Microbial Removal of Cyanide Compounds and Soil Cyanide by *Klebsiella oxytoca*

Siyanür Bileşiklerinin ve Topraktaki Siyanürün *Klebsiella oxytoca* ile Mikrobiyal Giderimi

Research Article

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ABSTRACT

In this study, *Klebsiella oxytoca* ATCC 13182 degraded potassium cyanide, potassium hexacyanoferrate(II) trihydrate, potassium tetracyanonickelate(II) hydrate and sodium ferrocyanide decahydrate with the efficiencies of 100%, 87%, 78.5% and 27.5%, respectively. Additionally, optimal conditions for cyanide biodegradation were found as 30°C, 100 rpm and pH=7.0 at the concentration of 0.5 mM potassium cyanide in the biodegradation medium. Furthermore, *K. oxytoca* degraded potassium cyanide in the presence of different ions (Mg, Ni, Co, Fe, Ca, Cr, As, Cu and Zn) and as a result the growth amount of *K. oxytoca* decreased as the ion concentrations increased. It is also observed that 5:10 (v:v) concentration of sterile crude extract of *K. oxytoca* degraded 73.5% of the cyanide content in the biodegradation media in the first 24 hours. Finally, it is examined that 6 $mgkg^{-1}$ and 240 $mgkg^{-1}$ cyanide in soil samples were also degraded partially by *K. oxytoca*'s culture and sterile crude extract.

Key Words
Klebsiella oxytoca, cyanide, sterile crude extract, biodegradation.

ÖZ

Bu çalışmada, *Klebsiella oxytoca* ATCC 13182 suşunun potasyum siyanür, potasyum hekzasiyanoferrat(II) trihidrat, potasyum tetrasiyanonikelat(II) hidrat ve sodyum ferrosiyanür dekahidratı sırasıyla %100, %87, %78.5 ve %27.5 oranında bozunduğu belirlendi. Buna ek olarak, siyanür biyobozunma optimal koşullar 0.5 mM derişimde potasyum siyanür içeren biyobozunma besiyerinde, 30°C, 100 rpm ve pH 7.0 olarak bulundu. Ayrıca, *K. oxytoca* potasyum siyanürü farklı iyonların varlığında da (Mg, Ni, Co, Fe, Ca, Cr, As, Cu ve Zn) yıktığı gözlendi ve sonuç olarak *K. oxytoca*'nın üreme miktarının iyon derişimi arttıkça azalma gösterdiği belirlendi. *K. oxytoca*'nın 5:10 (h:h) derişimdeki steril kültür süpernatanının da ilk 24 saatte biyobozunma besiyerindeki siyanürün %73.5'ünü parçaladıgı gözlendi. Son olarak, *K. oxytoca* kültür ve steril kültür süpernatanının 6 $mgkg^{-1}$ ve 240 $mgkg^{-1}$ siyanür içeren toprak örneklerindeki siyanürü de kısmen bozunmaya uğratabildiği belirlendi.

Anahtar Kelimeler
Klebsiella oxytoca, siyanür, steril kültür özütü, biyobozunma.

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INTRODUCTION

By means of discharging untreated wastewaters from industrial processes and cyanide formation by different organisms such as bacteria, algae, fungi, plants and animals, cyanide accumulation becomes an important issue for environmental health because of its toxic, carcinogenic and mutagenic effects [1-5]. In this respect, it is significant to take precautions against cyanogen compounds in nature in order to protect environment.

In cyanide treatment processes, both of chemical and biological systems are being used in order to treat cyanogen compounds efficiently. Among them, although chemical methods detoxify cyanide in a short time, these methods use toxic chemicals, produce toxic sub products, require extra equipment and need high processing costs. However, when biological methods are used in cyanide treatment procedures, cyanide removal may be done with low costs and end products are generally nontoxic. Therefore, biological treatment methods are known as environmentally friendly [6-8]. In this respect, *Pseudomonas pseudoalcaligenes*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas aureofaciens*, *Azotobacter vinelandii*, *Bacillus pumilus, Paracoccus sp.* and lots of other microorganisms were investigated according to their cyanide removal capabilities in different researches and as a result, these microorganisms are being used as removal agents for cyanide and some other toxic compounds [2,8,9-12].

