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Biodegradation of plastic by some fungi as influenced by physical factors

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ABSTRACT

Polyethylene and plastic are resistant to degradation and decay. Their accumulation in the environment produces environmental problems. So, the purpose of this research is to evaluate the ability of several fungal species to destroy plastic materials, choose the most active strain, and investigate some physical parameters influencing ligninolytic enzyme productivity. Twenty-six fungal isolates were recovered from benzene station soils, the Petroleum refinery area, and the Botany Department, Faculty of Science, Fayoum University. All of the tested fungi showed lignin peroxidase activity in both soil and liquid cultures. *Aspergillus niger* and *Penicillium chrysogenum* were more capable of producing ligninolytic enzymes. Hence, they were chosen for the following studies. The two fungi under investigation assessed several parameters to maximize the production of lignin peroxidase enzymes, such as Temperature, pH, and moisture. The temperature range from 15°C to 45°C was tried and the optimum temperature of the two fungi under study were 40°C for *Penicillium chrysogenum* and 35°C for A.niger. Maximum enzyme activity was reached at pH 5 and 6 for *Penicillium chrysogenum* and *Aspergillus niger*, respectively. From one to five days, The amount of enzyme production is greatly increased as a result of the constant rise in the moisture content of plastic materials, it reached 9.9 units⁄ml and 11.0 units⁄ml for P.chrysogenum and A.niger, respectively after 5 days of incubation of plastic materials in moisture content.

1. Introduction

Plastic materials are inexpensive, lightweight, and water-resistant that can be used for a variety of purposes [1]. This man-made polymer can be found in nearly every facet of life [2]. The use of plastic products is predicted to grow by 9% per year [3- 5]. Plastic contains a number of dangerous compounds that are toxic to biological organisms[6 - 8]. Plastic consumption is known to be increasing in response to changing lifestyles and a growing population[9]. However, because it is non-degradable, it has a negative influence on the environment [10]. Plastics are accumulating in the environment as a result of the increased use of plastic polymers [3]. because these pollutants require a very long time to be completely degraded in nature, these pollute the water, land, and air [11, 12]. Currently, The industry is divided into two types, organized and unorganized [13]. The organized sector produces high-quality products, whereas the unorganized sector produces low-quality and low-cost products. Acrylics, silicones, polyesters, polyurethanes, and halogenated plastics are among the most prominent categories in these classifications of plastics based on the chemical structure of their backbones and side chains [14 - 16]. Both land and aquatic habitats have been influenced by plastic waste [17]. Plastic waste is typically disposed of by landfill, incineration, or recycling. [18]. Landfill plastic waste processing takes a long time without degrading [19, 20]. Toxic gases will be released into the environment as a result of the incineration treatment [19, 21, 22]. Furthermore, recycling plastic waste is extremely expensive [19, 21, 22]. The goal of this study is to isolate and identify microorganisms in soil that are related to polyethylene and plastic polymers.

2. Materials and Methods

2.1. Microorganisms Used

Twenty-six isolates were tested for their ability to degrade plastic materials as follows: a- Seven fungal strains namely as, *Aspergillus flavus, Alternaria alternata, Aspergillus fumigatus, Aspergillus parasiticus, Fusrarium fusarium, Penicellium chrysogenum,* and *Aspergillus niger* were obtained

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from the Mycological Laboratory, Botany Department, Faculty of Science, Fayuom University. b- Nineteen fungal isolates were isolated from soil petroleum refinery and benzene station of Fayoum Governorate, Egypt. The soil samples were collected in sterile containers at a depth of nearly 5 cm and air-dried at room temperature in the laboratory.

2.2. LDPE Powder Preparation

Low-density polyethylene (LDPE) sheets were immersed in xylene and heated for 15 minutes to thoroughly dissolve. The residue was then physically crushed while wearing gloves. The crushed residue was allowed to evaporate before being dried overnight in a hot air oven at 60 °C. The powder obtained was maintained at room temperature [23].

