Investigation on the Biodegradative Potential of *Pseudomonas aeruginosa* on Water-based Paints

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Abstract: A strain of *Pseudomonas aeruginosa* (OB-6) isolated from biodeteriorated water-based paint samples was screened for its plasmid profiles and antimicrobial susceptibility patterns. The organism possessed two plasmids which ranged in molecular sizes from 0.030 to 0.112 kilobases (kb). Antimicrobial resistance patterns showed resistance to Tobramycin, Amikacin, Gentamycine and industrial biocides ZN467 and ZN489. After curing experiments with 0.002% (v/v) sodium dodecyl sulphate (SDS), the organism was observed to have lost the two plasmids and the resistance to Tobramycin, Amikacin and Gentamycin. The organism therefore, seemed to bear plasmids that encoded genes for deterioration and antibiotic resistance as determined by the curing experiments. A comparison of the biodegradative potential of the wild and the cured strain on the physico-chemical properties of fresh, sterile paints showed the wild strain to have 25% degradative potential, while the cured strain had 17% degradative potential. [Researcher. 2010;2(1):57-67]. (ISSN:1553-9865)

Key words: plasmid, paints, biodegradative potential

1. Introduction

Plasmid profile analysis has been used to aid in the identification of a source of deterioration and evaluate the efficiency of control measures (Olukoya and Oni, 1990). The mechanisms of bacterial resistance to some antibacterial agents such as antibiotics are reasonably well understood (Morton *et al*., 1998). The biodeterioration of many industrial products by microorganisms is a problem of great economic importance. Economic losses due to microbiological spoilage has been an increasingly significant problem for paint industries in recent years (Da Silva, 2003). Many components of paint formulation such as natural oils, plasticizers and emulsifying agents can be used by bacteria and fungi as carbon sources (Gillatt, 1992). Substances such as starch, protein and various synthetic compounds are used as binders in the paint industry and these substances are also subject to microbiological attack and spoilage (Gillatt, 1992; Da Silva, 2003). Candal and Eagon (1984) studied a total of sixteen isolates of *Pseudomonas* strains that were cultured from biocide-treated kaolin slurries and, in one instance, from a water-based latex paint with respect to their resistance to four industrial biocides. Many of the isolates showed multiple resistance patterns to more than one biocide as well as to certain heavy metals or other antimicrobial agents, and many of the isolates were resistant to agents to which they had no previous history of exposure. One to four plasmids were detected in each of the biocide-resistant isolates that were examined. The experimental data were highly suggestive that resistance to the industrial biocides was due to plasmid-encoded mechanisms. Thus, the purpose of this study was to determine whether the genes that encode for paint deterioration and resistance to antibiotics were chromosomal or plasmid-borne.

2. Materials and Methods

2.1 Microbial strain and cultivation

The microbial strain used for this study was a paint degrader, *Pseudomonas aeruginosa* (OB-6), previously isolated from biodeteriorated water-based paint samples obtained from a reputable paint industry in Lagos, Nigeria (Obidi, 2008). The culture was maintained on nutrient agar (NA) slants and stored at 4^0C when not in use (Tiligada *et al*., 1999). In order to generate inoculum cultures for this study, the organism was grown in 4ml tryptic soy broth in a tube at 37^0C overnight. The density of the inoculum was standardized by dilution with sterile saline to a density of 10^8 cells per ml. Since the presence of plasmids has been linked to biodegradation of organic molecules (Warren *et al.,* 2004), which is evidenced in paints by deterioration of aesthetic features and physico-chemical properties of paint samples, we screened organism for presence of plasmid.

2.2 Detection of Plasmids

Pseudomonas aeruginosa (OB-6) which was isolated from biodeteriorated paint samples was screened for presence of plasmids along side isolates obtained from fresh paints in previous studies (Obidi *et al*., 2009) using the method of Birmboim and Doly (1979). Ethanol-precipitated DNA obtained from the lysate was dissolved in 10 mM Tris (pH 7.5) and 1 mM Na_2 EDTA. The plasmid DNA extract was then subjected to agarose gel electrophoresis at 10v/cm for 4 h using Tris Borate-Na_2EDTA as buffer and 0.1% bromophenol blue as tracking dye. Gels were stained in 7 µl ethidium bromide solution and examined under UV transilluminator using protective goggles. Amplified plasmid DNA appeared as sharp bands that fluoresced when excited with UV light. The molecular weights of the plasmids were then determined by comparing them with the λ DNA Hind 111 digested marker.

2.3 Curing Experiment

The curing procedure was done as described by Sonstein and Baldwin (1972) to determine whether the plasmids isolated from *Pseudomonas aeruginosa* (OB-6) {(renamed OB-6 (W) because it is the wild strain} encoded the spoilage trait or not. To achieve this, *Pseudomonas aeruginosa* [OB-6(W)] was grown on yeast extract trypticase soy (YETS) (0.3% yeast extract and 1.5% agar). It was subsequently harvested from the YETS plates and emulsified in normal saline at a density of about 10^5 cells per ml. This was then inoculated into YETS broth containing 0.002% (v/v) SDS and incubated with constant shaking at 37 ^oC for 18 h. After incubation, the developed colonies were screened for loss of the plasmids by repeating the DNA extraction process.

