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Study on Degradation of Nylon 6 by thermophilic bacteria *Anoxybacillus rupiensis* Ir3 (JQ912241)

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Abstract

Polyamide-6 (also known as nylon-6) is one of the biodegradable-resistant synthetic polymers used in the manufacturing of commodity plastic materials. The environmental effects of the persistence of this material in landfill pose a global problem of disposal system. Knowledge of the microbial pattern of interaction with this plastic will provide the biological resources and scientific basis for the development of sustainable disposal and treatment method. *Anoxybacillus rupiensis* Ir3 (JQ912241) is a novel thermophilic bacterium, it was isolated from hydrocarbon contaminated soil in Iraq and showed good ability to utilize aromatic compounds, It is represented a new carbazole-degrading bacterium. In an attempt to investigate the ability of this bacterium to degrade nylon6, the strain Ir3 was grown on the nylon6 separately as a sole source of carbon and nitrogen. Results showed that strain Ir3 was able to degrade this compound. Optimum conditions for degradation of nylon6 were investigated. It was found that these conditions are growing this bacterium in chemical define media CDM containing (0.5%) nylon6, and incubated with shaking (180rpm) at 65°C for 7 days, and to confirm the ability to degrade nylon6, analytical experiments HPLC (High Performance Liquid Chromatography) and FTIR (Fourier Transmittance Infrared Spectroscopy) were used. The 6-aminohexanoic acid as intermediate products in the culture medium was mentioned by using HPLC, while biodegradation of the nylon6 was monitored by using FTIR.

Keywords: Microbial degradation, Nylon6, *Anoxybacillus rupiensis*

Introduction

Aliphatic polyamides, also known as nylons (e.g., Nylon-6; Nylon-66; Nylon-612; Nylon-46; Nylon-12; *etc.*) are among the most important commodity polymers (Palmer, 2003). Polyamides are heterochain polymers containing amide groups in the macromolecular backbone. This large polymer encompasses thermoplastics of extremely broad range of available properties which are used in the production of films and fibers, moulding compounds, *etc.* (Shropshire, 2000) and (Estes and Schweizer, 2011).

The majority of polyamides are semicrystalline and generally very tough materials with good thermal and chemical resistance. The different nylon types give a wide range of properties and are used in many applications due to the combination of outstanding mechanical and electrical properties, particularly toughness and wear resistance. Nylons also have excellent chemical resistance and can be used in high temperature environments. Heat stabilised reinforced systems allow sustained performance at temperatures up to 185 °C (Prasad et al., 2012).

Nowadays, more attention is focused on the attractive class of bio-based polyamide thermoplastics, which are partly or wholly made from renewable resources (Brehmer, 2013) and (Kuciel et al., 2012). In the production of such polyamides, bio-based monomers derived from castor oil or mass produced by fermentation are applied. Synthetic pathways to obtain bio-based polyamides are basically the same as to synthetic polyamides and there are a number of commercial products available on the market (Thielen, 2010).

Nylons are available for processing *via* injection moulding, rotational moulding, casting or extrusion into film or fibery. For industrial uses, polyamides persistently replace traditional materials in applications ranging from showpiece examples such as artificial organs and construction materials for moon-based space stations through to the more routine but equally important uses, including high load bearings, wear pads, support and guide wheels, buffer pads and gears, and many more (Herzog et al., 2013).

Biodegradation of synthetic polyamides is generally known to be poor, although its chemical structure (presence of amide bonds in the main chain) resembles those of natural proteins and synthetic polypeptides (Murthy et al., 2012), (Albertsson and Karlsson, 1992) and (William, 1989). The high resistance to degradation of synthetic polyamides is caused mainly by the high symmetry of their molecular structures and strong intermolecular cohesive force caused by hydrogen bonds between molecular chains, which results in highly crystalline morphology (Negoro, 2005) and (Morales et al., 2010).

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).

The microbial species are associated with the degrading materials were identified as bacteria (*Pseudomonas, Streptococcus, Staphylococcus, Micrococcus, Moraxella*), fungi (*Aspergillusniger, Aspergillus glaucus*), *Actinomycetes* sp. and *Saccharomonospora* genus (Swift, 1997) and (Chee et al., 2010). Specifically some white rot fungi also can degrade toxic compounds by the secretion of extracellular enzymes. Microbial degradation of plastic caused by oxidation or hydrolysis using microbial enzymes that lead to chain cleavage of the high molecular weight polymer into low molecular weight oligomer and monomer by aerobic or anaerobicmetabolism (Kumar et al., 2013).(Inan et al., 2011). reported that *Anoxybacillus*species are widely distributed and readily isolated from geothermal heated environments, with continually increasing industrial interest in their thermostable gene product .The isolating new strains of this novel bacterial genus is not a taxonomy concern, but also a necessity in order to exploit its biotechnological potential completely.

