4.1 Introduction

Plastics are synthetic long chain polymer molecules (Scott *et al.* 1999) and its consumption is growing at a rate of 12% per annum globally and approximately 0.15 billion tones of synthetic polymers are produced worldwide annually (Premraj *et al.* 2005, Leja *et al.* 2010, Das *et al.* 2014). Accumulation rate of plastic waste in the environment is 25 million tons/year (Orhan *et al.*2000, Nayak *et al.*2011, Baruah *et al.*2011, Kaseem *et al*.2012) and is consequently considered a serious environmental peril (Sivan *et al.*2006,Thompson *et al.*2004).Currently used polyethylene which is used in product packaging as sheet and thin plastic films are Polyolefin-derived plastics. Out of these LDPE constitutes ~60 % of the aggregate plastics production of plastic bags and most prevalent solid waste. LDPE is characterized by good strength, resistance to chemicals, plasticity, and limpidity. Hydrophobicity interferes its availability to microorganisms (Albertsson *et al*. 1993). These are characteristically inert so they persist in the nature (Potts, 1978). Its recalcitrant nature is due to its high molecular weight, complex three-dimensional structure. (Nanda *et al.* 2010). Land filling and incineration are prevalent operations for the management of PE but they have various environmental constraints so biodegradation appears as the best option for the PE waste management. (Juan-Manuel Restrepo- Florez *et al*. 2014). Biodegradation is the tendency of a polymer to get break down into its components by microorganisms. Degradation of polyethylene with fungi is preferred over bacteria because of their high production potential and good penetrating ability (Hanaa *et al* .1998). Biodegradation of polyethylene by fungi has formerly been reported using *Mucor rouxii* NRRL 1835 and *Aspergillus flavus* (Hanaa *et al*.1998), *Penicillium simplicissimum* YK (Yamada-Onodera *et al*.2001) and *Phanerochaete chrysosporium* (Liyoshi *et al*. 1998). However there are reports on biofilm formation on

the polyethylene by soil fungi such as *Aspergillus*, *Fusarium*, *Rhizopus arrhizus*, *Penicillium* sp etc (Mahalaxmi and Niren 2012, Raaman *et al.* 2012, Das and Santosh 2014). Biodegradation of Polythene bag and plastic cup by *Rhizopus* sps have been reported (Kannahi *et al*.2013). Degradation of high density polyethylene (HDPE) and low density polyethylene (LDPE) by *Rhizopus oryzae* has not done yet. In the present study lab isolate *Rhizopus oryzae* was used for the biodegradation studies of HDPE and LDPE films.

4.2 Results and Discussion

4.2.1 Identification of fungal isolate

The identification of the selected fungal isolate designated as NS5 was established via molecular characterization. The phylogenetic analysis which was grounded on BLAST search applying 18S rDNA gene sequence demonstrated maximum homology (100%) with the fungus

Rhizopus oryzae strain with gene bank Accession Number: AY213625.1. Based on the cladistic analysis as well as homology valuation, it was concluded that the selected fungal isolate could be regarded as *Rhizopus oryzae* NS5 (Fig.4.1). The sequence of *Rhizopus oryzae* NS5 has been deposited in NCBI with accession no. KT160362. Fungus *Rhizopus oryzae* is known for potential degdradation of complex compound (Alberts, 2009).

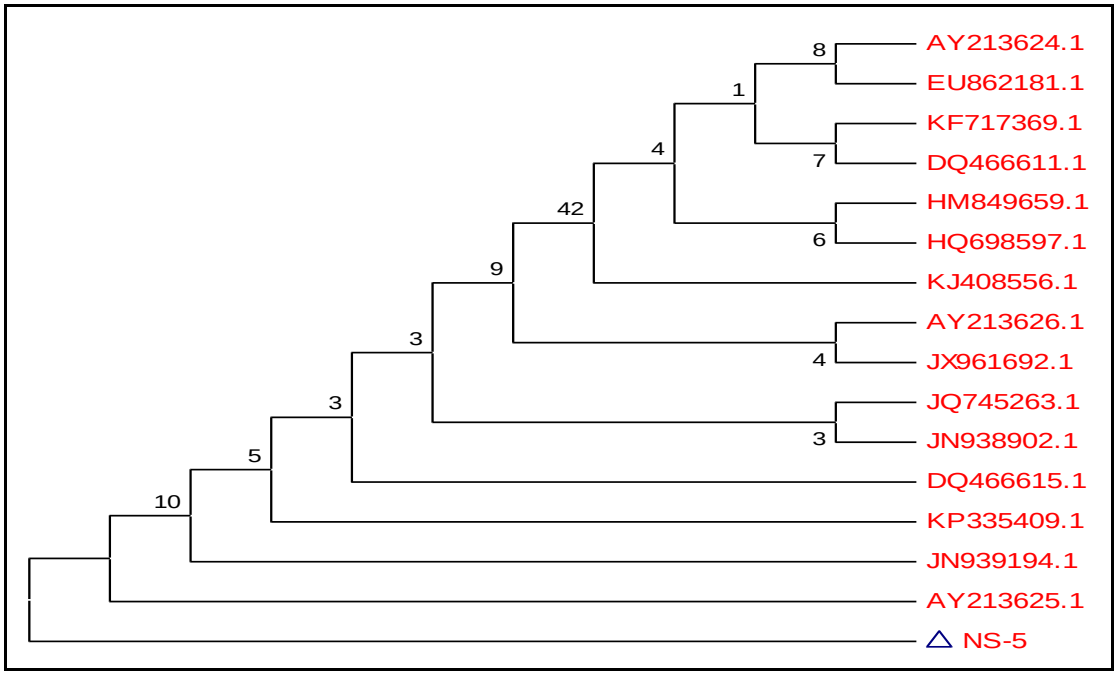

Fig.4.1 Phylogenetic tree of Rhizopus Oryzae NS5

4.2.2 Measurement of weight loss

To quantify the HDPE and LDPE degradation efficiency of *Rhizopus oryzae* NS5 the reduction in weight was measured at different time intervals in 30 days incubation. There was time dependent weight loss of PE films. Over a period of 30 days 5.9±0.3% of HDPE and 8.7±0.3% of LDPE was found to be degraded by fungus (Fig.4.2a-b). However weight loss was not observed in control experiment. Therefore this result showed the reduction in weight was due to utilization of PE films as a sole carbon source by *rhizopus oryzae* NS5. ANOVA results indicate that reduction in weight of HDPE ($F=602$ and $P<0.001$) and LDPE ($F=934$, $P<0.001$) with incubation time was significant.

