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Microbial solutions for plastic pollutants: Caprolactam, Polyvinyl alcohol, and surgical face masks

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Abstract Caprolactam and polyvinyl alcohol (PVA) are plastic pollutants that in excess concentrations affect the environment adversely. The purpose of this study was to identify the microorganisms with the potential to break down biodegradable polyvinyl alcohol synthetic plastic polymer caprolactam, and face masks. Caprolactam and PVA-degrading bacteria were isolated from the soil. The identification of the isolate was done using morphological, biochemical and 16S rRNA sequencing and identified as *Pseudomonas aeruginosa.* Colorimetric and reverse-phase high-performance liquid chromatography analysis revealed the degradation of 61 and 96 % caprolactam (1%) and PVA (0.1%) respectively by the isolate. The degradative product of caprolactam and polyvinyl alcohol was identified as adipic acid and fumaric acid based on the mass spectroscopic analysis. Field emission scanning electron microscopy of the control and bacterial-treated face mask was carried out to check the degradation potential of the strain. Microbial face mask degradation showed 97.89 % reduction in the diameter of fibers, which proved the potential use of *Pseudomonas aeruginosa* in the remediation of bioplastic, synthetic and microplastic polluted areas.

Keywords: Caprolactam, face mask, microplastic, polyvinyl alcohol, *Pseudomonas*

1 Introduction

Polyvinyl alcohol (PVA) is a water-soluble and biodegradable synthetic polymer that possesses excessive biocompatibility and many applications in paper coatings, fiber sizing and biomedical devices (Goodship and Jacobs 2009). The applications include water treatment, dyes, laundry detergents, agricultural chemical substances, disinfectants, and industrial cleansing chemical substances. Polyvinyl alcohol is extensively used in textile yarn and papers, especially to make the latter extra resilient to oils and grease. Polyvinyl alcohol breaks into ketones, fatty acid and alcohol. PVA is a refractory organic pollutant due to its low biodegradability which is removed from

wastewater with the help of methods such as ozonation, oxidation, radiation (Sun *et al*. 2017). Natural degradation of biopolymer PVA is not satisfactory (Marusincová *et al*. 2013). Symbiotic, mixed cultures of microorganisms have been studied for the bioremediation of PVA (Halima 2016, Bian *et al*. 2019).

Caprolactam is an organic aggregate with the formula $C_6H_{11}NO$. This achromatic solid is a lactam of caproic acid (Linde and Fisher 2004). The global demand for this compound is in million tons per year (Dahlhoff *et al*. 2001). This has caused significant pollution of the environment and serious toxicity to living organisms (Shama and Wase 1981). Caprolactam is the predecessor of many industrial chemical productions such as in nylon, plastic, paint, lysine synthesis and crosslinking of polyurethane industries. The toxicity of these chemicals has been demonstrated in various studies, including mutagenesis effects and inhibition of plant growth (Tin *et al*. 2009). The oligomer containing waste generated during caprolactam production contains unreacted monomer and cyclic oligomers (Baxi and Shah 2002). Biological treatment of caprolactam using micro-organisms could therefore be a prominent substitute to the current waste utilization techniques. Different types of microorganisms such as *Pseudomonas jessenii* (Otzen *et al*. 2018), *Pseudomonas aeruginosa*, *Alcaligenes* and *Acinetobacter* are reported to degrade caprolactam and their by-products adipic acid which further converted to 3 Oxoadipyl CoA (Baxi and Shah 2002). There are reports where plasmid bearing *Pseudomonas aeruginosa* capable of degrading caprolactam (Esikova *et al*. 1990).

Surgical facemasks are made of polypropylene, poly-butylene terephthalate, and poly-tetrafluoroethylene with filter particles larger than 5 µm (Santarsiero *et al*. 2020). The contents of the face mask have been reported to have ecotoxic effects due to its plastic and metal contents too (De-la-Torre *et al*. 2022). To avoid, the negative consequences of face masks, researchers are trying to make ecofriendly or biodegradable masks.

This study aimed to isolate polymer tolerating strain *Pseudomonas aeruginosa* and to see the utilization potential of the strain for the degradation of caprolactam, polyvinyl alcohol (PVA) and surgical face masks.

2 Material and Methods

2.1 Isolation and identification of caprolactam and polyvinyl alcohol degrading bacteria

Soil sample was collected from municipal waste disposal site, for the isolation and identification of the isolate with the potential to degrade caprolactam and polyvinyl alcohol. Minimal salt medium (MSM, Hi media) was used for isolation [MSM (g/l) K_2HPO_4- 0.173, KH_2PO_4-0.068, NaCl-0.01, $MgSO_4.7H_2O$ -0.68, $FeSO_4.7H_2O$- 0.003, $CaCl_2.2H_2O$-0.002, NH_4NO_3-0.1, agar-5] supplemented with 1g plastic source (polyvinyl alcohol or caprolactam). The Petri plates were incubated for 24 h at 30 degree centigrade temperature. The isolate was streaked on MSM media and were

selected for further biodegradation studies. Biochemical tests were carried out by using Bergey's Manual of Determinative Bacteriology (Bergey 1994). The isolate was identified based on biochemical and 16s rRNA sequencing method. The phenol/chloroform extraction procedure was used to isolate genomic DNA, and then the 16S rRNA gene was amplified using universal primers 16F27 (Sambrook 1989). According to the manufacturer's instructions, the amplified 16S rRNA gene PCR product was purified by PEG-NaCl precipitation and immediately sequenced on an ABI®3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA). Essentially, additional internal primers were used to perform sequencing from both ends, reading each place at least twice. Assembly was carried out with the Laser gene package, and identification was done with the EzBioCloud database (Yoon et al. 2017).

