

Isolating and Identifying Soil Bacteria Impact on Contamination of Automobile Colors

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Abstract

In the automotive industry, using artificial colors has contaminated natural ecosystems such as groundwater and living organisms. Vehicle colors are one of the most dangerous pollutants of the environment. Biodegradation and using bacteria in order to reduce paint contamination is a cost-effective method, and it is a favorable method for the environment. Therefore, screening of decomposing bacteria of colored compositions from contaminated soils by automotive color was carried out in Fars province. In this study, 12 strains of negative and positive gram bacteria were isolated. Using diagnostic kits, identification of isolates indicated that they included bacterial species such as Streptomyces and bacilli. In order to evaluate the efficacy of these isolates, it was done in liquid culture medium in colorless condition and with the absorbance rate of 260 nm (at 1 to 24 hours); by using the spectrophotometer and in the presence of white metallic and oily paints, isolates were examined on the 6th, 9th and 12th days. The highest percentage of strains was Streptomyces that was classified as the degrading bacteria for oily and metallic paints (91.66%, 81.66%) respectively; and the least percentage of strains was bacillus species (5.15%, 7.33%) respectively. Based on the percentage of bleaching, the most color degradation was observed at 30 ° C and PH7. The data show that the conditions of the culture medium can have a great impact on the growth and color degradation by the color degrading bacteria.

Keywords: Automobile Color, Pseudomonas, Biodegradation, Streptomyces.

Introduction

Color is a mixture of pigmentation fluid that, after its introduction as a thin film, turns into a non-transparent surface. The pigment is a very fine particle that is used to make the color and insoluble in the base essentially. The base is referred to a liquid component which includes a color former resin, a volatile material in the color, and everything else solving in the liquid portion of the color. The shades and paint coatings are provided by pigmentation; therefore, in the definition of color, it was said that pigment should not be shade necessarily (Abadulla et al., 2000). The automobile color should have this feature that is drying in the air quickly. For this reason, the pigment is dissolved in highly volatile organic solvents such as amyl acetate, ethyl acetate, and butyl acetate. Metallic pigments were used for metallic colors (Abraham et al., 2007). Since 1856, when the first synthetic color, Mavin, has been made; 100,000 synthetic colors, with an annual volume of 0.7 million tons, are being manufactured in the world. Synthetic colors are used in textile industry, paper, foodstuff, cosmetics and sanitations (An et al., 2002). Aromatic compounds have high thermodynamic stability because of their high negative resonance energy. Micro-organisms, mainly bacteria, have enzymatic systems that destroy the benzene structure in aerobic and semi-aerobic conditions. Semi-aerobic metabolism or depend on Oxygen that is common in Azo compounds are made up of two main and secondary routes. The main route is to convert large different components into a few central intermediaries. In the aerobic metabolism, the initial reaction, with aromatic ring groups and hydroxyl groups, occurs after the breakdown of the graft due to the arrival of two oxygen atoms. These reactions are catalyzed by hydroxylases and oxygenates. Under the semi-aerobic conditions, broken down the ring created due to ring-restoring which involves carboxylation, revived de-hydroxylation, and increasing reactions that do not occur in aerobic metabolism (Bromley et al., 2004). In an anaerobic environment, decolorization of the Azo dyes leads to the formation of

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aromatic amines, and it is believed that aromatic amines are more resistant to degradation in the conditions. However, mineralization of a small number of aromatic amines, under methanogen conditions, was reported which include three 2-isomers and four amino phenols, 2-amino benzoate, 4-dihydroxy aniline and 5-aminosalicylic acid. Complete degradation of de-salicylate Azo and partial mineralization of orange Montant Azo under methanogen conditions were reported (Zhang et al., 2004); Aerobic degradation of aniline and p-amino benzoate had been done (Zhang et al., 2004).

Biodegradation of colors occurs both in aerobic and anaerobic conditions. Aerobic treatment is one of the common methods but it is not effective than continual aerobic-anaerobic treatment. In a study on four strains of bacteria including *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Redo bacterosphaeroid* *Sevoroid* and *Bacillus cirulcance*, it was stated that a two-stage reactor system (anaerobic-aerobic) required to decolorization of Azo color (sulfate diazo Remazol black as The model) (Dafale et al., 2008).

