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Recovery of plastic utilizing bacteria by electromagnetic radiation treatment

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Present study determines that multispecies microbes involved in plastic degradation can be detached in intact form by exposing them to different energies (wavelength) of electromagnetic radiations in a novel experimental setup. Detailed output parameters that were statistically performed include: a) DNA quantity (85 to 98 % more DNA was obtained in light-treated samples compared to control), b) total bacterial count using DAPI (4',6-diamidino-2-phenylindole) dye by epifluorescence (2.8×10^5, 6.1×10^5, 6.7×10^5 for control, blue light treated, and red light treated samples, respectively after 48 h of treatment), and c) cultivation of plastic-degrading microbes on modified Bushnell Hass agar supplemented with LDPE in 0.8 % saline water (obtained microbial growth for blue and red light treated samples, but no growth for control samples). Based on experimental findings, microbial community involved in plastic degradation can be successfully regulated and detached from plastic surface by electromagnetic radiation treatment and could be used for further analysis as natural or intact best plastic degrading microorganisms.

[**Keywords**: Electromagnetic radiation, Metagenomic DNA, Modified Bushnell Hass agar, Plastic degrading microbes]

Introduction

Starting from the initial period of discovery, plastic production has been increasing continuously hitting high records due to its user-friendly applications and uses in various sectors. In the initial phase, the production capacity of plastic was around 2 million metric tons in the 1950s, and it has reached 381 million metric tons in 2015 and is projected to double in the next 15 years if the same growth rate continues[1]. However, the continuous generation of huge amounts of plastic waste is difficult to handle due to a lack of recycling and remediation strategies. These plastic molecules are very difficult to degrade, and remnants of plastic waste, which may be degraded by any of physical, chemical or biological methods may gather in the environment and interfere with normal environmental processes. Most of the plastic waste also reaches to the oceans from dumping grounds through sewage or river or rainwater run-offs. Due to the difficulty in its degradation, plastic particles may accumulate within organisms and transfer from one organism to another at the tropic level, especially in the marine ecosystem; hence, bioaccumulation and biomagnification takes place[2].

Upon introduction of plastic into the ocean, certain microorganisms evolved and developed the mechanism to utilize plastic as a source of nutrients by developing a gene that codes plastic degrading enzymes. Various enzymes, especially oxidoreductase or lipase[3], have been reported that can degrade large plastic molecules into smaller ones, which can further metabolized into cellular materials, especially lipids and carbon dioxide[4]. In order to develop suitable plastic waste recycling strategies, one should understand the exact mechanism of degradation of plastics by microbes. In this investigation, sampling was performed in the marine environment, since a large amount of plastic waste enters the ocean every year, and the inhabitant marine microbes facing extreme conditions, develop abilities to degrade plastic and utilise it as a source of nutrients.

Once microorganisms locate a substrate, they attach to it. The attached microbes started expressing genes of enzymes or proteins required in the

degradation of the complex substrate to make it soluble (*e.g.*, microbial degradation of plastics). These soluble substrate molecules are selectively consumed by microbes and metabolized. Depending on the nature of the byproducts obtained during metabolism, other organisms attach symbiotically to the plastic degrading microorganisms creating a symbiotic community. For instance, if a substrate is polyethylene, CO_2 is generated as a byproduct of degradation[5] and phytoplankton or macroalgae, which provide O_2, attach to the plastic degrading microbes and collectively become part of the biofouling community in marine environments. Plastic-degrading microbes are very hard to detach[6], and little is known about microbial interactions on plastic.

