Section A-Research paper

Biosurfactant Synthesis by Aerobic bacteria that Degrade Synthetic Polyethylene: Screening and Characterization.

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Article History: Received 15.06.2023 Revised: 20.07.2023 Accepted: 24.07.2023

Abstract

Due to their numerous applications, plastic manufacturing and consumption are rising. Because there is no practical method for properly disposing of plastics, they frequently collect in the environment and constitute a problem. Even banning plastic covers does not stop the production of plastic garbage. This paper presents findings from a preliminary analysis of surface chemistry employing biosurfactants to speed up the biodegradation process. The biodegradation investigation used synthesized biosurfactants from isolated soil bacteria *Alcaligenes feacalis.* Tween 20 was found to be the hydrocarbon that was most thoroughly emulsified out of all those employed to examine the emulsion of biosurfactants. In this investigation, the percentage weight loss of synthetic polymers was utilized to gauge the degree of biodegradation, and the isolated *Alcaligenes feacalis* with biosurfactants added showed the maximum weight loss of 22.2%, compared to 13.3% when used alone.

Key words: Synthetic polymer, biosurfactants, degradation, *Alcaligenes feacalis,*

Introduction

Synthetic organic polymers such as plastics are utilized worldwide due to their stable properties and longevity (Syranidou *et al.,* 2017). The worldwide production of plastics is anticipated to reach 500 million tons by 2050, from 300 million tons in 2014 (Lohr *et al.,* 2017; Wang *et al.,* 2019). Plastic litter contributes significantly to the widespread ocean contamination (Katsnelson, 2015). Due to poor recycling rates and improper disposal, plastic is not easily biodegradable, and as a result, resistant plastic wastes have accumulated in the environment and are harmful to the ecosystem. Every year, rivers release between 1.15 and 2.41 million tons of plastic trash into the ocean (Lebreton *et al.*, 2017).The top twenty contaminated bodies of water are primarily found in Asia and make up around 67% of the worldwide total. The majority of plastic waste is made up of low-density polyethylene, which is often commonly used in carry bags (Harshvardhan and Jha 2013). Due to its hydrophobic properties, large molecular weight, and lack of functional groups that microbes can identify, polyethylene, one of most popular hydrocarbon plastic, remains in the environment (Nowak *et al.*,2011) The impacts of breakdown and biofilm formation on plastics in the marine environment must be clearly understood, said several scientists recently (Karlsson *et al.,* 2018).Recognizing the interactions among polymeric materials and elements involved in polymer structural changes is essential, as is being aware of the mechanisms underlying polymer degradation. Very little is known about how polymer materials break down and how that affects the environment (Hersztek *et al.,* 2019).

Surfactants are chemical substances derived from petroleum that can reduce the surface tension between two or more liquids because to their amphiphilicity. A hydrophobic and hydrophilic moiety make up the primary structure of a surfactant molecule (Juhasz *et al.,* 2009; Varjani *et al.,* 2017). A polar fragment can be made by a carbon chain, whereas a polar moiety can be formed by

carbohydrates, amino acids, carboxylic acids, phosphate, or alcohol (Silva *et al.*, 2017). Biosurfactants, on the other hand, are metabolites produced by filamentous fungi, yeast, and bacteria. By fermenting hydrocarbons, these extracellular, amphiphilic molecules were identified in the 1960s and compromise a number of benefits over chemical surfactants. Biosurfactants have attracted a lot of interest in recent years due to their low toxicity, high selective biodegradability, low critical micellar concentration (CMC), and stability under harsh pH, salinity, and temperature conditions (Araujo *et al.*, 2013; Souza *et al.*, 2018).As in process of forming a film at an interface, microorganisms release biosurfactants which alter the waters' ability and other properties.

On the deterioration and degradation of polyethylene, several scientists have worked. There hasn't been much research on the use of biosurfactants in the microbiological deterioration and breakdown of synthetic polymers. Because of this, we have attempted to partial degradation of polyethylene in our study utilizing screened microorganisms and biosurfactants that were isolated from dumpsite soil.

