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RESEARCH ARTICLE

Biodegradation of Cyanide under Alkaline Conditions by a Strain of *Pseudomonas putida* Isolated from Gold Mine Soil and Optimization of Process Variables through Response Surface Methodology (RSM)

Mahboubeh Moradkhani[1], Soheila Yaghmaei[1*], Zahra Ghobadi Nejad[2]

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Abstract

In regard to highly poisonous effects of cyanide ion, concerns have been focused recently on treatment of such compounds in different ways. Four bacterial strains (C1-C4) capable of using cyanide as nitrogen source were isolated from contaminated gold mine soil samples under alkaline conditions at 30 °C, pH 9.5-10.5, and agitation speed 150 rpm. The gram-negative bacterium C3 (identified as Pseudomonas parafulva NBRC 16636(T) by 16S rRNA gene sequencing) was able to tolerate cyanide up to 500 ppm besides removing 93.5% of 200 ppm cyanide in 13 days which was confirmed by microorganisms growth. The addition of basal salts enhanced the removal efficiency of C3 by 16%. Cyanide removal efficiency of co-culture was 30% less than C3. Optimization of three significant parameters including temperature, pH, and glucose concentration for cyanide biodegradation was studied using response surface methodology (RSM). The optimum conditions for maximizing cyanide biodegradation were temperature (32.23 °C), pH (9.95), and glucose concentration (0.73 g/l).

Keywords

biodegradation, indigenous microorganisms, cyanide removal, alkaline conditions

[1] Department of Chemical and Petroleum Engineering
Biotechnology Research Center,
Sharif University of Technology, Tehran, Iran
P.O. Box 11155-1399, Tehran, Iran

[3] Biochemical and Bioenvironmental Eng. Research Center (BBRC),
Sharif University of Technology, Tehran, Iran

[*] Corresponding author, e-mail: yaghmaei@sharif.edu

1 Introduction

Cyanides are technically defined as compounds with a negative charge of one, consisting one carbon atom in the +2 oxidation state along with one nitrogen atom in the -3 oxidation state. Cyanide is produced by a number of fruits, seeds, leaves, and roots of various plants such as cassava, a number of soil bacteria, fungi, and various species of invertebrates [1, 2]. Nevertheless, industrial wastes of human activities contribute the largest portion of environmental cyanide pollution [3, 4]. The most important application of cyanide is in the mining of precious metals (e.g. gold and silver). Gold and silver are precious metals that are insoluble in water. Instead, they form soluble complexes, $[Au(CN)_2]^-$ and $[Ag(CN)_2]^-$, that require an alkaline environment (pH > 10) to keep cyanide in its ionic form and prevent the formation of volatile toxic hydrogen cyanide [3].

Hydrogen cyanide is extremely toxic, and is able to deactivate the enzyme cytochrome oxidase, the last enzyme in the respiratory cycle, which causes cell suffocation. Due to its excessive toxicity to the ecosystem, especially that of hydrogen cyanide (HCN), the level of cyanide compounds permissible in the environment, is below 1 ppm. Therefore, environmental protection necessitates the treatment of industrial cyanide-containing waste streams prior to their release into the ecosystem. The most conventional cyanide treatment method is alkaline chlorination. Although this technique is successful in free cyanide detoxification; it is incapable of treating metallic-cyanide complexes (MCNs). Moreover, other physical or chemical treatment techniques are rather expensive. Consequently, despite toxicity of cyanide compounds to living microorganisms, biological treatment, however, can be just as effective as it is becoming less costly [5-7].

Numerous organisms use these toxic compounds as their energy or nutrient source. A number of these organisms use cyanide solely as their nitrogen source [1, 8, 9], while for some others, the previous compound provides both carbon and nitrogen sources [10-12]. Biodegradation of cyanide compounds may occur in the presence of various carbon sources including simple carbohydrates, e.g. glucose [13], fructose [8], acetate [14, 15], and methanol [16]. Cyanides such as free cyanide

[17, 18], metal complexes [17, 19], and thiocyanates [17], are the exclusive nitrogen source for numerous microbial strains.

Extensive research has been conducted on bacteria capable of using cyanide, *Klebsiella*, *Serratia, Noraxella*, *Pseudomonas sp.* [20], *Pseudomonas pseudoalcaligenes* [15], and *Agrobecterium tumefaciens* [21], are the related examples. A recent bacterial species "*Rhodococcus* UKMP-5M" capable of degrading cyanide to less toxic compounds has been isolated from petroleum-contaminated soils [1]. In another study, two fungi mixtures in which one comprising *Fusarium solani* and *Trichoderma polysporum*, and the other consisting of *Fusarium oxysporum*, *Scytalidium thermophilum*, and *Penicillium miczynski*, as well as both being capable of consuming iron- or nickel-cyanide complex, as their nitrogen source, under acidic or neutral conditions were isolated from acidic soils of gas-production factories [22].