In the present study, favourable results were obtained that support the hypothesis that external lights of suitable wavelengths and intensity regulate plastic degrading microbial community. In this study, pre-incubated low-density polyethylene (LDPE) film was treated with blue Light Emitting Diode (LED), red LED, and control with dark treatment. Results were confirmed by re-suspending plastic-degrading bacteria, analyzing DNA amounts obtained, taking Total Bacterial Counts (TBC) using an epifluorescence microscope, and cultivating microbes on modified Bushnell Hass agar supplemented with fine LDPE powder. DNA amounts and TBC have values significantly greater in light-treated samples compared with control samples. Plastic-degrading microbial growth was only found on the modified Bushnell Hass agar plates containing light (blue and red) treated samples, whereas no growth was found in control samples. Hence, resuspension of plastic-degrading microbial community occurs only in light-treated samples.

Materials and Methods

Methodology

Incubation of LDPE

LDPE film was cleaned with 70 % isopropanol and fitted in three different stainless-steel frames (15 cm × 15 cm) separately and incubated underwater around 20 feet deep in the Arabian Sea at latitude 19°29′25.728″ N and longitude 72°49′14.412″ E. After six months of incubation from September 2020 to February 2021, the LDPE film was brought to the laboratory under proper aseptic conditions in an ice box and processed immediately[8].

Experimental setup using lights of different wavelengths

Three separate chambers were used, with blue LED and red LED lights fitted in chambers 1 and 2, respectively, and no light fitted in chamber 3 to serve as a control (Fig. 1). Collected LDPE sample was cut into 4 cm × 4 cm strips, aseptically. Four inoculated LDPE strips were placed in each of the three 1000 ml sterile Erlenmeyer flasks within the chambers, with 400 ml of autoclaved 0.8 % saline solution in each flask. These flasks with plastic strips and saline were

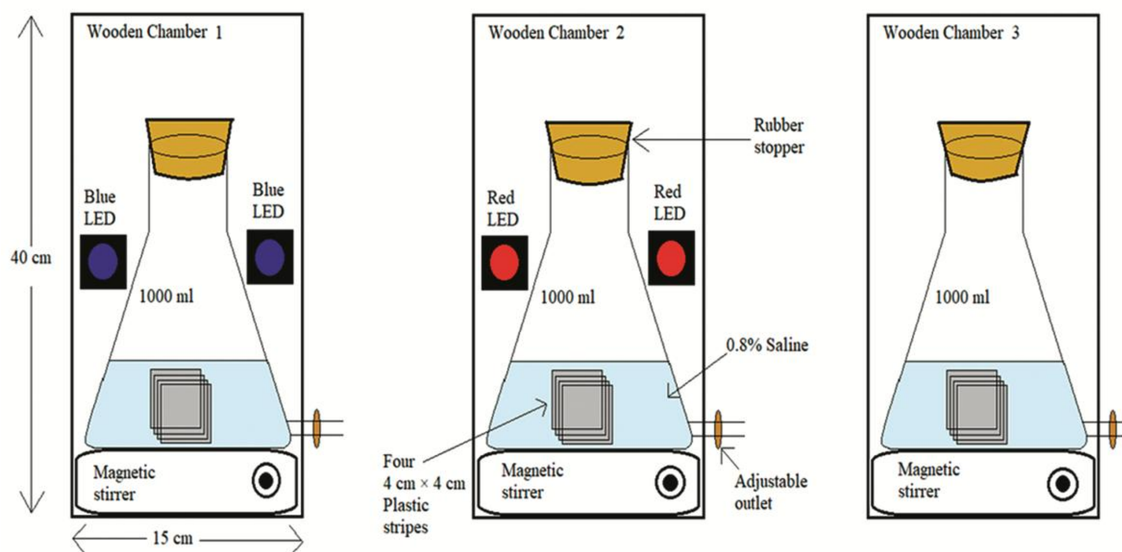

Fig. 1 — Schematic diagrams for experimental setup using lights of different wavelengths. Chamber 1 is incorporated with 4 Watt blue LED lights. Chamber 2 is incorporated with 4 Watt red LED lights. Chamber 3 has no light and is dedicated to no light treatment. All the flasks contain 0.8 % saline with plastic stripes with associated microbes on it

then separately incubated in the three chambers at 23 – 28 °C, and the same experiment was repeated three times. Each chamber was equipped with a magnetic stirrer for proper circulation of samples and evenly distribution of light to each plastic stripe among four for proper detachment of microbes in response to light treatment. Samples were analyzed from each flask after 24 and 48 h of incubation in light and dark conditions. Plastic degrading bacteria detached from all four plastic strips to saline solution corresponding to each light wavelength were filtered using 0.22 µm pore size millipore filter paper, and DNA were extracted from the filtered bacterial colonies. DNA were analyzed by metagenomics using whole genome sequencing of each sample.

