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Survey bioremediation of 4-Chlorophenol by yeast and mold isolated from industrial and petroleum wastewaters (Imam Khomeini seaport, Mahshahr)

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ABSTRACT

Chlorophenols are the most toxic pollutants of water and wastewater. Since 4-chlorophenol is a high soluble compound in water, it is found in water and wastewater abundantly. Because of high costs, high energy consumption and in some cases environmental inconsistency in chemical and physical removal methods, biochemical degradation of 4-chlorophenol is very important. In the present study, 13 strains of bacteria and 6 strains of yeast and mold were purified and isolated from wastewater treatment plant (Imam Khomeini seaport, Mahshahr), which lasted about 15 days. Then, the ability of each microorganism isolated in the presence of 100ppm of 4-chlorophenol was studied and two microbial species suitable for TY_1 and TY_2 were selected for use in mixed microbial culture. In this research, one of the most important factors affecting 4-chlorophenol degradation was by mixed microbial culture including glucose concentration with 2 and 5g/l was investigated. After examination, the microbial strains suitable for TY_1 and TY_2 were able to completely remove the 100ppm of 4-chlorophenol, so that the TY_1 strain was removed completely after 45 hours and by TY_2 strain after 21 hours and also, using a mixture of TY_1 and TY_2 strains (50/50) and in the presence of 2g/l glucose, 100ppm of 4-chlorophenol was completely removed after 18 hours. The significance and impact of this study was the use of indigenous strains isolated from wastewater treatment plant in petroleum refineries and petrochemical industries for the biodegradation of chlorophenol.

1. Introduction

Today, protecting the environment and purging it from a variety of pollutants is one of the most important human tasks. Phenolic derivatives are the main and most important group of pollutants in wastewater that needs to be removed using wastewater treatment processes. One of the most important human goals in recent decades was protecting the environment against the growing trend of pollutants. Water resources are one of the most important biological resources that protecting and cleaning them from pollutants is one of the most important tasks. Phenol and phenolic derivatives are considered among the most

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significant pollutants in the environment by the Environmental Protection Agency (Solyanikova and Golovleva, 2005). These compounds are used in several industrial processes for the production of chemicals such as pesticides, explosives, drugs, textiles, paints and resins. Phenolic compounds are produced not only from human activities, but also naturally by the decomposition of leaves and wood.

Phenolic compounds are produced not only from human activities, but also naturally by the decomposition of leaves and wood. As a result, these materials are found in soils and sediments, and often lead to contamination of groundwater and wastewater (Pradeep et al., 2015; Zhang et al., 2017).

Due to the high toxicity of these compounds and pathogenic properties of some of them, various methods have been used to remove and analyze these toxic compounds, among which biodegradation methods are one of the most effective and easy methods for the removal and decomposition of this type of pollutants. In these methods, microorganisms are used as decomposing agent of toxic compounds.

Various methods have been used for the elimination and analysis of these toxic compounds due to the high toxicity of these compounds and pathogenic properties of some of them, among which biodegradation methods are one of the most effective and easy methods for the elimination and decomposition of this type of pollutant. In these methods, microorganisms are used as decomposing agents of toxic compounds. Phenolic derivatives are one of the most toxic pollutants in the environment. Chlorophenols are among the most toxic pollutants in the water and wastewater. Since 4-chlorophenol is a compound with high solubility in water, it is found in water and wastewater, abundantly. 4-chlorophenol biodegradation is very important due to the high costs, high energy consumption and in some cases environmental mismatches, chemical, and physical removal methods.

1.1. Biodegradation of chlorophenols and effective microorganisms

Today, the biodegradation of aromatic compounds by microorganisms is of particular interest. Many microbial species, including bacteria and fungi, can remove chlorophenols as the only carbon source. Due to the use of degrading microorganisms in chlorophenols biodegradation, the microbial cultures are classified into two main groups, which are discussed below.

1.1.1. Pure microbial cultures

There are many microorganisms, including bacteria and fungi that can remove chlorophenols. In Table 1, a list of aerobic biodegrading microorganisms of chlorophenols is presented as the only carbon source (Tarighian et al., 2003; Monsalvo et al., 2009; Muftah at al., 2017).