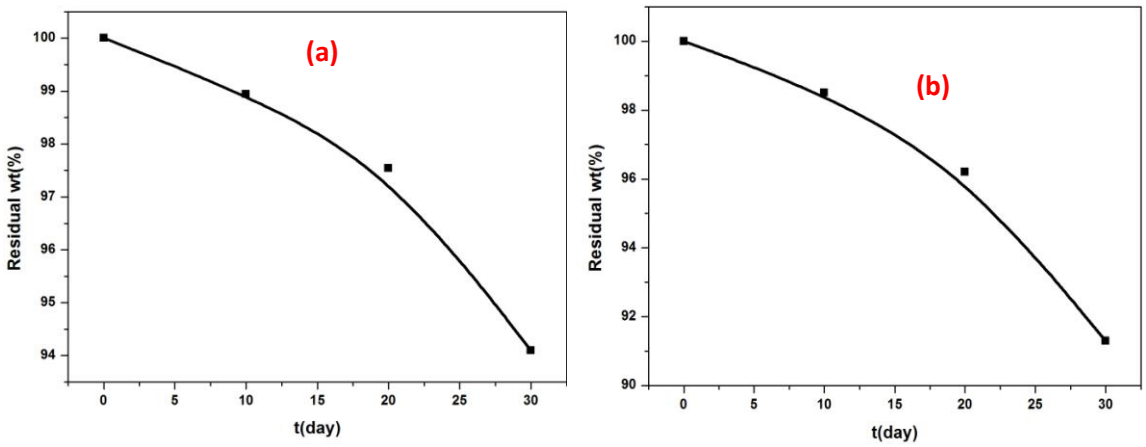

Fig.4.2 Change in weight of HDPE and LDPE (b)

Biodegradation of polyethylene was studied by Hanaa *et al* who explored the propensity of fungi and *Streptomyces* strains to rush the degradable polyethylene consisting of disposed polyethylene bags (Hanaa *et al*.1998). *Aspergillus flavus*, isolated from sanitary landfills was also found to be able to degrade polyethylene (Mendez *et al.*2007).Yamada *et al* have identified a fungus, *Penicillium simplicissimum* YK, that could degrade the untreated high-density polyethylene (Yamada-Onodera *et al.*2001). *Rhizopus oryzae* NS5 incubated polyethylene film showed 5.9±0.3 % 8.7 ±0.3 % reduction in weight of HDPE and LDPE respectively after 30 days of incubation. It is most likely due to the slow process of degradation. This biodegradation rate is in agreement with the earlier reports ranging from 3.5 to 8.4% for polyethylene incubated in soil for 10 years (Albertsson and Karlsson 1990). Mathur *et al* also studied the biodegradation of HDPE by *aspergillus niger* and found out only 3.44% wt loss. (Mathur *et al*.2011). This decrease in weight is in association to the others' findings Singh *et al*. carried out degradation of LDPE using *Aspergillus fumigatus* and *Penicillium* sp. According to their work, *A. fumigatus* was able to degrade 4.65 % of polyethylene and *Penicillium* sp. degraded 6.58 % (Singh *et al*. 2012).

4.2.3 Measurement of change in tensile strength

In the present study, approximately 50% reduction in tensile strength of HDPE and 54% reduction in LDPE was observed after 30 days incubation (Fig 4.3a&b). There was no change in tensile strength of blank HDPE and LDPE films after 30 days. ANOVA result indicates that reduction in tensile strength ($F=156$ and $P<0.001$) of HDPE and ($F=191$, $P<0.001$) of LDPE with incubation time was significant. Mathur *et al.* 2011 reported 61% loss in tensile strength after 1 month incubation with *aspergillus niger*. In a study by Vijaya and reddy (2008) 18.48% TS reduction in HDPE and 12.15% in LDPE in 4 months by *A. glaucus* has been reported. There are reports similar to our results by Hanaa *et al*., Lee *et al*. and Nowak *et al*. they reported that biodegradation reduces the percentage elongation of polyethylene films (Hanaa *et al*. 1998, Lee *et al.* 1991& Nowak *et al*. 2011). In a study by orhan and buyukgungor (2000) 56% reduction in TS in 3 months was observed. Our results are also in agreement with earlier studies by Jakubowicz *et al*. on thermally-treated-PE films, which showed a reduced elongation (%) after incubation with compost microorganism (Jakubowicz *et al*. 2006). It has been reported by konduri *et al* (2011) that 51% reduction in TS in 3 months with the incubation with *A. Oryzae.*

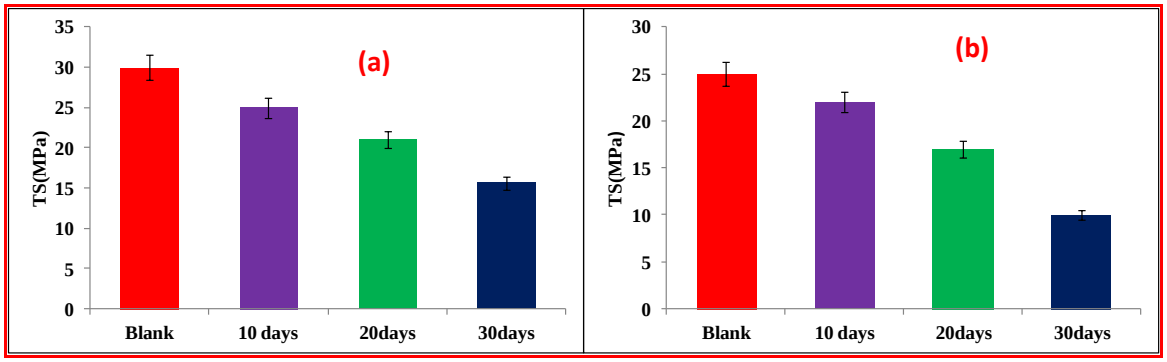

Fig.4.3 Change in tensile strength of HDPE (a) and LDPE (b)

4.2.4 Measurement of change in pH

The Initial pH of media was 5.1±0.27 while pH after 30 days incubation was measured as 4.4±0.30 and 4.2±0.30 for HDPE and LDPE respectively while pH remained unchanged after 30 days incubation in blank experiment (Fig.4.4a-b). Reduction in pH validates that the culture is still metabolically active and PE film is utilized for its growth. The reduction in pH affirms the consumption of the polyethylene film as their sole carbon source. (Duddu *et al.*2015, Das *et al.*2015, Arutchelvi *et al.*2008). ANOVA result indicates that reduction in pH of HDPE ($F=22.8$ and $P<0.001$) and of LDPE ($F=42.2$, $P<0.001$) incubated media with incubation time was significant. The reduction in pH not only confirms the usage of the polyethylene sheets as its source of carbon. However, it also paves the way for the idea regarding the production of several monomers which has possibly been produced post-degradation. Reduction in pH from 7.3 to 5 after 90 days of incubation of HDPE and LDPE films with *Penicillium oxalicum* NS4 and *Penicillium chrysogenum* NS10 has been reported by Ojha *et al.*2017.