Light intensity and wavelength measurements

Light intensity was measured using a Lutron LX-101A digital lux meter. Values obtained were converted to $\mu mole m^{-2} s^{-1}$ using conversion of 1K lux = 19.5 $\mu mole m^{-2} s^{-1}$[ref. 7]. Light wavelength was measured using a spectrofluorometer (RF-6000: SHIMADZU).

DNA extraction and Total Bacterial Count (TBC)

DNA of detached microbial community from LDPE stripes to saline solution was extracted from each flask (incubated in respective blue, red, and no light chambers) after 24 and 48 h. For that, 150 ml saline was filtered using millipore 0.22 µm filter papers. These filters were then aseptically cut into pieces using sterile scissors. DNA was extracted from filter papers by a Qiagen DNeasy power soil pro kit using the standard protocol provided by the kit manufacturers. DNA concentration and purity were confirmed using Qiaxpert, Nanodrop 2000, and Qubit for all triplicates and the average was reported. TBC was performed after samples were incubated for 48 h. One millilitre saline from each incubated flask that is light and dark treated was mixed with 10 µl DAPI in a dark environment. This mixture was filtered using Whatman nucleopore track-etch membrane filter paper along with 1 ml of phosphate buffered saline. Then the filter paper was placed on a glass slide and viewed under an Olympus BX 53 epifluorescence microscope, using Olympus Cellsens standard software for TBC and images. Images were taken at 40X magnification.

Genome sequencing and analysis

Light treatment experiment was performed in triplicate, and three times DNA were extracted per sample (light and dark treated). All three DNA per sample were pulled into a single Eppendorf 2 ml centrifuge tube for each sample that is the Control, Red-light, and Blue-light treatments and were sequenced using HiseqX with a read length of 151 bp. The samples were processed for whole genome metagenome analysis. Adapter trimming was performed using Fastq-mcf. Initially, the reads were filtered for human DNA contamination using Burrows-Wheeler Alignment Tool (BWA-MEM). The filtered reads were then aligned to bacterial, fungal, viral, and archaeal genomes using BWA-MEM.

Modified Bushnell Hass agar supplemented with fine LDPE powder

UV-treated three gm/L fine LDPE powder was added to autoclaved Bushnell Hass agar with 0.8 % saline to grow plastic-degrading marine microbes specifically. 100 µL of 48 h light (blue and red) treated and without light (control) treated samples were spread on separate petri plates with culture media containing LDPE as the only carbon source for 48 h at 30 °C. Magnified phase contrast images for showing the growth of microbes on LDPE-enriched media were examined with a FLoid™ Cell Imaging Station. 100 µL of each sample was spread on Zobell's marine agar to check the growth of symbiotic associated microbes.

Results

To detach plastic-associated microbes, different wavelengths of LED lights were used while keeping light intensity constant (around 230 $\mu mole\ m^{-2}\ s^{-1}$) for both 4 Watt blue and red LED lights (Fig. 2). Concentration of DNA (ng/µl) obtained after 48 h of treatment along with purity of DNA (A260/280-absorbance ratio at 260 and 280 nm) and Total Bacterial Count (TBC) values are given in Table 1.

