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"Antimicrobial activity of amylases extracted from a thermophilic fungus, *Aspergillus fumigatus*"

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Abstract: Extract, describe the properties of a thermophilic fungal amylase, *Aspergillus fumigatus* which isolated from different soil samples and explore its antimicrobial activity. Six distinct fungal strains were isolated from Mansoura University soil samples using standard media, examined for characteristics, and tested for amylase production using a modified Czapek-Dox medium supplemented with starch. Results: Using Czapek's agar media, the isolated fungi were screened for the synthesis of amylase. One fungus strain was chosen and identified as Aspergillus sp. based on the pink color zone that developed. The most active species was *Aspergillus fumigatus*, which was identified from soil samples. After 30 minutes at 50 °C and pH 7.0, the organism that had been cultivated under submerged fermentation conditions generated amylase. In conclusion, Since *Aspergillus fumigatus* was isolated from soil samples demonstrating substantial amylase activity, it can be further used for the commercial manufacture of amylase. Amylase is an antibacterial agent that works well.

Key words: *Aspergillus fumigatus*, Amylase, Optimization; Purification; Antimicrobial

Introduction:

Amylases (EC.3.2.1.) are enzymes that break down starch and help break down internal polysaccharide glycosidic bonds while keeping the alpha-anomeric structure of the products intact [1]. They exist in every kind of species, regardless of kingdom. For years, the brewing sector has employed amylases derived from plants and microbes. Oriental food is frequently prepared with the use of fungus amylases [2]. Amylases from bacteria, fungus, and viruses are being explored more and more because they are extracellular in nature and hence easier to generate on a large scale with lower downstream costs than amylases from plants and animals [3]. These enzymes are important because industry will use them at some point. Pharmaceutical enzymes are helpful medications for treating a number of illnesses, including myocardial infarction and cancer [3]. Amylases are used in the starch processing industry to hydrolyze polysaccharides like starch into simpler sugar components. Amylase is being utilized in analytical chemistry as well as pharmaceutical and medicinal applications due to the increasing opportunities in biotechnology [4]. Approximately 25% of the world market for enzymes is made up of amylases, one type of industrial enzyme. As per Kali et al. (2021) [5], Anticancer medications are made from several fungal species, including *Aspergillus* and *Penicillium*. It has been studied if these compounds are effective against lung, pancreatic, breast, and uterine cervix tumors, among other cancers. A few enzymes that are highlighted are laccase, α-amylase, β-glucosidase, and asparaginase; these enzymes either promote or inhibit the proliferation of cancer cells.[6].

Commercially available antitumor enzymes for clinical use are obtained from *Escherichia coli* and *Erwinia carotovora* and are usually elspar, oncaspar, ervinase, and hydrolase. It is marketed as Hydralase and has both negative and positive side effects [7-10]. Marine Fungi

may be a source of anticancer agents in the maritime environment, a vast range of fungi containing bioactive secondary metabolites (MNPs) are present. They include compounds with antiviral, antibacterial, and anticancer properties. Alkaloids, polyphenols, and polysaccharides are only a few of the potent anticancer bioactive compounds found in marine fungi [8]. The Solanaceae family and genus Solanum contain the therapeutic herb *Solanum nigrum* [9]. Along with these names, it is sometimes referred to as "Black Nightshade" and "Makoh." S. nigrum is used extensively across the world to cure a variety of ailments, including inflammation, discomfort, ulcerations, diarrhea, convulsions, jaundice, and various eye infections. Flavonoids, phenolic chemicals, tocopherols, polysaccharides, glycoalkaloids, and glycoproteins are found in *Solanum nigrum* (fruit and leaf portions), and because of their immunomodulatory qualities, they exert antiproliferative actions. By stimulating pro-apoptotic proteins or blocking important transcription factors, flavonoids and glycoalkaloids also have antifungal and anti-inflammatory properties [8]. Numerous investigations have demonstrated the effectiveness of SN as an anticancer agent against human endometrial cancer cells, human colon carcinoma cells, human ovarian carcinoma cells, and human hepatocellular carcinoma cells in vitro. [11]. The creation of natural antibiotics and antifungal drugs, as well as the growing commercial need for therapeutic enzymes in the treatment of cancer, offer new research options. The use of plants as an easily available and inexpensive source of enzymes has gained popularity, especially when it comes to obtaining large concentrations of amylase that exhibit strong antibacterial impact. Salivary α-amylase showed an anti-proliferative impact upon exposure to human breast cancer cell primary cell cultures [12].

