Isolation and Identification of Resistant Microorganisms From Automotive Paint Sludge

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Abstract

**Background:** Paint coating systems are widely implemented on different surfaces for both aspects of decoration and protection against corrosion. Due to the presence of organic compounds, the growth of microorganisms is more likely to take place in paints, such as automotive paint. In the process of automotive painting, 20% - 60% of the paint does not expose to the automotive body, which is washed using water and would lead to the painting sludge formation. Paint sludge is considered one of the hazardous wastes from the automotive industry, which is finally landfilled or incinerated.

**Objectives:** Despite the presence of inhibiting compounds in paint sludge, such as heavy metals and biocides, the objective of this study was to isolate and identify microorganisms in the sludge culture.

**Methods:** The microorganisms were isolated using serial dilutions, direct cultivation, and enrichment methods in basic salt cultivation media. Then, their biochemical and molecular specifications were investigated.

**Results:** The number of microorganisms counted in paint sludge was approximately around 1 × 10⁴ CFU/mL, and six isolated colonies were finally obtained.

**Conclusions:** The main isolated microbial consortium from paint sludge included *Pseudomonas aeruginosa*, *Staphylococcus haemolyticus*, *Micrococcus yunnanensis*, *Rothia amarae*, *Gordonia terrae*, and *Brevibacillus agri*. Nearly 83% of the isolated strains were Gram-positive.

**Keywords:** Paint Sludge, Automotive Industry, Cultivation Media, Six Bacterial Colonies, Resistant Microorganisms

1. Background

Paint is a synthetic material used as a substrate in the texture of furniture and other things (1). Paint is applied using a brush, roller, or spray as a thin layer on wood, metals, and stones to protect against corrosion (2) and create excellent decoration effect. The vehicle paint is a mixture of binders, pigments, solvents, and additives (3). Binders cause adhesion in paint particles, pigments create color and prevent corrosion, solvents lead to the dispersion of paint, and additives strengthen brushing and resistance properties (4). Automotive paint consists of a multilayer coating: primer as an anti-corrosion layer, a base coating that paints the automotive body, and a clear coating for a radiant appearance and UV protection (5). To produce the final automotive color, two types of paints are required: water-based and solvent-based (6). Methods for painting the automotive body include overspray, immersion, and powder methods (7). In overspray staining, only 50% - 80% of the paint reaches the automotive body (6), and 20% - 60% of the paint is transmitted as surplus (8) to the room with airflow and is successively removed using rotary washing water. The detergents added to the wash water separate the flocculants and coagulants from excess paint and contribute to the separation of paint from water (6). The mixture of water and spray paint, called paint sludge, is collected in a sludge pit (7).

Today, with the increasing number of vehicles around the world, the production of paint sludge is growing (6). Each factory produces about 10 - 15 tones sludge per day, containing some elements and contaminants (9). The combination of volatile organic compounds in paints can cause environmental pollution in the short or long-term (10). Ingredients such as antifouling Tributyltin (TBT) in the paint are highly toxic to aquatic environments (2).
Paint sludge is also a serious hazard to the health and environment, and in most cases, its production is inevitable (8). Eighty percent of environmental concerns in automotive painting factories are related to volatile organic compounds (VOCs), carbon dioxide (CO\textsubscript{2}), and heavy metals. As a waste, paint sludge contains heavy metals and dissoluble organic carbon (DOC) of over 3,700 mg/L and, thus, is extremely hazardous. Paint sludge is classified in the EU code for waste disposal 080113 and is considered a waste with hazardous characteristics (6).

Salihoglu et al. investigated the possibility of composting automotive water-based paint sludge with sewage sludge of the same industry as substrate and corncob as a bulking agent in sex reactors. Their results showed that carbon and nitrogen, organic matter and BTEX, nickel and tin, the ratio decreased (11). Ghomi Avili et al. (12) investigated chromium removal from automotive paint sludge using biological sludge and Eisenia fetida worms. The results showed that the Cr\textsuperscript{6+} concentration fell from 6 mg/kg to less than 0.2 mg/kg, pH decreased from 7.8 to 7.3, volatile solids decreased from 80.4% to 37%, and the C/N ratio decreased from about 27 to 14.3 after 90 days.