2. Materials and Methods:

2. A. Collection of samples and separation of microorganisms:

A conventional approach was used to evaluate a soil sample taken from the area of a local dumpsite in the city of Raichur in the state of Karnataka. A pure culture of the identified isolated microbe was retained in the lab for further investigation after it was tested for biosurfactant synthesis.

2.1.1. Screening technique for Biosurfactant assay:

2.1.1. a. Preliminary test:

2.1.1. a. i. Phenol: H_2SO_4 method:

One ml of bacterial supernatant was added with 5% one ml phenol and 2-5 ml of Conc H_2SO_4 was added carefully drop by drop until orange color persisted which indicated the presence of lipids containing biosurfactant. (Sushma *et al.*, 2018).

2.1.1. a. ii. Biuret test:

This test was performed to detect the lipopeptide containing biosurfactant. Two ml of bacterial supernatant was heated at 700℃ and then was mixed with one ml of 1 M NaOH solution. Few drops of 1% $CuSO_4$ was slowly added until violet/pink ring developed. Formation of violet or pink ring indicated the presence of lipopeptides containing biosurfactant. (Sushma *et al.*, 2018).

2.1.1. a. iii. Phosphate test:

Ten drops of 6M HNO_3 was added to 2ml of culture supernatant, and was heated at 70℃, 5% (W/V) ammonium molybdate was added to this mixture drop by drop slowly until the formation of yellow color and then the yellow precipitate. This indicates the presence of phospholipids containing biosurfactant. (Kalyani *et al*., 2014).

2.1.2. b. Secondary/ Confirmatory test:

Biosurfactant assay was determined for the pure culture of bacterial culture by different methods namely, Haemolysis, drop collapse, Oil spreading technique, Emulsification index.

2.1.2. b.i. Haemolysis activity:

Biosurfactant assays of the isolate was evaluated by the hemolysis test on blood agar and the plates were incubated at 28℃for 7 days. Plates were observed for zone of clearance around the colonies.

2.1.2. b.ii. Drop collapse method:

A clean glass slide was taken at the end of the slide indicator mixed oil drop was added, then 10 microliter cell free culture was added to the oil drop. After 2min the drop was collapsed indicating the presence of biosurfacatnt in the cell free suspension. (Yousaf *et al.,* 2013)

2.1.2. b.iii. Oil- spreading technique:

5ml of distil water was taken in the petriplate, 2ml of crude oil (petrol), 1ml of olive oil was added onto the petriplate and 1ml of culture filtrate was placed on the petriplate at the center of the oil layer. The presence of biosurfactant would displace (spread) the oil and a clear zone on the oil surface would be visualized under visible light and UV after 30 sec. The zone increased after 10min from 3mm to 5mm diameter which is also known as displacement activity (Vimala & Mathew 2016).

2.1.3. b.iv. Emulsification index/assay:

Emulsification assay was carried out using petroleum as described by Revathi *et al.,* 2018. 2ml of cell-free supernatant was taken in the test tube, 4ml of water and 6ml of petrol (hydrocarbon)was added and vortexed for 2min to ensure the homogenous mixing of the liquids to obtain maximum emulsification. After, 24-48h emulsification index (Ellaiah *et al.,* 2021,).

The emulsification activity was observed after 24h and it was calculated by using the formula given below:

$$\% \text{ E24} = \frac{\text{Total height of the emulsion}}{\text{Height of the aqueous layer}} \times 100$$

2.1.4. b.vi. Blue agar plate method (BAP):

BAP method is another semi-quantitative method for detection of biosurfactants in which CTAB and Methylene blue are used. Bacteria grown on the plate produced dark blue halos. Minimal agar medium with glucose (2%), cetyl-trimethyl ammonium bromide (CTAB, 0.4 mg/ml), and methylene blue (0.2 mg/ml) were used to detect extracellular biosurfactants (Adnan *et al.,* 2018). 25µL of culture supernatant was added to each well and incubated for 48-72 h at 37°C. Formation of dark blue color halos around the well confirmed the presence of biosurfactant.