2.4 Biocide and Antibiotic Sensitivity

The strain of *Ps. aeruginosa* which was isolated from biodeteriorated paint samples and observed to possess plasmids [OB-6(W)] was tested for sensitivity to industrial biocides ZN467 and ZN489 obtained from the paint industry by means of the Kirby Bauer disc diffusion technique (Bauer, 1966). This was done to determine the agar disc diffusion zone diameters and hence the susceptibility of the plasmid-bearing *Pseudomonas aeruginosa* strain [OB-6(W)] isolated from spoilt paints to biocides used in inhibiting spoilage in the industry. The overnight cultures were harvested from NA plates and emulsified in tryptose soy broth. The tubes were incubated aerobically for 2 – 5 h at 37 ^0C to produce a bacterial suspension of moderate cloudiness. The density of the suspension was standardized by dilution with tryptose soy broth to a density of about 10^8 cells per ml. A sterile cotton wool swab was dipped into the suspension and the surplus removed by rotation of the swab against the sides of the tubes above the fluid level. Mueller Hinton agar medium was inoculated by even streaking of the swab over the entire surface of the plate. Sterile filter paper discs soaked in 1% of each of the industrial biocides were then placed one per plate in the center of the plates using sterile forceps. The control plates had no biocide discs. All plates were subsequently incubated aerobically for 16 – 18 h at 37 ^0C. Single discs (Oxoid Ltd Basingstoke Hampshire, England) containing tetracycline (10 µg), gentamycin (10 µg), ceftazidime (30 µg), piperacillin (100 µg), ofloxacin (5 µg) ticarcillin (75 µg), amikacin (30 µg) and tobramycin (10 µg) were also employed for the sensitivity test on both the wild (W) and the cured (C) strains of the organism. The zones of inhibition produced by the test organism (plasmid-bearing strain) were measured to the nearest millimeter with a millimeter rule and compared directly with that of the control strain (the cured prototype). The zones of inhibition observed around the antimicrobial discs were taken as indication of sensitivity and were interpreted based on the Clinical Laboratory Standards Institute (CLSI, 2006) guidelines. The degradative potentials of the wild and the cured strains were determined by inoculating one ml each of the overnight cultures of the wild and cured strains into 9 ml fresh, sterile paint samples (PS-1 — P S-6) and incubated at room temperature (30$\pm$ 2^0C). Physico-chemical parameters including specific gravity, optical density, transmittance, pH, and viscosity were then determined at two weeks intervals.

2.5 Determination of physico-chemical parameters

Specific gravity (SG): Specific gravity was determined by pycnometry as described by Ohwoavworhua and Adelakun (2005). A sterile pycnometer of 50 ml capacity was weighed and the weight recorded as M1. Paint sample (50 ml) was transferred into the pycnometer. The pycnometer and its content were weighed and the weight recorded as M2. The pycnometer containing the paint sample was filled with distilled water and shaken many times to allow all trapped air within the pycnometer to be expelled. The weight of the pycnometer and its content was recorded as M3. The pycnometer was emptied, washed and refilled with distilled water and the weight recorded as M4. Specific gravity was then calculated using the formula:

$$SG = \frac{(M2-M1)}{(M4-M1)(M3-M2)}$$

2.6 Optical density (OD) and transmittance (TR):

This was determined colorimetrically (Rieck *et al.,* 1993) with a photoelectric colorimeter (Model: AE-11C Tokyo Erma Optical works Ltd, Japan). The colorimeter was standardized by adjusting it to read 100% light transmittance with 5 ml distilled water in a 1 cm light path cuvette placed in it at 600 nm. The colorimeter had two scales. The bottom scale displayed the absorbance while the top scale, % transmittance.

2.7 Mean pH:

The pH of the paint samples was determined with a pH meter (Model: Jenway M50/Rev CE350EU) in 1: 200 solution of the paint samples in distilled water. The pH meter was calibrated using phthalate buffer (pH, 4.0) and phosphate buffer solutions (pH, 7.0).

2.8 Viscosity:

Viscosity was determined using a glass capillary tubular viscometer (Model: Capirograph Toyoseikl Seisaku-Sho Ltd) as described by Rammohan and Yassen (2003). The paint sample was allowed to flow through an outlet tube. Two annular reference marks on the measuring tubes were used. The time it took the sample meniscus to drop from the upper to the lower reference mark was measured manually with a stop-watch. The viscosity was then calculated by multiplying the measured time by the viscometer calibration factor at room temperature (30 ± 2^0C).

