Republic of Iraq
Ministry of Higher Education
And Scientific Research
Al-Nahrain University
College of Science

Isolation, Identification and Genetics of Nylon6 Degrading *Pseudomonas putida*

A thesis

Submitted to the Collage of Science of Al-Nahrain University in partial of Fulfillment of Requirements for the Degree of the Master of Science in Biotechnology

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Acknowledgment

First of all praise to **Allah** the lord of the universe, peace be upon **Mohammed** the Messenger of **Allah** and upon his **Relatives**.

First and foremost my thanks must go to my Supervisor**Dr. Majed H. Al-Gelawi**for his kindness and great support throughout my studies. I specially appreciate their warm and gentle approach to my supervison, as well as their immense patience in all their dealings with me during my time at the laboratory. The knowledge I gained from them and both academic and non academic matters have been invaluable and will definitely be beneficial to my future career.

I am grateful toDr. Salah Mahdi, Mr. Sameer Naji and Mss.GhydaaHussain/Biotechnology research center for help and support.

Its pleasure to thank my teachers, Dr. RashaSadiq,Mr. HamedJehadMrs. RoaaNizar, Mrs.Muntaha, Mr. Zaid, Miss. Oroba, Mrs. Ayat, Miss. Raghad and Mrs. Suham and how that I didn‘tmentioned.

My Sincere thanks and appreciation go to my best friends Mohammed Redah, SamerImad, HayderHussain,RiyamBasim, Noor Dhia Russell Abdelrazaq, Aseel Ali and Marwa Sabah their understanding, endless love, encouragement and great friend ship that made easier for me to overcome difficulties in all hard times.

My special thanks to pattern in life uncle Dr. AlaaAmeen and at last I would like to thank my mother, father, brother and sisters, and I want to know that I am very grateful for their unreserved love and encouragement through out my studies, to which have been source of inspiration.

Summary

Sixty-four soil samples (contaminatedwith nylon waste for many years) were collected from different places in Baghdad. From these samples, forty-sevenisolates were obtained as a degrader for nylon6 film.These isolates were repeatedly tested in mineral salt medium supplemented withnylon6 film to ensure their degradation ability; only 27 isolates appeared tohave degradation ability fornylon6 filmas a sole source of carbon and nitrogen. These isolates (27 isolates) were screened for their ability to degrade nylon6 film according to growth density;it was found that these isolates were varied in their growth density. Themost efficient isolates were T1, M3, S3A, S12 andS17; however, S3A isolate is the best among them.

According to growth density, 20 isolates were selected for identification; they were identified depending on morphological, cultural, and biochemical characteristics.Results showed that 16 isolates belonged to*Pseudomonas* spp.(*Pseudomonas putida* (14) isolates, *Pseudomonas stutzeri* (1) isolate and *Pseudomonas* sp. (1) isolate, while the other four belonged to*Moraxella* spp.

The plasmid profile for *Pseudomonas putida*S3A was studied. Results showed that this isolate harbored small plasmid DNA bands.In order to study the role of its plasmid in degradation of nylon6 film, curing experiment was performed by using sodium dodecyl sulfate (SDS) and showed that two colonies had lost their ability to degrade nylon6 film as a sole source of carbon and nitrogen. Plasmid DNA extraction from one of these colonies indicated the loss of plasmid DNA bands,

Summary

and this referred that the plasmid DNA bands could be responsible for degrading nylon6 film in *P. putida* S3A.

Optimum conditions for degradation of nylon6 film by *P. putida* S3A were investigated. It was found that these conditions are growing this bacterium in mineral salt medium (pH 6.5) containing 0.1% of nylon6 film and incubated with shaking (180rpm) at 37 ˚C for seven days. It was found also that this bacterium was able to survive up to 0.7%of nylon6 film.

In an attempt to investigate the ability of bacteria to degrade crude nylon, the efficient five isolates(T1, M3, S3A, S12 and S17) were grown on crude nylon6 and nylon66 as a sole source of carbon and nitrogen. Results showed that nylon66 is more susceptible to degrade than nylon6 by these isolates, and maximumgrowth was recorded by*P. putida* S3A.

To insure isolation of bacteria capable to degrade crude nylon6, nylon6 was processed by UV irradiation and transferred to film, and this film was used to isolate bacteria.From the Fourier Transformer Infrared Red Spectroscopy (FTIR) results, it can be concluded that the irradiated nylon6 became less difficult to degrade because U.V light photodegradenylon6 into small pieces, and reduced its molecular weight. It can be concluded also that nylon6 film was degraded by*P. putida* S3A, which used the (N-H, C=O and C-H)groups as carbon and nitrogen source.

Summary

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List of Abbreviations

Abbreviations	
rpm	Round per minute
v/v	Volume per volume
w/v	Weight per volume
PA`S	Polyamides
PET	Polyethylene-terephthalate
COD	Chemical oxygen demand

Chapter One

Introduction

and

Literatures Review

Chapter one

Introduction and Literature Review

1.1. Introduction:

Rapid developments in the chemical industry have lead to the distribution of a wide variety of synthetic compounds into the environment (Negoro, 2000).

Synthetic polymers form the base for the more than 55% of all textile material with a worldwide fiber production of 3.3 million tones (Engelhardt, 2003). Approximately 140 million tones of synthetic polymers are produced worldwide every year. Since polymers are extremely stable, their degradation cycles in biosphere are limited. Environmental pollution by synthetic polymers, such as waste of plastic and water-soluble synthetic polymers in waste water has been recognized as a major problem (Premraj and Doble, 2005).

Depending upon the efficiency of the polymerization process, the amount of this solid waste generated can vary from one plant to another and it ranges typically from 20 to 180 metric tones /year. This method of waste disposal has the disadvantage of possible leaching of waste constituents into surrounding soil or ground water. The toxicity of caprolactam is one of the constituents of the solid oligomeric waste to animals and human (Gross, 1984; Schonborn, 1986).

Modern intensive industrial production leads to considerable increase of environmental stress, which is evinced by significant contamination of environment with toxic compounds, among the most widespread pollutants in the contemporary world are a polymer based on 6-aminohexanoic acid and caprolactam (polycaproamide) and polymer materials caprone, nylon6 and nylon (Ponamoreva *et al*., 2010).

The biodegradation of unnatural synthetic compounds which have been released into the natural environment with the development of the chemical industry provides a suitable system for investigating how microorganisms evolve the enzymes essential for the degradation of such xenobiotic compounds (Yasuhira *et al*., 2007a).

Biodegradation or detoxification of xenobiotic compounds has been recognized as a useful way to eliminate environmental pollutants. However, the efficiency of removal is highly dependent on the specific enzyme that can catalyze the desired degradation reaction (Negoro, 2002).

Research on the microbial degradation of xenobiotic polymers has been underway for more than 40 years. It has exploited a new field not only in applied microbiology but also in environmental microbiology and has greatly contributed to polymer science by initiated the design of biodegradable polymers (Fusako, 2010).

According to important use of nylon, and because of limited studies of nylon biodegradation, this study was aimed to:

- Isolation and identification of bacteria that capable of degrading nylon6
- Screening the bacteria for their ability to degrade nylon6 and select the efficient isolate(s)
- Determine the plasmid(s) profile of the efficient isolate(s)
- Determine the role of plasmid(s) in nylon6 degradation process via curing and/or transformation experiments.
- Study some optimum conditions for nylon6 degradation by efficient isolates

1.2. Literature Review

1.2.1. History of nylon

Nylons are a class of polymers know as polyamides (PA`s) by the condensation reaction of diamines and dibasic acid, nylons were first synthesized by Carothers and scientist at Due Pont, in 1930 and in 1939 PA`s become first truly synthetic fiber to be commercialized (Mark and Whiby 1940; Stevens1990).

A polyamides (PA`s) also referred to as nylons, are the class of thermoplastic polymers which contain the amide repeated linkage in the polymer backbone (Kohan, 1973).

Caprolactam is monomeric precursor used for process of manufacture of nylon 6 (Kulkarni and Kanekar, 1998a).

Nylon is a polyamide fiber, derives from a diamine and a dicarboxylic acid, because a variety of diamines and carboxylic acids can be produced, there are very large number of polyamide materials available to produce nylon fiber. The two most common versions are nylon 66 and nylon6 (Galanty and Bujtas, 1992).

1.2.2. Types of nylon

The regularity of amide linkages along the polymer chain defines the two main classes of PAs (AB and AABB). AB polyamides are formed in a polycondesation reaction from cyclic amides (lactams). Type AABB, where the amide linkage alternate in orientations along the background, are formed diacids and diamines in a polycondensation reaction (Kroschwit *et al*., 1996).

Polyamide-6 (PA-6) and polyamides 6, 6 (PA-6, 6) are examples of AB and AABB types, respectively as shown in Figure (1-1). PA-6 is produced by the ring opening polymerization of ε-caprolactam initiated by water. Small

specific amount are added to polymerization to control molecular weights and catalyze reactions (Schwartz, and Goodman, 1982).

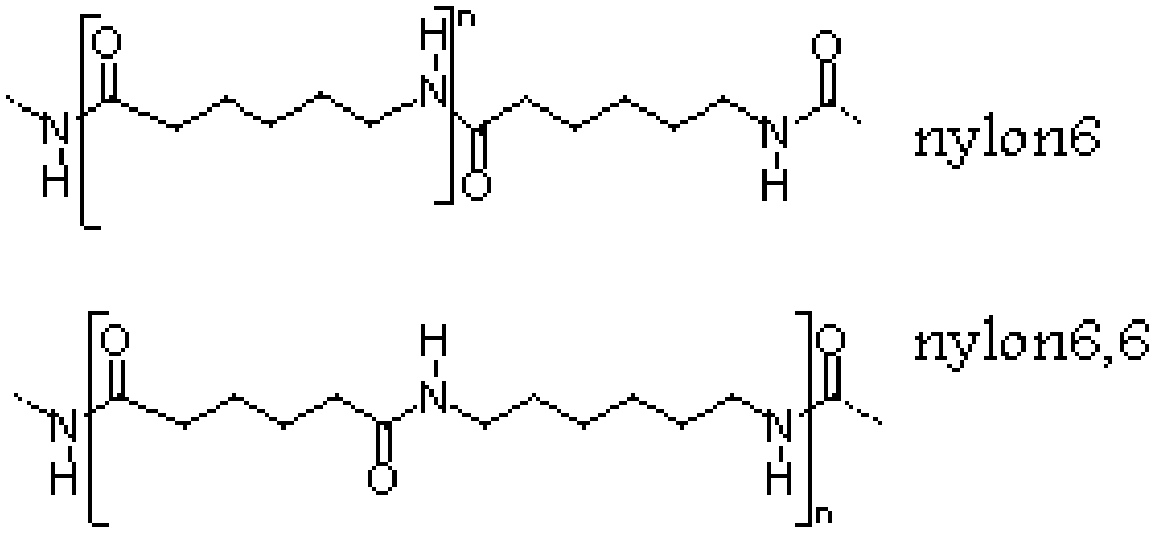


Figure (1-1): Repeating unit of polyamides 6 and polyamides 6, 6 reaction (Kroschwitz *et al*., 1996).

-Nylon6

Nylon6 have several commercial names (perlon, nylon and steelon) and produced from □-caprolactam by ring cleavage polymerization. It consists of more than 100 units of 6-aminohexanoic acid. During the polymerization reaction, some molecules fail to polymerize and as, oligomers, while other undergo head to teal condensation to form a cyclic oligomers (Negoro, 2000; Kotowa, 2004).

Polymers bearing recurring amide groups in their backbone are defined as polyamide. Aliphatic polyamides can be synthesized by condensation of bi-functional monomers. The two commercial polyamides are poly hexamethylene adipamid (polymer of adipic acid and hexanmethylene diamine) (nylon66) and polycaprolactam (nylon6) (Sudhakar *et al*., 2007).

1.2.3. Properties of nylon

PA ‘s are semi-crystalline they consist of crystalline and amorphous phases crystalline results from the polar amide groups, which undergo hydrogen bonding between carbonyl and NH groups in adjacent section of the PA chains. The regular spatial alignment of amides groups that allows a high degree of hydrogen bonding to develop when chains are aligned together the more random amorphous regions have a much lower degree of hydrogen bonding (Xenopoulos and Clark, 1995).

A semi crystalline structure gives rise to a good balance of properties the crystalline regions contribute to the hardness, yield strength, chemical resistance, creep resistance and temperature stability. The amorphous areas contribute to the impact resistance and high elongation. Because of their good chemical resistance, PA‘s are insoluble in common organic solvents at room temperature. However, they are soluble in formic acid, phenols mineral acids and fluorinated alcohols such as 2, 2, 2-triflurotoethanol (Tuzar, 1991).

The high melting point of PA‘s are a function of the strong hydrogen bonding between the chains. PA-6 and PA-6, 6 are isomers that share the same empirical formula, density and other properties but differ in melting point. PA-6 melts at 255˚C and PA-6, 6 at 265˚C. This is due to the different in the alignment of molecular chains and crystallization behavior (Grigg, 2006).

Polyamides are hydroscopic as water hydrogen bonds to the polar amide groups (Grigg, 2006).

Water absorption is greater in the amorphous regions due to greater availability of amide groups. Moisture strongly affects properties of polyamides and acts as a plasticizer increasing the flexibility and toughness. Polyamides are also degraded by hydrolysis at elevated temperature (Kroschwitz *et al*., 1996).

The dyeing efficiency of nylon fibers is enhanced due to the end groups -COOH and -NH2, which exhibit polar and hydrophilic characteristics. Dye diffusion into fibers is closely related to the rate of dyeing, level of dyeing through dye migration, and wet fastness properties of dyes. It is generally believed that dye diffusivity is independent on dye concentration, with some exception (Shibusawa, 1996).

The -COOH and -NH_2 end-groups in nylons are sensitive to light, heat, oxygen, acids and alkali. When exposed to elevated temperatures, unmodified nylons undergo molecular weigh degradation, which results in loss of mechanical properties. The degradation is highly time and temperature dependent. By adding heat stabilizer, nylon can be used at elevated temperature for long-term performance. Exposure to UV light results in degradation nylon over an extended period, it appear that adding carbon black can reduce the radiation degradation (Garf and El kemry, 1997).

Both nylon 6 and nylon 66 are semi-crystalline polymers. These linear aliphatic polyamides are able to crystallize mostly because of strong intermolecular hydrogen bonds through the amide groups as shown in Figure (1-2) (Dasgupta *et al*., 1996).

