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Biological Treatment of Cyanide by Using *Klebsiella pneumoniae* Species

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Summary

In this study, optimization conditions for cyanide biodegradation by *Klebsiella pneumoniae* strain were determined to be 25 °C, pH=7 and 150 rpm at the concentration of 0.5 mM potassium cyanide in the medium. Additionally, it was found that *K. pneumoniae* strain is not only able to degrade potassium cyanide, but also to degrade potassium hexacyanoferrate(II) trihydrate and sodium ferrocyanide decahydrate with the efficiencies of 85 and 87.5 %, respectively. Furthermore, this strain degraded potassium cyanide in the presence of different ions such as magnesium, nickel, cobalt, iron, chromium, arsenic and zinc, in variable concentrations (0.1, 0.25 and 0.5 mM) and as a result the amount of the bacteria in the biodegradation media decreased with the increase of ion concentration. Lastly, it was also observed that sterile crude extract of *K. pneumoniae* strain degraded potassium cyanide on the fifth day of incubation. Based on these results, it is concluded that both culture and sterile crude extract of *K. pnemoniae* will be used in cyanide removal from different wastes.

Key words: Klebsiella pneumoniae, cyanide, biodegradation

Introduction

Untreated effluents of industrial processes are mainly responsible for environmental pollution with various forms of toxic substances, especially free cyanides and metal cyanide complexes (*1–4*). Since cyanide is highly reactive and forms different complexes with transitional metals, it is a dangerous toxic compound for living organisms (*4–6*). Additionally, cyanide is also the inhibitor of cytochrome oxidases in electron transport chain, which is a crucial pathway in the respiratory system of bacteria and other organisms (*1,3,5,7,8*). In this respect, treatment of cyanide in wastewaters and in contaminated areas is an important issue for scientists.

In order to protect the environment from the toxic effects of cyanogen wastes at low costs, different microorganisms are being used in the treatment of cyanogen compounds. Based on the investigations obtained from the literature, *Burkholderia cepacia* (*2*), *Pseudomonas pseudoalcaligenes* (*6*), *Pseudomonas putida* (*1*), *Agrobacterium tumefaciens* (*9*), *Klebsiella oxytoca* (*3*), *Bacillus pumilus* (*10*), *Fusarium oxysporum* (*11*), *Rhizopus oryzae* (*12*) and *Trichoderma* sp. (*13*) are known as some of the cyanide-biodegrading microorganisms.

Klebsiella sp., which is a natural colonizer of humans, vertebrates, birds, reptiles and even insects, is also isolated from contaminated areas such as soil and wastewaters (*14–17*). Accordingly, *Klebsiella* sp. is used as a remediation agent in research of the removal of azo dyes, phenols, hexahydro-1,3,5-trinitro-1,3,5-triazine, organochlorine insecticides, aromatic amines and many other toxic substances (*16–19*). However, biodegradation of different cyanide sources, optimization of medium containing cyanide and effect of different ions on cyanide biodegradation using *K. pneumoniae* have not been studied yet. Therefore, in this study, the aim is to investigate the cyanide biodegradation abilities of 17 different *K. pneumoniae* strains

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and to select the most efficient one, as well as to determine the effects of optimization conditions for cyanide biodegradation process. Additionally, biodegradation efficiency of *K. pneumoniae* in the presence of different cyanide compounds (potassium hexacyanoferrate(II) trihydrate, potassium tetracyanonickelate(II) hydrate and sodium ferrocyanide decahydrate) was also determined. Furthermore, the effects of different ions as contaminants in the biodegradation medium and the efficiency of sterile crude extract of *K. pneumoniae* strain as an extracellular product on cyanide biodegradation were also investigated.

Materials and Methods

Klebsiella pneumoniae strains and growth conditions

Seventeen different *Klebsiella pneumoniae* strains were investigated according to their cyanide degradation efficiencies. Accordingly, *K. pneumoniae* strains were inoculated into the biodegradation medium and incubated at 30 °C and 150 rpm for 5 days (Certomat BS-I; Sartorius, Tokyo, Japan). At the end of the incubation, the most effective strain in cyanide degradation was identified by using 16S rRNA analysis which showed similarity to *K. pneumoniae* ATCC 13883 strain (Refgen, Ankara, Turkey). Accordingly, this Kp2 strain was used for the rest of the study.

Biodegradation medium

K. pneumoniae strains were incubated in the Luria Bertani broth (enrichment medium) at 37 °C and 150 rpm for log phase (Certomat BS-I; Sartorius). After the incubation, absorbance of the culture was adjusted to 1.0 at λ=600 nm spectrophotometrically (UV 1700; Shimadzu, Tokyo, Japan) (*3*). *Klebsiella* culture was inoculated in the biodegradation medium (pH=7) containing (in g/L): glucose 1, K_2HPO_4 0.5, KH_2PO_4 0.5, $MgSO_4$ 0.05 (*20*) at the volume ratio 1:10. Incubation was performed at 30 °C and 150 rpm for 5 days (Certomat BS-I; Sartorius). The experiment was performed in triplicate.

