Role of Some Selected Fungi Cultures on Bioremediation of Herbicide Chlorsulfuron

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This study aims for the performance of certain soil fungi for bioremediation of chlorsulfuron herbicide solutions on chemical oxygen demand (COD), biochemical oxygen demand (BOD), total organic carbon (TOC) and active ingredient parameters under agitated culture conditions. Soil fungi were isolated from chlorsulfuron-free soil in an agricultural field. Based on the laboratory experiment results, *A. Alternata* had the highest removal rate for all parameters about 5-6 days. Additionally, it was observed that *M. chlamydosporia* showed the lowest COD and active ingredient removal efficiency. On the other hand, TOC removal rates were similar with *P. simplicissimum, M. chlamydosporia* and *S. chartarum* species nearly 53%. It was demonstrated that, there were a suitable isolated fungi species for bioremediation of chlorsulfuron in agricultural soil media. It was conducted that all isolated fungi demonstrated up to 50% ability to degrade chlorsulfuron on important environmental parameters.

Key Words: Chlorsulfuron, Fungi, Chemical oxygen demand, Total organic carbon, Biochemical oxygen demand

Bazı Seçkin Mantar Kültürlerinin Klorsulfuron Herbisitinin Biyoremediasyonundaki Rolü

Bu çalışma bazı toprak mantarlarının çalkalanmış kültür koşullarındaki klorsulfuron herbisit çözeltilerinin kimyasal oksijen ihtiyacı (KOİ), biyokimyasal oksijen ihtiyacı (BOİ), toplam organik karbon (TOK) ve aktif madde parametrelerinde biyoremediasyon performansını bulmayı hedeflemiştir. Toprak mantarları klorsulfuronsuz tarım arazisinden izole edilmilştir. Laboratuvar deney sonuçlarına göre *A. Alternata* bütün parametrelerde yaklaşık 5-6 günde en yüksek giderim verimine sahiptir. İlaveten *M. chlamydosporia* en düşük KOİ ve aktif madde giderim verimini göstermiştir. Diğer bir taraftan TOK giderim oranları *P. simplicissimum, M. chlamydosporia* ve *S. chartarum* türlerinde yaklaşık %53 olarak benzerdir. Klorsulfuronun biyoremediasyonu için tarım toprağı ortamında izole edilmiş uygun mantar türlerinin mevcut olduğu görülmüştür. Yapılan araştırmaya göre, izole edilmiş bütün mantarlar, klorsulfuronu önemli çevresel parametreler bakımından %50 den yüksek bir parçalama göstermiştir.

Anahtar Kelimeler: Klorsulfuron, Mantar, Kimyasal oksijen ihtiyacı, Toplam organic karbon, Biyokimyasal oksijen ihtiyacı

Introduction

Chemical methods for weed control are effective tools that can compensate for the low endeavour. Today in our world, it is understood that chemical pesticides are not free of problem, so scientists are working on integrated pest management, which brings together all the possible methods of pest control (Labrada, 1997.). Bioremediation is one of the most useful method for pesticide control. Bioremediation is a cost and effective method that involved the usage of bacteria and fungi in treating pesticides (Megharaj et al., 2011). However, there are some limitations for pest control in using bioremediation techniques such as poor microbial capacities, fewer bioavailability of pesticides on spatial and temporal scales, for efficacy testing of bioremediation (Megharaj et al., 2011).

Soil contamination resulting from agricultural activities has caused high problems in last years (Ha et al., 2014). Pollutants such as pesticides entering the water or soil reveals an important threat to human health and natural habitat (Zeng et al., 2013). Most of pesticides are used simultaneously on most agricultural activities, which lead to a higher environmental problems and increased pollution (Pino and Peñuela, 2011). Pesticides entering the soil can bring environmental problems, and effect biochemical and microbial aspects of soil properties. On the other hand, bioremediation of soils contaminated with pesticides has exhaustes considerable research interest (Chen et al., 2015). For developing new methodologies to prevent pesticide contamination from soils is an important issue. Most of microorganisms can use these compounds including pesticides for their

mineralize, growth and detoxify them (Belal et al., 2008).

