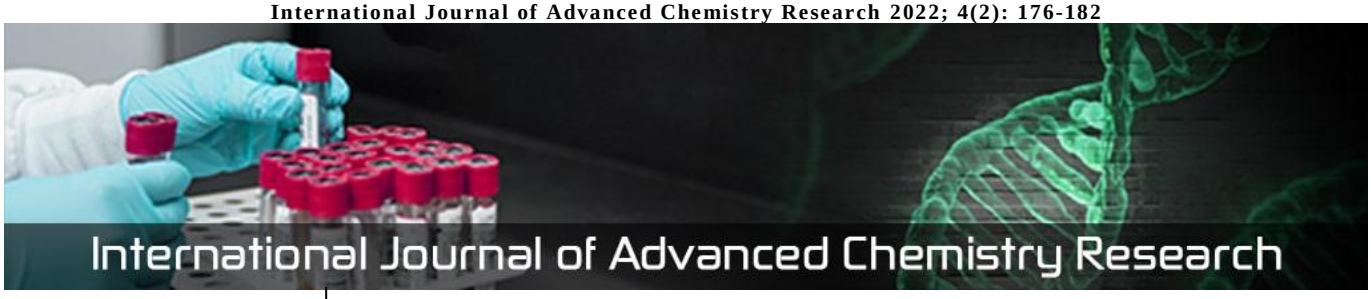

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A study on microbial deterioration of low-density polyethylene

Anila Sadan, Archana Vaishnava and Varsha Mondal

Abstract
Plastics have been widely used as a packing material in the form of low-density polyethylene (LDPE) they are very versatile in nature, cost efficient and facile. However, they are not decomposable and resistant to microbial attack which is a major cause of persistent and long-term environmental pollution. In this study, the isolation of polythene degrading microorganisms was done from plastic wastes dumped site. The effect of the pre-treatment on the biodegradation of polythene was analyzed during one month of incubation period in liquid (shaker) culture method. To check the efficiency of biodegradation, weight method was performed. The bacterial species found associated with the degrading materials were found to be as Gram-positive cocci and Gram-negative rods; and these were identified as *Staphylococcus simiae* and *Proteus vulgaris* with the help of an online advanced bacterial identification software. Several biochemical assays were performed to determine the effective biodegradation of polyethylene bags. This work reveals that *Proteus vulgaris* possess greater potential to degrade polythene as compared to *Staphylococcus simiae*. *Proteus vulgaris* showed better degradation efficiency in case of both pre-treated and untreated samples, proving to be a potent microbe in degrading polythene.

Keywords: Poly ethylene, *in vitro*, biodegradation, enzymatic degradation, bio-based plastics, microbial degradation

Introduction
During the past 3-decades, plastic materials have been increasingly used in food, clothing, shelter, transportation, construction, medical, and recreation industries. Plastics are highly versatile and are widely used as they are strong, light weight, and durable. However, the aggregation rate of plastic waste has caused a serious environmental peril, as they are resistant to biodegradation, they cannot be incinerated as that leads to toxic fumes which causes air pollution, polythene causes severe environmental hazard. The successful production and marketing of biodegradable plastics will help alleviate the problem of environmental pollution. In the past 10 years, several biodegradable plastics have been introduced into the market. However, none of them is efficiently biodegradable in landfills. For this reason, none of the products has gained widespread use [1]. Hence, there is an urgent need to develop efficient microorganisms and their products to solve this global issue. The polythene is the most commonly found non-degradable solid waste that has been recently recognized as a major threat to marine life. The polythene could sometimes cause blockage in intestine of fish, birds and marine mammals [2, 3].
Plastics can be degraded by chemical, thermal, photo or biological degradation. Any physical (like weight loss of sample, tensile strength) or chemical change (like carbon dioxide production) in the material suggests biological degradation by microorganisms. The degradation of a polymer is affected many factors like temperature, moisture, oxygen, sun light, stress, living organisms and contaminants [4].
Low density polyethylene is one of the major sources of environmental pollution. Polyethylene is a polymer and made of long chain monomers of ethylene. The worldwide utility of polyethylene is expanding at a rate of 12% per annum and approximately 140 million tons of synthetic polymers are produced worldwide each year [5]. Low–density Poly-ethylene (LDPE) accounts for 60% of the total plastic production and the most commonly found solid waste is the non-degradable polythene carry bags. Most of the municipal and garbage sites are littered with large quantity of this highly recalcitrant waste material. Due to their slow degradability in natural environment, microorganisms have been utilized to study

their effect on the biodegradation of plastics as bacteria & fungi have been reported for the degradation of both natural and synthetic plastics [6]. These synthetic polymers are normally not biodegradable until they are degraded into low molecular mass fragments that can be assimilated by microorganisms.