So far, various isolator microbial species have been isolated and identified from paint (13). Some organic and inorganic compounds are toxic to microorganisms. High quantities of heavy metals (14) and biocides in the paint can inhibit the metabolic activity of bacteria and fungi. Biocides can affect the growth of microorganisms through degradation of the cell membrane, inhibition of metabolic reactions, alteration of intracellular pH, and accumulation of toxic anions (15). Despite the presence of inhibitors in automotive paint sludge, the possibility of the isolation and identification of resistant microorganisms in the sludge was investigated in this study.

2. Objectives

Lots of research have been conducted on the isolation and identification of microorganisms from petroleum sludge, sewage sludge, contaminated coastal soils, mine soils, contaminated sediments, municipal waste, and wastewater sites for use in bioremediation. In the current study, the possibility of isolation and identification of resistant microorganisms in paint sludge was investigated.

3. Methods

3.1. Sampling

Paint sludge samples were collected from the site of an automotive painting factory under aseptic conditions using sterile spatula gathered inside 100 mL sterile Falcon tubes. The samples were then transferred to the laboratory at a temperature of 4°C.

3.2. Methods for Isolation of Microorganisms

3.2.1. Isolation by the Enrichment Method

Conventional salt-based media such as Bushnell Haas medium (BHMS) (16), mineral salt medium (MSM) (17), 9KFe\textsuperscript{2+} (18), 9Ks, and 9KNa\textsubscript{2}S\textsubscript{2}O\textsubscript{3} (19) were used to isolate a wide range of bacteria. Culture media were prepared according to Table 1. Subsequently, 2% (w/v) of sterile paint sludge was added as a single source of carbon and energy. With some modifications in the amount of inoculation, 15 g of a paint sludge sample under aseptic conditions was added to the medium and incubated at 30 ± 2°C in a shaker incubator at 110 rpm for five consecutive days. To create colonies, 10 - 15 g of agar and 2% (w/v) sterile paint sludge were added to produce a solid medium. Then, 0.1 mL of each salt medium of Erlenmeyer cultured (triplicate of each medium) in nutrient agar (NA), potato-dextrose agar (PDA), and plate count agar (PCA) using the pour plate and steric methods and incubated at 30 ± 2°C for five days (20, 21).

3.2.2. Isolation by Direct Cultivation and Serial Dilution Method

Ten g of paint sludge sample was poured into a 250 cc Erlenmeyer under aseptic conditions, and 90 mL of sterile distilled water was added. The Erlenmeyer was covered with sterile cotton and homogenized with a shaker. Then, a portion of 1 mL was added to a tube containing 9 mL of sterile distilled water along with shaking. It continued serially to a dilution of 10 - 5. Then, 1 mL of each dilution and direct sample were cultured (triplicate of each dilution) on a plate containing NA and PDA. The plates were incubated at 30 ± 2°C for five days (22).

3.3. Morphological Properties

Gram-positive and Gram-negative strains were detected by Gram staining and microscopic observation.

3.4. Biochemical Properties

Species were isolated according to Bergey’s manual of determinative bacteriology (23).

3.5. Molecular Identification

The 16SrRNA gene sequencing was performed by genomic extraction using Favorgen’s Mini Kit Genetic DNA extraction kit. A polymerase chain reaction (PCR) was done to determine 16SrRNA gene proliferation using primers 4F: 5’-TATCGGAGAGTTTGATCCTGG-3’ and 1541r: 5’-AAGGAGGGATCCAGCCGCA-3. The PCR program was performed in 35 cycles (24), as given in Table 2. The
Table 1. Composition of Salt Media for Isolation of Microorganisms