3. Results and Discussion

A summary of the results with respect to biocides is presented in Plates 1 & 2. The disc diffusion techniques revealed that *Ps. aeruginosa* (OB-6) demonstrated substantial resistance to biocide ZN467 (Plate 1). This probably is as a result of its plasmid possession. Bacterial plasmids have been reported to play a central role in the evolution of bacterial populations and provide a means of gene duplication, thereby, creating an opportunity for accumulated mutational change (Warren *et al.,* 2004). *Ps. aeruginosa* (OB-6) showed least resistance to biocide ZN489 (Plate 2). This is indicative of the differences in the efficacy of industrial biocides used in the paint industry. To investigate whether resistance was limited to industrial biocides which *Pseudomonas* populations in paints were repeatedly exposed to for periods of weeks and months, or it was of more general occurrence, we examined the resistance of *Ps. aeruginosa* (OB-6) that had been isolated previously from biodeteriorated paints to selected antibiotics. The resistance patterns of the plasmid-bearing *Ps. aeruginosa* [OB-6(W)] and the cured strain [OB-6(C)] measured against a range of antibiotics are presented in Figure 1. The susceptibility classification was based on the zone-size interpretative chart for disc diffusion susceptibility testing of the Clinical laboratory Standards Institute (CLSI, 2006). It was clear that the resistance of *Ps. aeruginosa* (OB-6) to industrial biocides was a general phenomenon as resistance was similarly observed with antibiotics. However, the analysis of the antibiotic resistance patterns of the wild and the cured strains revealed a remarkable relationship between possession of plasmids and paint degradation. These findings led us to carry out a comparative evaluation of the spoilage abilities of the wild and cured strains on fresh, sterile paints. The results as shown in Figures 2-6 revealed higher degradation (25%) in paint samples inoculated with the wild strain and less degradation (17%) in the paints inoculated with the cured strain. This observation evidenced in the physico-chemical properties and hence aesthetic features of the paint samples indicate possibility of genetic linkage. The possession of plasmids in *Ps. aeruginosa* OB-6, now [OB-6(W)] may also explain its ability to grow in many environments and degrade a wide range of organic molecules. The plasmid isolation techniques revealed that prior to the curing experiments, *Pseudomonas aeruginosa* [OB-6(W)] harbored 2 plasmids of different molecular weights ranging from 0.030 – 0.112 Kb. Plate 3 shows the plasmid bands on lane 7 consisting of *Pseudomonas aeruginosa* (OB6) as observed under an ultraviolet transilluminator. The other lanes consist of organisms (OB-4 and OB-5) isolated from previous studies (Obidi, 2008; Obidi *et al*, 2009). However, after the curing experiments, *Pseudomonas aeruginosa* [OB-6(W)] was observed to have lost the 2 plasmids that it possessed earlier on (Plate 4). Therefore, growth in YETS broth containing 0.002% v/v SDS resulted in the complete elimination of the plasmids. This result suggests that SDS, employed to eliminate the two plasmids detected in *Pseudomonas aeruginosa* [(OB-6(W)] is an effective curing agent with rates of curing far surpassing rates obtained with ethidium bromide and elevated temperatures (Sonstein and Baldwin, 1972). Various methods of eliminating plasmids from microorganisms have been reported (Kulkarni and Kanekar, 1998). These methods effect curing either by taking advantage of differences in the rates of plasmid and chromosomal DNA replication during growth at elevated temperatures (Ghosh *et al*., 2000) or by the use of agents such as the acridine dyes (Tomas and Kay, 1984) and ethidium bromide (Hein *et al*., 2006) which are believed to inhibit the synthesis of DNA. It appears that SDS may be a useful agent in the study of

extra chromosomal genetic elements and their relationships to the cell membrane because of its ability to disrupt biological materials and cause a permanent, heritable alteration in genetic materials. The comparative evaluation of the spoilage potentials of the plasmid-bearing and the plasmid-cured strains on the physico-chemical properties of fresh, sterile paints, further proved that the plasmid-bearing strain had higher degradative potentials than the plasmid-cured strain (Figures 2 – 7). It was therefore, thought that the genes for degradation may be enclosed in the plasmid. The plasmid-bearing strain [OB-6(W)] was resistant to ceftazidime, amikacin, tobramycin, gentamycin and tetracycline. However, after the curing experiments, it was observed to be sensitive to tobramycin, gentamycin and amikacin. This further confirmed that the gene for degradation of paint may be plasmid-borne. Sodium dodecyl sulphate, probably affected the genes for degradation and spoilage. Since SDS is known to cause disruption of biological materials (Sonstein and Baldwin, 1972), the possibility exists that SDS as a curing agent cured the organism of its plasmid by disrupting the membrane sites of plasmid attachment. The present sensitivity of the cured strain [OB-6(C)] to tobramycin, gentamycin and amikacin was thought to be due to the loss of these plasmids. *Pseudomonas aeruginosa* (OB-6) might have acquired the antimicrobial resistance plasmid to survive in the biodeteriorated paints.

