ABILITY OF SOME FUNGI TO BIODEGRADE CARBENDAZIM FUNGICIDE

S. S. Raheem[1] M. A. Al-Dossary[1] H. T.AL-Saad[2]
Researcher Assit. Prof. Prof.
[1]. Department of Ecology, College of Sciences, University of Basrah, Iraq
mustafa.najem@uobasrah.edu.iq[1] shaymaa.raheem@uobasrah.edu.iq[1]
[2]. College of Marine Science, University of Basrah, Iraq
htalsaad@yahoo.com[2]

ABSTRACT

This study was amid to isolate soil fungi from agriculture soil and study their roles in the biodegradation of carbendazim fungicide at different concentrations were explored for two periods of incubation. Results showed the highest degradation rates of carbendazim at 4 part per million (ppm) concentration were observed by using single culture of *Aspergillus niger* (69.66% and 99.96% after 10 and 20 days, respectively).In the mixed cultures of *Exerohilum* sp., *Fusarium* sp., and *A. niger*, the maximum degradation rate (98.34%) was achieved at the same concentration after 10 days. The mixed culture of *Exerohilum* sp. and *Fusarium* sp. demonstrated the highest degradation rates of (92.14% and 55.74%) at 8 and 12 ppm concentrations, respectively, after 10 days of incubation.

Keywords: Carbendazim, soil fungi, biodegradation, mixed cultures

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مجلة العلوم الزراعية العراقية -2021 :52 (1):259-267 رحيم وآخرون

قابلية بعض الفطريات على التكسير الحيوي للمبيد الفطري CARBENDAZIM

شيماء سعيد رحيم مصطفى عبد الوهاب الدوسري حامد طالب السعد
باحث استاذ مساعد استاذ

المستخلص

يهدف هذا البحث الى عزل بعض فطريات التربة ودراسة دورها كعزلات مفردة ومختلطة في التكسير الحيوي للمبيد الفطري كاربندزايم وبتراكيز مختلفة ولفترتين زمنيتين من الحضن. اظهرت النتائج ان اعلى نسبة تكسير حيوي للمبيد الفطري في تركيز4 جزء بالمليون لوحظت في المزرعة المفردة للفطر *Aspergillus niger* وكانت (69.66 % و 99.96 %) على التتابع بعد 10 و20 يوم من الحضن. اما المزرعة الفطرية المكونة من الفطريات *Exerohilum* sp. *A. niger* و *Fusarium* sp., فقد سجلت اعلى نسبة تكسير حيوي وبلغت (98.34%) بعد 10 ايام من الحضن ولنفس التركيز السابق. سجلت المزرعة المختلطة للفطرين *Exerohilum* sp. و *Fusarium sp.* فقد سجلت اعلى نسبة تكسير حيوي بلغ (92.14 % و 55.74 %) بتركيز 8 و 12 جزء بالمليون على التوالي بعد عشرة ايام من الحضن.

الكلمات المفتاحية : كاربندزايم، فطريات التربة،التكسير الحيوي، عزلات مختلطة.

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NTRODUCTION

Pests (viruses, nematodes, fungi and bacteria i.e.,) and diseases can affect crops, thereby decreasing the economic outputs of farms. Disease organisation and prevention are best performed through integrated pest management using combined methods, which involve the use of pesticides (18,23,24,27, 28). Carbendazim fungicide (methyl 1 *H*-benzimidazol-2-ylcarbamate) is a systemic wide-spread benzimidazole used in managing a broad range of pathogenic fungi affecting various plants and treating infected soil (11,12,39). World health organization categorised carbendazim as a dangerous chemical, as one of the main contaminants frequently detected in food and the environment, carbendazim has severe effects on humans and other forms of life because of its low degradation rate and persistence in soil and water for prolonged periods, which may reach 12 months (25,32,37). Carbendazim accumulates in the environment after frequent use and negatively influences soil nature and human health (4). Therefore, alternative approaches must be developed for the management of fungicide contamination. In general, conventional treatment is nearly ineffective in pesticide removal because of the toxicity of pesticides (26,33). Many microorganisms, including fungi, play an important role in the degradation of unwanted compounds or wastes and convert them into safe, acceptable, or valuable products. The application of fungal technology for the clean-up of contaminants has shown promise since 1985 (5, 6 , 19 , 20). Several previous studies deal with the biodegradation of carbendazim by fungi such as (2,3,17).Given the widespread use of this fungicide and its harmful impact on the environment, the current study was conducted for the investigation of the role and capability of some soil fungi in the degradation of this pollutant and remove it from the soil.

