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BIODEGRADATION OF LOW-DENSITY POLYETHYLENE (LDPE) BY HALOPHILIC BACTERIA ISOLATED FROM BAY OF BENGAL WATER.

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ABSTRACT

A saltpan is an extreme environment, inhabited by organisms that survive in very high salinity withstands temperature and severe solar radiationhave been isolated from halophilic bacteria various hypersaline conditions such as salt lakes, natural and artificial solar saltpans. Currently in research, a total of two overlying saltpan water samples were collected from Digha West Bengal, from which 2 distinct halophilic bacterial isolates were found. Its optimization the growth parameters of the isolated halophilic bacteria were carried out to determine the optimal NaCl, temperature, pH and LDPE source required for their growth. Optimum NaCl, temperature, pH, and the LDPE source required for their growth is as follows: 20%, 40°C, pH 9-10 and 0.5% LDPE source of the 2 isolates tested, only two showed some measure of hydrophobicity. Clear zone assay was performed to detect the biodegradation of LDPE (Low Density Polyethylene). Halophilic bacteria. 2/3 (66%) of the 2 isolates showed clearance around the colony their potential to degrade LDPE. Synthetic is one of the most common types the polymer found in solid waste is low density polyethylene (LDPE). LDPE is one of the most inert plastic materials, a characteristic which is mainly contributed by its high molecular weight, three-dimensional structure and hydrophobicity as evidenced by various long-term degradation studies (Hadad et al. 2005). Polymers are used by biodegradation microorganisms as a source of carbon and energy for them growth (Albertson et al. 1997). Microbial degradation is involved using extracellular and intracellular depolymerase organisms break down organic matter. Exoenzymes from microorganisms induce a chain cleavage of the polymer and that can be absorbed to produce oligomers and monomers used for microbial metabolism. Most germs that polyethylenedemonstrated its ability to degrade identified as bacterial species belonging to genera pseudomonas, Streptococcus, Staphylococcus, Micrococcus, and the fungal species Aspergillus and Trichoderma.

Key Word: Degradation of polythene, low density polythene, plastic degradation bacteria, Gram staining, Biochemical test.

INTRODUCTION

Widely distributed in hypersaline environments earth's continents where they exist as natural waters bodies such as permanent saline lakes or artificially created solar salt (Litchfield 2002). Bacteria grow in them environments known as halophilic bacteria. Halophilic archaea are characterized as competent organisms growing from about 8% (1.5 M) sodium chloride (NaCl). of approximately 36% (5 M) NaCl, for which the conch NaCl They are chemoorganotrophic, using amino acids or carbohydrates as carbon sources and occur ubiquitously in nature where salt concentrations are high (Grant 1989, Thongthai 1992). Plastics are synthetic polymers or man-made polymer. Commonly used is plastic polyethylene (LDPE, MDPE, HDPE and LLDPE), polyethylene terephthalate (PET), and polybutylene terephthalate (PBT), nylon, polypropylene (PP), polysty rainy (PS), Polyvinyl Chloride

(PVC), and Polyurethane (PUR). They are synthetic polymers that accumulates in the environment due to absence of skills procedures for safe disposal and a cumulative inventory environmental threats to flora and fauna. A major start to facilitate isolation and subsequent degradation LDPE is directly degraded by microorganisms using polymers as the sole carbon sources(Roy 2008). Several previous studies have reported on biodegradation degradation of polyethylene by bacterial and fungal species (Cathyresan 2003, Kim 2003). The present study was therefore undertaken to the isolation and identification of halophilic bacteria from saltpans their ability to induce biodegradation of LDPE (less density polyethylene).