Analysis of potassium cyanide biodegradation products

Residual cyanide concentration was assessed by using modified picric acid method (*21*) as follows: 0.5 mL of 0.5 % (by mass per volume) picric acid solution and 0.5 mL of 0.25 M Na_2CO_3 solution were added into 0.5 mL of culture supernatant. This mixture was boiled for 5 min, then diluted to 10 mL with 8.5 mL of distilled water and cooled under tap water for 30 min, after which its absorbance was measured spectrophotometrically at λ=520 nm (model UV 1700; Shimadzu).

Modified nesslerization method (*22*) was also used to evaluate the concentration of ammonia in the biodegradation medium. Accordingly, 0.5 mL of culture supernatant was diluted with 0.5 mL of distilled water, 0.05 mL of EDTA (0.01 mol) and 2 mL of Nessler's reagent (Merck, Darmstadt, Germany) were added into the diluted sample and after 10 min the absorbance of the solution was measured spectrophotometrically at λ=425 nm (UV 1700; Shimadzu).

Finally, growth of *K. pneumoniae* Kp2 strain in the biodegradation medium was measured spectrophotometrically at λ=600 nm (UV 1700; Shimadzu).

Effects of physiological conditions on potassium cyanide biodegradation

Effects of incubation period (1 to 4 days), initial pH value (3 to 10), incubation temperature (20 to 50 °C), initial KCN concentration (0.25 to 2 mM) and rotation speed (0 to 200 rpm) were investigated in order to determine optimal potassium cyanide biodegradation conditions by *K. pneumoniae* Kp2 strain. The experiments were performed in triplicate.

Effect of ions on potassium cyanide biodegradation process

The effect of magnesium, nickel, cobalt, iron, chromium, arsenic, copper and zinc ions on the biodegradation of potassium cyanide was also investigated. In this respect, ion concentrations of 0.1, 0.25 and 0.5 mM were added into the biodegradation medium separately and *K. pneumoniae* Kp2 strain was inoculated into it at the volume ratio 1:10. Incubation was carried out at 25 °C and 150 rpm for 3 days (Certomat BS-I; Sartorius). The experiment was performed in triplicate.

Biodegradation of different cyanide sources by K. pneumoniae

In order to investigate the biodegradation ability of *K. pneumoniae* against different cyanide compounds (potassium hexacyanoferrate(II) trihydrate, potassium tetracyanonickelate(II) hydrate and sodium ferrocyanide decahydrate), biodegradation medium was prepared by using 0.5 mM of each cyanide compound separately. *K. pneumoniae* Kp2 was inoculated into this medium at the volume ratio 1:10 and incubation was performed at 25 °C and 150 rpm for 3 days (Certomat BS-I; Sartorius). The experiment was performed in triplicate.

Effect of K. pneumoniae crude extract on potassium cyanide biodegradation

K. pneumoniae Kp2 was incubated into the biodegradation medium at the volume ratio 1:10 and incubation was performed at 25 °C and 150 rpm for 3 days (Certomat BS-I; Sartorius). After incubation, cultures were centrifuged at 2200×*g* for 5 min (Eppendorf Centrifuge 5417R; Hamburg, Germany). Culture supernatant was taken and sterilized by using 0.45 μM cellulose acetate filter (Sartorius) to obtain sterile crude extract of *K. pneumoniae* Kp2. Accordingly, different mass per volume ratios of this sterile crude extract (10, 20, 30, 40 and 50 %) were inoculated into the biodegradation medium separately and incubation was performed at 25 °C and 150 rpm (Certomat BS-I; Sartorius) in order to examine the biodegradation efficiency of sterile crude extract. The experiment was performed in triplicate.

Results and Discussion

Industrial discharge of cyanide and cyanogen compounds may have serious effect on living organisms (*3,4,23*). Therefore, the contaminated wastewaters or soils must be treated before disposal in order to protect the environment (*5*). Accordingly, different chemical treatments

are used in order to detoxify cyanide and its derivatives. However, when chemical treatments are used, additional methods are required to detoxify the by-products that are produced. In contrast to chemical treatment, biological treatments form nontoxic end products such as ammonia, so they are seen to be more advantageous and successful than the chemical ones (*1,4,5,7,10,24–26*). In this respect, biodegradation of cyanide by using different microorganisms including bacteria, fungi and plants are used in cyanide biodegradation processes (*27,28*).

