2.1 Material:

Polyamide is a polymer, which contains recurring amide groups (R-CO-NH-R') as integral part of the main polymer chain. Synthetic polyamides are produced by a condensation reaction between monomers, in which the linkage of the molecules occurs through the formation of the amide groups.

The type of plastic used is polyamides (nylons) as follows;

1. Nylon 6
2. Nylon 6, 6

NYLON 6

Nylon 6 or polycaprolactam is a polymer developed by Paul Schlack at IG Farben. Unlike most other nylons, Nylon 6 is formed by ring-opening polymerization. It is a semi-crystalline polyamide. Caprolactam molecule used to synthesize Nylon 6 by ring opening polymerization.

Fig. 5. Structure of Caprolactam.

Synthesis:

Nylon 6 begins as pure caprolactam. As caprolactam has 6 carbon atoms, when caprolactam is heated at about 533 K in an inert atmosphere of nitrogen for about 4-5 hours, the ring breaks and undergoes polymerization. Then the molten mass is passed through spinnerets to form fibers of Nylon 6.
Fig.6. Formation of Nylon 6 by Polymerization process.

During polymerization, the peptide bond within each caprolactam molecule is broken, with the active groups on each side re-forming two new bonds as the monomer becomes part of the polymer backbone.

**Properties:**

Nylon 6 fibers are tough, possessing high tensile strength, as well as elasticity and luster. They are wrinkle-proof and highly resistant to abrasion and chemicals such as acids and alkalis. The fibers can absorb up to 2.4 % of water, although this lowers tensile strength.

**Applications:**

Nylon 6 is used as thread in bristles for toothbrushes, surgical sutures, and strings for acoustic and classical musical instruments, including guitars, violins, violas, and cellos. It is also used in the manufacture of a large variety of threads, ropes, filaments, nets, and tire cords, as well as hosiery and knitted garments. It can also be used in gun frames.

**Nylon 6, 6:**

Nylon 6, 6 is a type of nylon comes in many types; the two most common for textile and plastics industries are Nylon 6 and Nylon 6, 6.

**Composition:**

Nylon 6, 6 is made of hexamethylened diamine and adipic acid, which give Nylon 6, 6 a total of 12 carbon atoms, and its name.
Unlike Nylon 6, 6, in which the direction of the amide bond reverses at each bond, all Nylon 6 amide bonds lie in the same direction. This difference has little effect on the polymer's mechanical or chemical properties, but is sufficient to create a legal distinction.

**Physical properties:**

1. Nylon 6, 6 has a melting point of 265 °C, high for a synthetic fiber. This fact makes it resistant to heat and friction and enables it to withstand heat setting for twist retention.

2. Its long molecular chain results in more sites for hydrogen bonds, creating chemical “springs”, making it very resilient.

3. It has a dense structure with small, evenly spaced pores. This means that Nylon 6, 6 is difficult to dye, but once dyed it has superior colorfastness and is less susceptible to fading from sunlight and ozone and to yellowing from nitrous oxide.

**Synthesis:**

1. Hexamethylene diamine and adipic acid are combined with water in a reactor. This produces nylon salt. The nylon salt is then sent to an evaporator where excess water is removed.

2. The nylon salt goes into a reaction vessel where a continuous polymerization process takes place. This chemical process makes molten Nylon 6,6.

3. The molten Nylon 6, 6 undergoes a spinning process, where the Nylon 6,6 is extruded and sent through a spinneret, which is a small metal plate with fine holes. The nylon is then air-cooled to form filaments.
4. Nylon's chemical formula is \( n\text{HOOC-}(\text{CH}_2)_4\text{-COOH}+n\ \text{H}_2\text{N-}(\text{CH}_2)_6\text{-NH}_2----> [-OC-}(\text{CH}_2)_4\text{-CO-NH-}(\text{CH}_2)_6\text{-NH-}] \ n+2n\text{H}_2\text{O} \) and the part -CO-NH- will stick together becoming Nylon 6, 6.

**Applications:**

Nylon 6, 6 is mainly used in making Carpet fiber, Apparel, Airbags, Tires, Ropes, Conveyor Belts and Hoses. Nylon 6, 6's longer molecular chain and denser structure qualifies it as a premium nylon fiber, specified most often by professional architects and designers for use in commercial settings like offices, airports, and other places that get a lot of wear and tear. It is also an excellent choice for residential carpet applications.