Plastic is a ubiquitously used polymer and is a wonderful invention of modern science which is included as one of the essentials in the lifestyle of development of human civilization commodity polyethylene is classified into three groups according to their density as low density Polyethylene (LDPE), Linear Low Density PE (LLDPE) and High Density Polyethylene (HDPE). Per Polyethylene group is used ubiquitously but LDPE is used due to extremely minimal weight and it can easily withstand a lot of pulling before breaking completely. Today's people can think nothing but plastic carry bags are used for light to medium weight items because a plastic the weight of the carry bag is very low which is negligible compared to other products. Not plastic it is naturally degradable and does not absorb any liquid so it is widely used in wrapping, preventing the flow of any liquid etc. LDPE is widely used in plastic carry bags, squeezes bottles, wire and cable insulation, wash bottles, various laboratory instruments, food storage containers, etc. But this amazing discovery of science is a big environmental threat to the world because LDPE the cause of pollution is scattered here and there as garbage. Polythene bags, wrappers can prevent water ingress drains which create unsanitary environment, are accidentally eaten by herbivores while grazing, large aquatic animals such as various types of fish, invertebrates etc. mistakenly eat plastic as their own hunting and plastics in every case pose their own metabolic hazards because no animal has a metabolic system plastic degrading enzymes. LDPE, also known as Petroleum Plastic chain which makes it complex in structure. If plastic is burned to destroy it, many toxic gases are released because it is a carbon rich compound. Hence biological degradation methods are very cost effective and the role of halophile is very effective in this environment friendly. Halophilic bacteria are also readily available hyper salinity aquatic environments that use plastic as a source of carbon in their metabolic systems plastic degrades LDPE with enzymes that are synthesized through their metabolic pathways. Halophilic bacteria belong to the group of extremophiles that prefer to live in environments where salt density is too high. 2/3 of it is covered by oceans and salt lakes where halophile survive and if the salt concentration somehow becomes low, it becomes difficult for them to grow and carbon accumulation is essential for the growth and development of developmental bacteria Provides energy for metabolism. Polyethylene is an inert material with very strong hydrocarbon bonds and stable so it is not affected by any natural factors and remains for a long time unmodified halophiles use polyethylene as a major carbon source for monomeric and oligomer units. In their metabolic pathway they synthesize hydrolysing enzymes that initiate the depolymerisation reaction and work from the surface to the core and break down the entire structure. In case Hydrophilic bacteria they can bind to both hydrophilic and hydrophobic surfaces but in the case of hydrophobic bacteria they can only bind to hydrophobic surfaces. There are several halophilic bacteria which can easily degrade LDPE as Bacillus cruluoechia, Bacillus pseudoformus, Prolinoborus fasciculus, Nesiotobacterexalbescens, Bacillus vietnamensis, Halobacteriumsalinarium, Halobacillussalinus, Vibrio Fishery, Aeromonas spp., Staphylococcus epidermidis etc. LDPE has a hydrophobic surface and there many halophiles are hydrophobic in nature, so halophiles can bind very tightly to LDPE surfaces and forms a biofilm on it that begins to degrade in about 50 to 60 days.

Plastics are long-chain synthetic polymers used in a variety of applications fields as paper and other cellulose-based substitute’s products for packaging due to their bioactivity and excellent moisture barrier properties (Andrady 2011). Annually worldwide the demand for plastics has steadily increased

over the years and currently stands at around 245 million tonnes. Unfortunately, the plastic waste accumulates in the environment pollution, reduces soil fertility, reduces water circulation the ability of plants to threaten animal life by ingesting plastic, and releases harmful chemicals that lead to health problems (primula and Ramesh 2015). This negative effect is clearly visible since plastic is resistant to natural processes of degradation. LDPE is one of the most inert plastic materials, a characteristic which is mainly contributed by its high molecular weight, three-dimensional structure and hydrophobicity as evidenced by various long-term degradation studies (Hadad et al. 2005). Other study, no signs of biodegradation were observed polythene sheets have been covered in moist soil for 12 years. More, Polyethylene films have been poured into the soil for 32 years partial degradation. Some biodegradation studies, however, have reportedthermal-potentials Pre-treatment based on increasing its biodegradation rate in Long-Term Studies on Polyethylene Biodegradation Conducted in soil, UV-irradiated 14C-labeled polyethylene sample, after 10 <0.5% carbon dioxide by weight is released consistent with the inert nature of the year, the non-radiative 14C- Labeled polythene sheets are sprinkled on the ground for the same length time evolved <0.2% carbon by weight. It has been suggested that UV exposure and heat treatment generates macro radicals in the amorphous area of polyethylene film and it is through a series of reaction, these free radicals will then be converted to carbonyls groups that can be used in degradation by microorganism's process.

