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作品名稱 **Preparation of a Specific Detector for Aspergillus Niger in Swimming Pools**

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Abstract

Swimming pools are one of the transmission routes of superficial and cutaneous fungal infections. Maintenance of environmental hygiene in different parts of swimming pools is of great importance, especially the hygiene of water (1). The conventional fungal detection methods include direct smear preparation, culture, and pathological examinations. However, these methods are not fast enough or do not have sufficient sensitivity (2). Therefore, the present research introduces a novel method for detecting *Aspergillus niger* in pool water through creating optimal conditions for this fungus, which leads to the citric acid production by the fungus and pH changes of the related culture medium.

Four experiments in 10 steps were performed to find the optimal conditions for fungal growth. According to our results, adding each of the variables sucrose, soy, and ferrous sulfate can lead to favorable results. Moreover, the shaker speed increase and fungal aeration are important. Also, we showed that soybean led to the best results compared to other variables. Considering the obtained results, including the shortened detection duration and cost-effectiveness, this method can be presented to the swimming pool owners and pathobiology laboratories as the method of choice for *Aspergillus niger* detection.

1- Introduction

Fungi can grow under almost any condition, even at non-physiologic temperatures. These micro-organisms cause various diseases, such as allergies. The spores of opportunistic fungi, especially species of the *Aspergillus* genus, such as *Aspergillus fumigatus*, *Aspergillus niger*, and *Aspergillus flavus*, are considered as the most common fungal aeroallergens (2). During the last 3 decades, the prevalence of a group of systemic and opportunistic fungal infections, such as those caused by *Aspergillus*, has increased. As the causative agents of several diseases, species of the Aspergillus genus are associated with Allergic Bronchopulmonary Aspergillosis (ABPA), fungal keratitis, otomycosis, nasal sinusitis, and invasive pulmonary infections (4)

Swimming pools are water recreation centers with a vast number of visitors over the year. These places can be a potential transmission route for superficial and cutaneous fungal infections. Therefore, maintenance of environmental hygiene in different parts of swimming pools is of great importance, especially the hygiene of water and physical rooms of the swimming pool (1). The most common fungi found in the pool water include *Aspergillus*, *Candida*, and *Rhizopus* genera (4). The conventional fungal detection methods include direct smear preparation, fungal culture, and pathological examinations. However, these methods are not fast enough or do not have sufficient sensitivity. The immunologic detection methods are relatively fast but do not meet the needed specificity and accuracy (2). Other detection methods include molecular methods usually based on the Polymerase Chain Reaction (PCR). Despite recent advances, the application of this fast and cost-effective method for detecting acute and dangerous fungal infections is still problematic. Currently, routine use of these tests is not extensively practiced (5).

Aspergillus niger is a species of the genus *Aspergillus*. Typically, this fungus is a saprophyte, with its spores extensively found in the environment while attached to the organic material and soil. Moreover, *Aspergillus niger* has industrial uses in producing various enzymes and organic acids through fermentation (6).The present study introduces a novel method for detecting *Aspergillus niger* in pool water. This method is faster than the conventional methods used and can be useful in detecting the contamination of the water pool with *Aspergillus niger*. If any contamination is found, the factors effective on fungal growth in pool water can be changed, resulting in improved health of the swimmers and prevention of fungal infections. The present study aims to

detect the *Aspergillus niger* by creating optimal conditions for this fungus, which leads to the citric acid production by the fungus and pH changes of the related culture medium.

General Objective: Preparation of a specific detector for *Aspergillus niger* in swimming pools

Applied Objectives: Presentation to water recreation centers, public and private swimming pools, and pathobiology laboratories

2- Literature Review

For the first time, *Aspergillus niger* was described in 1867 in a manuscript entitled "physiologiedes mucedinees" by the French botanist Philippe Édouard Léon Van Tieghem. He isolated this fungus from molded galls when investigating gallic acid production through fungal fermentation (7).

In 1971, James Corey, a food chemist, found that the hyphae of *Aspergillus niger* produced high concentrations of citric acid while growing in an environment with carbohydrates (8).

Hang and Woodams (1984) investigated the yields of citric acid production by 5 strains of *Aspergillus* using apple pomace, reporting that *A. niger* NRRL567 produced the highest amount of citric acid in the presence of 4% methanol. The reported yield was 88% of the used sugar (9).

Nanbakhsh, Diba, and Hazrati Tapeh (2002) studied fungal contaminations and some other physical and chemical factors in indoor swimming pools located in Urmia, Iran, reporting that 12.5% of the samples had fungal contamination. The most common fungi included *Aspergillus*, *Candida*, *Rhizopus*, other filamentous fungi, and yeast species (10).

Hosseini, Rezazad Bari, and Alizadeh Khaledabad (2009) tried to optimize the citric acid production from apple pomace using a new surface culture method. They reported that *Aspergillus niger* could produce a maximum amount of 14.951 gr citric acid per kg of dried pomace (11).