1.1.2. Mixed microbial cultures

Mixed microbial culture is a group of different species of microorganisms that act as a population together. Examples of microbial collections are found in active sludge ponds; biofilms such as those found in trickling filters and found in various soil ecosystems. Mixed cultures have been used in fermentation processes, such as food fermentation such as fermentation and alcohol production, production of yogurt and cheese, as well as in wastewater treatment and contaminated water, from very early times. In microbial collections, organisms work together in a complex system, all of which utilize each other's activities in the population. For example, microbial collections are more efficient in complex organic waste degradation than single species or even mixtures mixed with microorganisms and with a greater variety of metabolic capabilities (Tarr, 2003; Gaufeng et al., 2004).

2. Materials and Methods

General stages of the project

- Sampling
- Stages of phenol degrading microorganism culture
- Isolation of phenol degrading yeast and mold
- Measuring chlorophenol removal
- Determining the identity of selected strains

2.1. Nutrient and mineral culture mediums

The culture media used in this study can be used for the isolation of fungi from bacteria

which are Sabouraud Dextrose Agar (SDA), Sabouraud Dextrose Agar with Chloramphenicol (SC) and Potato Dextrose Agar (PDA). In the next step, for the final purification of yeast from mold, specific culture media, such as yeast peptone agar (YPG), Malt Agar Extract (YM) were used.

Mineral Salt Medium (MSM) and Trace elements used in enrichment and biodegradation of pollutants are shown in Tables 2, 3 (Lee et al., 2007). Due to the growth of microorganisms in 4-chlorophenol mineral environments, glucose was used as a substrate for growth supplement or primary substrate in the enrichment and recovery phases of the wastewater. In cases where glucose supplement substrate was added to the environment, its concentration was considered 2 and 5g/l (Yang et al., 2008).

2.2. Sampling

Since the main objective of the study is the elimination of 4-chlorophenol from the native microorganisms, the wastewater treatment unit has been used as a source of microbial isolation.

The wastewater and sludge used in these experiments were prepared from wastewater treatment plant (Imam Khomeini seaport, Mahshahr) (Figure 1).

Fig1. Sampling location

2.3. Pre-treatment of microbial source and selection of microorganisms

Due to the increased ability to remove pollutants by microorganisms in wastewater and sludge, a Series of 4-chlorophenol pollutant adaptation and enrichment were performed on it as follows.

Table 1. List of chlorophenols and their degrading microorganisms

Microorganisms	Chlorophenols
Desulfovibrio dechloracetivorans (ATCC700921), *Alcaligenes sp., Ralstonia sp., Azotobacter sp., P. putida, Cystobacteri sp., P.cepacia*	2-Chlorophenol
Desulfomonile tiedjei	3-Chlorophenol
P. putida, Comamonas testosteroni JH5, P.cepacia, Rulstonie eutropha, Alcaligenes sp., Azotobacter sp., Ralstonia sp., Candida tropicalis, Fusarium flocciferium Penicillium, Aspergillus, Graphium, Phanerochaete, Fusarium sp., Trichosporon sp.	4-Chlorophenol
Desulfitobacterium dehalogenans, Desulformonile tiediei, Ralstonia sp., Clostridium sp. Burkholderia cepacia, P. pickettii (DTP0606).	2,4 Dichlorophenol
Desulfomonile tiedjei, Desulfovibrio dechloracetivorans	2,5 Dichlorophenol
Desulfitobacterium dehalogenans(JW/IU-DC1), *Mycobacterium chlophenolicum, P.cepacia Azotobacter sp., P.pickettii* (DTP0606), *Desulforibrio dechloracetivorans, Ralstonia sp.*	2,6 Dichlorophenol

Table 2. Specifications of mineral salt medium used in the experiments

Mineral salt	NH_4NO_3	$MgSO_4.7H_2O$	$CaCl_2.2H_2O$	$KHPO_4$	KH_2PO_4
Salt concentration (gr/lit)	0.5	0.2	0.02	0.5	0.5