Zimmerman et al. (1982) studied on differentiated of Azo color degradation base on their structures. The researchers noted that Azo compounds with a hydroxyl or amino group would be more easily degraded than Azo compounds with methyl, methoxy, sulfa or nitro groups (Zimmermann et al., 1982). In 1996, Bannat et al used an enrichment method to isolate the strains involved in decolorization, and after several months, they began some re-cultivation experiments to adapt microorganisms of samples with Azo. In this report, by selecting colored samples and wastewater containing different colors, the competing strains were separated in less time (Banat et al., 1996). Bragger et al (1997) discovered a strain that was able to decolorized and use azosulfonate (4-carboxy-4-sulfo-azobenzene) as the only source of carbon and energy (Bragger et al., 1997).

Beydilli used different physical, chemical and biological methods to remove color from wastewater containing color components. Physical-chemical techniques include membrane filtration, coagulation/flocculation, sedimentation, absorption, ionic exchange, mineralize with ultrasound waves, electrical decomposition, advanced oxidation and chemical revive (Beydilli et al., 2000). Bras et al. (2001) found that pH ranges from 6 to 8 provide optimal conditions for the decomposition of Azo colors (Bras et al., 2001).

Methods

Collecting samples from soil

Collecting samples of soil was carried out of 7 automobile painting workshops located in different parts of Shiraz which are referred them in Table (2-2) with geographical location. During the samples collection, sterilize conditions was followed and soil samples were collected from the depths of 5 cm from the soil surface and placed in sterilized and autoclaved plastics. The collected samples were kept at 4 ° C and transferred to the microbiological laboratory for isolation of the organisms and their initial identification.

Table 1: separation areas of isolates

number	Sample separation areas	latitude	Longitude	Height
1	Akbar Abad	41' 12.21" N °29	32' 59.65" E °52	1715m
2	Javadiyah	42' 9" N°29	29' 15.17" E°52	1649 m
3	Passenger terminal of Yaghutin Street	36' 40.32" N°29	29' 3.4" E°52	1567m
4	Aboozar Ghaffari Dehpayalah	34' 32.16" N°29	32' 58.92" E°52	1507 m
5	Pottery	34' 41.159" N°29	33' 38.88" E°52	1502 m
6	Nasr Street	35' 0.032" N °29	36' 56.66" E°52	1486m
7	Quran Gate	38' 46.52" N°29	34' 11.29" E°52	1634m

Sampling and counting bacteria:

After transferring the samples to the laboratory, bacteria were counted by cunt total viable plate method. In this method, soil samples from the workshops were prepared with a dilution physiology serum of 10^{-1} to 10^{-6} and cultivated in a plat cont agar 0.5 grams/liter of oily white and metallic white on the surface; and also the suspension was re-cultivated in a non-colored culture medium. After 24 to 42 hours, it was incubated at 30 ° C. Numbers of colonies was counted in colorless and colored media.

Isolates screening

After 24 hours, the colonies were selected for purification; from 200 isolates, 12 organisms that were different in term of morphology, the pigment and the colony arrangement evaluated in the aspect of decolorization and incubated in a pure agar medium for 24 hours at 30 ° C. Then, each of the isolates, based on the hot test, was classified into two groups, positive gram and negative gram and after

identification of the selected samples, the samples were transferred to the broth neutralizing medium. As such, by using a sterile loop, the samples were added to 5 ml of the culture medium and incubated for 24 hours at 30 ° C. After 24 hours, a hazy environment, which is a sign of the complete growth of bacteria, as compared with McFarland's half and it was used for further experiments.

Preparing a specific culture medium for evaluation of decolorization

To study the effect of decolorization, MSM (mineral salt medium) was used as a specific environment for decolorization test.

Isolation of microorganisms from soil

To isolate the microorganisms, first, the dried soil samples were screened to separate the coarse particles. Then, in 7 sterilized tubes, 9 ml of sterile physiological serum was added and then with 1 gram of screened soil added to the first tube and dilutions were sequentially prepared to 10⁻⁶ (Fig. 1). Then, white oily broth and metallic broth were used to enrichment. 0.1 ml of suspension was taken from each tube and spread on a white oily broth medium and metallic broth. For purification and identification of microorganisms, after 24 hours, the colonies were selected for purification. The bacteria that were morphological, pigment and colony arrangement were different, were evaluated.

