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ORIGINAL PAPER

Isolation and identification of low-density polyethylene degrading novel bacterial strains

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Abstract

Plastics are usually made up of low-density polyethylene (LDPE) that serve as the environmental nuisance. The recalcitrant nature of plastics is a huge concern, whereas the increasing demand has made it difficult to handle the plastic waste that eventually leads to plastic pollution. In recent years, due to increasing demand and high pressure for its safe disposal, plastic biodegradation has gained a lot of attention. In the current study, four bacterial strains were isolated from the solid-waste dumpsites of Faisalabad, Pakistan, using enrichment culture technique. The isolated bacterial strains were capable of growing on media having polystyrene as the sole carbon source. Based on 16S rRNA gene sequencing and phylogenetic analysis of the isolated strains *Serratia* sp., *Stenotrophomonas* sp. and *Pseudomonas* sp. were identified as the potential strains for the biodegradation of LDPE. *Serratia* sp. resulted in 40% weight loss of the LDPE plastic pieces after 150 days of treatment. *Stenotrophomonas* sp. and *Pseudomonas* species resulted in 32 and 21% weight loss of the treated piece of plastics (LDPE), respectively. Polyethylene pieces were characterized by Fourier-transform infrared spectroscopy (FTIR) analysis before and after biodegradation. The FTIR spectra indicated that the isolated bacterial strains have a good potential to degrade LDPE. Future studies are required to investigate the bacterial genetic makeup, mechanisms of LDPE biodegradation and the factors that can enhance the biodegradable characteristics of these indigenously isolated bacterial strains.

Keywords Biodegradation · LDPE · Plastic pollution · PET · Polystyrene · FTIR · Synthetic polymers · PHAs

Introduction

Plastics are petrochemical-based polymers that are integral part of our society because of their indispensable use in electronics, manufacturing of various equipment and in packaging material (Koller 2019). Due to their chemical inertness and resistance, plastics are highly employed in industrial manufacturing for a wide range of commodities and domestic products (Godfrey 2019; Muneer et al. 2020). Plastics are categorized into macro, micro and nano plastics depending upon the size of their constituent particles, all of these types have polluted the water reservoirs, resulting in serious threats to the aquatic and marine life (Yoshida et al. 2016; Allen et al. 2019). In environment, plastic products are persistent due to the complete absence or low activity of the catabolic enzymes that have the potential to degrade them (Alshehrei 2017). Various studies have concluded that the high ratio of aromatic components in the plastics make them resistant to degradation (Yoshida et al. 2016).

Currently, the rate of plastic production in the world is more than 410 million tons per annum, of which polyethylene terephthalate (PET) alone constitutes more than 70 million tons (Hahladakis et al. 2018; Palm et al. 2019). According to an estimation, more than 13 million tons of plastics entered the ocean from land-based sources in 2010 (Beaumont et al. 2019). Due to serious environmental effects of the plastic pollution, international forums such as the United Nations environmental program (UNEP) has included it in the list of global problems (Ivanova et al. 2018). Human activities can increase the declining threshold of biological diversity and extinction rates of biological species of fauna and flora (Villarrubia-Gómez et al. 2018). Because of poor waste management, major share of plastics enter into the

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marine ecosystem and have adverse effects to marine and human life. Polyethylene bags in oceans and rivers have life threatening effects on fish, turtles, jellyfish and various other aquatic species (Beaumont et al. 2019). Mismanagement of the plastic waste leads to the accumulation of plastics into the oceans and the fragmentation of such plastics results into production of micro-plastics (Wang et al. 2019). Recently, the microscopic remnants of plastic objects have been found in the intestinal tracts of various invertebrates, fish and birds that have a lot of impacts on their physiological activities, such as growth and development (Yu et al. 2018; Barboza et al. 2018). Plastic items also cause aesthetic problems, which is a nuisance rather than a hazard. Studies have shown that the used fishing nets and plastic bags thrown in the oceans and water bodies result in chocking and clogging of the respiratory tracts of aquatic animals such as fish, turtles and jellyfish. Micro and nano plastic particles are ingested by marine animals and remain in their digestive tract, which results in their retarded growth and improper physiological functions (Rhodes 2018; Muneer et al. 2021a).