Methods and Materials:

Sampling soil samples and identifying thermophilic fungus isolation:

The fungi utilized in this investigation were separated from soil samples that were gathered from several locations within Mansoura University. The dilution plating method was used to isolate soil fungus strains [13–14]. To sustain isolated organisms, the modified Czapek Dox medium was employed, which contained the following (g/L of distilled water): 2.0 $NaNO_3$, 10.0 starch, 1.0 K_2HPO_4, 0.5 $MgSO_4 \cdot 7H_2O$, 0.5KCl, 2.0 KH_2PO_4, 0.5 $MgSO_4.7H_2O$, 0.01 $FeSO_4.7H_2O$, 2.0 yeast extract, and 20.0 Agar at pH 7.0.

Microscopic identification of the isolated fungi:

After the resulting fungal colonies were subcultured on Czapeck-Dox agar medium, each pure culture was examined under a microscope to determine its morphological and microscopic features, as well as its color and sporulation. The inspection was conducted using needle mount preparation, as detailed by [15–16]. On a sanitized glass slide, the piece of the culture's sporing surface was removed using a needle and a drop of alcohol. A drop of lactophenol was added to the fragment to stain it, and then a cover slip was carefully attached, taking care to prevent air bubbles. The preparation was then examined under a light microscope. The isolated, pure fungus was identified down to the species level if possible. The following generally recognized keys for the identification of various isolated fungi were used to assist identify the fungal genera and species [17–18].

Screening of Amylase producing fungi:

By using a plate test, the fungi that were separated from the preceding procedures were quickly screened for the synthesis of amylase [19]. "0.2% NaNO3, 1% Starch, 0.2% KH2PO4, 0.052% KCl, 0.052% MgSO4.7H2O, 0.005% ZnSO4.7H2O, 0.003% FeSO4.7H2O, 0.1K2HPO4, 0.2 Yeast extract and 1.8% agar" were the ingredients of the pH 6.2 modified Czapek Dox's (mCD) medium, which was employed for fungi. The starting pH was 6.2. The control plates were made of mCD medium with a carbon source (starch) and a nitrogen source (NaNO3). After plating, the six selected fungal isolates were cultured for 48 hours at 40°C. The isolates whose colonies showed a colorless zone following the addition of Lugol's solution were used as indicators of the amylase enzyme's synthesis in order to measure the enzyme activity.

Amylase production by submerged fermentation:

Amylase was produced by submerged fermentation with a liquid medium that was altered from Czapek Dox. Using a primary-screened organism, 100 mL of the suitable media were added to an Erlenmeyer flask for infection. The flasks were incubated for different amounts of time (24–168 hours) at 40 °C. A control method is an inoculation medium. Using Whatman No.1 filter paper, the cultures were collected. To calculate the enzyme's activity, the culture filtrate was used as a crude enzyme.

Assay of Amylase activity:

Using Miller's D.N.S. (Dinitrosalicylic acid) test technique, amylase activity was measured by measuring the quantity of reducing sugars produced in a combination containing 1.0 mL of soluble starch in phosphate buffer, pH 6.0, and 1.0 mL of enzyme extract [20]. After 10 minutes of incubation at 50 °C in a water bath, the reaction was halted using 1.0 mL of (D.N.S.) reagent. For fifteen minutes, the mixture was brought to a boil. After allowing the test tubes to cool, the absorbance at 540 nm was measured using a UV spectrophotometer. The released glucose concentration was compared to a glucose standard. One enzyme activity is defined as the amount of enzyme that, under assay conditions, released one mole of reducing sugar per minute per milliliter.

