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Production Of A– Amylase By Fungi Isolated From Oil-Contaminated Soil In Karbala, Iraq

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ABSTRACT

Objective: The present study aims to isolate and diagnose of fungi from soil polluted with oils and investigate their ability to produce α-amylase.

Methods: Soil samples were collected from different areas of oil-polluted soils in Karbala, Iraq for the period from October to November 2019. 0.5 g of each sample was diluted with distilled water and cultured on potato dextrose agar (PDA). A week later, the purification processes were carried out to obtain pure colonies of isolated fungi. The isolated fungi were diagnosed and their own codes were given. The fungal isolates were then implanted in the nutrient broth to determine the susceptibility of these isolates to produce of α-amylase. After 10 days of incubation, the filtration and centrifuge processes were measured for production media. The amount of the enzyme was measured by using kit of α-amylase in the Reflatron.

Results: Four fungal genera were obtained in this study and are belong to five fungal species (45 Isolates), including: Aspergillus niger (20 isolates) , Aspergillus flavus (8 isolates) , Rhizopus stolonifera (5 isolates) , Phoma medicaginis Malbra var. pinodella (9 isolates) and Mucor sp. (3 isolates).

Twelve isolates were found in the production of α-amylase produced. The two isolates, Phoma medicaginis Malbra var. pinodella (D7) and Aspergillus niger(A19), were the most productive α-amylase, with the amount of enzyme produced (746 and 321) U/L, respectively.

Conclusions: The fungal species found in the soil polluted with oils in the city of Karbala are five fungal species including Aspergillus niger, Rhizopus stolonifer, Phoma medicaginis Malbra var. pinodella, Aspergillus flavus and Mucor sp. The highest production of α- amylase was through the fungal isolation of Phoma medicaginis Malbra var. pinodella D7.

Keywords: Fungi, Soil , Oil , α – amylase, Karbala.

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INTRODUCTION

Fungi are very felicitous residents of soil , due to their high plasticity and their ability to take over various forms in response to opposite favorable conditions. Because it can produce extracellular enzymes of many types, it analyzes the soil components, and it destroys their organic materials of all kinds.s [1]. Many fungi are pathogenic and some may be useful in bio –exploitation [2].

Fungi are well related with the dissolution of hydrocarbons by the production of various enzymes among catalase, lactases, and peroxidase are immense importance [3]. Fungi are considered to be very important for crude oil or polyaromatic hydrocarbon handling based on their capacity to discredit insurrectionary longer handcuffed or multiple ring s hydrocarbons [4]. Petroleum and its derivatives are one of the most pollutant organic matter in all parts of the soil, due to its wide spread all over the world [5]. For the purpose of removing petroleum hydrocarbons from the soil, this is done through advanced physical and chemical treatment technologies such as burning, thermal absorption, soil washing, extraction and testing of solvents[5]. Several petroleum hydrocarbon degradation pathways have been well established . However , compounds in petroleum are not all utilized equally , an important consideration in bioremediation [6]. The components of petroleum or crude oil can be divided into five fractions by liquid column chromatography : aliphatics , aromatics , polar , resins and asphalts [5].

Diabetes mellitus is a group of metabolic disease describe by hyperglycemia resulting from disorders in insulin excretion, insulin action , or both [7]. The harm and dysfunction in addition to the long failure of the various organs, especially the kidneys, nerves, eyes, and blood vessels, are all linked to chronic hyperglycemia with diabetes [7]. In urban residents, the prevalence of diabetes is higher compared to that of villages and rural areas [8]. Diabetes is largely undiagnosed and untreated particularly in the rustic setting. The universal load of diabetes has increased twelve fold between 1985and 2011 [9].

The relationship between human alpha-amylase pancreatic activity and post-eating glucose levels is a positive correlation [10]. The capacity of alpha – amylase enzyme inhibitors to obviate dietary starch blockers [11]. Only a mild pancreatic alpha amylase inhibition action is recommended [12].

Secondary metabolites can be defined as compounds that do not belong to the primary metabolites exciting quibble which has not ever ceased [13]. Generally, the most acceptable concept, in line with Kossel's view, is that the chemical components necessary for the functioning of living organisms are primary metabolites, while secondary metabolic compounds are limited to a group of species or genera [14]. Most often secondary metabolites are produced as families of compounds associated with limited parts of the life cycle with production that are often associated with the stage of morphological differentiation, and that secondary metabolites are biologically active and of low molecular weight [15]. For example, marine fungal strains produce biologically active secondary metabolites, such as alkaloids derived from polypeptides, turbines, peptides, and mixed biosynthesis compounds produced by fungi as representative groups [16] . In addition to that micro-organisms such as bacteria and fungi that live with plant tissues without causing any immediate negative

public effects to these plants were found, as isolated products from these micro-organisms were tested for use in medicine, industry and agriculture as more vitally active products [17].