**2.1.1 Collection of Polymer Sample:**

For experimental purpose the Nylon 6 and Nylon 6, 6 samples were purchased from Sigma Aldrich Company. The sample used was in the form of plastic pellets.

**2.1.2 Soil Sample Collection for the Isolation of Polyamide Degrading Microbes:**

Sample of soil was collected from the site such that waste of various types such as plastic bags, plastic covers, plastic bucket, manure of all animals, fossils, waste of plants, and other domestic waste etc. are dumped or long duration.

Soil collected from three sites from Kolhapur city namely;

1. **Kasba Bavda, Kolhapur (B1):**

This is the site, where, the wastes of various types including plastic were dumped for years. This site is more contaminated with plastic bags. Dry soil sample was collected from this place as land and soil collected named as ‘B1’.

2. **Kadamwadi area, Kolhapur (K1):**

The soil sample taken from open running drainage water, in which, the plastic bags are trapped on the walls of the open channel flow. The plastic bags carrying moist soil inside, is used as sample named as ‘K1’.
Fig. 8. $B_1$ soil sample collection area.

Fig. 9. $K_1$ soil sample collection area
3. Kadamwadi Road, Kolhapur (K$_2$):

The dry soil sample collected from the same site near Open Channel Flow. The soil from this area is more contaminated with plastic bags and threads. The land is digger for about 10cm and soil is collected named as ‘K$_2$’.

2.1.3 Microorganisms used for the biodegradation study:

1. *Phanerochaete chrysosporium:*

Description and significance:

*Phanerochaete chrysosporium* is a model white rot fungus because of its specialized ability to degrade the abundant aromatic polymer lignin, while leaving the white cellulose nearly untouched. *Phanerochaete chrysosporium* releases extracellular enzymes to break-up the complex three-dimensional structure of lignin into components that can be utilized by its metabolism. The extracellular enzymes are non-specific oxidizing agents (hydrogen peroxide, hydroxyl radicals) used to cleave the lignin bonds.

Due to *Phanerochaete chrysosporium* specialized degradation abilities, extensive research to enhance the bioremediation of a diverse range of pollutants. Therefore, *Phanerochaete chrysosporium* is the first member of the Basidiomycetes to have its complete genome sequenced.

Cell structure and metabolism:

*Phanerochaete chrysosporium* is a crust fungus, which forms flat fused reproductive fruiting bodies instead of the mushroom structure. This fungi exhibit a septet hyphae, giving a stronger line of defense in times of distress. The hyphae network has some branching, with diameters ranging from 3-9 μm. At the ends of the hyphae rests chlamydospores, thick-walled spores varying from 50-60 μm. The conidiophores gives rise to round asexual blast conidia, which are 6-9 μm in diameter.

Degradation of lignin and pollutants is made possible by the production of extracellular enzymes. Components such as lignin peroxidase and manganese peroxidase take part in the remediation of various pesticides, polyaromatic hydrocarbons, PCBs, TNT, carbon tetrachloride and various poisons.
Ecology:

Recent studies have revealed an association of a certain bacteria found in conjunction with this strain of fungi. *Agrobacterium radiobacter* was isolated as coexisting with the fungi, and very difficult to separate. Discovery of how bacteria and fungi affect each physiologically is yet to be conclusive, but further research could give further evidence of mutualism, and its effect on bioremediation.

Pathology:

*Phanerochaete chrysosporium* is a saprophytic fungus capable of organic breakdown of the woody part of dead plants. Symptoms may include white patches of cellulose due to the disappearance of lignin from the plant structure. This fungus is not a known pathogen of humans or animals.

Application to Biotechnology:

*Phanerochaete chrysosporium* useful because of its biodegradation of harmful chemicals by means of extracellular enzymes, its ability to leave pure white cellulose has been important in the industry of paper. Bio pulping would remove brown lignin, which this fungus does naturally, in production of paper. Incorporation of this natural alternative would limit the amount of pollution produced by machines and also decrease the amount of chemicals used for the bleaching of paper.

2. *Trametes versicolor*:

Description and significance:

*T. versicolor* also known as *Coriolus versicolor*. *T. versicolor* has a trimitic hyphal system. The vegetative hyphae range in thickness from 3-10 µm and do not possess any cross-walls or clamp connections. Anamorphic manifestations of this species include oidia and chlamydomspores.