Isolation and purification of color degrading bacteria

To purify and identify of color degrading bacteria, a culture medium of white oily broth and metallic broth base was applied.

Choosing a superior bacterial strain with decolorization feature

For this purpose, two culture medium were used that the isolated bacteria were cultured in a non-colored culture medium and in a culture medium containing white oily broth and metallic broth. Initially, the color was solved in an organic solvent (acetone) (color as a food source for color degrading bacteria). Sterile culture medium was used to blanch. Then the bacteria culture medium was added to it. For each 100 ml of culture medium, 10 ml of cultured bacteria were cultured from the last night (in a basic environment it was cultured for 24 hours) and then by UV-VIS spectrophotometer (Lombda 25 Model, Perkin Elmer, made in the USA) At 540 wavelengths, optical absorption (OD) is read for identified bacteria.

The amount of absorbance for each cultured bacterium in a color-containing environment was investigated for 12 days. Also, it was read on the 6th, 9th and 12th days of absorption again, and recorded; the amount of decolorization was measured by using the following formula:

$$\text{Decolorization \%} = (\text{OD initial} - \text{OD control}) / \text{OD initial} \times 100$$

Then, color degradation level by bacteria in a culture medium containing color compared with the growth of bacteria in a non-colored medium (culture medium) was evaluated. As it was observed, the bacteria grew in a non-colored base culture medium for 24 hours, but in a medium containing color, the growth time was 12 days. Strains with a higher speed of decolorization were investigated for further identification and determination of decolorization power in actual waste containing this color. The bacterial identification was performed based on the shape, biochemical and genetic characteristics of SrRNA 16 Coughlin et al., 2002).

Optimizing decolorization conditions:

Decolorization was tested in the presence of carbon source, suitable concentrations of color (0.5-1 mg / L) and pH values (5-7-9), which contained nutrients in the basic and selective culture media. The proper pH is considered 7 because the bacterial mortality increases with increasing pH. Because the bacteria were mesophilic and isolated at a temperature of 30 ° C, 30 ° C temperature was used for this purpose, however, 20 ° C and 40 ° C temperatures were considered. The optimum growth temperature was considered to be 30 ° C. Since these bacteria were neither acidic nor alkaline, they were considered pH = 7 at all stages of growth, and the best growth was at pH = 7. But it was also used to check the pH (9-5) more accurately.

Phenotypic identification of isolated microorganisms from wastewater of color workplace

After the bacteria were evaluated for their ability to decolorization, identification of each strain was performed by using biochemical tests. Initially, with the help of organisms' gram in the aspect of a positive or negative gram and morphology, they were studied. Then, due to the differentiation of germs into two gram-positive and negative groups, gram-positive bacteria were stained with acid and

phosphorus and then catalase and oxidase tests were done. Other tests were used to detect gram-positive bacteria including starch hydrolysis, hemolysis, hydrolysis, nitrate revive test, growth in 7% NaCl and Oxidative-Fermentative test, and Api 20E kit was used to detect negative gram bacteria; (Baroon et al., 1990).

Experiment results

Bacteria counting

The most number of bacteria in the presence of color related to Abozar Ghafari Dehpialeh's sample and the least number of bacteria in the presence of color related to the sampling station in Kozegari. The most number of bacteria in the absence of the color related to the Javadiieh sampling station and the least number of bacteria in the absence of the color related to the sampling station of the Quran Gate.

Table 2: counting bacteria in different regions

Number	Sample separation areas	Without color	With color
1	Akbar Abad	55*10 ⁶	10*10 ³
2	Javadiyah	65*10 ⁵	55*10 ²
3	Passenger terminal of Yaghutin Street	45*10 ⁴	15*10 ³
4	Aboozar Ghaffari Dehpayalah	20*10 ⁵	45*10 ³
5	Pottery	35*10 ⁴	5*10 ³
6	Nasr Street	25*10 ⁵	15*10 ³
7	Quran Gate	15*10 ⁴	10*10 ³

Identification of gram-positive microorganisms

Gram-positive isolates were identified by using biochemical tests. Based on this, five kinds of bacteria were gram-positive, belonging to Bacillus, Streptomyces and Enterococcus bacteria.