MATERIALS AND METHODS

Samples collection

Soil samples from four agricultural areas were collected. From each area, six soil samples were collected from the surface layer (0-15cm) .All soil samples were air dried and stored in sterile plastic bags at 4 °C until use. Carbendazim residues in soil samples were determined (Table1) as described previously by (10).

Table 1. Carbendazim residues in stations soil

No. of station	Name of station	Range µg/g	Mean µg/g	±St.D	Longitude	Latitude
St.1	Abu-Al-Kaseeb	0-2.67	1.615	0.961	N: 30˚ 27' 28"	E: 47˚ 58' 40"
St.2	Al-Hartha	0.58-1.92	1.200	0.480	N: 30˚ 38' 46"	E: 47˚ 45' 3"
St.3	Al-Zubair	0.46-2.09	1.291	0.555	N: 30˚ 25' 57"	E: 47˚ 41' 2"
St.4	Shatt-Al-Arab	0-3.05	0.931	1.215	N: 30˚ 34' 9"	E: 47˚ 48' 58"

Culture media

Three types of culture media, namely, malt extract agar (MEA), corn meal agar (CMA) and potato dextrose agar (PDA, Hi Media Company, India), were used for the cultivation and preservation of fungal isolates. In the biodegradation experiments, a Czapeks-Dox broth medium was used. This medium was prepared as follows: $FeSO_4{\bullet}2H_2O$, 0.1 g;$MgSO_4{\bullet}7H_2O$, 0.1 g; KCl, 0.5 g; K_2HPO_4, 1 g; $NaNO_3$, 3 g; sucrose, 30g; were weight and dissolved in one liter of D.W., all media were sterilised by autoclaving at 121 °C under 15pounds/inch^2 for 15 min.

Chemicals

Carbendazim was purchased from (Toronto company, Canada). A standard stock solution from its 2000 ppm concentration was prepared by dissolving 2 mg of the standardin1ml of dimethyl sulfoxide and stored until use at 4 °C. Residual pesticides from liquid media or soil were extracted using solvents and chemicals purchased from Biosolve (France), J. T. Baker (Germany) and Himedia (India).

Fungal isolation and identification

Fungi were isolated from soil samples through the dilution method (38). Approximately 10 g from each soil sample was added to 90 ml of sterile physiological saline in a 250 mL flask to 10^{-1} dilution, from which a serial dilution of up to 10^{-3}was prepared. From each dilution, 1 ml was transferred to sterile petridishes. MEA, CMA and PDA were then added separately to each sample, Furthermore, 250 mg/l chloramphenicol was added for each media to inhibit bacterial growth. All the culture media were incubated for 7–14 days at 25± 2 °C. The fungi were purified and identified on the basis of their morphological features depending on (14,30). A live culture from each fungal species was transferred to a PDA slant and stored at 4 °C until use

Preliminary screening for fungal isolates

The ability of fungi to tolerate different concentrations of carbendazim was tested as previously described by (1). Firstly, all the fungi were activated by culturing them on PDA media. The PDA media which was supplemented with 1, 2 and 3ppm carbendazim were inoculated by a piece obtained by a 6 mm cork borer from each fungal isolate. The culture medium without fungicide served as the control. The experiment was performed in triplicate for each fungi and concentration. All of the plates were incubated for 7 days at 25 ± 2 °C. The tolerance of fungi to carbendazim was calculated as follows (16):

$$\textbf{Inhibition \%} = \frac{the\ growth\ in\ control(mm) - the\ growth\ in\ test(mm)}{the\ growth\ in\ control\ (mm)} \times 100$$

Ability of single isolate to biodegrade carbendazim: The biodegradation of carbendazim in liquid medium at 4 ppm for two periods(10 and 20 days) was performed as previously described by (3) by using fungi showing maximum resistance against carbendazim in the previous test. Conical flasks (250 ml)containing100 ml of Czapeks-Dox broth with 4 ppm carbendazim were inoculated with a piece from the fungal isolates previously selected using a 6mm cork borer. The experiment was performed in triplicate for each fungi. The flasks were then incubated in shaker incubator at 120 rpm, 25 °C for 10 and 20 days. To exclude contamination, a control flasks containing 4 ppm carbendazim without any fungi were prepared.