In this study, it is aimed to determine the cyanide biodegradation efficiencies of different *K. oxytoca* strains and to investigate optimization conditions and activator/inhibitor effects of different ions as contaminants on this biodegradation process of most effective cyanide biodegrading strain. Additionally, the usability of sterile crude extract of *K. oxytoca* strain was also examined in cyanide removal instead of *K. oxytoca* culture. In the last part of this study, different concentrations of cyanide containing soil samples were prepared and the efficiencies of *K. oxytoca* strains' culture and sterile crude extract on cyanide removal of these soil samples were also investigated.

MATERIALS and METHODS

Bacterial Strains

Cyanide degradation efficiencies of 6 different *K. oxytoca* strains were investigated in this study. Accordingly, *K. oxytoca* was inoculated into Luria Bertani Broth and incubated for log phase. After the incubation period, the absorbance of the culture was adjusted to A_{600nm}=1 and it was inoculated with the concentration of 1:10 (v:v) into the biodegradation media (pH=7) containing (gL^{-1}) 1 glucose, 0.5 K_2HPO_4, 0.5 KH_2PO_4, 0.05 $MgSO_4$ [10]. Incubation period was carried out at 30 °C, 100 rpm for 5 days (Certomat BS-I; Sartorius, Tokyo, Japan). At the end of the incubation period, the most effective strain in cyanide degradation process was selected in order to use in the rest of this study. Additionally, this strain was identified by using 16S rRNA analysis (Refgen, Ankara, Turkey) and it is determined as 99.9% similar to *K. oxytoca* ATCC 13182 [13].

Analysis of Biodegradation Products

Residual cyanide concentration was assessed by using picric acid method [14] and nesslerization was used to evaluate the concentration of ammonia in the biodegradation medium [15]. Additionally, the growth amount of *K. oxytoca* cultures was measured by spectrophotometrically (UV 1700, Shimadzu, Tokyo, Japan) at A_{600nm}.

Biodegradation Capability of *K. oxytoca* Against Different Cyanide Sources

Selected *K. oxytoca* strain was inoculated into the biodegradation media containing different cyanide sources (potassium cyanide, potassium hexacyanoferrate(II) trihydrate, potassium tetracyanonickelate(II) hydrate and sodium ferrocyanide decahydrate) separately with the concentration of 0.5 mM and incubation period was carried out at 30°C, 100 rpm for 5 days (Certomat BS-I; Sartorius, Tokyo, Japan). The experiment was performed in triplicate.

Investigation of Optimal Cyanide Biodegradation Conditions for *K. oxytoca*

Incubation period (1 to 6 days), initial pH value (3 to 10), incubation temperature (20°C to 50°C), initial KCN concentration (0.25 mM–2 mM) and

rotation speed (0 to 200 rpm) were investigated in order to examine the optimal conditions for cyanide biodegradation by using *K. oxytoca* strain. All of these experiments were performed in triplicate.

Effect of Ions on Cyanide Biodegradation Process

In this part, activator and inhibitor effects of different concentrations (0.1 mM, 0.25 mM and 0.5 mM) of ions (Mg, Ni, Co, Fe, Ca, Cr, As, Cu and Zn) on cyanide biodegradation process were investigated. Accordingly, *K. oxytoca* strain (A_{600nm}= 1.0) was inoculated with the concentration of 1:10 (v:v) into the ion supplemented biodegradation media and incubation period was carried out at 30°C, 100 rpm for 5 days (Certomat BS-I; Sartorius, Tokyo, Japan). The experiments were performed in triplicate.

Effect of Sterile Crude Extract of *K. oxytoca* on Cyanide Biodegradation Process

Biodegradation capability of sterile crude extract of *K. oxytoca* was also investigated in this study. Accordingly, *K. oxytoca* was inoculated into cyanide biodegradation media with the concentration of 1:10 (v:v) and incubated in a rotary shaker (Certomat BS-I; Sartorius, Tokyo, Japan) at 30°C, 100 rpm for 5 days. After the incubation period, culture was centrifuged at 4000 rpm for 5 minutes (Eppendorf Centrifuge 5417R; Hamburg, Germany). Supernatant was taken and sterilized by 0.45 μM cellulose acetate filter (Millipore) in order to obtain sterile crude extract of *K. oxytoca*. Accordingly, different concentrations of sterile crude extract [1:10, 2:10, 3:10, 4:10 and 5:10 (v:v)] were inoculated into the biodegradation media separately. Incubation period was carried out at 30°C and at 100 rpm (Certomat BS-I; Sartorius, Tokyo, Japan). The experiment was performed in triplicate.