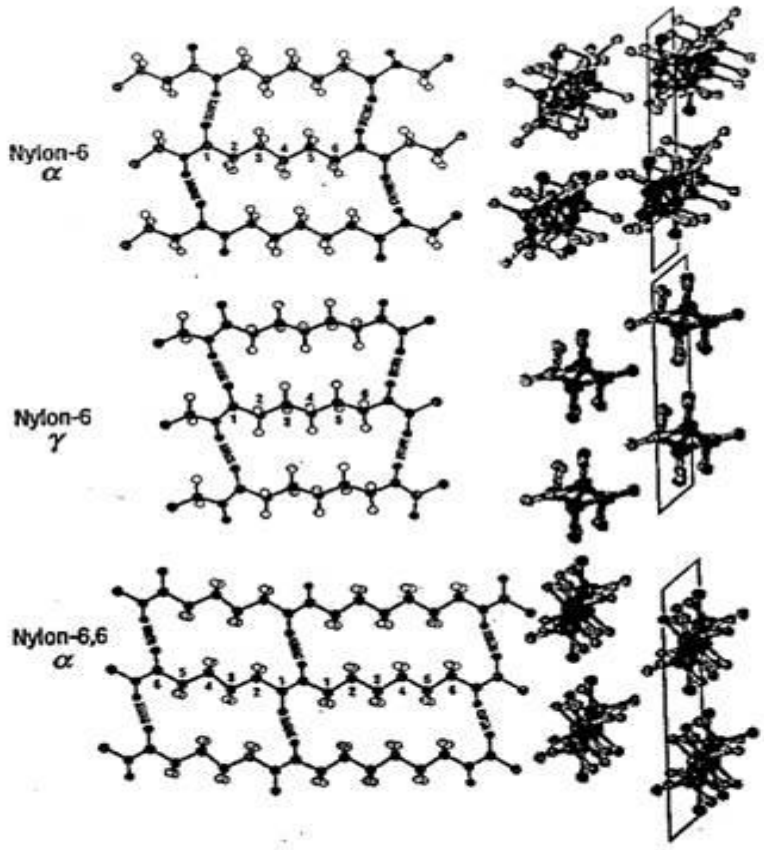


Figure (1-2): The left side of the figure shows hydrogen-bonding planes, and the right side shows the view down of chain axis for nylon6 and nylon6, 6 (Dasgupta *et al*., 1996).

1.2.4. Applications of nylon

Polyamides are significant commercial polymeric materials, representing approximately 4% of the total world consumption of principal polymer. PA-6 accounts of approximately 54% of polyamides produced while PA-6, 6 accounts for approximately 36% (Kroschwitz *et al*., 1996).

Almost 75% of polyamides are used as fibers while about 15% are used as engineering plastics. Fiber uses include carpet, apparel, home furnishings and industrial applicatons (Meplestor, 1997).

Engineering uses vary from wheel covers, handles, radiator end and fuel hoses in the automotive industry to hair dryers, lawnmowers, gears and bearings (Dasgupta *et al.*, 1996).

In 2005, consumption of nylon was reached 10% and 10.4 % by 2007. Figure (1-3) shows the forecasted consumption of nylon fiber in nonwoven Industry until 2007 (Www. Ifj.com).

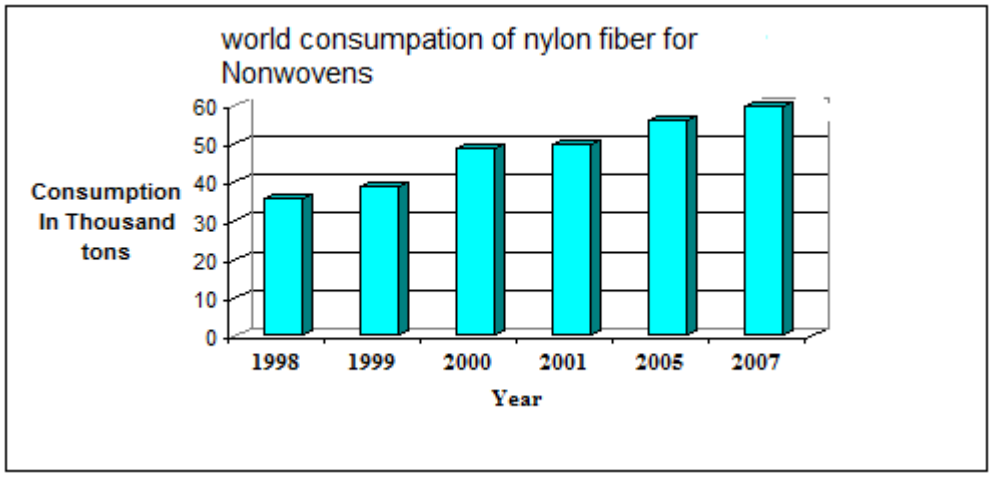


Figure (1-3): the forecasted consumption of nylon fiber in nonwoven Industry until 2007. (Www. Ifj.com)

In certain applications, the performance of nylon fiber is hard to beat. However, because of its higher cost, it is used in specialized applications where its performance can justify the increased cost. It is used as a blending fiber in some cases, because it conveys excellent tear strength. The resiliency and wrinkle recovery performance of a nonwoven produced from nylon is not as excellent as that from PET fiber. This polymer is used in moderate quantities, because it is more expensive than polyester, polypropylene, or rayon. Some particular applications are as follows:

- It can be mostly found in garment interlinings and wipes where it supplies strength and resilience.

- In Ni/H and Ni/Cd batteries, nylon fibers are used as nonwovens separators.

- Nylon fibers are used for the manufacture of split table-pie fibers. These fibers find application in high performance wipes synthetic suede, heat insulators, battery separators and specialty papers.

- Nonwovens developed from nylon are found in automotive products, athletic wear and conveyor belts.

It is notable that a large number of biodegradable plastics are categorized as polyester many examples of plastic degradation have been documented (Matsumura, 2002).

1.2.5. Polymer blends

The blending of polymers has steadily increasing in the scope and importance in the last three decades. Mixing of the two polymers, have different properties, to give a product with some desirable intermediates property, some of benefit of poly-blending include enhanced process ability, increase modules for elastomers, decrease modules for thermoplastics, improve thermal properties and improvement in impact resistance (Kienzel, 1988). Kyrikou and Briassoulis, 2007 reported that polymer do not consist of only one chemical homogeneous component, but contain different polymers (blend) or low molecular weight additives (plasticizers) which can serve as a good nutrients for the ambient microorganisms developed on polymer surfaces.

1.2.6. Oxidation of polymer

The service life of polymer is limited by their degradation, which can be caused by a number of environmental factors, e.g. temperature, humidity, impurities, mechanical load, irradiation, microorganisms, chemicals and air degradation is an undesirable process in the majority of polymeric

applications as it generally leads to changes in the chemical and physical structure of the polymer resulting in the loss of many useful properties such as molecular weight, mechanical strength, impact resistance and colur (Grassie and Wire, 1965; Ghaemy *et al*., 1981; Girois and Scott, 1997 ; Rychly *et al*., 1997).

Hoffman (1861) mentioned that oxidation is one of the most important degradation process and made the first report on oxidative degradation in 1861. Zwiefel (2001) elucidated that oxidation occur in every stage of the life cycle of polymer: during manufacture and storage of the polymer resin, as well as during processing and end use of the plastics article produced. Numerous oxidation products are formed as the result of degradation of the polymer such as peroxide, alcohols, ketones, aldehydes, acids, peracids, perestres and γ-lactones. Elevated temperatures, irradiation (e.g. UV) and catalysts such as metal ions increase oxidation rates. Also most polymers have structural elements that are particularly pron to oxidative degradation reactions.

When a hydrocarbon polymer is exposed to oxygen the first goes through an apparent induction period during which the rate of formation of oxidation products dose not seem to increase as shown in Figure (1-4) (Scott, 1993).

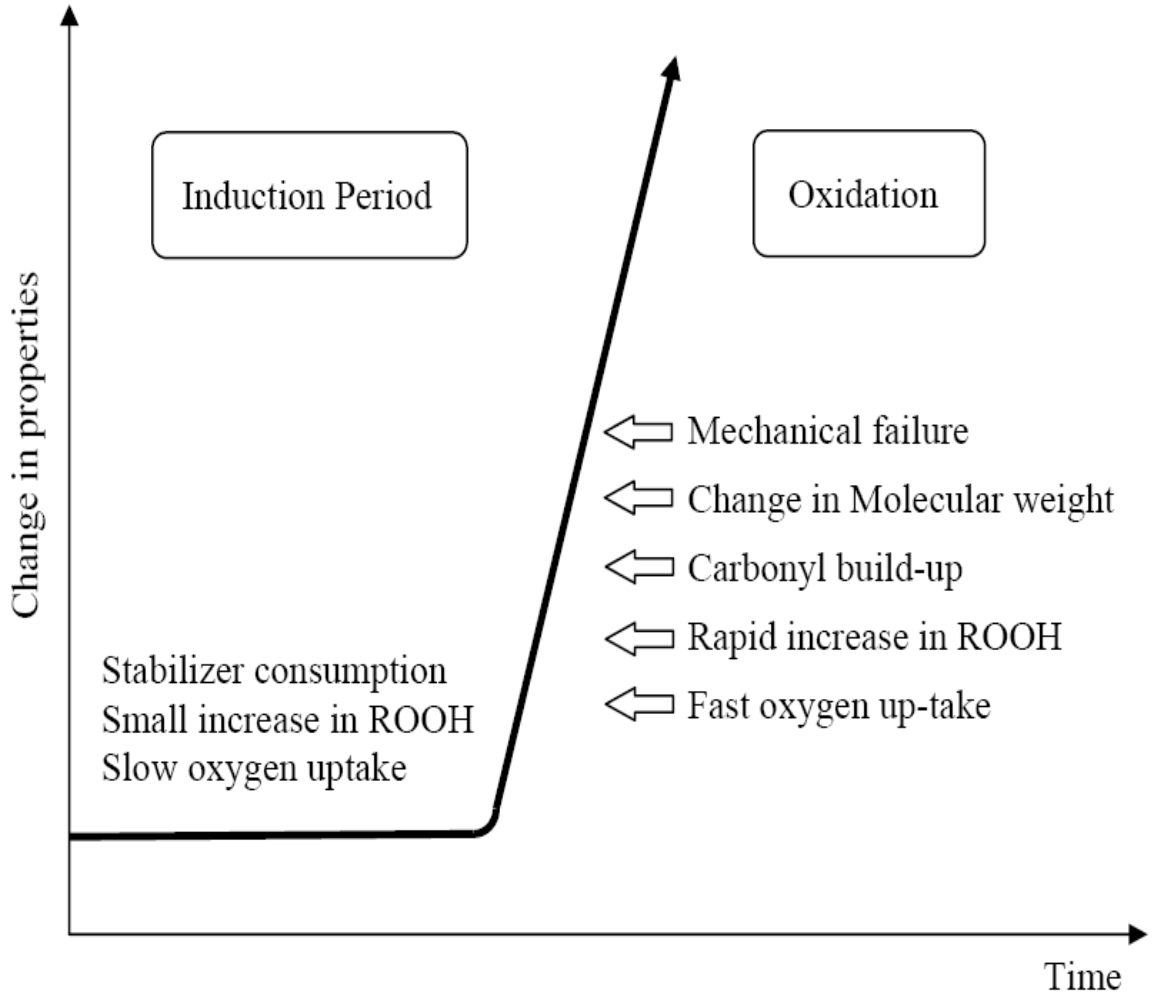


Figure (1-4): Changes in the polymer properties during aging of polymers (Scott, 1993)

1.2.7. Isolation of nylon degrading microorganisms.

Microorganisms are highly adaptive to environment and secret both endoenzymes and exoenzymes that attack the substrate and clave the molecular chains into segments (Huang *et al*., 1990; Albinas *et al*., 2003).

A few groups of microorganisms are able to degrade certain oligomers of nylon 6 (Fukumura, 1966; Kohan, 1973; Kinoshita *et al*., 1975).

Jayasekara *et al.,* (2005) and Zheng *et al.,* (2005) reported that synthetic polymeric materials can suffer from different forms of deterioration, including chemical (e.g., oxidation), physical (e.g., UV light), and biological.

Flemming (1998) decleared that microorganisms can damage the structure and functions of synthetic polymers by biological coating masking surface properties, increase leaching of additives and monomers that are used as nutrient, production of metabolites (e.g., acids), enzymatic attack, physical penetration disruption, water accumulation, and execration of pigment.

Nylons, synthetic polyamides, are barely biodegradable, but nylon oligomers occurring as byproduct during the synthesis of nylon can be biodegraded by microorganisms. Linear and cyclic oligomers of ε-aminocaproic acid (byproduct of nylon manufacturing) are assimilated by *Flavobacterium* sp. K172 and *Pseudomonas* sp. NK87 that utilized them as sole carbon and nitrogen source (Kinoshita *et al*., 1975; Kanagawa *et al*., 1989).

Alkanophilic nylon oligomer- degrading bacteria, *Agromyces* sp. KY5R and *Kocuria* sp. KY2 were isolated. It was also found that the genetic organization of nylon oligomer degrading enzymes is similar to that of strain K172, albeit with some rearrangements (Yasuhira *et al*., 2007a; Yasuhira *et al*., 2007b).

Negoro *et al.*, (2005) and Yasuhira *et al.*, (2010) explained that the mutations rate is not the same for all synthetic polymers, since polyethylene or polypropylene are only slightly biodegradable, although they have been used for approximately the same period of nylon, catalytic mechanisms and evolution of enzymes from ancestor enzymes NYLB and NYLA based on X-ray analysis of crystallized.

Tomita *et al*., (2003) reported that a thermophilic bacterium, *Geobacillus thermocatenulatus* has been suggested as a possible degrades of nylon-12 and nylon-66 but not nylon-6, while Sudhakar *et al*., (2007) found that marine bacteria *Bacillus cereus, Bacillus sphericus*, *Vibrio fumisii* and *Brevundimonas vesicularis* were able to degrade nylon6 and nylon66 in mineral salt medium at 35˚C and pH of 7.5 under submerged enrichment

conditions with polymer as the sole carbon source, maximum degradation was observed with the *Bacillus cereus* for both nylons. Average molecular weight decreased by 42% and 31% and weight decreased by 7% and 2% in the case of nylon66 and nylon6.

Fischer *et al*., (2004) and Heumann *et al*., (2008) reported that *Noccardia farcinica* related to its potential to hydrolyze water insoluble polyamide oligomers and polymide 6. In the study of Deguchi *et al*., (1998) they reported that the fungus IZU-154 significantly degraded nylon-66 membrane under ligninolytic conditions. Nuclear magnetic resonance analysis showed that four end groups, CHO, NHCHO, CH3, and CONH2, were formed in the biodegraded nylon-66 membranes, suggesting that nylon-66 was degraded oxidatively.

Baxi and Shah (2001) illustrated that *Alcaligenes faecalis* G utilized 95-97% of 5-15 g of ε-caprolactam in 24-48 h over a pH range of 6-8.5 and at 23-40 ˚C without complex nutrient requirement, the chemical oxygen demand (COD) of the wastewater of nylon-6 plant was mainly due to its caprolactam content. *Alcaligenes faecalis* G decreased the caprolactam content and the COD of the waste water by 80-90% of the original, inspit the wastewater having higher caprolactam content.

Baxi and Shah (2007) reported that three caprolactam degrading bacterial isolates grew in liquid synthetic medium containing solid waste of a nylon-6 production plant as the sole source of carbon and nitrogen, *Alcaligenes faecalis* the most potent caprolactam-degrading bacterium of the three isolates. Kulkarni and Kanekar (1998a) shown that *Pseudomonas aeruginosa* strain MCM- B407 isolated from activated sludge, able to remove ε-caprolactam with simultaneous reduction in chemical oxygen demand (COD). Kulkarni and Kanekar (1998b) also reported that a strain *Pseudomonas putida* MCM- B408 capable to utilize ε-caprolactam (monomer of nylon6) as

sole carbon and nitrogen source and found to harbour a single 32-kb plasmid with same electrophoretic mobility as that of pARI180.

