International Journal of Applied Research 2017; 3(3): 693-695

ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 5.2
IJAR 2017; 3(3): 693-695
www.allresearchjournal.com
Received: 24-01-2017
Accepted: 25-02-2017

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Production and screening of depolymerising enzymes by potential bacteria and fungi isolated from plastic waste dump yard sites

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Abstract
The most alternative plastic waste treatment method is enzymatic degradation. These kind of depolymerising enzymes are effectively produced by some microorganisms. This study revealed that, microorganisms produce depolymerising enzymes during biodegradation of polyethylene carry bag (PCB). For that, in sterile minimal broth, pure culture of various isolates were added, pieces of mass polyethylene carry bag films were added and incubated for a month. After the incubation the broth was centrifuged at 10,000 rpm for 20 minutes at 4 °C. The bacteria *Pseudomonas aeruginosa*, *Bacillus* sp and *fungi Fusarium graminearum* were found producing depolymerising enzymes *viz.*, amylase, lignin /manganese peroxidase and laccase followed by others. These potential bacteria and fungi might be applied for bioremediation in the polyethylene-contaminated environments.

Keywords: Polyethylene, depolymerase, *Fusarium graminearum* and Bioremediation

1. Introduction
Polymeric materials were subjected to degradation by biological, chemical and or physical (mechanical) actions in the environment. Endo and exo-enzymes are produced by both prokaryotic and eukaryotic, most often these enzymes can effectively interact and breakdown the solid macromolecules to digest them. In polyethylene biodegradation microorganisms are producing specialized enzymes of its own secretions for depolymerization of substances. The enzymatic degradation of plastics by hydrolysis is a two-step process: first, the enzyme binds to the polymer substrate then subsequently catalyzes a hydrolytic cleavage. Polymers are degraded into low molecular weight oligomers, dimers, monomers and finally mineralized to CO_2 and H_2O, which can pass through the microbial cell membranes and act as a source of carbon and energy (Frazer A. C. 1994) [5]. Many microbial enzymes are involving in plastic degradation like microbial production of Laccase, lignin & manganese peroxidase, lipase, esterase, amylase etc. Typical examples related to biodegradation are biological hydrolysis by hydrolase enzymes and oxidation by oxido-reductase enzymes. The hydrolase enzyme is responsible for the hydrolysis of ester, carbonate, amide and glycosidic linkages of the hydrolysable polymers producing the corresponding low molecular weight oligomers. The oxido-reductase enzyme is responsible for the oxidation and reduction of ethylenic, carbonate, amide, urethane, etc (Matsumura, 2005) [6]. However, the degradation process precedes both by abiotic and biotic actions in the environment. The manganese peroxidase and lignin degrading enzyme partially produced from *Phanerochaete chrysosporium* are reported to degrade high molecular weight polyethylene. This research investigated the production of various microbial enzymes involving in depolymerization and screened their biodegradation activity in polyethylene carry bag isolated from plastic waste dump yard sites.

2. Materials and Methods
2.1 Screening of microbial isolates for their enzyme activity
For production of enzymes by microorganisms, minimal broth was prepared in 100 ml Erlenmeyer flasks and sterilized. Pieces of mass polyethylene carry bag films were added in each culture and incubated for a month at favorable temperature of every individual cultures. After the incubation period the polyethylene films were removed and the broth was

centrifuged at 10,000 rpm for 20 minutes using cooling centrifuge, temperature was reduced to 4 °C.

2.2 Manganese peroxidase assay (Vares *et al.*, 1995) [7]

Manganese peroxidase (MnP) activity was monitored with phenol red as substrate at 30 °C. Reaction mixtures contained 25 mM Lactate, 0.1 mM MnSO4, 1 mg of bovine serum albumin, 0.1 mg of phenol red and 0.1 ml of culture filtrate in 20 mM sodium succinate buffer (pH 4.5) in a total volume of 1 mL. The reaction was started by the addition of H_2O_2 to final concentration of 0.1 mM and was stopped after 1 min with 50 µl of 10% NaOH and A610 was measured. Maximum peroxidase activity was expressed as the increase in A610 min^{-1} ml^{-1}.

2.3 Lignin peroxidase assay (Tien and Krick, 1984) [8]

Lignin peroxidase was measured using veratryl alcohol at 25 °C. The reaction mixture consists of 0.1M sodium tartrate buffer (pH 3.0). 0.4 mM veatryl alcohol and 1.65 ml of culture filtrate in a total volume 0.3 ml. The reaction was started by adding H_2O_2 to a final concentration 0.2 mM and A310 were monitored.

2.4 Screening of laccase and manganese peroxidase assay (Viswanath *et al*., 2008) [9].

The isolated bacterium was screened for the laccase production using laccase screening medium (LSM). Bacterium was inoculated in LSM agar plate and the plate was incubated for 7 days in dark condition. The substrate utilized reddish brown color in screening medium indicates the positive strain for laccase.

2.5 Amylase assay (Annamalai *et al,* 2011) [10]

The reaction mixture was prepared by mixing 0.5 ml of 1% soluble starch (substrate) in 1.2 ml of 50 mM potassium phosphate buffer (pH 7) and added 0.3 ml of the supernatant (enzyme) thereafter. The assay was performed using four test tubes, namely, Test (T), Substrate Control (SC), Enzyme Control (EC) and Blank (B). In Substrate control, substrate and buffer were taken while enzyme and buffer were added into Enzyme control test tube. Blank contained only 2 ml of buffer. All the test tubes were incubated at 40 °C for 15 minutes followed by the addition of 1 ml of freshly prepared DNSA.