Table 3. Specifications of trace elements used in the experiments

Trace elements	$MnSO_4.H_2O$	$FeSO_4.7H_2O$	$CuSO_4.5H_2O$	$ZnSO_4.7H_2O$	$NaMoO_4$	$CoCl_2.7H_2O$
Salt concentration (gr/lit)	0.025	0.15	0.025	0.02	0.034	0.053

2.3.1. Adaptation of sludge and wastewater of Imam Khomeini Petrochemical Plant

Since the microorganisms of the wastewater at first had a negligible growth, the Adaptation operation was done in presence of substrates on the sludge and wastewater to enhance the ability to remove and then isolate the microbial species. Aeration was done each time to work with the wastewater and to activate its microorganisms (Loh et al., 1998; Saravanan et al., 2008).

To initiate the experiments, salt culture medium and trace elements salt solution was used. To strengthen the wastewater and sludge samples and the necessity of existence of a stimulus substrate for the growth of microorganisms along with toxic contamination of 4-chlorophenol, 2 and 5g/l glucose were also used.

Adaptation was performed in 5 stages for 60 days. In each stage, 10% of the previous culture was transferred to the fresh mineral culture medium containing pollutants (El-Sayed et al., 2009). Finally, the microorganism was separated from the final Adaptation stage. The pollutant concentration used at each stage of the various habituation operations was from low to high concentrations and finally, 100ppm.

2.3.2. Sludge and wastewater enrichment in Imam Khomeini Petrochemical treatment unit

The enrichment operation was carried out in 5 stages for 15 days. In each step, 10% of the previous culture was transferred to the fresh mineral culture medium containing pollutants and ultimately the enriched sludge and effluent was obtained from the culture of the final enrichment stage. The concentration of the pollutant was constant and 100ppm at each stage of enrichment. The Erlenmeyer tested were placed in incubator shaker with 200rpm and at 30 °C.

2.4. Isolation and purification of microorganism

After performing the adaptation and enrichment on sludge and effluent and ensuring the removal of 4-chlorophenol, sabouraud dextrose agar (SDA), Sabouraud Dextrose Agar with Chloramphenicol (SC) and potato dextrose agar (PDA) were used to isolate fungi from bacteria. In the next step, the yeast strains from mold from specific culture media, such as yeast peptone agar (YPG), Malt Agar Extract (YM) were used for the final purification. Then, each of the pure colonies was examined macroscopically and microscopically.

2.5. 4-Chlorophenol biodegradation using pure microbial culture

To perform 4-chlorophenol biodegradation tests, all degradation tests were first performed on pure microbial species and then, after obtaining the desired results, the main experiments were carried out on mixed microbial culture. All experiments were carried out in this section under operating conditions of 30 °C, 200rpm in 100ml Erlenmeyer flask and in two replications (Farrell et al., 1999).

2.6. 4-Chlorophenol biodegradation using mixed microbial culture

At this stage, a mixture of isolated microorganisms from the enrichment phases on sludge and wastewater was used as a mixed microbial culture, which is described below.

After ensuring the 4-chlorophenol decomposition by isolated species from the enrichment stages, subsequent experiments were carried out using mixed culture of isolated microbial species. All experiments were carried out at a concentration of 100ppm of 4-chlorophenol and 2g/l of glucose (Zouari et al., 2002; Herrera et al., 2008; Hiroaki. 2015).

2.7. 4-chlorophenol measurement method

In this study, the 4-chlorophenol measurement method is based on the 4-aminoantipyrine method (Farrell et al., 1999). The well known 4-aminoantipyrine spectrophotometric method developed in 1943 by Emerson is still in common use mainly due to special features like speed, cost-effectiveness and absence of laborious steps. 4-aminoantipyrine is the most widely used analytical reagent for the estimation of phenol. Phenolic compounds were determined by buffering the sample to a pH of 10.0 and adding 4-aminoantipyrine to produce a yellow or amber colored complex in the presence of ferricyanide ion. Finally, after 15 minutes, the absorbance is measured at 500 nm. The color is intensified

through extraction of the complex into chloroform. Measurement of this color quantitatively determines the phenol concentration of the sample (Jones et al., 1973; Fiamegos et al., 2002).