Ecology:

*T. versicolor* is found ubiquitously in temperate to sub-tropical forests throughout the world where it serves as a primary decomposer of hardwood. *T. versicolor* causes a de-lignifying soft-rot of wood biopolymers and is therefore functionally characterized among the white-rot basidiomycetes.
Chapter II

Material and Methods

Fig. 10. Lignolytic fungus, *Phanerochaete chrysosporium*

Fig. 11. Fungus, *Trametes versicolor*
**Application in biotechnology:**

*Trametes versicolor* produces a number of enzymes that are involved in the degradation of wood lignocellulose including: sixteen isoforms of lignin peroxidase (LiP), five isoforms of manganese peroxidase (MnP), laccase, carboxymethyl cellulase, etc. The copper trafficking enzymes *tah A* and *Cta A* regulate the production of laccase by controlling the intracellular availability of this co-factor during protein folding. *T. versicolor* secretes oxalic acid to chelate potentially toxic metals and stabilize the Mn(III) used by MnP.

The laccase produced by *T. versicolor* can be used to detoxify a variety of recalcitrant xenobiotics including: polychlorinated biphenyls, textile dyes and polyaromatic hydrocarbons. This enzyme can also be applied to generate or degrade synthetic polymers. The pulp and paper industry has engineered fermentation systems using *T. versicolor* to bio bleach Kraft pulp and to detoxify softwood extractives. *T. versicolor* cultures can synthesize or degrade antibiotics, produce carotenoid derived flavor compounds or bioremediation high molecular weight asphalting components of petroleum.

**3. Pseudomonas aeruginosa:**

It is a Gram negative, aerobic, rod-shaped bacterium with unipolar motility. It is an opportunistic human pathogen; and it is also an opportunistic pathogen of plants

*Pseudomonas aeruginosa* is a common bacterium that can cause disease in animals, including humans. It is found in soil, water, skin flora, and most man-made environments throughout the world. It thrives not only in normal atmospheres, but also in hypoxic atmospheres, and has, thus, colonized many natural and artificial environments. It uses a wide range of organic material for food; in animals, the versatility enables the organism to infect damaged tissues or those with reduced immunity. It is also able to decompose hydrocarbons and has been used to break down tar balls and oil from oil spills. Some members of the genus, *Pseudomonas* are able to metabolize chemical pollutants in the environment, and as a result can be used for bioremediation.
2.1.4 Collection of Fungi sample for experimental study:

The fungal cultures were collected from the National Centre for Industrial Important Microorganisms (NCIM), National Chemical Laboratory, Pune. The cultures of microorganisms were maintained by using Potato Dextrose Agar.

Fungi used for study are as follow:

*Phanerochaete chrysosporium* NCIM 1073, *Phanerochaete chrysosporium* NCIM 1106, *Trametes versicolor* NCIM 1086

Bacteria used for study is *Pseudomonas aeruginosa* NCIM 2242

2.1.5 Instruments used for study:

a) Micrometer:

A micrometer sometimes known as a micrometer screw gauge is a device incorporating a calibrated screw used widely for precise measurement of small distance, along with other metrological instruments such as dial, vernier, and digital calipers. This instrument was used to measure the thickness of plastic sheets.

**Operating principles:**

Micrometers use the principle of a screw to amplify small distances that are too small to measure directly into large rotations of the screw that are big enough to read from a scale. The accuracy of a micrometer derives from the accuracy of the thread from that is at its heart.

b) Inverted Microscope:

An inverted microscope is a microscope with its light source and condenser on the top, above the stage pointing down, while the objectives and turret are below the stage pointing up. It was invented in 1850 by J. Lawrence Smith, a faculty member of Tulane University. The stage on an inverted microscope is usually fixed, and focus is adjusted by moving the objective lens along a vertical axis to bring it closer to or further from the specimen. The focus mechanism typically has a dual concentric knob for coarse and fine adjustment.
Depending on the size of the microscope, four to six objective lenses of different magnifications may be fitted to a rotating turret known as a nosepiece. These microscopes may also be fitted with accessories for fitting still and video cameras, fluorescence illumination, confocal scanning and many other applications. In present study the morphological changes occurred in Nylon 6 and Nylon 6, 6 were observed by using inverted microscope. These plastic sheets were observed under 40X for changes occurred during degradation as change in colour of sheet, change in surface morphology etc.

c) Infrared spectroscopy (IR):

Infrared spectroscopy (IR) is one of the most powerful analytical techniques which offer the possibility of chemical identification. It is one of the most common spectroscopic techniques used by organic and inorganic chemists. It is the absorption measurement of different IR frequencies by a sample positioned in the path of an IR beam. The main goal of IR spectroscopic analysis is to determine the chemical functional groups in the sample. Different functional groups absorb characteristic frequencies of IR radiation. Using various sampling accessories, IR spectrometers can accept a wide range of sample types such as gases, liquids, and solids. Thus, IR spectroscopy is an important and popular tool for structural elucidation and compound identification.