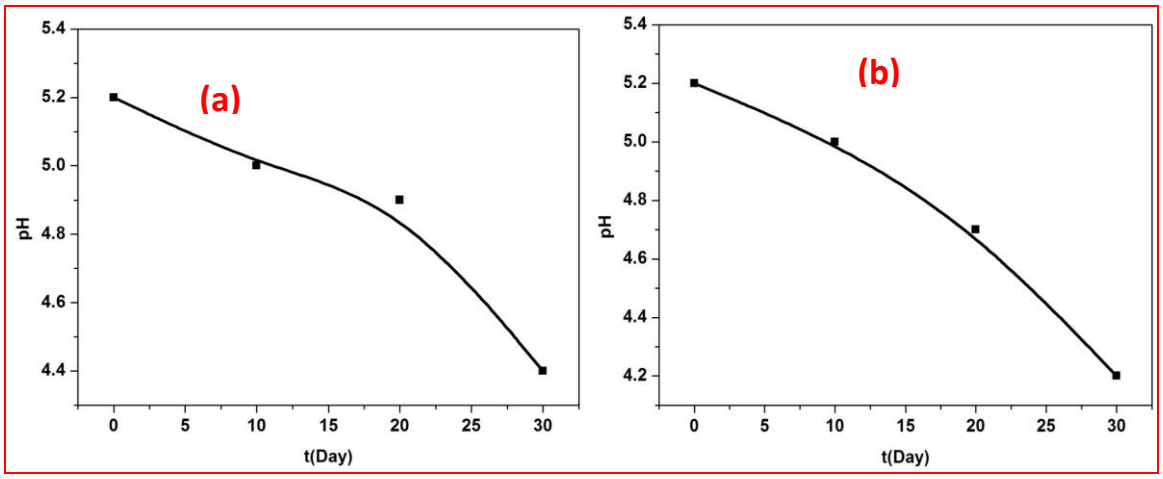

Fig.4.4 Change in pH of HDPE (a) and LDPE (b) film

4.2.5 Measurement of change in contact angle

The initial contact angle of the HDPE and LDPE film was 102.3±2.6 and 98.6±2.6° respectively (Fig.4.5a & 4.6a).After abiotic treatment angles decreased to 95.2±2.6 and 91.5±2.6 ° respectively (Fig.4.5b & 4.6b). It shows that there was an increase in hydrophilicity of the surface of the films as a result of thermal treatment. Further there is a decrease in contact angle after incubation with fungal isolate which is 86.6±2.6 and 67.0±2.6° for HDPE and LDPE respectively(Fig.4.5c & 4.6c). The values represent average of the triplicates. The wettability and the corresponding hydrophilicity of the polyethylene surface was increased with the decrease in contact angles of the films as a result of enzymatic activities of fungus. There was, though, no decrease in the contact angle for the control set of sample. This indicates that the polyethylene surface became relatively hydrophilic with increasing immersion period, which was also reported earlier by several authors (Sonak *et al.* 1995, Sudhakar *et al.* 2008).

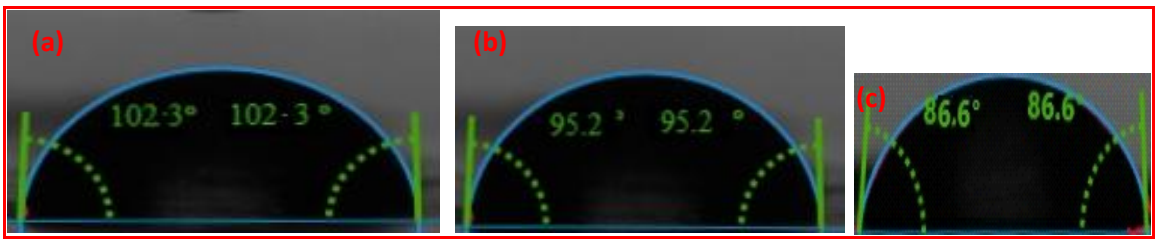

Fig.4.5 Contact angle of blank HDPE (a), thermally treated HDPE (b) and biologically treated HDPE film (c)

Fig.4.6 Contact angle of blank LDPE (a), thermally treated LDPE (b) and biologically treated LDPE film (c)

4.2.6 Change in surface morphology

4.2.6.1 Scanning Electron Microscopy (SEM)

The surface morphology of Polyethylene film was observed by SEM and AFM micrographs (Fig. 4.7, 4.8, 4.9 & 4.10).There were cracks and grooves around the fungal cells on the surface of films after 30 days incubation of the polyethylene. At the same time there was no any growth of fungus on the surface of control film. It is possibly because r*hizopus* secretes lipase, (Coenen *et al.*1997) tyrosinase, peroxidase (León-Santiesteban *et al.*2008) and laccase (Shinkafi *et al*.2014) enzymes capable of degrading polyethylene, and consequence of such enzymatic activities is the grooves formation. Since the initial attack generally begins with a surface colonization. Scanning electron microscopy (SEM) allows direct observation of biodegradation. Superficial growth of fungal hyphae on the biodegraded HDPE films is presented in Figure 4.7(b-c). The biodegradation of polyethylene was evidenced through the formation of cavities on the surface as well as the hyphae penetration and adherence of hyphae and spores to the surface, as shown in Figure 4.7(a-c) & 4.8(a-c).The microbial adhesion to the surface of polymer is a fundamental step for biodegradation to take place (Moriyama *et al.*1993). After 15 days of incubation with *Rhizopus oryzae* showed hyphal growth (Fig. 4.8b) because it has been identified that the fungus also undergo change in shape upon biofilm formation (Raaman *et al.*2012).There are several reports demonstrating the core. Microorganisms colonize the polymer surface and adhere by extracellular polymer production. The results obtained are in close association with earlier reports (Manzur *et al.* 2004, Sepulveda *et al.* 2002).This change in growth rate could be a cellular response to the change in surface topography of the LDPE film during degradation whereby pits are formed on the film surface due to enzymatic digestion.