Cyanide Removal in Soil

Soil samples were collected from Beytepe/Ankara, Turkey and sterilized with autoclave at 121°C, 1.5 atm for 15 min. After the sterilization period, 6 $mgkg^{-1}$ and 240 $mgkg^{-1}$ cyanide containing soil media were prepared in order to examine cyanide removal efficiencies of *K. oxytoca*'s culture and its sterile crude extract. Accordingly, culture and sterile crude extract of *K. oxytoca* strain were inoculated into the biodegradation media with the concentrations of 1:10 (v:v) separately and incubation period was carried out at 30°C, 100 rpm and 5 days in a rotary shaker (Certomat BS-I; Sartorius, Tokyo, Japan). The experiment was performed in triplicate.

RESULTS and DISCUSSION

By means of discharging untreated wastewaters through the soil and water from different industrial processes, cyanide compounds dissociate in water to its ions and form complexes with heavy metals rapidly which represent different toxicity and stability [16]. Accordingly, these metal cyanide complexes metabolize or transform into nontoxic end products by microorganisms as a carbon and nitrogen source [8]. Therefore, in this study, 6 different *K. oxytoca* strains were investigated according to their cyanide biodegradation abilities and Kox1 strain which was identified as *K. oxytoca* ATCC 13182 by using 16S rRNA analysis (Refgen, Ankara, Turkey), was found to be the most effective one in cyanide removal (Figure 1).

In this study, it is observed that, *K. oxytoca* degraded tetracyanonickelate with an efficiency of 27.5% under unoptimized conditions (Figure 2). Yanase et. al. [17] found that tetracyanonickelate was degraded by using cell free extracts of bacteria and fungi and these microorganisms degraded it with the efficiencies of 20-30% and 100% in about 1 month, respectively. On the other hand, whereas hexacyano complexes of iron, which is known as ferro cyanides, are stable and recalcitrant cyanide compounds [18], *K. oxytoca* strain also degraded potassium hexacyanoferrate(II) trihydrate and sodium ferrocyanide decahydrate in unoptimized conditions (Figure 2). Accordingly, our results indicated the usability of *K. oxytoca* in the biotreatment processes of wastewaters contaminated with different cyanogen compounds.

Furthermore, although in different researches, it is determined that *A. tumefaciens* degraded cyanide with a percentage of 75 [19], *P. fluorescens* with a percentage of 78.9 [8] and

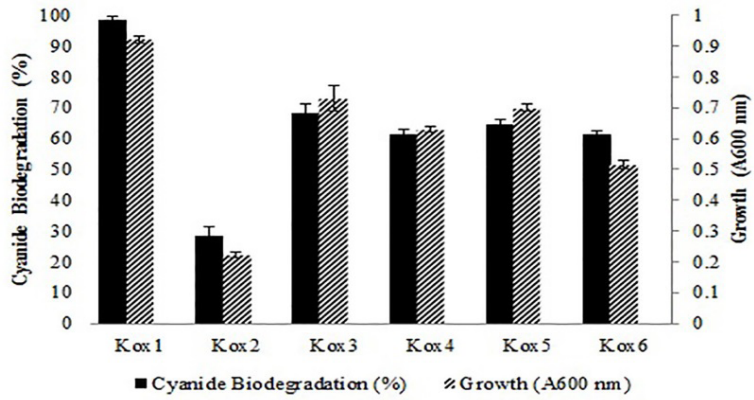


Figure 1. Biodegradation of potassium cyanide by different *K. oxytoca* strains.