The microorganisms are the basis of accelerated rates and their ability to degrade specific compounds such as pesticides. Many scientists have reported that indigenous strains of microorganisms can enchange the biodegradation of pesticides in wastewaters. Bioremediation/biodegradation methods were mainly use the degradation effect of microorganism to remove the contaminants. It is called the environment-friendly alternative technology since it has safe, low-cost and rapid advantages (Brian et al., 2002). To handle with environmental difficulties, efficient, low cost and safe environmentally waste treatment technologies are needed (Kelly and Zhen 2014). Fort his, the bioengineered technologies adapted for each type of organic wastes such as pesticides are required to succeed high performance treatment efficiencies. Biodegradation is an efficient bioremediation method that grow in different ecosystems with microorganisms. These microorganisms are able to survive even in divergent conditions (Castillo et al., 2011).

Chlorsulfuron is a specific herbicide that controls selected undesirable grasses and broadleaf weeds. This herbicide inhibits cell division in plant roots which causes harmful plants to stop growing. Chlorsulfuron is the most frequently used herbicide in sunflower and wheat cultivation in Marmara region, Turkey (Erguven and Yildirim 2016).

The aim of this study is to determine reduction of synthetic chlorsulfuron on chemical oxygen demand (COD), biological oxygen demand, total organic carbon and chlorsulfuron active ingredients during fungal incubation.

The objectives of the present study were to investigate the ability of *Penicillium thrichoderma, Penicillium simplicissimum, Penicillium talaromyces, Metacordyceps chlamydosporia, Stachybotrys chartarum* and *Alternaria alternata* fungi types on the degradation of the herbicide chlorsulfuron in synthetic culture solutions with some important environment parameters. The study was conducted in two phases (1) Isolation of fungi from soil sample with no background of chlorsulfuron (2) Bioremediation of the herbicide under agitated culture media with some parameters.

Materials and Methods

Chemicals

Chlorsulfururon active ingredient was purchased from Sigma-Aldrich, Turkey with 99.9% purity. All reagents used for GC studies were of analytical grade. Chlorsulfuron, sold under the trade name "Hammer Extra 75 DC", was supplied by an agricultural products shop. The physical and chemical properties of chlorsulfuron are given in Table 1. This trade product contains 75% active chlorsulfuron. Malt extract agar, potato dextrose agar, dichloran rose bengal chlorinated agar, sabouraud dextrose agar were used for isolation and malt extract was used for enrichment studies (Cruikshank, 1972). These media were purchased from Sigma Aldrich, Turkey.

Field sampling studies

The size of the agricultural field was 4,4 hectares which was used for wheat culcivation in Turgutbey village of Lüleburgaz district of Kırklareli province (41° 27' 30.9024'' North, 27° 23' 15.5580'' East), in Trakya region and no chlorsulfuron or other sulfonylurea herbicides was used previously on this field. According to world reference base for soil resources (WRB) classification (FAO, 2006), the soil type is silty clay loam. In the agricultural field, representative samples were taken from ten points. The points were determined by a "zig-zag" shaped sampling pattern be walked through the field. In each of these ten points, the soil was dug in a "V" shape to 20 cm depth and in a depth of 6 cm soil was collected as a composite sample and was transferred into a sterile glass container. Soil samples collected from ten points were mixed and then 1000 g soil was transfered from this mixture labelled and packaged up for transport to the laboratory for herbicide residue analyses. After the analysis, we sure that and there were no background of Chlorsulfuron or other sulfonylarea herbicides in the studied field and then, this soil sample used for isolation of fungi.

Isolation and identification of fungi

All media used for isolation and enrichment studies were autoclaved at 121 ^{0}C for 15 min. to ensure a sterilized solution. After cooling, 10^{-4} times diluted agricultural soil was added to petri dishes. Isotonic 0,8% NaCl solution used for

dilution studies. The pH of medium was adjusted to 7.0 and temperature was set at 25 ^{0}C to isolate and enrich fungi species (Cruikshank, 1972).