2.3. Isolation of plastic degrading fungi

One gram of soil samples were serially diluted up to 10-7 . 1.0 ml of dilutions (10-5 ,10-6 , 10-7) were spread on Czapek's dox agar medium plates [24]. Plates were incubated at 25°C for five days. The soil dilution plate method was used for fungal isolation, in accordance with[25]. A Czapek-Dox agar medium was employed for this purpose. This medium contained the following elements (g/l): Sucrose, 20 $NaNO_3$, 2 : K_2HPO_4, 1 : KCl, 0.5: $MgSO_4$. $7H_2O$, 0.5: $FeSO_4$. 7 H_2O, 0.01 and agar, 15, Streptomycin(30 µg/ml) was added to the aforementioned medium after sterilization by autoclaving at 121 °C and 1.5 bars for 20 minutes. To isolate the fungal species, each dilution of A Czapek-Dox agar medium plate was cultured at 25°C for 4-7 days. The grown colonies were isolated and sub-cultured repeatedly to obtain pure culture, which was then stored as slants at 4°C .

2.4. Screening of plastic degrading microorganisms

To isolate the biodegrading microorganisms, they were grown in mineral salt agar plates with 0.1% (w/v) low-density polyethylene powder [26]. The microorganisms with a clearance zone around their colonies were chosen for further research due to their ability to degrade plastic samples [27]. Plates were incubated in a static incubator at 30˚C. The diameter of the clear zone of each plate was measured after 3, 6, and 9 days.

2.5. Determination of dry weight of the residual polyethylene (PE).

The weight loss of plastic material was determined in two ways, the conical flask test method and the Petri dish test method.

2.5.1. Conical flask test method.

Pre-weighed 100 mg polyethylene discs were aseptically transferred to a conical flask holding 100 ml of culture broth medium inoculated with fungal strains [28]. In the microbe-free media, plastic discs were employed as a control. Separate flasks were retained for each treatment and placed in a static incubator. After three weeks, the plastic discs were collected, cleaned with distilled water, shade-dried, and weighed to determine their final weight. The weight loss of the plastics was calculated using the data obtained.

2.5.2. Petri dish test Method

Bottom media agar was made by combining 15 g of agar with 1 L of basal medium and autoclaving for 20 minutes before pouring into petri plates. Each test strain (5.0 mL of spore suspension) was placed on top of sterilized polyethylene film pieces (100 mg). The polyethylene film was removed after 3 weeks of incubation at 30°C and washed several times with distilled water before being dried overnight at 80°C. The weight change of the polyethylene film was calculated as weight loss = Initial weight - Final weight [29].

2.6. Clear zone test Method.

Two agar media layers were put onto 10 cm plates, the lower (15 mL) layer contained baseline medium agar, and the upper (10 mL) layer contained a polymer suspension (4 g/L suspended polymer powder and 14 g/L agar). Each test strain (5 mL spore suspension) was placed into the center of the plate and cultured at 30 °C for 14 days before measuring and recording the clear zone diameter [27].

2.7. Effect of some physical factors on production of extracellular ligninolytic enzymes

This was done by testing some environmental conditions that affect fungal ligninolytic enzyme production to determine the best condition for the production of enzyme by two fungal strains namely, *Penicillium chrysogenum* and *Aspergillus niger*. The fermentation medium was modified to be suitable for studying the respective factors as follows:

2.7.1. Moisture

1 cm diameter fungal discs were obtained from the periphery of 7-day-old cultures grown on the modified basal mineral salts medium at pH 7 in triplicate flasks [30, 31]. The flasks were incubated at 30°C for 10 days under static conditions. The filtrate was used to calculate extracellular lignin peroxidase production as well as the final pH value. To determine the dry weight, the mycelial mat was oven-dried at 80°C until it attained a consistent weight. Polyethylene discs were soaked in dist. water for 1, 2, 3, 4, and 5 days before being incubated. The enzyme activity was measured after ten days of incubation.