2.2.1. Extraction of Biosurfactants:

2.2.1. a. Acid precipitation method:

Incubated cultures were centrifuged at 4000rpm at room temperature for 30 min. To the supernatant obtained 1M H_2SO_4 was added to adjust the pH at 2. Chloroform: Ethanol was added in the ratio of 2:1. These mixtures were shaken well to ensure proper mixing and were left overnight for evaporation (Vimala & Mathew 2016, Kannahi & Sherley 2012).

2.2.1. b. Production of Biosurfactants

For the production of biosurfactants by the strains was determined by using MSM, recommended by Zhang *et al*., 2005. The strains were activated twice in nutrient broth, and then bacterial suspension was adjusted to pH 2. Finally, they were inoculated into MSM at the ratio of 1/20. Bacterial cultivation was performed in 500 mL flask containing 200 mL medium at 35°C, stirred in an orbital rotary shaker at 180rpm for 7 days (Kaskatepe *et al.,* 2015).

2.2.1. c. Purification of Biosurfactant

After seven days of incubation period, pH was adjusted to 8.0 (using 10M NaOH), and biomass was removed by centrifugation for 20 min at 9000rpm. The pH of supernatant was adjusted to 2 (using 6 M H_2SO_4) and it was kept overnight at 4℃. Next day equal volume of chloroform: methanol (2:1) mixture was added. The resultant suspension was shaken for 10 min rotary shaker and organic phase was removed. The extraction process was repeated one more time. The obtained biosurfactant was concentrated from pooled organic phases by using a rotary evaporator, the product was dissolved in analytical grade methanol and filtered for spectrophotometric analysis. (Kaskatepe *et al.,* 2015).

2.3.1. Characterization of Biosurfactant

The extracted biosurfactant were melted in chloroform: methanol (9:1). 2µL of the sample was applied onto TLC plates (Merck) with Chloroform: Methanol: Acetic acid (65:15:2) solution as the developing solvent. The TLC plates were dipped in 15% H_2SO_4 in ethanol and incubated at 100℃. Appearance of brown dot was considered as presence of glycolipids. (Kaskatepe *et al.,* 2015).

2.3.2. Application of Biosurfactant in degradation

Biosurfactants were added to the production medium to facilitate in the biodegradation of LDPE. LDPE bags were cut into 2x2 cm strips, weighed, sterilized for 40 minutes by placing in 70% ethanol, thoroughly washed with distilled water, and then dried at 40°C in an incubator. The experiment was set up as following, in a 250 ml conical flask with 150 ml of biosurfactant production media and sterile LDPE strips were added, a microbial culture was inoculated and incubated for 30 days at 200 rpm. A disc of LDPE was aseptically removed from the flasks, washed

in 2% sodium dodecyl sulphate (SDS) for three hours, completely rinsed in distilled water, and dried in an incubator for five days as part of the experimental method. (Nnaji *et al.*, 2021)

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

3. Results

3.1. Preliminary test:

3.1. a. Phenol: H_2SO_4

The bacterial isolate developed orange color which indicated the production of glycolipids containing surfactants which is a positive result (Figure 3.1.a).

3.1. b. Biuret test:

On addition of NaOH and $CuSO_4$ to the bacterial isolate, violet ring developed indicates lipopeptide containing surfactants producing capacity (Figure 3.1.b.).

3.1. c. Phosphate test:

After addition of 5% W/V ammonium molybdate solution drop by drop to the isolates containing 6M HNO_3, yellow precipitate was observed in the bacterial isolate. This indicates the presence of phospholipid containing surfactant producing ability. (Figure 3.2.c.).

3.2. Confirmatory test

3.2. a. Oil spreading technique:

When the isolates supernatant was applied to the center of an oil layer spread over a layer of water, a clear zone emerged that displaced the oil layer, indicating the presence of biosurfactants (Figure 3.1.d.)