Fungi incubation took about one week in the incubator at 25 ^{0}C. After fungal growth, the agar medium were screened for any colonies that were visually different than the others. After incubation, the cultures were placed carefully in an enrichment media (malt extract) and kept there for seven days and allowed to grow under the same temperature and shaken at 150 rpm continuously (Cruikshank, 1972).

To ensure the reproduction from sport fungi, the fungi marked on the petri dishes (From F1 to F6) were sown in PDA (Peptone Dextrose Agar) petri dishes by streak plates. The fungi that were grown from single colony isolated at room temperature were taken to other PDA dishes and until they reached the appropriate size for DNA isolation studies, were kept at room temperature. A sterile blade used for scratch the growing fungi and they crushed with liquid nitrogen in sterile conditions, after which, DNA was isolated from the fungal hyphae.

For PCR (Polymerase Chain Reaction), an ordinary taq polymerase was conducted for many combinations of ITS (Internal Transcribed Spacer) region primers, which are often used in the definition of DNA. The PCR conditions were;

Heat cycle conditions: 1 cycle: 94°C -3 min 35 cycles: 94°C - 15 s, 55°C - 30 s, 72°C - 30 s / 1 cycle: 72°C 1 - 5 min.

Final concentrations (total 25 µL reaction volumes): 1X Taq polymerase buffer / 1.5 µM $MgCl_2$ / 0.4 µM forward primer / 0.4 µM reverse primer / 0.5 µM dNTP / 1 U(unit) Taq polymerase (F1, F4, and F6) or 1.25 U Taq polymerase (F2, F3, and F5) and 200ng DNA.

In the PCR, One-Taq polymerase was used for F2 (*Penicillium simplicissimum*), F3 (*Penicillium Talaromyces*) and F5 (*Stachybotrys chartarum*) and the expected length of the bands was obtained for F1 (*Penicillium thrichoderma*), F4 (*Metacordyceps chlamydosporia*) and F6 (*Alternaria alternata*). The designed three primers gave two results (Avcioglu-Dundar 2014). These tapes, which cleaned (in the case of multiple bands) or as a single band were cut from the agarose gel, were sent directly for sequence analysis for PCR reaction. For cleaning of the bands cut from the agarose gel, a Thermo-Scientific Gene JET Gel Extraction Kit was used. At last, primers, sequence and references used to identify the fungi are presented in Table 1.

Table 1. Primers, sequence and references used to identify the fungi (Erguven, 2015).

Fungi Code and Approximate species identity	First Primer 5’-3’ sequence and reference	Second Primer 5’-3’sequance and reference
Penicillium thrichoderma (F1)	ITS3 GCATCGATGAAGAACGCAGC (White et al., 1990).	ITS ATCCCTACCTGATCCGAGGTC (Avcioglu-Dundar 2014).
Penicillium simplicissimum(F2)	ITS6 GAAGGTGAAGTCGTAACAAGG (Cooke et al., 2000).	ITS ATCCCTACCTGATCCGAGGTC (Avcioglu-Dundar 2014).
Penicillium talaromyces (F3)	ITS4 TCCTCCGCTTATTGATATGC (White et al., 1990).	ITS6 GAAGGTGAAGTCGTAACAAGG (Cooke et al., 2000).
Metacordyceps chlamydosporia(F4)	ITSTRfw GAGACCGCCACTGTATTTCG (Avcioglu-Dundar 2014).	ITS3 GCATCGATGAAGAACGCAGC (White et al., 1990).
Stachybotrys chartarum (F5)	ITS1 TCCGTAGGTGAACCTGCGG (White et al., 1990).	ITS ATCCCTACCTGATCCGAGGTC (Avcioglu-Dundar 2014).
Alternaria alternata (F6)	ITS3 GCATCGATGAAGAACGCAGC (White et al., 1990).	ITS ATCCCTACCTGATCCGAGGTC (Avcioglu-Dundar 2014).