3. Results

3.1. Isolation of microorganisms

In this study, after adaptation and enrichment of collected samples and purification of strains, a total of 19 microorganisms (13 strains of bacteria and 6 strains of yeast and mold) were isolated and purified which were morphologically examined (Table 4). Figure.2 shows macroscopic forms (Observation) and Figure.3 shows the microscopic forms of isolated strains.

Finally, 2 microbial strains in screening showed the best results in 4-chlorophenol biodegradation. Table5 shows the macroscopic and microscopic characteristics of the selected microorganisms. It has been shown that strains TY_1, TY_2 separated from Total refinery effluent flow are a yeast species.

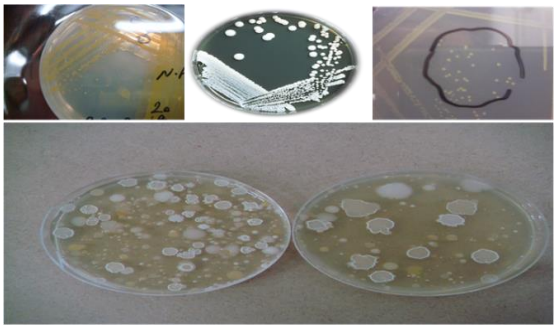


Fig 2. Macroscopic Observations of isolated microorganisms

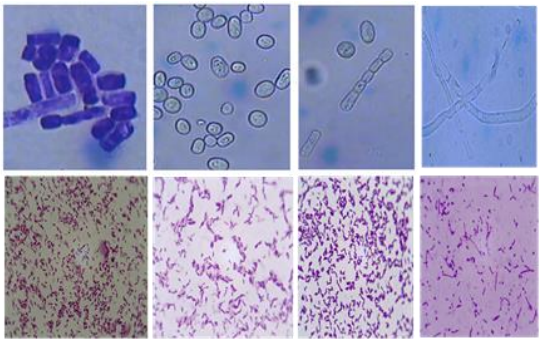
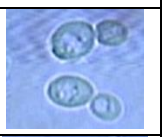
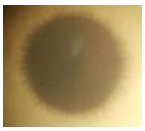


Fig 3. Microscopic Observations of isolated microorganisms

Table 4. Microscopic form and isolation site of isolated strains

Isolation site	Microscope form	Sample
Storage tank (interstitial water)	Gram negative bacilli	TB_1
Location of oil drainage of reservoirs to return to the refinery system	Gram positive bacilli	TB_2
Oil storage tank	Gram positive bacilli	TB_3
Oil storage tank	Gram positive coco-bacillus	TB_4
Storage tank	Gram positive bacilli	TB_5
Accumulated oil sludge	Gram positive coco-bacillus	TB_6
Accumulated oil sludge	Gram positive bacilli	TB_7
Total refinery effluent flow	Gram negative bacilli	TB_8
Total refinery effluent flow	Gram positive bacilli	TB_9
Total refinery effluent flow	Gram negative bacilli	TB_{10}
Total refinery effluent flow	Gram negative bacilli	TB_{11}
Total refinery effluent flow	Gram positive bacilli	TB_{12}
Total refinery effluent flow	Gram positive bacilli	TB_{13}
Total refinery effluent flow	Yeast	TY_1
Total refinery effluent flow	Yeast	TY_2
Total refinery effluent flow	Yeast	TY_3
Total refinery effluent flow	Mold	TY_4
Total refinery effluent flow	Mold	TY_5
Total refinery effluent flow	Yeast	TY_6

Table 5. Macroscopic and microscopic characteristics of selected strains

Name of microorganism	The appearance and color of the clones	Appearance of the clones	Cell shape	Cell shape
TY_1	Spherical and white		Oval with sprout	
TY_2	Spherical cream color with a small hyphae around the colony		Long string-like hyphae	

3.2. 4-Chlorophenol degradation using TY_1 isolated microorganism

After examining the apparent form of the TY_1 isolated microorganism, the microorganism growth curve was examined and drawn up. In Figure 4 the cellular growth curve of the TY_1 isolated microorganism is shown.