Material and Method:
Sample collection: Here Sea water of costal area was used as a source of halophilic bacteria from Digha, West Bengal, India.

Collection of substrates: Low density polyethylene (LDPE) was collected in the form of plastic carry bag from the local market of Tarakesware, Hooghly.

Optimization of growth parameters: By determining the temperature, ph, NaCl concentration and LDPE source the optimization of growth parameters was done because all of them are required as important parameters for the growth of halophilic bacteria.

NaCl concentration: Five plates of nutrient agar media with salt concentrations were prepared 10%, 15%, 20%, 25% and 30% respectively where bacterial isolates were inoculated and incubated at 40°C for 24 hours and observed

Temperature: 150 mL of seawater was taken as 50 mL in three sterile conical flasks 20% salt concentration and then incubated at different temperatures of 30°C, 37°C and 40°C. 24 hours and their growth curves were observed using UV-VIS spectrophotometer. It will help determine their optimum temperature for growth.

pH: 150 mL of seawater was taken in a sterile conical flask with a salt concentration of 20% and incubated at 40% for 24 hours. Here Na2CO3 is mixed to give three different ph as 8, 9 and 10 to adjust the pH value. The growth curve was observed using UV-VIS spectrophotometer.

LDPE as a carbon source: Wherein 150ml sea water was taken in sterile conical flask the concentration was kept as 20% and incubated at 40°C for 24 hours. Here is the density of LDPE 0.5%, 1% and 2% were kept which will help in determining the best source of carbon growth pf halophilic bacteria. The growth curve was observed using UV-VIS spectrophotometer.

Preparation of LDPE powder: The collected samples were cut into 1cm2 shape and boil with xylene for 10 to 15 min and then the boiled pieces were mixed at 3,000 rpm for 10 min. LDPE powder by the

way. It was extracted and washed with 70% alcohol to remove xylene. Then keep it open in a container from 2 to 3 h for evaporation of ethanol and then dried overnight in a hot air oven at 62 °C and stored room temperature in a closed container. Now the substrate is ready for testing.

Enrichment of the culture: Mineral salt agar media for enrichment of halophiles culture 300 ml prepared where separately autoclaved Na2CO3 was added to maintain the medium at pH 10 which was cooled at room temperature before addition. Then 300mg LDPE powder was added to the broth medium. The sample was mixed well with 10 ml of seawater and left for incubation at 40°C for 5 days on a shaker. After 5 days of incubator incubation, 1 ml samples were taken from the culture and serially diluted up to 10-9 dilution.