ITS: Internal transcribed spacer, TRfw: Thrichoderma forward primer

Microbial Biodegradation Studies

In order to determine the capacity of fungi on biodegradation of chlorsulfuron active ingredient, the quantification of this active ingredient was carried out using a Perkin Elmer Clarus 500 gas chromatograph. HP-5MS capillary column with a dimension of 30 m x 0.25 mm x 0.25 µm was used in the equipment. Chlorsulfuron active ingredient was detected using an electron capture detector (ECD), and the oven temperature program used in the analysis was at 70°C (2 min), 25°C min^{-1} to 145°C, 3°C min^{-1} to 190°C, and finally it was hold at 190°C for 5 min. The temperature of inlet and outlet of detector were 250°C and 320°C respectively. The make-up gas was ultra-pure nitrogen at a 30 ml min^{-1} flow rate and ultra-pure helium (He) was chosen for the carrier gas at 1.2 ml min^{-1} flow rate. Oven program was run for a total of 25 min. For calibration process, five standards were prepared with concentrations between 1 and 50 ng $µl^{-1}$.

COD, TOC and BOD_5 reduction for six fungi species, (approximately 1 x 10^7 CFU ml^{-1} each) were incubated under liquid culture conditions with 1 ml of Hammer Extra 75 DC (including 0,75g ml^{-1} of chlorsulfuron)

To prepare the liquid media, 1 ml of hammer extra 75 DC and 1 ml of enriched culture were added to 98 ml 0.8 % isotonic saline water. The solutions were prepared in the concentration that is actually used in the field for manufacturer instructions (75 mg l^{-1} per decare).

In biodegradation studies, liquid samples were monitored at 24-hour intervals for COD, TOC and BOD_5 levels. Closed reflux titrimetric method (standard method 5220C) was used for determinate COD reduction.[18] TOC measurements were performed according to standard method 5310B High temperature combustion method (APHA, 2009) and BOD5 test was conducted with Standard Method 5210B (5 day BOD_5 test) (APHA, 2009). All experiments were performed triplicate.

Results and Discussion

Reduction of Chlorsulfuron on COD, Active ingredient, TOC and BOD_5

The results of reduction of Chlorsulfuron in liquid media by *P. thrichoderma, P. simplicissimum, P. talaromyces, M. chlamydosporia, S. chartarum* and *A. Alternata* are presented in Figure 1, Figure 2, Figure 3, Figure 4, Figure 5 and Figure 6 respectively.