Table 3: Biochemical tests

Chemical tests	Bacillus	Streptomyces	Enterococcus
Hot staining	+	+	+
Fast acid staining	-	+	-
Urease test	-	+/-	-
Nitrate revive test	+	+	-
Potpsy hydrolysis test	+	+	+
Growth in NaCl 7%	-	+	+
Catalase test	+	+/-	-
Oxidase test	+	+/-	-
Citrate test	+	+/-	-

Identification of gram-negative microorganisms

Gram-negative isolates were identified by using API 20E kits, based on this, Pseudomonas and Citrobacter were reported.



Pseudomonas



Citrobacter

Relative frequency and genera percentage of color degrading bacteria

Five genera of bacteria, according to the diagram (1-4), the genus *Pseudomonas* and *Bacillus* showed with 33.33% of the most isolated genera (from 12 isolates, 4 pseudomonades identified), and the bacteria of *Citrobacter* and *Streptomyces* have the least frequency with 8.33%.

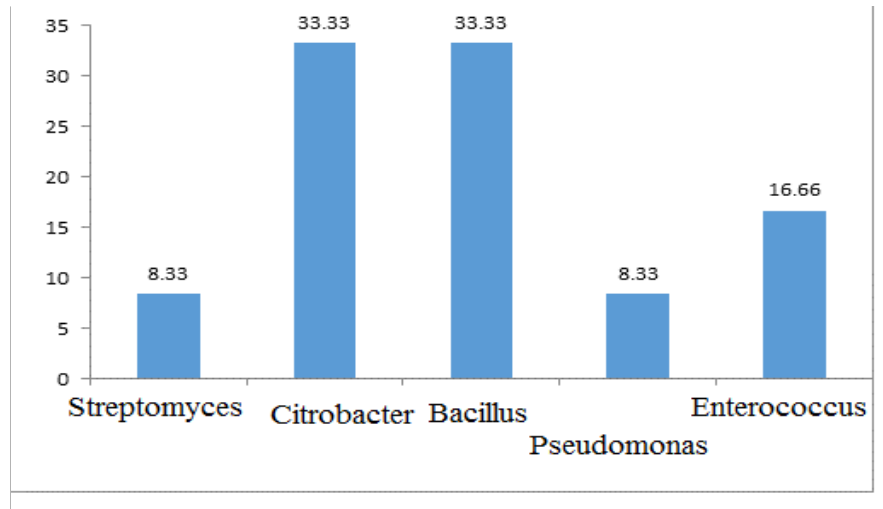


Fig. 2. Relative frequency and genera percentage of color degrading bacteria

Evaluation of decolorization by isolates

The decolorization levels of the isolates at the concentration of 0.5 g/l of the used color at 30 ° C at pH = 7 after the 6th, 9th and 12th days were calculated by using the formulas mentioned in the previous chapter, and all isolates had different percentages of the ability to test the color, while the highest percentage of decolorization was on day 6th, 9th and 12th and belongs to bacteria number B1, which was 15.83% - 79.16% - 91.66% respectively. Also, in the absence of color according to the 24-hour analysis, B12 bacteria had the highest growth rate and B2 had the least growth. Regarding the examination of samples in the presence of color and absence of color, we find that the growth rate of bacteria is quite different from their degradation rate.

The effect of organisms on decolorization with different concentrations

Increasing the concentration of all colors at 1 g/L at 30 ° C was applied to all isolates. Accordingly, the obtained data are shown in the diagram (5-4-3-2). Also, the results of the decomposition of oily white and metallic white in relation to increasing the color concentration indicate that the percentage of decolorization decreases with increasing color concentration, which may be due to the color structure and probably the effect of color on the microorganisms.

Based on the data obtained from the degradation of the oily white color on the 6th and 9th days, isolate 1, isolate 3 and isolate 10 had the highest percentage of decolorization on the 6th and 9th days, but on the 12th day, isolate 1, isolate 10 and isolate 12 had the highest percentage of decolorization.