Biodegradation has been considered as a natural process in the microbial world where polymers can be used as carbon and energy sources for their growth and plays a key role in the recycling of these materials in the natural ecosystem [7]. The microbial degradation of plastics is caused by certain enzymatic activities that lead to a chain cleavage of the polymer into oligomers and monomers. These water soluble enzymatically cleaved products are further absorbed by the microbial cells where they are metabolized. Aerobic metabolism results in carbon dioxide and water [8], whereas anaerobic metabolism results in carbon dioxide, water and methane as the end products, respectively [9]. Various mediums or environment as a whole is used for biodegrading polymers. Due to various physical as well as biological forces depolymerization can be observed. The physical forces such as temperature, moisture, pressure cause mechanical damage to the polymers [10]. A large number of microorganisms have found to produce enzymes which degrade plastics and many more metallic as well as nonmetallic compounds.

The pre-treatment of polyethylene is very significant for its biodegradation. Physical rupturing of polyethylene and chemical washing by ethanol might have added value to its degradability [11]. The mixing of small amount of cellulose can bring some changes in the polymer properties and lead to its microbial degradation [12]. Microbial degradation of plastics is caused by certain enzymatic activities that lead to a chain cleavage of the polymer into oligomers and monomers. These water soluble enzymatically cleaved products are further absorbed by the microbial cells where they are metabolized.

Against these backdrops, the purpose of the present study was to isolate polyethylene degrading microorganism (bacteria) from polyethylene dumped sites, identification of the high potential microorganism that degrade the polyethylene, to study the effect of the pre-treatment on the biodegradation and also to study the efficiency of the microorganism to degrade the pre-treated polyethylene strips.

Materials and Methods

Sample preparation

Screening and molecular identification of bacterial isolate:

Polyethylene sample was collected from the plastic waste dumped site of city in a sterile plastic bag. Isolation of microorganisms from their natural environment is very useful for dealing with many biotechnological problems therefore the isolation usually begins with a solution culture leading to an increase in number of bacteria of desired character. Considering this fact, the bacterial pure colonies were obtained using standard protocol on Nutrient Agar Media (NAM) and NB (Nutrient Broth) media (Hi-media, Pvt. Ltd, Mumbai). The sample was serially diluted and spread to obtain isolated colonies and was streaked to obtain pure cultures as described by Aneja [13]. Bacterial pure cultures were maintained on NAM slants and stored at 4 ˚C.

Identification of the isolates were performed according to their morphological, cultural and biochemical characteristics by following Aneja [13], Bergey's Manual of Systematic Bacteriology [14]. Various biochemical tests for the identification of isolates were performed according to Cappucinno and Sherman [15].

Characterization of bacterial isolates was done on the basis of colony morphology, cell morphology (Gram staining) and biochemical tests.

Low density polyethylene and its in-vitro degradation study

Polyethylene bags were cut into small strips & transferred to fresh solution containing 18ml tween, 10ml bleach (5 gm NaCl, 5 gm NaOH, 10 ml glacial acetic acid), and 225ml of distilled water & stirred for 30-60mins in the orbital shaker. Strips were then transferred to the beaker containing distilled water & stirred again for one hour. They were then aseptically relocated to ethanol solution 70%v/v for 30 mins. Finally, the polyethylene strips were transferred to the culture broth under aseptic conditions. Ethanol was used as disinfectant & also to remove any organic matter adhering to its surface.