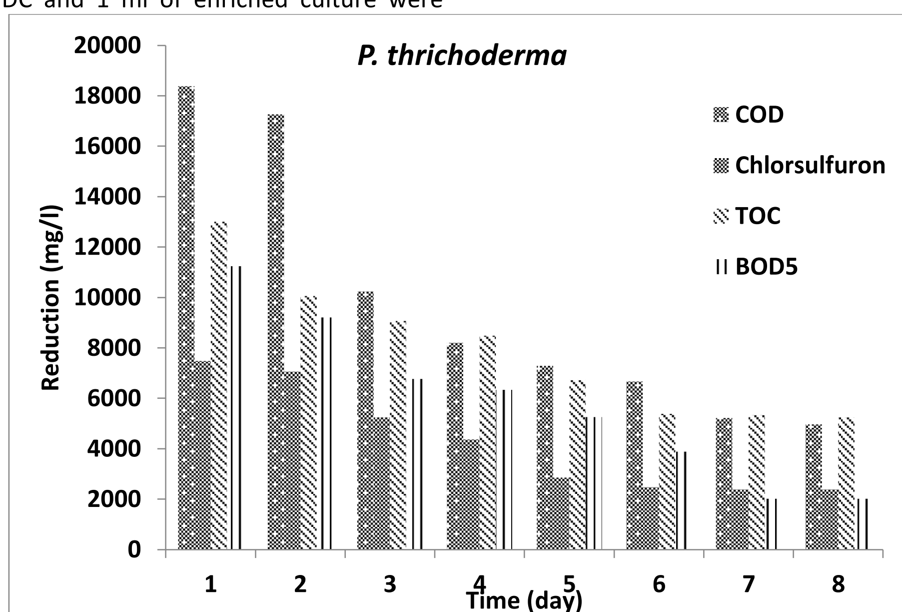

Figure 1. Reduction of chlorsulfuron by *P. thrichoderma*

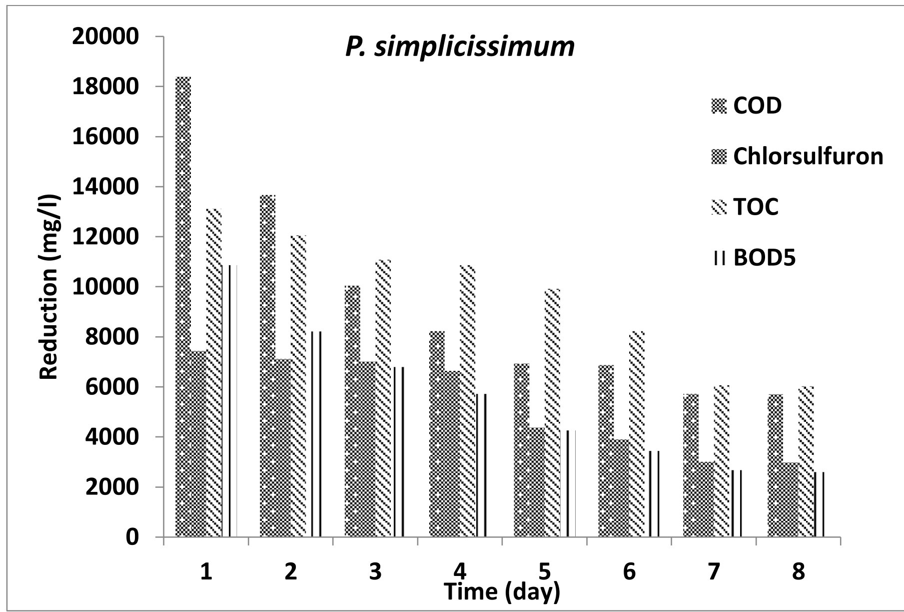

Figure 2. Reduction of chlorsulfuron by *P. simplicissimum*

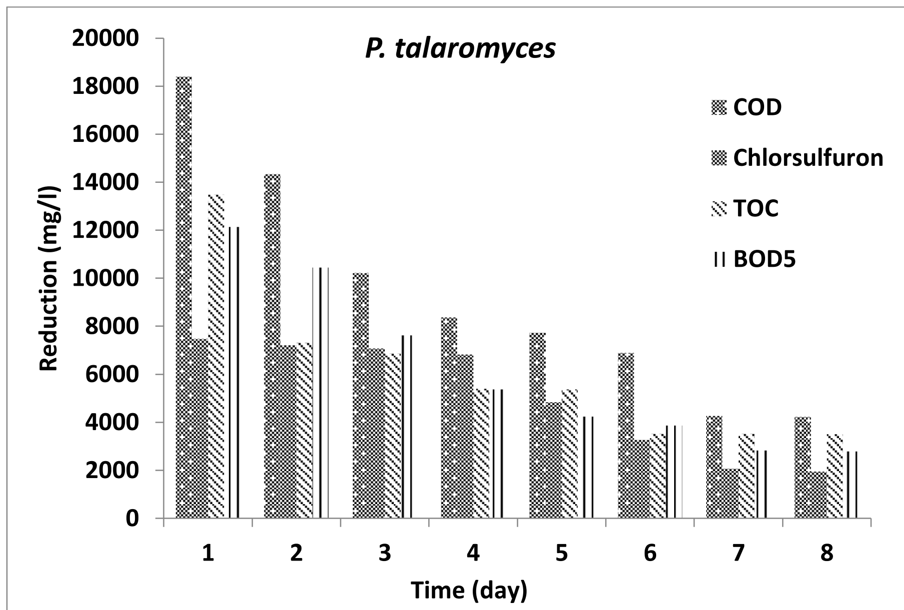

Figure 3. Reduction of chlorsulfuron by *P. talaromyces*

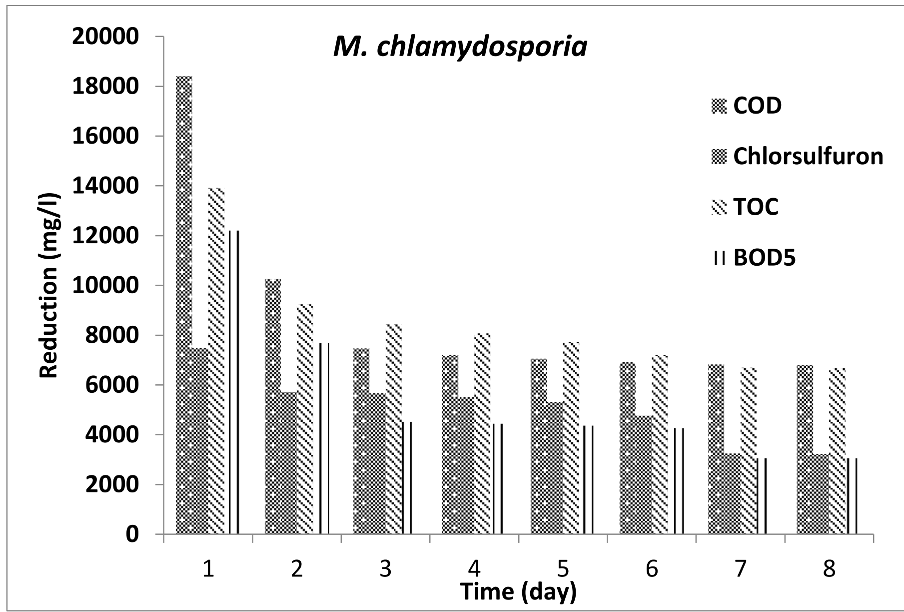

Figure 4. Reduction of chlorsulfuron by *M. chlamydosporia*

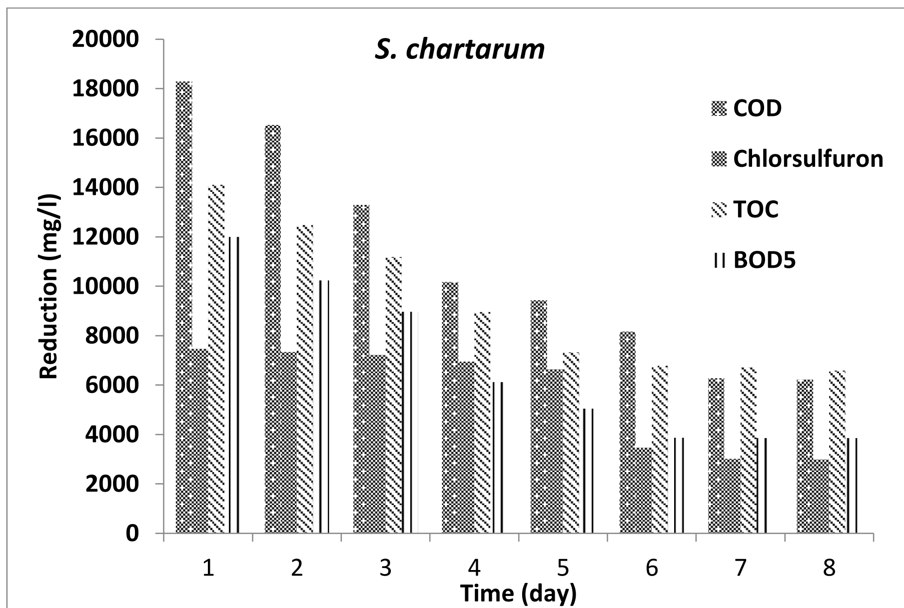

Figure 5. Reduction of chlorsulfuron by *S. chartarum*

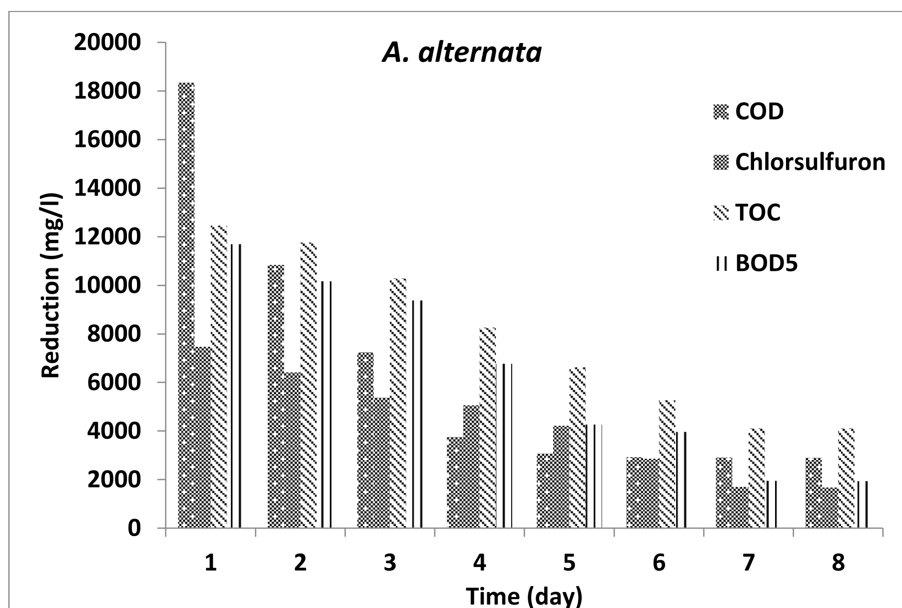

Figure 6. Reduction of chlorsulfuron by *A. alternata*

All reduction rates in the liquid media have showed different results depend on differences in fungal species. The COD reduction efficiencies of *P. thrichoderma, P. simplicissimum, P. talaromyces, M. chlamydosporia, S. chartarum and A. Alternata* species were 73, 69, 77, 63, 66 and 84%, respectively. Chlorsulfuron active ingredient reduction rates