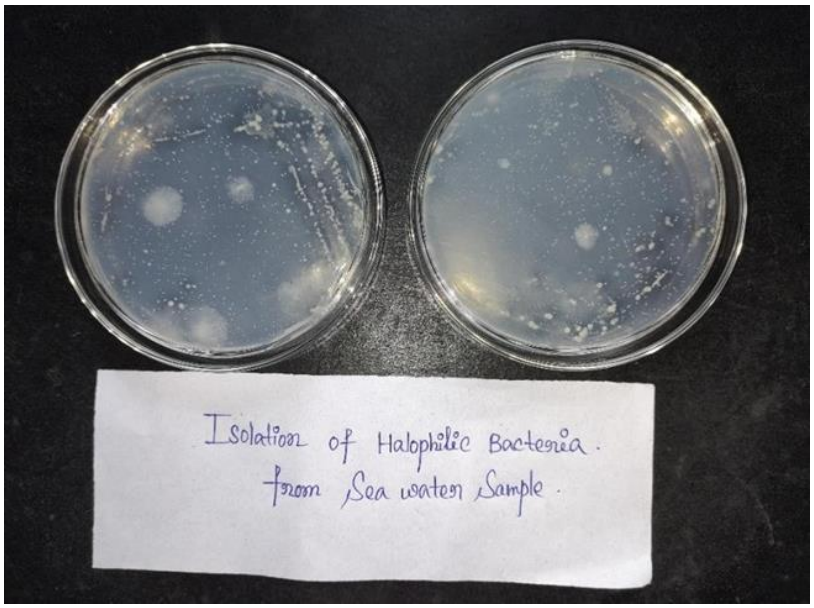

Figure 1: Isolation of Halophilic Bacteria from Sea water sample

Isolation of halophilic bacteria: Culture samples from 10-9 dilution were inoculated by a loopful spread the plate technique through minimal salt agar and incubate at 40°C for 24 to 48 hours. Next colonies present on the day were subjected to gram staining and mobilisation was a colony observed for pigmentation and isolates were characterized by standard biochemical methods catalase test, oxidase test. Purified cultures were stored in 40% glycerol stock solution at -40°C.

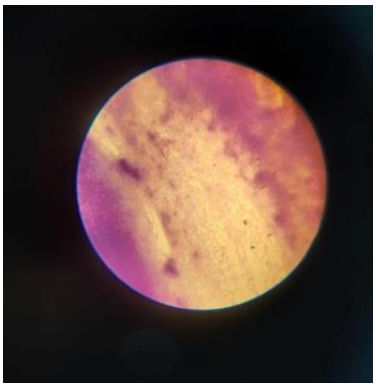

Figure 2: Gram staining.

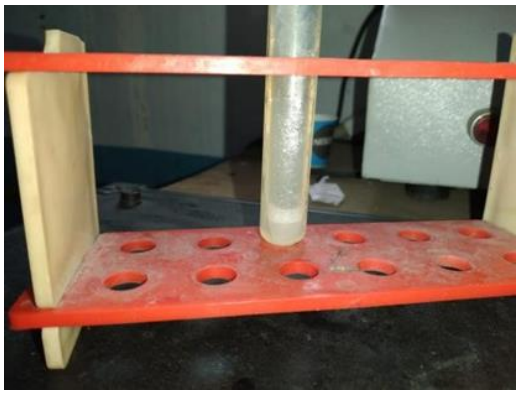

Figure 3: Catalase test

Identification of LDPE degrading halophiles by 16s rRNA sequencing and Phylogenetic Analysis:

Screening of extracellular Hydrolytic Enzymes:

Amylase activity: Bacterial cultures were inoculated onto starch agar medium containing 20% (w/v). In total salt and incubated for 24 h at 40 °C. After incubation the plates were flooded with iodine monitoring for solutions and clearance zones. Clearance indicated amylase activity.

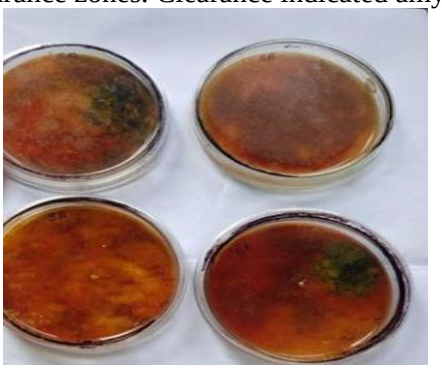

Figure 4: Amylase

Protease activity: Bacterial cultures were inoculated onto gelation agar medium and incubated at 40°C, after incubation for 24 h the plates were flooded with mercuric chloride. Isolates showing its zone clearance after treatment with mercuric chloride was considered positive for protease producing bacteria.