Yamano *et al*., (2008) shown that two strains identified as *Pseudomonas* sp. are able to degrading nylon4 and these two strains can degrade nylon4 in 24 h and produced γ aminobutyeric acid (GABA) as degradation product.

There are many mechanisms responsible for polymer respiration as shows in the Figure (1-5) (Yoshito and Hedito, 2000).

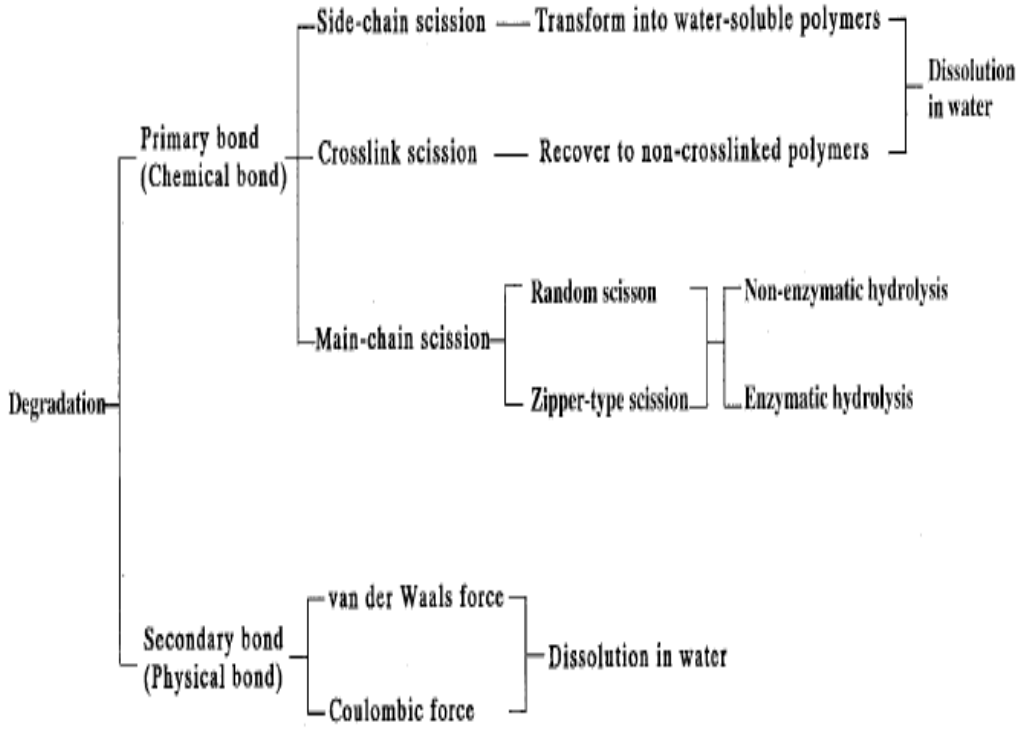


Figure (1-5): Modes of resorption of polymers in the nature (Yoshito and Hedito, 2000)

1.2.8. Genetics and Enzymes of Nylon Degradation

Bacteria are known to pocesses plasmids, which encode part or the entire degradative pathway of hydrocarbon (Focht, 1988). Friefelder (1987) and Dale (1998) reported that plasmids are extrachromosomal genetic elements

are wide spread in bacteria, they have their origin of replication, and autonomously replicates with respect to chromosomal DNA and stable inherited. Normally plasmids contain genes that may be essential in certain environments. Frantz and Chakrabarty (1986) reported that in *Pseudomonas*, several degradative pathways such as those for toluene, camphor, salicylates, alkanes and naphthalene, are dependent on "degradative plasmids" called TOL, CAM, SAL, OCT and NAH plasmids, respectively.

Farell and Chakrabarty (1979) reported that degradtive plasmid represents a group of naturally occurring plasmids, that code for the dissimilation of complex organic compounds.

Negoro *et al*., (1980) and Negoro and Okada (1982) explained that *Flavobacterium* sp. K172 harbors three kinds of plasmids pOAD1 (39.7 kbp), pOAD2 (43.6 kbp) and pOAD3 (56.4 kbp) and on pOAD2 the EI and EΠ enzymes are encoded. Kinoshita *et al*., (1977) reported that *Flavobacterium* sp. K172 has three enzymes, 6-aminohexanoate-cyclic-dimer hydrolase (EI). EI responsible for degradation of 6-aminohexanoate oligomers and it was homodimer enzyme with subunit of *M*r 52 kDa, it is active only toward the cyclic dimer, 6-aminohexanoate dimer hydrolase. Kinoshita *et al*., (1981) shown that EΠ is also homodimer enzyme with subunit of *M*r 42kDa and was active on 6-aminohexanoate oligomers ranging from dimer to hexamer and 6-aminohexanoat oligomer hydrolase. Negoro *et al*., (1992) and Kakudo *et al*., (1993) explained that EIII enzyme was either a homodimer or a trimer with subunit of *M*r 37kDa. It was active on the cyclic tetramer and pentamer and on linear oligomers higher than trimer.

Two kinds of repeating sequence (RS-I and RS-Π) were detected on pOAD2. The sequence of five RS-I regions identified on the plasmid were identical over 880-bp, except that the 420-bp regions at the 3′terminous of the 880-bp regions was duplicated in RS-IB (Negoro *et al*., 1983; Kato *et al*., 1994).

Okada *et al*., (1983) explained that RS-Π has two regions on pOAD2 (RS-ΠA and RS-ΠB). RS-ΠA contained the *nyl*B gene, while RS-ΠB contained the analogous *nyl*B′ gene. The *nyl*B and *nyl*B′ had the same length ORF (1, 176-bp) the same Shine- Dalgarno sequence and the same position of initiation and termination codons. Of 392 amino acid residues encode by the ORF, only 46 residues were different.

Kato *et al*., (1995) found that the plasmid pOAD2 comprised 45,519 bp with a G+C content of 66.6%.

Pseudomonas sp. NK87 harbored six kind of plasmid, namely: pNAD1 (20Kb), pNAD2 (23Kb), pNAD3 (51Kb), pNAD4 (57Kb), pNAD5 (76Kb) and pNAD6 (80Kb). Cloning and hybridization experiments revealed that the *nyl*A and *nyl*B genes of NK87 were located on pNAD2 and pNAD6, respectively (Kanagawa *et al*., 1989).

The EI from NK87 (P- EI) was composed of 493 amino acid residues, which was identical to the length of EI of *Flavobacterium* sp. K172 (F-EI). The amino acid sequence of the P-EI enzyme was almost identical to the sequence of the F-EI (99% homology) (Tsuchiya *et al*., 1989).

The homology between the EΠ sequence of K172 (F- EΠ) and of NK87 (P-EΠ) was only 35% with respect to the amino acid sequence (Kanagawa *et al*., 1993).

EIII (*nyl*C gene product) hydrolyzed the linear trimer, tetramer, and pentamer of 6-aminohexanoate by an endo-type reaction, and this specificity is different from that of the El (*nyl*A gene product) and EII (*nyl*B gene product) as shown in the Figure (1-6). Amino acid sequencing and sodium dodecyl sulfate-polyacrylamide gel electro- phoresis of the purified EIII demonstrated that the enzyme is made of two polypeptide chains arising from an internal cleavage between amino acid residues 266 and 267 (Kakudo *et al*., 1993).

Biodegradation of caprolactam oligomers beings with the hydrolysis of amide bonds by specific hydrolysis with the formation of 6-aminohexanoate, which is then sequentially transformed by enzymes of the caprolactam catabolic pathway into adipic semialdehyde, adipate and Krebs cycle (Boronin *et al*., 1984).

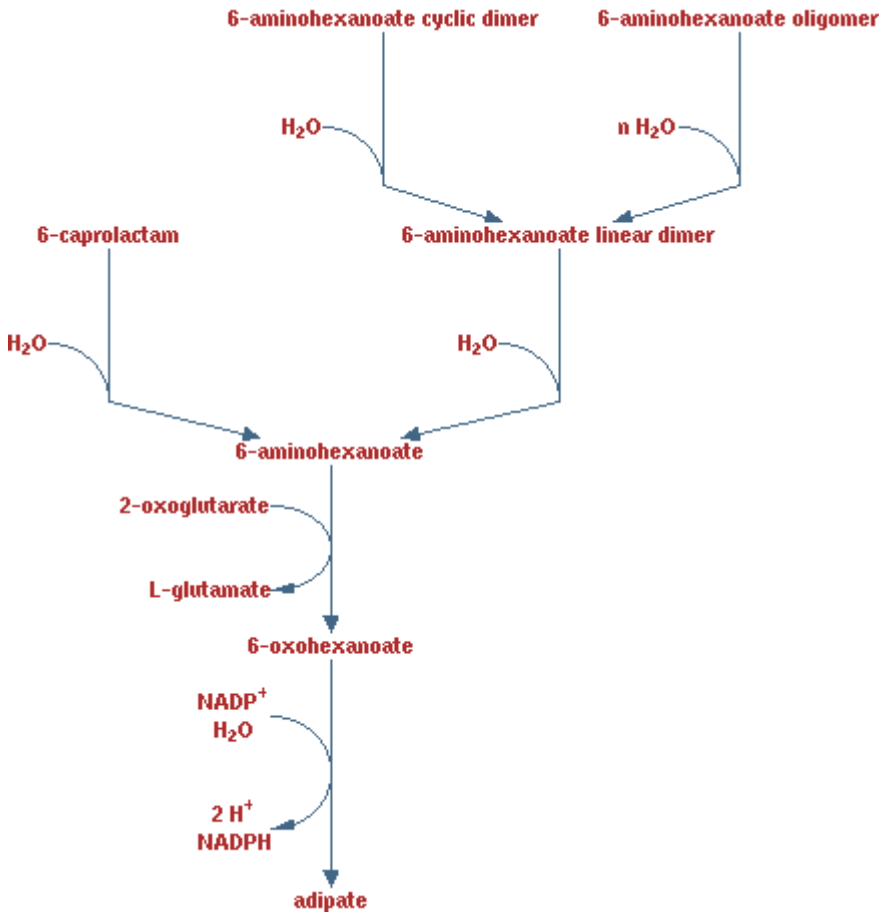


Figure (1-6): Nylon 6 oligomer degradation pathway (Kakudo *et al*., 1993).

1.2.9. Molecular basis for adaptation toward a xenobiotic compound.

Several molecular mechanisms are possible for microorganisms to adapt toward non-physiological substrates, (1) alteration of substrate specificity of an enzyme (Clarke and Slater 1986) (2) activation of a cryptic gene by

mutation (Hall and Clarke 1977) (3) alteration of regulator specificity (Ramos *et al*., 1986) and (4) alteration of uptake specificity.

Plasmid pOAD2 in *Flavobacterium* sp. K172 has five transposable elements all these five elements are identical with 764bp, externally imposed stress such as high temperature, exposure to poison or starvation can activated transposases. The presence transposases in such numbers on the plasmid suggests that the plasmid is designed to adapt when bacterium under stress (Truman, 2003).

Two hypotheses for the birth of nylon oligomer degradation genes where proposed namely:

- The EΠ enzyme is specify by an alteration ORF of preexisting coding sequence that originally specified a 472- residue –long arginine-rich protein, and a frame shift mutation in the ancestral gene creates the new gene (Oho, 1984)

- There is some special mechanism, which protects a nonstop frame (NSF), namely a long stretch of a sequence without chain-terminating base triplets, from mutations that generate the stop codons on the antisense strand; and such a mechanism enables the NSF to evolve into new functional genes (Yomo *et al*., 1992).

Chapter Two

Materials

and

Methods

Chapter two materials and methods

2.1. Materials

2.1.1. Equipment and apparatus

The following equipments and apparatus were used in this study:

Equipment	Company (origin)
Autoclave	Hirayama (Japan)
Incubator	Sanyo (Germany)
Distillator	GFL (Spain)
Millipore filter unit	Millipore corp (Spain)
pH- meter	Martini (Germany)
Centrifuge	Hermile (Germany)
Shaker incubator	Gallenkamp (Germany)
Sensitive balance	Denver (Germany)
UV-transilluminator	Viber lourmat (USA)
Cooling centrifuge	Hettich (Germany)
Micropipette	Ependrof
Water bath	Grant (England)
Minimal electrophoresis apparatus	Major science
Spectrophotometer	Cecil (England)
vortex	Stuart (England)
Balance	Satorius (Germany)
Laminar air flow hood	Sanyo (Germany)
Compound light microscope	Olympus (japan)
Fourier transformed infrared spectroscopy	Q panel company (U.S.A)
weather-o-meter, Q.U.V. tester	Q panel company (U.S.A)
Hot plate magnetic stirrer	Gallenkamp (England)

2.1.2. Chemicals

The chemicals used in this study are the following classified according to the manufactured company

Materials	Company(origin)
Chloroform, Urea, Peptone, Iodine, KH_2PO_4, NaOH, NaCl, $MgSO_4$. $K_2HPO_4.7H_2O$, KI, Formic acid	BDH-(U.K)
Agar, Gelatin, Boric acid	Biolife (Italy)
Starch	Difco (USA)
Sodium dodecylsulfate(SDS), Tris (hydroxyl methyl), Aminomethan base (Trise-base), Ethylenediaminetetraactic acid (EDTA), Methyl red, Hydrogene peroxide, Crystal violet and $FeSo_4.7H_2O$	Fluka (Switzerland)
Boric acid, Bromophenol blue, Cetrimide	Riedel-Dehaeny (Germany)
Nylon6, Nylon66, Ethidium bromide, Agarose	Sigma (USA)

2.1.3. Bacterial strain

Bacterial strain *(E. coli* HB101) harboring pBR322 (Ap^r, Tc^r) was obtained from Department of Biotechnology, Al-Nahrain University.

2.1.4. Soil samples

The soil samples used in this study were taken from five main regions of Baghdad (Topjy, Sholla, Bayaa, Jamylla and Mahmodya). These regions were contaminated with nylon and waste product of nylon.

2.1.5. Media

2.1.5.1 Ready to use media

The following media were ready-made media prepared according to the manufactured `s instructions:

Media	Company/(origin)
Trypticase soya broth	BDH (England)
trypticase soya agar	BDH (England)
Simmon citrate agar	Difco (USA)
Nutrient agar	Biolife (Italy)
Nutrient broth	Biolife (Italy)
TSI agar	Biolife (Italy)
MR-VP medium	Difco (USA)

2.1.5.2. Laboratory prepared media

- **King A (Starr *et al.*, 1981)**

Peptone	20g
K_2SO_4	10g
$MgCl_2$	1.4g
Glycerol	10ml
Agar	15g
Distilled water	1000ml

pH was adjusted to 7.2 and sterilized by autoclaving at 121˚C for 15 min.

- **King B (Starr *et al.*, 1981)**

Peptone	20g
$MgSO_4.7H_2O$	10g
K_2SO_4	1.4g
Glycerol	10ml
Agar	15g
Distilled water	1000ml

pH was adjusted to 7.2 and sterilized by autoclaving at 121˚C for 15 min.

- **Starch agar (Atlas *et al.*, 1995)**

Soluble starch	10g
Nutrient agar	23g
Distilled water	1000ml

pH was adjusted to 7.2 and sterilized by autoclaving at 121˚C for 15 min.