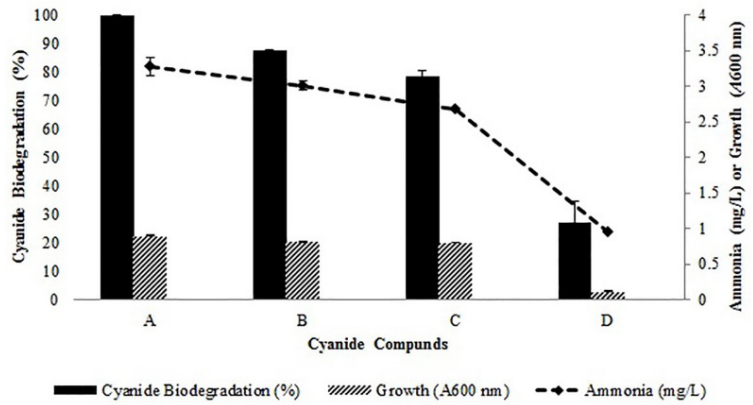
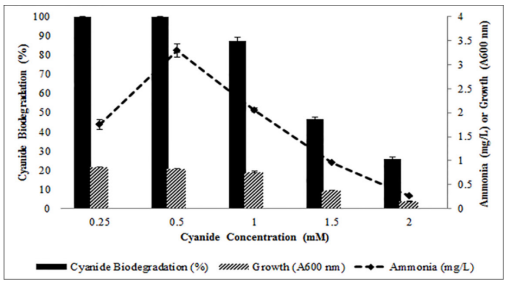


Figure 2. Biodegradation ability of *K. oxytoca* strain against different cyanide compounds.
(A:Potassium cyanide; B: Potassium hexacyanoferrate(II) trihydrate; C: Sodium ferrocyanide decahydrate and D: Potassium tetracyanickelate (II) hydrate).

these strains are indicated as usable agents in cyanide biodegradation processes, *K. oxytoca* degraded over 70% of the cyanide content in the biodegradation media in the first 24 hours of the incubation period in this study (Figure 3a). Additionally, in previous studies *P. fluorescens* degraded 0.25 mM KCN and it is found that growth and cyanide degradation amount decreased as the concentration increased [20]. However, in this study *K. oxytoca* degraded 1 mM KCN over 80% efficiency and it also degraded 1.5 mM and 2 mM KCN with a rotation speed of 100 rpm (Figure 3b and Figure 3c). In this respect, these results indicated that *K. oxytoca* strain is an important agent for biodegradation processes.

Additionally, pH of the industrial wastes in which cyanide are being used or cyanogen compounds are formed, is approximately 7.5 to 10.0 [21]. In this respect, microorganisms which may be used in these cyanide degradation processes must have a wide range of pH toleration to grow and to form biodegradation enzymes. In this study, *K. oxytoca* exhibited a great pH tolerance since this strain degraded cyanide over 70% between the pH values of 3-10 and over 90% between the pH values of 6-8 (Figure 3d) which is resemble with other researches which found as pH= 7.5 for *Cryptococcus humicola* [22], pH= 7-8.5 for *Azotobacter vinelendii* [10] and pH= 7-8 for a consortium including *Bacillus sp., Klebsiella sp.* and *Pseudomonas sp.* [23].

In previous researches, optimal cyanide biodegradation temperatures were found as 25°C for *Cryptococcus humicola* [22] and 35°C for *Serratia marcescens* [24]. Accordingly, in this study whereas optimal temperature for cyanide biodegradation by using *K. oxytoca* is found as 30°C, it is also examined that this



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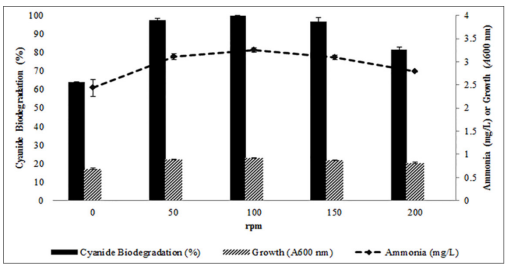