Degradation of plastics by physical and chemical methods is expensive and is not safe for the environment because of the production of persistent organic pollutants (POPs) known as dioxins and furans (Alshemmari 2021). The byproducts of plastic degradation by physical and chemical methods are toxic irritants and harmful gaseous foams that not only damage the ozone layer in the upper atmosphere but also destroy the underground water resources, cause infertility of soil, prevent degradation of normal substances and are proved dangerous to human, animals and the ecosystem (Windsor et al. 2019). The ecofriendly methods to resolve plastic pollution is either biodegradation of plastics using microbes or bioplastic production using biomass that can be obtained from lignocellulosic plant materials or from polyesters produced by different microbes (Muneer et al. 2021b, c). Recent research suggests that there are a lot of microbial species that can degrade synthetic polymers at faster rates due to the presence of natural catalytic enzymes that break apart the strong hydrocarbon bonds in the plastic materials (Charnock 2021). The enzymes like lipases excreted by *Rhizopus arrhizus*, *Achromobacter* sp., *Rhizopus delemar* and *Candida cylindracea* and esterases from hog liver have potential to degrade poly(ε-caprolactone) and polyethylene adipate (Alshehrei 2017). It has been reported earlier that certain bacteria like *Rhodococcus ruber*, *Brevibacillus borstelensis* and some fungi such as *Fusarium solani*, *Penicillium simplicissimum* YK, *Penicillium oxalicum* NS4 (KU559906) and *Penicillium chrysogenum* NS10 (KU559907) have the potential to degrade natural and synthetic polyethylene (Ojha et al. 2017). Bioplastic research has also emerged as an alternative and ecofriendly solution to counter plastic pollution (Khan et al. 2019). Biobased polymers and polyesters such as polyhydroxyalkanoates (PHAs) and polyhydroxybutyrate (PHB) derived from microorganisms have shown a great potential to replace the conventional synthetic plastics. The search for new microbes, enzymes and catalytic systems is of global interest (Yuan et al. 2020). Due to the sophisticated techniques and expensiveness of the methods employed for the production of such biopolymers and polyesters, highly advanced research is required (Muneer et al. 2021b; Karan et al. 2019).

The aim of this study is to isolate and identify some novel bacterial strains that are capable of plastic degradation. The present study also aims to set up a biological method for the biodegradation of synthetic plastics using the indigenously isolated bacteria. Phylogenetic analysis of these isolated bacterial strains is also included as a major part of this study. The current study will pave the way for developing innovative strategies for plastic degradation and will moderate the environmental impacts of the plastics with an economically feasible approach. In the future, the isolated bacterial strains can be further investigated for the purification of the catalytic enzymes that have the potential to degrade synthetic plastics.

Materials and methods

Isolation of plastic-degrading microorganisms

To obtain plastic-degrading microbes samples of decomposing plastic bags from solid waste-dumping sites of Faisalabad, Pakistan, were collected. The collected plastic samples were dipped in 0.9% NaCl (saline) solution and gently shaken for 24 h to remove attached soil and dust particles. Under aseptic conditions, 10 ml of the saline solution was inoculated into 40 ml liquid carbon-free basal medium with plastic pieces as the sole carbon source for microorganisms. The cultures were incubated at 37 °C for 14 days. The microbes obtained from previous inoculation were streaked on Luria Broth (LB) medium solidified with 1.5% agar. These agar plates were incubated at 37 °C for 24 h. Colonies of microorganisms obtained from this step were streaked on separate LB-agar plates. The liquid carbon-free basal medium had KH_2PO_4 (0.7 g/L), K_2HPO_4 (0.7 g/L), $MgSO_4{\cdot}7H_2O$ (0.7 g/L), NH_4NO_3 (1.0 g/L), NaCl (0.005 g/L), $FeSO_4{\cdot}7H_2O$ (0.002 g/L), $ZnSO_4{\cdot}7H_2O$ (0.002 g/L), and $MnSO_4{\cdot}H_2O$ (0.001 g/L). The microbial colonies obtained were separately streaked on LB-agar plates to maintain the inoculum of pure cultures.

Screening of microbes having plastic degradation potential

Microorganisms from the inoculum were grown on growth media having 0.016% K_2HPO_4, 0.02% KH_2PO_4, 0.002%

$CaCl_2.2H_2O$, 0.01% $(NH_4)_2SO_4$, 0.02% $MgSO_4.7H_2O$, 0.005% $ZnSO_4.7H_2O$, 0.005% $MnSO_4.H_2O$, and 0.001% $FeSO_4.7H_2O$, which was solidified with 1.5% agar, having polystyrene powder as carbon source at final concentration of 0.1% (w/v).