Microbial susceptibility testing:

Agar well diffusion method:

A volume of the microbial inoculum is applied to the whole surface of the agar plate to inoculate it. Subsequently, a 9 mm diameter hole is aseptically punched using a sterile corn borer or tip, and 100 µL of the necessary concentration of sample is added to the well. Agar plates are then incubated, depending on the test microorganism, under the appropriate conditions. The microbial strain under investigation is prevented from growing as the antimicrobial drug diffuses throughout the agar media [18]. "*Salmonella typhi*", "*Bacillus cereus*", "*Escherichia coli*","*Bacillus subtilis*", "*Klebsiella pneumoniae*" and "*Staphylococcus aureus*" were among the bacterial strains that were tested.

Determination of Minimum Inhibitory Concentration (MIC):

By gradually reducing the bacterial suspension at different concentrations, the minimum inhibitory concentration (MIC) in the nutritious broth medium was determined. The control group just had inoculation broth and was incubated for 24 hours at 37 °C. The MIC end point is the lowest concentration of bacterial suspension at which no observable growth occurs in the tubes. The visual turbidity of the tubes was observed both before and after incubation in order to confirm the MIC value, and the result was confirmed by measuring the O.D. at 600 nm [21–22]. The strain of bacteria that was tested was called "*Bacillus cereus*".

Results And Discussion:

Isolation and screening of microorganisms

From the soil sample, six different fungal cultures were recovered. The cultures were identified morphologically as "*Aspergillus fumigatus*", "*Penicillium chrysogenum*", "*Penicillium digitatum*", "*Penicillium purpurogenum*", "*Rhizomucor miehei*" and "*Rhizomucor pusillus*". The identified fungi were displayed Amylase activities. A qualitative, straightforward, and quick method of evaluating Amylase production is the plate method.

The fungus that makes amylase was tested using plate assay technique. The assessment of fungal amylase production using the plate assay method, with a specific focus on "*Aspergillus fumigatus*" (**Figure 1).** Below is a summary of the main ideas: The plate assay method was used to examine the fungus that produces amylase. The evaluation of *Aspergillus fumigatus* production of fungal amylase through the use of the plate assay method. A synopsis of the key concepts is given below: Amylase Plate Assay: Using this method, a colorless zone that surrounds fungal colonies on plates containing starch needs to be watched. The fungus's capacity to make amylase is indicated by the size of this zone; larger zones (diameters greater than 15 mm) imply more amylase output. Test for Iodine: Starch is detected using Lugol's solution, an iodine-potassium iodide solution. The creation of polyiodide complexes is what gives iodine its blue-black color when it combines with starch. The helical structure of polysaccharides

is responsible for this particular color change. such as glycogen, dextrin, or amylose. Iodine by itself is insoluble in water; however, when combined with potassium iodide, it produces iodide ions, which can then react to produce polyiodide ions, such as triiodide and pentaiodide. These polyiodide ions interact with the helical glucose chains of polysaccharides to cause noticeable color changes, depending on the length and structure of the chains. Effects of Solvent and Temperature: Temperature and the presence of organic substances that are soluble in water, such as ethanol, can have an impact on the color intensity of the iodine-starch combination. **(Figure 2).**

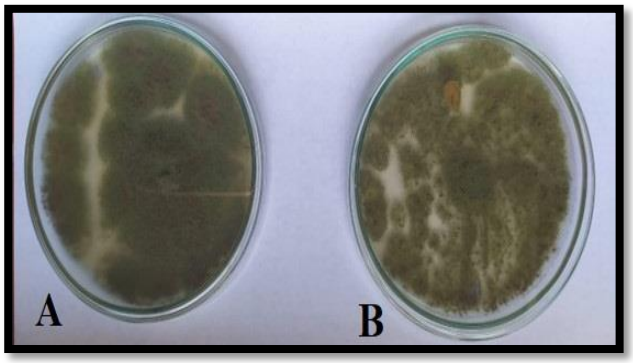

Figure 1: Plate showing the colony of Aspergillus fumigatus.