The literature is dominated by studies on cyanide microbial degradation under acidic or neutral conditions [22-24]. Under these conditions, cyanide is converted to hydrocyanic acid, a weak acid with pKa = 9.2, which causes serious problems, and hence there is a great interest in biological treatment of cyanide compounds under alkaline conditions. The fungi *F. solani* [25], bacterium *P. pseudoalcaligenes* [13, 15, 26], and strain *Burkholderia cepacia* [8] exemplify species with the capacity of producing enzyme biodegrading cyanides under alkaline conditions. The bacterium *Burkholderia cepacia* biologically degrades cyanides at pH = 8-10 with the highest removal rate, 1.85 mg CN/h [27].

There is a broad use of cyanide in extracting gold; however, it was for the first time in my country that indigenous microorganisms were used to degrade cyanide in soil sample of a gold mine under alkaline condition. Those microorganisms offered a practical solution for cyanide biodegradation. Therefore, it is necessary to search for new and more efficient strains in order to overcome the growing issues caused by these toxic contaminants.

The main purpose of this study was to isolate and identify cyanide degrading bacteria from soil samples of Takab gold mine under alkaline conditions, which increased the safety and accuracy of experiments by preventing HCN formation and cyanide evaporation. Furthermore, the optimum growth conditions of isolated species were found in the presence of toxic cyanide compound.

2 Experimental Protocol

2.1 Isolation and purification of microorganisms

In order to isolate and purify microorganisms to be able to remove cyanide, a sample from contaminated gold mine soil (Takab gold mine, Urmia, Iran) was selected. The desired microorganisms were isolated by enrichment in nutrient broth containing 30-100 ppm cyanide. For this purpose, at first a 90 ml saline solution containing 0.1% Tween 80 was added to a 500 ml Erlenmeyer flask. This compound facilitated the transfer of microorganisms from the soil to aqueous phase. Then, 10 g soil was added to the saline solution and was mixed for 1 h at 30 °C and 150 rpm. After precipitation of soil, the supernatant liquid was added to the autoclaved nutrient broth supplemented with 30 to 100 ppm cyanide. The microorganism enrichment was done every 4 days by 10% V/V inoculation into a fresh medium, with steps increasing cyanide concentration between 100-500 ppm. During all steps pH was controlled above 9.5 by adding 0.1 N NaOH. 10 ml of culture was inoculated into a mineral medium to isolate cyanide degrading bacterium.

The mineral medium contained NaOH 0.1 N, K_2HPO_4 4.35 g/l, 10 ml solution of trace salts ($FeSO_4.7H_2O$ 300 mg, $MgSO_4.7H_2O$ 180 mg, $CoCl_2$ 130 mg, $CaCl_2$ 40 mg, $MnCl_2.4H_2O$ 40 mg, and MoO_3 20 mg dissolved in 1 liter deionized water) and 0.1% yeast extract [28]. The pH was adjusted in the range of 9.5-10.5. The medium was autoclaved at 15 psi and 121 °C for 20 min, then was supplemented with filter-sterilized glucose (1 g/l) as carbon source. Filter-sterilized KCN (50-130 ppm) was added to the mineral medium as nitrogen source every 5-6 days. Incubation was done at 30 °C and 150 rpm using sealed shake flasks besides controlling of pH. Cultured microorganisms were then purified by single cell clonal isolation in nutrient agar. Four microorganisms (C1-C4) were successfully separated from cyanide-contaminated gold mine soil and then were purified. The isolated bacterial strains were able to grow and biologically degrade high cyanide levels under alkaline conditions. The microorganisms (C1-C4) were characterized by several biochemical and bacteriological tests (catalase and oxidase), lactose, sucrose, and glucose fermentation, a starch hydrolysis test, and finally a Gram staining test.

2.2 Cyanide degradation capability of microorganisms

In order to assess cyanide removal, a desired amount of soil was autoclaved and 10 g of the soil samples were dispensed in sterile sealed plates. Then, 3 ml of autoclaved basal salt solution containing 0.5 g/l KH_2PO_4, 0.5 g/l K_2HPO_4, 0.5 g/l $MgSO_4.7H_2O$, 0.01 g/l $CaCl_2$, in addition to 1 g/l filter sterilized glucose and KCN (100 and 200 ppm) were added into the samples. These salts were nutritive which promoted microbial growth. In order to evaluate cyanide removal capacity of each strain, the isolated microorganisms were cultured in nutrient broth for 24 h at 30 °C and agitation speed of 150 rpm, and then they were inoculated into soil samples. The initial bacteria count was 1×10^7 CFU/ml. Soil moisture was controlled daily by weighing the samples. A single strain was selected based on its higher efficiency in removing cyanide, then the ability of this microorganism was analyzed in different initial cyanide concentrations, 100 - 500 ppm. In all steps, non-inoculated medium served as control and all experiments were performed in duplicate.