- **Urea agar (Colline and Lyne, 1987)**

Urea agar base	24g
Distilled water	950ml

pH was adjusted to 7.0 and sterilized by autoclaving at 121˚C for 15 min, then 50ml of 40% (w/v) urea (sterilized by filtration) was added to 950 ml media.

- **Cetrimide agar (Stolp and Gadkari, 1984)**

Peptone	20g
$MgCl_2$	4.5g
K_2SO_4	10g
Cetrimide	0.3g
Agar	15g
Distilled water	1000ml

pH was adjusted to 7.2 and sterilized by autoclaving at 121˚C for 15 min.

- **Gelatine medium (Stolp and Gadkari, 1984)**

It was prepared by adding (12% w/v) gelatine to trypticase soya broth then sterilized by autoclaving at 121˚C for 15 min.

- **Modified mineral salt medium (EM) (Negoro *et al*., 1980)**

KH_2PO_4	3g
K_2HPO_4	1g
$MgSO_4.7H_2O$	0.25g
NaCl	2g
$FeCl_3$	0.8mg
Distilled water	1000ml

pH was adjusted to 7.0 and sterilized by autoclaving at 121˚C for 15 min.

- **LB (Luria-Bertani) broth (Maniatis *et al*., 1982)**

Bacto-tryptone	10g
Bacto-yeast extract	5g
NaCl	5g
D-glucose	1g
Distilled water	1000ml

pH was adjusted to 7.0 and sterilized by autoclaving at 121˚C for 15 min.

2.1.6. Reagents

- **Catalase reagent (Atlas *et al*., 1995)**

A solution of 3% hydrogen peroxide was prepared

- **Oxidase reagent (Colline and lyne, 1987)**

This reagent was prepared by dissolving 1g of tetramethyl-p-phynylene diamine dihydrochloride in 100ml D.W.

- **Starch hydrolysis reagent (Cruickshank *et al*., 1975)**

- Potassium iodide (KI) 2g
- Iodine (I_2) 1g
- H_2O 300ml

- **Methyl red (Colline and Lyne, 1987)**

This indicator was prepared by mixing 0.1g of methyl red, 300ml of ethyl alcohol and 200ml D.W

- **Vogas-Proskauer (VP) (Harely and Prescott, 1996)**

It consists of two solutions:

A- KOH (16g) in 199ml D.W

B-α–naphtholamine (6g) in 100ml of 95% ethyl alcohol

2.1.7. Buffers and solutions

2.1.7.1. Plasmid extraction buffers and solutions

Buffers and solutions were prepared according to the salting out method (**Kieser, 1995**)

- **SET buffer**

  Tris-HCl 20mM

  EDTA 25mM

  NaCl 75mM

  pH was adjusted to 8.0 and sterilized by autoclaving.

- **NaCl solution(5M)**

  It was prepared in distilled water and sterilized by autoclaving

- **SDS solution**

  It was freshly prepared by dissolving 10 g in 100ml of sterile distilled water.

- **Lysozyme solution**

  This solution was freshly prepared by dissolving 1mg lysozyme enzyme in 1ml of sterile D.W

- **TE buffer**

  Tris-OH 1mM

  EDTA 10mM

  pH was adjusted to 8.0 and sterilized by autoclaving

2.1.7.2. Electrophoresis buffers (Sambrook and Russell, 2001)

- **Trise-Borate-EDTA 5X (TBE) (pH 8.0)**

  Trise-base 54g

  Boric acid 27.5g

  EDTA (0.5M) 20ml

  Distilled water to 1000ml

- **Ethidium bromide**

Prepared in concentration of 10 mg/ml in D.W. and stored at 4˚C.

- **Gel loading buffer (6X)**

Brromophenol blue (0.25% w/v)

Sucrose in H_2O (40% w/v)

2.1.7.3. Curing solution (SDS solution)

It was prepared as stock solution of 10% SDS in D.W.

2.2. Methods

2.2.1. Sterilization methods

- Culture media (liquid and solid), and solutions were sterilized by autoclaving at 121 ˚C, 15Ib/in^2 for 15 minutes.
- Glasswares (cylinder, petri dishes, etc…) were sterilized in electric oven at 180-200 ˚C for 2 hours.
- Thermolabile components or materials (such as urea) were sterilized by filtration through Millipore filter paper (0.22μm).

2.2.2. Sample collection

The soil samples were collected from different regions in Baghdad (Topjy, Sholla, Bayaa, Jamylla and Mahmodya). In these regions nylon waste was the main waste for many years ago. Soil samples were transferred to the laboratory using sterile plastic bags to isolate nylon6 utilizing bacteria.

2.2.3. Nylon6 Film preparations

Film of nylon 6 was prepared by dissolving 0.25g of nylon6 in 25 ml of formic acid, which is used to prepare polymer films. The films were prepared by casting and evaporation techniques at room temperature for 24 hrs. The film samples were fixed on a special holder for irradiation, which is an aluminum plate (0.6mm) in thickness supplied from Q-panel Company (U.S.A.).

- Irradiation experiments

Polymer irradiation experiment was performed at polymer research unit, Department of chemistry, College of Science, Al-Mustansirya University. The accelerated weather-o-meter was used for irradiation of polymer films. The accelerated weathering tester contains stainless steel plate with two cavities in the front and rear sides. Each side contains four lamps, type (U.V.B. 313), located horizontally, giving spectrum range of wavelength between 290 to 360 nm, and the maximum intensity is located at 313 nm.

The polymer film holders are fixed vertically and paralleled to the lamps, so that U.V radiation is vertically incident on the polymeric samples. The irradiated sample holders were changed in positions from time to time to ensure that all samples received the same intensity of light. The temperature of the tester chamber is constant at 50°C for all samples. During the irradiation process, the degree of photodegradation of the polymer films was monitored by taking different exposure times.

Nylon film preparations before and after irradiation were subjected to FTIR analysis (see section 2.2.14).

2.2.4. Isolation of Bacteria

Fifty militer of Mineral salt medium (EM) (2.1.5.2) were dispensed in the 250ml Erlenmyer flasks supplemented with 1g/L of nylon6 film (2.2.3) as a sole source of carbon and nitrogen, autoclaved at 121˚C for 15min. One percent (w/v) of soil samples were added to the flasks and incubated at 37˚C with shaking (180rpm) for seven days. Samples (0.1) ml of appropriate dilutions were spread onto plates of trypticase soya agar, incubated at 37˚C for 48hrs. A single colony was picked with a sterile loop to prepare a pure subculture in a fresh trypticase soya agar plate by streaking. Pure isolates were grown on the mineral salt medium containing nylon6 film to ensure there ability to degrade nylon6 film as a sole source of carbon and nitrogen.

2.2.5. Screening of nylon6 film degrading bacteria

To determine the efficient bacterial isolates capable to degrading nylon6 film, 20 milliliter of mineral salt medium (EM) were dispensed in Erlenmyer flasks (100ml), supplemented with 1g/L of nylon6 film, and sterilized by autoclaving. Flasks were inoculated with 1% of fresh culture (18hr) and incubated in shaker incubator (180rpm) at 37 ˚C for 7 days. Bacterial growth was measured as mentioned in (2.2.7). Control was made by inoculating flasks containing the same mineral salt medium (EM) but without any source of carbon and nitrogen (nylon6 film). Triplicates were made for each bacterial isolates.

2.2.6. Degradation of crude nylon6 and nylon66

Efficient isolates grown on nylon6 film were used to study their ability to grow on crude nylon6 and nylon66. Twenty milliliter of Mineral salt medium (2.1.5.2), supplemented with 1g/L of crude nylon6 or nylon66 as a sole source of carbon and nitrogen, were dispensed in 100ml Erlenmyer flasks and

sterilized by autoclaving at 121˚C for 15min. Triplicates were performed to each one of the bacterial isolates and all the flasks were inoculated with 1% fresh culture (18hrs.), and incubated in shaker incubator (180rpm) at 37˚C for 7 days. Bacterial growth was measured as mentioned in (2.2.7).

2.2.7. Measurement of bacterial growth

Growth of bacteria was monitored by measuring the optical density of the liquid culture in spectrophotometer (Spectronic20) at 600nm.

2.2.8. Identification of bacteria

Bacterial isolates were subjected to the following morphological, cultural and biochemical tests for their identification.

2.2.8.1 Morphological Characteristics (Atlas *et al.*, 1995).

A single colony was transferred by a loop onto clean slide. The smear was stained with crystal violet, treated with iodine, decolorized with absolute alcohol and counterstained with safranine, then examined under microscope to detect gram reaction, shape, spore forming and capsule of isolated bacteria.

2.2.8.2. Cultural Characteristics

Shape, color, edge, odor and viscosity of the colony were studied on the trypticase soya agar plate after incubation at 37˚C for 48hrs.

2.2.8.3. Biochemical Tests

The following biochemical tests were performed:

- **Growth on the Kligler`s iron agar (Atlas *et al.*, 1995).**

After inoculation and incubation for 24hrs, bacteria that only ferment glucose produced an alkaline (red slant) and acid (yellow) butt. Bacteria that

ferment lactose or sucrose as well as glucose produced an acid (yellow) slant and acid (yellow) butt, bacteria that do not ferment glucose, lactose or sucrose produced an alkaline (red) slant and an alkaline (red) butt, bacteria that produced gas during fermentation form bubbles or cracks in the medium. Bacteria that produced H_2S gas turn the medium black. This test was used to detect fermentation of glucose, lactose, sucrose, H_2S production and gas formation.

- **Simmon citrate utilization (Atlas *et al*., 1995)**

Slant of simmon citrate was inoculated with single colony, incubated at 37˚C overnight. The appearance of blue color indicates that the pH is alkaline end product and positive citrate test. This test was used to examine the ability of bacteria to utilize citrate as sole source of carbon.

- **Methyl- red and Vogas Proskauer (Maza *et al*., 1997)**

Inoculated the liquid medium (MR-VP) tubes and incubated at 37˚C for 48hrs. Both tests are performed from the same inoculums suspension, which is divided for testing. The methyl -red test was performed after adding about five drops of the methyl –red reagent (2.1.6), positive is bright red and negative test is yellow, while Vogas-Proskauer test was performed after adding the Vogas-Proskauer reagent (2.1.6), positive test has a pink color. This test was performed to determine the pH of the end products of glucose fermentation. Vogas-Proskauer test detects acetoin.

- **Growth on the King A (Cruickshank *et al*., 1975)**

Inoculated the bacteria on the plate by streaking and incubated at 37˚C for 24hrs. This test was performed to study the production of the characteristic pigment, pyocyanin.

- **Growth on the King B (Cruickshank *et al.*, 1975)**

Inoculated the bacteria on the plate by streaking and incubated at 37˚C for 24hrs. Then the plates were exposed to U.V. This was performed to study the production of the characteristic pigment, fluorescein.

- **Starch hydrolysis test (Harely and Prescott, 1996)**

The bacteria was inoculated on the plate by making a single streak of bacteria and incubated at 37˚C for 48hrs. After incubation the plates were flooded with iodine solution. Development of yellow color around the colonies indicates the hydrolysis of starch. This test was used to examine the extacellular enzyme α-amylase that catalyzes the breakdown of starch to maltose.

- **Urease production test (Atlas *et al.*, 1995)**

Christensen urea agar slants were inoculated with bacteria, incubated at 37˚C for 24-48hrs, the appearance of pink color slant indicate a positive reaction. This test was used to examine production of urease enzyme, which hydrolysis the urea to ammonia and CO_2.

- **Growth on cetrimide agar (Greenwood *et al.*, 1997)**

This medium was used as a selective medium for *Pseudomonas* spp. The plate was inoculated with bacteria by streaking and incubated at 37˚C for 24hrs.

- **Gelatin hydrolysis test (Harely and Prescot, 1996)**

Inoculated the tube of gelatin medium with bacteria by stabbing, and then the tubes were incubated at 37˚C for five days. This test was performed to demonstrate the ability of bacteria to hydrolyze gelatin.

- **Catalase production test (Maza *et al*., 1997)**

This test performing by adding drops of hydrogen peroxide (3%) on a single bacterial colony grown on trypticase soya agar. The production of gaseous bubbles indicates the ability of bacteria to produce catalase enzyme.

- **Oxidase production test (Maza *et al*., 1997)**

Filter paper was saturated with oxidase reagent (2.1.6), and then colony of bacteria to be tested was rubbed on the filter paper with a sterile wooden applicator stick. An immediate color change to a deep blue indicates the ability of bacteria to produce oxidase enzyme.

2.2.9. Maintenance of bacterial isolates

Maintenance of bacterial isolates was performed according to Sambrook and Russell (2001) and as follows:

- **Short –term storage**

Colonies of bacteria were maintained for periods of few weeks on the surface of agar media; the plates were tightly wrapped in parafilm and stored at 4˚C.

- **Medium –term storage**

Bacterial isolates were maintained in the stab culture for a long periods of a few months. Such as cultures were prepared in small screw capped bottles containing 2-3 ml of agar medium, the cultures were inoculated using a sterile straight platinum wire that is dipped into dense liquid culture of bacteria and then stabbed deep into agar medium. After proper incubation, the cap was wrapped tightly with the parafilm to prevent desiccation of the medium and kept at 4 ˚C.

- **Long-term storage**

Bacteria can be stored for many years in the medium containing 15% glycerol at low temperature without significant loss of viability. This was done by adding 1.5ml of glycerol to an exponential growth of the bacteria in a small screw-capped bottle with final volume 10 ml and stored at -20˚C.

2.2.10. Plasmid extraction method (Kieser, 1995)

Plasmid isolation from *P. putida* S3Awas performed by harvesting 20ml of bacterial culture, in nutrient broth incubated in shaker incubator (180rpm, 18hrs, 37˚C), by centrifugation (6000, 15min). The pellet washed with 3ml of SET buffer and recentrifugation (6000, 15min). Cells were resuspended with 1.6 ml of SET buffer, then freshly prepared lysozyme (final concentration 1mg/ml) was added and incubated at 37˚C for 30min. One ml of 10% SDS was added and mixed by inversion, then incubated at room temperature for 30min, after that 2 ml of 5M NaCl was added and mixed by inversion at room temperature. An equal volume of chloroform was added, mixed by inversion for 15min., then centrifuged (6000rpm at 4˚C) for 20min. The aqueous phase was transferred to another sterile tube and 0.6 volume of isopropanol was added, mixed by inversion and kept at room temperature for 5min., centrifuged at 13000 rpm for 15min at 4˚C. Isopropanol was discarded and the precipitated DNA dissolved in 100 µl of TE buffer and stored at -20˚C.

2.2.11. Agarose gel electrophoresis (Sambrook and Russell, 2001).

Agarose gels (0.7%) were run horizontally in tris-borate-EDTA (TBE1X). Samples of DNA were mixed with 1/10 volume of the loading buffer and added to the wells on gel. Generally, gel was run for 2-3hrs at 5v/cm and the

gel buffer added up to level horizontal gel surface. Agarose gels were stained with the ethidium bromide by immersion them in the distilled water containing the dye at final concentration of 0.5µg/ml for 15-20min. DNA bands was visualized by U.V illumination at 302nm, gels were de-stained in distilled water for 30-60min to get ride of background before photographs were taken.

