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Evaluation of the ability of some local soil fungi to produce oxalic acid under different conditions

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***Abstract*---**The aim of this study was to isolate fungi from soil and evaluate their ability to produce oxalic acid, then determine the most productive fungal isolate and the optimal conditions for acid production. All fungal isolates were oxalate producers, and the most efficient fungal species was *A.niger*, followed by *A.flavus*, then *Rizoctonia solani* with production rates of 4.05% , 3.54% , and 3.42% respectively , the rest isolates gave variable productivity. The optimum conditions for oxalate production were as follows ; (4%) sucrose as the best carbon source, (0.25%) ammonium sulfate as a best nitrogen source, (0.1%) hydrated magnesium sulfate as a best salt , (3%) which is equal to 1×10^6 spores/ml was the best inoculum size, the optimal pH value was equal to 6.5 , and 25°C represent an optimal temperature degree , for 7 days incubation period.

Keywords---oxalic acid, A.niger, local soil fungi.

Introduction

Oxalic acid or Oxalate (OA): it is an important and a simplest di-carboxylic organic acid, the chemical formula is $C_2H_2O_4$ (1). It is found in most living organisms such as plants, animals, fungi, and microorganisms (2), OA has different uses, including in the pharmaceutical industries such as tetracycline and phenobarbital production (3). It is also used in detergents manufacture, as included in cleaning and rust-removing solutions, works as a polishing reagent for marbles and metals, as a decolorizer in leather manufactures, and for fabrics printing (4). It is also used in metals extraction and purification (5), and sewage treatment (6). Chemically, the acid is prepared by heating the sodium format to

360°C in the presence of sodium carbonate or sodium hydroxide , which leads to the formation of sodium oxalate, which is converted to calcium oxalate that is treated with sulfuric acid to give OA , this is an ancient but still adopted method . Recently , There's a trend toward using microorganisms in the production of organic acids including OA by exploiting the microbes' fermentation (a biological processes) instead of chemical methods , as the biological method are easy , cheap , and safe to work , inapposite to the chemical methods which is difficult and expensive , as well as the potential of increasing the microbial productivity through controlling the environmental conditions and genetic engineering (7) .

Many microorganisms can produce OA , like some bacterial species such as, *Pseudomonas fluorescens* , *Bacillus licheniformis* (8) , and fungal species such as , *Aspergillus niger* , *Aspergillus flavus* , *Aspergillus oryzae* , *Trichoderma viride* , *Penicillium janthinellum* , *Rhizoctonia sp.,* and *Conidiobolus sp.* (9). Fungi are one of the most important microorganisms that are used in OA production, and because of their productive efficiency, there are many studies aimed to reaching the highest production at the lowest cost, this is happen through determining the most productive fungal species and growing them on cheap available raw materials (10), and to provide appropriate fermentation conditions (11). Due to its importance, this study aimed to produce OA compound by a local fungal isolate by achieving the following lines:

- Isolation and diagnosis of fungi from soil, then determining the most efficient isolate in oxalate production.
- determining the most appropriate fermentation conditions for oxalate production.

Materials and Methods

Samples collection

One kg of soil was taken for each sample, and from different gardens, placed in clean tightly closed nylon bags and transferred to the lab where the dilution method was done according to (12).

Isolation & diagnosis of fungal species

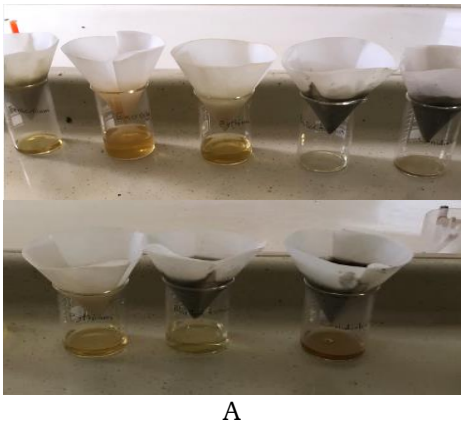
Fungal isolates were purified according to (13), by touching and transferring the mixed colonies separately to new SDA plates, that were prepared according to (14), then incubated at 25 ° C for 7 days, the process was repeated until pure colonies have been obtained. Fungal diagnoses are based on both morphologic features, such as the shape, color, and growth behavior, as well as the microscopic features, by preparing methylene blue stained smears and noting the colonies' features, such as the hypha (divided or not-divided), conidial shape, and the sporangium, the diagnosis depended on the references (15,16,17).