The technique is based upon the simple fact that a chemical substance shows marked selective absorption in the infrared region. After absorption of IR radiations, the molecule of a chemical substance vibrate at many rates of vibration, giving rise to close-packed absorption bands, called an IR absorption spectrum which may extend over a wide wavelength range. Various bands will be present in IR spectrum which will correspond to the characteristics functional groups and bonds present in a chemical substance. Thus an IR spectrum of a chemical substance is a fingerprint for its identification.

Band positions in an infrared spectrum may be expressed conveniently by the wave number \( \bar{\nu} \), whose unit is cm\(^{-1}\). The relation between wave number \( \bar{\nu} \), wavelength \( \lambda \) and frequency \( \nu \) is as follows.
\[ V = \frac{C}{\lambda} \]

OR \( \frac{v}{(cm^{-1})} = \frac{v}{c} = \frac{1}{\lambda} \)

Infrared spectroscopy (IR) is found to be very useful in qualitative and quantitative analysis of chemical substance. The functional group of substance is also analyzed by IR spectroscopy.

In the present study the IR spectroscopy was used to detect chemical changes occurred in Nylon 6 and Nylon 6, 6 during treatment with microorganisms. The chemical changes specially related with the functional group were detected by using IR spectroscopy.

d) Thermo Gravimetric Analysis:

When matter is heated it undergoes certain physical and chemical changes. These physical and chemical changes take place over a wide temperature range. For instance physical changes such as melting or boiling may occur at widely varying temperatures depending on the material involved. Chemical changes such as decomposition or reaction, may also take place at very different temperature.

During the past few years, the methods of thermal analysis have been widely accepted in analytical chemistry. The term ‘thermal analysis’ incorporates those techniques in which some physical parameter of the system is determined and / or recorded as a function of temperature. Based on the above definition, the various techniques of thermal analysis are used to detect the changes in material structure.

However, only Thermo Gravimetric (TG), Differential Analysis (DTA), Thermometric titrations, Differential Scanning Coulometer (DSC), Pyrolysis- Gas Chromatography (Pyrolysis-GC) and Thermo mechanical analysis (TMA) these are the different techniques in the thermogravimetry.

Thermal events are usually studied by recording the change in thermal property as the temperature is varied to give a thermal analysis curve or thermo gram.

Thermogravimetry:

Thermogravimetry (TG) is as a technique whereby the weight of a substance, in an environment heated or cooled at a control rate, is recorded as a function of time or
temperature’. From the above definition it follows that the basic requirements are a method of heating (or cooling) and a means of weighing there are three types of thermogravimetry.

a) Isothermal or static thermogravimetry:- In this technique the sample weight is recorded as a function of time at constant temperature

b) Quasistatic thermogravimetry:- In this technique, the sample is heated to constant weight at each of a series of increasing temperatures

c) Dynamic thermogravimetry: - In this technique, the sample is heated in an environment whose temperature is changing in a pre-determine manner, generally at a linear rate. Most of the studies are generally carried out with dynamic thermogravimetric. Therefore, it is generally referred to as Thermogravimetry.

**Recording of Results:**

The instrument for thermogravimetry is a precision balance programmed for a linear rise of temperature. This instrument is known as thermo balance.

Thermo analytical techniques are used for characterization of glass transition and melting temperatures, thermal stability and other properties as a function of temperature of polymers and fibers. Thermo gravimetric analysis was carried out in a high resolution thermo balance (NETZSCH STA 409 C / CD instrument).

TGA is a technique whereby the weight of a substance in an environment is recorded when the sample is heated or cooled at a controlled rate and change of weight is measured as a function of temperature or time. Approximately five –six mg of sample was heated from room temperature to 1400 °C at a heating rate of 20 °C / min. with a continuous N₂ flow. The sample weight and its rate of weight loss were continuously measured as a function of temperature. The thermal stability of the selected polymeric materials was studied from TGA curves.

