well documented [5,6].

One of the the principle mechanism which prevents the accumulation of these chemicals in the environment is microbial degradation. But, when pesticide degradation is too rapidly, pest control may be less effective. One way to increase the rate of microbial degradation of pesticides in soil is one or more previous applications of the same type of pesticide with a similar chemical structure. This phenomenon is known as accelerated or enhanced degradation and can result in economic losses to farmers [7].

Persistence of pesticides makes bioremediation of such soils challenging. It is regarded that it requires a sequence of anaerobic–aerobic conditions and a presence of a co-substrate [8]. During anaerobic phase, the pesticides are dechlorinated into more susceptible metabolites to further degradation. Sequestered fractions of these compounds show reduced toxicity, reduced mobility and are resistant to degradation [9]. For soil contaminated with chlorinated pesticides the research focuses mainly on ecotoxicological aspects. There is little studies have done to recognize its significance for their bioremediation [10].

The side effects of pesticides on the soil microflora were studied by several authors [11]. Pesticides may affect the microbial population by controlling the survival and reproduction of individual species. On the other hand, several microorganisms were reported to degrade some pesticides [12]. Population size, enzymatic activity and biodiversity of certain systematic and physiological groups of microorganisms may serve as bioindicators of changes

taking place in the soil following herbicide application [13,14]

Bioremediation mediated by fungi and other organisms is considered to be a more environmentally approach for the detoxification of persistent organic substances in comparison with traditional chemical and physical methods, and there are several efficient potential biotechnological applications, e.g. organic pollutant biodegradation [15]. Fungi possess extracellular enzymes, many of which are highly potent and relatively non-specific to the chemical composition of the substrate, and may be induced by nutrient-limiting conditions [16].

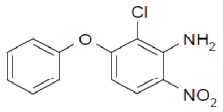
Microorganisms are thought to play an important role in the removal of pesticides from the environment. Many bacteria that are able to degrade pesticides have been isolated from soil around the World [17]. Many members of different groups of soil microorganisms (Bacteria, Fungi, Actinomycetes and Algae) isolated from the soil are capable to degrade pesticides [18]. The goal of this work was to monitoring the removal of Aclonifen with isolated soil bacteria and fungi as COD parameter day by day and investigate the colony number related with Aclonifen and non Aclonifen media in time.

II. Materials and Method

A. Chemicals

The Aclonifen herbicide active ingredient, sold under the trade name "Chekic 600", was supplied by an agricultural products shop. The physical and chemical properties of Aclonifen is given in Table I. pH of Aclonifen was 6.5 and temperature was 250C. This herbicide contains 600 gr L-1 of Aclonifen. All media for the isolation and enrichment of bacteria and fungi were obtained from Sigma Aldrich.

Table 1: Physical and Chemical Properties of Aclonifen

Properties	Unit	Value	Reference
Molecular formula	-	C12H9ClN2O3	[25]
Molecular structure	-		
Molecular mass	g/mol	264.7	
Visual at standard temperature and pressure	-	Yellowpowder (99.6% pure, 99.4 % technicalmaterial	
Vapor pressure	Pa	1.6×10^{-5} Pa 20 °C (%99.3 pure)	
Water solubility	g/l	1.4 mg/l 20 °C pH 5, 7 ve 9 (%99.7pure)	
Melting point	^{0}C	81.2 °C (%99.6 pure)	

B. Media Preparation

Plate Count Agar (PCA), Malt Extract Agar (MEA), Dextrose Casein Peptone Agar, Potato Dextrose Agar, Dichloran Rose Bengal Chlorinated Agar, Sabouraud Dextrose Agar, Malt Extract and Sabouraud dextrose broth media were prepared according to manufacturer's instructions (Sigma Aldrich-USA) and were autoclaved at 1210C for 15 min to ensure a sterilized solution. After cooling, diluted agricultural soil (containing no trace of Aclonifen) in an isotonic solution was added to petri dishes. The medium pH was adjusted to 6.5 and temperature was 250C.