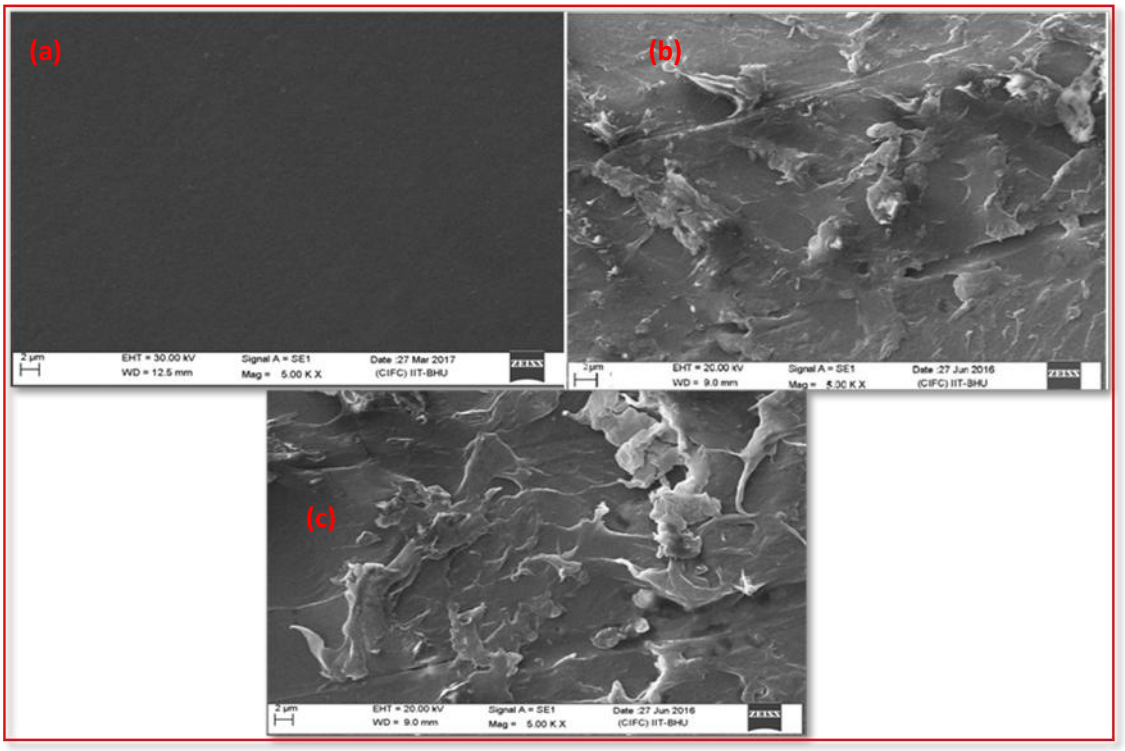

Fig.4.7 SEM of HDPE film blank (a), biologically incubated film after 15 days (b) and biodegraded film after 30 days (c)

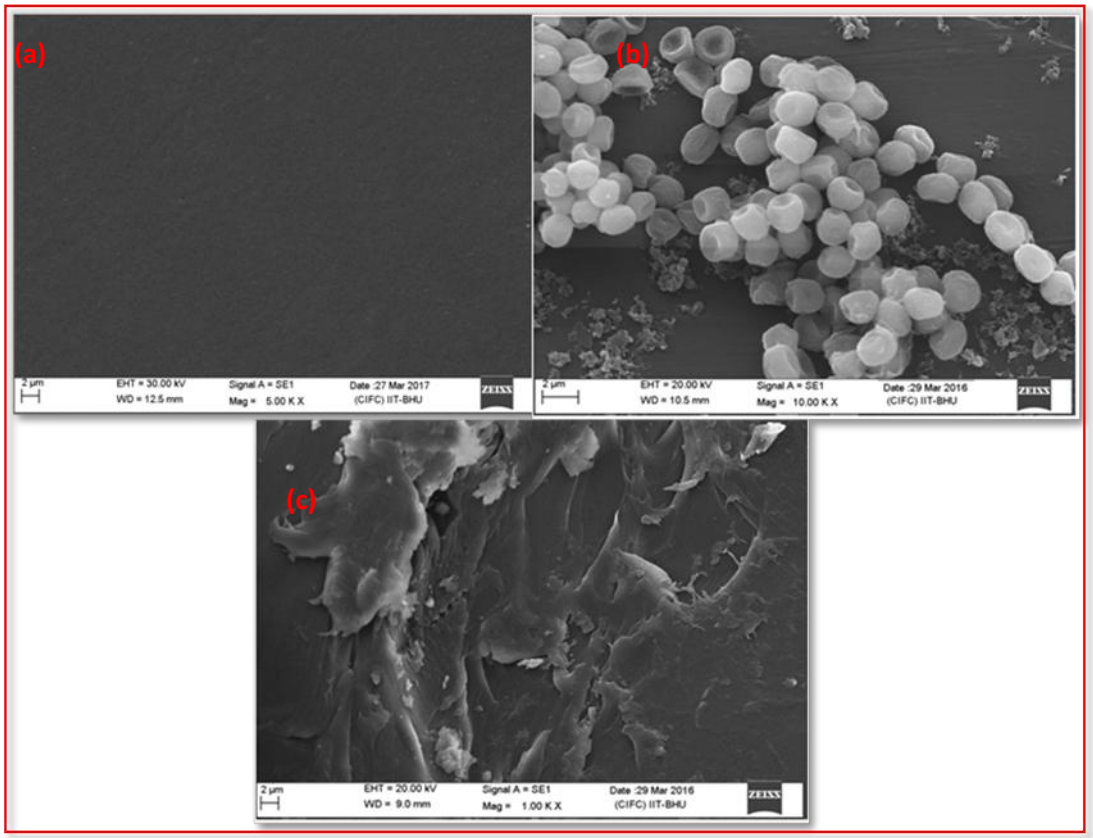

Fig.4.8 SEM of LDPE film blank (a), biologically incubated film after 15 days(b) and biodegraded film after 30 days (c)

4.2.6.2 Atomic Force Microscopy (AFM)

Change in surface roughness was observed by AFM analysis. Roughness of films increased after 30 days incubation (Fig.4.9 (a-b) and 4.10 (a-b)). For HDPE roughness of the film was increased from 32.2 to 41.7nm and the roughness of LDPE film was increased from 30.1 to 49.9nm.Similar changes in surface topology have also been noticed from AFM analysis while biodegradation with *Pseudomonas* sp.AKS2 (Tribedi *et al*. 2013).These results by surface morphology confirm the biodegradation of PE films.

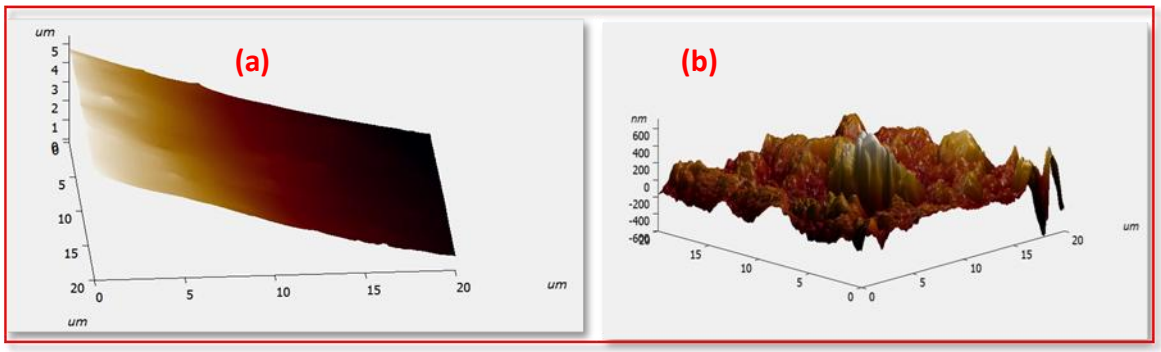

Fig.4.9 AFM of blank HDPE (a) and biodegraded HDPE (b) film

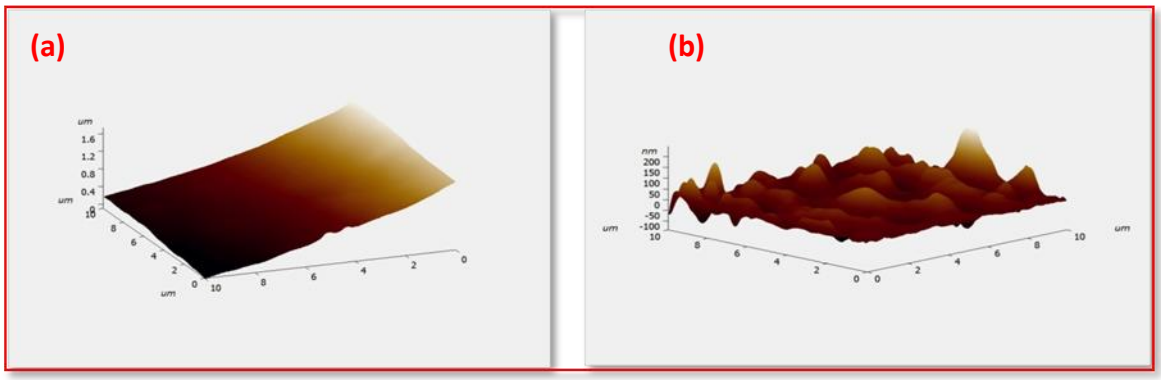

Fig.4.10 AFM of blank LDPE (a) and biodegraded LDPE (b) film

4.2.7 FTIR

A number of peaks are present in the control films (HDPE and LDPE) manifesting the complex nature of the PE (Fig. 4.11a & 4.12a). In control HDPE film bands are present at 721 (C–H bend-mono), 839, 1475 and 2627 cm^{-1} and for control LDPE