Evaluating the percentage of OA production

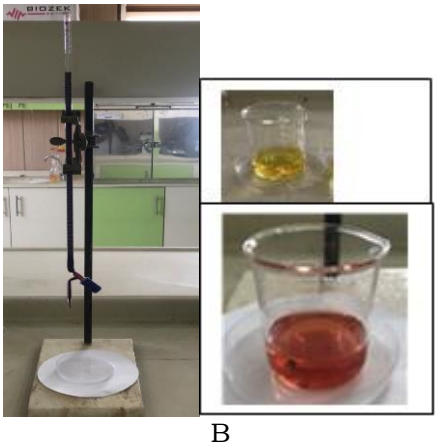
After isolation, purification, and diagnoses, the SDA medium was inoculated with the fungal isolate, incubated at 25°C for 7 days, then the percentage of OA was

estimated according to (18,19), were about (25 ml) of fungal filtrate titrated with potassium permanganate $KMnO_4$ (0.02 N) and stopped when the pink color appeared, since every 1 ml of potassium permanganate (0.02 N) equal to 1.2653 mg of OA. figure (1)

Oxalic acid (%) = potassium permanganate consumed volume × 1.2653



A



B

Figure 1. (A): the filtration of cultured media, (B): the titration with potassium permanganate

Determining the optimal conditions for OA production

Some conditions affecting the production of OA were studied and the most efficient fungal isolate was selected for the following experiments:

The effect of carbon source

SDB broth was prepared and distributed to three flasks (250 ml), and (4%) concentrations of the three carbon sources (glucose, fructose and sucrose), were added separately. The media was then inoculated with the fungal isolate and

incubated at 25°C for 7 days to determine the best carbon source for OA production.

The effect of nitrogen source

To determine the best source of nitrogen three nitrogen sources (ammonium phosphate $(NH_4)_2HPO_4$, ammonium sulfate $(NH_4)_2SO_4$, and potassium nitrate KNO3) were added separately to three flasks (250 ml) of SDB broth, then inoculated with the fungal isolate and incubated.

The effect of temperature

The fungal isolate was inoculated in a three SDS broth containing flasks (250 ml) and incubated at three different temperatures (30, 25, 20) °C for 7 days.

The effect of pH

SDB broth was prepared and distributed to three flasks of 250 ml, then the pH was adjusted to 6.5 by adding drops of HCL (1N), and to the values 7, 7.5 by adding drops of NaOH (1N), then the medium were inoculated with a fungal isolate and incubated at 25°C for 7 days.

The effect of salt

Three salts (potassium di-hydrogen phosphate KH_2PO_4, potassium chloride KCL, and hydrated magnesium sulfate $MgSO_4.7H_20$) , all at 0.1% concentration was added separately to three flasks (250 ml) of SDB broth, inoculated with a fungal isolate and incubated at 25°C for 7 days.

The effect of inoculum size

Spore suspension were prepared according to (20) and as the following formula (21):

$$\text{The average number of spores (X)} = \text{average number of spores in 5 squares} \times \text{dilution factor} \times 10^4$$

Then SDB broth was prepared and distributed into three flasks, each inoculated separately with the following concentrations (1 , 3 , and 5 %) , and incubated at 25 ˚ C for 7 days.

Results and Discussion

Fungi isolation and identification

About 51 fungal isolates were obtained and diagnosed. These isolates included within the following fungal species ; (14) isolates (27.45%) of *A.niger* , (8) isolates (15.69%) of *Penicillium* sp. , (6) isolates (11.76%) of *Rhizoctonia solani*, (5) isolates

(9.80%) of *Trichoderma viride*, (4) isolates (7.84%) belonging to *Emercella* sp., (3) species (5.88%) *A.oryzae*, two isolates (3.92%) from each of (*Pythium* & *Conidiobolus*), and one fungal isolate for each of (*Aspergillus flavus*, *Cladosporium sphaerosperum,* and *Beauveria bassiana*) with isolation ratio of (1.96 %) for each, fig (2).