2.7.2. Effect of Incubation temperature

The pH of the mineral salt media was set at 7.0. Sterile plastic components were added to the flasks at a rate of 1%. Fungal species disc 1 cm in diameter were induced in triplicate flasks. Control flasks were created with the above medium but without fungal strains. Flasks were incubated for 21 days at temperatures ranging from 15°C to 45°C. At the end of the incubation period, enzyme activity was evaluated [28].

2.7.3. Effect of pH

The pH of the fermentation medium was changed to the values 3, 4, 5, 6, 7, 8, and 9. Following autoclaving, 1% cleaned sterile plastic materials were added to the flasks. In triplicate flasks, discs of fungal species from a seven-day culture of *P. chrysogenum* and *A. niger* were inoculated. Control flasks with the above medium but without tested fungal species were prepared. Flasks were incubated for 21 days at 40°C for *P. chrysogenum* and 35 °C for *A. niger* (the best incubation temperatures from the previous test). At the end of the incubation periods, the enzyme activity was evaluated.

2.8. Quantitative Lignin Peroxidase Assay

Methylene Blue Assay: 3.0 ml of assay mixture was used: 2.2 ml diluted supernatant, 0.1 ml of 1 mM methylene blue, and 0.6 ml 0.5 M sodium tartrate buffer (pH 4.0). The reaction was started by adding 0.1 ml of 2.7 mM H_2O_2. The absorbance drop at 664 nm was utilized to calculate the dye conversion to Azure C. The absorbance change per minute was reported, and the activities were given as U /L [32].

3. Results and discussion

3.1 Isolation and screening of plastic degrading fungi

The fungi used in this investigation were obtained from the following places, cultures from No.1 to 7 which are; *Aspergillus flavus, Alternaria alternata, Aspergillus fumigatus, Aspergillus parasiticus Fusrarium fusarium, Penicillium chrysogenum,* and *Aspergillus niger*, came from the Mycological Laboratory, Botany Department, Faculty of Science, Fayuom University. Cultures from No. 8 to 16 listed in Table (1) were isolated from benzene stations obtained from several areas of Fayoum Governorate. Cultures from No. 17 to 26 came from the petroleum refinery area. The enzyme activity of different fungal isolates cultured on a solid mineral medium supplemented with 0.06% polyethylene powder suspension is shown in Table (1). Plastics were employed as the only source of carbon and nitrogen. A thin clear zone encircled the fungal colonies, indicating that the degrading enzymes adhere to the fungal cell wall rather than being released in the media [33, 34].

Table 1. Diameter of fungal colonies and clear zones (mm) of each fungal species after various incubation periods in static cultures under laboratory conditions

Source of Isolates	No.of Isolates	Diameter of fungal colonies and clear zones diameter on LDPE powder (mm)		
		3 days	6 days	9 days
Botany Dep.Fac.of Science	1	6* (-)**	7 (18)	28 (30)
	2	5 (-)	17 (19)	23 (24)
	3	8 (-)	15 (17)	25 (26)
	4	5 (-)	16 (18)	22 (24)
	5	8 (-)	15 (17)	17 (21.4)
	6	6 (-)	15 (18)	21(23)
	7	7 (10)	22 (23)	27 (29)
Benzene Stations Fayoum Governorate	8	8 (11)	19 (18)	23 (21)
	9	5 (7)	11 (13)	15 (17.5)
	10	6 (9)	7 (18)	12 (21)
	11	11 (11)	12 (12.5)	14 (14.4)
	12	4 (-)	9 (-)	11 (-)
	13	6.5 (6.5)	7 (7.5)	9 (12.0)
	14	12.5 (4)	13 (14.5)	15.5(21)
	15	11.2 (8)	13(13.5)	17(18)
	16	4 (-)	7 (-)	9 (-)
Petroleum refinery area	17	_	4 (5.5)	6 (7.8)
	18	8 (-)	10 (10)	20 (20)
	19	_	_	6 (-)
	20	_	4 (-)	7 (-)
	21	13 (-)	14 (14)	17 (17)
	22	9 (9)	11 (13)	14 (14.5)
	23	12(12.2)	13 (13.7)	19 (20.1)
	24	11 (-)	14(-)	18 (-)
	25	_	2 (5)	6 (8)
	26	_	4 (-)	11(-)