Species of *Anoxybacillus* are widespread in geothermal springs, manure, and milk-processing plants. The genus is composed of 22 species and two subspecies, but the relationship between its lifestyle and genome is little understood (Goh et al., 2014).

The *Bacillaceae* family members are a good source of bacteria for bioprocessing and biotransformation involving whole cells or enzymes. In contrast to *Bacillus* and *Geobacillus*, *Anoxybacillus* is a relatively new genus that was proposed in the year 2000. Because these bacteria are alkali-tolerant thermophiles, they are suitable for many industrial applications. More than a decade after the first report of *Anoxybacillus*, knowledge accumulated from fundamental and applied studies suggests that this genus can serve as a good alternative in many applications related to starch and lignocelluloses biomasses, environmental waste treatment, enzyme technology, and possibly bioenergy production (Goh et al., 2013).

Anoxybacillus rupiensis Ir3 (JQ912241) is a novel thermophilic bacterium, it was isolated from hydrocarbon contaminated soil in Iraq and showed good ability to utilize aromatic compounds, It is represented a new carbazole-degrading bacterium (Al-Jailawi et al., 2013).

This research was aimed to study the ability and some optimum conditions of nylon6 degradation by *Anoxybacillus rupeinsis* Ir3 (JQ912241)) as carbon and nitrogen source, In addition to use FTIR and HPLC analysis to confirm this ability.

Materials and Methods

Materials

Commercial grade Nylon 6 was provided by Sigma Aldrich. The material is in the form of pellet. All chemicals and solvents used in all experiment were

AR grade. A thin sheet of nylon 6 was prepared from nylon 6 pellets by melting and pressing the pellets of Nylon 6.

Bacterial isolate *A. rupiensis* strain Ir3 (JQ912241)

The bacterium used in this study (*Anoxybacillus rupeinsis* Ir3 (JQ912241)) is a novel strain and has the able to utilize different aromatic compounds. It was isolated in pervious study from hydrocarbons-contaminated soil in Iraq (Al-Jailawi et al., 2013).

Media

Luria-Bertani (LB) medium (Nazian et al., 2001) and the chemical define media CDM (Al- Dousary, 2004) were used for the growth of the microorganism and degradation respectively. Nylon6 was used as the sole source of carbon and nitrogen in all experiments.

Sterilization of the sample

The sample sheets were sterilized before they were inoculated into the test medium. The nylon sheets were dipped in absolute alcohol for a few hours. Washed with distilled water and later dried. No physical or chemical changes were observed in the sample sterilization treatment.

Biodegradation assay of nylon6 by *A. rupiensis* strain Ir3 (JQ912241)

To determine the ability of the *A. rupiensis* strain Ir3 to degrade nylon 6, 100 milliliter of chemical define media (CDM) were dispensed in Erlenmeyer flasks (250ml) and sterilized by autoclaving. After sterilization, the flasks were supplemented with 5g/L of nylon 6 (disinfected30 min in ethanol and air dried for 15 minutes in laminar air flow chamber), inoculated with 1% of fresh culture (18hrs. old) of *A. rupiensis* strain Ir3 and incubated in shaker incubator (180rpm) at 65 °C for 7 days. Control was made by inoculating flasks with bacterial strain; these flasks containing the same chemical define .performed in triplicates. The degradation ability of this bacterium was determined by monitoring the growth density of the liquid culture inspectrophotometer at 600nm.

Optimization of nylon6 biodegradation

Effect of pH

The effect of pH on the ability of *A. rupiensis* strain Ir3 to utilize nylon 6 as a sole source of carbon and nitrogen was determined by supplemented chemical define media (CDM) with 0.5% of nylon 6 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 65 °C for seven days. The optimum pH value was employed in the subsequent experiment.

Effect of Temperature

To determine the effect of temperature on the ability of *A. rupiensis* strain Ir3 to degrade nylon6, chemical define media (CDM) (pH 7.0) supplemented with 0.5 % of Nylon6 film was inoculated and incubated in shaker incubator (180 rpm) at different temperatures (40, 45, 50, 55, 60, 65°C) for seven days. Optimal temperature was subsequently employed, dependingon the growth density measurement.