Biodegradation assay

Pure cultures of microbes obtained by above screening (Sect. 2.2) were inoculated in mineral salt medium (MSM) along with plastic pieces as carbon source. The composition of MSM used was; 0.01% yeast extract, 0.016% K_2HPO_4, 0.02% KH_2PO_4, 0.002% $CaCl_2.2H_2O$, 0.01% $(NH_4)_2SO_4$, 0.02% $MgSO_4.7H_2O$, 0.005% $ZnSO_4.7H_2O$, 0.005% $MnSO_4.H_2O$, 0.001% $FeSO_4.7H_2O$. Each reaction contains 45 mL of growth medium along with 5 mL of microbial culture and 50 mg (5 plastic pieces of 10 mg) of polyethylene pieces. The biodegradation assay was established for polyethylene. The microorganisms were cultured in MSM along with plastic pieces for 5 months at 30 °C and 120 rpm in a shaking incubator. During incubation, one plastic piece was separated from the reaction mixture (each flask) after interval of 30 days to determine the weight loss. The media was also refreshed after fifteen days. The percentage weight loss was calculated by following formula

$$\text{Weight loss}(\%) = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

FTIR analysis

After incubation of 150 days, the plastic pieces were subjected to FTIR analysis to determine the change in polymer bonds. For FTIR analysis samples were submitted to Central High Tech Laboratory, Government College University Faisalabad. Spectrum Two™ Perkin Elmer FTIR was used to carry out the FTIR analysis.

Identification of isolated microorganisms

Polymerase chain reaction and gel electrophoresis

The genomic DNA was extracted from the microbial cultures and were amplified separately for 16S rRNA gene (Mohan et al. 2016). For PCR followed the method as adopted by (Dey et al. 2020). DNA extracted from four different types of microbial cultures were subjected to 16S rRNA gene amplification and the PCR results were confirmed by gel electrophoresis. The gel electrophoresis of these strains confirmed the amplification of 16S rRNA. The sequencing and amplification was done using universal primers 785F (5'-GGATTAGATACCCTGGTA-3'), 907R (5'-CCGTCAATTCMTTTRAGTTT-3') and 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), respectively (Hussein et al. 2015; Dey et al. 2020). Each illuminated band on gel represented the amplified 16S ribosomal gene of each strain. These samples were then delivered to a Korean company "Macrogen" to get sequences of 16S rRNA of AK-1, AK-2, AK-3 and AK-4.

Phylogenetic analysis

PCR products of four different strains obtained in the study, named as AK-1, AK-2, AK-3 and AK-4 were delivered to Macrogen Korea for 16S rRNA gene sequencing. After sequencing, results were visualized by chromas (https://technelysium.com.au/wp/chromaspro/) (Treves 2010). DNA Baser Sequence Assembler was used to assemble both the forward and reverse sequence files and the obtained sequences were subjected to NCBI BLAST separately to search the most similar sequences. Sequences that showed the highest similarity were selected for further analysis. The phylogenetic analysis was performed by a computational tool, MEGA7 (Kumar et al. 2016). ClustalW aligned the data sets for each strain, then the phylogenetic tree for each strain was constructed by Neighbor-Joining method (Li 2003). The evolutionary distances among the bacterial strains were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site.

Results and discussions

Isolation of plastic-degrading microorganisms

Eight different types of colonies were observed (Supplementary Fig. A1) when microbial strains from enrichment culture were streaked on agar plates and allowed to grow for 24 h at 37 °C. Each obtained colony was separately streaked on agar plate to get pure cultures of microorganisms. When these eight strains were grown on media having polystyrene powder as sole carbon source, only four of these strains showed the ability to grow in the presence of polystyrene as sole carbon source in MSM (Supplementary Fig. A2).

The technique of utilizing plastic-degrading bags for isolation of microorganisms has been previously reported on several times. Kowalczyk et al. (2016) isolated a polyethylene degrading bacterium, *Achromobacter xylosoxidans,* from polyethylene bags collected from waste sites. The current study first utilized the enrichment culture technique to isolate the microorganisms having potential to grow in the presence of plastic pieces as sole carbon source (Kowalczyk et al., 2016). According to the hypothesis, only those microbes will survive in the given environment that can utilize plastics.