In this study, biodegradation abilities of seventeen different *K. pneumoniae* strains were investigated and Kp2 was selected as the most effective one (Fig. 1). This strain was determined to be similar to *K. pneumoniae* ATCC 13883 by 16S rRNA analysis.

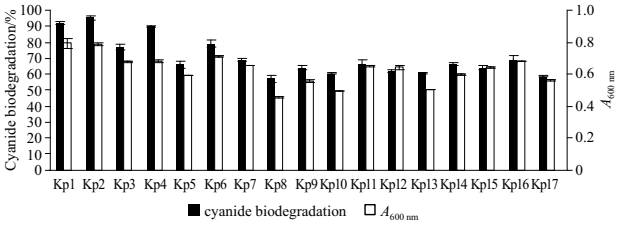

Fig. 1. Biodegradation of potassium cyanide by different *K. pneumoniae* strains (Kp1–Kp17)

Optimization conditions for cyanide degradation by *K. pneumoniae* Kp2 were investigated for application in different contaminated soils and wastewaters including cyanogen compounds. As a result, complete degradation of potassium cyanide was observed on the third day (Fig. 2) under the optimal conditions of 0.5 mM initial potassium cyanide concentration (Fig. 3), initial pH=7 (Fig. 4), rotation speed of 150 rpm (Fig. 5) and incubation temperature of 25 °C (Fig. 6). Growth of *K. pneumoniae* Kp2 under different conditions and formation of ammonia by degradation of potassium cyanide are also shown in Figs. 2–6.

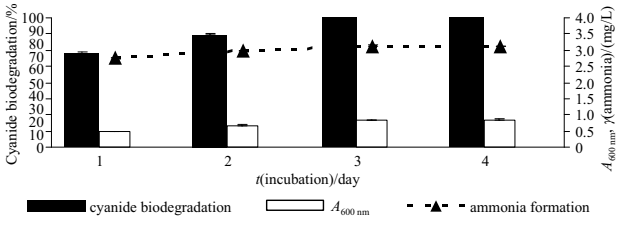

Fig. 2. Effect of incubation period on cyanide biodegradation by *K. pneumoniae* Kp2

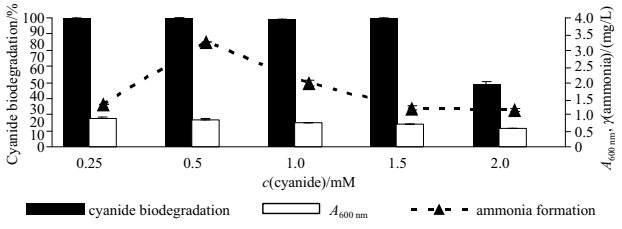

Fig. 3. Effect of initial cyanide concentration on cyanide biodegradation by *K. pneumoniae* Kp2

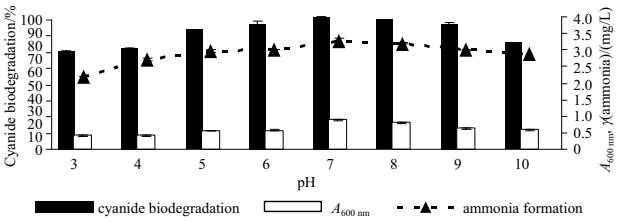

Fig. 4. Effect of initial pH on cyanide biodegradation by *K. pneumoniae* Kp2

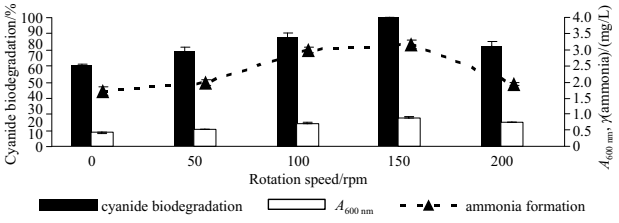

Fig. 5. Effect of rotation speed on cyanide biodegradation by *K. pneumoniae* Kp2