C. Isolation and Enrichment of Bacteria and Fungi

Bacteria and fungi were isolated from the soil samples using serial dilution on different media plates. Bacteria incubation took about three days, while fungi took about one week at 250C. Other isolation studies were also done at 40C and 350C, but best growing seen at 250C. After growing, the plates were screened for any colonies that were visually different from the others. After incubation, the cultures were placed carefully in an enrichment media for seven days to grow with the same temperature of taken soil samples before application of Aclonifen at 250C, and were shaken continuously.

D. Isolation Fungi-Bacteria Molecular Characterization Studies

Molecular characterization studies were implemented according to the Wizard Genomic DNA Purification Kit. For fungi; "Isolating Genomic DNA from Yeast", for bacteria, "Isolating Genomic DNA from Gram Positive and Gram Negative Bacteria" methods used [19].

Fungi Studies

The fungi marked on the petri dishes were shown in PDA petri dishes by streak plate to ensure the reproduction from sport fungi. The fungi that were grown at room temperature and from a single colony isolation were transferred to other PDA petri dishes and were kept at room temperature until they reached the appropriate size for DNA isolation. Growing fungi were scratched using a sterile blade and crushed with liquid nitrogen in sterile conditions, after which, DNA was isolated from the powder hypes.

An ordinary Taq polymerase was conducted for PCR using many combinations of ITS (Internal transcribed spacer) region primers, which are often used in the definition of DNA. The PCR conditions were:

Final concentrations (total 25 µl reaction volumes): 1X Taq polymerase buffer / 1.5 mM MgCl2 / 0.4 µM forward

primer /0.4 µM reverse primer / 0.5 mM dNTP / 1U Taq polymerase (*Metacordyceps chlamydosporia*) or 1.25 U Taq polymerase (*Penicillium simplicissimum*) and 200ng DNA.

Heat cycle conditions: 1 cycle: 940C -3 min / 35 cycles: 940C - 15 s, 550C - 30 s, 720C - 30 s / 1 cycle: 720C 1 - 5 min.

In PCR, the expected length of bands were obtained for *Metacordyceps chlamydosporia*. For the *Penicillium Talaromyces*, One-Taq polymerase was used. These tapes, which were cut from the agarose gel and cleaned (in the case of multiple bands) or as single band, PCR reaction were sent directly to the sequence analysis. A Thermo-Scientific GeneJET Gel Extraction Kit was used in the cleaning of the bands cut from the agarose gel. In cases of a sequence reaction on the bands (cut from the agarose gel) not performing well, re-amplification was made (by One Taq polymerase)

Bacterial Studies

Phire Hot Start II DNA Polymerase was used for PCR, given that it allows making no DNA isolation. Then, longer PCR bands of various lengths (1000–3000 bp) were obtained through bacterial 16S ribosomal general primers. The pipette instructions and cycling protocols were:
For final concentrations: (total 20µL reaction volume); 1X Phire Animal Tissue PCR Buffer (includes dNTPs and MgCl2) / 0.5µM forward primer / 0.5µM reverse primer / Phire Hot Start II DNA polimeraz and H2O.

Heat cycle conditions: 1 cycle: 98oC – 5 min / 40 cycles: 98oC – 5 s, 72oC – 20 s / 1 cycle 72oC – 4 min/4oC-∞

Bacteria were identified using 16sRNA Universal Primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′; Escherichia coli positions 8-27) [20]. 16S rRNA universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′; Escherichia coli positions 8–27) [20]. 1492 R 5'TACGGYTACCTTG GTTACGACTT 3' positions 1492–1512) [21, 22].

Microbial Biodegradation Studies

In order to assess the degradation ratio of the pesticide from the fungi and bacteria, 4 different aerobic consortia (1ml each of them) were developed through the enrichment technique.

Studies in Liquid Media

In the liquid media study, 1 ml of chekic 600 and 1 ml of enriched cultures (approximately 2 x 107 microorganism/ml) were added to 98 ml of 0.8 % isotonic saline water. Aclonifen was prepared in the same concentration as used in the field (200ml/1000m2). The growing media used in the experiments were the previously isolated and the enriched bacteria and fungi species, with 1 ml of the solutions obtained from the separately enriched solution (only fungus or bacterium) used in the experiments.