After 24 h light treatment of plastic with light (blue and red) and dark incubation, the amount of DNA was similar for both the light and dark incubation treatments (Fig. 3), around 23±2 ng/µL when measured by the NanoDrop 2000. This is inferring that a strongly attached plastic-degrading community might not detach until 24 h of treatment. DNA obtained in this case may be due to the detachment of a weakly attached symbiotic community, and the detachment of weakly attached symbiotic microbes need not necessarily require any external treatment. Concentrations of DNA obtained after 48 h of incubation in all the triplicates in blue and red light increased remarkably compared with dark incubation,

Table 1 — Concentration, purity of DNA, and TBC after 48 h of treatment

Sample No.	Treatment	DNA quantification with different instruments			A260/280 Ratio	Total Bacterial Count (TBC)
		Qiaxpert (ng/µL)	Nanodrop2000 (ng/µL)	Qubit (ng/µL)		
1	Control (Without light)	29.5	30.1	17.7	1.86	2.8×10^5
2	Blue light	45.9	45.3	32.8	1.88	6.1×10^5
3	Red light	47.3	47.7	35.2	1.86	6.7×10^5

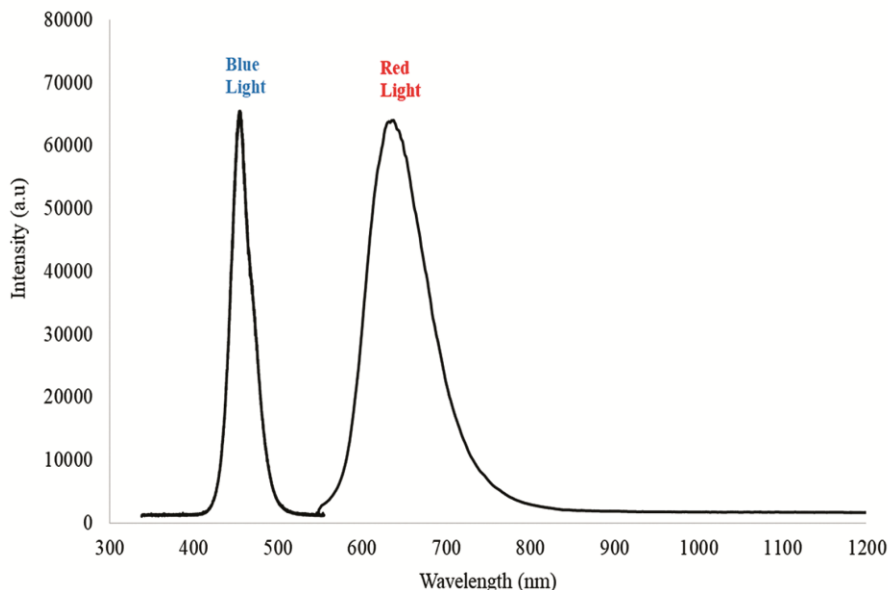

Fig. 2 — Emission wavelength range for blue and red LED lights used for the treatment of LDPE film to detach plastic-degrading microorganisms

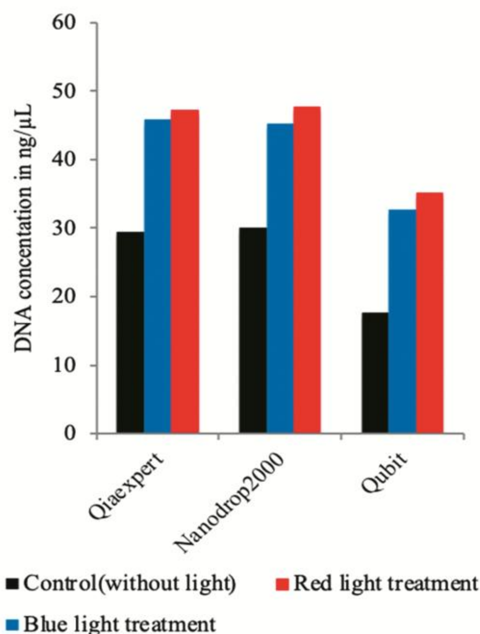

Fig. 3 — DNA quantification of treated plastic samples with different instrument

as shown in Table 1, Figure 3 and Figure 4. This indicates that strongly attached plastic-degrading microbes started detaching from plastic after 24 h of light treatment once the loosely attached symbiotic community was released completely. This meant that light is available in sufficient intensity for the plastic-degrading community.