Table (1): Amylase enzyme activities from thermophilic fungal strains.

Fungal strains	OD	Activity U/mL
Aspergillus Fumigatus	0.431	0.796
Penicillium crysogenum	0.111	0.203
Penicillium digtatum	0.039	0.07
Penicillium purpurogenum	0.126	0.231
Rhizomucor miehei	0.230	0.424
Rhizomucor pusillus	0.013	0.022

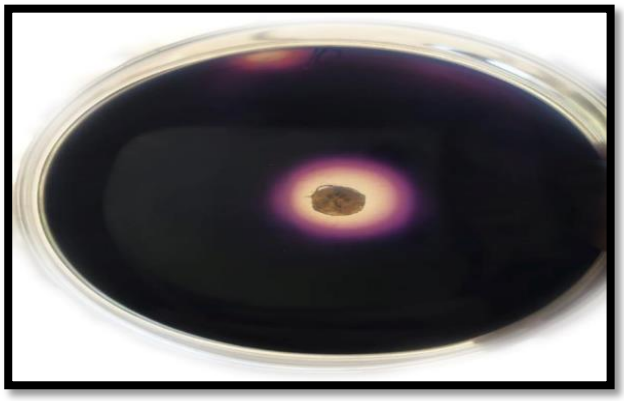

Figure 2: plate showing the colorless zone of amylase activity.

Figure 3: Growth of *Aspergillus fumigatus on* modified Czapek Dox's liquid media.

"Effect of pH on Amylase activity (U/mL)":

The purified Amylase was active over a wide pH range from (4 to 9), with an optimum activity **2.617** U/mL at pH **6**. The enzyme activity dropped off at higher pH levels. Even at pH 8.0 and pH 9.0, the enzyme maintains 1.466 U/ mL and 2.200U/ml respectively, of activity.**(Figure 4). (table 2)**

"Effect of temperature on Amylase activity (U/mL)":

The purified Amylase was active over a broad temperature range of 10 to 70 °C with an optimal Amylase activity of **3.057** U/mL at 40 °C and lower Fungal Amylase activity observed at lower temperatures **(Figure 6). (Table 3).**

Table2: "Effect of pH on Amylase activity "(U/mL)".

PH	Enzyme activity (U/mL)
PH4	2.191
PH5	2.132
PH6	2.617
PH7	2.459
PH8	1.466
PH9	2.200

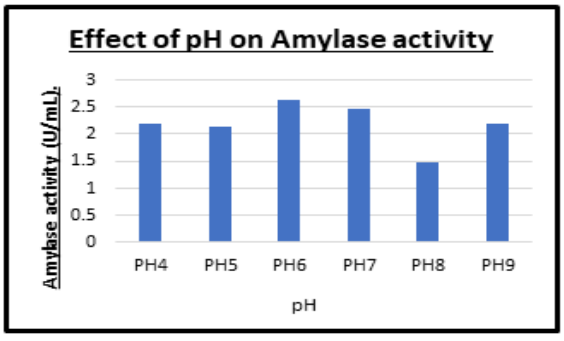

Figure 4: "Effect of pH on Amylase activity (U/mL)".