In this phase, solution samples were monitored at 24-hour intervals on the basis of the chemical oxygen demand (COD) parameter with the standard method 5220C Closed reflux titrimetric method [23] . According to this method, 1.5 ml of standard potassium dichromate digestion solution (K2Cr2O7) and 3.5 ml of 0.0176M Ag2SO4 solution were added to sample of 2.5 ml volume. After that, these samples heated at Velp WTW CR3200 thermoreactor for 2 hours at 1500C. After cooling, samples were taken to flasks and 2-3 drops of Ferroin indicator (FeSO4.7H2O) added to samples. Then, samples were titrated with 0.25M standard ferrous ammonium sulfate (FAS) titrant and COD results were calculated.

E. Monitoring of Turbidity and Colony Number

Studies were done to determinate starting time of removal of Aclonifen with Turbidity and increasing population by bacteria and fungi [24]. 8 different culture media with blanks, total volume of 100 ml was prepared. It includes 98 ml of styreline isotonic water, 1 ml of herbicide solution (includes 6.00 mg/ml Aclonifen) and 1 ml of culture solution taken from enrichment culture media. This enrichment media was prepared for bacteria took about 3 days, for fungi, took about 7 days. In blank media there was no Aclonifen. For carry out experiment studies in aerobic media and gaining oxygen from air, caps of the styreline bottles were limited open. Prepared culture media was placed in a shaker incubator (Gallenkamp Orbital Incubator) controlled 250C. Turbidity measurements were done at 650 nm on Photolab 6600 UV-VIS spectrophotometer. Inoculation of bacteria and fungi were done with serial dilution method (10-7,10-8,10-9) to PCA and MEA culture media for generate a colony. Inoculated petri dishes were put into an incubator controlled at 250C. Incubation time was carried on for bacteria in media with Aclonifen 1 day, in media without Aclonifen 3 days; for fungi, in media without Aclonifen 7 days, in media with Aclonifen 10 days.

III. RESULTS

A. Identification of Fungi and Bacterial Studies

The species of fungi obtained according to the results of primers, sequence and references used to identify the fungi are given in Table II. The identified bacterial codes and their species are given in Table III.

Table II: Primers, sequence and references used to identify the fungi

Fungi Code and Approximate species identity	First Primer 5'-3' sequence and reference	Second Primer 5'-3'sequence and reference
Penicilliumtalaromyces	ITS4 TCCTCCGCTTATTGATATGC [26]	ITS6 GAAGGTGAAGTCGTAACAAGG [27]
Metacordycepschlamydosporia	ITS3 GCATCGATGAAGAACGCAGC [26]	ITS3 GCATCGATGAAGAACGCAGC [26]

Table III: Identified bacterial codes and their species

Accession Number	Bacterial Code and Approximate Species Identity	Identity	Reference
KC634108.1	*Micrococcus yunnannesis*	99%	[28]
KF555623.1	*Micrococcus luteus*	99%	[29]

Table IV: Results of increasing of the bacteria and fungi population number in media with and without Aclonifen as Turbidity Parameter