The DNSA reagent was prepared by mixing two components: 60% Rochelle salt (sodium potassium tartarate) dissolved in distilled water. 5% 3, 5-dinitrosalicylic acid dissolved in distilled water containing 2 Mol/L sodium hydroxide. These test tubes were then subjected to boiling at 100 °C for 5 minutes. The volumes of Substrate control and Enzyme control tubes were made equal with the solution in the test by adding 0.5 ml of substrate into enzyme control and 0.3 ml of enzyme into substrate control. The test tubes were cooled and their optical density (O.D.) readings were noted at 540 nm. The amount of reducing sugar formed was determined with the help of a standard curve (25 µg/ml to 250 µg/ml).

3. Results and Discussion

The isolated bacteria *Pseudomonas aeruginosa, Alcaligenes sp, Bacillus sp, Bacillus anthracis, Shigella sonnei, Rhodococcus ruber and Streptomyces griseus* and fungi *Aspergillus flavus, Aspergillus niger and Fusarium graminearum* from the plastic wastes dump yard sites were screened for their ability to produce the enzymes amylase, manganese peroxidase, lignin peroxidase and laccase. These enzymes have the ability to cleave the carbon bonds of the polyethylene films to be utilized by microorganisms. Production of these enzymes induces the biodegradation of PCB (polyethylene carry bag). The enzyme producing microbial isolates were identified and listed in table 1.

Table 1. Screening of isolates for depolymerising enzyme production

Isolates	Name of the microorganisms	Amylase	laccase	Lignin peroxidise	Manganese peroxidase
PBIV	*Pseudomonas aeruginosa*	+	+	+	+
PBVI	*Alcaligenes sp*	+	-	-	-
PBIX	*Bacillus sp*	+	+	+	+
PBXV	*Bacillus anthracis*	+	+	+	-
PBXVI	*Shigella sonnei*	-	-	-	-
PFI	*Aspergillus flavus*	+	+	+	-
PFV	*Aspergillus niger*	-	+	+	+
PFVII	*Fusarium graminearum*	+	+	+	+
PAI	*Rhodococcus ruber*	-	+	+	+
PAIII	*Streptomyces griseus*	+	+	+	+

Nb: PB denotes polyethylene degrading bacteria, PF denotes polyethylene degrading fungi and PA denotes polyethylene degrading actinomcetes.

Table 2: Effect of microbial isolates on enzymatic depolymerisation of Polyethylene carry bag

Isolates	Name of the microorganisms	Amylase (%)	Laccase (%)	Lignin peroxidase (%)	Manganese peroxidase (%)
PBIV	*Pseudomonas aeruginosa*	31	46	27	39
PBVI	*Alcaligenes sp*	36	NP	NP	NP
PBIX	*Bacillus sp*	48	32	13	18
PBXV	*Bacillus anthracis*	26	13	21	NP
PFI	*Aspergillus flavus*	20	39	44	NP
PFV	*Aspergillus niger*	27	43	53	47
PFVII	*Fusarium graminearum*	19	35	32	40
PAI	*Rhodococcus ruber*	NP	62	41	51
PAIII	*Streptomyces griseus*	33	38	39	48
SE_D		3.31	4.88	4.62	4.89
CD		0.290	0.226	0.438	0.326

Nb: PB denotes polyethylene degrading bacteria, PF denotes polyethylene degrading fungi and PA denotes polyethylene degrading actinomcetes.

Fig. 1: Treatment for enzyme production

Fig. 2: Amylase production plates

The maximum range of amylase production was observed in (B1) *Bacillus* sp (48%), followed by others, the actinomycetes *Rhodococcus ruber* not produced the enzyme amylase. The maximum range of Laccase was observed in *Rhodococcus ruber* (62%) and followed by *Pseudomonas aeruginosa* (46%) and others the *Alcaligenes* sp. not produced the laccase. The maximum range of lignin peroxides was produced in *Aspergillus niger* (53%) followed by *Aspergillus flavus* and others the *Alcaligenes* sp. not produced that enzyme. The maximum range of manganese peroxidise was produced in *Rhodococcus ruber* (51%) followed by *Aspergillus niger* (47%) and the other bacterial isolates *Alcaligenes* sp., *Bacillus anthracis* and fungal *Aspergillus flavus* not produced the enzyme. The quantitative productions of depolymerising enzymes were detailed in table 2. Bholay *et al*. (2012) [3] reported that the lignin peroxidase enzyme activity of the isolates was in the range of 30% to 76%. Among all the isolates highest activities were obtained for *Pseudomonas aeruginosa* and *Serratia marcescens* which were 75.67% and 59.45%
Demonstrated that there are three bacteria producing, some veratryl alcohol but no 2, 4-dichlorophenol lignin peroxidase. We can then assume that each of the bacterial cultures was lignolytic. Polycaprolactone (PCL), an important polymer due to its strong mechanical properties, biodegradability, and miscibility with a number of other polymers (Kim and Rhee 2003 [3]; Labet and Thielemans 2009) [13], has been investigated for its degradation in terrestrial and aquatic environments. A number of PCL-degrading bacterial and fungal strains *viz*., *Alcaligenes, Clostridium, Aspergillus, Penicillium, Fusarium,* and *Streptomyces* have been isolated (Benedict *et al*. 1983 [2]; Tokiwa *et al*. 2009) [14].

4. Conclusion

The accumulation of plastic is one among the worst menaces our environment is facing. Disposing them by natural method is the need of the hour. Harnessing the power of polyethylene depolymerizing enzymes obtained from microbes would be an effective and cheap method. The microbes viz., *Rhodococcus ruber* and *aspergillus niger* found to various PE depolymerases and can be utilized by mixing with suitable carrier material to enhance biodegradation under controlled environment.

5. Acknowledgement

The authors thank UGC-GUCC for giving financial support under XII Plan innovative project to carry out the above research.

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