Table 3: "Effect of temperature on Amylase activity (U/mL)"

Temperature of incubation of fungi(°C)	Enzyme activity (U/mL)
10	2.570457455
20	2.936806619
30	3.010816552
40	3.057072759
50	2.762883279
60	2.670370864
70	0.945939445

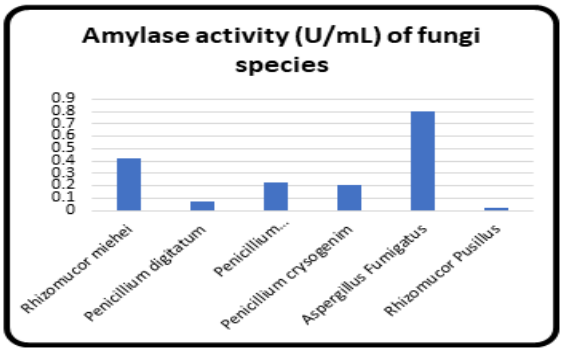

Figure 5: Amylase activity (U/mL) of the isolated fungi.

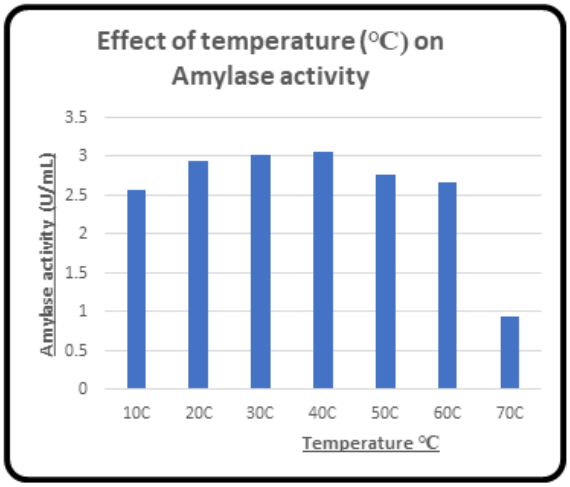

Figure 6: "Effect of temperature on Amylase activity (U/mL)"

Submerged fermentation and Amylase activity of fungi species:

Amylase production peaked at 0.79 U/ml after **5 days of incubation, at 50**°C. As shown in **(Figure 5). (Table 1).**

"Effect of Time on Fungal Amylase activity(U/mL)":

Further extensions of the incubation time resulted in a decline in enzyme production rather than any discernible increase. Therefore, 30 minutes was determined to be the ideal time for enzyme synthesis at **PH 6.0** and **40°C**. following immunization. **(table 4). (Figure7).**

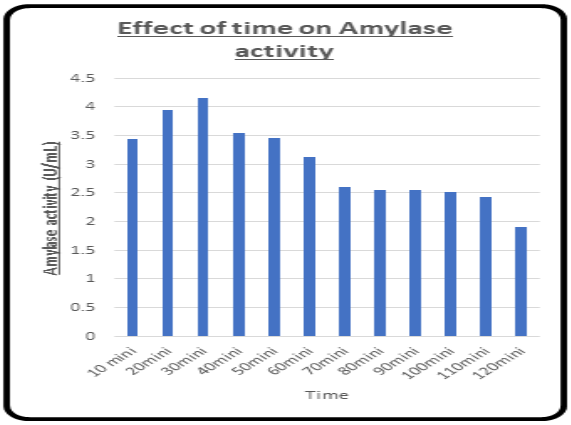

Figure7: Effect of time on Amylase activity extracted from *Aspergillus fumigatus* (U/mL).

Table (4): The optical Density and the enzyme activity in period incubation time at pH 6.0 and temperature 40°C.

Incubation time (mini.)	O. D	Enzyme Activity(U/ml)
10	1.856	3.432
20	2.136	3.950
30	2.246	4.154
40	1.92	3.550
50	1.87	3.458
60	1.688	3.121
70	1.41	2.607
80	1.382	2.555
90	1.347	2.540
100	1.36	2.514
110	1.31	2.422
120	1.034	1.911