MATERIALS AND METHODS

The method described by Ogbonna *et.al* was followed to isolate the fungi found in soil polluted with oils [18] with some modification in some steps:

Collection of specimens: Collection of five specimens of soil polluted with crude oil from the surface and sub surface regions of the soil for different regions in industrial district in Karbala province-Iraq during October and November 2019 and putting them into collection cup. Placing the specimens in the plastic bags being sterile and clean as well as dry. Transporting the specimens to the laboratory as soon as possible to retaining on the fresh properties to the organisms.

Culturing of specimens: Prepare 25 pin tube and marked them then put them in the rack. Take 0.5 g of soil specimen from each soil specimen that polluted with crude oil. Melting the 0.5 g of polluted soil specimen in 5 ml of distilled water then doing a series of dilutions (doing 5 dilutions). Take 0.5 ml of tube 1 and add this amount to 4.5 ml from the second dilution in the second tube then repeat this steps to the ending of dilutions with attention to mixing the tube before use. After that take 0.1 microliter of the third dilution and fourth dilution to all five specimen and spread this amount on petri dishes (The sterilizing potatoes dextrose agar media with chloramphenicol) by micropipette. Incubate the plates at 28° C for one week until appearance fungal growth on the plates then predisposing to the purification method.

Purification procedure: Educate the dishes from the incubator after the appearance of fungal growth. Take a small portion of the periphery of the fungal colony by loop or scalpel with care do not open the cover of the plat farness about the benzene burner for the purpose of decontamination. Transport this small piece of the colony to another plate and put this piece in the reverse form on the plate. Repeat this process up on the number of fugal colonies that appear in the plate. Incubate the plates at 28° C for few days.

Preparation of broth media process: Prepare 25 tube and marking them according the specimen name. Prepare the amount of nutrient broth according to specific calculation and add to the mixture of broth media. A particular amount of antibiotics before placing the broth in the autoclave device for the purpose of sterilization. Near to the benzene burner pour 10 ml from the broth to each tube after cooling the broth. For few minutes after educating it from the autoclave device. Sterilize the loop until it becomes red and it cooled at the periphery of the plate. Make the loop touch the periphery of the fungal colony and transport very small amount of it then make stabbing inside the tube that contain pure broth media. Incubate the tubes at 28° C in the incubator for at least 10 days.

Filtration procedure: Near to the benzene burner place filter paper inside a glass funnel and put the funnel inside pin tube then place the tube inside beaker. Pour the growing broth that educated from the incubator inside the funnel that founds in the pin tube. Wait until all the broth reeves down to the bottom of the tube. Repeat this process for all tubes after that doing the filtration process by centrifuge. To ensure from the complete filtration for all tubes doing the centrifugation to all tubes . Place the tubes inside the centrifuge for five minutes at 3000 rpm. By sterile syringe or micropipette pull the up layer of the filtrate and leave about 2ml from the bottom of the tube and transport this amount to another clean dry pin tube. Repeat these steps for all samples and then put the new tubes in the rack then put them in the refrigerator until use.

Measurement of α – amylase by spectrophotometer device: The method described by [19 , 20, 21 , 22 and 23] was followed to estimate the amount of α – amylase enzyme as shown below:

1- Remove the experiment strip from the container. Tightly recap the container directly after removing a test strip.
2- Exfoliate off the aluminum covering foil taking care not to droop the test strip.
3- Take about 30 micro liter volume from each sample that contain filtrate.
4- Stratify the wanted volume of sample on to the center of the red application zone using a pipette (reflotron pipette) being careful not to contact the application zone. Avert air bubbles.
5- Open the sliding cover within 15 second of applying the sample , put the exam strip on to the guide and slide it forward horizontally until it looks into place. Close the skidding cover.
6- The exam parameter abbreviations is shown on the display, if the exam strip has been correctly inserted and the magnetic code has been read. The result is displayed relying on the setting of the instrument.

Statistical analysis: The statistical analysis of tables to determine the significant differences between the factors studied in this study by using the Chi – square test and α = 0.01 (the probability level) [24].