Effect of nylon 6 concentration

In order to determine the optimum concentration of nylon 6 film that can be degraded by *A. rupiensis* strain Ir3, this film was added at different concentrations (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 65 °C for seven days. Bacterial growth was measured and the optimal concentration was employed later on.

High Performance Liquid Chromatography (HPLC) analysis.

Chemically defined media (100ml in 250 ml flask) containing (0.5% nylon 6) were inoculated with fresh culture of efficient bacterial strain*A.rupiensis*Ir3 (JQ912241) and incubated at 65°C, pH 7.0, for 60 days shaking at 180 rpm. After incubation, the bacterial cells were harvested by centrifugation at 13000 rpm for 10min. at 4°C. The resulting cell-free supernatant was analysed by HPLC for degradation products at Ministry of Industry and Minerals, IBN SINA STATE COMPANY. The standard was made up of caprolactam and 6-aminohaxanoic acid (50:50 v/v). The mobile phase consisted of, 0.1% formic acid: acetonitrile 60:40% (v/v). The analyses were performed on (ShimadzuLC- 10 AVP binary delivery. Pump. Monitor by LC-10A UV-Vis spectrophotometer Japan, Icoyota) system; UV detection at 250 nm and the flow rate were 1ml/min in C18 column (50×4.6 mmvd, 3mm particles size).

Extraction Procedure

The inoculated flasks were extracted by using separating funnel in presence of ethyl acetate as a solvent. One ml of culture supernatant was taken from cultures growing with (nylon 6) and extracted with 3ml of ethyl acetate. The ethyl acetate solvent was evaporated and the residue was dissolved in 1ml ethanol (Akbar, 2008).

Characterization of nylon 6

In order to characterize the chemical nature and structure of nylon 6, and analyze the change innylon6structure after incubation with *A. rupiensis* strain Ir3, Fourier transformed infrared spectroscopy (FTIR) (Shimadzu) analysis was done at Ministry of Industry and Minerals, IBN SINA STATE COMPANY. *A. rupiensis* strain Ir3 was grown on chemical define media (CDM) pH 7.0 containing 0.5% of nylon 6 with shaking (180 rpm) at 65 °C for60 days. After incubation, samples of nylon6 were taken and subjected to FTIR analysis.

Results and Discussion

Biodegradation assay of *A. rupiensis* strain Ir3 (JQ912241)

In order to test the ability of *A.rupiensis* Ir3 to utilize nylon6 as a sole source of carbon and nitrogen, the growth experiment were performed by inoculating the tested bacterial strain in CDM media and incubated in shaker incubator (180 rpm) at 65 °C for 7 days. These experiments revealed that *A. rupiensis* Ir3was able to grow with nylon6 yielding optical density of(1.51) after 7 days of incubation. This observation indicated that this bacterium has the ability to utilize nylon 6 as a sole source of carbon and resulting in partial degradation of plastics.

Nylon-6 was generally regarded as a xenobiotic polymer (Oppermann et al., 1998). Its reported degradation by a thermophilic bacterium *Bacillus pallidus* (Tomita et al., 2003) and marine strains of *Bacillus cereus, Bacillus sphaericus, Vibrio furnisii,* and *Brevundimonas vesicularis* (Sudhakar et al., 2003) brighten the hope of the prospects for microbial degradation of polymer. However, the degree of microbial degradation has been shown to be lower in the larger molecule (Prijambada et al., 1995).

Nylon6 could also biodegrade by fungi and bacteria, (Deguchi et al., 1997) and (Deguchi et al., 1998) reported thatthe white rot fungi strain IZU-154, a kind of lignin-degrading microorganisms, degraded Nylon6 films through oxidative processes. *Geobacillus thermocatenulatus* could also provide Nylon-12 and Nylon-66 biodegradation (Tomita et al., 2003). Bacterial degradation of Nylon-12 is usually associated with the enzymatic hydrolysis of amine bonds, which is accompanied by the formation of 12-amino dodecanoic acid. (Tomita et al., 2003) reported on a thermophilic strain isolated from 100 soil samples by enrichment culture technique at 60 °C which is capable of degrading Nylon-12. At this temperature, the strain grew on Nylon-12, accompanied by a marked decrease in molar mass of Nylon-12. The strain is also capable to degrade Nylon-6 as well as Nylon-12, but not Nylon-66.(Yasuhira et al., 2007) found that the accumulations of nylon waste in soil increase the adaptation of the soil bacteria to degrade nylon or develop the degradation capability of nylon by gene mutation (Anderson and Purdon, 2009).