DTA involves heating or cooling a test sample and an inert reference under identical conditions, while recording any temperature difference between the sample and reference. This differential temperature is then plotted against time, or against temperature. DTA curves are recorded simultaneously with TGA curves. In the DTA trace, the base line remains unchanged so long as there is no thermal transition in the sample. First – order transitions, namely crystallization ($T_c$) and melting ($T_m$), appear
as peaks in the exothermic and endothermic directions, respectively. After melting the material may undergo decomposition reactions at higher temperatures \(T_d\) which give broad peaks; these may be generally exothermic but are sometimes more complex in shape. The measure transition temperatures \(T_m\) and \(T_d\) were detected from the DTA.

This instrument is found to be more important for analyse the changes occurred in polymer during degradation. As thermal changes observed by melting point and degradation of compound. In present study the changes occurred in Nylon 6 and Nylon 6, 6 during biodegradation treatment was observed by TGA. As the change in structure eof polymer during biodegradation was indicated by temperature change at corresponding weight loss.

**Differential Scanning Calorimetry (DSC):**

Melting behavior of the PA – 6 samples was evaluated by differential scanning calorimetry (DSC) analysis using a Perkin Elmer pyris 1 instrument. A heating rate of 10 \(^\circ\)C min. used in the range from 20 to 250 \(^\circ\)C under nitrogen. Melting temperatures \(T_m\) and enthalpies of fusion \(\Delta H\) were measured during the first heating scan.

**2.2 Methodology:**

**2.2.1 Preparation of Sheets of Nylon 6:**

A sheet of Nylon 6 was prepared from pellets of Nylon 6. Sheet was prepared with the help of two iron blocks in which one iron block was heated on burner and with the help of another iron block the sheets were prepared by hammer at much specified pressure such that Nylon 6 pellet was press from all sides with equal thickness.. The sheets were pressed with constant pressure.

**2.2.2 Preparation of Sheets of Nylon 6, 6:**

A sheet of Nylon 6, 6 was prepared from pellets of Nylon 6, 6. Sheet was prepared with the help of two iron blocks in which one iron block was heated on burner and with the help of another iron block the sheets were prepared by hammer at much specified pressure such that Nylon 6, 6 pellet was press from all sides with equal thickness. The sheets were pressed with constant pressure.
2.2.3 Preparation of Nylon 6 Powder:

A powder of Nylon 6 sheets was prepared for isolation purpose. A mixture made with 3g of cut Nylon 6 films and 3g NaCl respectively was ground for 10 minutes using pestle and mortar. The mixture of ground Nylon 6 film and NaCl was transferred into a cylinder containing distilled water. The floating layer of Nylon 6 particles were collected on filter paper, washed three times with distilled water and dried in an oven at ~60°C overnight. Dried powder of Nylon 6 was then passed through a sieve mesh (No 100) (Daniel Burd, 2008). Nylon 6 pellets from Sigma Aldrich were used. These powders serve as sole source of carbon in growth, culture and final media.

2.2.4 Preparation of Nylon 6, 6 Powder:

A powder of Nylon 6, 6 sheets was also prepared for isolation purpose. A mixture made with 3g of cut Nylon 6, 6 films and 3g NaCl respectively was ground for 10 minutes using pestle and mortar. The mixture of ground Nylon 6, 6 film and NaCl was transferred into a cylinder containing distilled water. The floating layer of Nylon 6, 6 particles were collected on filter paper, washed three times with distilled water and dried in an oven at ~60°C overnight. Dried powder of Nylon 6 and Nylon 6, 6 were then passed through a sieve mesh (No 100) (Daniel Burd, 2008). Nylon 6, 6 pellets from Sigma Aldrich was used. These powders serve as sole source of carbon in growth, culture and final media.