Ability of mixed cultures to biodegrade carbendazim: Depending on the results of the prior experiments, three fungal isolates that demonstrated excellent degradation rates were selected, and their capability to degrade carbendazimin in mixed culture for 10 days was studied as previously described by (8). Conical flasks (250 ml)containing100 ml of Czapeks-Dox broth with 4,8 or12 ppm carbendazim were used in the degradation tests. The flasks were inoculated with a mixture of the three fungal isolates following a previously described method. All possible mixtures were prepared

Extraction and quantification of carbendazim residues: Carbendazim residues were extracted from the single and mixed liquid cultures with 100 ml of dichloromethane in a separating funnel, shook well numerous times and left to settle until two layers were formed; the organic layer was obtained, and residual water was removed by passing the organic layer in anhydrous sodium sulphate (3). The extract was stored in clean container at 4 °C until analysis.

HPLC analysis: The extract samples were injected in an High-performance liquid chromatography (HPLC) type Shimadzu LC solution equipment for the identification and quantification of carbendazim residues. The column was C18 (250 mm, 25 cm, 4.6 mm), the mobile phase consisted of acetonitrile/water (90:10 v/v), the flow rate was 0.5 ml/min, the injection volume was 20µl and the UV/vis wavelength was 254 nm. The carbendazim degradation percentage was calculated as follows (21):

$$\textbf{Degradation\%} = \frac{ppm\ of\ pesticide\ in\ control - ppm\ of\ pesticide\ in\ test}{ppm\ of\ pesticide\ in\ control} \times 100$$

Statistical analysis

One-way ANOVA was applied using the Minitab ver.16. Relative least significant difference (RLSD) values were calculated for the identification of significant difference in fungal degradation rate. A complete randomised design was employed.

RESULTS AND DISCUSSION

Fungal identification: A total of 23 fungal species belonging to 11 genera were isolated from the soil samples (Table 2). The number of isolated fungi was moderate compared with other studies on agricultural soils. One of the possible reasons was high air temperature, which may have reached 50 °C and negatively affected fungal growth in soil, during the isolation periods; the use of different fungicides also negatively affected their growth (15, 36) . Anamorphic fungi came in the first position in their appearance, This finding was in accordance with other studies (13,29) ; this group of fungi have good resistance to harsh environments and produce a large number of reproductive cells; these features enable them to readily diffuse in all environments (29).

Table 2. Numbers and Occurrence (%) of the Fungal Species Isolated from soil samples

No.	Species	N. of samples appeared in	Occurrence %
1	*Alternaria alternata*	6	25
2	*Alternaria* sp.	8	33.3
3	*Aspergillus candidus*	15	62.5
4	*A. flavus*	9	37.5
5	*A. fumigatus*	17	70.8
6	*A. niger*	24	100
7	*A. terreus*	12	50
8	*A. vesicolor*	6	25
9	*A. wentii*	1	4.1
10	*Chaetomium elatum*	1	4.1
11	*C. globosum*	2	8.3
12	*C. madransense*	4	16.6
13	*C. semon-citrilli*	2	8.3
14	*Cladosporium herbarum*	4	16.6
15	*Exserohilum* sp.	5	20.8
16	*Fusarium* sp.	4	16.6
17	*Humicola grisea*	1	4.1
18	*Microascus trigonosporus*	8	33.3
19	*Myrotheciumgramineum*	1	4.1
20	*Penicillium*sp1.	5	20.8
21	*Penicillium* sp2.	2	8.3
22	*Stachybotrys sansevieria.*	1	4.1
23	*Ulocladium* sp.	6	25