Optimization of nylon6 degradation by *A. rupiensis* Strain Ir3 (JQ912241)

Effect of pH

Chemical defined 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 *A.rupiensis* Ir3 (JQ912241) on nylon6. The obtained results as shown in Figure (1) elucidated that an optimum growth was occurred at pH 7; the optical density for bacterial growth was reached 0.95, while bacterial growth was decreased at other pH values compared with growth at pH 7 after seven days. (Kinoshita et al., 1977] described that the pH for nylon 6 oligomer degradation by *Flavobacterium* sp. K172 ranging between pH 5.5 to pH 8.5. The optimum pH required to degrade nylon 6 oligomer was 6.3 by *P. aeruginosa* NK87 (Kanagawa et al., 1989). While suitable pH for nylon 4 film degradation by *Stenotrophomonas* sp. and *Fusarium* sp was found to be 7.2 (Tachibana et al., 2010). (Baxi and Shah, 2001) found that pH 7.2 was suitable for caprolactam degradation by *Alcaligenes faecalis.*

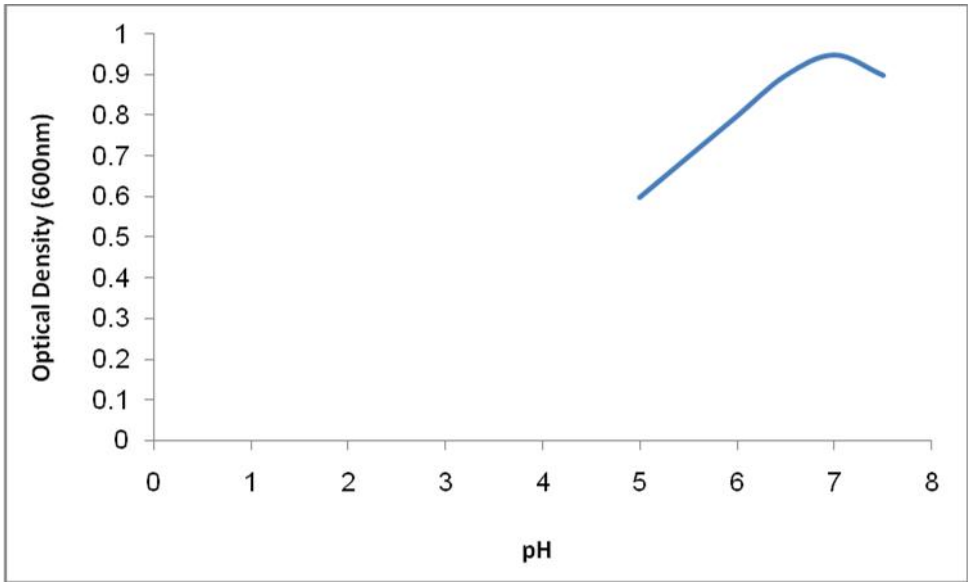

Figure (1): The effect of pH on *Anoxybacillus rupiensis* Ir3 grew on 0.5% of nylon 6

Effect of temperature

A.rupiensis Ir3 (JQ912241) was grown and incubated at different temperatures (40, 45, 50, 55, 60, 65°C). Results shown in Figure (2) pointed out that the optimum temperature for growth in presence of nylon6 was 65°Cand the optical density of bacterial growth at 65^{o}C was 1.9 after seven days of incubation, which was suggested as the optimum temperature for bacterial growth. Relative result of bacterial growth was recorded at 65^{o}C. Whereas, at 45 °C, bacterial growth was lower than at other incubation temperatures. This result similar to (Tomita et al., 2003), in which species to *Geobacillus thermocatenulatus*, having a growth optimum at 65^{o}C and, capable of degrading nylon 66. (Negoro et al., (1980) pointed that *Flavobacterium* are able to degrade 6-aminohexanoic cyclic dimer (waste of nylon 6) 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).