samples, the characteristic absorption bands are present at 719 cm^{-1} (C–H bend-mono), 1472 (C=C stretch), 1375 (due to side chain $–CH_3$ branching), 2660 (- CHO stretch), 2919 and 2850 cm^{-1} (both due to C–H stretch).

After abiotic (thermal) treatment a new peak appeared as carbonyl group at 1720cm^{-1} in HDPE and at 1760 cm^{-1} in LDPE (Fig 4.11b & 4.12b). These results were similar to Gajendiran *et al*, Das and Kumar and Balasubramanian *et al.* (Gajendiran *et al.* 2016, Das and Kumar 2015, Balasubramanian *et al*. 2010). After incubation with Fungus the appearance of new peaks in HDPE was observed at 839, 1087 and 3324 cm^{-1}, there was total disappearance of peak at 1720cm^{-1}. Peak at 3324cm^{-1} was due to the presence of oxidised groups like alcohol and acids which were resulted from the oxidation of film by fungal enzymes (Fig 4.11c). Similar results were reported by (Bhatia *et al*. 2014), whereas functional groups appeared in biodegraded LDPE film were 839 cm^{-1} (C-H), 1107 cm^{-1} (C=C) and at 3400 cm^{-1} (alcohol and acids) (Fig 4.12c). This supports the depolymerization activity of the fungal isolate. The change in the peak values of almost all functional groups confirming the configurational changes on PE surface (Das *et al.* 2015, Gilan *et al.* 2004, Drímal *et al.* 2007, Hadad *et al.* 2005**,** Arboleda *et al*. 2004).

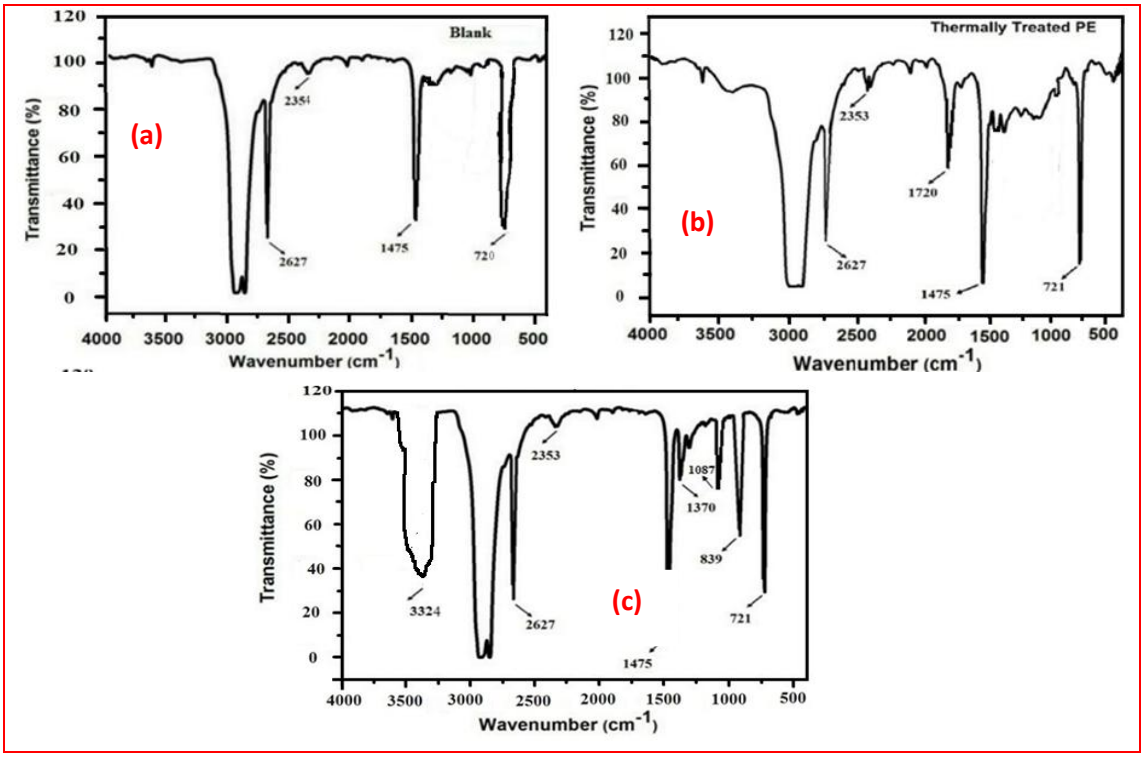

Fig.4.11 FTIR of blank (a), thermally treated (b) & biodegraded HDPE (c)