Microbial Degradation of Plastics in Laboratory Condition Determined by the Weight loss. **[16]**Pre-weighed discs of 1-cm diameter weighing 50 mg were prepared from polythene bags. These were then aseptically transferred to the conical flask containing autoclaved 50 ml of culture broth (nutrient broth) medium. Control was maintained with plastic discs in the bacteria-free medium. Different flasks were maintained for pre-treated and without pre-treated plastic strips. The flasks were then inoculated with different bacterial species and left in a shaker at 37 ˚C and at 150 rpm for a period of 1 month. The plastic discs were collected at a regular interval of a week for a month, after which these were washed thoroughly using methanol and distilled water, shade-dried and then weighed for final weight.

From the data collected, weight loss of the plastics was calculated.

Analysis of the LDPE film biodegradation

Weight reduction measurement

The weight after 1month incubation was compared to the mass before incubation. For comparison the mass of film in the control was also measured. The percent weight reduction was calculated with the formula:

$$\% \text{ Weight reduction} = \frac{W_1 - W_2}{W_1} * 100$$

where W_1 is the weight of polyethylene film before incubation and W_2 is the weight of polyethylene film after 1-month incubation.

Result

Various kinds of biochemical tests were done on specially prepared media by inoculating the two bacterial isolates on the basis of colony morphology on nutrient agar plates and nutrient agar slants, simple staining & arrangement of the cells of the bacterial isolates and Gram Staining, into them. The tests included starch hydrolysis test, casein hydrolysis test, gelatin hydrolysis test, carbohydrate fermentation test, triple sugar-iron agar test, IMViC test, hydrogen sulfide

production test, urease test, litmus milk reactions, nitrate reduction test, catalase and oxidase test.

Both the bacterial isolates A and B showed positive results for casein hydrolysis, gelatin hydrolysis, carbohydrate fermentation, triple sugar-iron agar test, hydrogen sulfide test, urease test, litmus milk reactions, and nitrate reduction. In the IMViC test, both the isolates showed positive result for indole, MR and citrate test while negative result for VP test. For oxidase isolate A was negative and B was positive and for catalase and coagulase test both the isolates gave negative result.

ABIS (Advanced bacterial identification software) online software was used for the identification of the unknown bacterial isolates. It is an advanced bacterial identification software which is a laboratory tool for bacterial identification, based on morpho-biochemical characters, cultural characteristics and growth conditions. The program allows a great flexibility in choosing biochemical tests and is an alternative to commercial systems, code-books or identification tables. The identified microorganisms were-

Isolate A: *Proteus vulgaris* (85% match; 30% accuracy).

Isolate B: *Staphylococcus simiae* (81% match; 22% accuracy).