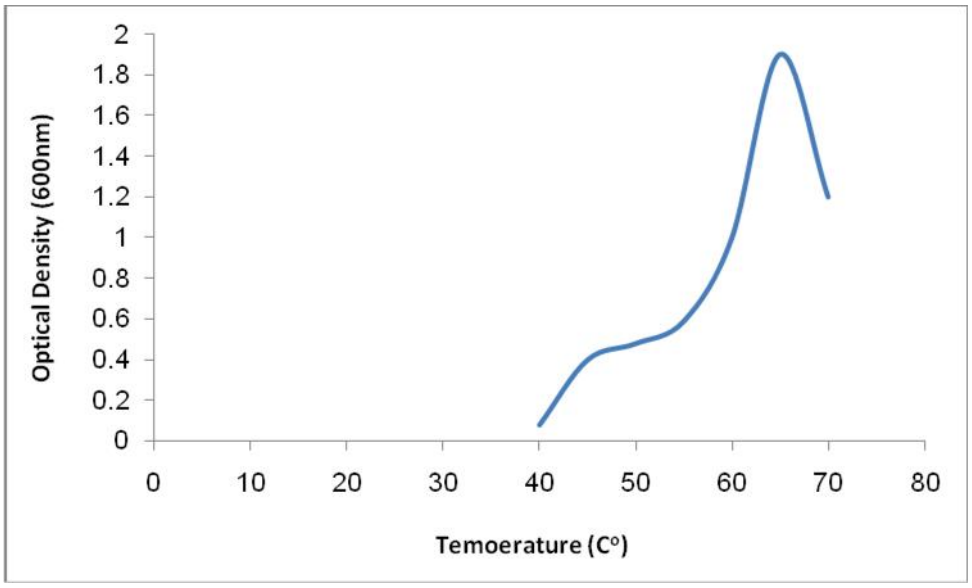

Figure (2): The effect of temperature on *Anoxybacillus rupiensis* Ir3 grew on 0.5% of nylon 6 and pH 7.0

Anoxybacillus is a mild thermophile. The closest genus to *Anoxybacillus* is *Geobacillus*, based on the 16S rRNA phylogeny and concatenated sequence similarity, yet the genomes of the latter genus are larger and the cells grow at higher temperatures, while *Anoxybacillus* has one of the smallest genomes in *Bacillaceae*. Based on the genome annotation,

the thermophily of *Anoxybacillus* is attributable to many features that stabilize proteins, DNA, and RNA. The presence of adaptive genes is sufficient for the cells to live in an alkaline environment with the presence of organic nitrogen and carbohydrates and to overcome the threats from UV radiation. In addition, for *Anoxybacillus* to survive under extreme conditions, we think that genetic exchange, especially uptake of genetic material via HGT, is important (Pikuta et al., 2000) and (Saw et al., 2008)and original.

Effect of nylon 6 concentration

Different concentrations (between 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, and 1%) of nylon6 were used to grow *A.rupiensis* Ir3 (JQ912241) in order to determine the optimum concentration. Results in figure (3) indicated that the optimum concentration for bacterial growth was 0.5%, in which the optical density of bacterial growth, after seven days of incubation was 1. Figure (3) showed also that gradual increasing of nylon 6 concentration accompanied with increasing of bacterial growth, and then the growth reached to its optimum at a concentration of 0.5%, while nylon 6 concentrations higher than 0.5% showed decrease in bacterial growth. This bacterium was able to survive with up to 1 % of nylon6.