2.2 Biodegradation studies

Biodegradation investigations were conducted in independent trials using minimal salt broth containing 1% caprolactam and 0.1% PVA for 10 days at room temperature. The decrease in the concentration of polyvinyl alcohol was estimated as per the colorimetric method described by Vaclavkova *et al*. (2007). For colorimetric assay, 1ml cell-free broth was mixed with 2 ml of boric acid solution and 0.5 ml of iodine solution. After incubation at room temperature for 5 minutes, absorbance was measured at 450nm. The standard dose response curve of standard PVA was prepared in the range of 1-10 mg/ml.

Caprolactam was estimated using a hydroxylamine reagent based colorimetric method (Bergmann 1952). A volume of 1 ml of standard caprolactam solution was mixed with 2 ml of alkaline hydroxylamine reagent, then covered the tubes and heated for 7 hours in a water bath at 60^0C . After cooling, 1 ml of 3.5 HCL and 1 ml of 0.74M $FeCl_3$ were added. The absorbance was measured at 540 nm. The decrease in the concentration of caprolactam was estimated using the standard dose-response curve of caprolactam (1-10mM/ml). Bacterial growth was monitored by taking absorbance at 600 nm. The kinetic model during the biodegradation of PVA and caprolactam was assessed using Computer Assisted Kinetic Evaluation (CAKE) software version 3.6 (https://cake-kinetics.org/).

2.3 Characterization of biodegradative products of caprolactam and polyvinyl alcohol

The fermented broth containing caprolactam was centrifuged and supernatant was collected. The supernatant was concentrated and the residue was dissolved in methanol for High performance liquid chromatography (HPLC) studies. The solvent extraction was carried out using methanol (CH_3OH): water (50:50 V/V) as the solvent system. The residual concentration of caprolactam was assessed by using reverse phase HPLC with a flow rate of 1.0 mL/min.). The volume of the standard was 100.0 µg/ml, and the detection wavelength was fixed at 205 nm. Biodegradative metabolites of caprolactam and polyvinyl alcohol were identified using high-resolution mass spectrometry

(HRMS). Bruker Impact HD instrument (Japan) was used for the analysis of mass of standard and its biodegradative metabolite extracted from fermented broth. A dual electrospray ionization source was operated in positive ion mode to acquire full scan mass spectra from 50 to 1200 m/z with a scan rate of 4.0 spectra/s. The source gas temperature was set at 200°C and 7.01 l/min flow rate. The sample injection volume was 10 µl. The nebulizer pressure was adjusted to 1.7 bars. The data was analyzed using Bruker Compass Data Analysis 4.2 software (Seripracharat *et al*. 2022).

2.4 Biodegradation studies of face mask using microscopic method

In minimal salt media, strips of face mask were added as a carbon source. Fermentation was done using 1% bacterial inoculum (10^9cells /ml) and 30 days incubation period at room temperature (30^{o}C). Strips of face masks were intermittently checked for the weathering effect. Face masks were observed under stereomicroscope and the 30th day mask was further processed for field emission scanning electron microscopic studies as per the method of Zhao *et al*. (2007).

3 Results

3.1 Identification of caprolactam and polyvinyl alcohol degrading bacteria

The isolates were identified based on the morphological, biochemical characters (negative methyl red test, and positive catalase, oxidase, indole, and citrate tests) and then confirmed by 16S rRNA sequencing.

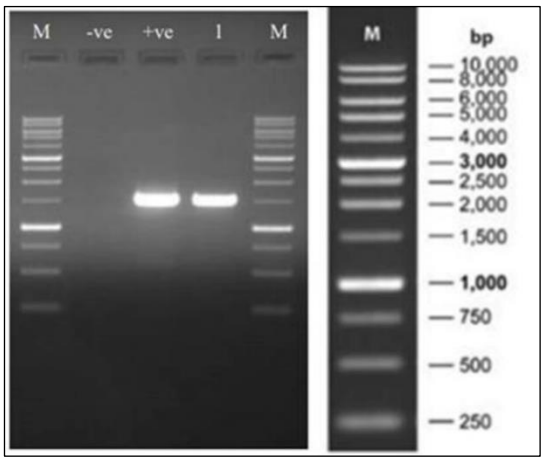

Fig 1. Agarose Gel (1%) image of PCR product of bacterial DNA using DNA ladder of 250 to 10000bp (-ve: negative control, +ve: positive control)

The isolate was confirmed to be *Pseudomonas aeruginosa* by 16S rRNA sequencing using the EzBioCloud Database with the extent of the 200-10000 bp sequence

homology (Yokoyama *et al*. 2003). PCR product of genomic DNA was processed for gel electrophoresis given in sample lane (Figure 1).

DNA bar-coding data was analyzed by editing and assembling the forward and reverse sequencing results into one full-length sequence. The sequence of the selected bacterial isolate was deposited in GenBank with accession number of PP800223.1. Data was analyzed using Sequence Scanner software 2 as given in Figure 2.