Preliminary screening

The growth rates of some fungal species, including *Aspergillus flavus*, *A. fumigatus*, *Exserohilum* sp. and *Ulocladium* sp., were unaffected and grew well at all concentrations, achieving 0% inhibition rate. By contrast, some fungal species that were unable to tolerate carbendazim at different concentrations showed inhibition rates ranging from 7.55 to 91.17 (Table 3, Figs.1 and 2). carbendazim in solid medium was allowed to degrade for the selection of the best degrading species and exclusion of non-degrading species. The results showed the variable inhibition rates of fungal groups and species related to the same genus possibly because of the enzymatic activity of each species and their tolerance to fungicide(16) .The tolerant nature of some fungi, such as *A. fumigatus* and *A. flavus*, are suspected to be due to their adaptation to environmental stress caused by the repeated incorrect use of pesticides. This result is similar to previous findings (16,19,21,34). statistically significant differences ($p<0.01$) were shown in the ability of fungi to tolerate different carbendazim concentrations.

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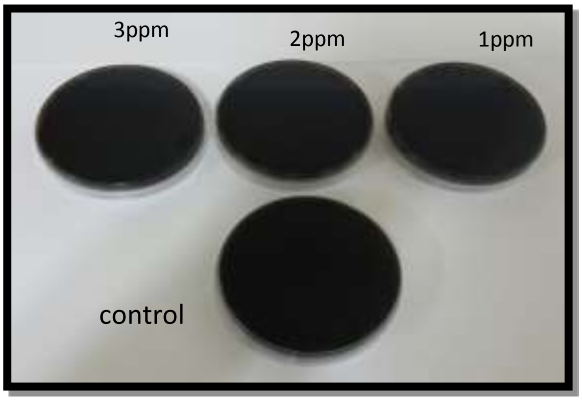

Fig..1-Growth of *Exerohilum* sp. at 1,2and 3ppm of carbendazim, the growth was completely not affected when compared with control

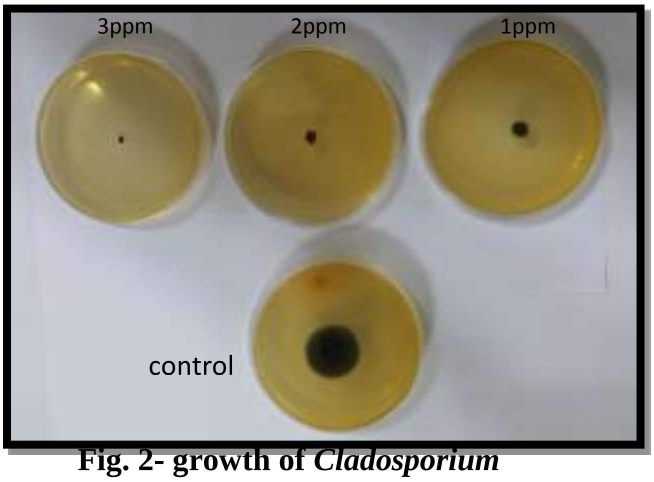

Fig. 2- growth of *Cladosporium herbarum* at 1,2and 3ppm of carbendazim compared with control, , the growth was highly affected.

Ability of a single fungal isolate to biodegrade carbendazim

The biodegradation ability of each of the six fungal isolates that showed high resistance to carbendazim were examined. The results showed that *A. flavus* exhibited the lowest degradation rate (23.39%), with high residual pesticide concentration of 1.610 µg/ml after 10 days. *A. niger* demonstrated the highest degradation rate (69.66%) and lowest residual concentration (0.637µg/ml).The other fungi showed degradation rates ranging from 38.30% to 60.50% (Fig. 3). The six isolates showed good degradation rates ranging from (93.52%) for *A. fumigatus* with the highest residual concentration (0.136µg/ml) to (99.96%) for *A. niger* with the lowest residual concentration (0.0007 µg/ml, Fig. 4) after 20 days. These fungi showed heavy mycelial growth in contrast to other species and the control, which may cause increase in contact between the fungal cells and pesticide molecules in the media. Such increase accelerates the pull and entry of pesticides inside cells or promotes contact between pesticides and extracellular enzymes secreted by fungi; thus, degradation rate also increases (16,19, 22). Statistical analysis showed no significant differences (p>0.05) in the residual concentration and degradation rate during the 10 days. However, significant differences in degradation rate and residual pesticide concentration were found after 20 days (p<0.01).Significant differences (p<0.01) in the degradation rate of carbendazim pesticide at 4ppm between 10and 20days were also observed.