3.2. b. Drop collapse method:

As the interfacial tension between the culture supernatant drop and the hydrophobic surface is diminished, the droplet spreads over the hydrophobic surface. Yet, in the absence of surfactant, the droplet stays beaded or spherical (Figure 3.1.e.)

3.2. c. Blue agar plate method:

An accurate method to find anionic surfactants is the BAP agar test. The test is based on the notion that the production of an insoluble ion pair with diverse compounds can be used to identify anionic surfactants in aqueous solutions. These isolates have a blue halo zone around the isolate, indicating that they are positive for BAP testing (Figure 3.1.f.)

3.2. d. Haemolysis activity:

Biosurfactant assays of the isolate was evaluated by the haemolysis test on blood agar and producing clear zone around the colonies, indicating the presence of biosurfactant (Figure 3.1.g.).

(g) Phenol:H_2SO_4

(a) Biuret test

(f) Phosphate test

(c) Oil spreading test

(e) Blue agar plate method

(d) Haemolysis test

(b) Drop collapse method

Fig.3.1.a. to 3.1.g. Preliminary and Confirmatory tests for biosurfactant

3.2. e. Emulsification index:

The emulsification activity of the isolates supernatant was assessed against olive oil, benzene, toluene, Tween 20, and gasoline, and their relative E24 indices was measured. The isolates were cultured in a crude oil-containing medium. The isolates had the highest emulsification activity for Tween 20, followed by other hydrocarbons (Fig.3.2).

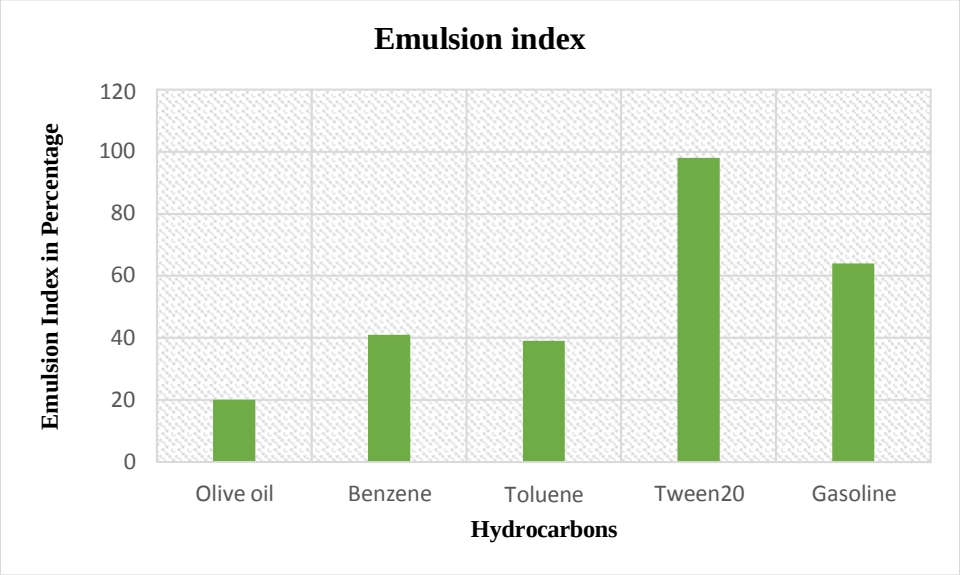

Fig.3.2. Emulsification index

3.3.Acid precipitation method:

After vigorously shaking mixes of acid, chloroform, and ethanol and allowing them to sit overnight to evaporate, white colored precipitate was observed at the interface between the two liquids, demonstrating the presence of biosurfactants. After drying, weights were measured after the mixtures were once more centrifuged to obtain biosurfactants in the pellet form (Table 3.1).

Table.3.1: Weight of extracted biosurfactant

Name of the organisms	Weight of petridish (g)	Weight of BS+ petridish (g)	Weight of BS (g)
RG1	27.04	27.88	0.84

3.4.Characterization of Biosurfactants

The TLC outcomes are exhibited in the image below; each isolated organism allowed a indication on the TLC plates, suggesting that it was capable of producing glycolipids (Fig.3.3).