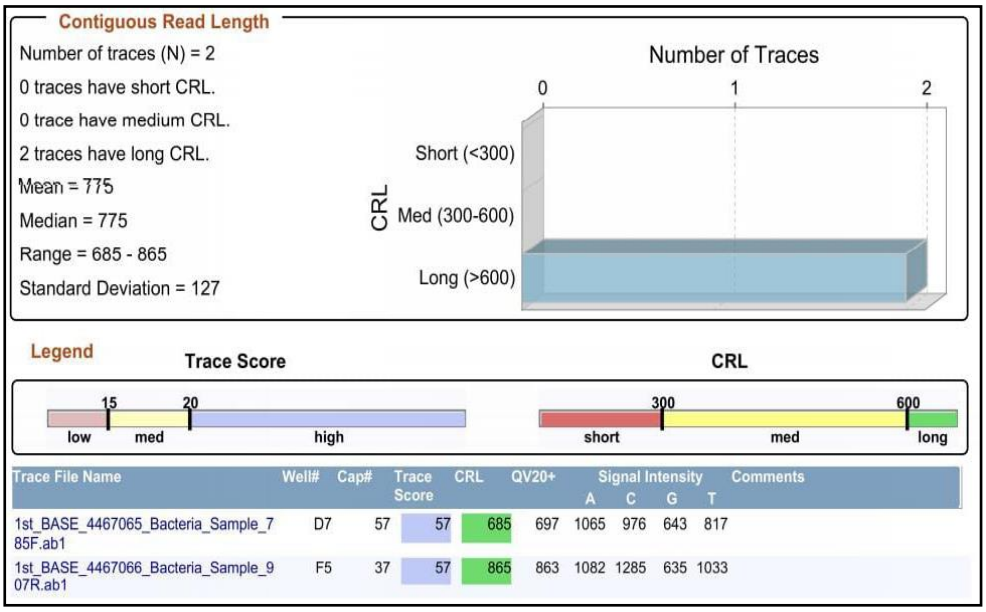

Fig 2. Electropherogram analysis of the 16s rRNA data of *Pseudomonas aeruginosa.*

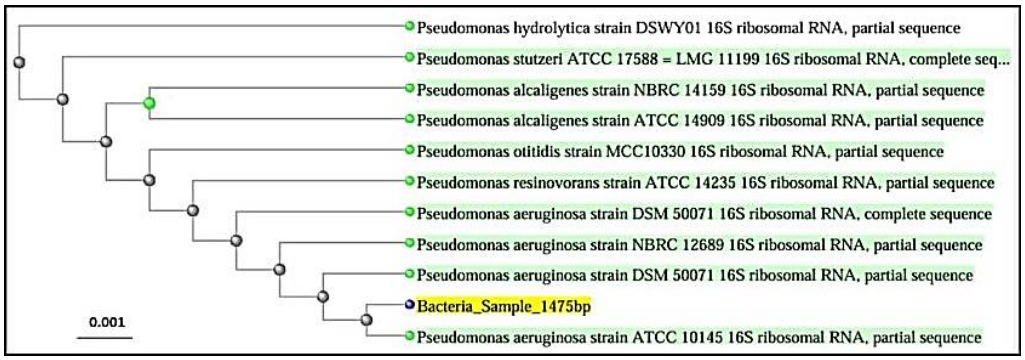

Fig 3. Phylogenetic tree of *Pseudomonas aeruginosa* based on the neighbor joining analysis.

The strain homology with G+C content was found to be 46.24%. The evolutionary history of selected 10 isolates were inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown (Figure 3). The tree is drawn to scale, with

branch lengths of 0.001 in the phylogenetic tree.) by NCBI Blast Tree Method where the isolate showed 99% matching with *Pseudomonas aeruginosa*.

3.2 Biodegradation studies

During the biodegrading of caprolactam and polyvinyl alcohol, there was an increase in the biodegradation with the increase in the cell concentration, and incubation period.

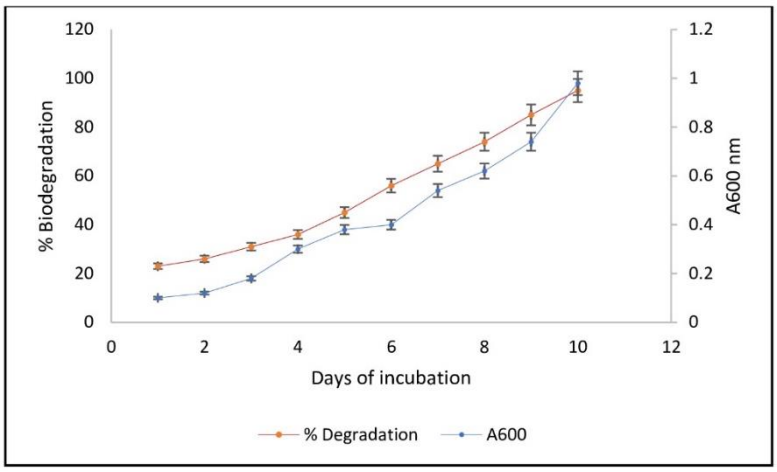

Fig 4. Time course effect of biodegradation of polyvinyl alcohol by *Pseudomonas aeruginosa*

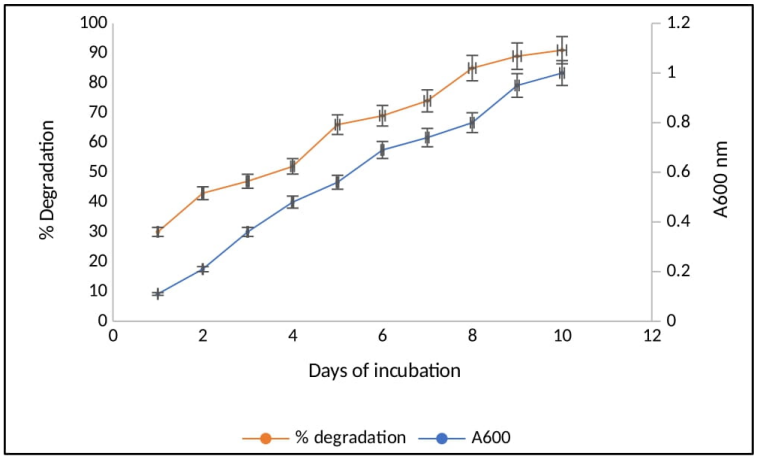

Fig 5. Time course effect on biodegradation of Caprolactam by *Pseudomonas aeruginosa*