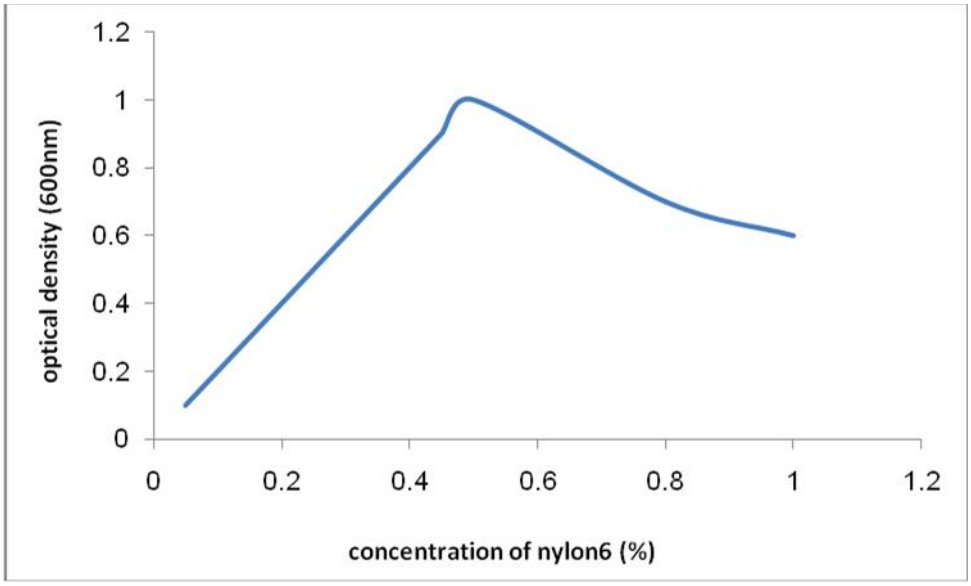

Figure (3): The effect of nylon6 concentrations on *Anoxybacillus rupiensis* Ir3 growth

HPLC Analysis

The HPLC chromatograms of the standards and the supernatant of the culture treated with *A.rupiensis* Ir3 (JQ912241) is as shown in the Figure 4 (a, b). Figure 4a shows the peaks of the standards. The 6-aminohexanoic acid resolved with1.343 minutes retention time (Rt.) while the Caprolactam resolved with2.848 minutes retention time (Rt.).

Figure (4-a): HPLC of the standards: 6- aminohexanoic acid with retention time 1.343 and caprolactam with retention time 2.848

The chromatogram of the*A.rupiensis*Ir3 treated sample (figure 4b) showed the presence of the 6-aminohexanoic acid at retention time of 1.328 minutes with peak area of 46145mAs. So that the major detected degradation product was 6-aminohexanoic acid.

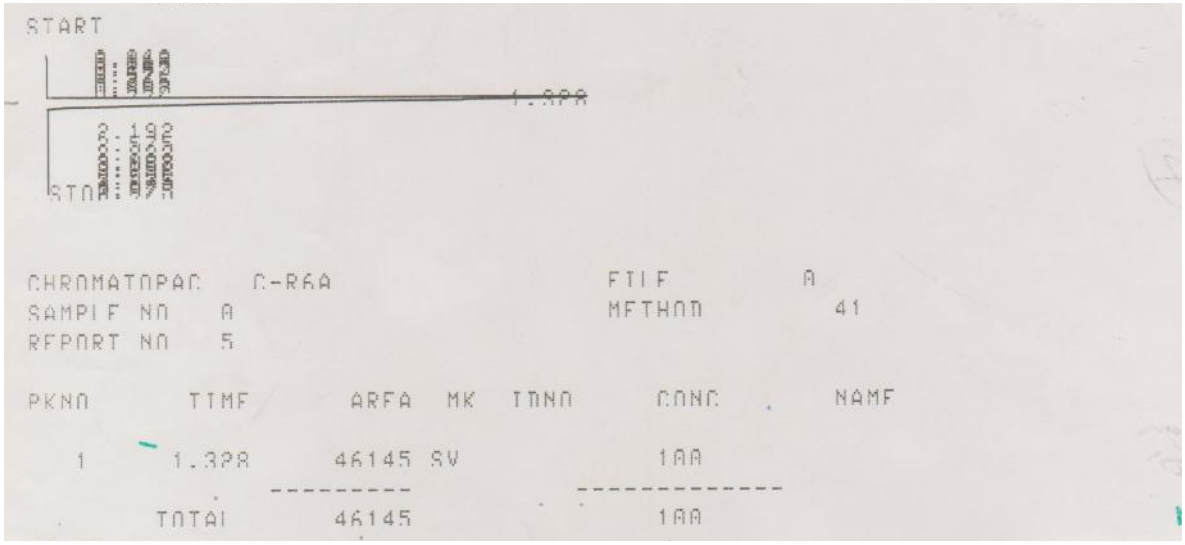

Figure (4-b): HPLC of the *A.rupiensis* Ir3 (JQ912241) treated nylon6 supernatant, showing 6- aminohexanoic

FTIR analysis

In order to confirm the ability of the bacteria to degrade nylon 6 film, *A.rupiensis*Ir3 was grown CDM (pH 7.0) containing 0.5% of nylon 6 film at 65 ˚C for 60 days, after that nylon 6 film was subjected to FTIR to detect its structure. From the results of FTIR spectrum (figure 5a), compared with spectrum of nylon6 film (figure 5b) before bacterial growth, It can be concluded that nylon 6 film was degraded by *A.rupiensis*Ir3, which used the (N-H, C=O and C-H) groups as carbon and nitrogen source. The strength of characteristic bands of C (O) NH occurring around 3500-2654, 1690-1630, 1550 and 1018 cm-1 decreased after 60 days. Formation of new groups like CH_3, $CONH_2$, CHO and COOH, may be formed due to hydrolysis and oxidation.