Plastic was also treated using 4 Watt incandescent light in a separate chamber. After 48 h of treatment, results showed less DNA as compared to blue and red-light LED treatment. Incandescent light treatment resulted in 27 ng/µL (measured by NanoDrop 2000), an amount of DNA approximately equal to samples with no light treatment (30.1 ng/µL). This result may be due to the intensity of incandescent light (30 µmole m^{-2} s^{-1}), which is significantly less compared to the intensity of the LED lights used (around 230 µmole m^{-2} s^{-1}).

Epifluorescence microscope images (Fig. 4) show that fewer microbes are stained in control samples (without light treatment) even after 48 h. This may be due to the detachment of symbiotic microbes only. In

the case of both blue and red light treatment samples, dense microbial communities were stained by DAPI dye after 48 h of treatment. This indicates that plastic-degrading microbes detaches successfully by light treatment.

The pre-processed reads are first aligned to the human genome (hg19) using the BWA-MEM aligner to remove human genome contamination from the samples. The uncontaminated sequences were then taken for further alignment with known bacteria, archaea, fungi and viruses genomes using the BWA-MEM aligner. Around 4.45 – 15.65 % of reads were mapped to the human genome, and 46 – 53 % of reads were mapped to bacterial genomes, as shown in Table 2 and Figure 5.

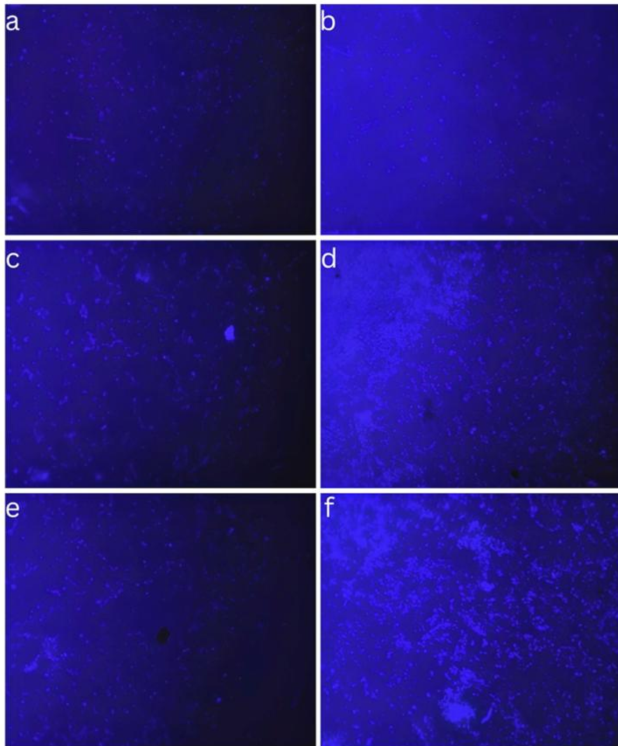

Fig. 4 — Epifluorescence microscope images of DAPI stained plastic (low density polyethylene) film indicating detachment of multispecies microbial community. a, b) Images taken after 24 and 48 h, respectively with no light incubation treatment (control); c, d) Images taken after 24 and 48 h, respectively with blue light incubation treatment; and e, f) Images taken after 24 and 48 h, respectively with red light incubation treatment

Further, the growth of plastic-degrading microbes and symbiotic microbes are shown in Figure 6. In each petriplate, 100 μL of saline resuspenssion containing bacteria was spread from light and dark-treated samples separately. Microbial growth was observed after 10 days of incubation (since plastic is slow to degrade) at 30 °C in LDPE powder supplemented with Bushnell Hass culture media.