The absence of color in the two-hour period was considered and presented in the table that at 12 O'clock, the growth rate was less than 24 O'clock, and the superior isolates at 12 o'clock were isolate 12, 1 and 3, respectively; but at 24 o'clock, the highest growth rates were observed, the highest isolates were: isolate 12, isolate 1, and isolate 10 respectively. Accordingly, the bacterial growth rate will increase over time.

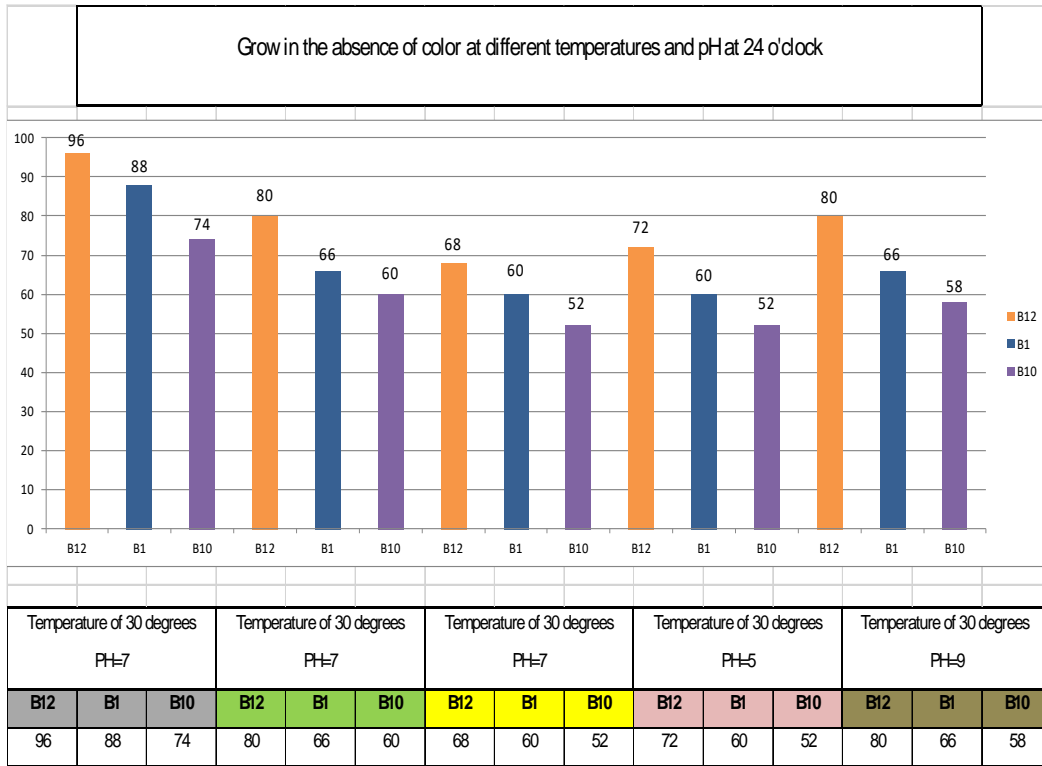


Diagram 1: growth percentage of 12-1-10 bacteria in the absence of color

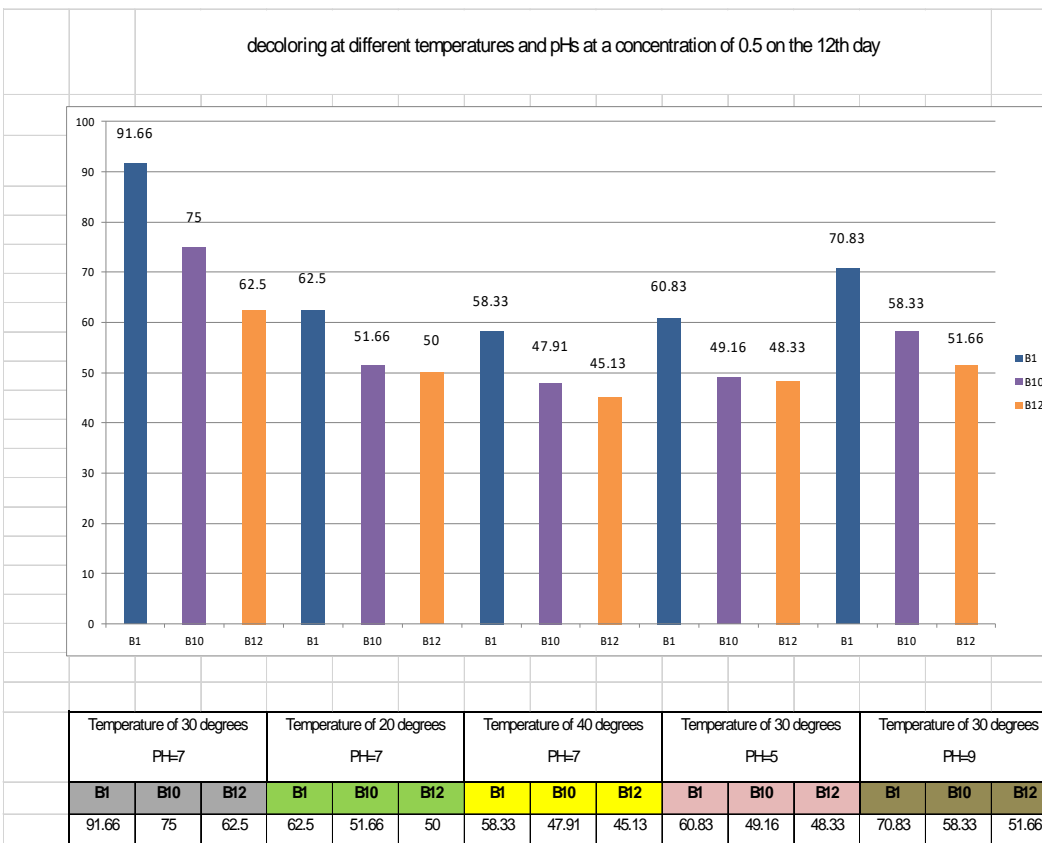


Diagram 2: percentage of decolorization of bacteria 1-10-12 in the presence of color

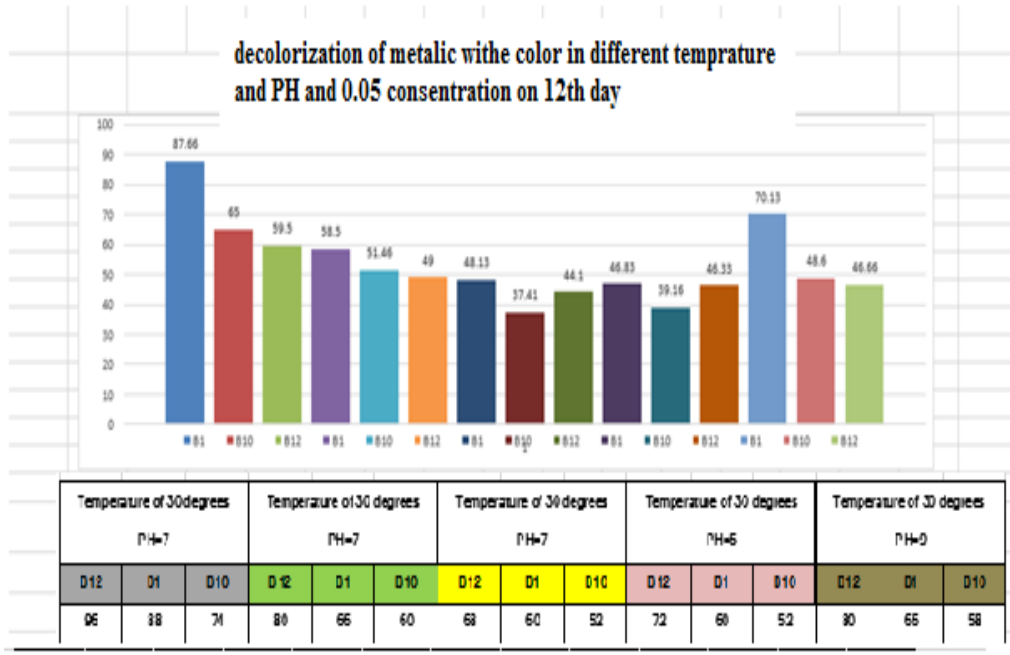


Diagram 3: decolorization percentage of bacteria 1-10-12 in the presence of color