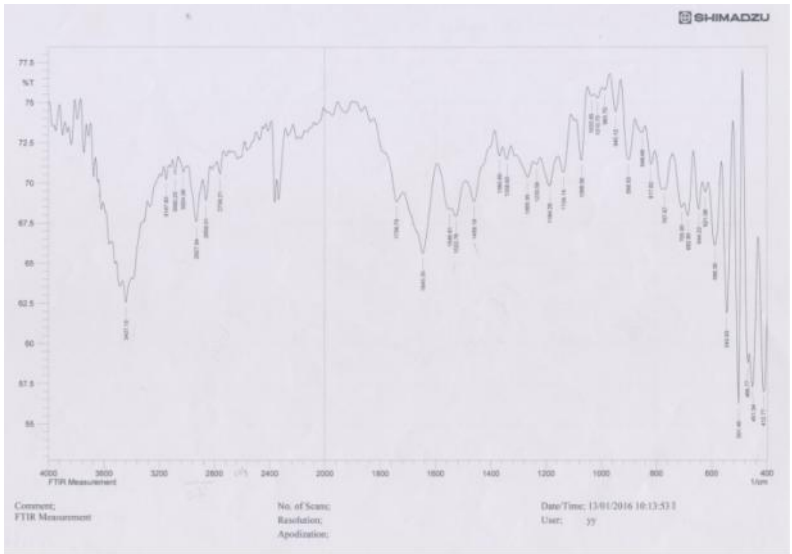

Figure (5-a): FTIR spectra of nylon6 untreated

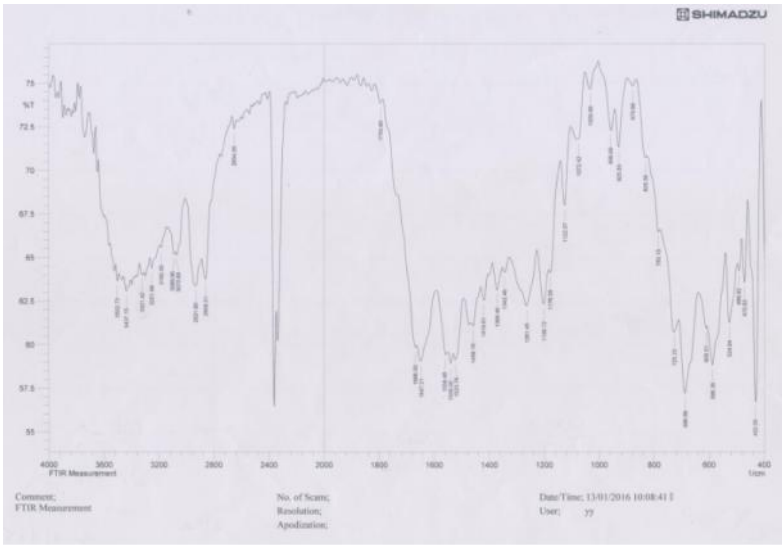

Figure (5-b): FTIR spectra of nylon6 treated

(Sudhakar, 2007) referred that the FTIR for both nylon 6 and nylon 66 which degraded by marine bacteria showed formations of 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, CH_2 and $CONH_2$ group. (Tomita et al., 2003) demonstrated that some of white rot fungal strains can degrade nylon 66 and make changed in the surface on nylon 6 fiber.

Conclusion

In the present study, it was concluded that the novel bacterial isolate *A.rupiensis* Ir3 (JQ912241) was able to grow in medium containing nylon6 as a sole source of carbon and nitrogen. The optimum conditions for the growth are growing this isolate in the chemical define media CDM (pH 7) containing 0.5% of nylon 6 and incubated with shaking (180rpm) at 65 °C for seven days. In addition, the 6- aminohexanoic acid as intermediate products in the culture medium was mentioned by using HPLC, while biodegradation of the nylon6 was monitored by using FTIR. Thus, further molecular study is needed to determine the catabolic genes resident in the novel strain *A.rupiensis* Ir3 that were responsible for the nylon6-utilizing ability. Also it is recommended to understand the mechanism responsible for the biodegradation of nylon 6.

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