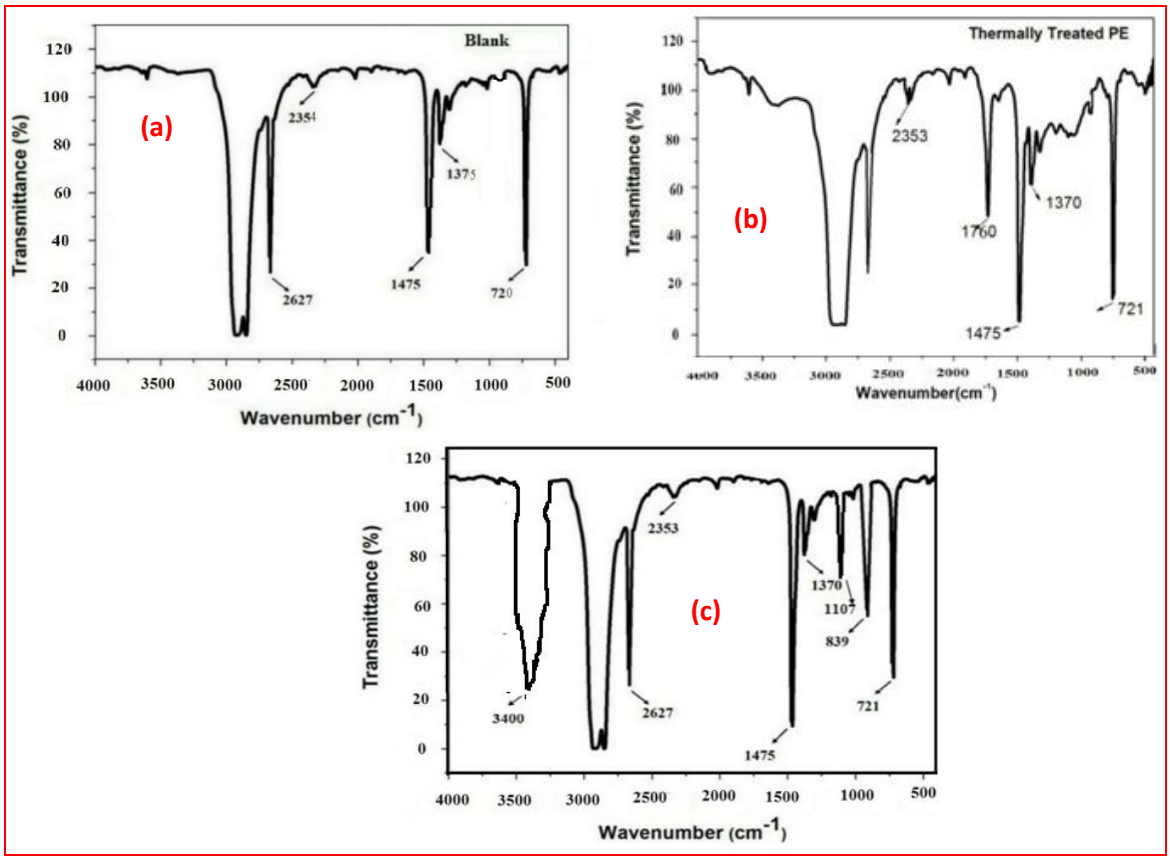

Fig.4.12 FTIR of blank (a), thermally treated (b) & biodegraded LDPE (c)

4.2.8 Optimization of physico-chemical parameters

4.2.8.1 Optimization of pH

pH is one of the most important factors controlling the growth and enzymatic activities of microorganisms. Effect of pH on biodegradation was observed at different pH values ranging from 3 to 10. Figure 4.13 (a-b) shows the effect of pH on degradation of HDPE and LDPE films. It was observed from the figures that wt loss achieved at pH 3 was very less (i.e.0.6±0.3 and 2.3±0.3%) for HDPE and LDPE respectively. Whereas degradation efficiency improved drastically, when pH was increased from 5.0 and wt loss of 8.7% ±0.3% and 5.9%± 0.3% was observed for HDPE and LDPE respectively at pH 6.0. The reason might be optimum pH for the growth of *R. oryzae* is 3.4-6.0 (Kurniawati *et al.* 2014).

With further increase in pH, efficiency of degradation again reduced and only 2.9±0.3, and 5.9±0.3% wt loss was observed for HDPE and LDPE respectively at pH 9 and 1.1±0.3, 1.8±0.3% at pH 10.

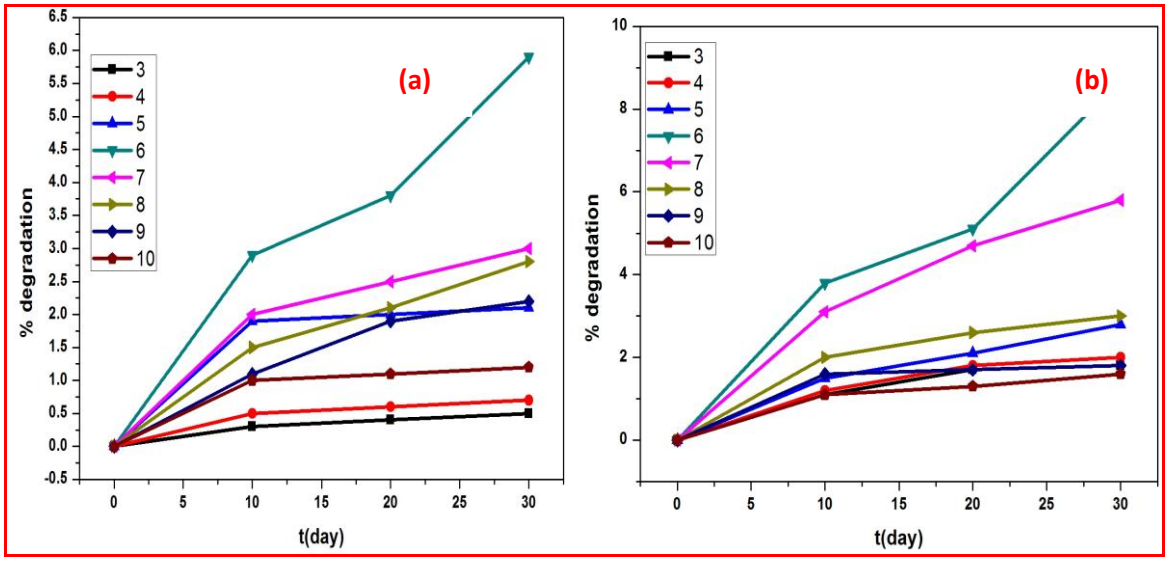

Fig.4.13 Effect of various pH on biodegradation of HDPE (a) and LDPE (b) by *R. Oryzae* NS5 in Potato Dextrose Broth. Conditions: shaking condition, 10% (v/v) inoculum

4.2.8.2 Optimization of temperature

Temperature imparts a vital role in growth and performance of fungal strain. So, the effect of temperature on biodegradation of PE films by *R. Oryzae* NS5 was evaluated in the range of 25–50°C as shown in Fig. 4.14(a-b). It is clear from this figure that ~ 0.4% and 2.5% degradation was obtained at 25°C for HDPE and LDPE respectively. However, with increase in temperature from 25 to 40°C there was an enhancement in the percent wt loss by fungal culture, and 5.7, 8.5% wt. loss of HDPE and LDPE respectively was observed at 40°C. With further increase in temperature, efficiency started decreasing resulting only 0.5% and 2.5% wt loss at 50 °C. The lessened degradation efficiency beyond 40°C is possibly due to lowered enzymatic activity. The wt loss rate depends on temperature because at very low and high temperature (severe conditions) inactivation of enzymes takes place. Increasing the temperature resulted in further decrease in metabolic activity of fungus at 50°C. It can be explained by the statement that the temperature optima for intra and extracellular laccase was reported to be 40^{o}C (Salleh *et al*.1993)