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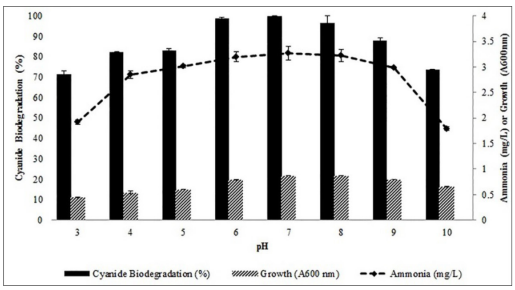

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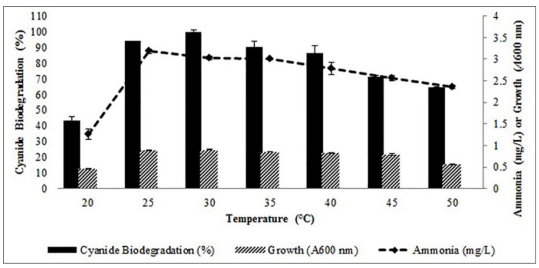
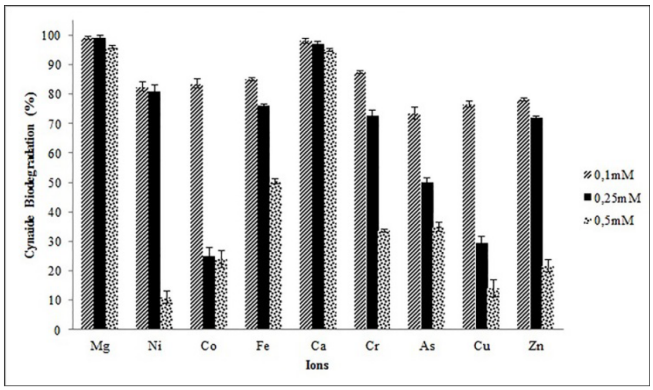


(e)

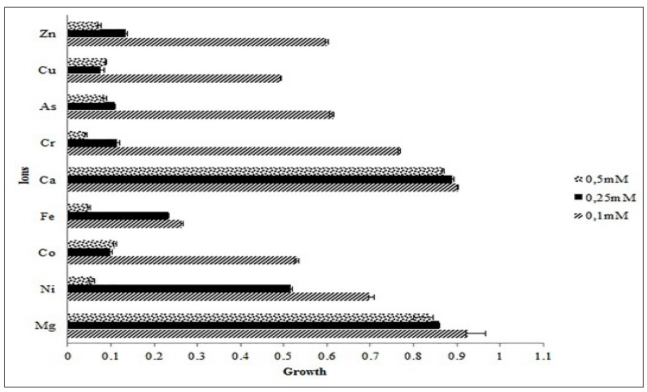
Figure 3. Optimization conditions for potassium cyanide biodegradation by *K. oxytoca* (a) Incubation day; (b) Cyanide Concentration; (c) Rotation Speed; (d) pH and (e) Temperature.

strain degraded over 70% of the cyanide content in the biodegradation media between the incubation temperatures of 25-45°C too (Figure 3e). Therefore, these results also indicated that enzymes of *K. oxytoca* synthesize effectively in different temperatures and it is seen as advantageous for using *K. oxytoca* strain in cyanide biodegradation processes which requires both of low and high temperatures.

Steel, coal and mining industries discharge wastes including heavy metals such as nickel, copper, iron and zinc [25,26]. Therefore, it is significant to select stable microorganisms in order to biodegrade cyanide in the presence of different ions in variable concentrations. In different researches, it is observed that, arsenic,



(a)



(b)

Figure 4. Effects of different ions on (a) potassium cyanide biodegradation, (b) growth.
(Mg: Magnesium; Ni: Nickel; Co: Cobalt; Fe: Iron; Ca: Calcium; Cr: Chromium; As: Arsenic; Cu: Copper; Zn: Zinc).