* Fungal colony diameter (mm), ** Clear Zone diameter (mm)

3.1.Qualitative screening for enzyme activity of 7 fungal species, on polyethylene powder agar plates.

However, only seven isolates were recognized at the genus and species levels. When examined using three different proposed test methodologies, they showed considerable degradation potential, including the petri dish test method, the liquid static culture method, and the clear zone method Table (2). Our results are similar to previous studies where these strains generated significant weight loss in the polyethylene powder employed in the agar plate cultures, reaching up to 63 mm in the case of *A.niger*. *Alternaria alternata*, on the other hand, produced a clear zone diameter only 44.0 mm and 55.0 mm in case of *P.chrysogenum*.

Table 2. Clear zone diameter (mm) by selected 7 fungi on minimal salt medium at pH 7 and temperature 30 °C after 14 days incubation

Fungal species	Clear zone diameter method	
	Diameter of clear zone (mm)	Colony mean diameter (mm)
A. niger	63.0±1.7	72.0±1.5
A. flavus	27.0±1.2	33.0±1.7
Alternaria alternata	44.0±2.1	48.0±1.2
Fusarium fusarium	32.0±2.9	37.0±0.6
Penicillium chrysogenum	55.0±0.5	61.0±2.1
A. fumigatus	11.0±0.6	24.0±2.5
A. parasiticus	13.0±0.8	35.0±0.8
LSD (at 0.01)	6.759	5.836
LSD (at 0.05)	4.821	4.163

3.2. Conical flask test method

In this experiment, polyethylene pieces were infected in a liquid culture medium containing fungal strains and stored for three weeks to determine the percentage of weight loss induced by fungal species. The results demonstrate the degradation ability of the fungal strains after three weeks of incubation. *A.niger* and *P.chrysogenum* had the highest percentage of weight loss due to deterioration (18.6, 13.7), respectively Table (3). They have a higher degrading potential than other fungal species. This experiment resulted in the identification of seven fungal species with the potential to degrade polyethylene, as measured by growth rates reached on basal medium amended with polyethylene materials as the sole source of carbon and nitrogen.

Table 3. Percentage weight loss of polyethylene samples by selected 7 fungi on minimal salt medium at pH 7 and temperature 30 °C after 3 weeks incubation.

Fungal species	Difference of weight loss (mg)	% Weight loss/3 weeks
Control	0.13 ±0.01	0.13 ±0.01
A. niger	18.6±0.20	18.6±0.2
A. flavus	9.4 ±0.06	9.4 ± 0.06
A. alternata	11.9±0.4	11.9 ±0.4
F. fusarium	10.3± 0.12	10.3 ±0.12
P. chrysogenum	13.7 ± 0.06	13.7±0.06
A. fumigatus	9.2 ±0.12	9.2±0.12
A. parasiticus	8.5±0.06	8.5±0.06
LSD (at 0.01)	0.689	0.689
LSD (at 0.05)	0.496	0.496

3.3. Determination of % weight loss by petri dish test technique.

Only seven isolates demonstrated breakdown activity of polyethylene film in the petri dish test technique Table (4). The highest degradation activity was obtained by *P.chrysogenum*, which caused 17.8 % loss in weight of polyethylene film pieces, and *A.niger* showed activity of about 12.7%. *A. fumigatus* showed minimal activity of about 7.2 %. It is clear that *A. niger* and *P. chrysogenum* produced the most extracellular enzymes with the highest activity under static cultural conditions. As a result, they were chosen for further studies.