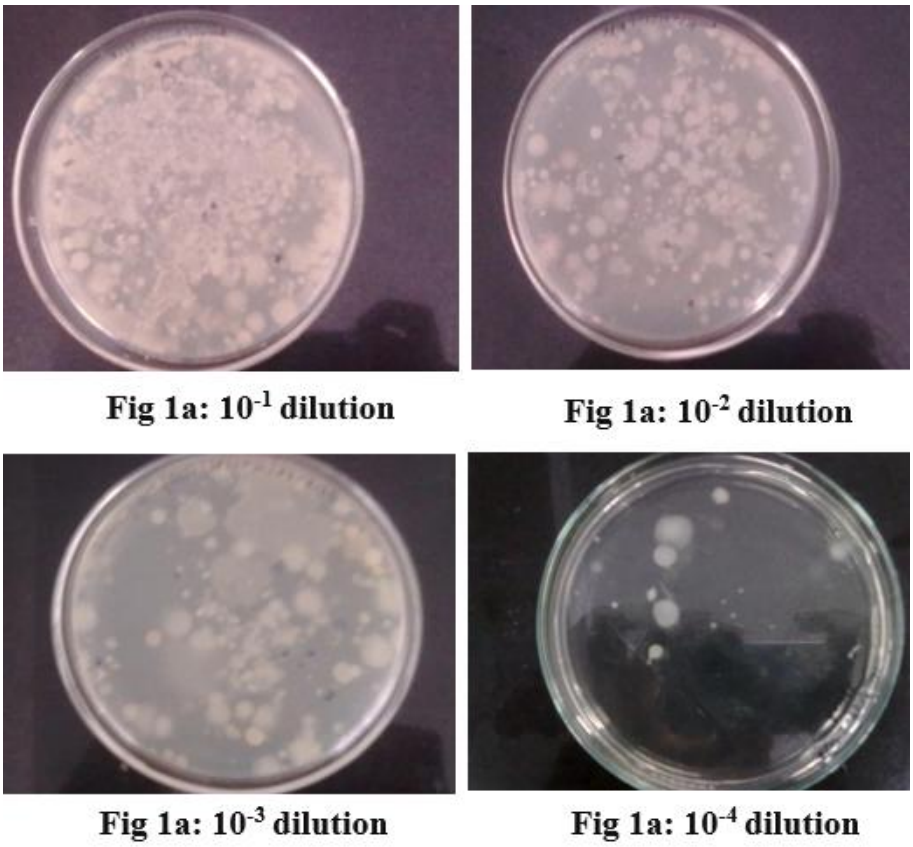

Fig 1: Serial dilution plates

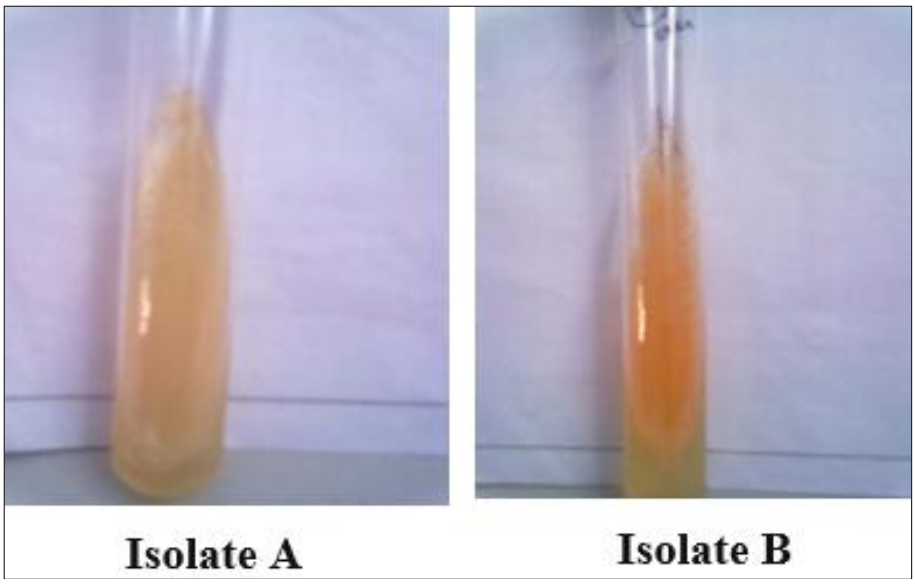

Fig 2: Nutrient Agar Slants

Table 1: Biochemical test results of the bacterial isolates

S. No.	Biochemical tests	Isolate A	Isolate B
1	Starch hydrolysis	-ve	+ve
2	Casein hydrolysis	+ve	+ve
3	Gelatin hydrolysis	+ve	+ve
4	**Carbohydrate Fermentation**		
4.1	Glucose	+ve (gas production)	+ve (gas production)
4.2	Lactose	+ve (gas production)	+ve (gas production)
4.3	Sucrose	+ve (gas production)	+ve (gas production)
5	Triple Sugar- Iron Agar test	Acid butt, Acid slant	Acid butt, Acid slant
6	**IMViC test**		
6.1	Indole test	+ve	+ve
6.2	MR test	+ve	+ve
6.3	VP test	-ve	-ve
6.4	Citrate	+ve	+ve
7	Hydrogen Sulfide	+ve	+ve
	Motility test	+ve	+ve
8	Urease test	+ve	+ve
9	Litmus milk reactions	+ve, pink,curd formation	+ve, pink,curd formation
10	Nitrate reduction	+ve	+ve
11	Catalase	-ve	-ve
12	Oxidase	-ve	+ve
13	Coagulase	-ve	-ve

In order to study the effect of pre-treatment on polyethylene strips. The strips were weighed before and after the pre-treatment. No change in the weight of the polyethylene strips was observed.