Pseudomonas could degrade 61% and 96% respectively from the initial contractions of caprolactam (1%) and PVC (0.1%) in the medium during 10 days of incubation, The effect of time course on degradation of polyvinyl alcohol and caprolactam by *Pseudomonas aeruginosa* is shown in Figures 4 and 5 respectively.

Polyvinyl alcohol and caprolactam biodegradation kinetics were studied using CAKE software to find out kinetic models and the time (days) required for the degradation. PVA biodegradation follows First Order Multi-Compartment (FOMC) kinetic; the model assumes that the chemical degrades at different rates depending on the conditions (Table 1). Caprolactam biodegradation follows simple first-order (SFO) kinetic which suggests that a fixed proportion of the pollutant decays in each time (Table 1).

Table 1. Kinetic analysis of polyvinyl alcohol and caprolactam biodegradation

	PVA biodegradation kinetics	Caprolactam biodegradation kinetics
Kinetic Model	First Order Multi-Compartment (FOMC)	Simple first order (SFO)
M0	87.5	100
Chi-sq error	9.23	25.9
50% Decay Time DT_{50} (days)	3.67	6.93
90% Decay Time DT_{90} (days)	12.2	23

3.3 Characterization of biodegradative products of caprolactam and polyvinyl alcohol

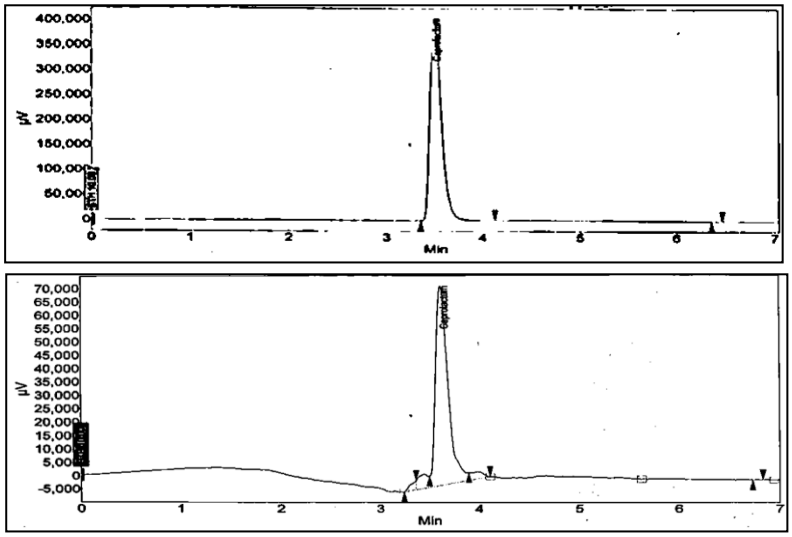

Fig 6. (a) Reverse phase HPLC chromatography of standard caprolactam (top), and, (b) Reverse phase HPLC chromatography of degradative metabolite caprolactam (bottom).

The residual concentration of caprolactam was assessed using HPLC (Figure 6a). It was observed that 61% of caprolactam was degraded on the 10th day. Reverse phase HPLC chromatogram of extracted degradative metabolite of caprolactam showed retention time of 3.9 and 6.783 whereas standard caprolactam with 3.608 retention time (Figure 6b).

Bruker Impact HD instrument was used for the analysis of the mass of standard polyvinyl alcohol. The chemical formula of PVA is $(C_2H_4O)_x$ and molecular weight (MW) was found to be 1,15,000. The HRMS analysis of standard, control and test polyvinyl alcohol are shown in Figures 7 a, b and c respectively. HRMS spectra of PVA biodegradative products were compared with the spectra of standard PVA and control broth. The MW 116.9 g/mol obtained from the fermented broth showed that the degradative product of polyvinyl alcohol is fumaric acid.