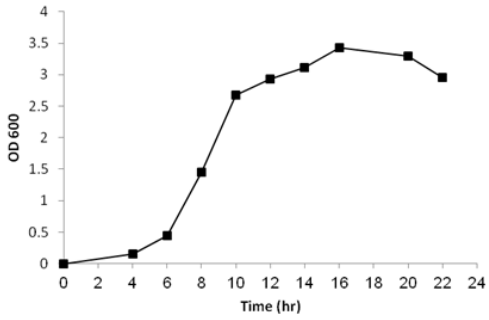


Fig 4. TY_1 isolated microorganism growth curve

After examining the growth conditions and selecting the end of the logarithmic phase of growth as an appropriate age for microbial inoculation, the 4-chlorophenol 100ppm decomposition was performed by TY_1 microbial species. In Figure.5 the 4-chlorophenol biodegradation process is shown in terms of time by TY_1 isolated microorganism. The TY_1 microorganism is capable of completely degrading 100ppm of 4-chlorophenol in about 45 hours.

3.3. 4-Chlorophenol degradation using TY_2 isolated microorganism

After examining the appearance of the TY_2 species, the microorganism growth curve was examined and drawn up. Figure 6 Shows the TY_2 isolated microorganism growth curve. After examining the growth conditions and selecting the end of the growth logarithmic phase as an appropriate age for microbial culture, the 100ppm 4-chlorophenol degradation was performed by TY_2 microbial species.

In Figure 7 shows the 4-chlorophenol biodegradation process in TY_2 isolated microorganism in terms of time. The TY_2 microorganism is capable of completely degrading 100ppm of 4-chlorophenol in about 21 hours. This strain is a superior microorganism among microorganisms isolated from wastewater, which is able to remove 4-chlorophenol in the shortest time compared with other isolated microbial species.

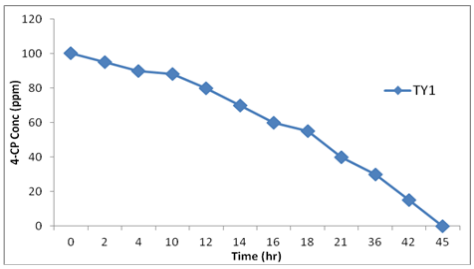


Fig 5. Changes in 4-chlorophenol concentration by isolated microorganism TY1

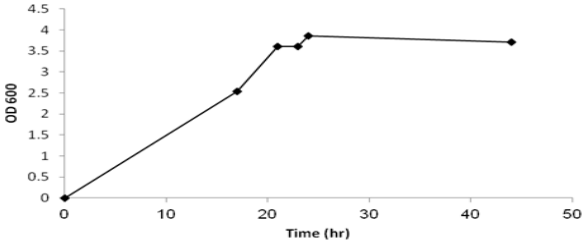


Fig 6. Growth curve of TY2 isolated microorganism

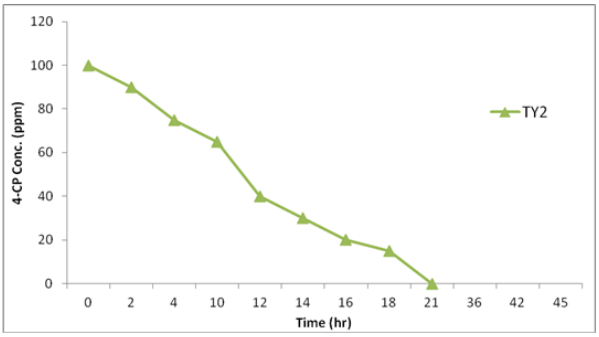


Fig 7. Changes in 4-chlorophenol concentration by isolated microorganism TY2

3.4. 4-Chlorophenol degradation using TY_1TY_2 mixed microbial culture

After isolation of wastewater microorganisms and purification and performing removal tests, two microbial species TY_1TY_2 were available for examination of 4-chlorophenyl degradation experiments. Biodegradation experiments were done using mixed culture with two TY_1TY_2 microorganisms combined percentage of each (50/50 TY_1TY_2) and in the presence of 100ppm of 4-chlorophenol and 2g/l of glucose as a substrate. In these experiments, 4-chlorophenol degradation was compared by mixed culture and its degradation by pure strains. Figure 8 shows the changes in the biomass growth of pure cultures and mixed microbial culture in the presence of 100ppm of 4-chlorophenol and 2g/l of glucose. Two TY_1TY_2 microbial species were able to completely degrade 100ppm of 4-chlorophenol in approximately 18 hours.