Identification of the C3 strain was performed in Iranian Biological Resource Center (IBRC) by using microorganisms bank. Total DNA of the selected microorganism (C3) was extracted according to modified Marmur method [29]. PCR reaction was carried out using 27F [5'-AGAGTTTGATCMTGGCTCAG-3'] and 1492R [5'-GGTTACCTTGTTACGACTT -3'], universal primers, to amplify the gene coding for 16S rRNA.

2.3 Cyanide biodegradation assay

Cyanide removal efficiency of a mixture of four microorganisms was compared with the selected strain. For this purpose, a mixture of four isolated bacterium was cultured in nutrient broth for 24 h and then was inoculated into soil samples supplemented with 200 ppm cyanide. The amount of cyanide removal was detected from the 9 to 13th day.

The effect of yeast extract, as a nutritive source for microbial growth, on the removal of cyanide was evaluated. For this purpose, at 200 ppm initial cyanide concentration, 1 g/l yeast extract was also added to soil samples.

All the experiments were done in duplicate at 30 °C, pH=9.5-10.5, and agitation speed of 150 rpm. Control experiments (without bacterial inoculation) were also performed.

2.4 Bacterial Growth

The growth of isolated microorganism was analyzed by using one of the most reliable techniques which is culturing by pour plate technique and counting of colonies. For this purpose, firstly, 1 ml of grown microorganisms in soil samples which were prepared previously (Section 2.2), was resuspended in 9 g/l NaCl in order to carryout serial dilution. Then 1 ml of each dilution was added into sterile plates containing 15 ml nutrient agar. They were incubated for 24 h at 30 °C (each dilution was performed in duplicate). The plates containing 30-300 colonies were used for counting viable cells as colony forming units/ml (CFU/ml) [28].

2.5 Analytical methods

Measurement of residual cyanide was done using a DR 5000 Spectrophotometer UV-VIS (HACH, Germany) and cyanide test-kit (24302-00) according to the method 8027 (Pyridine-Pyrazalone (0.002 to 0.24 ppm CN^-)) developed by the HACH company. The blue compound formed during the determination of cyanide was quantified by measuring absorbance at 612 nm [30]. Variation in pH was measured using a pH meter (827 Metrohm, Swiss). The number of colonies was counted using a microscope (JENUS, china) and a rotary shaker-incubator (Wise cube, South Korea) was utilized for all experiments.

2.6 Experimental design and optimization

Response Surface Methodology (RSM) was employed for the optimization of process variables to improve the degradation of cyanide using design expert 7.1.5 version (Stat-Ease, Minneapolis 2008) to analyze the results. RSM is an empirical modelization technique for studying the influence of different variables on responses by varying them simultaneously and conducting a limited number of experiments. In this study, central composite design (CCD) was used to study optimum variable levels in cyanide biodegradation, temperature (25-40 °C), pH (9-12), and glucose concentration (0.1-1.0 g/l). The experiments in the central point were done in triplicate for 7 days.

3 Results and Discussion

3.1 Isolation and purification of microorganisms

Table 1 shows characteristics of isolated microorganisms. In order to compare cyanide removal efficiencies of C1-C4 strains, they were inoculated into soil samples. After 6 days of incubation, at initial cyanide concentration of 100 ppm, the removal efficiency of the four strains (C1-C4) was about 51, 39, 58 and 47% and at 200 ppm it was 38, 40, 63.5 and 49%, respectively. *Pseudomonas parafulva* C3 was selected as the best strain based on its higher removal efficiency and was used in the subsequent steps. The 16S rRNA gene sequence analysis of the C3 strain showed that the isolate composed of 1406 nucleotide had 99.4% identity with *Pseudomonas parafulva* NBRC 16636(T) accession number BBIU01000051.

Table 1 Characterization of the separated microorganisms

Microorganism	C1	C2	C3	C4
Appearance	Thin rod -shaped	Long rod-shaped	Thin rod-shaped	rod-shaped
Gram staining	Negative	Negative	Negative	Positive
Catalase test	Positive	Negative	Positive	Negative
Oxidase test	Positive	Negative	Negative	Negative
Starch hydrolysis test	Positive	Positive	Positive	Negative
Glucose, lactose, and sucrose fermentation test	Only ferments glucose	Only ferments glucose	Only ferments glucose	Only ferments glucose
H_2S gas production	Negative	Negative	Negative	Negative

3.2 Cyanide degrading experiments

3.2.1 Removal efficiency of *Pseudomonas parafulva* C3

The effect of the initial cyanide concentration was analyzed by adding 100, 200, 300, 400, and 500 ppm cyanide into the soil samples and cyanide concentration was monitored every day consecutively.