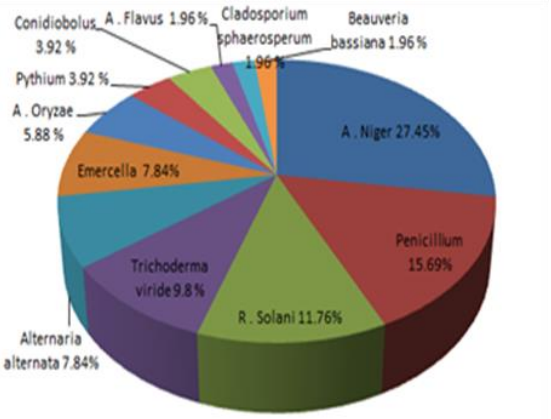


Figure 2. The studied fungal species with their percentages

The percentage of OA production

The ratios of OA production shown in table (1) were the highest percentage of OA production was (4.05 %) that obtained by *A.niger*, followed by *A.flavus* (3.54%), then *Rhizoctonia solani* (3.42), followed by the rest isolates in different proportions. So, *A.niger* was at the top of OA producers, this is due to its rapid growth rate and simplest nutritional requirements, and it can consume different carbon sources (10).

Table 1
The percentages of OA production

Isolated fungi	The ratios of oxalate production %
Aspergillus niger	4.05
Aspergills flavus	3.54
Rhizoctonia solani	3.42
Penicillium sp.	1.52
Pythium spp.	1.27
Trichoderma	1.12
Conidiobolus	1.01
Emercella	0.89
Alternaria alternate	0.76
Aspergillus oryzae	0.63
Cladosporium sphaerosperum	0.38
Beauveria bassiana	0.25

Determining the optimal conditions for OA production

The effect of carbon source

Fig (3) show that the best carbon source for OA production was Sucrose where its productivity reached (5.69%), in comparison with glucose and fructose. As their production rates were 4.43% and 3.67% respectively. Carbon sources are very necessary for fungal growing and production of different secondary metabolites, and sucrose is a disaccharide made of glucose and fructose, its water soluble and easy to consumption by microorganisms (22). It has been confirmed that using carbon in lower concentrations leads to the production of OA in large quantities instead of citric acid (23).

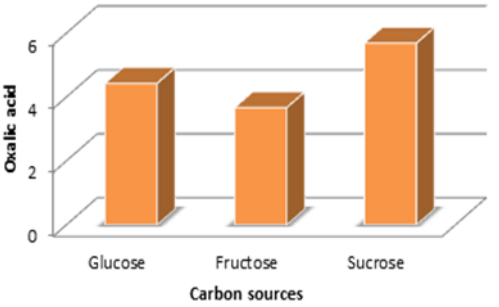


Figure 3. The effect of Carbon source on OA production

The effect of nitrogen source

The results in fig (4) show that ammonium sulfate was the best N-source with a percentage of 5.19 %, followed by potassium nitrate, then ammonium phosphate with a productivity of 3.16 and 0.89 % respectively. In comparison with other sources ammonium sulfate is a cheap available N-source. Nitrogen is an important growth factor, as it is a structural unit in proteins and nucleic acids (24), the amount of nitrogen needed to produce OA must be in limited quantities, and large quantities of C-source must be provided during OA production, that is achieved by the cell membrane enzymes (25).

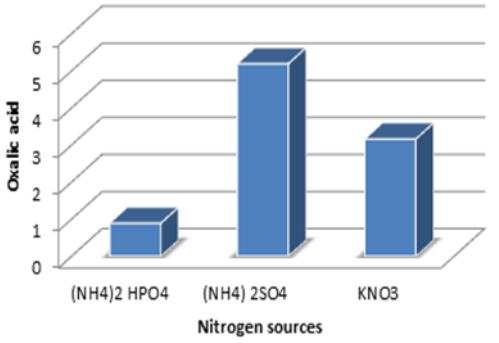


Figure 4. The effect of Nitrogen source on OA production

The effect of temperature

The results in Figure (5) show that the highest productivity of OA was achieved at 25°C followed by 30°C, then 20°C, as the percentage of OA formation was 5.06%.4.30%, and 3.16% respectively. The microbial fermentation process is controlled by temperature degree, but the optimum temperature needed for building compounds differs from that needed for growth, as high temperatures decrease the dry biomass and carbon consumption, high temperatures also inhibit enzymes responsible for OA production, and it is mentioned previously that the amount of OA decreases when the temperature rises up to 25 °C (26).