In 2014, Fangfang Wang investigated the characteristics of the species of *the Aspergillus* genus, showing that the black-to-brown color of *Aspergillus niger* spores under the light microscope can be used for differentiating the *A. niger* from other species of the same genus (8).

Samad Louei and Gharanjik (2015) tried to optimize the methods used for citric acid production from the species of *Aspergillus* genus, reporting that increased source concentration (soy protein), carbon source (sucrose) increase, and other factors can boost the citric acid production yields (12).

3- Methods

Phases 1 and 2: data collection and pool water sampling

Using the available facilities, data were collected on the research topic. A total of 4 liters of water were collected from 4 different house pools.

Phase 3: Tryptic Soy Broth (TSB) medium preparation.

The TSB medium is a liquid and highly nutritious culture medium used for general applications. This formulation of this medium is based on the coordinating pharmacopeial methods.

Table 1. The materials available in the TSB medium and its final pH.

Casein peptone	17 gr/L
Sodium Chloride	5 gr/L
Soy peptone	3 gr/L
Dipotassium phosphate	5.2 gr/L
Dextrose	5.2 gr/L
Final pH	3.7 at 25 ° C

Phase 4: test tube sterilization and addition of pool water to test tubes.

First, about 16 test tubes were disinfected and numbered for water sample evaluations. The tubes were arranged in order.

Each pool water sample was added to 3 test tubes, with 20 ml of water in each tube. The classification of the test tubes is presented in the following table.

Table 2. Addition of pool water samples to test tubes.

Pool water sample	Test tubes
No. 1	1, 2, 3
No. 2	4, 5, 6
No. 3	7, 8, 9
No. 4	10, 11, 12

Phase 5: Culture of *Aspergillus niger*

We used the samples from pool water because their contamination with *Aspergillus niger* was highly probable. However, in addition to 12 tubes in which the pool water was added, we cultured *Aspergillus niger* in another 4 tubes.

Phase 6: Addition of variables to the cultures.

Each of the variables of sucrose, soy, and iron nanoparticles were added to some tubes as presented in Table 2 in order to find the best variable for the *Aspergillus niger* detection kit. These materials could act as an *Aspergillus niger* growth factor. Moreover, in case of fungal growth in the test tubes, the pH of the medium will change. Also, none of the variables were added to Tube 16.

Table 3. Addition of study variables to test tubes.

Sucrose	Weight	Soy	Weight	Iron nanoparticles	Volume
1, 4, 7, 10, 13	2 gr	2, 5, 8, 11, 14	2 gr	3, 6, 9, 12, 15	4 µL

The initial pH of the tubes was measured and recorded. Afterward, the tubes were incubated in an incubator shaker because the samples, especially those containing soy, needed aeration. The temperature was set at 24.1 ° C. The pH of the tubes was measured every 6 hours.

Phase 7: Repetition and accuracy assessment

Step 1: The pool water samples were tested for linearity to ensure their contamination with *Aspergillus niger*. Only the pool water samples from Pool 1 had fungal contamination.

Figure 1. The colony of *Aspergillus niger* after incubating the water sample from Pool 1 for 30 hours.

Step 2: In this step, phases 1, 2, and 5 were repeated. However, we used 50-ml beakers and Erlenmeyer flasks rather than test tubes to increase the contact surface with air. The containers were numbered in the same way as the test tubes. Moreover, we used 1 gr of ground soy rather than 2 gr. Also, 4 mL of ferrous sulfate solution (1 M) was added to each container instead of the iron nanoparticles. When the cultures were ready, they were covered with paraffin.

Step 3: The pH of the containers was measured every 2 hours.

Step 4: In this step, 2 Erlenmeyer flasks were numbered as containers 17 and 18. Then, 20 ml of water from Pool 1, which had confirmed fungal contamination, was added to Container 17. Moreover, 20 ml of the TSB medium was added to Container 18. The mentioned medium was sterilized under standard conditions, and *Aspergillus niger* was cultured in it. These 2 containers underwent shaking at 25 ° C, with a speed of 140 rpm. Then, the following materials were added to containers 17 and 18: 1 gr of sucrose, 1 gr of ground soy, 2 ml of ferrous sulfate solution (1 M).

Figure 2. Sample preparation for step 4.

Step 5: The pH of containers was measured 1, 2, 4, 24, and 30 hours after incubation.

Step 6: As in the previous step, we used Erlenmeyer flasks in this step for better aeration. 3 flasks were added with the water from Pool 1, which had confirmed fungal contamination. Moreover, 3 other flasks were added with the TSB medium, and 1 flask was only added water and ethanol. The detailed information is presented in Table 4.

Table 4. Addition of variables to the Erlenmeyer flasks.

Medium	Pool water sample			With culture medium			Negative control sample
Container	Erlenmeyer flask 1	Erlenmeyer flask 2	Erlenmeyer flask 3	Erlenmeyer flask 4	Erlenmeyer flask 5	Erlenmeyer flask 6	Erlenmeyer flask 7
Material (variable)	Sucrose	Soy	Ferrous sulfate solution (1 M)	Sucrose	Soy	Ferrous sulfate solution (1 M)	Water and ethanol
Amount	2 gr	1 gr	2 ml	2 gr	1 gr	2 ml	20 ml

Step 7: The pH of the containers was measured every 3 hours.