copper, iron and zinc are effective heavy metals on bacterial growth and bacterial enzymes for degradation processes [6,18]. Additionally, in a previous study it is found that cyanide degradation by *Burkholderia cepacia* directly correlates with the concentrations of Ni, Co, Mn and Mo ions [27]. In another study, it is found that Ni, Cu and Zn ions didn't affect cyanide biodegradation but 20% inhibition was observed in the presence of Pb and Cd ions and 30 - 35% inhibition was also observed in the presence of Fe by a bacterial consortium [21]. In a different study, it is found that in the presence of Cu and Ca, growth and cyanide degradation of a bacterial consortium were also inhibited. However, when Mg and Mn are used, the growth amount didn't affect from these ions but cyanide degradation decreased [23]. In this study, *K. oxytoca* degraded potassium cyanide even in the presence of different concentrations (0.1 mM, 0.25 mM and 0.5 mM) of all ions (Mg, Ni, Co, Fe, Ca, Cr, As, Cu and Zn) and the growth amount of *K. oxytoca* decreased as the ion concentrations increased (Figure 4a and Figure 4b). In this respect, results indicate that our strain is capable of degrading cyanide compounds in the presence of different concentrations of variable ions and as a result this strain may be used in biodegradation processes of cyanide contaminated areas.

Microorganisms degrade different compounds by synthesizing intracellular and extracellular enzymes. Therefore, not only bacterial cultures but also bacterial crude extracts are also effective in biodegradation processes. In the literature, among all *Klebsiella* species, *K. ozaneae* is the first investigated species for degrading cyanide with its extracellular enzyme called cyanide hydratase [28]. In this study, it is found that 73.5% of the cyanide content in the biodegradation media including sterile crude extract with a concentration of 5:10 (v:v) was degraded in the first 24 hours. Additionally, fully degradation

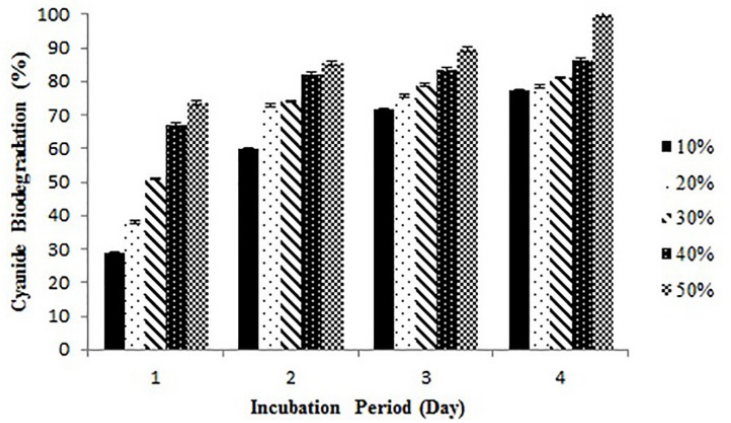


Figure 5. Biodegradation capability of sterile crude extract of *K. oxytoca*.

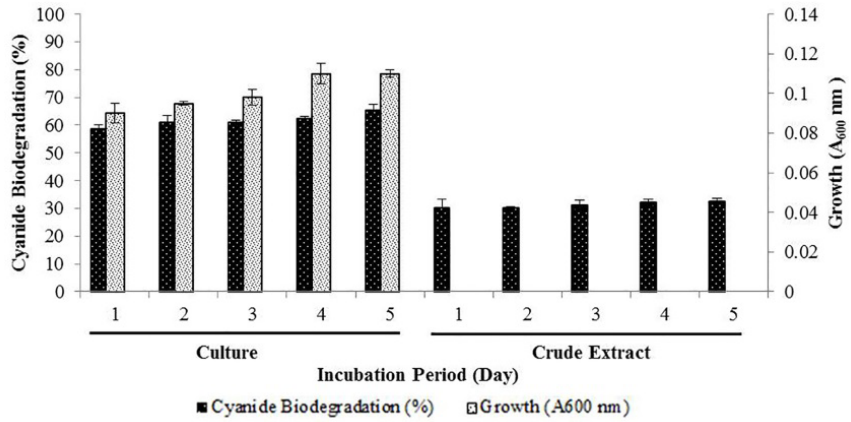


Figure 6. Cyanide removal in 6 mgkg^{-1} cyanide containing soil media with cultures and sterile crude extracts *K. oxytoca*.

was observed at the end of the 4th day of the incubation period (Figure 5). In this respect, it is observed that sterile crude extract of *K. oxytoca* is also active in cyanide biodegradation process.