Fungal Amylase are utilized as food additives. Due to fungi's capacity to use inexpensive media and produce the enzyme extracellularly, which facilitates the purification procedure, they are significant. Out of all the tested fungal isolates, "*Aspergillus fumigatus*" produced the most Amylase in the current study. "*Aspergillus fumigatus*" Amylase production was identified in the current study by the formation of a colourless zone around the colonyBy measuring the width of the clear zone (zone of hydrolysis) that formed around the fungal colonies on starch agar medium, researchers were able to identify that the colorless zone was created when Amylase broke down the glycosidic bond in the starch in

the growth medium to make glucose [23]. (Figure 2). As the incubation period was extended up to 50 minutes, "*Aspergillus fumigatus"* Amylase activity gradually increased. However, a longer incubation period with the substrate led to a decrease in Amylase activity, which may have been caused by the product's inhibition. At 30 minutes of incubation. **(Table 4).**

The antimicrobial test by disc diffusion assay of Amylase extracted from "*Aspergillus fumigatus"* against **7** different pathogenic bacteria with Tetracycline as +ve control showed significance inhibition zones (mm) against "*Bacillus cereus"*, "*Staph. epidermidis"* "*Staphylococcus aureus"*, and "*Klebsiella pneumoniae"*, as shown in (**Figure 8)** and (**Table 5)**. So it has been chosen to carry out next experiments.

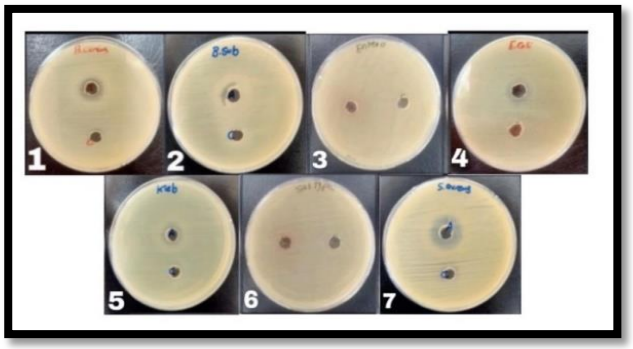

Figure 8: Antimicrobial activity of Amylase extraction against 1. *Bacillus cereus*, 2. *Bacillus subtilis*, 3. *Enterobacter cloacae*, 4. *Escherichia coli*, 5. *Klebsiella pneumoniae*, 6. *Salmonella typhi*, and 7. *Staphylococcus aureus*.

Table 5: Antimicrobial activity of Amylase extraction against different bacterial species.

Bacterial strains	Diameter of clear zone by Amylase extraction (mm)	Control (buffer)
1. *Bacillus cereus*	**14**	**-ve**
2. *Bacillus subtilis*	**-ve**	**-ve**
3. *Enterobacter cloacae*	**10**	**-ve**
4. *Escherichia coli*	**-ve**	**-ve**
5. *Klebsiella pneumoniae*	**11**	**-ve**
6. *Salmonella typhi*	**-ve**	**-ve**
7.*Staphylococcus aureus*	**22**	**-ve**

The MIC value was taken as the minimum concentration of Amylase extracted from *Aspergillus fumigatus* against bacterial growths of *Bacillus cereus* at which no microbial growth was observed.

The MIC of Amylase was 0.416 g/mL against bacterial growth. It is common practice to evaluate plant or microbial extracts' antibacterial activity using the Agar well diffusion method. Using a volume of the microbial inoculum, the entire surface of the agar plate is inoculated; this process is similar to the disk-diffusion strategy. Next, a sterile corn borer or tip is used to aseptically punch a hole with a diameter of 9 mm. Subsequently, the well is filled with 100 µL of the extract solution or antimicrobial agent at the specified concentration. After that, depending on the test microorganism, agar plates are incubated under the proper circumstances. As the antimicrobial medication permeates the agar media, it stops the microbial strain that is the subject of the inquiry from developing. [24].

Conclusions:

This summary provides an overview of the experimental data and findings related to Amylase from *Aspergillus fumigatus*, highlighting its potential industrial applications based on its robust enzymatic properties under various conditions. This enzyme has many excellent qualities that make it highly valuable to be used as a potent antimicrobial and anticancer agent, Moreover, based on the findings of this investigation, the isolated *Aspergillus fumigatus* holds promise as a potential source of Amylase.[25].

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