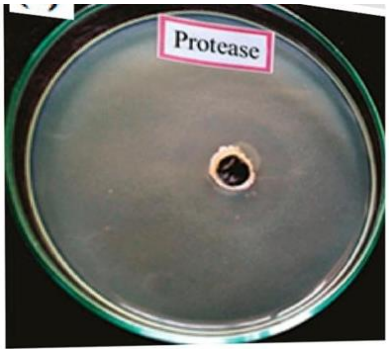

Figure 5: Protease

Lipase activity: Bacterial cultures were inoculated onto tributary in agar medium and plated incubated for 24 h at 40 °C. Isolates that showed clear zones of hydrolysis were considered lipase bacterial production.

Figure 6: Lipase

Screening for biodegradation of LDPE by Halophilic bacteria:
Colonization studies of LDPE: LDPE sheets were cut into small pieces corresponding to 1 cm X 1 cm weight, sterilized with 70% ethanol for 30 min and transferred to sterile distilled water for 20 minutes then dry in a laminar air flow chamber for 15 minutes. Dry sheets were treated Mineral oil for easy attachment of bacteria. Then there was LDPE sheet of the same weight placed in a conical flask containing 300ml of sterile seawater. It was inoculated with 1ml of bacteria 24 hour culture. These were incubated at 40°C and the results were observed after 1 week 10 days.

Dry weight estimation: To facilitate accurate measurement of residual weight Polyethylene, polyethylene sheets were taken for degradation after 60 days of incubation and the bacterial biofilm from the polymer surface was washed with 2% (v/v) aqueous sodium dodecyl sulfate solution, a surfactant that denatures and thoroughly washes the cells surface (using shaker) for 4 hours, followed by distilled water. Then add chloroform again SDS gel removes mineral oil. Then finally wash with 70% ethanol to ensure the maximum possible remove cells and debris. The cleaned polymer pieces were placed on a filter paper and dried overnight at room temperature before weighing. Weight loss was calculated by this formula.

Weight loss (%) = initial wt-final wt X 100 / initial wt.

SEM analysis of LDPE sheets: After a period of 60 days there were untreated and treated samples after repeated washing with 2% (v/v) aqueous SDS and distilled water, subjected to SEM analysis wash with 70% ethanol to remove excess by gentle shaking for several minutes cells to expose maximum surface area for visualization. is pasted onto the sample SEM sample stubs using a carbon tape and samples were analyzed under high resolution scanning Electron - microscope.

Cell surface hydrophobicity: The hydrophobicity of bacterial cells BATH (Bacterial adhesion to hydrocarbons) was tested where bacterial culture was performed on mineral salt agar after the broth and incubation period for 24 h, this broth culture was centrifuged at 10,000 rpm for 10 min. and then wash twice with phosphate-urea-magnesium (PUM) buffer. Then discarding Supernatant pellets were suspended in PUM buffer. Its absorbance was then measured 400 nm. 0.3 ml of hexadecane was

added and shaken for 20 min. The test tubes were kept intact for 5 min, which forms two phases i.e., organic and aqueous phase. Aqueous layer adsorption measurements were made at 400 nm by UV-VIS spectrophotometer. Culture-free buffer was used as a blank.

Clear zone assay: Mineral salt agar media for the clear zone assay was prepared by mixing LDPE Powder with a concentration of 0.5%. Then sonicated for 2h and sterilized at 121°C for 20min and pressure of 15lb/in2. Then allowed to be plated and solidified. Then pre-preparation Halophilic bacterial cultures were inoculated and left for incubation at 40°C. Plate after 7 days the comais was flooded with blue solution and left in that position for 30 minutes. Then treatment is done with 10% acetone for decolonization. Clear areas were visible after colonization indicates biodegradation of LDPE by halophiles.

RESULT

LDPE Degradation: For this test sea water samples were collected from Digha coastal area of Bay of Bengal Hydrophobicity was tested to reveal that three bacterial isolates gave positive reactions for LDPE Biodegradation assay.