Table 2: Effect of the pre-treatment on the weight of the polyethylene strips

Treatment	Weight of PE (mg)		Weight of PE degraded (mg)	% of PE degraded
	Initial	Final		
Without any treatment	50 mg	50 mg	0 mg	0%
Chemical- alkali Treatment (NaOH)	50 mg	50 mg	0 mg	0%

The weight loss of the polyethylene strips was noted at the end of each week of both the bacterial isolates for the pre-treated and also the untreated strips (Table 3 & 4).

Table 3: Weight of polyethylene degraded in each week by the bacterial isolates for both pre-treated and untreated polyethylene strips

Sample	Isolates	Weight of PE (mg)				
		Initial	After 1st week	After 2nd week	After 3rd week	After 4th week
Without any treatment	Isolate A	50 mg	49 mg	47 mg	46 mg	45 mg
	Isolate B	50 mg	50 mg	50 mg	49 mg	48 mg
	Control	50 mg	50 mg	50 mg	50 mg	50 mg
With Treatment	Isolate A	50 mg	48 mg	47 mg	45 mg	43 mg
	Isolate B	50 mg	49 mg	48 mg	48 mg	47 mg
	Control	50 mg	50 mg	50 mg	50 mg	50 mg

Table 4: The overall reduction in the weight of the polyethylene strips observed after 1-month period by both the bacterial isolates A and B for both treated and untreated strips

Sample	Name of the microbe	Weight of PE (mg)		Weight of PE degraded (mg)	% of PE degraded
		Initial	Final		
Without any treatment	Isolate A	50 mg	45 mg	5 mg	10
	Isolate B	50 mg	48 mg	2 mg	4
	Control	50 mg	50 mg	0 mg	0
With Treatment	Isolate A	50 mg	43 mg	7 mg	14
	Isolate B	50 mg	47 mg	3 mg	6
	Control	50 mg	50 mg	0 mg	1