Microorganism	Media withAclonifen			Media withoutAclonifen		
	Time (min)	Turbidity (NTU)	Colonynumber ($x10^9ml^{-1}$)	Time (min)	Turbidity (NTU)	Colonynumber ($x\ 10^9ml^{-1}$)
Micrococcusyuannesis	0	0,116	0,53	0	0	0,02
	10	0,135	0,60	10	0,016	0,05
	15	0,139	0,63	15	0,073	0,09
	20	0,288	0,65	20	0,094	0,24
	25	1,2	1,81	25	0,137	0,62
	30	1,304	2,01	30	0,271	0,87
	60	1,369	2,27	60	0,471	1,13
	70	1,503	2,29	70	0,662	1,74
	75	1,726	2,44	75	0,669	1,88
Micrococcusluteus	0	0,002	0,43	0	0,011	0,01
	15	0,003	0,44	15	0,024	0,03
	30	0,004	0,46	30	0,028	0,04
	45	0,005	0,51	45	0,034	0,07
	60	0,007	0,89	60	0,139	0,12
	75	0,01	1,07	75	0,157	1,18
	90	0,016	1,33	90	0,169	1,31
	105	0,018	1,68	105	0,194	1,44
	120	0,019	2,04	120	0,206	1,88
Metacordyceps chlamydosporia	0	0,081	0,01	0	0,003	0,01
	40	0,124	0,04	40	0,003	0,01
	45	0,371	0,07	45	0,009	0,02
	50	0,522	0,21	50	0,015	0,02
	55	0,641	0,48	55	0,022	0,03
	60	0,773	1,31	60	0,028	0,04
Penicilliumtalaromyces	0	0,001	0,002	0	0,001	0,01
	5	0,002	0,04	5	0,001	0,02
	10	0,003	0,05	10	0,004	0,07
	15	0,004	0,06	15	0,006	0,08
	20	0,007	0,07	20	0,009	0,08
	25	0,017	0,09	25	0,013	0,11

B. Bioremediation Results

COD removal rate was observed between 93% and 70% with Aclonifen. According to these results, *Micrococcus yuannesis* has the best removal performance. The COD which was calculated as 15600mg/l decreased to 1090 mg/l at the end of day 5. The poorest removal performance was observed with *Micrococcus luteus*, decreasing the COD from 15600mg/l to 4680 mg/l (Table IV).

When the results were examined, it was observed that as the turbidity increased in all the cultures, the population of microorganisms also increased. In studies using culture media with and without herbicide, although the differences between the values of the turbidity caused by the cultures and the number of population were identified, it was impossible to interpret these differences in terms of turbidity, and the number of microorganisms showing the best and the worst removal, according to the results obtained from COD studies. However, the increase in the value of turbidity and the number of population in the media with herbicide starts later than those in the blank media without herbicide. This period may extend from minutes to hours. This refers to the adjustment periods of the microorganisms (Lag phase) to the media with herbicide. It also leads to the increase in the number of microorganisms using carbon and phosphorus and the value of turbidity caused by the microorganisms, along with the decomposition of herbicide.

In media with Aclonifen, results of COD by *Metacordyceps chlamydosporia* and *Penicillium talaromyces* is given in Figure 1, results by *Micrococcus yuannesis* and *Micrococcus luteus* is given in Figure II.

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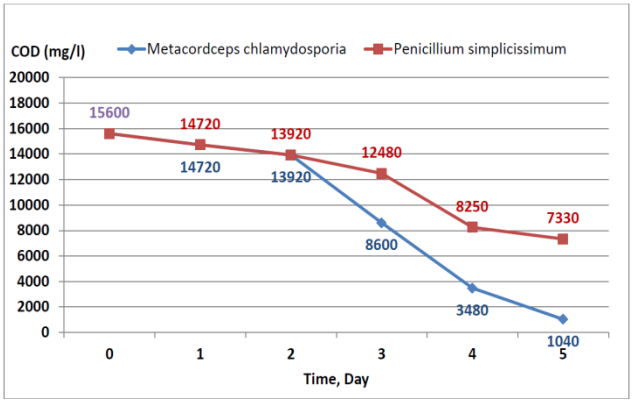

Fig.1. Removal Rate of Aclonifen by *Metacordyceps chlamydosporia* and *Penicillium talaromyces*

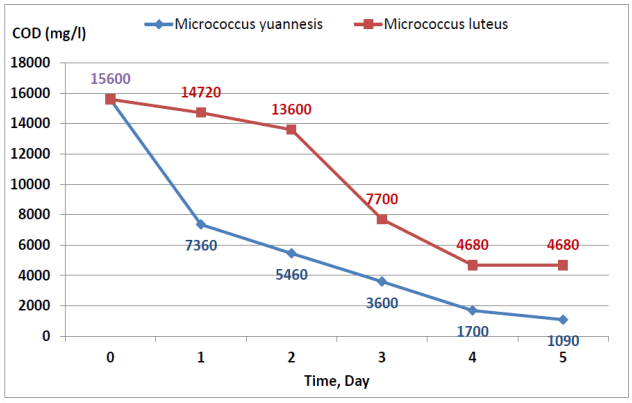

Fig.2. Removal Rate of Aclonifen by *Micrococcus yuannesis* and *Micrococcus luteus*