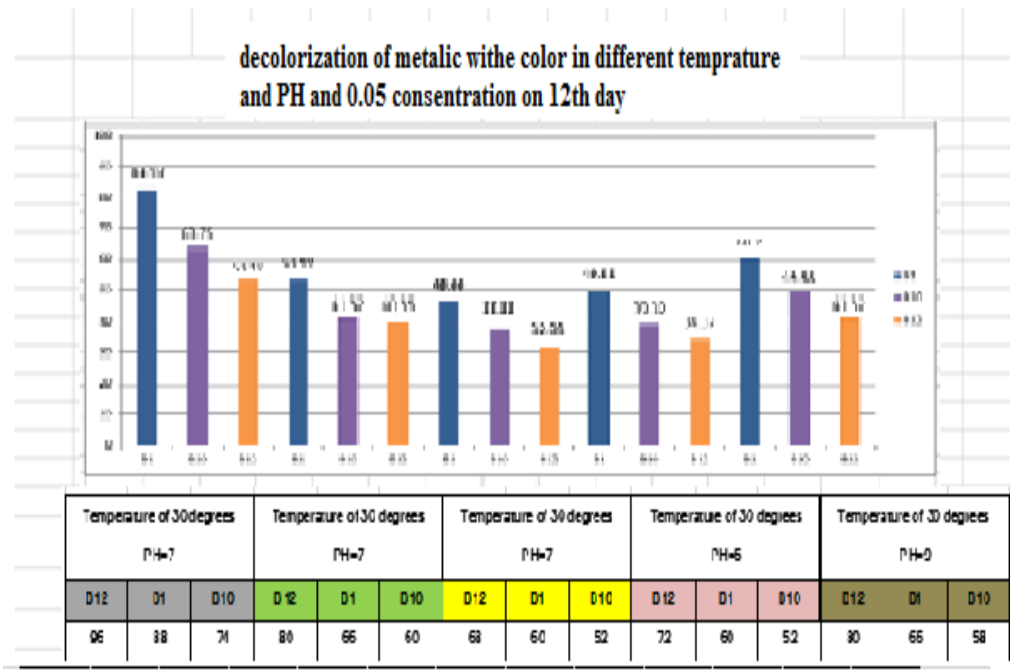


Diagram 4: decolorization percentage of bacteria 1-10-12 in the presence of color