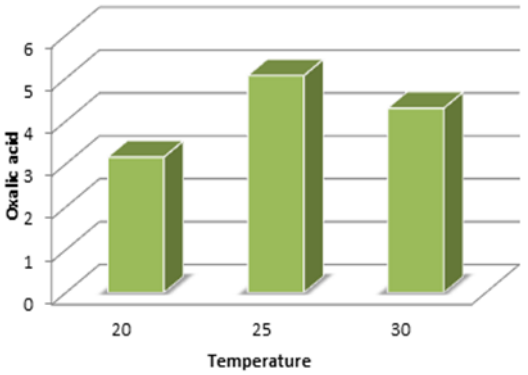


Figure 5. The effect of incubation temperature on OA production

The effect of pH

The best pH for OA production was (6.5) , as the percentage of acid was 5.69% , while the productivity decreased at 7 and 7.5 pH values , with production rates of 4.05% and 3.92% respectively, fig (6). Most filamentous fungi grow well at acidic pH, and through affecting different enzymatic activities, pH also has a role for secondary metabolites production, the activity of Oxaloacetate acetylhydrolase (an enzyme responsible for OA formation) increases when pH value increases (27).

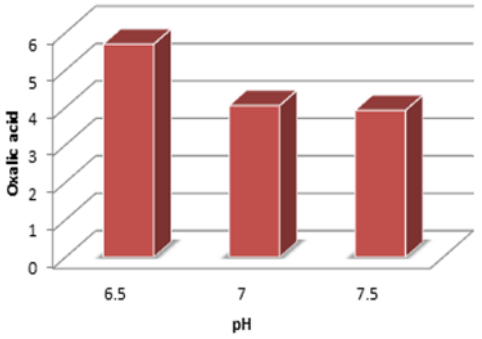


Figure 6. the effect of pH values on OA production

The effect of salt

Fig (7) show that magnesium sulfate gave best productivity for OA, which reached 5.06 %, followed by potassium di-hydrogen phosphate, then potassium chloride with a production rates of 3.92% and 2.66% respectively. It is clear that salts have a clear effect on fungal growth as well as the production of secondary metabolites, as salt-containing media are the best media for fungi to grow, and produce the OA (28).

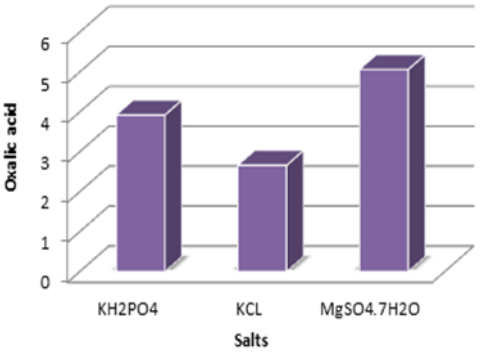


Figure 7. The effect of salt on OA production

The effect of inoculum size

Results shown in Fig (8) indicated that the maximum production of OA happens when 3% inoculation volume added, as the productivity of OA was 5.44%, followed by 5% , then 1% with a production rates of 4.81% and 3.16% respectively. Inoculation volume affects the initial growth rate, as the larger the inoculation, the rapid the growth, and faster consumption of carbon sources, which is reflected in OA production (29).

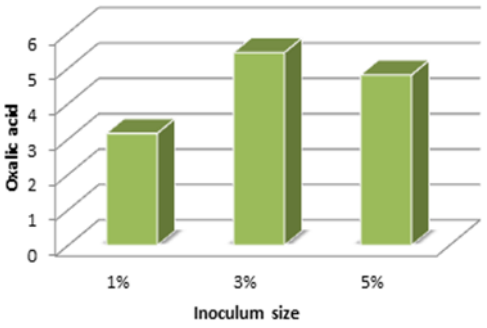


Figure 8. The effect of Inoculation size on OA production

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