C. Microbial Activity and Colony Number Results

Experiment results of media with and without Aclonifen by bacteria and fungi species as Turbidity and colony number is given in Table IV. According to the obtained results, graphic related with *Micrococcus yuannesis* and *Micrococcus luteus* bacteria is given in Figure III and Figure IV, related with *Metacordyceps chlamydosporia* and *Penicillium talaromyces* fungi given in Figure V and Figure VI respectively.

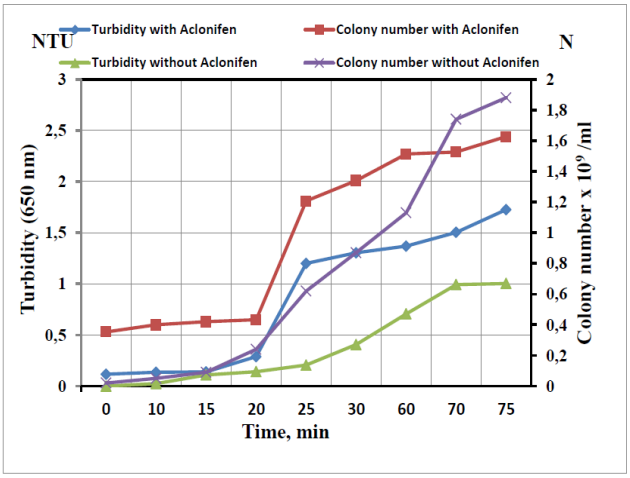

Fig.3. Monitoring Increasing of population number of Micrococcus yuannesis in media with and without Aclonifen by Turbidity (NTU: National Turbidity Unit)

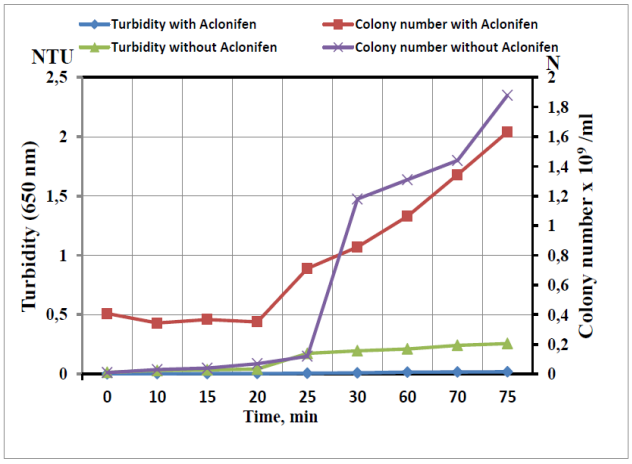

Fig.4. Monitoring Increasing of population number of *Micrococcus luteus* in media with and without Aclonifen by Turbidity

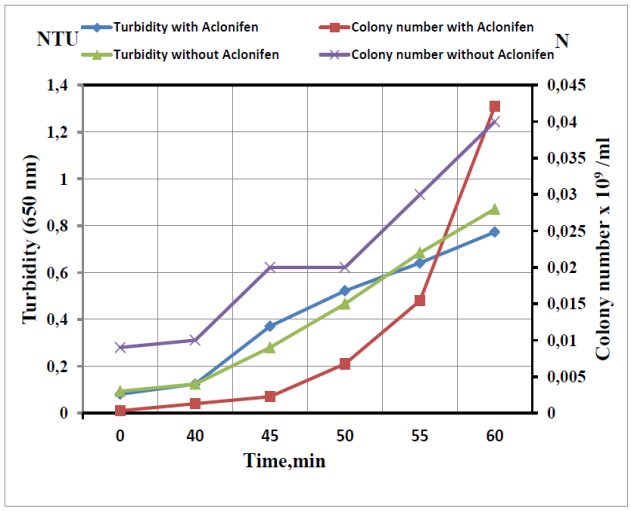

Fig.5. Monitoring Increasing of population number of *Metacordyceps chlamydosporia* in media with and without Aclonifen by Turbidity