Samples from light-treated plastic had growth, whereas no growth was observed on the petriplates for the dark-treated samples, even after 10 days of incubation. From these results, it can be concluded that plastic-degrading microbes can easily detach intact when plastic samples are subjected to light treatment (with required intensity and wavelength). Hence, this technique can be useful in growing and analyzing plastic-degrading microbial communities. As for the detachment of symbiotic microbial communities, there is no need for light treatment, as can be seen by significant microbial growth obtained in samples without light treatment as compared with light-treated samples on Zobell's marine agar after 48 h of incubation at 30 °C (Fig. 6).

Discussion

In this study, the application of artificial LED light in the detachment of the plastic-degrading microbial community was successful. This information was used for further analysis, cultivation, and treatment. Based on experimental findings, the next step in this work is to test the involvement of a symbiotic community associated with the plastic-degrading community. This is supported in the literature by Shimao, 2001[ref. 8], who states that the involvement of symbiotic microbes is associated with one of the synthetic polymer-degrading microbes. Together with the symbiotic microbial community, the plastic-degrading community (which takes part in the actual degradation of plastic) makes a plastic-associated microbial community. A strongly attached plastic-degrading microbial community can easily detach intact by using light treatment and can be cultivated for further analysis in media containing plastic as the only hydrocarbon source.

Table 2 — Sequence alignment summary

Sample_name	Human (%)	Bacteria (%)	Fungi (%)	Virus (%)	Archae_bacteria (%)
C-3 (Control)	15.65	50.02	0.02	0.0014	0.0158
PE-1 (Red light treated)	4.45	53.76	0.02	0.0013	0.0211
PE-2 (Blue light treated)	7.92	46.99	0.01	0.0012	0.0114

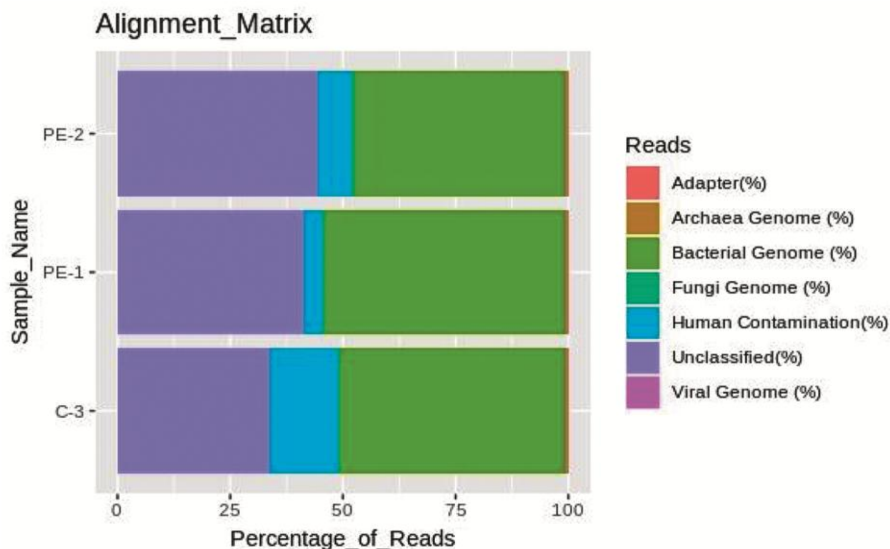

Fig. 5 — Percentage of alignment to bacteria, virus, fungi, archaea and human genomes. PE-2 is blue light treated; PE-1 is red light treated, and C-3 is control