| Media      | Values          |
|------------|-----------------|
| BHMS media |                 |
| MgSO\(_4\).H\(_2\)O, g | 0.2  |
| CaCl\(_2\).H\(_2\)O, g | 0.002 |
| KH\(_2\)PO\(_4\), g | 1     |
| K\(_2\)HPO\(_4\), g | 1     |
| NH\(_4\)NO\(_3\), g | 1     |
| FeCl\(_3\), g | 0.05  |
| Distilled water, cc | 1000  |
| pH         | 7               |
| 9KFe\(^{2+}\) media |        |
| (NH\(_4\))\(_2\)SO\(_4\), g | 3  |
| KCl, g     | 0.1             |
| K\(_2\)HPO\(_4\), g | 0.5  |
| MgSO\(_4\).7H\(_2\)O, g | 0.5 |
| Ca(NO\(_3\))\(_2\), g | 0.01 |
| Distilled water, cc | 700  |
| H\(_2\)SO\(_4\) (w/v), N | 10   |
| FeSO\(_4\).7H\(_2\)O, g | 44.22|
| Distilled water, cc | 300  |
| pH         | 1-2             |
| MSM media  |                 |
| K\(_2\)HPO\(_4\), g | 0.05  |
| MgSO\(_4\).H\(_2\)O, g | 0.5  |
| Ca(NO\(_3\))\(_2\), g | 0.01 |
| KCl, g     | 0.1             |
| Na\(_2\)SO\(_4\).10H\(_2\)O, g | 3. |
| (NH\(_4\))\(_2\)SO\(_4\) | 3     |
| Distilled water, cc | 1000 |
| pH         | 7 ± 0.2         |
| 9KS media  |                 |
| (NH\(_4\))\(_2\)SO\(_4\), g | 3  |
| KCl, g     | 0.1             |
| K\(_2\)HPO\(_4\), g | 0.5  |
| MgSO\(_4\).7H\(_2\)O, g | 0.5 |
| Ca(NO\(_3\))\(_2\), g | 0.01 |
| Sulfur or thiosulfate, g | 10   |
| Distilled water, cc | 1000 |
| H\(_2\)SO\(_4\), M | 2     |
| pH         | 6.5             |

PCR products were electrophoresed, and after observing the proper band, they were purified by Favorgen’s Purification Mini kit. The DNA concentration after purification was measured at 260 nm. The purified PCR products were sent by the Iranian Research Organization for Science and Technology to Bioneer Company in Korea for sequencing. The sequencing was performed using primers as follows: 16r339: 5'-ACTGCTGCTCCCGTAGGAG-3', 27f: 5'-GAGTTTGATCCTGGCTCAG-3', 704f: 5'-GTAGCGGTAAATGCGTAGA-3' and 16f358: 5'-CTCTCACGGGAGGCAGCAG-3'. The obtained sequences were compared with nucleotide sequences available in valid databases, including NCBI and Eztaxon. Then, the phylogenetic trees were plotted using Mega version 6 software (25, 26).

| Process    | Temperature, °C | Time |
|------------|-----------------|------|
| Initial denaturation | 95             | 3'   |
| Denaturation   | 93             | 45"  |
| Annealing   | 58             | 60"  |
| Extension   | 72             | 90"  |

Table 2. Polymerase Chain Reaction Program Protocol

4. Results

The number of microorganisms counted with the plate count agar method was 1 × 10\(^4\) CFU/mL. Six colonies were isolated on a saline culture medium, including BHMS, MSM, and 9KNa\(_2\)S\(_2\)O\(_3\), and nutrient agar medium using direct and serial methods., using the direct and serial methods. For Gram staining, an expansion of isolated colonies was prepared, and the isolation of species was performed based on Bergey’s manual. Some morphological and biochemical properties of the isolated microorganisms are given in Table 3.