were 68, 60, 74, 57, 60 and 78% respectively. The reduction rates for TOC were 59, 54, 74, 52, 52 and 67%, At the end of the same time, the changes for BOD_5 was 82, 76, 77, 75, 68 and 83% respectively. There were negligible changes in COD, active ingredient, TOC and BOD_5 parameter at the end of 6th day. Previous studies on fungal degradation of similar herbicides revealed that relatively few bacterial species were actually able to degrade herbicides. Yanga et al. (2014) studied a bacterial strain with the ability to utilize chlorimuron-ethyl as the sole carbon source in Phosphate-basal minimal medium (PBM) cultures. They found chlorimuron-ethyl was provided as the sole carbon source, the increase of growth rate of bacterial strain accompanied with the depletion of chlorimuron-ethyl, and more than 95% of chlorimuron-ethyl at an initial concentration of 50 mg l^{-1} was degraded at the end of a 4-day incubation period at 30 ◦C. Zhang et al. (2009) investigated the degradation of chlorimuron-ethyl in sterilized soils with or without the addition of bacterial strain. According to the results of their study, there was a significant difference in residual chlorimuronethyl between treatments with regards to the addition of bacterial strains. At the end of two monthsof incubation, only 1.38% of chlorimuronethyl was degraded in the sterilized soil not supplemented with bacterial strain. As indigenous microorganisms display some degradation ability towards chlorimuron-ethyl, the degradation rate was slightly higher (7.56%) in the uninoculated natural soil. Degradation of chlorsulfuron by Aspergillus niger was studied under laboratory conditions by Boschin et al. (2003). As a result, after 28 days, the chemical degradation excluded, were about 30% for chlorsulfuron. In addition, they found Aspergillus niger fungus seems to be able to hydroxylate the aromatic ring of chlorsulfuron.