The results (Fig. 1) showed that *Pseudomonas parafulva* C3, could remove 93.5% of cyanide with initial concentration of 200 ppm at 13 days. The bacterium C3 tolerated cyanide

concentrations, as high as 500 ppm, during this period. The cyanide concentration of 200 ppm recorded the highest removal efficiency in the shortest period of time, compared to other concentration values; so it was determined as the optimum initial concentration. Increasing the concentration beyond this value had a destructive effect on cyanide removal. Fig. 1 showed that cyanide was in fact as the substrate up to 200 ppm and its inhibitory effect was appeared in higher concentrations. So, biodegradation may started gradually at those concentrations regarding the microorganisms adaptation to this condition. No cyanide removal was detected in the absence of augmentation in the control samples.

In most of previous studies, researchers investigated cyanide removal using different bacteria under different conditions. Most of those bacteria were mesophilic while optimum temperature was about 25- 30 °C and the optimum pH varied from 5.5 to 11.5. It is evident that by increasing cyanide concentration, the amount of cyanide removal decreased or the required time increased markedly. Ezzi and Lynch [12] studied cyanide removal at concentration of 2,000 ppm and successfully removed total cyanide at 90 days. Other researchers investigated the effect of lower amounts of cyanide. For instance, Naveen et al. [18] removed 83% of cyanide with initial concentration of 150 ppm at 120 h. Özel et al. [31] investigated the use of fungi on cyanide removal and found that by using *S. commune*, *P. arcularis* and *G. luncidum* at pH 10.5 for 42 h, cyanide degradation was almost 100%, but the initial cyanide concentration was just 25 ppm. It showed that by increasing cyanide concentration up to 200 ppm, the removal efficiency decreased to less than 50%. The results of literature data are presented in Table 2.

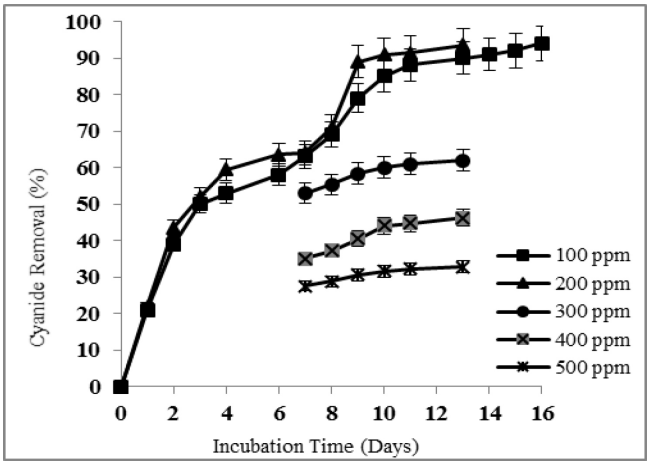

Fig. 1 Cyanide removal efficiency of the isolate C3 at different initial concentrations

3.2.2 Removal efficiency of mixed culture

In order to compare the C3 strain with co-culture, a mixture of the four isolated bacteria, C1-C4, was cultured in nutrient broth medium for 24 h and inoculated into soil samples supplemented with 200 ppm cyanide.

Fig. 2 shows the performance of a mixture of strains from C1 to C4 in degrading 200 ppm cyanide from the 9 to 13th day compared to that of individual C3, under the same conditions.

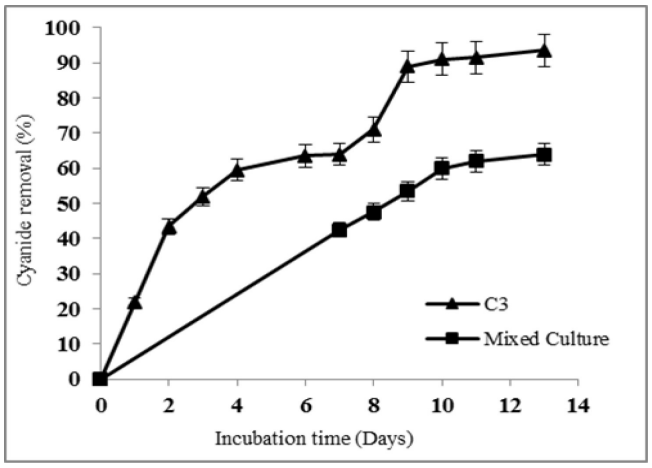

Fig. 2 Comparison between cyanide removal efficiency of the isolate C3 and C1-C4 mixture at 200 ppm initial cyanide concentration