Table 4. Percentage of weight loss of polyethylene film by selected 7 fungi on minimal salt medium at pH 7 and temperature 30 °C after 21 days of incubation.

Fungal species	Petri dish test technique	
	Weight Loss (initial-final) (mg)	% weight loss
Control	0.22±0.02	0.11±0.26
A. niger	25.4±0.023	12.7±0.12
A. flavus	20.8±0.06	10.4±0.23
Alternaria alternata	18.2±0.18	9.1±0.06
Fusarium fusarium	22.4±0.17	11.2±0.12
Penicillium chrysogenum	35.6±0.07	17.8±0.13
A. fumigatus	14.4±0.23	7.2±0.12
A. parasiticus	16.6±0.23	8.3±0.17
LSD(at 0.01)	0.755	0.664
LSD (at 0.05)	0.544	0.478

3.4. Effect of some physical factors on the production of ligninolytic enzymes by tested fungi

3.4.1. Effect of Moisture

Microbe development requires moisture, so it plays a vital role in the deterioration of plastic[35]. Microbe activation requires a sufficient amount of water. Increased moisture content increases microbial hydrolytic activity[9]. Because microorganisms require water for growth and multiplication. Moisture can affect polymer biodegradation in different ways. As a result of rapid microbial action, the rate of polymer degradation increases in the presence of sufficient moisture[36]. Furthermore, moisture-rich conditions encourage hydrolysis by enhancing chain scission activities. As a result, all of the studied fungal species exhibit substantial plastic degradation with increasing moisture content [37, 38]. After 5 days, the influence of moisture on lignin peroxidase synthesis by the investigated fungi is shown in Table (5). The progressive rise in moisture from one to five days resulted in a large increase in enzyme synthesis. It reached 9.9 units⁄ml and 11.0 units⁄ml for *P.chrysogenum* and *A.niger*, respectively after 5 days of incubation of plastic materials in moisture content [39, 40, 41].

Table 5. Effect of miosture of the incubation medium of Penicillium chrysogenum and Aspergillus niger on the final pH of the cultivation medium, fungal dry weight and lignin peroxidase activity

Moisture / days	Final pH		Dry Weight (mg/100ml)		Enzyme activity (U/ml)	
	Penicillium	*Aspergillus*	*Penicillium*	*Aspergillus*	*Penicillium*	*Aspergillus*
Control	7.7±0.17	7.8±0.12	70.0±2.52	82.0±1.15	7.8±0.12	8.4±0.21
1	7.8±0.25	8.0±0.29	75.0±2.08	84.4±0.23	7.8±0.15	8.5±0.31
2	7.9±0.12	8.1±0.06	81.0±1.15	85.5±0.29	8.3±0.17	8.5±0.15
3	8.0±0.12	8.2±0.20	81.1±0.06	86.0±1.15	9.2±0.12	9.3±0.12
4	8.2±0.06	8.3±0.17	83.2±0.12	90.0±1.53	9.2±0.12	11.0±0.12
5	8.2±0.06	8.4±0.21	84.0±2.08	90.1±0.06	9.9±0.12	11.0±0.06
LSD (at 0.01)	N.S		3.816		0.268	
LSD (at 0.05)	N.S		2.807		0.197	

N.S : Non significant

3.4.2. Effect of incubation Temperature

One of the most important parameters influencing enzyme secretion is the incubation temperature. The trials were designed to investigate the influence of various incubation temperatures on enzyme synthesis i.e. 15, 20, 30, 35,40, and 45°C to detect the most favorable temperature for the maximum enzyme production after 21 days for *Penicillium chrysogenum* and *Aspergillus niger*. The results in Table (6) showed that the optimum incubation temperatures for *Penicillium chrysogenum* and *Aspergillus niger* were 40°C and 35°C, respectively due to these fungi being mesophilic. The enzyme activity was 12.20 and 13.30 U/ml for *P.chrysogenum* and *A. niger*, respectively. These findings are consistent with[42, 43].