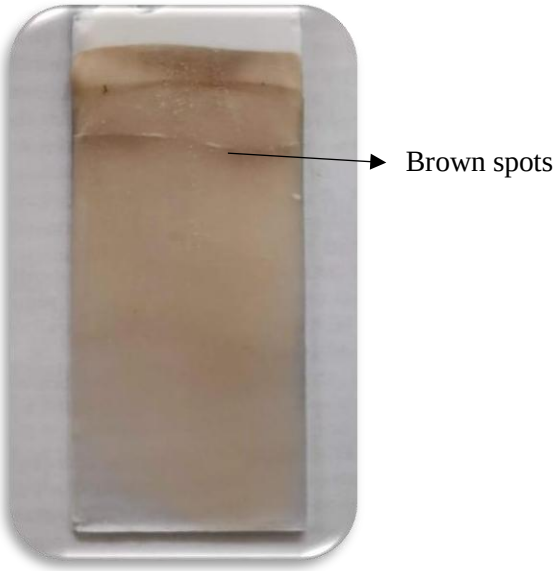

Fig.3.3. TLC of Biosurfactant extracted from cultures.

3.5.Application of Biosurfactant in degradation:

Weight loss over a period of 30 days, compared to the control shows no reduction in the weight during polythene degradation. The weight reduction of polyethylene after one month,

indicating that the organism used polyethylene for partial decomposition. They colonize to produce biofilm because of their hydrophobicity, which promotes polymer biodegradation activity. During the early degradation, the main chain cleaves, resulting in the formation of low-molecular-weight fragments (oligomers), dimers, or monomers. Degradation is brought on by the organisms secreting extracellular enzyme. These low-molecular-weight compounds are also sources of carbon and energy for the microbes (Fig.3.4).

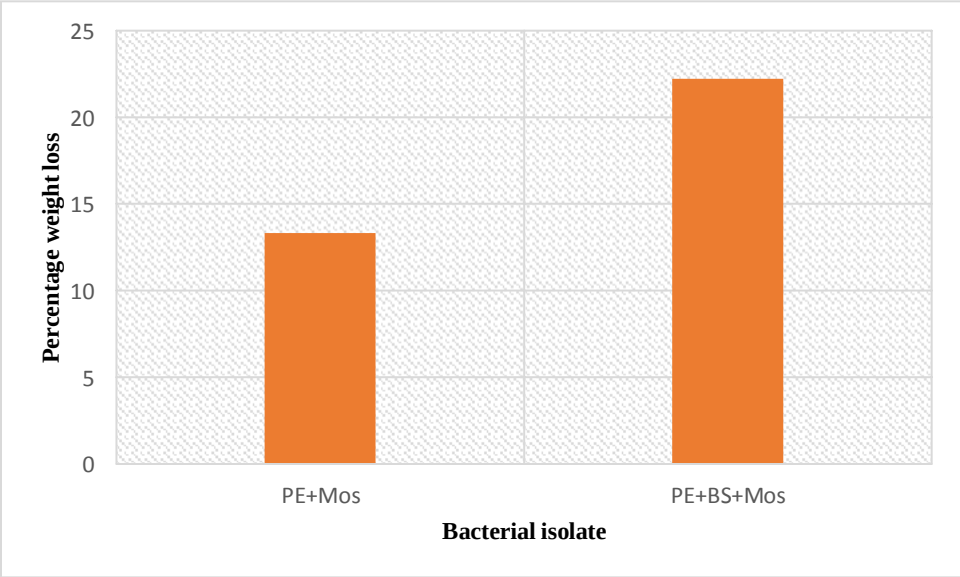

Fig.3.4. Biodegradation of LDPE by Biosurfactants

3.6. Discussion

Microorganisms had been reported to degrade LDPE for a number of years. The use of biosurfactants in the bioremediation process is still up for debate. The capacity of biosurfactant-producing microorganisms to degrade low density polyethylene in the current investigation suggests a tight relationship between these two processes. Abundant studies demonstrate that biosurfactants are more biodegradable than surfactants from synthetic

origins (Lima *et al.,* 2011). Tween 80 increased the adhesion and biodegradation of polyethylene by *Pseudomonas aeruginosa*, as demonstrated by Duddu *et al*., (2015); Albertsson *et al.,* (1995), and Yamada-Onodera *et al.*, (2001) found that the nonionic surfactant Triton X-100 enhanced *Penicllium simplicissimum* growth in a medium without the utilization of polyethylene by the fungus.