2.2.12. Role of plasmid in nylon6 degradation

- Curing of plasmid DNA (Trevors *et al*., 1986)

Curing experiments were performed on *Pseudomonas putida* S3A using SDS. This bacterium was grown in 10 ml of LB broth to mid log phase. Then 0.05ml inoculums of the culture were used to inoculate a series of 10ml fresh LB broth flasks containing various concentration of SDS (1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, and 10%). All tubes were incubated at 37˚C for 24-48hrs. The growth density of different tubes was observed by nicked eye and compared with the control to determine the effect of curing agent on bacterial growth. The lowest concentration of curing agent that inhibited the growth of bacterial isolate considered as the minimum inhibitory concentration (MIC).

Samples were taken from tube containing the highest concentration of SDS that allowed the bacterial growth, and diluted appropriately, then 0.1 ml from proper dilutions were spread on trypticase soya agar plates, incubated overnight at 37˚C to score the survival colonies. Those colonies were replica plated (using toothpick) on trypticase soya agar, and on selective medium. If colony was able to grow on the master plate but not on the selective liquid medium containing the nylon6 film, it means that, the cells of the colony are cured cells that lost the ability to grow on

nylon6 as a sole source of carbon and nitrogen. The percentage of cured cells was determined.

The DNA has been isolated from cured cells, and gel electrophoresis was performed in order to compare between the resulting bands with original one.

2.2.13. Optimization of nylon6 film biodegradations

Optimization experiments were carried out through dispensing of 50 ml of mineral salt medium (EM) in 250 ml Erlenmyer flasks, inoculated with 1% of mid- exponential phase of *Pseudomonas putida* S3A. The flasks were incubated in shaker incubator (180rpm) at 37 ˚C. After incubation period, bacterial growth was measured for each sample as mentioned in (2.2.7).

2.2.13.1 Effect of nylon6 film concentration

Nylon6 film was added at different concentrations (0.05%, 0.06%, 0.07%, 0.08%, 0.09%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1%), pH was adjusted to 7.0, and then incubated in a shaker incubator (180 rpm) at 37 ˚C for three and seven days. Bacterial growth was measured and the optimal concentration was employed later on.

2.2.13.2 Effect of Temperature

Mineral salt medium (EM) (pH 7) supplemented with 0.1% of nylon6 film was inoculated and incubated in shaker incubator (180 rpm) at different temperatures 30, 37 and 45 ˚C for three and seven days. Optimal temperature was subsequently employed, depending on the growth density measurement.

2.2.13.3 Effect of pH

Mineral salt medium (EM) supplemented with 0.1%of nylon6 film was prepared at different pH values (6, 6.5, 7, 7.5), in an attempt to determine the suitable pH value, then cultures were incubated in a shaker incubator (180 rpm) at 37 ˚C for three and seven days. The optimum pH value was employed in the later experiment.

2.2.14. FTIR analysis

FTIR analysis was done at the Chemistry Department at the College of Science/AL-Nahrain University. *Pseudomonas putida* S3Awas grown on mineral salt medium (EM) pH 6.5 containing 0.1% of nylon6 film with shaking (180 rpm) at 37 ˚C for seven days. After incubation, samples of nylon6 film were taken and subjected to FTIR analysis. Nature and chemical structure of nylon6 film, that degraded by bacteria, was examined by using Fourier transformed infrared spectroscopy (FTIR) in order to characterize the chemical nature of this compound.

Chapter Three

Results

and

Discussion

Chapter Three

Result and Discussion

3.1. Isolation of nylon6 film degrading bacteria

Soil samples were collected from different regions of Baghdad (Sholla, Topjy, Jamylla, Bayaa and Mahmodya), these regions were contaminated with nylon waste for many years. Sixty-four soil samples were used to isolate nylon6 film degrading bacteria; Degradation of nylon6 film was investigated in a liquid media (mineral salt medium) containing the nylon6 film as a sole source of carbon and nitrogen, and after 7 days of incubation.

Forty-seven of pure bacterial isolates were obtained, the bacteria isolated from Sholla samples termed S, while the bacteria isolated from Topjy termed T. As for Jamylla the isolates termed X, however Bayaa isolates termed B. Finally for the isolates from Mahmodya they were termed M.

These isolates were repeatedly tested in mineral salt medium supplemented with nylon6 film, in order to ensure their degradation ability. Out of 47 bacterial isolates, only 27 isolates showed ability to degrade nylon6 film (Table 3.1). The failure of twenty isolates to degrade nylon6 film may be attributed to their ability of benefiting from the metabolites of the other bacteria (nylon6 film degrader) and degraded it to less complex compounds (Negoro *et al*., 1980; Shama and Wase, 1981).

Results (table 3.1) indicated that out of 27 isolates, 22 were isolated from Sholla, while two were isolated from Jamylla, and one isolate was isolated from each one of Topjy, Bayaa and Mahmody.

The difference between these regions in their content of bacterial isolates that are capable to degrade nylon6 film may be related to the type, size

and history of pollution with nylon waste for these regions. It can be concluded that the main waste in Sholla samples was nylon6 byproducts waste for a long time. Accumulations of nylon waste in soil increase the adaptation of the soil bacteria to degrade nylon (Clark and Slater 1986) or develop the degradation capability of nylon by gene mutation (Hall and Clark, 1977; Kevin and Georgia, 2009).

Table (3.1) The growth density of bacterial isolates in mineral salt medium (pH 7) supplemented with 0.1% of nylon6 film along 7 days of incubation with shaking (180 rpm) at 37 □C.

Growth of bacteria (O.D600 nm)			
Bacterial Isolates	3 days	5 days	7 days
S11	0.044	0.078	0.09
M3	0.09	0.15	0.17
S13	0.049	0.07	0.1
S6	0.06	0.082	0.1
S8	0. 02	0.032	0.08
S12	0.087	0.121	0.17
S3	0.04	0.054	0.1
X1	0.03	0.06	0.05
S7	0.046	0.06	0.09
T1	0.095	0.128	0.14

S20	0.076	0.09	0.13
S22	0.048	0.063	0.077
S23	0.08	0.1	0. 13
S17	0.09	0.12	0. 189
S14	0.035	0.05	0.076
S1	0.065	0. 09	0. 134
B	0.07	0.1	0.114
X	0.031	0.047	0.058
S4	0.034	0.05	0.08
S3A	0.160	0.183	0.240
S7	0.015	0.03	0.056
S2	0.0356	0.054	0.078
S9	0.037	0.046	0.054
S15	0.0576	0.078	0.1
S10	0.0247	0.045	0.087
S19	0. 0386	0.0798	0. 09
S18	0.0590	0.0728	0.089

3.2. Screening of nylon6 film degrading bacteria

All the isolates (27 isolates) were screened for their ability to degrade nylon6 film according to growth density at 600nm along seven days of incubation (Table 3.1). Twenty isolates were selected for identification (section 3.3) which is showed good ability in degrading nylon6 film.

However, it is difficult to expect which molecular change can be expected by a specific microbe, since each group of microorganisms, even various strains of one genus, can alter a selected molecule differently (Alexander, 1980).

Result indicated that there was, in general, a lag phase of three days before growth ensured, as judged by an increased in the turbidity of the culture and that may be attributed to the various molecular mechanisms enable microorganisms to recruit genes and modified nucleotide sequence in the structural and regulatory genes to enhance expression and use synthetic compound as substrate (Chakrabarty, 1976). Kulkarin and Kanekar (1998a) reported that *Flavobacterium* sp. K172 and *Pseudomonas* sp. NK87 strains almost completely utilized 1% of the cyclic dimer of nylon6 within 24h.

A distinct difference was observed in growth rate and pattern of utilization of caprolactam, which *Alcaligenes faecalis* showed a better growth rate than *Alcaligenes citrus* and reach a stationary phase by 10h while *Alcaligenes citrus* approach the same stage by 40h (Baxi and Shah 2001). Fredrich *et al*., (2007) found that only about 10% of nylon6 fiber was damaged by fungal strain *Bjerkandera adusta* after 10 days.

It is known that all microbial degradation process is caused by enzyme and since all nylon6 and nylon6 byproduct degradation required efficient system for degradation. However, the efficiency of degradation is highly dependent on specific enzyme that can catalyze the desired reaction (Kawashima *et al*., 2009).

The result indicated also (Table 3.1) that the most efficient isolates were T1, M3, S3A, S12 and S17; however, S3A isolate is the best one. These isolates showed respectable density growth compared with other isolates throughout the incubation period, and this may be attributed to physiological and genetic properties of these isolates, these five isolates were used for further study.

3.٣. Identification of bacterial isolates

Twenty isolates were selected for identification according to their ability to degrade nylon6 film as mentioned above. These isolates were identified depending on morphological, cultural and biochemical characteristics. Bacterial isolates revealed different colonial appearance depending on the selective or differential media used.

Results (Table 3.2) showed that these isolates were characterized as a *Pseudomonas putida* (14 isolates) *Moraxella* spp. (4 isolates), *Pseudomonas stutzeri* (1 isolate) and *Pseudomonas* sp. (1 isolate). The morphological and biochemical test results (table 3.2) for these isolates were in agreement with Holt *et al.* (1994).

Table (3.2) Morphological, physiological and biochemical characteristics of isolated bacteria

Characters	Strains						
	S22	S12	X	S11	X1	B	S7
Colony color	White	White	White	White	White	White	White
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram stain	-	-	-	-	-	-	-
Catalase production	+	+	+	+	+	+	+
Oxidase production	+	+	+	+	+	+	+
Growth on King A	(+)*	(+)*	(+)*	-	(+)*	(+)*	(+)*

Growth on King B		(+)*	(+)*	(+)*	-	(+)*	(+)*	(+)*
Growth on Cetrimide		+	+	+	-	+	+	+
Gelatin hydrolysis		-	-	-	-	-	-	-
Growth on 4°C		-	+	+	-	+	+	+
Growth on 42°C		-	-	-	-	-	-	-
Urea hydrolysis		+	+	+	-	+	+	+
Starch hydrolysis		+	+	-	+	-	-	-
Citrate utilization		+	+	+	+	+	+	+
Methyl red		-	-	-	-	-	-	-
Vogas-Proskauer		+	+	+	+	+	+	+
Growth on Kilgher`S Iron agar	Slant	Alkaline	Acid	Alkaline	Acid	Alkaline	Alkalin	Alkaline
	Butt	Acid	Acid	Acid	Acid	Acid	Acid	Acid
	H_2S production	-	-	-	-	-	-	-
	Gas formation	-	-	-	-	-	-	-

Characters	Strains						
	S9	S19	S23	S6	S13	S1	T1
Colony color	White	White	White	White	White	White	White
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram stain	-	-	-	-	-	-	-
Catalase production	+	+	+	+	+	+	+
Oxidase production	+	+	+	+	+	+	+
Growth on king A	(+)*	(+)*	(+)*	-	-	-	(+)*
Growth on king B	(+)*	(+)*	(+)*	-	-	-	(+)*
Growth on Cetrimide	+	+	+	-	-	-	+

Gelatin hydrolysis		-	-	-	-	-	-	-
Growth on 4°C		+	+	+	-	-	-	+
Growth on 42°C		-	-	-	-	-	-	-
Urea hydrolysis		+	+	+	-	-	-	+
Starch hydrolysis		-	-	-	+	+	+	-
Citrate utilization		+	+	+	+	+	+	+
Methyl red		-	-	-	-	-	-	-
Vogas-Proskauer		+	+	+	+	+	+	+
Growth on Kilgher`S Iron agar	Slant	Alkaline	Alkaline	Alkaline	Acid	Acid	Acid	Alkaline
	Butt	Acid	Acid	Acid	Acid	Acid	Acid	Acid
	H_2S formation	-	-	-	-	-	-	-
	Gas formation	-	-	-	-	-	-	-

Characters	Strain					
	S17	S3A	M3	S18	S14	S20
Colony color	White	White	White	White	White	White
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod
Gram stain	-	-	-	-	-	-
Catalase production	+	+	+	+	+	+
Oxidase production	+	+	+	+	+	+
Growth on king A	(+)*	(+)*	(+)*	(+)*	(+)*	(+)*
Growth on king B	(+)*	(+)*	(+)*	(+)*	(+)*	(+)*
Growth on Cetrimide	+	+	+	+	+	+

Gelatin hydrolysis		-	-	-	-	-	-
Growth on 4 °C		-	+	-	-	-	-
Growth on 42 °C		-	-	-	-	-	-
Urea hydrolysis		+	+	+	+	+	+
Starch hydrolysis		-	-	-	-	-	-
Citrate utilization		+	+	+	+	+	+
Methyl red		-	-	-	-	-	-
Vogas-Proskauer		+	+	+	+	+	+
Growth on Kilgher`S Iron agar	Slant	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline
	Butt	Acid	Acid	Acid	Acid	Acid	Acid
	H_2S formation	-	-	-	-	-	-
	Gas formation	-	-	-	-	-	-

(+*)= growth without pigment

Pseudomonas putida = X, X1, B, S7, S9, S19, S23, T1, S17, S3A, M3, S18, S20, S14

Morexella spp. = S11, S1, S13, S6

Pseudomonas sp. = S12

Pseudomonas stutzeri = S22

The results indicated that 80% of identified bacteria were belonged to genus *Pseudomonas*; all of them were *P. putida* except two. *Pseudomonas* strains are known to exhibit a wide range of metabolic activity against the most natural and xenobiotic compounds because of their extreme nutritional versatility (Chakrabarty, 1976; Holloway *et al*., 1979; Ramos *et al*., 1994). Nasier *et al*., (2002) recorded that *Pseudomonas* spp. were the most abundant and the most efficient bacterial species in utilizing crude oil and different hydrocarbon

compounds in Iraqi soil. Several attempts have been made to degrade nylon6 and nylon6 byproduct, the degree of microbial degradation has been shown to be lower the larger molecule (Fredrich *et al*., 2007). Numerous microorganisms including the bacteria *Pseudomonas*, *Achromobacter, Corynebacterium*, and fungal genera *Absidia*, *Aspergillus*, *Byssochlamis*, *Penecillium*, *Rhodotorula* and *Trichosoron*, metabolize a nylon6 monomer (a cyclic form □- caprolactam or its linear form 6-aminohexanoic acid) (Fukumara 1966 ; Shama and wase, 1981). In an oligomer consisting of a small number of monomeric units of nylon6, biodegradation was observed only with *Pseudomonas* and *Flavobacterium* (Irfan *et al*., 1995; Negoro, 2000).

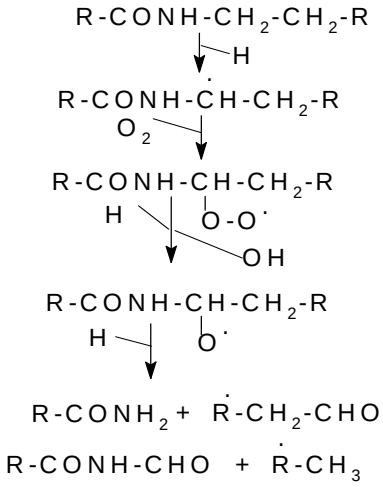
Tomita *et al*., (2003) found a thermophilic bacterium related to *Bacillus pallidus* that degrade nylon12 and nylon6 but not nylon66. Marine isolated bacteria *Bacillus cereus, Bacillus sphericus*, *Vibrio furnisii* and *Brevundimonas vesicular* were shown to degrade nylon6 and nylon66 in mineral salt medium (Sudhakar *et al*., 2007).