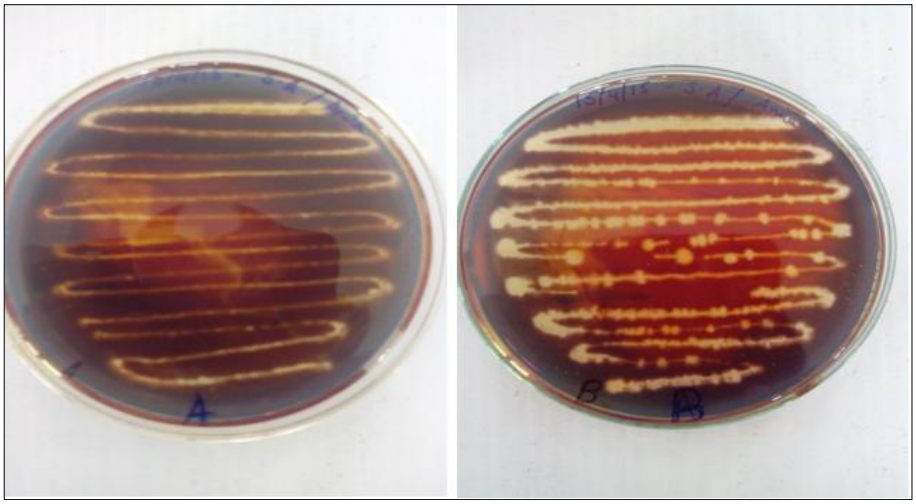

Fig 3: Starch Hydrolysis

Fig 4: Casein Hydrolysis

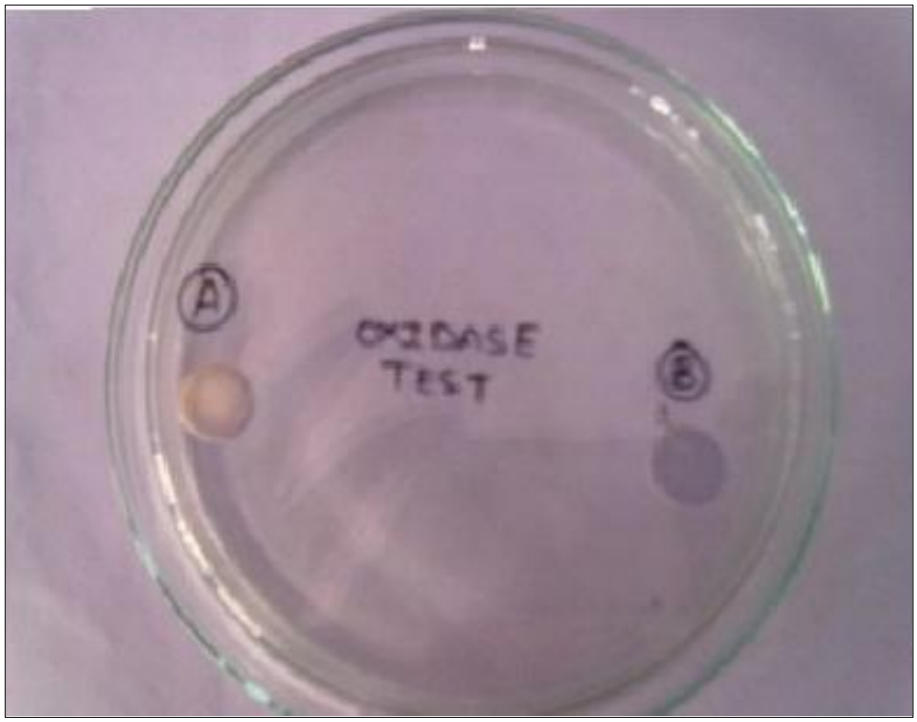

Fig 5: Oxidase Test

Fig 6: Catalase Test

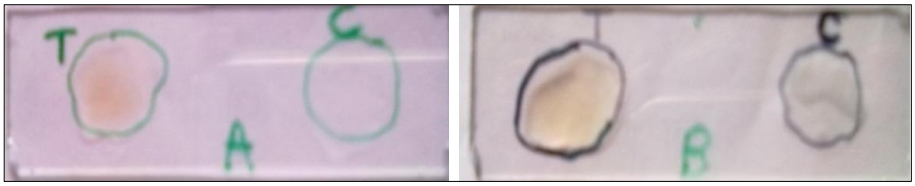

Fig 7: Coagulase Test

The weight loss in the polyethylene strips were recorded after a period of 1 month and the percentage of degradation was observed for both the isolates of both pre-treated and untreated polyethylene strips. The isolate A showed more efficiency in degrading the plastic strip than isolate B. Isolate A's biodegradation efficiency was found to be more in the case of treated polyethylene than in untreated polyethylene, the biodegradation efficiency of isolate B also increased profoundly in case of treated polyethylene strips however did not prove to be so effective in case of untreated polyethylene strips.

For the untreated polyethylene strips, the loss of 5mg was observed in the initial weight of the strips by the isolate A whereas a loss of 2mg was observed in the weight of the strips by the isolate B with an overall reduction of about 10% and 4% respectively and For the pre-treated polyethylene strips, the loss of 7mg was observed in the initial weight of the strips by the isolate A whereas a loss of 3mg was observed in the weight of the strips by the isolate B with an overall reduction of about 14% and 6% respectively.

Discussion

The present study deals with the isolation, identification and degradative ability of plastic degrading microorganisms from soil. Different types of changes are produced by the microorganism during morphological and biochemical analysis. Polythene samples were used to study their biodegradation by microorganisms isolated from polythene dumped sites.

Microbial degradation of a solid polymer like polyethylene requires the formation of a biofilm on the polymer surface to enable the microbes to efficiently utilize the non-soluble substrates by enzymatic degradation activities. Developments of multicellular microbial communities known as biofilm, attached to the surface of synthetic wastes have been found to be powerful degrading agents in nature. When the total biodegradation process of any organic substrate is considered the formation of microbial colony is critical to the initiation of biodegradation. Thus, the duration of the microbial colonization is an important factor that effects total degradation period.