Figure 9 shows changes in the concentration of 4-chlorophenol during the process of degradation by pure and mixed microbial culture.

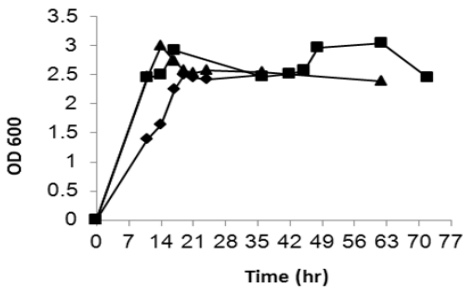


Fig 8. Comparison of cell mass growth in the 4-chlorophenol degradation process using mixed and pure culture of two microbial strains (TY2:♦, TY1:■, TY1TY2:▲)

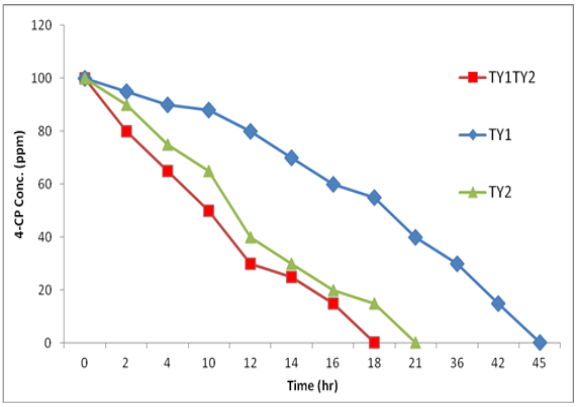


Fig 9. Comparison of changes in 4-chlorophenol concentration in the 4-chlorophenol degradation process using mixed and pure culture of two strains of TY1 and TY2 (TY2:▲, TY1:♦, TY1TY2:■)

3.5. Effect of carbon source concentration in 4-chlorrrophenol degradation

Considering that the enrichment operation was done in presence of 2g/l of glucose and the microbial species were isolated at this concentration, in addition to examining the degradation in concentration 5g/l, another concentration of glucose was also investigated because the high concentrations of the primary substrate can be a deterrent to 4-chlorophenol degradation and one of the important factors in the removal of pollutants. The primary effect of glucose as the primary substrate is on the growth of existing microorganisms, so at higher concentrations, we will necessarily have further growth of the biomass. In Figure 10, 4-chlorophenol degradation was shown at two concentrations of 2 and 5g/l at different times. It is concluded that the concentration of 2g/l of glucose was consumed at shorter time and resulted in the degradation of 4-chlorophenol in shorter time.

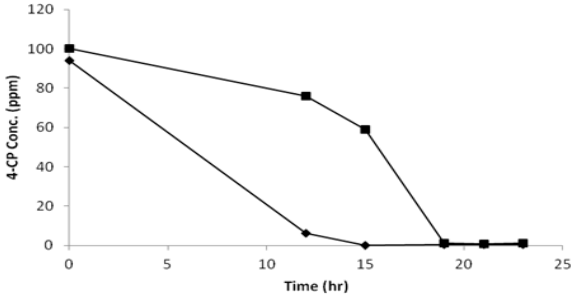


Fig 10. 4-Chlorophenol changes over time in different concentrations of glucose by TY1TY2 mixed culture with combination of 50% in presence of 100ppm of 4-chlorophenol (♦: 2g/l glucose concentration, ■: 5g/l of glucose concentration)

4. Discussion

From the appearance of the results of the experiments, it seems that the mixed microbial culture acts as an isolated single species, TY_2, so that the elimination time in both TY_2 microbial culture and TY_1TY_2 mixed culture is approximately the same. The complete removal time of 100ppm 4-chlorophenol by TY_2 strain is 21 hours, and the complete removal time of 100ppm 4-chlorophenol by mixed culture TY_1TY_2 in presence of 2g/l glucose was determined at about 18 hours.