Table 3. preliminary test of fungi against carbendazim

No.	Species	Control (mm)	Colony diameter (mm) at different concentrations of carbendazim(ppm)			Inhibition % at different concentrations of carbendazim			Inhibition mean
			1ppm	2ppm	3ppm	1ppm	2ppm	3ppm	
1	*Aspergillus flavus*	85	85	85	85	0	0	0	0a
2	*A. fumigatus*	85	85	85	85	0	0	0	0^{a}
3	*Exserohilum* sp.	85	85	85	85	0	0	0	0^{a}
4	*Ulocladium* sp.	85	85	85	85	0	0	0	0^{a}
5	*Fusarium* sp.	75	69.5	70	68.5	7.33	6.66	8.66	7.55^{a}
6	*A.niger*	85	85	75	67.5	0	11.76	20.58	10.78^{a}
7	*Microascus trigonosporus*	50	33.5	28.5	26.5	33	43	47	41.33^{bc}
8	*Alternaria* sp.	50	41.5	26	16	17	48	68	44.33^{bcd}
9	*A. alternata*	25	15	14	5	40	44	80	54.67^{cdef}
10	*Stachybotrys sansevieria*	30	19.5	11.5	8.5	35	61.66	71.66	56.11^{cdef}
11	*Cheatomium semon-citrilli*	25	13	10	8.5	48	60	66	58^{defg}
12	*C. elatum*	25	13	10	8.5	48	60	66	58^{defg}
13	*C.globosum*	25	13	10	8.5	48	60	66	58^{defg}
14	*Myrothecium gramineum*	54	31.5	20	11.5	41.66	62.96	78.70	61.10^{efgh}
15	*Penicillium* sp.1.	78	49	24.5	11.5	37.17	68.58	85.25	63.67^{efgh}
16	*Penicillium* sp.2.	85	20.5	34	18.5	75.88	60	78.23	71.37^{ghi}
17	*Cladosporium herbarum*	26	8.5	7	5.5	67.30	73.07	78.84	73.07^{hij}
18	*Humicola grisea*	85	36	20.5	11.5	57.64	75.88	86.47	73.33^{hij}
19	*A. candidus*	78	12.5	10.5	8.5	83.97	86.53	89.10	86.53^{jk}
20	*A. wentii*	75	10.5	9	6.5	86	88	91.33	88.44^{k}
21	*A. vesicolor*	77.5	10.5	9	7	86.45	88.38	90.96	89.10^{k}
22	*C. madransense*	41	4.5	4	4	89.02	90.24	90.24	89.78^{k}
23	*A. terreus*	85	9.5	7.5	5.5	88.82	91.17	93.52	91.17^{k}

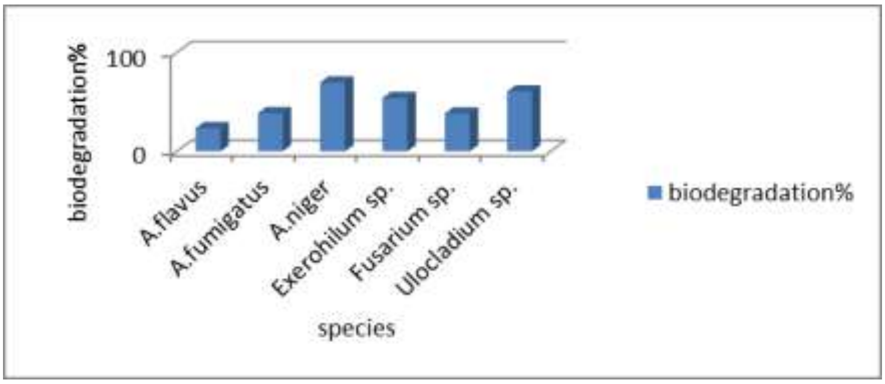

Fig. 3. Biodegradation of Carbendazim by Single fungal Isolates at 4ppm for 10 day(p>0.05).