It was observed that the removal efficiency of the mixture of the four microorganisms was 30% less than the isolate C3. Competition between individual species in a heterogeneous microbial community affected the removal of cyanide by releasing inhibitory side-products. On the other hand, the cyanide concentration was 200 ppm which was the known optimum concentration for *Pseudomonas parafulva* C3. It implied that cyanide was a substrate with no inhibitory effect on the growth of the C3 strain until it reached 200 ppm. It was reasonable that this amount would be different for the mixed culture and consequently, the C3 strain offered more efficient removal than the co-culture. In some studies, mixed culture would display higher efficiency on cyanide removal, if optimum substrate was provided. For example, Kang and Kim [20] studied the degradation of cyanide by a mixture of bacteria. They demonstrated that cyanide played a role as a substrate until 300 ppm in which the mixture recorded maximum growth rate and cyanide removal. Nwokoro and Dibua [32] examined the degradation of cyanide by single and mixed culture of *Pseudomonas stutzeri* and *Bacillus subtilis*, and they reported that *P. stutzeri*, *B. subtilis* and their mixture removed cyanide up to72, 66.9 and 88.5%, respectively.

3.2.3 The effect of nutrients on cyanide removal

Figs. 3 and 4 show cyanide removal yield in the 3rd, 6th, 9th, and 13th days of treatment to indicate the effect of inorganic basal salts and yeast extract, respectively, on microbial growth and their ability in promoting cyanide removal.

It is shown in Fig. 3 that inorganic basal salts were necessary as they facilitated C3 growth and the removal of cyanide. The presence of these salts improved the removal efficiency by 16%.

As illustrated in Fig. 4, yeast extract also improved cyanide removal capability of *Pseudomonas parafulva* C3 by enriching

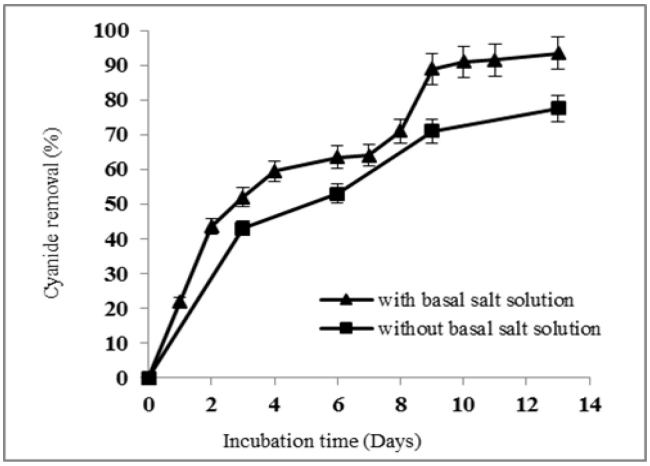

Fig. 3 Cyanide removal efficiency of the isolate C3 with or without adding inorganic basal salts at 200 ppm initial cyanide concentration

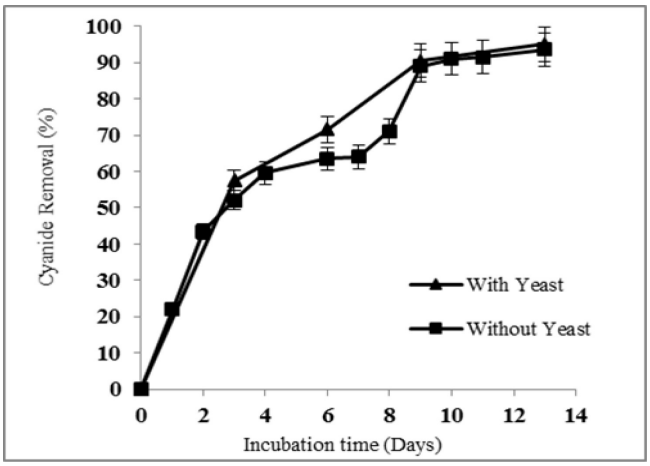

Fig. 4 Cyanide removal efficiency of the isolate C3 with or without adding Yeast extract at 200 ppm initial cyanide concentration