Later, the potential of microorganisms obtained from the enrichment culture was checked by growing microbes on medium containing polystyrene powder as sole carbon source. Microbes that have the potential to utilize polystyrene actively grow on the provided medium. Hussein et al. (2015) utilized this method to screen plastic-degrading microbes. Mehmood et al. (2016) isolated the low-density polyethylene degrading strains of various microbes from the solid-waste dumpsite using enrichment culture technique.

Biodegradation assay

In biodegradation assay, five pieces of polyethylene sheets having weight of 10 mg each were added in the mineral salt medium along with the microorganisms. Four different strains were isolated in the study therefore, biodegradation assay for each strain was established separately. After 150 days of incubation, the percentage weight loss of 10 mg plastic piece noted for AK-1 (*Stenotrophomonas* sp.) was 32%, AK-2 (*Serratia* sp.) 40%, AK-3 (*Pseudomonas* sp.) and AK-4 (*Pseudomonas* sp.) was 21% each.

Phylogenetic analysis

A computational tool, MEGA7, was used to perform the phylogenetic analysis for each strain. The phylogenetic analysis of AK-1 (MW898426), AK-2 (MW898428), AK-3 (MW898430) and AK-4 (MW898432) showed that these strains belong to *Stenotrophomonas* sp., *Serratia* sp., and *Pseudomonas* sp. respectively (Fig. 1). Two of the bacterial strains that had the potential to degrade plastic i.e. *Stenotrophomonas* sp. and strain *Serratia* sp. AK-2 has been previously reported in bacterial-mediated biodegradation of linear low-density polyethylene (Azeko et al. 2015). Sekhar et al. (2016), identified polystyrene degrading microorganisms by sequencing 16S rRNA gene and have reported *Stenotrophomonas* sp. for the degradation of low-density polyethylene. Closely related type strains of *P. stutzeri* and *S. marcescens* are on the same line because these share almost 100% similarity.

FTIR analysis

FTIR was employed for the detection of change in functional group or in the chemical structure of plastic pieces because of incubation with isolated strains. As plastic pieces were exposed to four different strains AK-1 (*Stenotrophomonas* sp.), AK-2 (*Serratia* sp.) AK-3 (*Pseudomonas* sp.) and AK-4 (*Pseudomonas* sp.) separately and results were presented in various FTIR graphs (Fig. 2b–e). The change was observed in the structure of all plastic pieces that were exposed to all isolates. It was noted that AK-1 (*Stenotrophomonas* sp.) and AK-2 represented the increase in transmittance at 1459.7 cm^{-1} and 872.96 cm^{-1} that indicated the reduction in bonds. These peaks represented reduction in CH_2 bends and skeletal C–C vibrations respectively. The change was also observed at 1107.66 cm^{-1}. The change at 1651.47–1670 cm^{-1}, at this point reduction