Two bacterial species from soil sample were isolated and characterized on the basis of their morphological and biochemical characteristics. The isolated microorganisms from polyethylene dumped areas can be interacted with polythene and undergo changes in mechanical properties of tensile strength, optical changes of cracking, erosion and decolorization [17]. It is clear that polymers can be degraded to some extent by microbes. In the present study pieces of plastics were inoculated in the liquid culture medium containing bacterial isolates and kept for 1 month to observe the percentage of weight loss by bacteria. The result shows the degradative ability of the microorganisms after one

month of incubation. Similar work done by Nayak & Tiwari [18]. and Pandey & Anbuselvi [19]. Has relevance with the work done in this dissertation that the microbes confirmed polyethylene degradation. Two isolates were identified by micro and macroscopically and confirmed by various biochemical tests. Based on the colony morphology, gram staining, biochemical tests, motility test and with the help of Software (ABIS) both isolates were identified as *Staphylococcus* and *Proteus*. This is in accordance with Dutt and Anbuselvi [20]. The biodegradation of polythene by isolated bacteria showed weight loss. *Proteus* showed more degradation than and *Staphylococcus.* These microbes confirmed its polyethylene degradation by weight loss. These findings can also be related to the work done by Nayak and Tiwari [18] that these two bacteria are involved in biodegradation.

The pre-treatment of polythene is very significant for its biodegradation. Physical rupturing of the polyethylene and chemical washing by ethanol might have added value to its degradability [11]. The weight loss in the polyethylene strips were recorded after a period of 1 month and the percentage of degradation was observed for both the isolates of both pre-treated and untreated polyethylene strips. But the pretreatment enhanced the biodegradation of polyethylene. This is in accordance with Hanaa *et al*., [21] and Hasan *et al*., [22] that pretreatment enhances the biodegradation of polyethylene. The isolate A showed more efficiency in degrading the plastic strip than isolate B. Isolate A's biodegradation efficiency was found to be more in the case of treated polyethylene than in untreated polyethylene, the biodegradation efficiency of isolate B also increased profoundly in case of treated polyethylene strips however did not prove to be so effective in case of untreated polyethylene strips.

Conclusion

The problem of plastic pollution is now really a mess for mankind. There is no part of the world untouched from its impact. In the present era of globalization some stress must be given to plan safe disposal of products before making it commercial. Making science to the leap and forgetting the other side of the coin lead to such conditions. Biodegradation is a novel procedure to degrade different kinds of artificial substances in a biological manner. This helps to maintain balance in the surrounding. Bacterial strains were successfully isolated from polythene garbage dumps.

In the present investigation, an attempt has been made to study the biodegradation of polythene in control laboratory conditions. The isolation and characterization of polyethylene degrading bacteria from polyethylene garbage was done. Identification of the isolated strains was performed on the basis of colony morphology, grams nature, and several biochemical tests. Polyethylene strips were subjected to biodegradation by isolated bacteria using nutrient broth medium. The degradation was observed by changes in weight of the polythene strips.

The bacterial strains were identified macroscopically by examining colony morphology, surface pigment, shape and size on nutrient agar plates. Microscopic examination including Gram's staining is used to study the staining behavior, shape and cell arrangement. Motility test was also performed. Further characterization was confirmed by performing the following biochemical tests such as Catalase, Gelatin hydrolysis, Triple sugar-iron agar, Indole, Hydrogen Sulfide, Urease test, Litmus milk reactions, Nitrate reduction, Coagulase, Oxidase, Methyl red, VP, Starch hydrolysis, Casein hydrolysis, Carbohydrate Fermentation, Citrate and Motility tests.

ABIS (Advanced bacterial identification software) online software was used for the identification of the unknown bacterial isolates. It is advanced bacterial identification software which is a laboratory tool for bacterial identification, based on morpho-biochemical characters, cultural characteristics and growth conditions. Isolated bacterial strains were identified as *Staphylococcus simiae and Proteus vulgaris.* The pre-treated samples were far better biodegraded than untreated samples. The effect of the pre-treatment on the polyethylene samples was seen to accelerate the degradation capacity of both *Proteus vulgaris* and *Staphylococcus simiae*. *Proteus vulgaris* showed better degradation efficiency in case of both pre-treated and untreated samples, proving to be a potent microbe in degrading polythene.

Biodegradation of plastics by bacteria can be made most efficient by altering the factors that govern the process. It promises a reduction in plastic pollution in the future.

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