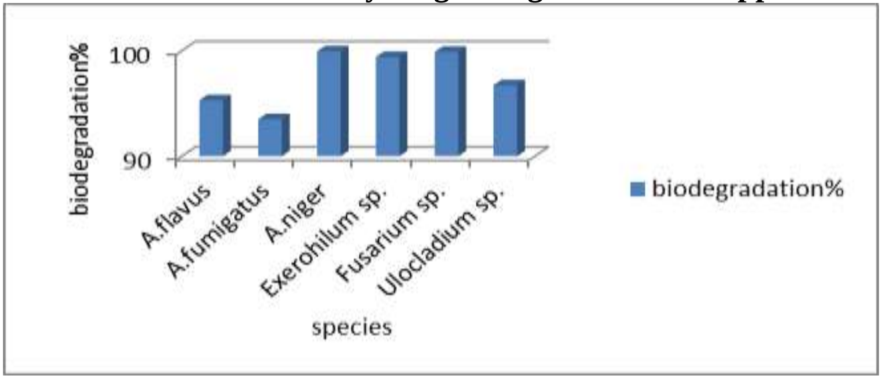

Fig. 4. Biodegradation of Carbendazim by Single fungal Isolates at 4ppm for 20 days (p<0.01).

Biodegradation of carbendazim by mixed cultures: The fungi that showed the excellent biodegradation ability in the previous experiment, namely, (A) *Exerohilum* sp., (B) *Fusarium* sp. and (C) *A. niger*, were used as mixed culture, and all possible mixtures were prepared. Mixture AB demonstrated the maximum residual concentration of 0.120 µg/ml at 4ppm and the lowest biodegradation rate of 94.28%. However, mixture ABC exhibited the lowest residual concentration (0.034µg/ml) and highest degradation rate of (98.34%, Fig. 5). All of the mixed isolates enhanced the biodegradation rate of carbendazim as compared with single isolates at 4ppm for 10 days. This result indicates that concentration did not greatly affect the fungi and the fungi adapted to grow in pesticide-contaminated soil; the fungi may have adapted through their synergistic effects when they grew together (35) thus, fungi enhances the biodegradation and removal of pesticides in the environment (14,35,41). By contrast, at 8and12ppm, variable degradation rates were observed among the mixed cultures, so as fungicide concentration increased, degradation rate decreased. This result indicated that increasing fungicide concentration negatively affects fungal growth and degradation rate. Mixture AB (*Exerohilum* sp. +*Fusarium* sp.) showed the highest degradation rate in both concentrations, at 8ppm, the mixture ABC showed the lowest degradation rate (53.99%), whereas mixture AB demonstrated the highest degradation rate (92.14%, Fig. 6). At 12 ppm, the results showed that mixture AB also achieved the lowest residual concentration of (3.024 µg/ml) and the highest degradation rate of (55.74%). Furthermore, mixture ABC showed the highest residual concentration of 4.479µg/ml with the lowest degradation rate of 34.44% (Fig. 7). It was appeared that mixture AB formed good growth and turbidity in the media possibly because of the synergistic enzymatic activities of the species. A single species cannot completely degrade pesticides at high concentrations, whereas the presence of two or more species may enhance biodegradation rate (35). Other mixtures did not enhance the biodegradation rate. However, some mixed isolates exhibited lower degradation rates than their corresponding single isolates possibly because of the antibiosis between the species in the media. This mechanism may have inhibited their growth and affected the degradation rate; furthermore, competition for nutrients in the media may have negatively affected the biodegradation rate (9,31). These findings are consistent with those of previous studies(2,7,40). Significant differences among the residual concentrations of pesticides of the media with 4,8 or 12 ppm fungicide concentrations ($p<0.01$) and among the degradation ratesafter10 days ($p<0.01$) were observed.