Accordingly, it is the first report for the extracellular enzyme of *K. oxytoca* in cyanide biodegradation process and it may be served as a source for future analysis of cyanide biodegradation by using extracellular enzyme of *K. oxytoca*.

Soil includes a wide range of organic and inorganic cyanide compounds as well as other organic and inorganic substances in its content [29]. Due to the usage of cyanide containing substances in daily life such as road salts and fire retardants and production of cyanide by a wide range of plants, algae and animals, cyanide accumulation in nature is increasing day by day [29-33]. Accordingly, accumulation of cyanide in soil and water may cause serious health risks on living organisms. Therefore, it is very important to take precautions for removal of cyanogen compounds. In this respect, in situ and ex situ cyanide removal techniques are being used in bioremediation of toxic substances in soil and other environmental areas. In this respect, biodegradation efficiencies of *K. oxytoca*'s culture and sterile crude extract on cyanide containing soil samples were also investigated in this study. As a result, it is found that *K. oxytoca* culture (65%) is more efficient than the sterile crude extract (33%) in the cyanide removal of 6 mgkg^{-1} cyanide containing soil sample (Figure 6). Additionally, when cyanide removal in 240 mgkg^{-1}

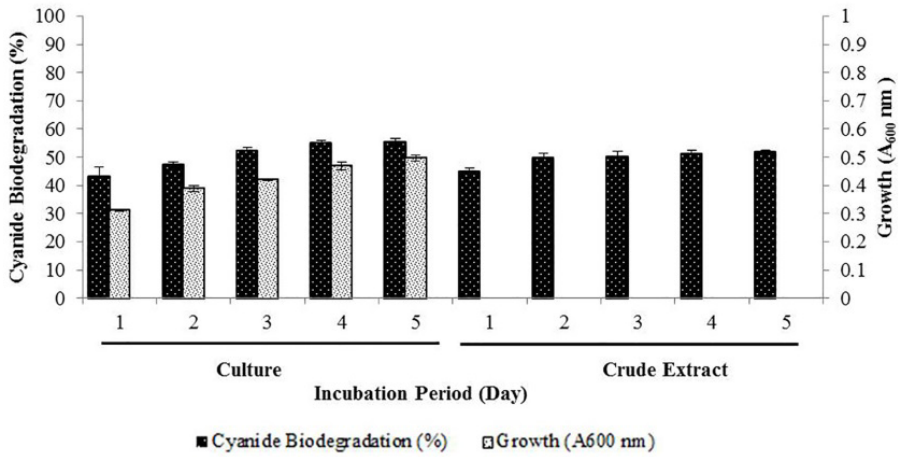


Figure 7. Cyanide removal in 240 mg.kg^{-1} cyanide containing soil media with cultures and sterile crude extracts of *K. oxytoca*.

cyanide containing soil sample is investigated, it is examined that both of the culture and sterile crude extract are also effective (55% and 52%, respectively) in cyanide removal (Figure 7). Accordingly, it is observed that both of *K. oxytoca*'s culture and sterile crude extract are efficient in the cyanide degradation of 6 mgkg^{-1} and 240 mgkg^{-1} cyanide containing soil samples. In literature, cyanide containing soil samples are generally treated by phytoremediation agents such as *Sorghum bicolor*, *Linum usitassium* [34], *Eichornia crassipes* [35], *Salix babylonica* [36] and *Zea mays* [37]. In parallel with our research, in another study it is found that *Bacillus subtilis* and *Pseudomonas stutzeri* degraded cyanide in soil (0.218 mg/g cyanide/soil) with the percentages of 66.9% and 72% in 10 days, respectively [38]. In this respect, *K. oxytoca* culture and sterile crude extract may be used in bioremediation of cyanide in contaminated areas too.

CONCLUSION

The results of this research demonstrated the importance of *K. oxytoca* in the removal of different cyanide compounds, stability against increasing cyanide concentration, pH and temperature, different concentrations of variable ions in cyanide degradation processes. Additionally, different than other researches, sterile crude extract of *K. oxytoca* was used in cyanide biodegradation processes. In this respect, the results of this study are also promising for the future researches on *K. oxytoca* strain's usability to degrade cyanide wastes contaminated with different concentrations of heavy metals by using its culture or crude extract.

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