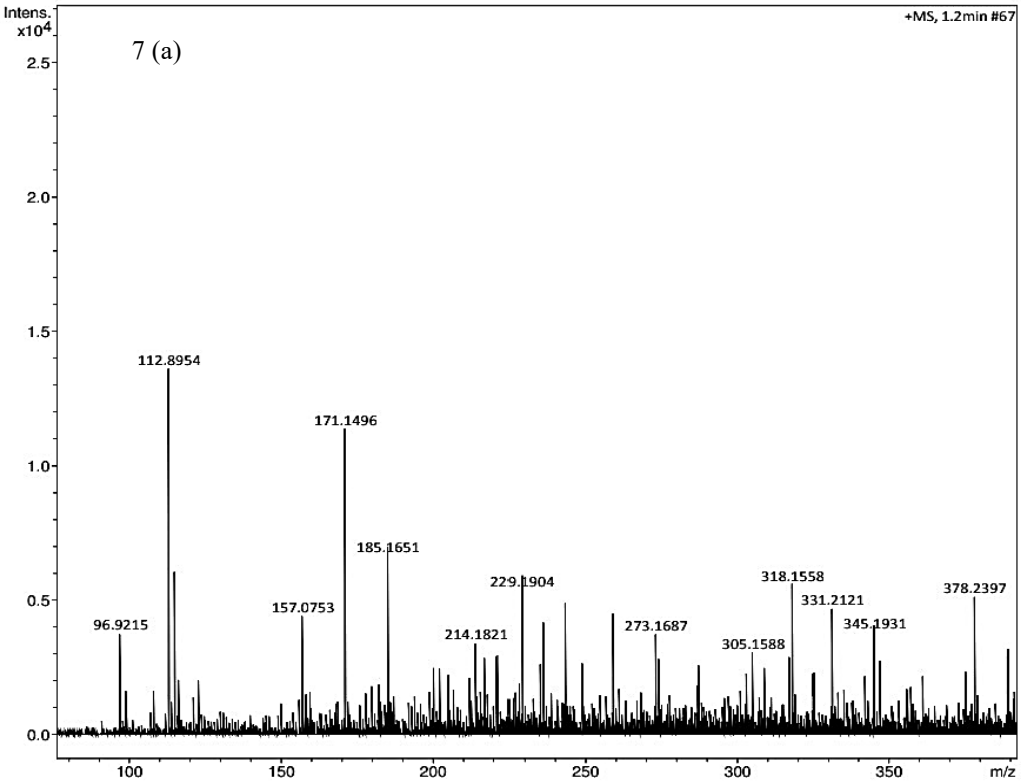

Fig .7 (a). HRMS spectra of PVA in uninoculated broth

Fig. 7 continued..

Figure 7. Continued...

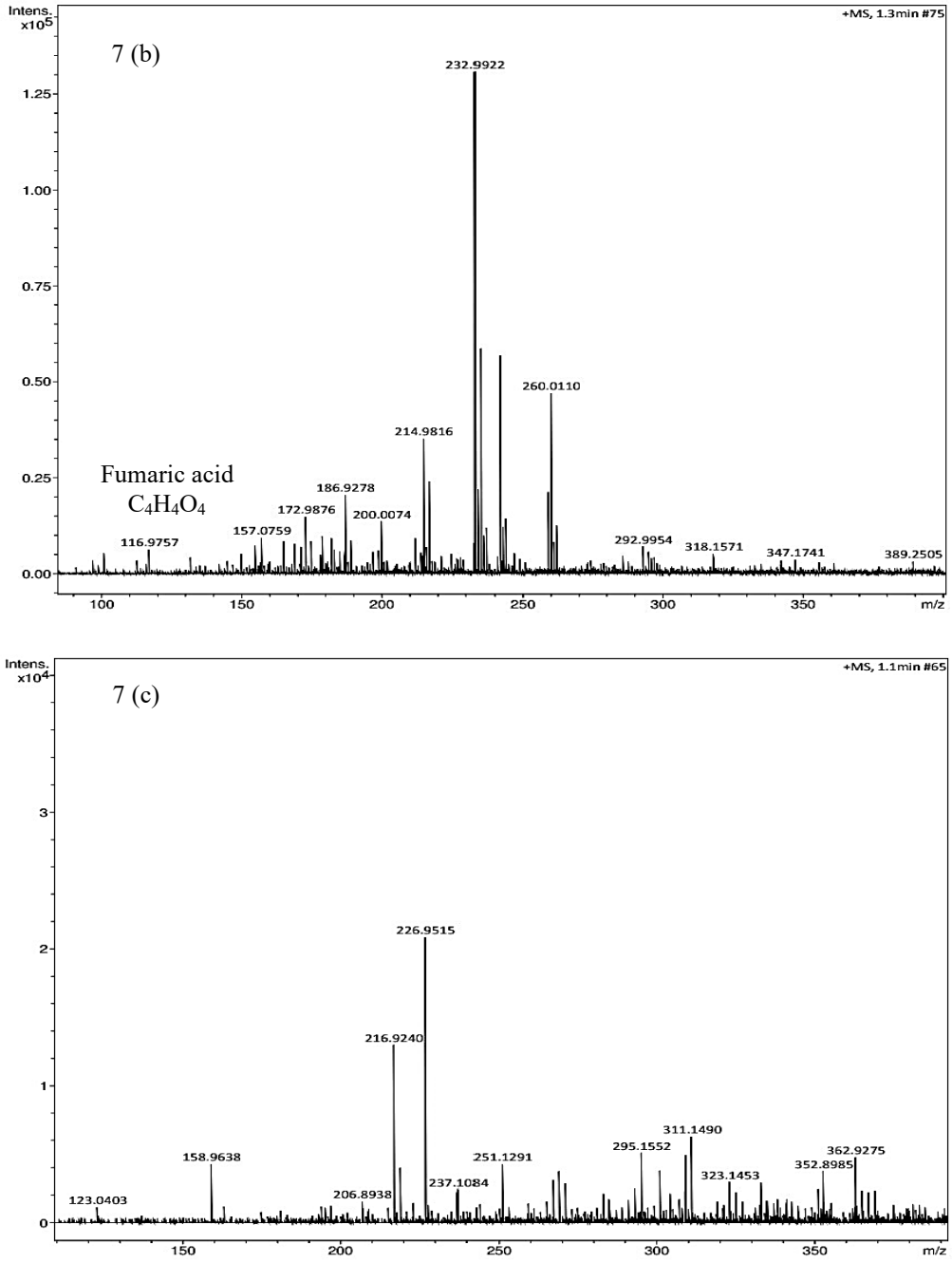

Fig. 7b. HRMS spectra of biodegradative product of PVA; 7c. HRMS spectra of standard PVA

Biodegradative product of caprolactam was identified using HRMS as adipic acid ($C_6H_{10}O_4$, molecular weight 146.4 g/mol) as represented in Fig. 8a and b.