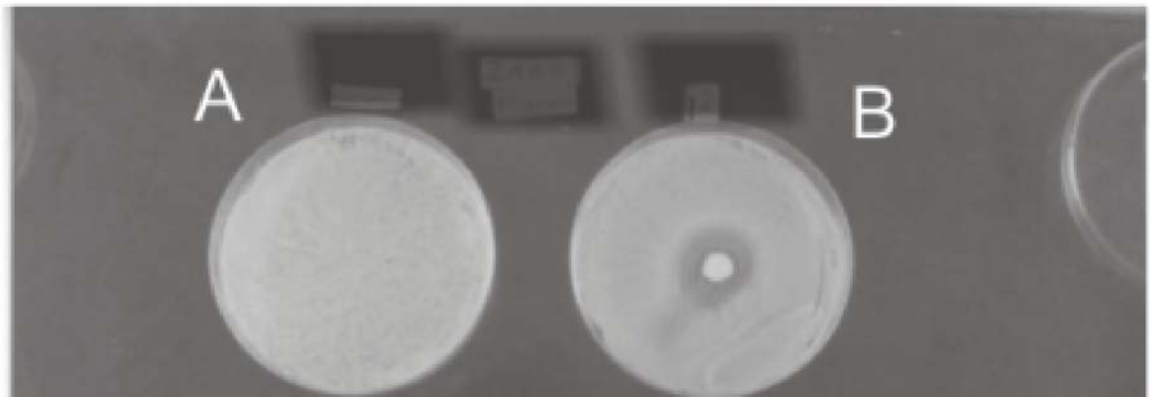


Plate 1. Effect of biocide ZN467 on *Pseudomonas aeruginosa* (OB-6)
A, control; B, 1% v/v.

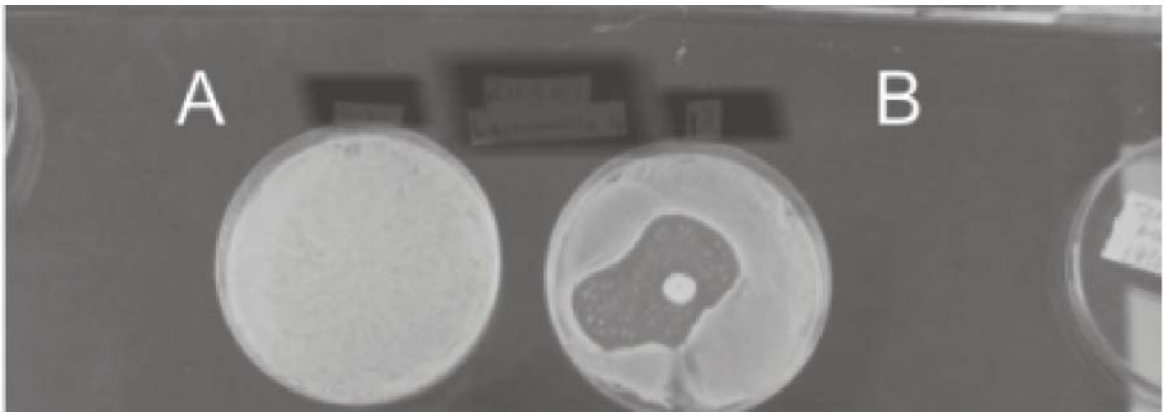


Plate 2. Effect of biocide ZN489 on *Pseudomonas aeruginosa* (OB-6)
A, control; B, 1% v/v.

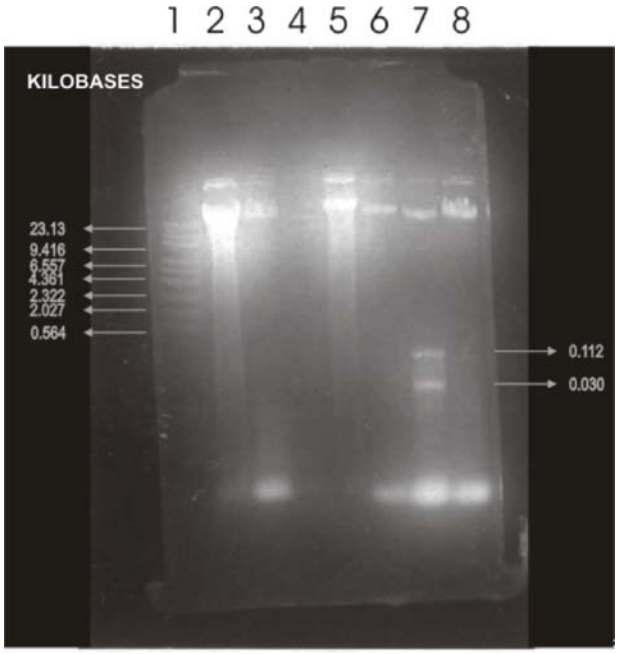


Plate 3. **Agarose gel electrophoresis plate showing plasmid DNA isolated from *Pseudomonas aeruginosa* [OB-6(W)] using the protocol of Birmboim and Doly (1979). Lanes: 1, λ DNA Hind 111 digested (marker); 2, OB-4; 3, OB-4; 4, OB-5; 5, OB-5; 6, OB-5; 7, [OB-6(W)]; 8, OB-4.**