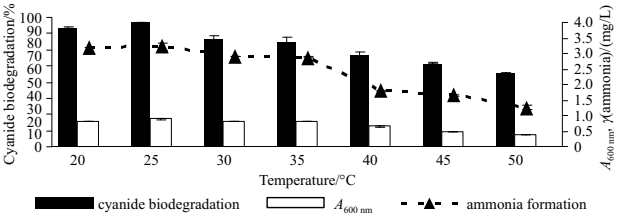

Fig. 6. Effect of temperature on cyanide biodegradation by *K. pneumoniae* Kp2

Literature shows that co-cultures of *Ralstonia* sp. and *Klebsiella pneumoniae* degraded tetracyanonickelate in 48 h (*29*). Another study concluded that *P. pseudoalcaligenes* degraded sodium cyanide in 14 days (*6*). It was determined that *Acremonium strictum* degraded tetracyanonickelate in 85 h and *Cryptococcus humicola* in 60 h (*30*). Previous research found that concentrated resting cells of *Klebsiella oxytoca* degraded 0.58 mM of potassium cyanide in 32 h and 0.9 mM of potassium cyanide in 80 h (*5*). Furthermore, immobilized cells of *K. oxytoca* degraded approx. 91 % of 1 mM KCN in 20 h (*4*) and anaerobically degraded tetracyanonickelate in 60 h (*8*). In accordance with this study, incubation temperature of 25–30 °C (*2–6,8,29*), pH= 6.5–7.5 (*3–5,8,29–31*) and rotation speed of 100–200 rpm (*8,29,31,32*) were determined as optimal conditions for biodegradation of different cyanide and cyanogen compounds in different research papers.

Additionally, in this study *K. pneumoniae* Kp2 degraded potassium hexacyanoferrate(II) trihydrate and sodium ferrocyanide decahydrate with the efficiencies of 85 and 87.5 % in three days, respectively (data not shown). Therefore, these results indicate that *K. pneumoniae* Kp2 can be used as alternative bacteria in the biotreatment of wastewaters contaminated with different cyanide sources.

In addition to cyanide, arsenic, chromium, copper, iron, nickel and zinc in gold mining industry, cadmium, chromium, copper, nickel and zinc in metal and electroplating industries, are the main contaminants of untreated industrial effluents (*3,33*). These ions bind with cya-

nide and form various compounds that exhibit different stability and toxicity (*31,20*). In this respect, different concentrations of ions (0.1, 0.25 and 0.5 mM) were added into the biodegradation medium and the correlation between cyanide biodegradation and the growth of *K. pneumoniae* Kp2 in the presence of ions was investigated. As a result, *K. pneumoniae* Kp2 was found to degrade KCN in the presence of different ions except for copper at concentration of 0.5 mM. Additionally, in accordance with the other studies the amount of the bacteria in the biodegradation medium was found to decrease with the increase of ion concentration (Fig. 7). However, this drop does not inhibit the biodegradation efficiency of *K. pneumoniae*, except for media containing 0.5 mM of arsenic (2 %), zinc (6 %) and chromium (8 %) (Table 1). Therefore, these results indicated that *K. pneumoniae* Kp2 strain can be used as a remediation agent in the treatment of cyanogen wastes.

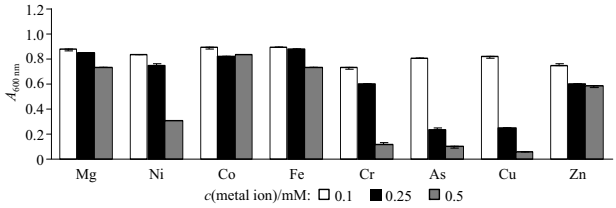

Fig. 7. Effect of different ions on the growth ($A_{600\,nm}$) of *K. pneumoniae* Kp2 in cyanide biodegradation process

Table 1. Cyanide biodegradation in the presence of different concentrations of ions

Ion	*c*/mM		
	0.1	0.25	0.5
	Cyanide biodegradation/%		
Mg	94	72	86
Ni	84	74	27
Co	98	86	88
Fe	100	99	72
Cr	72	63	8
As	56	43	2
Cu	83	20	0
Zn	79	68	6

Lastly, apart from full biodegradation of potassium cyanide by sterile crude extract of *K. pneumoniae* observed on the third day in this study, it was observed that 50 % of crude extract fully degraded potassium cyanide on the fifth day (Fig. 8). This result indicates that not only the culture of *K. pneumoniae* but also its crude extract have the ability to degrade KCN.

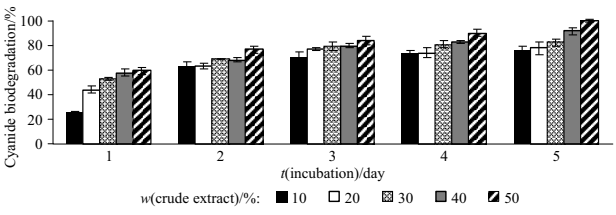

Fig. 8. Effect of incubation period on cyanide biodegradation by different concentrations of sterile crude extract of *K. pneumoniae* Kp2

Conclusions

The results of this research demonstrated the efficiency of *Klebsiella pneumoniae* and its sterile crude extract in the biodegradation of different cyanogen compounds. Additionally, *K. pneumoniae* Kp2 is a useful strain in the treatment of cyanogen wastes in the presence of different ions (magnesium, nickel, cobalt, iron, chromium, arsenic, copper and zinc) that are principal pollutants besides cyanide in wastes. Therefore, this strain and its sterile crude extract seem to be convenient agents for cyanide removal from wastewaters and soils.

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