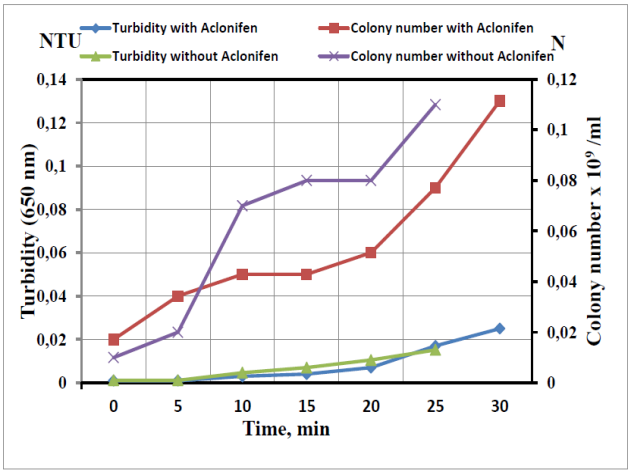

Fig.6. Monitoring Increasing of population number of *Penicilllium talaromyces* in media with and without Aclonifen by Turbidity

IV. Conclusion

In the current study, the rate of COD removal was observed to occur between 91% and 53%. According to these results, *Metacordyceps chlamydosporia* was observed to have the highest removal rate in terms of this parameter. By the end of the 5th day, the COD calculated as 15,600 mg/L was reduced to 1,040 mg/L by *Metacordyceps chlamydosporia*, and to 7330 mg/L by *Penicillum talaromyces*, having the lowest removal rate according to this rate. The data of the other four removal rates were observed to have varied between these two values.

When the experimental results of the monitoring of the microbial activity in the Aclonifen culture mediumthrough turbidity and population increase were examined, the increase in turbidity was observed to be dependent on *Micrococcus yuannesis* having the best COD removal rate in the Aclonifen medium, and *Metacordyceps chlamydospria* increasing in the population of microorganisms, particularly after the 25th and 40th minutes, respectively. It is understood from this that the adjustment period (Lag phase) of *Micrococcus yuannesis* occurs within a shorter period than that of *Metacordyceps chlamydosporia*. It is also understood that the same microorganisms behave differently in the medium without Aclonifen. It can be said that *Micrococcus yuannesis* propagates from the 10th minute, whereas *Metacordyceps chlamydoporia* is activated from the 40th minute. There is no substate, nutrient or Aclonifen in the medium. *Micrococcus yuannesis* started to propagate in the media without Aclonifen from the 10th minute, and barely reached the number of population obtained in the medium without Aclonifen, approximately from the 20th minute in the Aclonifen medium. While the value of turbidity in *Micrococcus luteus* barely reached 0,019 NTU from the 120th minute, this value was observed in the 15th minute in the medium without Aclonifen.

While the increase in turbidity and the number of microorganisms in *Metacordyceps chlamydosporia* had the best removal rate in the Aclonifen medium, as observed in the 40th minute, it was also observed in the 40th minute in the medium without Aclonifen that the population was four times larger than in the medium without Aclonifen. This fungus was observed not to have reached the number of microorganisms that it had reached in the Aclonifen medium, that it had in the medium without Aclonifen. The lack of nutrients in the medium caused this to happen.

While the increase in turbidity and the size of the population in *Penicillium talaromyces* having the worst removal rate in the Aclonifen was observed from the fifth minute in the Aclonifen medium, as it was also observed from the fifth minute in the medium without Aclonifen, the number of individuals was approximately two times higher than that in the medium without Aclonifen.

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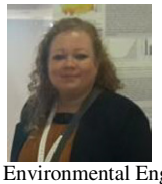

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