Dinamarca et al. (2007) reportad that, in polluted environments, bioremediation is an attractive technology for removal of herbicides. Selected s-triazine degrading strains have been applied for this processes. One of the interesting method for detection of striazine degrading microorganisms has been described in soils.

Previous study about biodegradation of the same herbicide, the COD reduction rates of *Bacillus simplex*, *Bacillus muralis, Micrococcus yunnanensis*, *Micrococcus luteus* and *Clostridium tetani* species were 94, 78, 79, 70 and 74%, respectively at the end of the 108th hour (Erguven and Yildirim, 2016). In a previous study about biodegradation of herbicide Aclonifen, COD removal rates were observed between 70 and 93%. According to these results, the highest COD reduction rate was achieved by *M. yunnanensis.* At the end of 5 days, 15600 mg l^{-1} of Aclonifen

was reduced to 1090 mg l^{-1} as COD. In this study, *M. luteus* displayed the lowest COD removal capacity as 70% (Erguven et al., 2015). In another study, the biodegradation ratios of trifluralin was found to be sufficiently high, especially in mixed fungi and bacteria cultures. In the liquid medium, they found the COD-removal efficiency of the culture medium varies according to microbial differences. The best degradation rate was seen by *Micrococcus luteus* and *Micrococcus yunnanensis* in four days, up to 91 %, and by *Bacillus simplex* and *Bacillus muralis* in five days. The results of the laboratory study showed important implication potential in the development of treatment systems for herbicide-contaminated aquatic environments (Erguven et al., 2016). Similarly with this study, we demonstrated COD reduction rates in the liquid media have showed different results depend on differences in bacterial species. The COD reduction efficiencies of *B. simplex, B. muralis, M. yunnanensis, M. luteus* and *C. tetani* species were 94, 78, 79, 70 and 74%, respectively. At the end of the 108th hour, there were negligible changes in COD (Erguven et al., 2016). Previous studies on microbial degradation of certain herbicides revealed that relatively few bacterial species were actually able to degrade these compounds.

Conclusion

One of the method of removing pesticides from liquid media is a bioremediation and bioremediation is a natural process. In this process, microorganisms can survive by degrading a herbicides. Most of the bacteria and fungi live in soil, air and water environments, but this situation is possible to change them to degrade herbicides with increasing speed. This properties of bacteria and fungi can be used as a cheap and useful technology to degrade herbicides. In addition, because using physical and chemical methods to degrade pesticide species are very expensive and hard, with useful of this technology, using fungi for this goal has been suggested by most of researchers. Because using fungi especially soil microorganisms is an economical and easy method so many researches have focused on biodegradation of herbicides with microorganisms. In recent years, most of studies regarding on biodegradation process of herbicides has been realized in fields where these herbicides are applied. Researches have been conducted around the world to determine pollutants and microorganisms in soil, water and air that have the ability to degrade pesticides. This research suggests biodegradation of herbicide chlorsulfuron with isolated some soil fungi from agricultural field so these fungi can improve the treatment.

In this study, it was observed that removal rates of chlorsulfuron in liquid media obtained by *P. thrichoderma, P. simplicissimum, P. talaromyces, M. chlamydosporia, S. chartarum* and *A. Alternata* were 73, 69, 77, 63, 66 and 84% as COD, 68, 60, 74, 57, 60, 78% as active ingredient, 60, 54, 74, 52, 53 and 67% as TOC, finally 82, 76, 77, 75, 68 and 83% as BOD_5 respectively. Based on these results, it could be concluded that *A. Alternata* had the highest removal rate.

As a result of this study, it was observed that *M. chlamydosporia* had the lowest COD and active ingredient removal efficiency. On the other hand, TOC reduction was similar with *P. simplicissimum, M. chlamydosporia* and *S. chartarum* species nearly 53%. There were a suitable fungi species for bioremediation of chlorsulfuron contaminated liquid media.

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