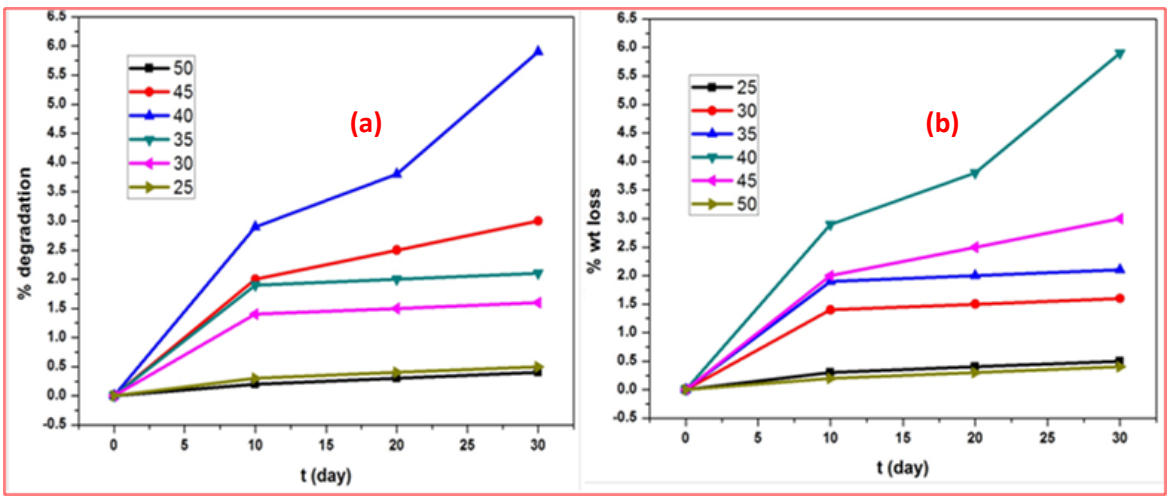

Fig.4.14 Effect of various temperatures on biodegradation of HDPE (a) and LDPE (b) by *R. Oryzae* NS5 in Potato Dextrose Broth. Conditions: pH 6 and 10% (v/v) inoculum

4.2.8.3 Optimization of agitation speed

Figure 4.15 (a-b) shows the effect of shaking speed of rotatory shaker on degradation of PE films. Agitation influences the tested fungi to absorb more nutrients by not only increasing the surface area of the microorganism for degradation of the polyethylene but also by booming the amount of dissolved oxygen in the cultivation medium. Agitation speed has also been proven to be a critical factor influencing mycelial biomass (Hamzah *et al.* 2012). Effect of agitation speed was studied in the range (100-150rpm) and wt. loss of 3.1 and 1.0% was noted for HDPE and LDPE respectively at 100 rpm. Because at slow agitation speed there is no proper absorption of nutrients and cell growth becomes slow in the lack of nutrients. Wt loss was increased with increase in agitation speed up to 120rpm. (5.5 and 8.3% for HDPE and LDPE respectively) Again there was decrease in wt loss while increasing agitation speed as there are greater mechanical or shear forces at high agitation speeds, which led to a higher rate of cell destruction, thus lowering enzyme production (Venkatadri & Irvine 1990). Agitation speeds that were higher or lower than 120 rpm resulted in decreased enzyme production and less fungal growth.

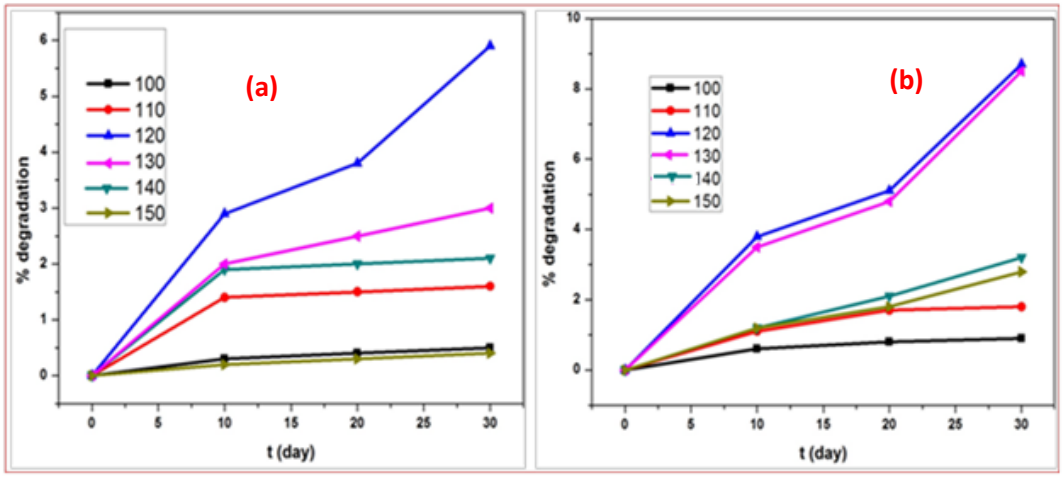

Fig.4.15 Effect of various agitation speeds on biodegradation of HDPE (a) and LDPE (b) by *R. Oryzae* NS5 in Potato Dextrose Broth. Conditions: Temp. 40°C, pH 6, 10% (v/v) inoculum

4.2.9 Kinetics of biodegradation of PE films by *Rhizopus oryae* NS5

Cell growth kinetics in a batch process can be represented by the eqn. $dX/dt = \mu X_0$ ..... (4.1)

The specific growth rate for HDPE and LDPE were determined from the slope of $\ln(X/X_0)$ against time, during the exponential growth phase (from day10 to 30) as shown in figure 4.16 & 4.17. Here X_0 represents the initial biomass, X is the biomass at different time (days) intervals. The results show that the specific growth rate for HDPE is 0.0423 day^{-1} while that for LDPE is 0.0631 day^{-1}.A fair fitting was obtained as shown in Figure 4.16(a-b) and 4.17(a-b). The parameters with their values are shown in table 4.1(a-b) with r^2 (regression coefficient) of 97%. It has been observed that there was decrease in the weight of the HDPE as well as LDPE films with time during the exponential growth phase (10 to 30 days) of fungus. It confirms that fungus is utilising PE films for its growth. Slope of the graphs 4.16b and 4.17b which represent degradation rate constant were found to be 5×10^{-5} and 8×10^{-5} $gday^{-1}$ for HDPE and LDPE film respectively.

4.2.9.1 Kinetics of biodegradation of HDPE film by *Rhizopus oryae* NS5

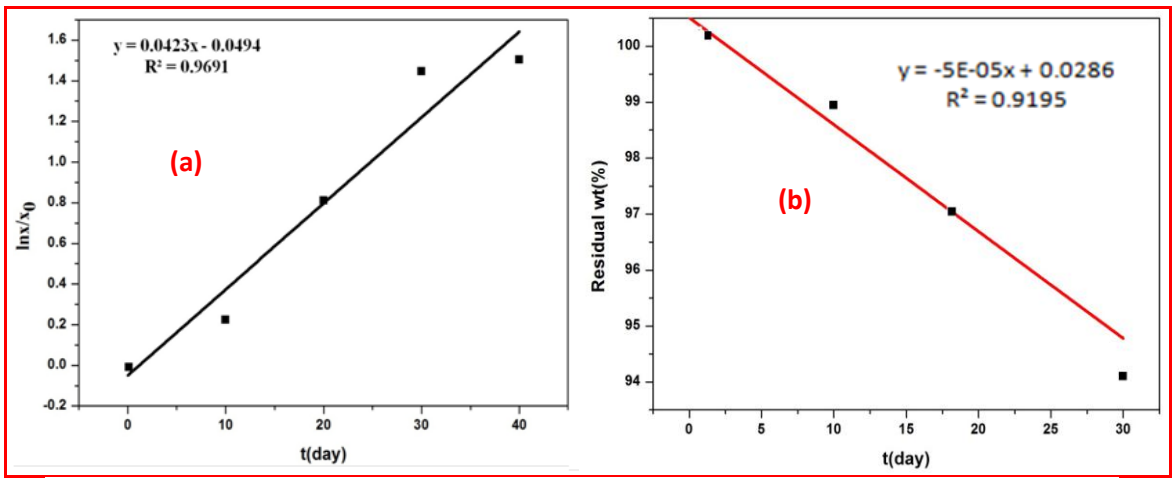

Fig.4.16 Increase in fungal growth (a) and decrease in weight (b) of HDPE film

4.1(a) Rate constants for cell growth and degradation of HDPE film

k ($gday^{-1}$)	μ(day^{-1})	r^2(film)	r^2 (Fungal growth)
5×10^{-5}	0.0423	0.9195	0.9691

The values are the means of three replicates with the standard deviation which was within 5% of the mean.