To investigate the PCR products, electrophoresis was performed, and 1,500 nucleotide fragments were observed, according to Figure 1. The DNA concentration after purification at 260 nm was measured, and the results are presented in Table 4. The analysis disclosed sequences with different nucleotides. These sequences were compared by basic local alignment search tool (BLAST) with the nucleotide sequences available in valid databases, such as the National Center for Biotechnology Information (NCBI), and the results of the similarity percentage of the strains with the existing ones are presented in Table 5. Figure 2 indicates an example of a phylogenetic tree using Mega version 6 software.
Table 3. Morphological and Biochemical Properties of the Isolated Microorganisms

| ID | aA | bB | cC | dD | eE | fF |
|----|----|----|----|----|----|----|
| Medium | BHMS | 9K | MSM | NB serial | NB serial | NB direct |
| Gram stain | Gram- | Gram+ | Gram+ | Gram+ | Gram+ | Gram+ |
| Morphology | Bacilli | Cocci | Cocci | Spherical | Coccoid | Rods |
| Colony shape | Circular | Circular | Circular | Circular | Rough | Flat |
| Colony color | Gray | White | Yellow | Cream | Orange | Gray |
| Size, mm | 2 - 2.5 | 0.5 - 1 | 0.15 - 3 | 1 | 2 - 2.5 | 2 - 4 |
| Catalase | + | + | + | + | + | + |
| Oxidase | - | - | - | - | - | + |
| Coagulase | + | - | ND | ND | ND | ND |
| OF | Oxidative Ferment | Inert Ferment | ND | + |
| Motility | + | ND | ND | ND | ND | + |
| Indole | - | ND | ND | ND | ND | - |
| Urease | ND | + | - | - | + | - |
| Hemolysis | ND | ND | ND | ND | ND | Alfa |
| Nitrate reduce | ND | + | + | - | + | - |
| Starch | ND | ND | ND | ND | - | + |
| Lipase | + | ND | ND | - | - | + |
| Gelatin hydrolysis | + | ND | ND | ND | ND | ND |
| Denitrification | + | ND | ND | ND | ND | ND |
| Hemolysis | ND | + | ND | ND | ND | ND |
| ONPG | ND | - | ND | ND | ND | ND |
| MR/VP | ND | ND | - | ND | ND | ND |

Figure 1. PCR product electrophoresis

5. Discussion

The present study showed that each salt medium provided growth conditions for some of the microorganisms found in paint sludge. *Micrococcus yunnanensis* was isolated from paint sludge in MSM, while in the same medium, Phulpoto et al. (27) isolated *Brevibacillus parabrevis* strains from oil-based paint sludge and Ashwini et al. (28) isolated *Pseudomonas*, *Staphylococcus*, and *Lactobacil-
Figure 2. The phylogenetic tree of the fF-Brevibacillus strain plotted with Mega software version 6.

Table 4. The Concentration of Purified DNA

| Strain Code | DNA Concentration, ng/µL |
|-------------|--------------------------|
| Strain A    | 154                      |
| Strain B    | 87.5                     |
| Strain C    | 89.6                     |
| Strain D    | 70.7                     |
| Strain E    | 93.8                     |

Table 5. The Comparison of the Similarity Percentage of Strains with National Center for Biotechnology Information

| Strain Code | 16s rRNA Sequence | Identification | Similarity, % |
|-------------|-------------------|---------------|--------------|
| Strain A    | 1496 nucleotides  | Pseudomonas aeruginosa JCM 5962(T) | 100          |
| Strain B    | 1516 nucleotides  | Staphylococcus haemolyticus MTCC 3383(T) | 99.86        |
| Strain C    | 1484 nucleotides  | Micrococcus yunnanensis YIM 65004(T) | 99.86        |
| Strain D    | 1492 nucleotides  | Rothia amarae JCM 11375(T) | 99.79        |
| Strain E    | 1483 nucleotides  | Gordonia terrae NBRC 100016(T) | 100          |
| Strain F    | 1495 nucleotides  | Brevibacillus agri DSM 6348(T) | 99.93        |

5.1. Conclusions

According to strains obtained in this study and the presence of resistant microorganisms in the automotive paint sludge, one can investigate the idea of the bioremediation using microorganisms existing in this type of sludge to reduce the pollution load and make the compliance with landfill standards.
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Footnotes

Authors’ Contribution: Fatemeh Honarjooy Barkusaraey performed the experiments. Fatemeh Honarjooy Barkusaraey, Roya Mafigholami, Mohammad Faezi Ghasemi, and Gholam Khayati performed the literature review, data collection, analyzed and interpreted the data, and prepared the manuscript text.

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