In the following, some previous studies have been cited in comparison with the present research. Butani et al. (2012) conducted a study on phenol degradation by a bacterial strain isolated from a phenol-infected place in India. A gram-positive bacteria strain isolated in this study was conducted to investigate the phenol biodegradation in the culture. Various physicochemical parameters such as pH, temperature, the initial concentration of phenol plus carbon and nitrogen sources were optimized for the highest phenol biodegradation conditions. Finally, a gram-positive bacteria isolate was selected as a candidate for the phenol wastewater recovery (Butani et al., 2012). Chandrakant et al. (2011) conducted an investigation into the removal of chlorophenol from contaminated water by *Bacillus cereus*. In this study, isolated *Bacillus cereus* was able to reduce the chlorophenol 50% ppm concentration by 98% for 60 hours (Chandrrakant et al., 2011). Gaufeng et al. (2004) used six halophilic bacteria strains isolated from soil contaminated with phenol to remove chlorophenol. The results of this study showed that the removal rate of chlorophenol 300 Mg/l at a concentration of 5% NaCl was more than 95% (Gaufeng et al., 2004).

In a study by Puhaka et al., (1995) mixed culture performance composed of three isolated microbial species was the same as the function of each of them alone (Puhakka et al., 1995). In another study by Sahinkaya and Dilek (2007), the data (specific rate of removal of 2, 4-dichlorophenol) showed that the yield of pure cultures isolated from mixed cultivation in 300 ppm of 2, 4-dichlorophenol was much better than mixed habituated culture (Sahinkaya et al., 2007).

In any case, the results of mixed microbial culture should be examined on a more detailed scale. In Table 6, the effect of mixed culture and pure microorganisms can be studied and compared in 4-chlorophenol degradation.

In the present study, microorganisms capable of biodegradation of 4-chlorophenol were first isolated and purified using adaptation and enrichment operations on wastewater and sludge. Due to the increased ability of wastewater and sludge microorganisms in using 4-chlorophenol as the only source of energy and carbon in all experiments, 2 g/l glucose was used as a substrate. The enrichment and adaptation operations lasted for about 60 days, and the microbial species obtained from the last stages of adaptation and enrichment operations were purified. The results showed that the adaptation and enrichment of the microorganisms in the petrochemical sludge and wastewater of the Imam Khomeini Petrochemical was very effective in increasing the 4-chlorophenol biodegradation.

After examining the growth process of all isolated species, by examining the elimination by them in the mineral microbial culture, isolated microbial species, suitable microorganisms for the 4-chlorophenol biodegradation were selected. In following, 4-chlorophenol biodegradation tests were performed by selected microorganisms and mixed culture, and the behavior of single and mixed microbial culture was compared. The results showed that mixed microbial culture was not significantly different from pure microbial species, but mixed culture would probably show this difference in the metabolic path of 4-chlorophenol degradation.

In the final stage of the experiments, the effect of the initial concentration of glucose with two concentrations of 2 and 5g/l was considered as a substrate. The results indicated that with increasing 4-chlorophenol concentration, its degradation time would be longer, but higher glucose concentration, despite the higher growth of microorganisms in its presence, had an inhibitory effect on microbial culture in 4-chlorophenol degradation.

Table 6. Comparison of biodegradation performance by mixed culture of the present study with other researchers

Scholar	Microbial culture	Concentration of 4-chlorophenol (ppm)	Substrate of growth supplement	Removal time (Hr)	Removal return (%)
Sahinkaya et al., 2007	Enriched mixed culture	300	Peptone	50	100
Yang et al., 2008	Isolated microorganism	100	Glucose or sodium acetate	96	100
Lee et al., 2007	Enriched mixed culture	50	Glucose	40	100
Wang et al., 1999	*P. putida*	200	Glucose	30	100
Lima et al., 2004	Algae collection	50	-	120	100
This research	Microbial mixed culture	100	Glucose (2g/l)	18	100
This research	Microbial mixed culture	100	Glucose (5g/l)	22	80

Refereces

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