3.4. Nylon6 film preparation

To insure isolation of bacteria capable to degrade nylon6, attempts were performed to make it more susceptible to attack by bacteria through processing it as mentioned in (2.2.3).

Figures (3.1) and (3.2) showed the FTIR of unirradiated nylon6 and irradiated nylon6 respectively.

Results of FTIR spectra indicated that when the nylon6 film samples, irradiated with wavelength (λ =313 nm), appearance of new carbonyl group after photodegradation of nylon6, which appeared in absorbance at the wave number 1720 cm-1 for aldehyde group and as following mechanisms for photodegradation of nylon6 (Scheme 3.1)



Scheme (3-1): Proposed mechanisms of nylon6 degradation by UV-Visible (Negoro, 2000)

In general, during UV-irradiation of polymers the concentration of functional groups on the chain ends and inside macromolecules (double bonds and carbonyl groups) increases. It probably makes polymers more susceptible to attack by bacteria in natural environment. It is also well known that the efficient main chain scission in irradiated polymers causes their mechanical deterioration and breaking on to small pieces. Thus, the access of oxygen and microorganisms is facilitated to the bulk of such destroyed products. In this way, polymers become biodegradable. (Unichiyama and Tabuchi, 1998; Zainab, 2006; Artham and Doble, 2007).

From the above results, it can be concluded that the irradiated nylon6 became more susceptible to degrade than unirradiation nylon6, where U.V light increases photodegradation of polymer (nylon6) into small pieces forms, then the molecular weight was reduced.

Successfully using of nylon6 film for isolation of nylon6 film degrading bacteria, as mentioned before (section 3.1), proved that nylon6 film was susceptible to attack and metabolize by soil bacteria. The use of

biodegradation offers a cheap method for recycling nutrients efficiently and, when optimized, at a faster rate than under natural conditions. It would appear to be low in its energy requirements (Tokiwa *et al*., 2009).

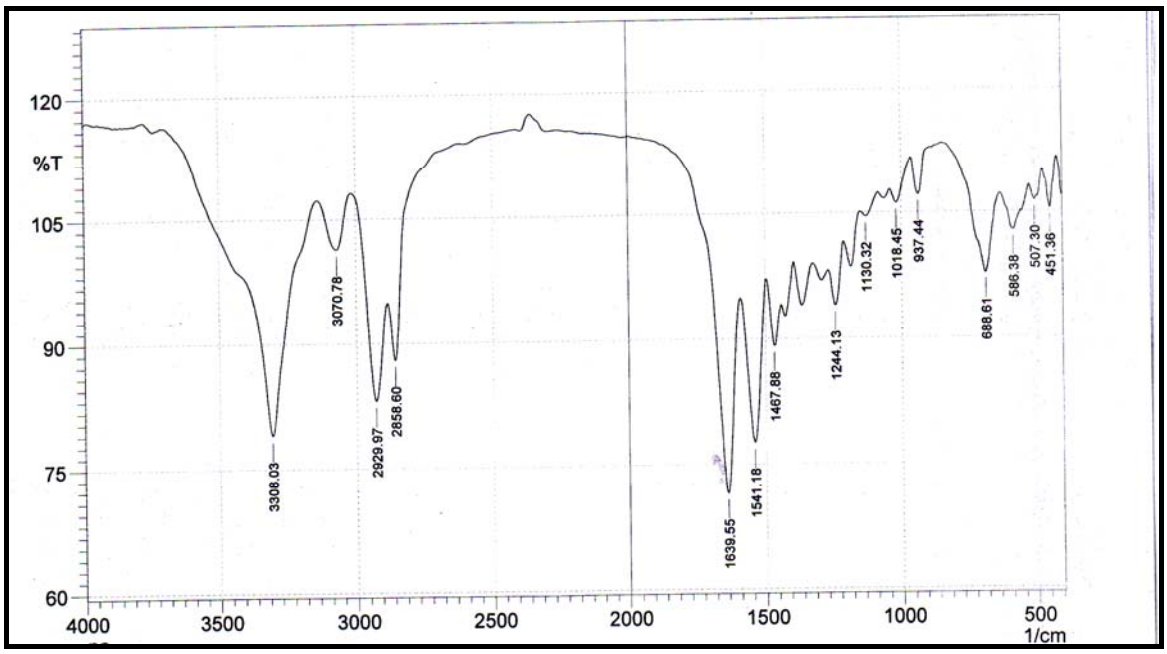


Figure (3.1): FTIR spectrum of unirradiated nylon6 film

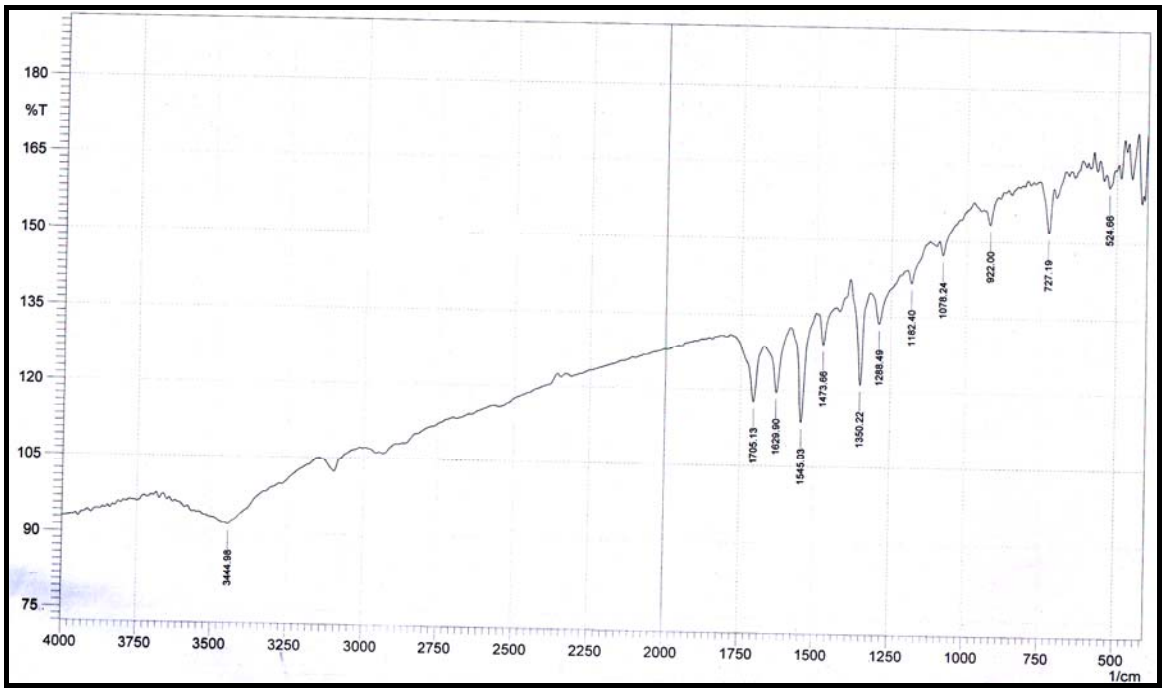


Figure (3-2): FTIR spectrum of irradiated nylon6 film.

3.5. Plasmid isolation

In order to determine the plasmid profile of *P. putida* S3A, a number of methods were used, the alkaline lysis and boiling methods (Sambrook and Russell, 2001), the alkaline lyses method that modified by Pospiech and Newman (1995) and salting out method (Kieser, 1995). Results indicated that the later method gave the best results. Figure (3.3) indicated that *P. putida* S3A containing a small plasmid DNA bands and these bands are approximately in the same size compared with the pBR322 plasmid (4.363Kb).

Several studies referred that the small plasmid DNA bands are prevalence in *Pseudomonas* sp. and responsible for degradation of different hydrocarbon and synthetic compounds (Kanagawa *et al*., 1989; Abd Al-Hussan, 2006; John and Okpokwasili, 2012).

Several studies indicated also that a different *Pseudomonas* isolates containing plasmids with same size but have different set of genes (Bayly and Barbour, 1984), or the plasmids are the same plasmids but there were transfered between the soil population of bacteria by conjugation or transformation (Devereux and Sizemore, 1982; Trevors, 1998).

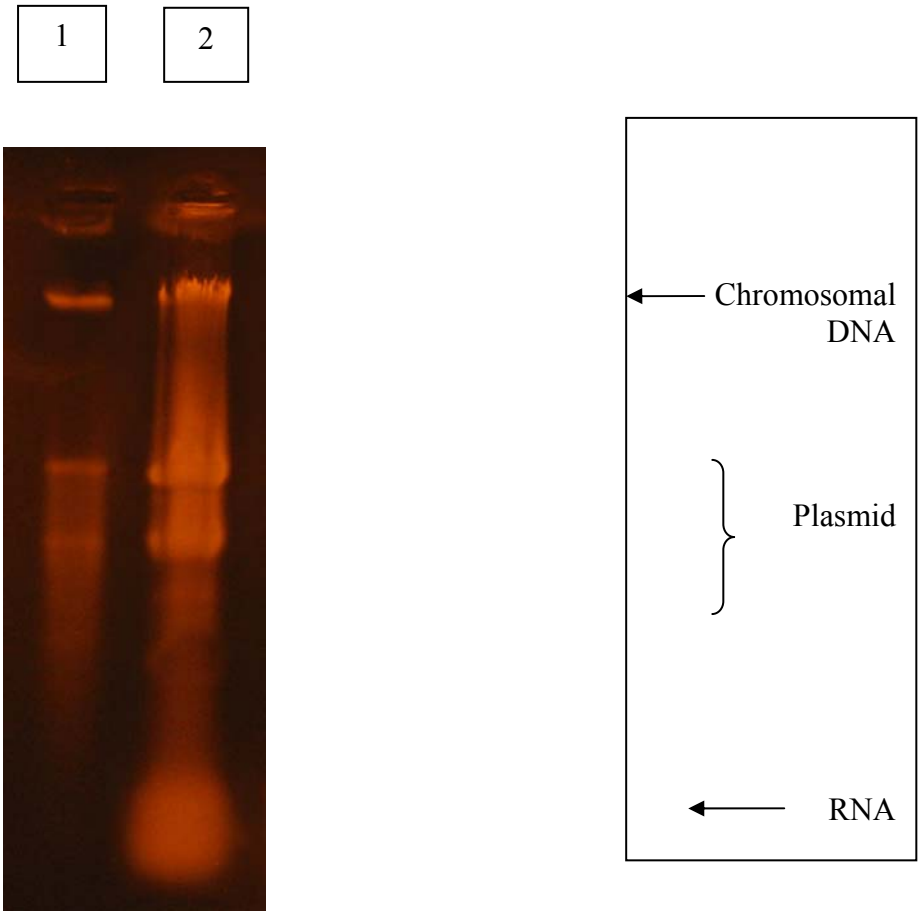


Figure (3-3): Gel electrophoresis of isolated plasmid from bacterial isolate *P. putida* S3A Migrated on agarose gel (0.7%) in TBE buffer at (5V/cm). 1) *E.coli* HB101, 2) *P. putida* S3A

3.6. The role of *Pseudomonas puti*da S3A plasmid(s) in degradation of nylon6 film

In order to study the role of plasmid(s) of *P. putida* S3A in degradation of nylon6 film, curing experiment was performed.

This bacterium was candidate to study the role of their plasmid(s) in degradation of nylon6 film, hence this isolate was the most efficient isolate, it was observed growth density and growth yield more than other isolates.

-Curing with SDS

Many attempts were made in order to cure *P. putiada* S3A using SDS. Results in (Table 3.3) indicated that the highest concentration of SDS that still allows the bacterial growth, was 6%. From this treatment of curing agent, appropriate dillutions were made and spread on trypticase soya agar plates, then 40 colonies were tested on selective media (mineral salts medium containing nylon6 film) in order to determine the cured colonies, which cannot degrade, nylon6 film.

After repeatedly plated of the selected colonies, to insure that these colonies are unable to degrade nylon6 film, results indicated that two colonies (5%) failed to grow on nylon6 film as a sole source of carbon and nitrogen.

To determine the failure of the colonies to grow on nylon6 film, was because plasmid elimination or not, plasmid isolation from one of the cured colonies was made and compared with that from original isolate. The results indicated that there was a change in the plasmid patterns between the cured and the original isolate. Cured isolate (*P. putida* S3AC1) was lost its plasmid DNA bands (Figure 3.4), and this referred that the plasmid DNA bands could be responsible for degrading nylon6

film in *P. putida* S3A. In other word, gene(s) responsible for degradation and/or regulation of nylon6 film are located on plasmid(s) in this bacterium.

Table (3.3) Effect of SDS on the growth of *P. putida* S3A.

SDS concentration (%)	Bacterial growth
1	+++
2	+++
3	++
4	++
5	+
6	±
7	-
8	-
9	-
10	-

(-): No growth.
(±): Slightly growth.
(+): Modorate growth.
(++): Good growth.
(+++): Very good growth.

Many studies reported that SDS was used successfully as a curing agent for plasmids of *Pseudomonas* spp. (Aislablie *et al*., 1990; Trevors, 1998; Nasier *et al*., 2004).

Plasmid involved in degradation of nylon6 and nylon6 by-product were isolated in previous studies. Okado *et al*., (1983) and Kato *et al*., (1995) found that Plasmid pOAD2 in *Arthrobacter* sp. encoded the enzyme that degrade, 6-aminohexanoate-linear-dimer, by-product of nylon6. It was found that *Pseudomonas* sp. NK87 harbored six kinds of plasmids and two of them are responsible for degradation of nylon6

(Kanagawan *et al*., 1989). Negoro *et al*., (1980) and Negoro and Okada (1982) indicated that *Flavobacterium* sp. K172 harbors three kinds of plasmids but only one plasmid was responsible for degradation of nylon6. Ponamoreva *et al.,* (2010) reported that *Pseudomonas putida* BS394 harbors plasmid (pBS268) that encoded enzymes responsible for caprolactam degradation.