Table 2 Cyanide removal efficiency using different microorganisms

Microorganism	Removed compounds	Initial Cyanide Concentration (ppm)	pH	Temperature (°C)	Process Time (days)	Removal Percentage (%)
***Trichoderma SPP.* and *Fusarium SPP.* [12]**	A stock solution contain CN^-	2000	6.5	25	90	100
CMN2 and CM5 specious of *pseudomonas* [11]	CN_{WAD} (weak acid dissociable)	400	9.2-11.4	30	5	93
Natural mixture of nine *pseudomonas* sp. [11]						67
***Pseudomonas parafulva* C3**	KCN	200	9.5-10.5	30	13	93.5
Rhizopusoryzae	Ferrocyanide	150	5.6	25	5	83
***Stemphylium loti* [18]**			7.2			90
***Agrobacterium tumefaciens* SUTS1 [21]**	KCN	25, 50	7.2	30	15	87.5
		150				97.9
***Fusarium solani* [25]**	KCN	50	9.2-10.7	30	6	100
Bacterial mixture [20] containing species of: the genera *Klebsiella*	KCN	70	8	28	2	100
Seratia						80
Noraxella						100
Pseudomonas						95
***Rhodococcus* UKMP-5M [1]**	Petrochemical contaminated soil	2.6	6.3	30	3	100

its growth. However, considering the negligible enhancement (~1.5%), feasibility studies and economic analysis should be conducted prior to the industrial application of yeast extract. Maniyam et al. [1] studied the biodegradation of cyanide by *Rhodococcus*. They analyzed the effect of glucose and yeast extract on bacterial growth and cyanide removal efficiency at two cases. First in the presence of both supplements simultaneously and second by adding only yeast extract to the culture medium. Results showed that in the presence of glucose, the bacterial growth was in a high level after 11 days of incubation, while the bacteria could not grow efficiently in the second case. Cyanide removal efficiency in the first case was four times more than in the second one. This indicated significantly higher effect of glucose than yeast extract, as cyanide degradation occurred partially by using yeast extract [1]. In addition, Dumestre et al. [25] examined the degradation of cyanide under alkaline conditions by a strain of *Fusarium solani* isolated from contaminated soils in both the presence and absence of yeast extract. It was concluded that yeast extract could affect formamide conversion, but the graph showed that this nutrient did not have any significant effect on cyanide removal.

Highly aerobic condition in the current study, ruled out the effect of chemical reaction (Kiliani-Fischer reaction) between glucose and cyanide [1, 12, and 33].

3.3 Pseudomonas parafulva C3 growth analysis

Fig. 5 shows that cyanide concentration had a noticeable effect on microbial growth as the bacteria count in medium containing 200 ppm cyanide was more than in medium containing 100 ppm cyanide in similar condition, which confirmed the absence of cyanide inhibitory effects up to 200 ppm. In other words, cyanide worked as a substrate in these concentrations which could promote microorganisms growth. The results of bacterial growth and cyanide removal were confirmed each other.

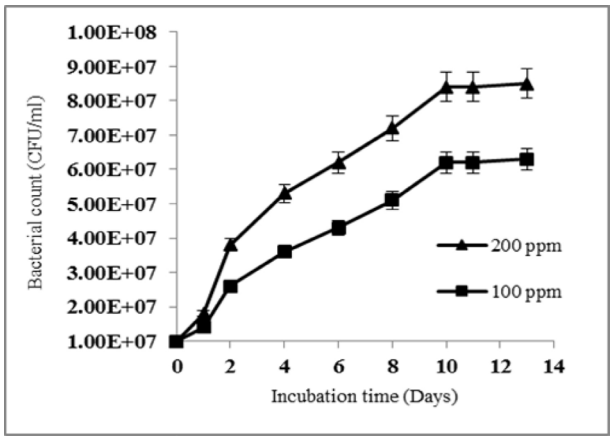

Fig. 5 C3 bacterial growth curve at 100 and 200 ppm initial cyanide

3.4 Response surface analysis for optimization of three factors

Data from Table 3 were processed by response surface method and the results were obtained by fitting to the quadratic model equation:

$$Y = 61.92 + 2.95A - 2.01B + 2.07C + 0.19AB + 0.063AC + 0.19A^2 - 0.28B^2 - 0.19C^2 \quad (1)$$

Where (Y) is the predicted removal percentage of cyanide. A, B, and C are the values of temperature, pH, and glucose, respectively. The results of Analysis of Variance (ANOVA) for response surface model are presented in Table 4.