The results of the current investigation showed that both mineral salt media with glucose as a carbon source and media containing polyethylene as a carbon source allowed the microorganisms to produce biosurfactants. The bacterial isolates produced positive findings throughout the initial and secondary screening processes. Fig. 3.1.a. shows the production of glycolipids presence whereas the fungal isolate did not show the orange color which is not a positive resut for glycolipids. Fig.3.1.b. shows the positive result by producing the violet color indicates biosurfactant presence. In Fig. 3.1.c. presence of biosurfactants is indicated by the yellow precipitate, of the bacterial isolate. BAP is an accurate method to find the anionic surfactants, the isolates showed the blue zone around its colony which is a positive result in the Fig. 3.1.f. in the screening of the biosurfactants the bacterial isolates produced biosurfactants by showing the clear zone around its colony which indicates the positive test for biosurfactant production. Biosurfactants emulsification index with bacteria using Tween 20- 98% followed by various hydrocarbons such as Olive oil 20%, Benzene 27%, Petrol / Gasoline 64%. Kavitha *et al.,* 2021, reported emulsification indices with hexadecane (16.66%), diesel (33.33%), kerosene (12%) and sesame seed oil (40%). Presence of biosurfactants confirmed in penetration assay as the hydrophilic liquid was able to break through the oil film barrier into paste. Singh & Sedhuram (2015), reported the formation of the white precipitate in 15 min as the upper phase changed from red precipitate. Table 3.1. shows the weight of extracted biosurfactant. Thin layer chromatography

was performed to characterize the extracted biosurfactant (Fig. 3.3.) all the identified bacterial isolates exhibited brown spots on the TLC plates, signifying the formation of glycolipids. In comparison to utilizing bacteria alone, supplementing with biosurfactants increased the rate of LDPE degradation in all tested organisms. From the findings, it is confirmed that the reduction in the polyethylene degradation by biosurfactants was slightly greater than the weight reduction by microbes alone in an UV treated polyethylene, there is a significant weight reduction in LDPE strips (Fig.3.4.). *Alcligenes feacalis* showed highest % weight loss when microbes were used alone along with biosurfactants augmentation (13.33% & 22.22%) these results were compared to Nnaji *et al*., 2021 and Harshavardhan *et al*., 2013.All species had seen an improvement in biodegradation efficiency of at least 1.2%, with the exception of Strain 2, where there was no apparent weight loss in the LDPE strip. When using only organisms or when supplemented with biosurfactants, Strain 1 experienced the greatest weight loss (1.9% and 3.3%, respectively), while *B. subtilis* experienced the least weight loss (1.4% when using only microbes and 2.6% when using biosurfactants) but experienced the greatest fold increase (1.86). These outcomes are close to those reported in Harshavardhan *et al*., 2013, when 1.75 & 0.06% weight loss of LDPE film was used.

3.7.Conclusion

Biosurfactants are less noxious, environmental friendly, recyclable, biosurfactant production is massively advantageous and competitive. Biosurfactants are widely use in the pharmaceutical, grease, paints, gasoline, food industry and polymeric deterioration. The isolated microorganisms yielded potential biosurfactants further they were chosen based on primary and secondary screening like the hemolysis method, drop collapse, Oil spread or oil displacement and emulsion index method, these were the procedures confirmed the synthesis of biosurfactants by the

microbes. Biodegradation of LDPE a synthetic polymer was encouraged by supplementing with the biosurfactants in additional to the use of microorganisms which also yielded the surfactants. Recognition of suitable organisms isolated from the dumpsite soil produced biosurfactant using polyethylene as carbon source.

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