Phase 8 and 9: Result classification, data analysis, and conclusion.

Phase 10: Introduction of the final detection kit.

According to the results of the previous phases, all 3 study variables can be used in an *Aspergillus niger* detection kit under specific conditions. However, soy is more available and cost-effective. A soybean includes 40% protein, 21% fat, 43% carbohydrate, and 4.9% ash (6). Therefore, soybean can be the best option for the detection kit.

4- Discussion and Conclusion

The results obtained from Phase 6 are presented in Table 5.

Table 5. The pH assessment of containers every 6 hours.

Pool Water Sample 1			Pool Water Sample 2			Pool Water Sample 3			Pool Water Sample 4			Pool Water Sample 5			Negative Control Sample
Sucrose	Soy	Iron nanoparticle	Sucrose	Soy	Iron nanoparticle	Sucrose	Soy	Iron nanoparticle	Sucrose	Soy	Iron nanoparticle	Sucrose	Soy	Iron nanoparticle	Water and ethanol
5	5	5	5	5	5	5	5	5	5	5	5	6	6	6	6
5	5	5.5	6	5	6	5.5	5	5	6	5.5	6	5	5	5	6
4.5	5	5	6	4	5	5	4.5	5	5	4	5	6	8	4	6

The results obtained from Phase 7 (Step 3) are presented in Table 6.

Table 6. The pH assessment of containers every 2 hours.

Pool Water Sample 1			Pool Water Sample 2			Pool Water Sample 3			Pool Water Sample 4			Pool Water Sample 5			Negative Control Sample
Sucrose	Soy	Iron nanoparticle	Sucrose	Soy	Iron nanoparticle	Sucrose	Soy	Iron nanoparticle	Sucrose	Soy	Iron nanoparticle	Sucrose	Soy	Iron nanoparticle	Water and ethanol
5	5	6	7	7	6	8	5	6	7	6.5	6	5	6	6	6
5	5.5	6	8	5.5	4	8	5	6	5	5	6	5	6	5	6
6	5.5	6	5	5	10	8	5	6	5	5	6	5.5	6	6	6

The results obtained from Phase 7 (Step 5) are presented in Table 7.

Table 6. The pH assessment of fungal growth media in Phase 7 (Step 5).

Duration	Container 17	Container 18
1 hour later	6	6
2 hour later	6	6
4 hour later	6	6
24 hour later	5	6
30 hour later	3	3

The results obtained from Phase 7 (Step 7) are presented in Table 10.

Table 10. The pH assessment of containers in Phase 7 (Step 7) every 3 hours.

Pool water sample			Positive control samples			Negative control sample
Sucrose	Soy	Ferrous sulfate	Sucrose	Soy	Ferrous sulfate	Water and ethanol
6	6	6	6	6	6	6
5	4	5	5	5	5	6
3	3	3	3	3	3	6

According to the results of Phase 6 (Table 5), the test conditions caused increased acidity, decreased acidity, or did not change the PH. Therefore, under specific conditions, pH can reduce due to fungal growth and activity. However, the used method does not meet the necessary accuracy, and there is a possibility of errors. The lack of any pH change in Tube 16, which contains water and ethanol and no fungal contamination, indicates that study variables can affect the pH of samples. Therefore, there is an association between study variables and pre-existed fungal contamination.

The results from the Phase 7 (Step 3) are similar to those from Phase 6, indicating that the method used does not meet the necessary accuracy, and there is a possibility of errors.

According to the results of Phase 7 (Step 5), *Aspergillus niger* could grow with all study variables, which was expected. The reduced pH observed in the containers indicates the fungal growth and citric acid production by *Aspergillus niger*. The use of Erlenmeyer flasks increased the aeration, which affected the results. According to our observations in Phase 7, the pH changes were not different between the pool water samples and culture media. In general, we can state that the method used meets the required accuracy and can detect *Aspergillus niger* at a minimum of 24 hours and a maximum of 30 hours.

According to the results of Phase 7 (Step 7), separate addition of materials led to favorable results. In this step, the shaker speed was increased, leading to better aeration. Moreover, it was found that soy led to the highest yields compared to other study variables. Citric acid production by *Aspergillus niger* increased the acidity of the fungal growth media. Therefore, *Aspergillus niger* can be detected in 6 hours.

Figure 3. The final *Aspergillus niger* detection kit.

5- References

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Suggestions

1. Accuracy assessment of citric acid production by *Aspergillus niger*
2. Evaluation of several water samples to achieve more accurate results and reduce the test errors
3. Increasing the sterilization level of culture samples to eliminate other pathogens and avoid interference with their growth
4. Repeating the experiment with the new detection kit

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It would be better to have more control experiments and verify whether colonies that grew on the plates are Aspergillus Niger or contaminations.

1. The data presented is very preliminary and no further data is available
2. The data should have statistical analysis