RESULTS

Isolation of fungi from soil polluted with oil: The results of isolating the fungi from soil contaminated with oils in different areas in the city of Karbala to isolate four fungal genera with Forty five isolates included *Aspergillus niger* (20 isolates) , *Aspergillus flavus* (8 isolates) , *Rhizopus stolonifer* (5 isolate) , *Phoma medicaginis* Malbra var. pinodella (9 isolates) and *Mucor* sp. (3 isolates) as show in Table 1 .

Table 1: Fungi species that isolated from soil contaminated with oil in the city of Karbala.

No.	Fungal Species	Total of Isolates	Symbol of isolates
1	*Aspergillus niger*	20	A1 ; A2 ; A3 ; A4 ; A5 ; A6 ; A7 ; A8 ; A9 ; A10 ; A11 ; A12 ; A13 ; A14 ; A15 ; A16 ; A17 ; A18 ; A19 ; A20
2	*Aspergillus flavus*	8	B1 ; B2 ; B3 ; B4 ; B5 ; B6 ; B7 ; B8

3	*Rhizopus stolonifer*	5	C1 ; C2 ; C3 ; C4 ; C5
4	*Phoma medicaginis* Malbra var. pinodella	9	D1 ; D2 ; D3 ; D4 ; D5 ; D6 ; D7 ; D8 ; D9
5	*Mucor* sp.	3	E1 ; E2 ; E3
Total		**45**	

$X^2 = 19.332$; P value = 0.0001

Production of α-amylase: The results in Table 2 show that the output of α- amylase by *Aspergillus niger* was by five isolates, which bear the following symbols: A3, A9, A12 , A14 and A19 as the amounts of production of the enzyme by these isolates are (106 , 210 , 112 , 99.5 and 321) U/L, respectively.

Table 2: Production of α- amylase by isolates of *Aspergillus niger* that isolated from oil-contaminated soil.

No.	Symbol of isolates	Amount of α-amylase U/L
1	A1	<29
2	A2	<29
3	A3	106
4	A4	<29
5	A5	<29
6	A6	<29
7	A7	<29
8	A8	<29
9	A9	210
10	A10	<29
11	A11	<29
12	A12	112
13	A13	<29
14	A14	99.5
15	A15	<29
16	A16	<29
17	A17	<29
18	A18	<29
19	A19	321
20	A20	<29
21	Control	<29

$X^2 = 1765.9$; P value=0.000 <29 means no production of α-amylase

The results indicate that among the eight isolates of *Aspergillus flavus*, there was one isolate with the ability to produce α-amylase and this isolate has the symbol B7, since the amount of the enzyme produced from this isolate is 209 U/L, as shown in Table 3.

Table 3: Production of α- amylase by isolates of *Aspergillus flavus* that isolated from oil-contaminated soil.

No.	Symbol of isolates	Amount of α-amylase U/L
1	B1	<29
2	B2	<29
3	B3	<29
4	B4	<29
5	B5	<29
6	B6	<29
7	B7	209
8	B8	<29
9	Control	<29

$X^2 = 587.7$; P value=0.000 <29 means no production of α-amylase

The results in Table 4 also indicated that one of five isolates belonging to the *Rhizopus stolonifer* was characterized by its ability to produce α-amylase, and this isolation is C3, and the amount of the enzyme produced by this isolate is 48.4 U/L.

Table 4: Production of α- amylase by isolates of *Rhizopus stolonifer* that isolated from oil-contaminated soil.

No.	Symbol of isolates	Amount of α-amylase U/L
1	C1	<29
2	C2	<29
3	C3	48.4
4	C4	<29
5	C5	<29
6	Control	<29

$X^2 = 9.73$; P value=0.90 <29 means no production of α-amylase

Also, the isolates belonging to the fungus *Phoma medicaginis* Malbra var. pinodella were distinguished by their high production of α- amylase, nine isolates were obtained from this fungus and four isolates were distinguished by their production of this enzyme and these isolates are D1 , D4 , D7 and D9 and the amount of the enzyme produced by them is (262, 74.5, 746 and 209) U/L, respectively. As in Table 5.

Table 5: Production of α- amylase by isolates of *Phoma medicaginis* Malbra var. pinodella that isolated from oil-contaminated soil.