The model for cyanide biodegradation by *Pseudomonas parafulva* C3 was significant ($p<0.0001$), indicating the adequacy and reliability in representing the actual relationship between response and variables [34, 35]. In regard to effective and removed terms, the model, Eq. (1), might be modified into Eq. (2):

$$Y = 61.92 + 2.95A - 2.01B + 2.07C + 0.19AB + 0.19A^2 - 0.28B^2 - 0.19C^2 \quad (2)$$

The fit of the models was controlled by the coefficient of determination R^2. According to the ANOVA results, the models reported high R^2 value of 0.9982 for cyanide biodegradation, which implied that it was a very good fit and 99.8% of the variation could be explained by the model [36]. Furthermore, the high value of the adjusted R^2 = 0.9965 proved a high significance of the model. Adequate Precision which was the ratio of signal to noise should have been more than 4 in order to indicate the adequacy of the signal; and it was 96.21, in this study. The diagnostic plot (Fig. 6) was also used for estimating the adequacy of the regression model [37]. Tendencies in linear regression in the graph showed a satisfactory correlation between predicted and experimental values.

Table 3 Three factors central composite design and experimental results

Run	A: Temperature (°C)	B: pH	C: Glucose concentration (g/l)	Removal efficiency (%)	
				Actual	Predicted
1	36.96	11.39	0.28	60	60.2
2	32.50	10.50	0.55	62	61.9
3	28.04	11.39	0.28	54	54
4	36.96	9.61	0.28	64	63.9
5	32.50	10.50	1.00	65	64.9
6	32.50	9.00	0.55	64.5	64.5
7	32.50	10.50	0.55	62	61.9
8	32.50	10.50	0.55	62	61.9
9	32.50	10.50	0.55	61.5	61.9
10	32.50	10.50	0.55	62	61.9
11	28.04	9.61	0.82	62.5	62.4
12	40.00	10.50	0.55	66.5	66.4
13	36.96	9.61	0.82	68	68.1
14	28.04	11.39	0.82	58	58.2
15	25.00	10.50	0.55	56.5	56.4
16	32.50	10.50	0.55	62	61.9
17	36.96	11.39	0.82	64.5	64.6
18	32.50	10.50	0.10	58	57.9
19	28.04	9.61	0.28	58.5	58.6
20	32.50	12.00	0.55	58	57.8

Table 4 ANOVA for the entire quadratic model

Source	Mean Square	F Value	p-value Prob > F
Model	26.09	610.68	< 0.0001
A-Temperature	119.03	2786.02	< 0.0001
B- pH	55.10	1289.71	< 0.0001
C- Glucose concentration	58.53	1369.99	< 0.0001
AB	0.28	6.58	0.0281
AC	0.031	0.73	0.4124
BC	0.031	0.73	0.4124
A^2	0.52	12.08	0.0060
B^2	1.11	26.01	0.0005
C^2	0.52	12.08	0.0060
Residual	0.043		
Lack of Fit	0.044	1.05	0.4790
Pure Error	0.042		

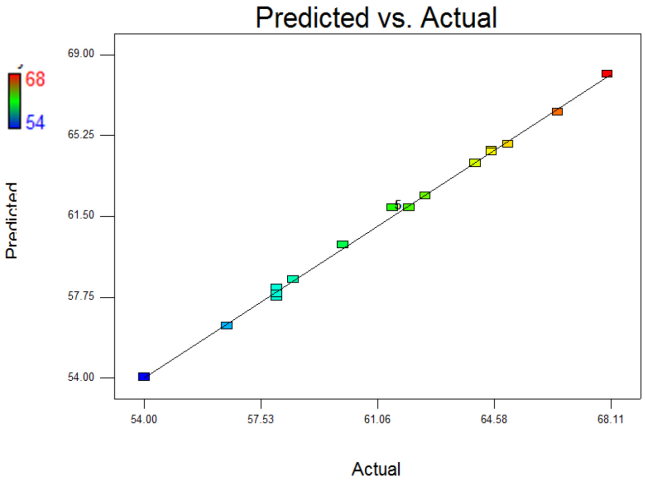

Fig. 6 The actual and predicted cyanide removal percentage

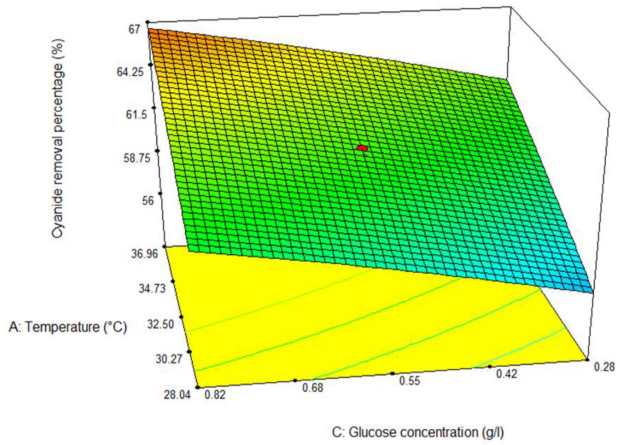

Fig. 8 Response surface plot showing interactive effect between temperature and glucose concentration on cyanide biodegradation

3.4.1 Interactive effect of processes of independent variables

RSM was used to investigate the interactive effect of temperature, pH, and glucose concentration on biodegradation of cyanide, and the results were shown in three dimensional (3D) plots, (Figs. 7, 8, and 9).