4.2.9.2 Kinetics of biodegradation of LDPE film by *Rhizopus oryae* NS5

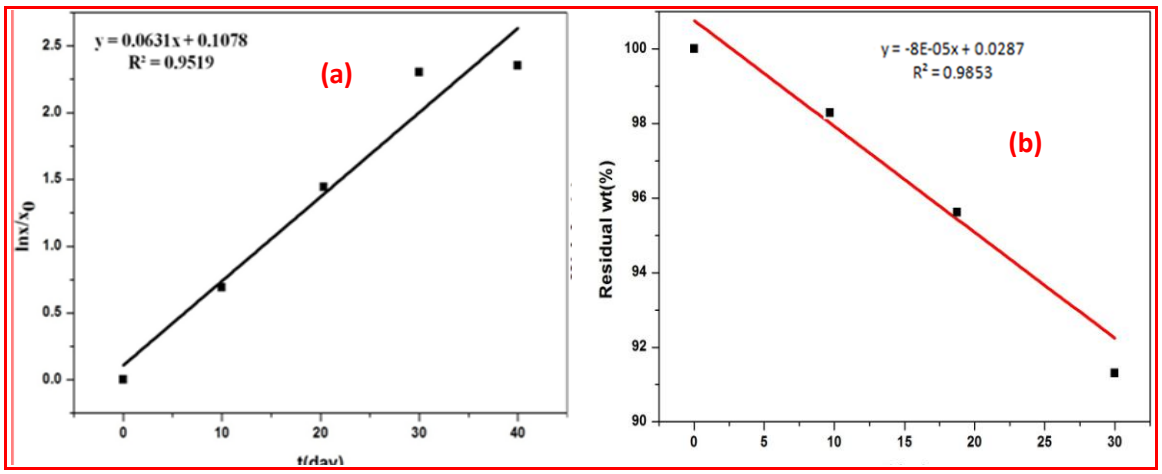

Fig.4.17 Rate of fungal growth (a) with decrease in weight (b) of LDPE film

Table 4.1(b) Rate constants for cell growth and degradation of LDPE film

k ($gday^{-1}$)	μ(day^{-1})	r^2(film)	r^2 (Fungal growth)
8×10^{-5}	0.0631	0.9853	0.9519

The values are the means of three replicates with the standard deviation which was within 5% of the mean.

4.2.10 Enzymatic activity of Laccase and Manganese peroxidase by *R. Oryzae* NS5

Rhizopus oryzae NS5 gives positive results for laccase and manganese peroxidase production (Shinkafi *et al.*2014). The laccase enzymes are known to degrade wood, plastic, paint, including jet fuel (Buddolla *et al.*2014).

Enzyme activity was calculated at regular time interval and an increasing pattern of enzyme activity with time for both of the enzymes was observed. Maximum enzyme activity was observed between 20 and 30 day which was consistent with the increase in weight of fungal mycelia (Fig.4.16a & 4.17a) and correspondingly decrease in wt. of PE films (Fig.4.16b& 4.17b). Laccase enzyme has been shown to degrade polyethylene due to conserved copper binding sites which couple the oxidation of a substrate with the cleavage of dioxygen bonds, leading to the capability to degrade plastics particularly polyethylene. The primary mechanism for the biodegradation of high molecular weight polymer (i.e. polyethylene) is the oxidation or hydrolysis by enzyme to create functional groups that improves its hydrophilicity. Consequently, the main chains of polymer are degraded resulting in low molecular weight polymer and feeble mechanical properties, thus, making it more accessible for further microbial assimilation (Albertsson and Karlsson 1990, Albertsson *et al.* 1987 & Huang *et al.* 1990).

4.2.10.1 Activity of laccase and manganese peroxidase for HDPE

Enzymatic activity of laccase and manganese peroxidise was calculated at regular interval of time. It was found that there was increase in enzyme activity with incubation time. Activity of manganese peroxidase (0.00092 IU/ml) was more compared to laccase activity (0.00087 IU/ml) after one month of incubation for HDPE (Table 4.2 a-b).

Table 4.2(a) Activity of laccase for HDPE

Days	10	20	30
Enzyme activity (IU/ml)	0.00029±0.0001	0.00043± 0.0004	0.00087± 0.0002

Table 4.2(b) Activity of manganese peroxidase for HDPE

Days	10	20	30
Enzyme activity(IU/ml)	0.00035 ±0.0003	0.00052 ±0.0001	0.00092 ±0.0002

4.2.10.2 Enzymatic activity of laccase and manganese peroxidase for LDPE by *R. Oryzae* NS5

For LDPE the activity of manganese peroxidase (0. 0.00098 IU/ml) was greater than laccase (0.00094 IU/ml) after one month incubation. (Table 4.3a-b). Activity of manganese peroxidase is more for LDPE than HDPE. It is consistent with the comparatively more loss in wt of LDPE than HDPE by *R. oryzae.* Similar observations have been reported by Sowmya *et al.* using fungal consortia with *Curvularia lunata, Alternaria alternata, Penicillium simplicissimum* and *Fusarium sp* (Sowmya *et al.* 2014).

Table 4.3(a) Activity of laccase for LDPE

Days	10	20	30
Enzyme activity(IU/ml)	0.00038±0.0001	0.00051± 0.0004	0.00094± 0.0002

Table 4.3(b) Activity of manganese peroxidase for LDPE

Days	10	20	30
Enzyme activity(IU/ml)	0.00040±0.0003	0.00055±0.0001	0.00098±0.0001

Conclusion

In the present study, lab isolate fungal strain, *Rhizopus oryzae* NS 5 (ITCC no. KT160362) capable of not only adhering to the surface of high density and low density polyethylene but also utilizing it as the source of carbon. The degradation has been confirmed by morphological changes, weight loss, mechanical properties changes and change in functional groups. Even though it is a slow technique, the prevailing examine gives an insight to the evidences of biodegradation of HDPE and LDPE. It shows that there is a remarkable possibility of finding microorganisms from the surroundings that may degrade artificial plastics. Knowledge of the enzyme device of *Rhizopus oryzae* NS5 will provide an insight to its role in biodegradation of PE. Further studies are underway for isolation of microorganisms with a ability of degrading untreated PE. Currently our efforts are focused on elucidating the pathway for the degradation of low density polyethylene and developing a new bioremediation strategy using *R. oryzae* NS5.