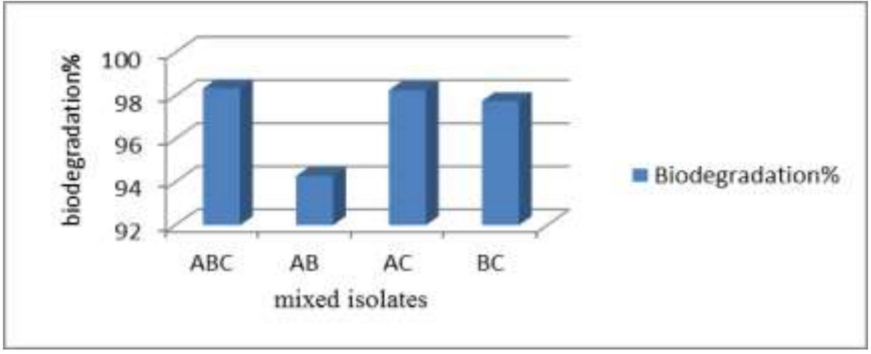

Fig. 5. Biodegradation of Carbendazim by Mixed fungal Isolates at 4 ppm for 10 days ($p<0.01$) . Where A: *Exerohilum* sp. , B: *Fusarium* sp. , C: *A. niger*

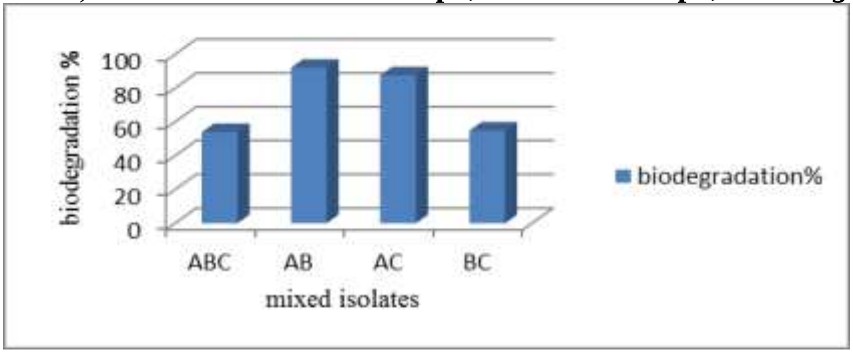

Fig. 6. Biodegradation of Carbendazim by Mixed fungal Isolates at 8 ppm for 10 days ($p<0.01$). Where A: *Exerohilum* sp. , B: *Fusarium* sp. , C: *A. niger*

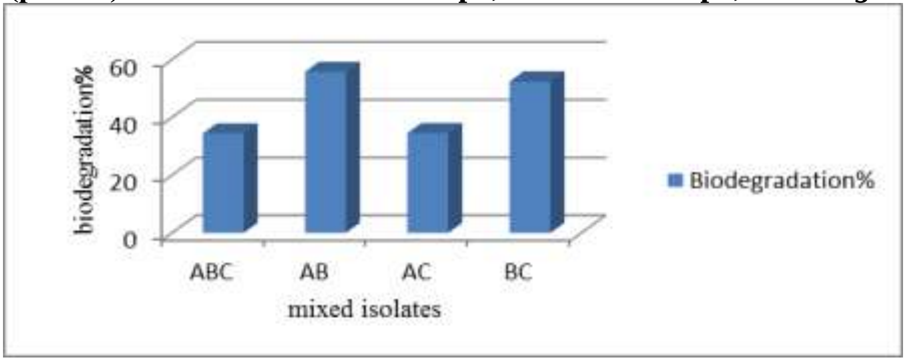

Fig. 7. Biodegradation of Carbendazim by Mixed fungal Isolates at 12 ppm for 10 days ($p<0.01$). Where A: *Exerohilum* sp. , B: *Fusarium* sp. , C: *A. niger*

CONCLUUSIONS

Most of the isolated fungi exhibit a good growth and tolerated the carbendazim concentration in the primary screening. As single isolate the fungus *A. niger* exhibited a high degradation potential with the ability to degrade 69.66% and 99.96% of total carbendazim fungicide after 10 and 20 days of incubation. As mixed isolates the mixture ABC exhibit a good degradation potential at 4ppm concentration with 98.34% degradation percentage. Where at 8 and 12 ppm, the mixture AB demonstrated the highest degradation rate with 92.14% and 55.74% respectively.

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