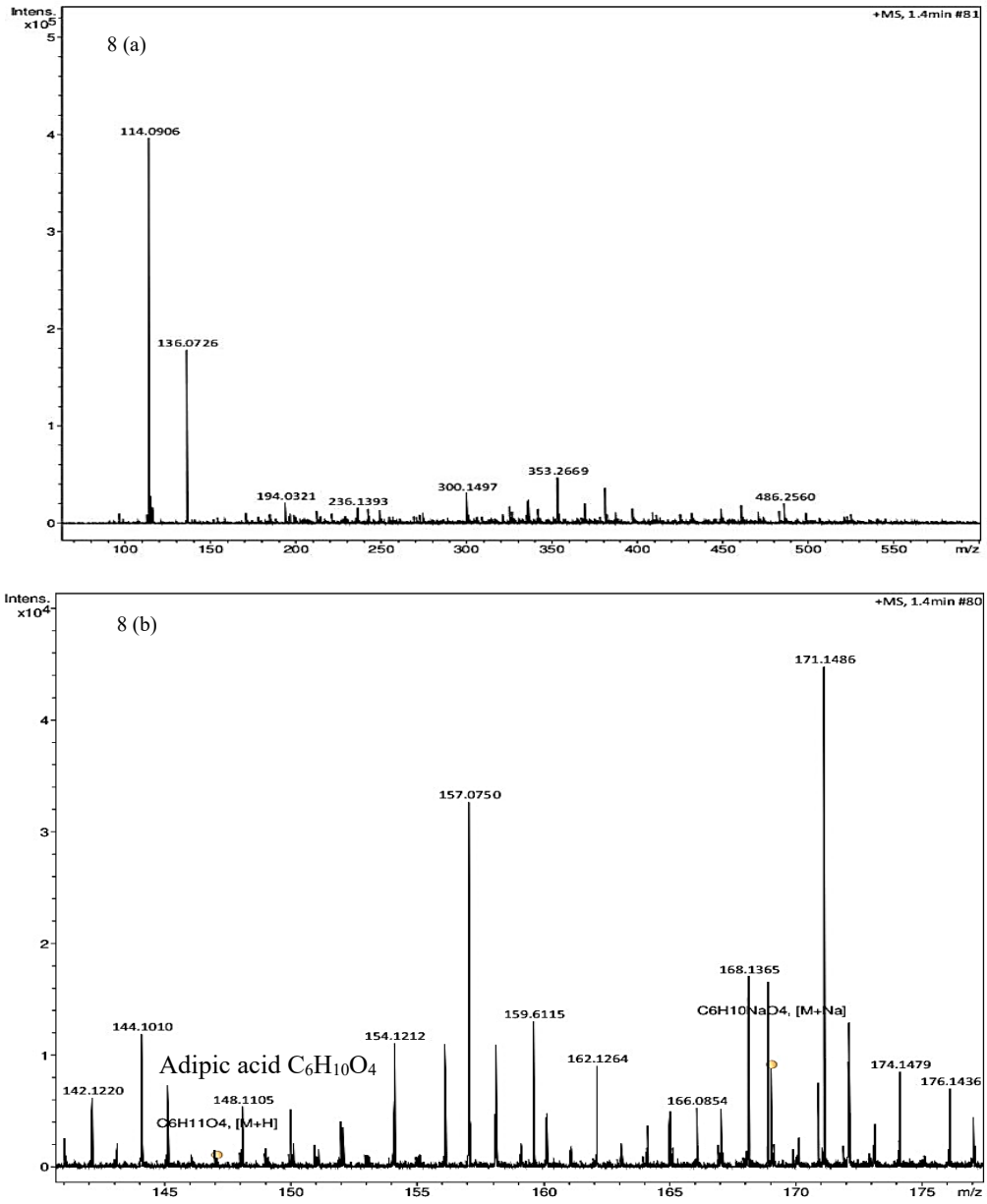

Fig 8a. HR MS of caprolactam; Fig 8b. HR MS of biodegradative metabolite of caprolactam (adipic acid)

3.4 Biodegradation studies of the surgical face mask using microscopic method

Bacterial-assisted weathering of surgical face masks was studied using stereoscopic and field emission microscopic data. Control and bacterial treated face mask samples showed the dispersion and compression of fibers which means the degradation of face mask has taken place. The before and after degradation of face mask images taken under stereomicroscope are shown in Figures 9 (a) and (b), respectively. Qualitative examination of the polymer weathering due to microbial action was done using the microscopic images. Further quantification of the fiber diameters was done using FE-SEM study.