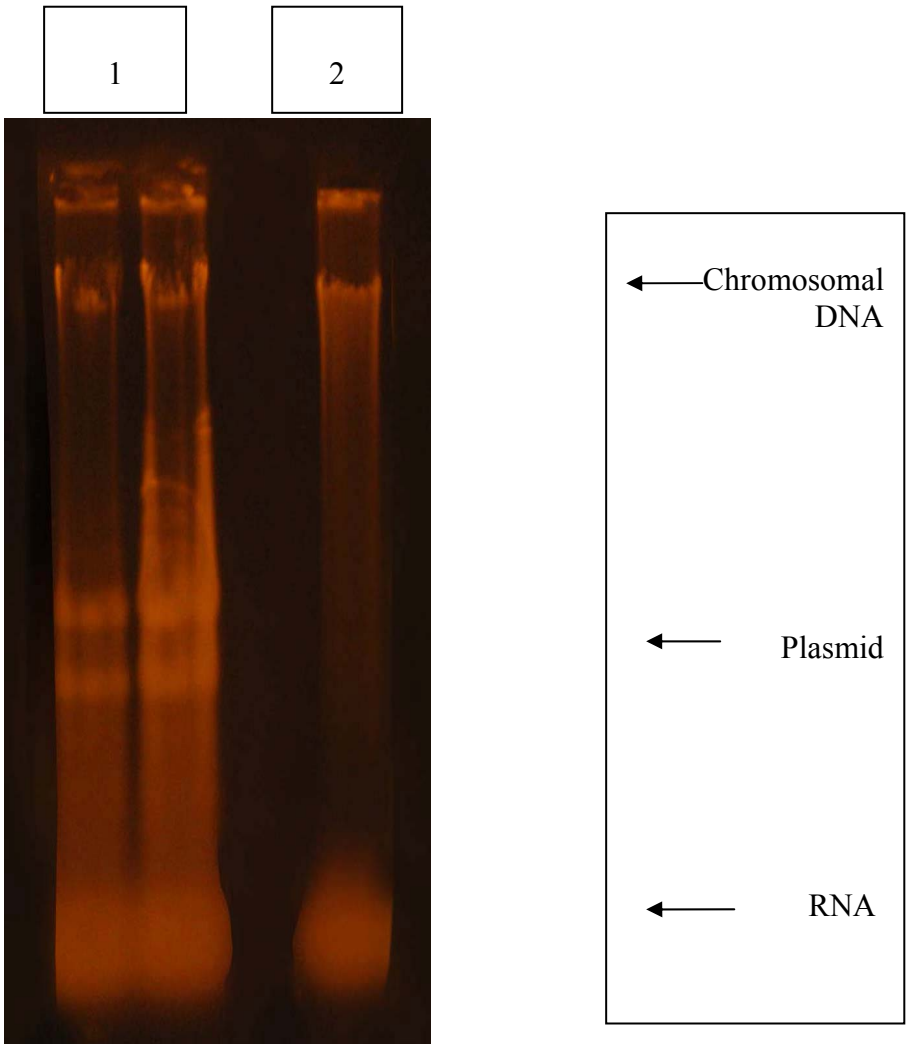


Figure (3-4): Gel electrophoresis of plasmid content of *P. putida* S3A before and after treatment with SDS on agarose gel (0.7%) in TBE buffer at 5V/ cm.

1) The original isolate, 2) Cured isolate (*P. putida* S3AC

3.7. Optimization of nylon6 degradation by *P. putida* S3A.

3.7.1. Effect of nylon6 film concentration

Different concentrations (between 0.05% and 1%) of nylon6 film were used to grow *Pseudomonas putida* S3A in order to determine the optimum concentration. Results in Figure (3.5) indicated that the optimum concentration for bacterial growth was 0.1% which the optical density of bacterial growth, at this concentration, after three and seven days of incubation were (OD_{600}=0.2) and (OD_{600}=0.267) respectively. Figure (3.5) showed also that gradual increasing of nylon6 film concentration accompanied with increasing of bacterial growth, and then the growth reached to its optimum at a concentration of 0.1%, while nylon6 film concentrations higher than 0.1% showed decrease in bacterial growth. Holmes (1988) and Sand (2003) reported that availability of water, temperature, oxygen usage, minerals, pH, redox potential, carbon and energy source influence the growth of microorganisms. Baxi and Shah (2001) reported that caprolactam concentration for screening of caprolactam degrading microorganisms was 1% (w/v) and 0.5% (w/v) for subsequent comparison of degradative ability.

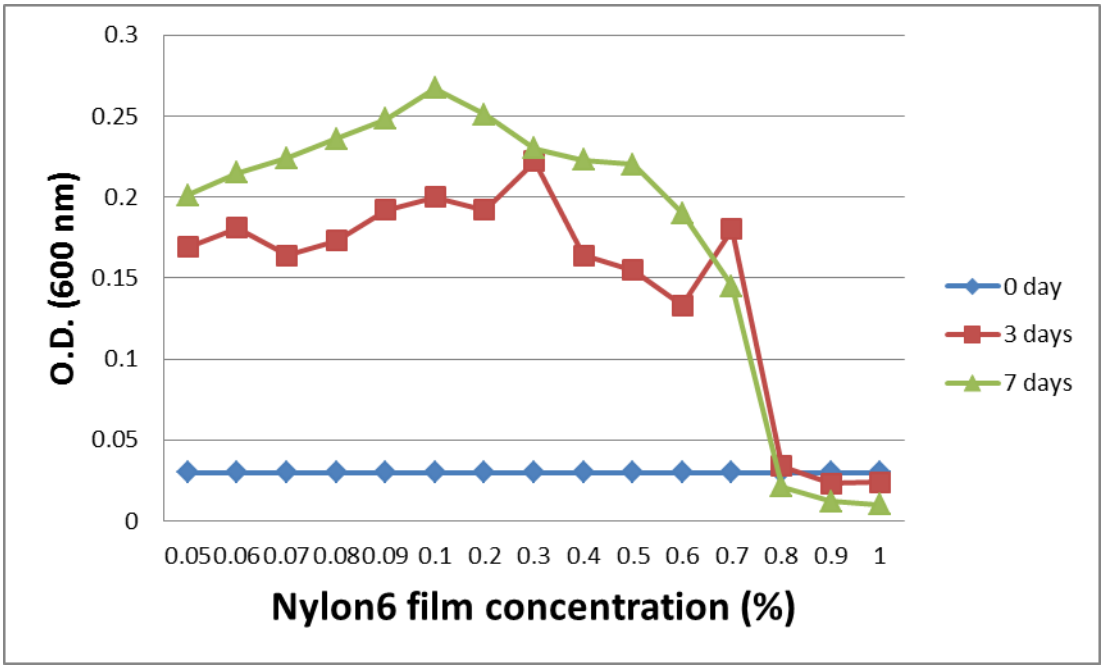


Figure (3-5): Effect of nylon6 film concentration on *Pseudomonas putida* S3A grown in mineral salt medium (pH 7) in shaker incubator (180rpm, 37°C) for 3 and 7 days.

3.7.2. Effect of temperature

Pseudomonas putida S3A was grown and incubated at different temperatures (30, 37 and 45 ˚C). Results shown in Figure (3.6) pointed out that the optical density of bacterial growth at 37 °C was OD_{600} = 0.21 and OD_{600} = 0.276 after three and seven days of incubation respectively, which was suggested as the optimum temperature for bacterial growth. Relative result of bacterial growth was recorded at 30 °C. Whereas, at 45 ˚C, bacterial growth was lower than at other incubation temperatures. Negoro *et al*., (1980) pointed that *Flavobacterium* are able to degrade 6-aminohexanoic cyclic dimer (waste of nylon6) at 30 °C. It was found that the optimum temperature for 6-aminohexanoate oligomer hydrolase enzyme was between 30°C and 45°C (Heumann *et al*., 2008). Optimum temperature for nylon6 degradation by *Alcaligenses* sp. was between 30°C and 35°C (Klun *et al*., 2003).

Irfan *et al*., (1995) noticed that optimum temperature for nylon6 degradation by *Pseudomonas aeruginosa*, which isolated from activated sludge of nylon6 factory, was 30°C.

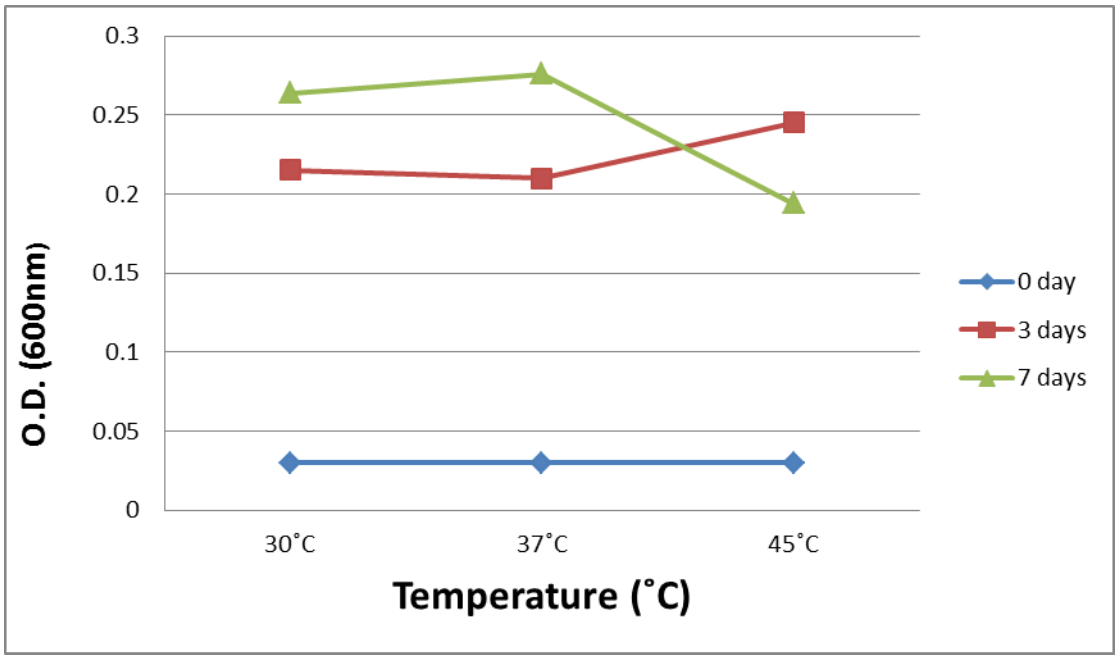


Figure (3-6): Effect of temperature on nylon6 film degradation by *P. putida* S3A grown in mineral salt medium (pH 7) containing 0.1% of nylon film in shaker incubator (180rpm) for 3 and 7 days.

3.7.3. Effect of pH

Mineral salt medium was prepared at different pH values (6, 6.5, 7 and 7.5) in an attempt to determine the optimum pH required for growth of *P. putida* S3A on nylon6 film. The obtained results as shown in Figure (3.7) elucidated that an optimum growth was occurred at pH 6.5; the optical density for bacterial growth was reached 0.319 and 0.430 after three days and seven days respectively. While bacterial growth was decreased at other pH values compared with growth at pH 6.5.

Within the pH 6 the optical density for bacterial growth was 0.215 and 0.197 after three and seven days respectively, while within pH 7 and pH7.5 was considered dramatic increase and decrease in the growth, with pH 7 the optical density for bacterial growth was 0.190 and 0.27 after three and seven days respectively, with pH 7.5 the optical density for bacterial growth was 0.242 and 0.198 after three and seven days respectively.

Kinoshita *et al*., (1977) described that the pH for nylon6 oligomer degradation by *Flavobacterium* sp. K172 ranging between pH 5.5 to pH 8.5. The optimum pH required to degrade nylon6 oligomer was 6.3 by *Pseudomonas aeruginosa* NK87 (Kanagawa *et al*., 1989). While suitable pH for nylon4 film degradation by *Stenotrophomonas* sp. and *Fusarium* sp was found to be 7.2 (Koichirio *et al.*, 2010). Baxi and Shah (2001) found that pH 7.2 was suitable for caprolactam degradation by *Alcaligenes faecalis*.

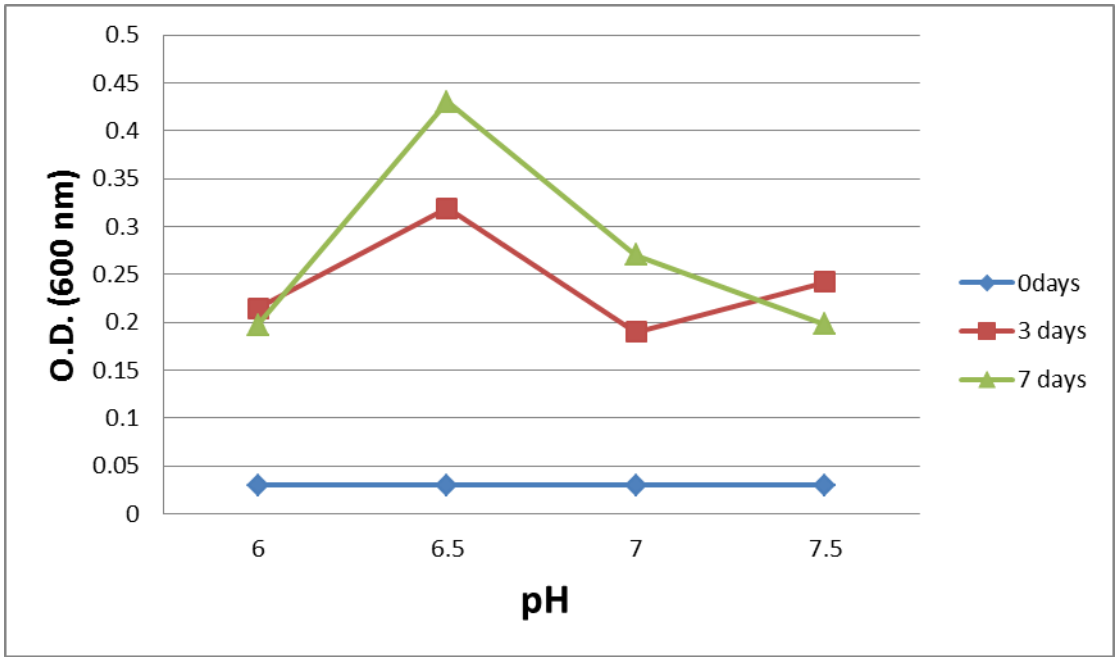


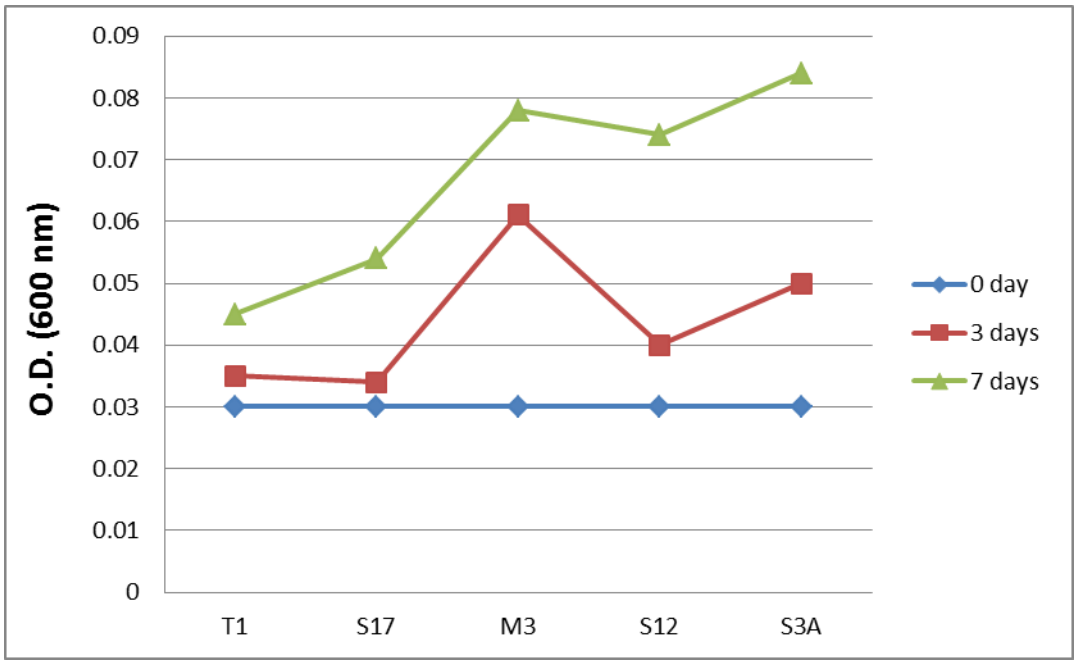
Figure (3-7): Effect of pH on nylon6 film degradation by *Pseudomonas putida* S3A grown in mineral salt medium containing 0.1% of nylon film in shaker incubator (180rpm, 37°C) for 3 and 7 days.