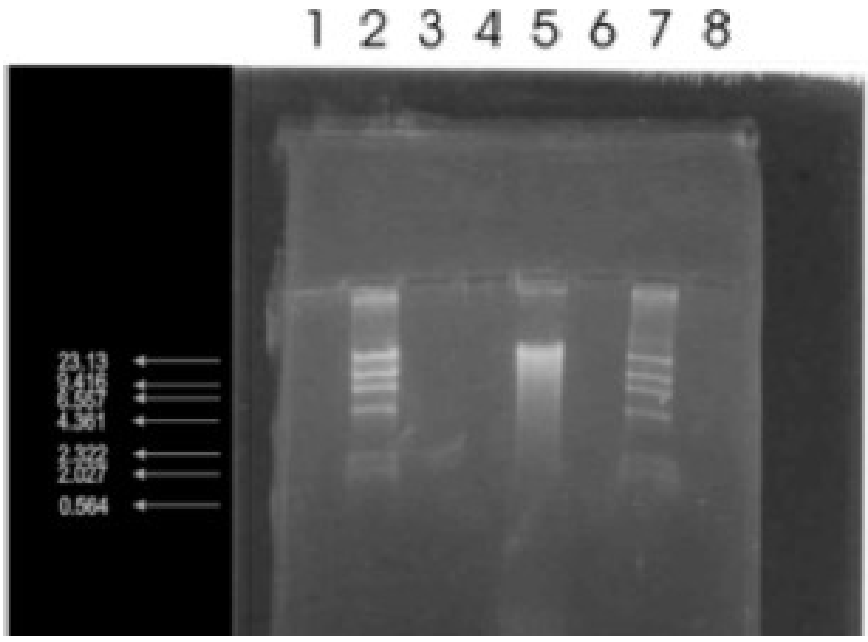
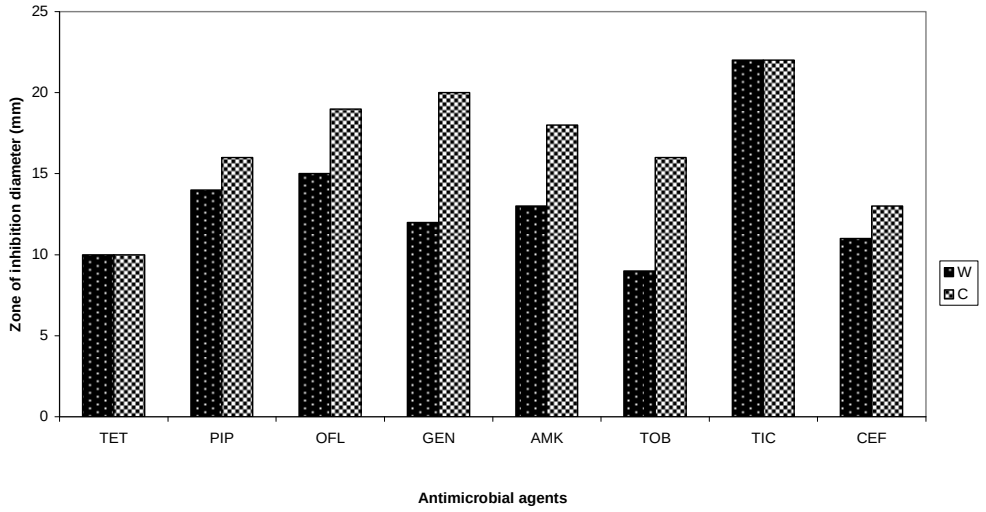


Plate 4: **Agarose gel electrophresis plate of cured plasmids of *Pseudomonas aeruginosa* [OB-6(W)] following the protocol of Birnboim and Doly (1979). Lanes: 2, λ DNA Hind 111 digested (marker); 5, *Pseudomonas aeruginosa* [OB-6(W)]; 7, λ DNA Hind 111 digested (marker).**
[OB-6(W)] showed loss of its plasmids



(10 µg) (100 µg) (5 µg) (10 µg) (30 µg) (10 µg) (75 µg) (30 µg)

Fig. 1: Antimicrobial sensitivity patterns of plasmid-bearing and plasmid-cured strains of *Pseudomonas aeruginosa*

TET, tetracycline; PIP, piperacillin; OFL, ofloxacin; GEN, gentamycin; TOB, tobramycin; TIC, ticarcillin; CEF, ceftazidime; AMK, amikacin; W, plasmid-bearing; C, plasmid-cured strain. The disc potency is indicated in parentheses.