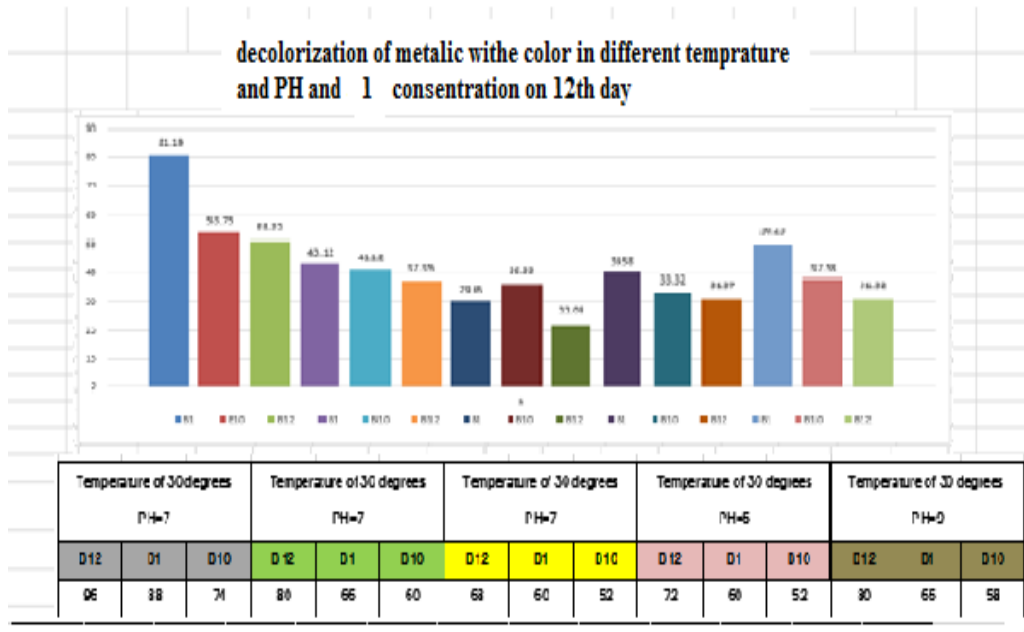


Diagram 5: decolorization percentage of bacteria 1-10-12 in the presence of color

Discussion

In this study, soil samples were collected from seven car painting workshops in Shiraz in order to isolate and diversify the color separating bacteria. Due to the variability in car colors and different applications, after assembling samples, the oily broth and metallic broth and MSM environments were used to enrich and isolate car coloring bacteria. In this study, 12 color degradation bacteria were isolated and after gram staining tests, catalase, oxidase, biochemical tests and DNA extraction by 16S rRNA method, and on the NCBI site, sequences of blast and bacteria were identified. In this study, because environmental bacteria were isolated at 30 ° C and pH 7, the effects and changes of temperature and pH in color degradation were evaluated by these bacteria. Therefore, due to the environmental and neutrality, we avoided to use the temperature difference very high or very low with an optimum temperature of bacteria, as well as high or very low pH with optimum pH, so that the temperature and pH effects on the car decomposition of the car were determined by the bacteria completely. In this study, also, a mixture of bacteria showed the ability to degradation of car color, while the Pseudomonas and Bacillus bacteria with the highest frequency of 33.33 percent were the most isolated genera. The bacteria of the Citrobacter and Streptomyces genera were the least and with the frequency of 8.33%. Separated bacteria were analyzed in terms of color degradation power. In this study, among 12 isolates, Streptomyces strain has a stronger enzyme in the color degradation. In optimal growth conditions, (temperature of 30 ° C and pH7) isolated bacteria in the presence of color showed that the degradation activity of Streptomyces xanthocidicus strain CSSP72 in the presence of oily white and metallic white color on the 6th day after culturing, with decolorization percentage 15.83 and 15.4 respectively, began the color degradation; the maximum degradation activity of oily white and metallic white on the 12th day, respectively, at 91.66, 81.66%, which showed the highest growth activity in the presence of colors at a concentration of 5 g/l.

Conclusion

The aim of this study was to investigate degradation bacteria of automobile color which results show that Bacillus and Pseudomonas and most importantly, Streptomyces, show the highest growth in the presence of color. Also, with changes in temperature and pH, the highest growth of bacteria was observed in the absence of color or in the presence of vehicle color at 30 ° C and pH7. Therefore, the general conclusion can be that:

1. The soil of automotive painting workshops contain hard colored compounds that can lead to environmental contamination.
2. Some of the simplest compounds that used in the color of the automobiles, degraded during photolysis process.
3. Complex compounds that are used in the combination of vehicle colors with the help of enzymes and by decomposing and with the help of bacteria and degrading fungi in the soil are broken down into simpler compounds.
4. Microorganisms, isolated from the soil in the car painting workshops in Shiraz, are able to degraded colors and use colors as carbon and nitrogen sources for their growth.

5. These microorganisms can be used to break down and decompose the colors and refine the sewage of the car paint workshops before being released to the environment.
6. It is a low-cost and cost-effective method to reduce and even eliminate sewage pollution from automobile paint workshops.

Suggestions

1. Since the project is being carried out for the first time in Iran, much more extensive studies in this field are necessary.
2. It is suggested that other car colors like oily colors with metallic gloss-coat be measured.
3. More genus and more different species of bacteria should be examined for color degradation.
4. Because most of the used paints are synthetic and artificial and have a very complex aromatic structure, bacteria should be selected with a broad spectrum degradation power for color degradation.
5. According to studies that color degradation has a direct relationship with temperature, pH and concentration and chemical structure, hence all the factors should be evaluated based on the genus and bacterial species in order to identify the best degradation strain of color.

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