3.8. Growth on crude nylon6 and nylon66

In an attempt to investigate the ability of bacteria to degrade crude nylon, the efficient five isolates that showed potential ability to grow on nylon6 film chose to grow on crude nylon6 and nylon66 as a sole source of carbon and nitrogen. Result in Figure (3.8) showed the growth of these isolates on the two nylon compounds for three and seven days of incubation. It was observed that both compounds supplemented the growth of *P. putida* S3A and maximum growth was recorded for this isolate compared with other isolates. It was observed also that *P. putida* T1 showed moderate growth on nylon6 but failed to grow on nylon66, while *P. putida* M3 showed opposite behavior. *Pseudomonas* sp. S12

showed moderate growth on nylon66 and slightly growth on nylon6, while *P. putida* S17 showed slightly growth on both compounds

(A)



(B)

Figure (3-8): growth of bacterial isolates in mineral salt medium (pH 7), incubated in shaker incubator (180rpm, 37°C) for 3 and 7 days and containing 0.1% of crude nylon6 (A) or crude nylon66 (B).

From these results, it was appeared that nylon66 is more susceptible to degrade than nylon6 by these isolates, which could possibly be due to its structure. Nylon66 is formed by condensation of hexamethylene diamine and adipic acid while nylon6 is formed by the ring opening caprolactam. So the repeated unit of the nlon66 has twice the number of C-N bond when compared to repeated unit of the nylon6 (Sudhakar *et al*., 2007).

Koichirio *et al*., (2010) referred that *Stenotrophomonas* sp. and *Fusarium* sp. were capable to degrade nylon4 but not nylon6 and nylon46. Friedrich *et al*., (2007) demonstrated that nylon6 disrupted by fungus *B. adusta* and that only whit root fungi are able to break down nylon6. It was found that *Brevundimonas vesicualris* able to grow on nylon66, reaching to stationary phase but no growth was seen on nylon6 (Sudhakar *et al*., 2007). Tomita *et al*., (2003) report that a thermophilic strain *Bacillus pallidus* capable of degrading nylon6 and nylon12 by enrichment culture technique at 60 °C, but not nylon 66.

3.9. Characterization of nylon6 film

In order to confirm the ability of bacteria to degrade nylon6 film, *Pseudomonas putida* S3Awas grown on mineral salt medium(pH 6.5) containing 0.1% of nylon6 film at 37 ˚C for seven days, after that nylon6 film was subjected to the Fourier Transformer Infrared Spectroscopy (FTIR) to detect its structure.

FTIR spectrum (Figure 3.9), compared with spectrum of nylon6 film (Figure 3.2) before bacterial growth, showed the following:

-The spectrum of nylon6 film before bacterial growth, showed band in absorbance at wave number 3303 cm^{-1}, which assaying to the stretching of N-H group, Bands at 2929 and 2860, which attributed to the aliphatic C-H stretching, and showed also bands at 1647, 1511 and 1373, which

represents C=O stretching of amide group (amide I), N-H bending (amide Π) and C-H bending respectively (Figure 3.2).

The intensity of N-H band, C=O band and C-H aliphatic band were lower and new groups were formed (Figure 3.9), and this mean that bacteria caused this effect.

It can be concluded that nylon6 film degraded by locally isolated *Pseudomonas putida* S3A, which used these groups (N-H, C=O and C-H) as carbon and nitrogen source. Sudhakar *et al*., (2007) referred that the FTIR for both nylon 6 and nylon66 that degraded by marine bacteria showed formations of the new groups. These new groups may be formed due to the process of hydrolysis as oxidation. Negoro *et al*., (1992) found that White rot fungus strain IZU154 was able to degrade nylon 66 with formation of CHO, NHCHO, CH2 and CONH2 group. Tomita *et al*., (2003) demonstrated that some of white rot fungal strains can degrade nylon 66 and make changed in the surface ofnylon6 fiber.

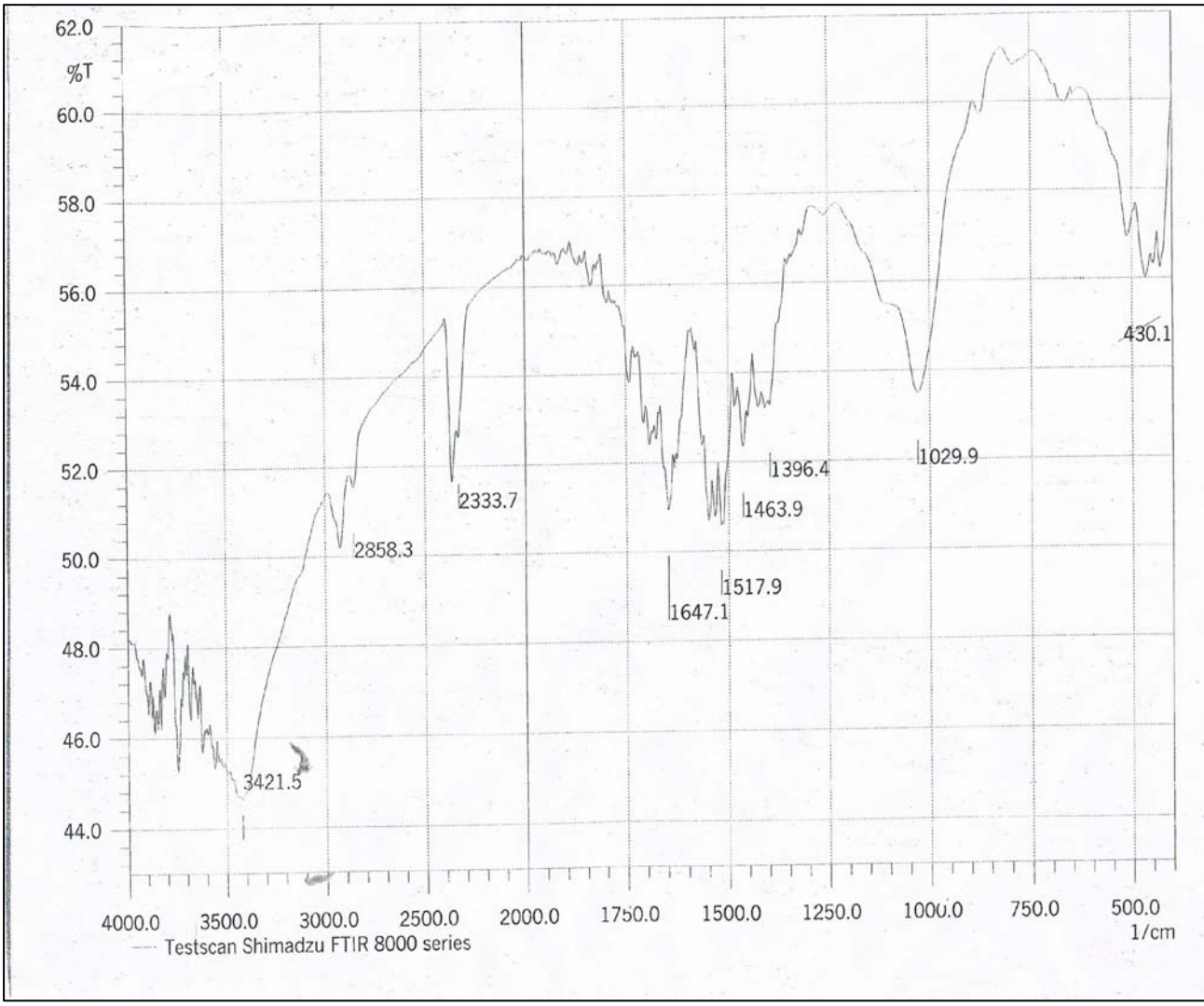


Figure (3-9): FTIR spectroscopy of nylon6 film after grown of *Pseudomonas putida* S3A in mineral salt medium (pH 6.5) containing 0.1% of nylon6 film in shaker incubator (180rpm, 37 ˚C) for seven days.

Chapter Four
Conclusions
and
Recommendations

4.1. Conclusions:

1. Soil contaminated with nylon waste was rich with bacteria capable to degrade nylon6 film.
2. The members of genus *Pseudomonas* were the most prevalence in degradation nylon6 film and *P. putida* S3A is the most efficient isolate.
3. Ability of *P. putida* S3A to degrade nylon6 film was enhanced under optimum conditions (growing this bacterium in mineral salt medium (pH 6.5) containing 0.1% of nylon6 film and incubated with shaking (180rpm) at 37 ˚C for seven days.)
4. The trait of degradation of nylon6 film is controlled by plasmid in *P. putida* S3A isolate.
5. Processing nylon6 and transferring it to a filmmakes itmore susceptible to degrade by bacteria. Nylon6 film was degraded by *P. putida* S3A, which used the (N-H, C=O and C-H)groups as carbon and nitrogen source.
6. Crude nylon6 and nylon66 can be degraded by some bacterial isolates and nylon66 is more susceptible to degrade.

4.2. Recommendations:

1. Screening the isolates for their ability to degrade other commonly used polymers.
2. Further studies are required to isolate other bacteria capable to degrade different types of nylon.
3. Studying the biochemical pathways of nylon6 degradation by microorganisms and characterize the intermediate compounds.
4. Characterize, purify and study the best conditions for nylon6 degrading enzymes of *P. Putida* S3A.
5. Determining the plasmid role in nylon degradationfor efficient isolates and confirm the role of *P. Putida* S3A plasmid by transforming the cured strain (S3AC1) with its plasmid DNA.
6. Attempts to develop efficient strains for degrading nylon via conjugation, transformation and genetic engineering techniques.

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الخلاصة

تم جمع ٦٤ عينة تربة (ملوثة بنفايات النايلون لسنوات عديدة) من أماكن مختلفة في بغداد.من هذه العينات تم الحصول على ٤٧ عزلةبكتيرية عدت محللة لرقائق النايلون٦. كرر أختبار هذه العزلات مرارا وذلك بتنميتها في وسط الاملاح المعدنية الحاوي على رقائق النايلون٦ لضمان قدرتها على التحليل؛وتبين أن ٢٧ عزلة فقط اظهرت القدرة على تحليل رقائق النايلون٦ كمصدر وحيد للكاربون والنايتروجين. غربلت هذه العزلات (٢٧عزلة)لقدرتها على تحليل رقائق النايلون٦تبعا لكثافة نموها، وقد وجد ان هذه العزلات كانت متباينة في كثافةالنمو. وان العزلات الأكثر كفاءة هي M3S3,S12, S17, T1,ولكنالعزلةS3Aهيالأفضلمن بين تلك العزلات.

وتبعا لكثافةالنمو،تماختيار ٢٠ عزلةلتشخيصها. شخصت تلك العزلاتاعتماداعلىالمواصفات المظهريةوالمزرعيةوالكيموحيوية. وأظهرتالنتائجأن ١٦ عزلة تعود لجنس *Pseudomonas* spp. (١٤عزلة*Pseudomonas putida*،عزلة واحدة لكل من*Pseudomonas stutzeri*و Sp.*Pseudomonas*)،فيحينأنالأربع عزلات الآخرىتعود لجنس.*Moraxella* spp.

درسالنمطالبلازميدي للعزلة*Pseudomonas putida*S3A. وأظهرتالنتائجأنهذه العزلة تحتوي على حزم دنا بلازميدية صغيرة. ولغرض دراسةدوربلازميد هذه البكتريا فيتحللرقائق النايلون6،تمتحييد هذه البكترياباستخدام مادةSDS،وأظهرتالنتائج أناثنينمنالمستعمراتفقدتقدرتهاعلىتحليل رقائقالنايلون6كمصدروحيدللكربونوالنيتروجين. عزل الدنا البلازميدي من أحدى العزلتين وتبين انها كانت فاقدةللدنا البلازميدي، مما يشير الى ان حزم الدنا البلازميدي ربما تكون مسؤالة عن تحلل و/أو تنظيم عملية تحلل رقائق النايلون٦في بكتريا*P. putida* S3A.

أختبرتالظروفالمثلىلتحلل رقائقالنايلون٦بفعل بكتريا*P. putida* S3A. وجدأنهذهالظروف هيبتنميةهذهالبكتيريافيوسطالأملاحالمعدنية (برقمهيدروجيني ٦.٥) الحاويعلى ٠.١٪ من رقائقالنايلون٦والحضنمعالتحريك (١٨٠دورة/دقيقة) بدرجة ٣٧°ملمدةسبعةأيام. وجدأيضاأنهذهالبكتيرياقادرةعلىالبقاءحتىتركيز ٠.٧% منرقائقالنايلون ٦.

وفيمحاولةلأختبارقدرةالبكتيرياعلى تحليلالنايلونالخام،نميت العزلات الخمسالكفؤة (T1،M3،S3A،S17 S12) علىالنايلون٦الخاموالنايلون٦٦الخام كمصدروحيدللكربونوالنيتروجين.

وأظهرتالنتائجأنالنايلون٦٦هوالأكثرعرضةللتحللمنالنايلون٦بفعلهذهالعزلات،وان اقصى نمو سجل بواسطة العزلة *P. putida* S3A.

لضمانعزلبكتيرياقادرةعلىتحليلالنايلون٦ الخام،تمتمعالجةالنايلون6بواسطةأشعةUVوحولإلىرقائق،أستخدمتلعزلالبكتيريا. ومن خلال نتائج اختبارطيف الأشعة تحت الحمراء (FTIR)،أمكنالأستنتاجبأنالنايلون6المشعةأصبحأقلصعوبةعلىالتحللوذلك لأن الأشعةفوقالبنفسجية قد حللت ضوئياالنايلون6إلىقطعصغيرة،وقللتوزنه الجزيئي. كماأمكنالأستنتاج أيضاان بكتريا*putida* S3A*P.* قد حللترقائقالنايلون٦ واستغلتمجاميع (N-H،C = Oو H-C) كمصدر للكربونوالنيتروجين.

إلى ...

عالم ومجتهد آل محمد(صلى الله عليه واله وسلم)

أهدي ثمرة جهدي المتواضع

علي

بِسْمِ اللَّـهِ الرَّحْمَنِ الرَّحِيمِ

وَلَقَدْ كَتَبْنَا فِي الزَّبُورِ مِنْ بَعْدِ الذِّكْرِ أَنَّ الْأَرْضَ يَرِثُهَا عِبَادِيَ الصَّالِحُونَ(١٠٥)

صدق الله العلي العظيم

سورة الأنبياء

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين

كلية العلوم

عزل وتشخيص و ووراثة *Pseudomonas putida* المحللة لنايلون٦

رسالة

مقدمة الى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل دراسة الماجستير علوم في التقانة الاحيائية

من قبل

علي عدنان جعفر الصراف

بكلوريوس تقانة احيائية

٢٠٠٩

بإشراف

أ.د. ماجد حسين الجيلاوي

شوال-١٤٣٣ ايلول- ٢٠١٢