No.	Symbol of isolates	Amount of α-amylase U/L
1	D1	262
2	D2	<29
3	D3	<29
4	D4	74.5
5	D5	<29
6	D6	<29
7	D7	746
8	D8	<29
9	D9	209
10	Control	<29

X^2 = 3170.7 ; P value=0.000 <29 means no production of α-amylase

Finally, the results showed in Table 6 that one of the three isolates belonging to *Mucor* sp. was producing an enzyme, and this isolation is E3, as the amount of the enzyme produced was 72.7 U/L.

Table 6: Production of α- amylase by isolates of *Mucor* sp. that isolated from oil-contaminated soil.

No.	Symbol of isolates	Amount of α-amylase U/L
1	E1	<29
2	E2	<29
3	E3	72.7
4	Control	<29

X^2 = 35.87 ; P value=0.000 <29 means no production of α-amylase

DISCUSSION

In the current study, it was observed that fungi isolated from soil contaminated with oils and spread in this soil are four fungi species : Aspergillus, Rhizopus, Phoma and Mucor with 45 isolates of these species were obtained. Also it turned out that twelve fungal isolates out of forty-five isolates were able to produce α - amylase enzyme, and the amounts of alpha amylase enzyme found differ from one isolate to another this may be due to several factors or reasons that include : the first , the collection area or place of the specimen may be do not contains fungal species capable of producing alpha amylase enzyme and the amount of production of alpha amylase enzyme may be due to the collection area of the specimen may be started work with a period may be not enough to production fungal strains that have unique ability to production this enzyme or it may be due to the fungal strains that cannot resistance environmental condition or high amount of oils that found in these soil or it may be due to improper technique of procedure. Similar anti diabetic activity by fungal isolated from *Adathoda beddomei* , however the mode of action less different [25]. In our study it had been used fungi isolated from soil polluted with oil to determine the inhibitory activity of alpha amylase enzyme where as in this study they used plant *Adthoda beddomei* was obtained from siddha institute , Chennai . The fresh leaf sample were used in this study [25]. In another study, fungal strains were enriched from soil samples contaminated with petroleum hydrocarbons. The focus was on fungal strains capable of secreting extracellular enzymes using existing hydrocarbons in that contaminated soil . Their results were isolated fungi were belong to 9 genera are *Aspergillus* , *Curvularia* , *Fusarium* , *Drechsiera*, *Hasiodiplodie* , *Mucor*, *Penicellium* , *Rhizopus* , *Trichoderma* – and two oil seed [26]. As well as in the study of natural plant extracts via inhibition of carbohydrates hydrolysis enzyme with emphasis on pancreatic alpha amylase [27], this study focus on inhibition of carbohydrates hydrolyzing enzyme is emerging as a useful tool for type 2 diabetes treatment . Finally the results of one study were set out isolate and identified filamentous fungi from flare pits across western and northern Canada and determine whether their processes the potential to degrade hydrocarbon while using this substrate as sole of carbon source. Sixty-four species of filamentous fungi isolated from five flare pits [28]. The most common species were *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus terreus*, *Emericella nidulans*, *Mucor racemosus*, *Mycosphaerella tassiana*, *Penicillium chrysogenum* and *Rhizopus stolonifera*, Forty-six species of fungi were tested to produce α-amylase, of which eight were of high amylase activity, and twenty seven were of moderate activity, while those of low activity were eleven. Starch was combined as a carbon source with ammonium sulfate as a nitrogen source to obtain the maximum amylase production at a temperature of 30 ° C and a pH of 6 and an incubation period of 6 days.[29].

Among the thirty isolates of endophytic fungi isolated from the *Alpinia calcarata* (Haw.) Roscoe isolate, *Cylindrocephalum* sp. (Ac-7) showed the highest haemolysis activity on the medium of glucose yeast extract peptone agar (GYP). [30].

CONCLUSIONS

According to the results obtained from this study, it is possible to conclude that the fungal species found in the soil polluted with oils in the city of Karbala are four fungal genera (five species) including *Aspergillus niger*, *Rhizopus stolonifer*, *Phoma medicaginis* Malbra var.

pinodella, *Aspergillus flavus* and *Mucor* sp. All fungal genera were characterized by their production of α-amylase, and the highest production of the enzyme was through the fungal isolate of *Phoma medicaginis* Malbra var. pinodella D7.

RECOMMENDATIONS

Recommendations can be summed up in the following studies:

1. Molecular studies to diagnose the most productive fungal isolates of amylase.
2. Studies on other fungal species isolated from other soils not contaminated with oils and evaluate the production of this enzyme.
3. Studies to obtain optimal production of alpha-amylase from fungi produced in the study are by changing the conditions and medium production.

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