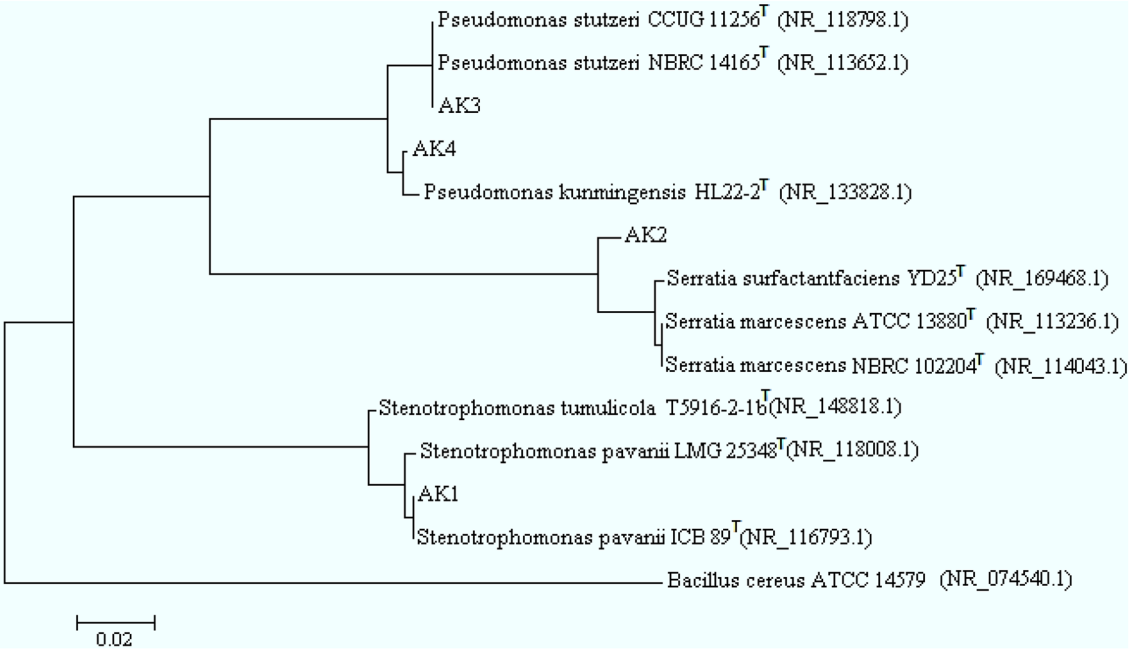

Fig. 1 Neighbor-Joining method was used to infer the evolutionary history of strains AK-1, AK-2, AK-3 and AK-4. *Bacillus cereus* ATCC 1459 is an out-group used to have a rooted phylogeny