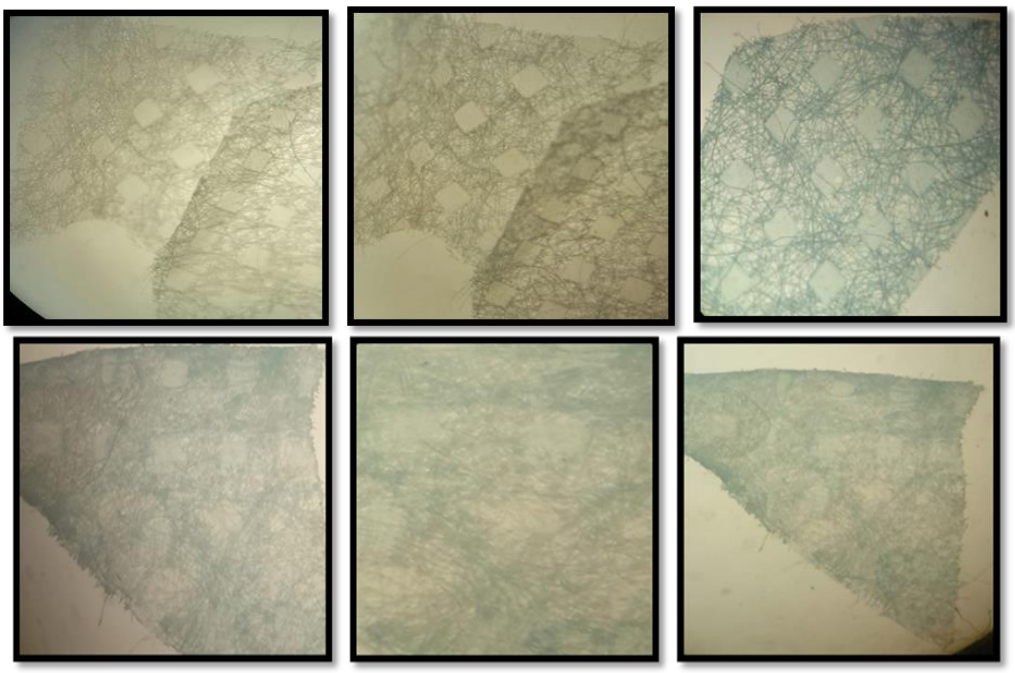

Fig 9. (a) Stereomicroscopic observations of face mask (control, top row) (mag. x 1000); (b) Stereomicroscopic observations (15th day) of face mask treated with bacteria (bottom row).

The FE-SEM images of before and after degradation of the face mask are shown in Figure 10 a and b respectively. The diameter of the fibers was found to be in the range of 55.50 µm to 178.5µm (control) and 117 -183.5 nm (after biodegradation). Microbial degradation showed 97.89% reduction in the diameter of fibers.

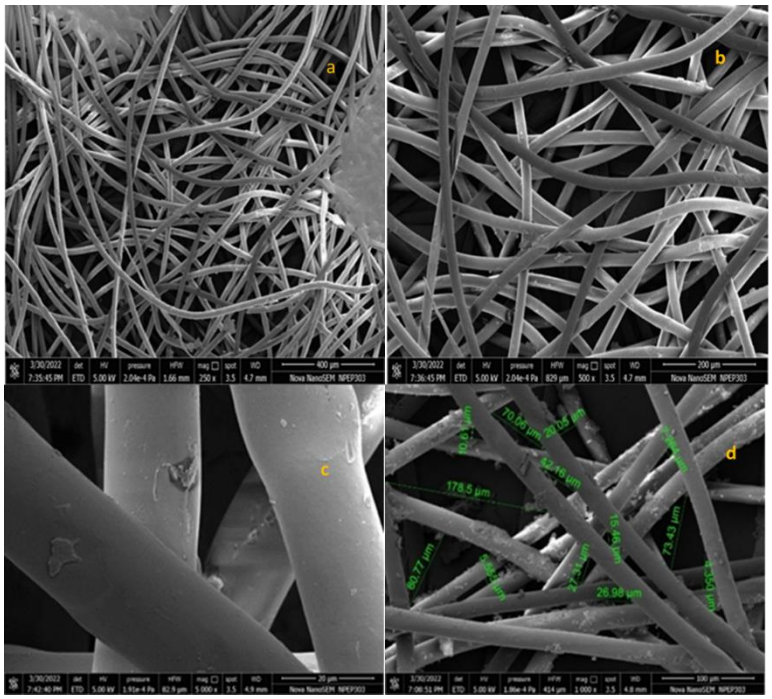

Fig 10a. FE-SEM images of the face mask (a: x 250, b: x 500, c: x 5000, d: x 1000 magnification)

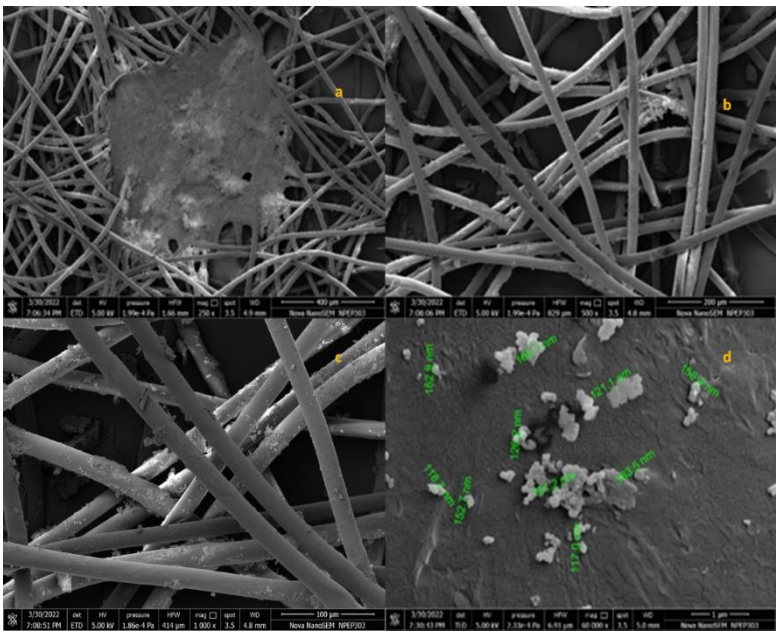

Fig 10b. FE-SEM images of face mask *Pseudomonas aeruginosa* treated (a: x 250, b: x 500, c: x 5000, d: x 1000 magnification).