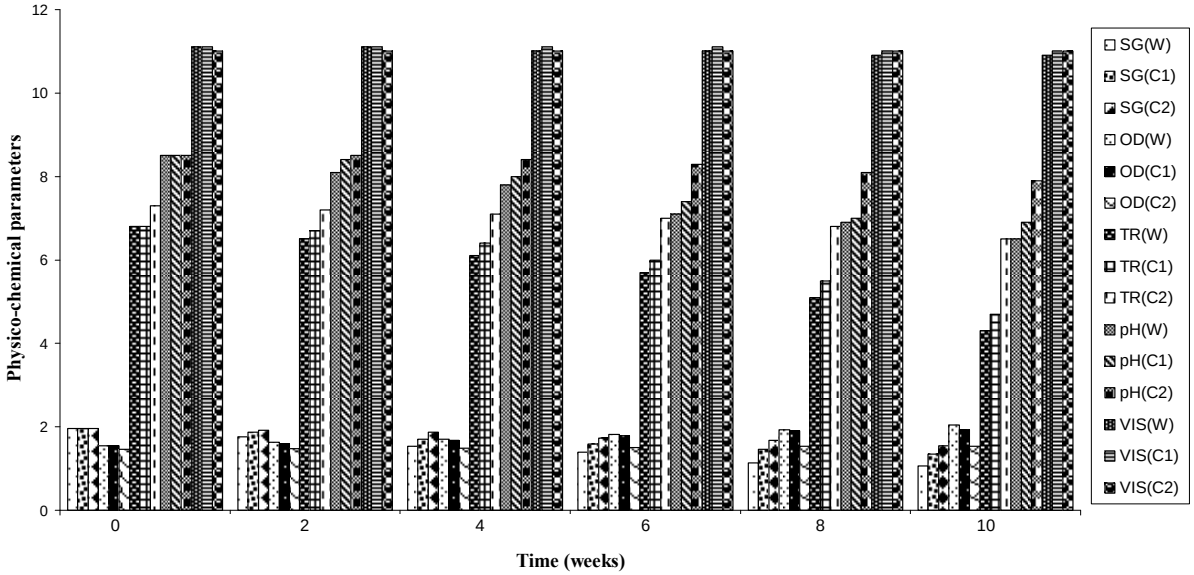


Fig. 3.44 Spoilage potential of the plasmid-bearing and plasmid-cured strains of *Pseudomonas aeruginosa* on the physico-chemical parameters of paint samples PS-1. VIS, viscosity (cst); TR, transmittance; OD_{600}; SG, specific gravity; (C2), control sample; (C1), plasmid-cured sample; (W), plasmid-bearing sample. Data represent the means of triplicate samples.

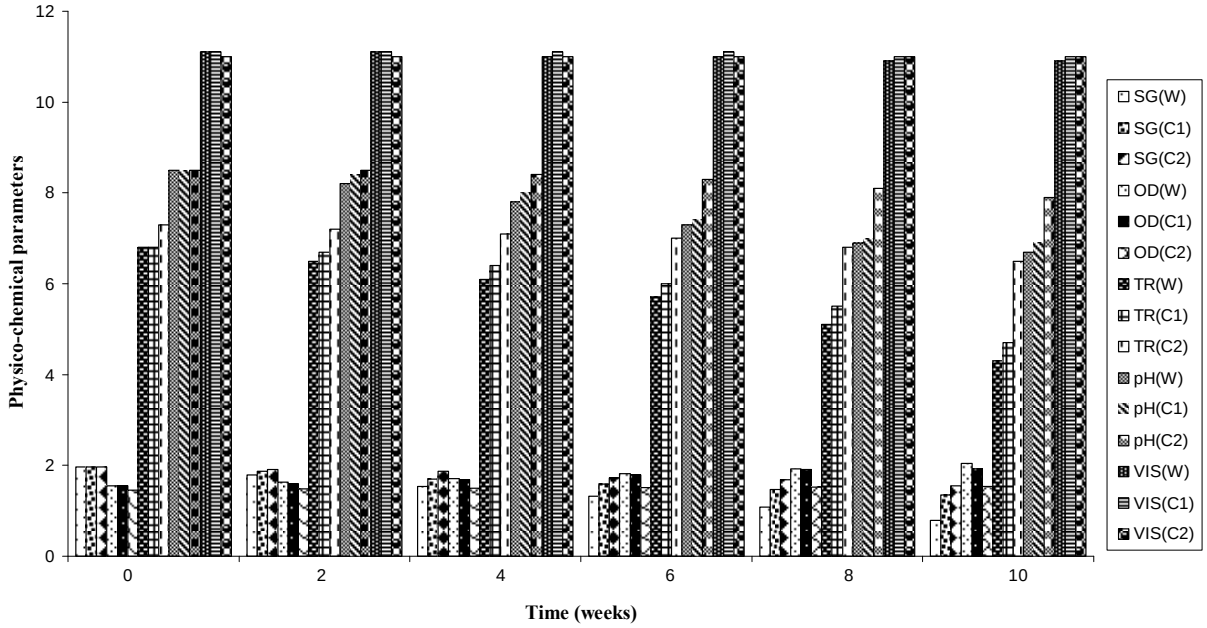


Fig. 3.45 Spoilage potential of the plasmid-bearing and plasmid-cured strains of *Pseudomonas aeruginosa* on the physico-chemical parameters of paint samples PS-2. VIS, viscosity (cst); TR, transmittance; OD_{600}; SG, specific gravity; (C2), control sample; (C1), plasmid-cured sample; (W), plasmid-bearing sample. Data represent the means of triplicate samples.