2.2.5 Preparation of Growth and Enrichment Media with Nylon 6:

The rationale behind the enrichment procedure was to create strong selective conditions using powder of Nylon 6 as a sole source of carbon. A growth medium consisting of 0.1% (NH₄)₂SO₄, 0.1% NaNO₃, 0.1% K₂HPO₄, 0.01% KCl, 0.02% MgSO₄ and 0.01% Yeast extract was prepared in one liter of distilled water. The enrichment medium was created by adding 0.2 grams of Nylon 6 to 100 ml of growth medium respectively. This media was autoclaved at 121°C for 15 minutes (Burd, 2008).
2.2.6 Preparation of Growth and Enrichment Media with Nylon 6, 6:

The rationale behind the enrichment procedure was to create strong selective conditions using powder of Nylon 6, 6 as a sole source of carbon. A growth medium consisting of 0.1% (NH₄)₂SO₄, 0.1% NaNO₃, 0.1% K₂HPO₄, 0.01% KCl, 0.02% MgSO₄ and 0.01% Yeast extract was prepare in one liter of distilled water. The enrichment medium was created by adding 0.2 grams of Nylon 6, 6 to 100 ml of growth medium respectively. This media was autoclaved at 121°C for 15 minutes. (Burd, 2008).

2.2.7 Isolation of Nylon 6 degrading microorganisms by enrichment procedure:

A few soil samples were collected at a local landfill in Kolhapur, Maharashtra. The three soil samples collected from three different places and named as ‘B₁’, ‘K₁’ and ‘K₂’; used for both Nylon 6. Each 1 gram of soil sample was added to sterile enrichment flask. This flasks re-named according to the soil samples and Nylon 6.

These flasks are further incubated on shaker incubator at 30°C for four weeks on 100 rpm. After four weeks of incubation, 10 ml suspension was removed and re-inoculated into 100ml of enrichment media. These flasks were kept for incubation under same condition and same duration. After four weeks of incubation, final enrichment culture (FEC) was filtered by filter paper and excess nylon powder was removed (Burd, 2008).

2.2.8 Isolation of Nylon 6, 6 degrading microorganisms by enrichment procedure:

A few soil samples were collected at a local landfill in Kolhapur, Maharashtra. The three soil samples collected from three different places and named as ‘B₁’, ‘K₁’ and ‘K₂’; used for both Nylon 6, 6. Each 1 gram of soil sample was added to sterile enrichment flask. This flasks re-named according to the soil samples and Nylon 6.

These flasks are further incubated on shaker incubator at 30°C for four weeks on 100 rpm. After four weeks of incubation, 10 ml suspension was removed and re-inoculates into 100 ml of enrichment media. These flasks were kept for incubation under same condition and same duration. After four weeks of incubation, final
enrichment culture (FEC) was filtered by filter paper and excess nylon powder was removed (Burd, 2008).

2.2.9 Measurement of ability for Nylon 6 and Nylon 6, 6 degradation:

Nylon 6 degradation was performed in flasks containing 50 ml of growth medium, 10ml of FEC, and weighed sheets of Nylon 6. Flasks were incubated on a shaker for 6 weeks at 30 °C. Control flasks containing 10 ml boiled FEC, to kill all microbes, 50 ml growth medium and weighed sheets of Nylon 6 was incubated under same conditions. Polyamide degradation was determined by measuring the weight loss (%) of the sheets of Nylon 6. After 6 weeks incubation, the average weight loss of sheets in control and sample flasks was checked. (Burd, 2008).

2.2.10 Measurement of ability for Nylon 6 and Nylon 6, 6 degradation:

Nylon 6, 6 degradation was performed in flasks containing 50 ml of growth medium, 10ml of FEC, and weighed sheets of Nylon 6, 6. Flasks were incubated on a shaker for 6 weeks at 30 °C. Control flasks containing 10 ml boiled FEC, to kill all microbes, 50 ml growth medium and weighed sheets of Nylon 6, 6 was incubated under same conditions. Polyamide degradation was determined by measuring the weight loss (%) of the sheets of Nylon 6, 6. After 6 weeks incubation, the average weight loss of sheets in control and sample flasks was checked (Burd, 2008).

2.2.11 Microbial identification:

2.2.11.1 Analysis of Final Enrichment Culture (FEC) with Nylon 6:

FEC was subjected to serial dilution and plating out on culture media in which sole source of carbon is Nylon 6 powders. Culture media with compositions was as such; 1 gram of Nylon 6, 1 gram of (NH₄)H₂PO₄, 0.1 gram of KCl, 0.1 gram of MgSO₄, 7H₂O, 0.1 gram of CaCl₂ for 1 liter of distilled water adjust pH 7.0 and add 6 grams of agar (Burd, 2008).