4 Discussion

Plastic waste disposal is one of the emerging problems. Biological clean-up of polymers is one of the extensively studied research topics (Halima 2016). Using various microorganisms, attempts have been made to solve the disposal issue of synthetic and biodegradable polymers and their subsequent bioconversion into less toxic products. PVA is a biodegradable polymer but its degradation in soil is not satisfactory (Marusincová *et al*. 2013). The present study was undertaken to find a microbial solution to remove or reduce the concentration of caprolactam, PVA and disposal face masks. Utilization of microorganisms at the polymer wastewater treatment plants is possible when the microorganisms can cope with the 1 to 2 g/l concentration of caprolactam (Kulkarniand 1997). Hence focus was given to the bioprospecting of bacteria which could tolerate high concentrations of polymer.

Pseudomonas aeruginosa has been reported as the promising candidate in the bioremediation of polyethylene (Mehmood *et al*. 2023), plastic additive 2,6-di-tert-butylphenol (Medić *et al*. 2019), Low-density polyethylene (LDPE) (Oluwole *et al*. 2022), and Polyurethane diol (Mukherjee *et al*. 2011). Reported caprolactam degraders are *Achromobacter* (10 g/l) (Baxi 2013), *Acinetobacter calcoaceticus* (19 g/l) (Rajoo *et al*., 2013), *Bacillus cereus* (35 g/l) (Mehta *et al*. 2014).

In the current study, *Pseudomonas aeruginosa* was found to tolerate 10 g /l concentration of caprolactam and could degrade 61% of the parent compound in 10 days which suggests that the strain is taking more incubation period for the biodegradation, but it can be considered as chassis strain. *Brevibacterium epidermidis* has been reported to degrade 91.8 % of 1000 ppm of caprolactam in 160 h and 6 aminohexanoic acid as product (Esikova *et al*. 2023). *Acinetobacter calcoaceticus* could degrade 19 g/l of caprolactam in 72 hours (Rajoo *et al*. 2013). *Bacillus cereus* has been reported with high caprolactam tolerance and degradation ability (Mehta *et al*. 2014). The potential of different strains of *Pseudomonas* to degrade caprolactam is due to CAP plasmid (Panov *et al*. 2013), enzymes (Ponamoreva *et al*. 2010) and extracellular polymers (Baxi 2013). According to reports, the caprolactam-degrading bacterium *Pseudomonas putida* BS394 (pBS268) uses the enzymes 2-oxoglutarate-6-aminohexanoate transaminase and 6-oxohexanoate dehydrogenase to break down caprolactam (Ponamoreva *et al*. 2010). By using ATP-dependent hydrolytic ring opening to produce 6-aminohexanoate, a strain of *Pseudomonas jessenii* degrades the nylon-6 precursor caprolactam (Palacio *et al*. 2019).

In the current study, the strain was found to tolerate a high concentration of PVA and 96% biodegradation potential with fumaric acid as the end product. Data suggests that the strain can be employed at the polymer-polluted wastewater treatment plants. Symbiotic biodegradation of polyvinyl alcohol (500 ppm) has been reported by *Sphingomonas* and *Rhodococcus erythropolis* in 2 weeks where *Sphingomonas* needs pyrroloquinoline quinone (PQQ) and growth factor from *Rhodococcus* (Vaclavkova, *et al*. 2007). Non-*Pseudomonads*, PVA degraders are *Bacillus niacin* (Bian *et al*. 2013), *Bacillus megatarium* (Mori *et al*. 1996), *Aspergillus niger* (Stoica-Guzun *et al*. 2011), *Penicillium brevicompactum* OVR-5 (Mohamed *et al*. 2022).

During the Covid 19 pandemic situation, kits with the content of fibers, nano plastic and microplastics were extensively used (Oliveira *et al*. 2023). The use of polyester face shields and polyolefin surgical masks greatly increases the amount of plastic pollution (Fu *et al*. 2023). Various methods like thermal degradation or the use of supercritical water have opted for its degradation. Considering the associated cost and associated ecotoxicity, we tried a microbial solution to solve the problem of the polymer. Evaluation of the degradation of face masks is generally carried out with the help of Scanning electron microscopy and energy dispersion spectrometry (SEM-EDX) to check microfibers fragmentation (De-la-Torre *et al*. 2022). In this study, we evaluated the morphological changes (fragmentation of fibers) in the surgical face mask due to bacterial activity. Very limited data is available on the biodegradation of the surgical facemask.

5 Conclusions

It was discovered that soil isolate *Pseudomonas aeruginosa* was effective at removing the plastic pollutants caprolactam and PVA as well as at weathering face masks in *in vitro* studies. This research work will be helpful for the future selection of microorganisms for the *in situ* remediation of the polymer contaminated sites. *Pseudomonas aeruginosa* has the potential to be used for the biological cleaning of plastic contaminants (caprolactam, PVA) and facemask. This study can be explored further with the help of transcriptomic analysis to find out the up and down regulation of genes involved during the degradation of biodegradable and synthetic plastics. Computational studies also can be accompanied by finding the QSAR (Quantitative structure activity relationship) and ecotoxicity studies. Strain improvement programs are needed to improve the efficiency of the strain to remediate the mixed polymer polluted sites.

Acknowledgements

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