Colony morphology:

Table 1: Colony morphology of the Halophilic Bacterial Isolates; W: Width, L: Length

Sr. No.	Name of isolates	Shape	Size (in micrometer)	Colour	Gram Nature
1	Enterobacter soli strain MP2.	Rod	W- 0.5-0.9	Yellow	Negative
			L- 1-2		
2	Enterobacterasburiae strain.	Rod	W-1	Fuzzy yellow/ slight yellow	Positive
			W-2		

Optimization of growth parameters: NaCl concentration: Three isolates were cultured on nutrient agar media containing five different NaCl concentrations as 10%, 15%, 20%, 25% and 30% with 20% concentration shown maximum colonization of halophiles.

Temperature: To determine the optimum growth temperature of halophile, 150 ml of sea water were taken in three sterile conical flasks with 20% salt concentration (named F1 and F2 and F3. Containing 50 ml of water and they were incubated at three different temperatures as 30°C. 37°C and 40°C for 24 h. After 24 hours their growth curve shows results in UV-VIS spectrophotometer and results suggest that 40°C is the optimum temperature for proper growth of halophiles.

pH: Three sample solutions as F1, F2 and F3 were prepared in the same temperature method determination and Na2CO3 were added to adjust the pH to 8,9 and 10 in F1, F2 respectively and F3 solution. And growth curve showed results in UV-VIS spectrophotometer.

LDPE as a carbon source: Polyethylene is a huge source of carbon needed by halophiles they degrade the LDPE film. Pre-sterilized and preserved LDPE powder is prepared for this test F1, F2 and F3 were mixed in tubes at 0.5%, 1% and 2% respectively and their growth curves observations were made using UV-VIS spectrophotometer.

Screening for biodegradation of LDPE by halophilic bacteria:

Colonization study: Halophilic bacteria are attached to LDPE sheets by adding mineral oil and then they created biofilms by which halophilic bacteria used these LDPE sheets as a source carbon which is an essential factor for the growth of bacteria is involved in production power. After 10 days of testing the sheets were released from the media and contained abundant bacteria was observed.

Dry weight estimation: Low density polyethylene is a hydrocarbon compound that is the backbone made of carbon. So since halophilic bacteria use carbon, LDPE sheets must lose them weight.

Table 2: Dry Weight Estimation of LDPE Degrading Halophilic Bacterial Sample

Sr. No.	Bacterial Sample	Initial Weight	Final Weight	Weight less
1	Enterobacter soli strain MP2.	1.5 g	0.23g	84.67
2	Enterobacterasburiae strain	1.5 g	0.18g	88

SEM analysis of LDPE sheets: Three isolates of halophilic bacteria (Enterobacter soli strain MP2, Enterobacterasburiae strain) showed positive results in colony studies indicating their affinity to LDPE sheets. After 60 days of incubation the LDPE sheets were observed under scanning electron microscope at high magnification. Resolution power and results showed that halophiles treated LDPE sheets got many pits and control LDPE sheet has fresh surface where cracked.

Table 3: OD value of LDPE degrading bacterial isolates; HSB- Halophilic bacterial sample

Sr. No.	Name of isolates	OD value before adding hexadecane	OD value after adding hexadecane
1	Enterobacter soli strain MP2.	0.18	0.9
2	Enterobacterasburiae strain.	0.35	0.16

Clear zone assay: After washing the LDPE sheets, they were flooded with coomasie blue solution observed under SEM and many clear zones were detected indicating its biodegradation LDPE by halophiles.