Table 6. Effect of incubation temperatures of *Penicillium chrysogenum* and *Aspergillus niger* on the pH of the cultivation medium, fungal dry weight and enzyme activity

Temperature (°C)	pH		Dry Weight (mg/100 ml)		Enzyme activity (U/ml)	
	Penicillium	*Aspergillus*	*Penicillium*	*Aspergillus*	*Penicillium*	*Aspergillus*
15	7.2±0.12	7.3±0.17	75.0±2.89	77.4±0.23	N.D	N.D
20	7.3 ±0.12	7.3 ±0.21	78.1±0.0.06	80.2±0.12	7.9±0.06	8.4 ±0.12
30	8.0±0.58	8.2 ±0.12	80.2±0.06	98.1±0.06	8.8±0.06	11.0 ±0.06
35	8.2± 0.06	8.3 ±	80.1±0.06	88.0±0.58	9.1±0.06	13.3± 0.12
40	8.5± 0.15	8.4 ±0.21	89.0±1.15	90.2±0.12	12.2 0±0.12	11.2±0.12
45	8.3 ±0.23	8.0 ±0.17	87.4±0.23	87.6±0.17	11.8±0.12	11.9 ±0.12
LSD (at 0.01)	N.S		3.288		0.179	
LSD (at 0.05)	N.S		2.419		0.132	

N.S : Non significant

3.4.3. Effect of pH

The fermentation medium was changed to various pH values of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 to study the effect of the pH of the medium on the production of enzymes by the tested fungal strains. The results given in Table (7) demonstrate the highest lignin peroxidase activity. The optimum pH for lignin peroxidase production by *P.chrysogenum* was 5.0, while that for *A. niger* was 6, below or above this level enzyme synthesis was reduced. These fungi prefer the acidic medium, these results are consistent with [44 - 47]. The greatest enzyme production was (11.2 U/ml) with *Aspergillus niger* and 9.9 U/ml with *P.chrysogenum*.

Table 7. Effect of different degree pH of the incubation medium of Penicillium chrysogenum and Aspergillus niger on the final pH of the cultivation medium, fungal dry weight and enzyme activity

Initial pH	Final pH		Dry Weight (mg/100ml)		Enzyme activity (U/ml)	
	Penicillium	*Aspergillus*	*Penicillium*	*Aspergillus*	*Penicillium*	*Aspergillus*
3	5.2±0.12	6.3±0.17	88.4 ±0.23	100.0±1.53	4.5±0.12	5.3±0.17
4	6.1±0.06	6.4±0.23	78.4±0.23	86.5±0.29	5.3±0.12	6.7±0.06
5	6.3±0.17	7.2±0.12	91.5±0.29	88.2±0.12	9.9±0.06	6.63±0.06
6	6.2±0.12	6.9±0.12	88.0±0.58	89.4±0.23	9.2±0.0.12	11.2±0.12
7	7.1±0.06	7.8±0.12	99.1±0.6	93.7±0.12	8.9±0.12	10.10±0.0.06
8	8.6±0.12	8.9±0.6	82.3±0.17	95.3±0.17	8.6±0.0.12	8.2±0.12
9	9.0±0.12	9.0±0.06	90.2±0.12	86.5±0.29	8.3±0.15	8.6±0.06
LSD (at 0.01)	0.199		1.736		0.225	
LSD (at 0.05)	0.147		1.284		0.166	

4. Conclusion

The results in the present work indicate that the plastic materials could be biodegraded by fungi, like *Penicillium chrysogenum* and *Aspergillus niger* for the use of the degradation products economically and the purified enzyme production in different industrial applications.

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