As indicated in Fig. 7, cyanide removal efficiency increased remarkably as temperature increased and, in contrast, pH decreased.

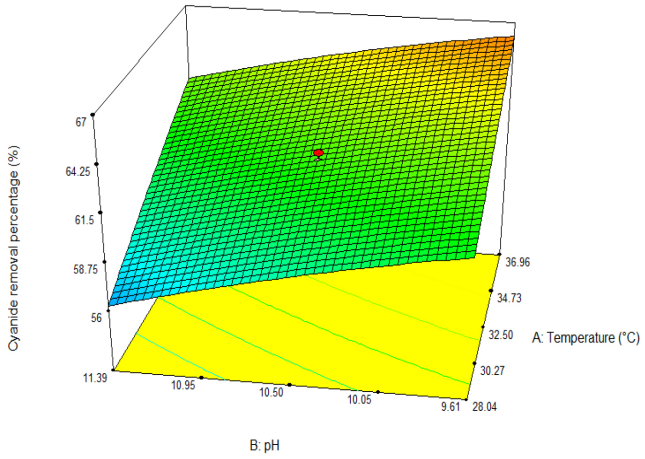

Fig. 7 Response surface plot showing interactive effect between temperature and pH on cyanide biodegradation

Fig. 8 shows the effect of temperature and glucose concentration on cyanide removal, simultaneously. As seen in Fig. 8, cyanide content decreased with increase in temperature and glucose concentration which resulted in increasing cyanide removal percentage.

The effect of pH and glucose concentration on the removal of cyanide is shown in Fig. 9. By increasing glucose concentration and decreasing pH, cyanide content decreased which implied that cyanide removal efficiency increased.

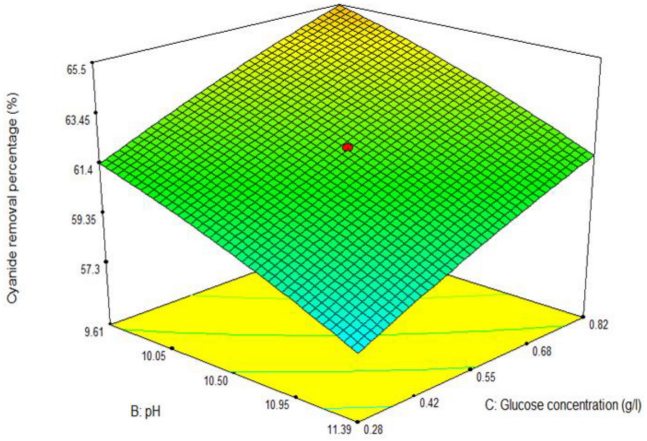

Fig. 9 Response surface plot showing interactive effect between pH and glucose concentration on cyanide biodegradation

3.4.2 Optimization experiment

The desired goal of the model was to remove cyanide as much as possible in order to achieve the most treated soil. At optimum condition of 32.23 °C, pH 9.95, and glucose concentration of 0.73 g/l, predicted cyanide removal efficiency was 66%, while the experimental result was 64%. This implied that the strategy for obtaining maximum cyanide removal was successful. Although at temperatures of 35-40 °C removal efficiency was more than 66%, decrease in cyanide content in control samples, without microorganism inoculation, showed that at those high temperatures, there were some other reasons for cyanide removal in addition to biodegradation.

4 Conclusions

Recent studies have shown that biological treatment of cyanide using living microorganisms was reliable, environmentally friendly and economical.

In the present study, indigenous microorganisms (C1 - C4) were isolated from contaminated soil, purified and their ability to remove cyanide was analyzed. The strain C3 was selected

as the most efficient strain in cyanide removal with a record of 93.5% cyanide degradation at concentration of 200 ppm after 13 days. This strain was able to survive and grow under alkaline conditions (pH > 10) and tolerated high cyanide concentrations up to 500 ppm, making it the most reasonable choice for biodegradation of cyanide-contaminated wastes. In the presence of glucose, as carbon source, *Pseudomonas parafulva* C3 displayed the highest cyanide removal yield. The addition of basal salts enhanced the cyanide removal efficiency by 16%. In addition, the results showed that the cyanide removal efficiency of the bacterium C3 was higher than a mixture of the four strains. The range of temperature, pH, and glucose concentration were established to optimize the cyanide biodegradation condition by RSM which could save experimental time and cost.

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