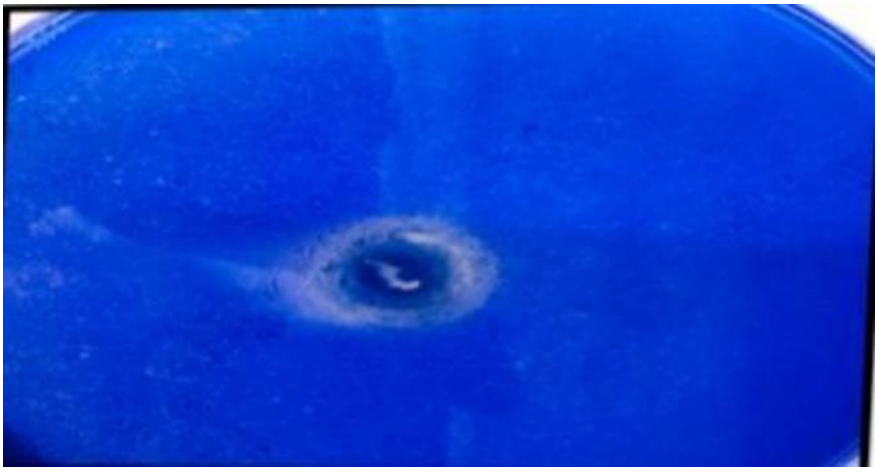

Figure 7: Clear zone assay

CONCLUSION

Low density polyethylene is very essential in daily life as well as it is very expert in causing pollution, a lot of water pollution. This is why its prevention is urgently needed and is possible only through biodegradation procedures otherwise preventing LDPE from forming, other processes can cause various types of contamination pollution. Halophilic bacteria are always available which can show effective and beneficial means in this regard case every biological process is carried out by the influence of different enzymes, proteinases compounds that catalyze the entire reaction remain unchanged at the end of the reaction. When Halophilic bacteria are inoculated into medium containing LDPE, where they begin to form biofilms use LDPE as the carbon source because polyethylene is a polymeric form of carbon that acts as a substrate.Their metabolic pathway is where it is broken down to provide amino acids and various other pools compounds that work together to form whole cells. When halophilic bacteria are inoculated in a medium containing small pieces of LDPE, they begin to form biofilms and the layer increases over time the biofilm thickens as more bacteria colonize and later for quorum sensing activity a period of about 60 days shows by SEM analysis that the surface of the LDPE sheet has become rough, full of holes, cracks and their dry weight is also reduced which is determined by carrying out dry weight assumptions that indicate its degradation. Bacterial colonization is confirmed by their execution colonization study LDPE which shows an increased weight of LDPE sheet. This is because after colonization Halophilic bacteria as biofilms synthesize various extracellular hydrolytic enzymes such as amylase, Protease, lipase etc. which degrade LPDE sheet help in carbon uptake. In the initial stage oxidation of the polymeric chain takes place leading to the formation of carbonyl groups and they are finally converted to carboxylic group which is then involved in β-oxidation. Then finally it involved in the citric acid cycle and releases CO2 and H2O which ultimately degrades LDPE. These complete reactions are catalyzed by several enzymes produced by halophilic bacteria.From these above experiments it can be concluded that halophilic bacteria are a good and beneficial source of hydrolytic enzymes. Both moderately halophilic bacteria and extreme halophilic bacteria are very beneficial moderately halophilic bacteria are of greater importance as a source of industrially required enzymes extreme halophilic bacteria cause extreme halophilic bacteria to grow in an extreme environment to be made which is very time saving and extreme condition in industrial production very rare made. There is no place on earth that can be found germ free. So germs community art can undoubtedly be used in the field. Amylase in industrial production.

FUTURE ASPECTS

As the prevalence of antimicrobial resistance increases, researchers are developing strategies to find new technologies and alternatives that reduce morbidity and mortality caused by disease MDR bacteria. Integrating present and future applications of natural product discovery multi-omics approach. Depending on the stage of the research, it predicts genomics, metagenomics, Transcriptomic, proteomics and metabolomics to reveal the biosynthetic capacity of a single organism Microorganisms or microbial communities in hypersaline environments. Most of what we use in daily life these days is artificial or artificial. All in all we know we have carelessly forgotten the effects of using these artificial products. Fresh and organic food is consumed of course, artificial colors can cause serious diseases like cancer. Its halophilic origin pigments can be used industrially for food coloring agents to be a good step towards health objectives being mentally and physically healthy is very important.

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