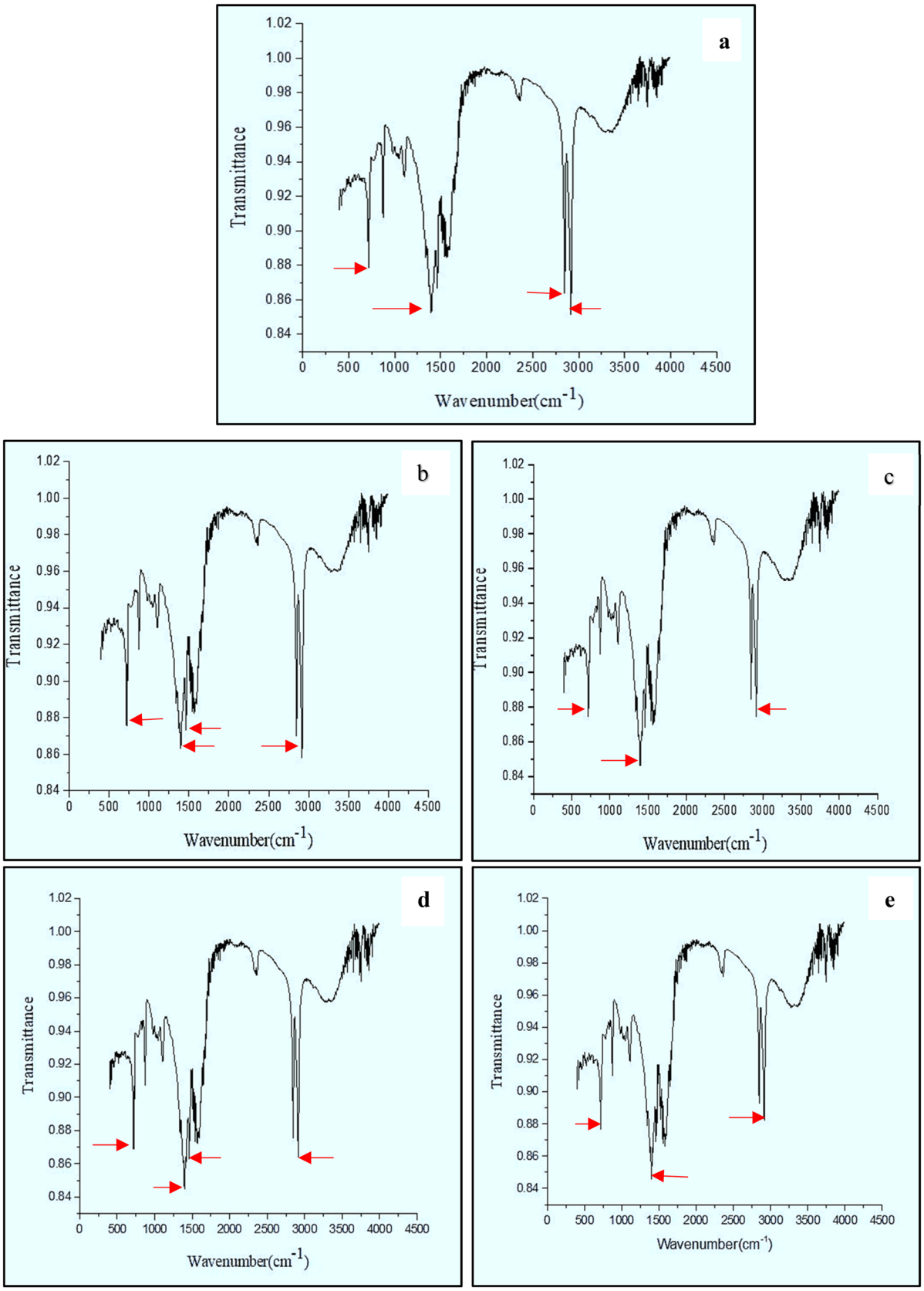

Fig. 2 **a** FTIR spectrum of controlled plastic piece (without bacterial treatment). FTIR spectra of plastic pieces treated with **b** AK-1 (*Stenotrophomonas* sp.) **c** AK-2 (*Serratia* sp.) **d** AK-3 (*Pseudomonas* sp.) and **e** AK-4 (*Pseudomonas* sp.)

in transmittance was observed. When plastic pieces were exposed to strain AK-2 (*Serratia* sp.), the increase in transmittance was observed at wavenumbers 2915.12, 2846.42, 1456.84, 874.39 cm^{-1}. These peaks represented –C-H stretch, –C-H bend, CH_2 bends and skeletal C–C vibrations respectively. Reduction in transmittance was observed at 1651.47–1670 cm^{-1}, when plastic pieces were degraded by *Serratia* sp. the decrease in transmittance observed at 1558.45, 1398.17 cm^{-1}, and 1103.36 cm^{-1} represented the increase in C = O, C–F, C–OH stretch. FTIR spectra of the plastic pieces treated with AK-3 and AK-4 strains showed less importance in terms of change in the plastic degradation when compared with the control (Fig. 2a–e.) of the same size and weight. By comparing all the spectra obtained from plastic pieces that were exposed to four different strains separately, it was concluded that the identified strains successfully reduced the C-H stretches and skeletal C–C vibrations. The range of approximately 1730–1650 cm^{-1} represented the presence of carbonyl group -C = O. The carbonyl group is formed by embodying an oxygen atom in the damaged structure of the polymer chains (Kowalczyk et al., 2016). In the present study, the decrease in transmittance was observed, which indicated the presence of carbonyl group that was a clear indication of the damaged structure of the polymer chains. The decrease in transmittance at 1100–1150 cm^{-1} was observed, that indicated the formation of new bonds (–C–O–C) due to weakness of C–C bonds. At 900–730 cm^{-1}, the increase in transmittance was observed. The peaks at 1150–1075 cm^{-1} represented the presence of ether bonds –C -O –C. This bond was formed because of oxygen atom embodying between the C–C bonds weakened due to the high electronegativity of oxygen atom. The peaks at 900–735 cm^{-1} and less than 700 cm^{-1} represented the skeletal –C–C vibrations. The higher absorbance indicated the weakness in structure of the test compound. This is because of cleavage and formation of new bonds, which indicated the change in structure of the low-density polyethylene. The structure becomes looser that reduces the energy level and enhances atomic vibrations (Kowalczyk et al., 2016).

Conclusion

Plastics are synthetic polymers having large spectrum of applications. Degradation of plastics by microorganisms, i.e. biodegradation, is known for several years. The current study was performed for the addition of plastic-degrading microorganisms by isolating from waste plastic bags taken from the soil waste-dumping sites of Faisalabad, Pakistan. Indigenously isolated bacterial strains can be effectively investigated for plastic degradation. Nature has a huge reserve for microbial organisms it is therefore of vital importance that these shall be studied for their plastic biodegradation characteristics. Efficient and non-pathogenic microbial strains that have the catalytic power to degrade synthetic polymers can be released in plastic dumpsites to effectively degrade the waste and rescue the environment from the environmental problems generated because of the plastics. To use bacteria for biodegradation purposes, it is highly required that novel bacterial strains must be employed in plastic degradation research and investigated for their degradation efficacy.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-021-02521-1.

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