Culture media was autoclaved at 121°C for 15 minutes. After sterilization, culture media is cooled till 55°C and poured in sterilized Petri-plate. Allow the Petri-plate for cooling and solidification. After plates are solidifying, streak the suspension made by using FEC by serial dilution. These plates are incubated at room temperature
21-28 °C for 1 week. Completion of 1 week, observe the isolated colony, colony number and colony characteristics (Burd, 2008).

2.2.1.2 Analysis of Final Enrichment Culture (FEC) with Nylon 6, 6:

FEC was subjected to serial dilution and plating out on culture media in which sole source of carbon is Nylon 6, 6 powders. Culture media with compositions was as such; 1 gram of Nylon 6,6 powder, 1 gram of (NH₄)H₂PO₄, 0.1 gram of KCl, 0.1 gram of MgSO₄·7H₂O, 0.1 gram of CaCl₂ for 1 liter of distilled water adjust pH 7.0 and add 6 grams of agar (Burd, 2008).

Culture media was autoclaved at 121°C for 15 minutes. After sterilization, culture media is cooled till 55°C and poured in sterilized Petri-plate. Allow the Petri-plate for cooling and solidification. After plates are solidifying, streak the suspension made by using FEC by serial dilution. These plates are incubated at room temperature 21-28 °C for 1 week. Completion of 1 week, observe the isolated colony, colony number and colony characteristics (Burd, 2008).

2.2.1.3 Purification of Microbial Strain from FEC:

Colony was picked up by wire loop, re-suspended in 0.2 ml of 0.85% NaCl and streaked onto the same culture media under sterile condition. To separate fungal and bacterial strain, this suspension is streaked onto nutrient agar for bacteria and potato dextrose agar for fungi plate at a time. Incubate for 24 hours and observe the growth. Thus, colony was picked up and again suspended on culture media containing Nylon 6 and the media containing Nylon 6, 6 as a sole source of carbon. Separate strain of fungi and bacteria is preserved on same media slants (Burd, 2008).

Purified strain was send to National Culture of Cell Science (NCCS), Pune for DNA sequencing and bacterial and fungal identification.

2.2.1.4 Microscopic and Macroscopic Identification of Microbial Strain:

The microbial strains were characterized according to their phenotype, based on colony morphology on culture media. Bacterial cell morphology was observed under microscope after gram staining and motility of strain (Bergey’s Manual of Determinative Bacteriology, 2000).
Gram staining is used to determine Gram status to classify bacteria broadly. It is based on the composition of their cell wall. Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50-90% of cell wall), which stains purple while Gram-negative bacteria have a thinner layer (10% of cell wall), which stains pink. Gram staining uses crystal violet to stain cell walls, iodine (as a mordant), and safranin (counter stain) to mark all bacteria.

2.2.1.5 Identification procedure carried out in National Centre of cell Sciences (NCCS), Pune:

Isolated microbial cultures were sent to NCCS laboratory, pune for identification of 16S r DNA sequences of different isolated organisms. They have their standard protocol for identification which is as follow;

1. DNA Extraction.
2. Column based PCR in which primer they as 16 S and 125 r. PCR amplification is done by denaturation, renaturation, annealing, extension and run for 35 cycles.
3. Agarose gel electrophoresis.
4. PCR purification by PEG NaCl based protocol.
5. Product obtained is cycle sequenced by provided ultimate condition 96°C for 10 minutes, 96°C for 10 seconds, 50°C for 10 seconds and 60 °C for 4 minutes then product is kept at 4 °C for infinity. They run this for 25 cycles.
6. Again the product is purified by sodium acetate purification technique.
7. The sample is DNA sequenced and output is in specific format of computational data i.e. ABI and SEQ format.
8. Chromus DNA sequence software has ability to read the specific file format.
9. The sequence obtained is copy and paste in BLAST (basic local alignment sequence tool) from National Centre for Biotechnology Information.

2.2.12 Biodegradation of Nylon 6 by selected fungi and bacterial culture

Sterilization of the sample:

The sample sheets of Nylon 6 were sterilized before they were inoculated into the test medium. The Nylon 6 sheets were dipped in alcohol for a few hours. Washed with distilled water thoroughly. No physical or chemical changes were observed in the sample after treatment of sterilization.
**Submerged Cultivation Procedure:**