1 Fig. 3

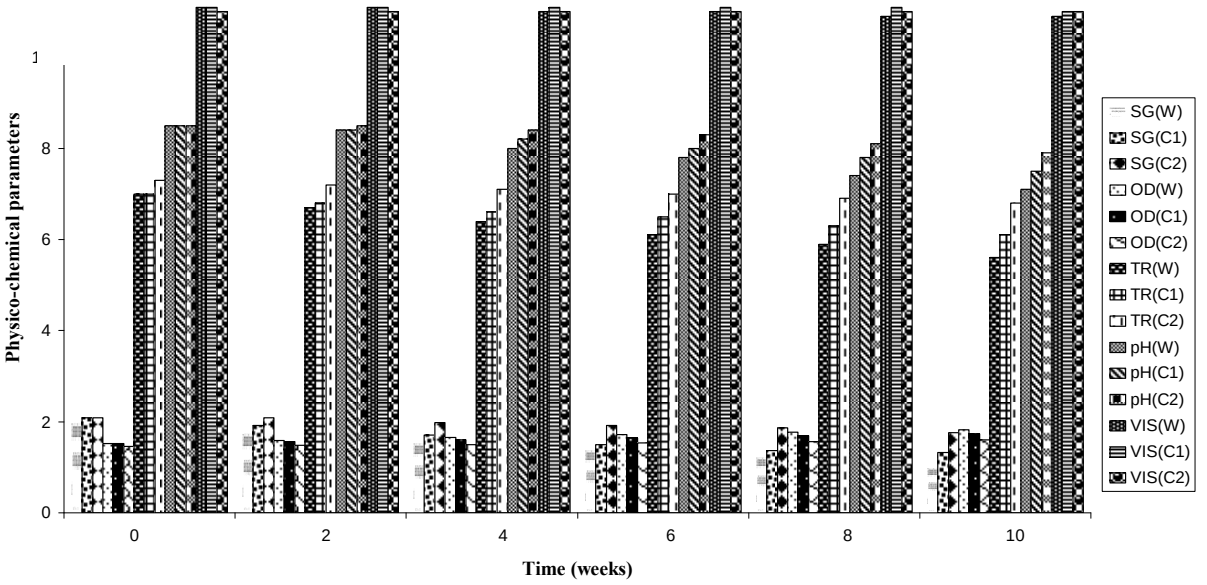


Fig. 3.46 Spoilage potential of the plasmid-bearing and plasmid-cured strains of *Pseudomonas aeruginosa* on the physico-chemical parameters of paint samples PS-3. VIS, viscosity (cst); TR, transmittance; OD_{600}; SG, specific gravity; (C2), control sample; (C1), plasmid-cured sample; (W), plasmid-bearing sample. Data represent the means of triplicate samples.

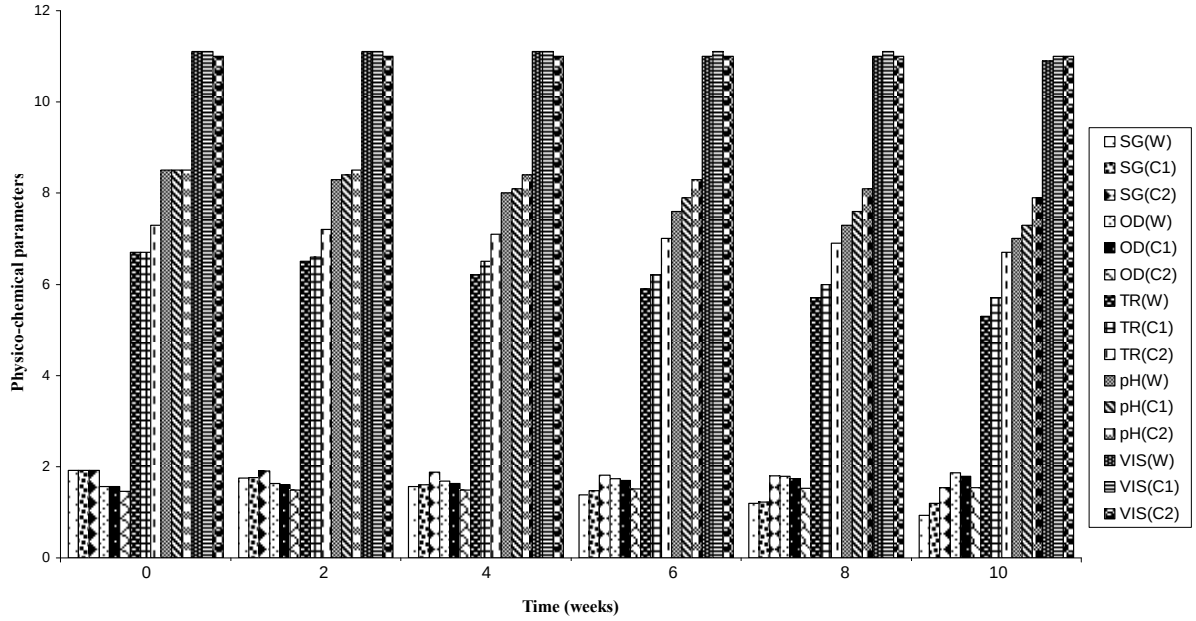


Fig. 3.47 Spoilage potential of the plasmid-bearing and plasmid-cured strains of *Pseudomonas aeruginosa* on the physico-chemical parameters of paint samples PS-4. VIS, viscosity (cst); TR, transmittance; OD_{600}; SG, specific gravity; (C2), control sample; (C1), plasmid-cured sample; (W), plasmid-bearing sample. Data represent the means of triplicate samples.