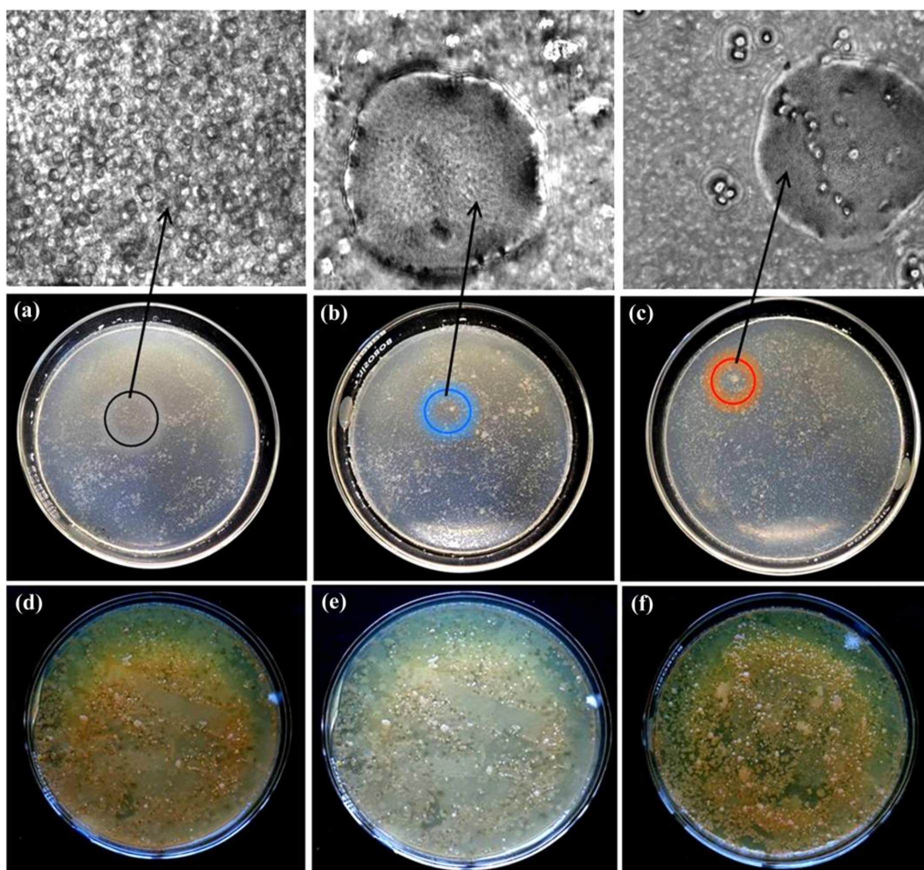

Fig. 6 — Growth of plastic-degrading microbes on Bushnell Hass agar supplemented with fine LDPE powder, after 48 h of light/dark treatment. a) Control plate without growth of microorganisms in which sample was kept in dark, b) Blue light treated plate with developed bacterial colonies, and c) Red light treated plate with developed bacterial colonies. Magnified phase contrast images for a, b, and c plates illustrated by arrows were examined with a FLoid™ Cell Imaging Station. d, e & f show microbial colonies on Zobell's marine agar plates for control, blue light, and red light treatments, respectively.

Conclusion

This is the first report of its kind that deals with the detachment of multispecies plastic-degrading microbes from plastic-associated microorganisms using different light wavelengths. Understanding the process of biodegradation of plastic is required in order to develop suitable plastic remediation strategies. To know what kind of microbes take part in the actual degradation of plastic, one should be able to detach these plastic-degrading microbes from the plastic surface in intact form. These plastic degrading microbes might be removed by scratching plastic with a rough surface; however, the chances of damaging microbial cells are more in this case. Hence, external electromagnetic radiations could be suitably applied to detach the microbes. Furthermore, the approach of using light to detach plastic degrading microorganisms can be widely used to study or treat particular microbial community (multispecies or single species). This can be applicable to any solid substrate associated microbial community analysis (*e.g*., metagenomics, cultivation).

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Conflict of Interest

Authors declare that there is no conflict of interest.

Ethical Statement

NA

Author Contributions

HSJ: Sampling, visualization, formal analysis, writing-original draft preparation; ABF: Conceptualization, methodology, writing-reviewing and editing, funding acquisition; MRJ: Data curation, formal analysis; and SS: Supervision, writing - reviewing and editing.

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