A sheets of Nylon 6 were exposed to submerged cultivation process Microbial degradation was performed in Erlenmeyer flasks in shaker condition. Each flask containing small sheet of Nylon 6 and 100 ml of the liquid glucose – salt medium at a pH 6.25. The medium contained per liter of deionised water, 10 gm of glucose, 1 g KH$_2$PO$_4$ 0.5 gm. of MgSO$_4$ 7H$_2$O and 0.5 gm of (NH$_4$)$_2$ SO$_4$ and 0.1 gm of CaCl$_2$. Aliquots (100 ml) of medium were poured into 500 ml Erlenmeyer flask and sterilized in an autoclave for 20 min at 121 °C and 1.2 atm, after cooling the medium was inoculated with 5 ml of the fungi spore suspension obtained by suspending spores from one agar slant tube in 20 ml sterile water. The fermentation broth was incubated on a rotary shaker at 30 °C and 90 rpm. Nylon 6 was the sole source of nitrogen in the medium.

Biodegradation of Nylon 6 was carried out by incubating the sheets into three known strains of fungus, one strain of bacteria. The three strains of fungus are *Phanerochaete chrysosporium* NCIM 1106, *Phanerochaete chrysosporium* NCIM 1073 (Ursa Klun, 2002) and *Trametes versicolor* NCIM 1086, and bacterial strain is *Pseudomonas aeruginosa* NCIM 2242. Incubation for about 4 - 12 months with day and night shaking provided at 22 - 27°C temperature (RT).

**2.2.13 Biodegradation of 6, 6 by selected fungi and bacterial culture**

**Sterilization of the sample:**

The sample sheets of Nylon 6, 6 were sterilized before they were inoculated into the test medium. The Nylon 6, 6 sheets were dipped in alcohol for a few hours. Washed with distilled water thoroughly. No physical or chemical changes were observed in the sample after treatment of sterilization.

**Submerged Cultivation Procedure:**

A sheets of Nylon 6, 6 were exposed to submerged cultivation process Microbial degradation was performed in Erlenmeyer flasks in shaker condition. Each flask containing small sheet of Nylon 6,6 respectively and 100 ml of the liquid glucose – salt medium at a pH 6.25. The medium contained per liter of deionised water, 10 gm of glucose, 1 g KH$_2$PO$_4$ 0.5 gm. of MgSO$_4$ 7H$_2$O and 0.5 gm of (NH$_4$)$_2$
SO₄ and 0.1 gm of CaCl₂. Aliquots (100 ml) of medium were poured into 500 ml Erlenmeyer flask and sterilized in an autoclave for 20 min at 121 °C and 1.2 atm, after cooling the medium was inoculated with 5 ml of the fungi spore suspension obtained by suspending spores from one agar slant tube in 20 ml sterile water. The fermentation broth was incubated on a rotary shaker at 30 °C and 90 rpm. Nylon 6, 6 was the sole source of nitrogen in the medium.

Biodegradation of Nylon 6, 6 was carried out by incubating the sheets into three known strains of fungus, one strain of bacteria. The three strains of fungus are *Phanerochaete chrysosporium* NCIM 1106, *Phanerochaete chrysosporium* NCIM 1073 (Ursa Klun, 2002) and *Trametes versicolor* NCIM 1086, and bacterial strain is *pseudomonas aeruginosa* NCIM 2242. Incubation for about 4 - 12 months with day and night shaking provided at 22 - 27°C temperature (RT).

### 2.2.14 Biodegradation of Nylon 6 under semi natural composting environment:

Biodegradation of Nylon 6 was carried out by keeping the sheets under semi-natural conditions of environment that is composting. Semi-natural is word used to describe the laboratory conditions. Its in-vitro composting method for degradation of polyamides. But in this semi-natural method composting was carried in Borosil glass tray. Composition of composting in which concentration of manure and soil as 1:3 ratios. Green and brown leaves, waste as carbon source, moisture condition moderately maintained.

Sheets of Nylon 6 under composting were kept for about 3 months for compost to complete. The compost soil samples 5gm and 45ml of distilled water were mixed, then the initial Pᴴ of the liquid was measured (Pᴴ meter). Then the composting process was progressed. Temperature and Pᴴ is calculated at regular interval of time (R. Mohee, 2007). Physico-chemical analysis of soil from composting after incubation was analyzed for concentration of organic carbon, phosphorus, potassium and electrical conductivity. Initial weight and initial thickness of sheets was calculated and final observation was done under inverted microscope. Also the chemical changes were recorded by using Infra-red Spectroscopy.
2.2.15 Biodegradation of Nylon 6, 6 under semi natural composting environment:

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