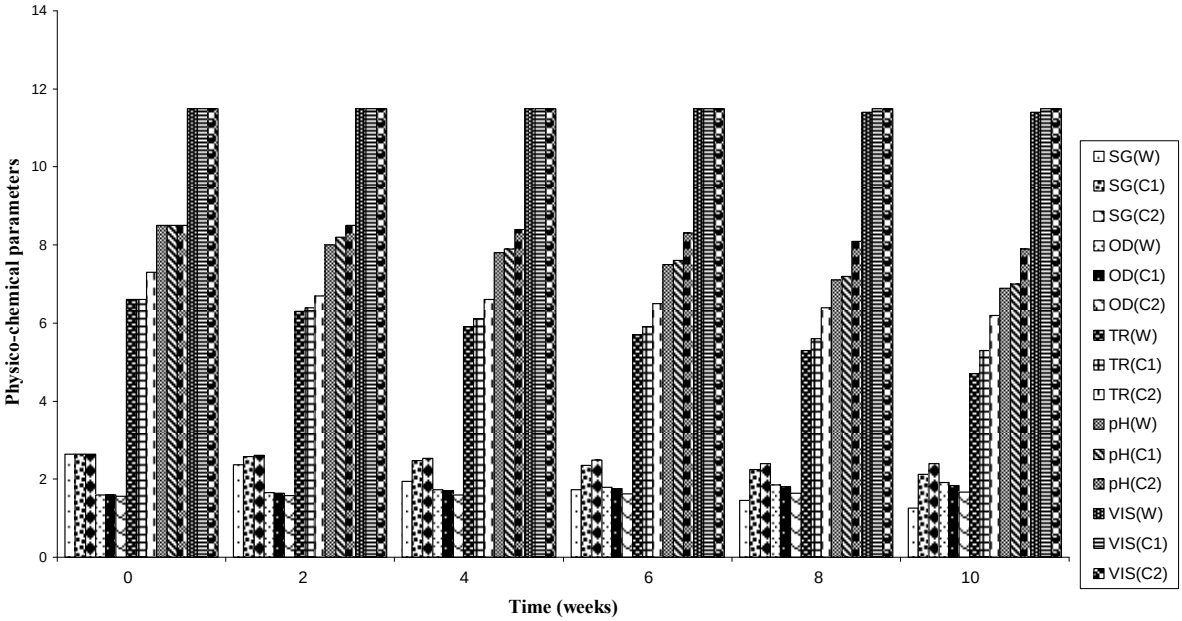


Fig. 3.48 Spoilage potential of the plasmid-bearing and plasmid-cured strains of *Pseudomonas aeruginosa* on the physico-chemical parameters of paint samples PS-5. VIS, viscosity (cst); TR, transmittance; OD_{600}; SG, specific gravity; (C2), control sample; (C1), plasmid-cured sample; (W), plasmid-bearing sample. Data represent the means of triplicate samples.

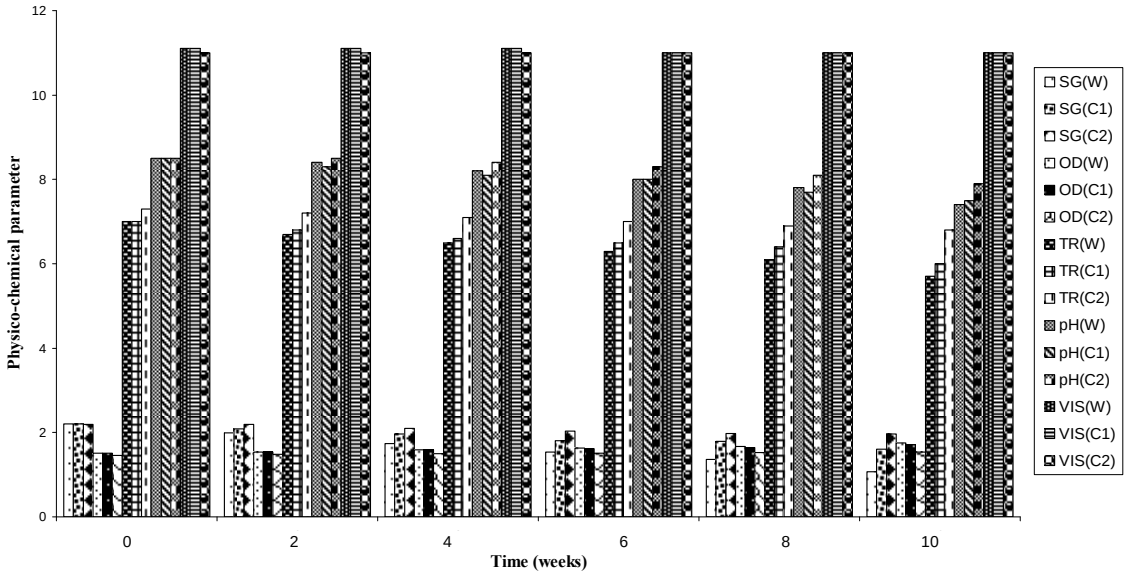


Fig. 3.49 Spoilage potential of the plasmid-bearing and plasmid-cured strains of *Pseudomonas aeruginosa* on the physico-chemical parameters of paint samples PS-6. VIS, viscosity (cst); TR, transmittance; OD_{600}; SG, specific gravity; (C2), control sample; (C1), plasmid-cured sample; (W), plasmid-bearing sample. Data represent the means of triplicate samples.

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