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Optimum Factors Estimation during Uranium Bioleaching Process from Three Different Samples using Two Different Fungal Strains

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Abstract: Bioleaching (biomining) is a mining and bio-hydrometallurgical technique that uses naturally existing microorganisms such as fungi to extract valuable metals from various ores. This process is beneficial for both humans and the environment. Uranium was bioleached using isolated fungal strains from Um Bogma formation in Gabal um Hamd, Southwestern Sinai, Egypt., Two fungal species were isolated from the different selected samples and, purified, then identified. The bio-dissolution studies revealed that *Aspergillus hollandicus* and *Penicillium citrinum* had the highest uranium leaching efficiency from the tested samples. The uranium grade and mineralogic elements of the ore material were discovered to have a vital role in the bioleaching process. By monitoring the process, the examined samples demonstrated that the optimal uranium leaching conditions are: 7 days incubation time, 3% pulp density at 30°C-35°C incubation temperature, and the appropriate pH value equals 3. Both fungal strains produced organic acids that were identified as oxalic, maleic, quinic, lactic, citric, and butyric acids in the culture filtrate, which participated in the bioleaching process as well as total protein excretion.

Keywords: Bioleaching process, *Aspergillus hollandicus*, and *Penicillium citrinum*, Optimum uranium leaching conditions, Organic acids.

1 Introduction

Nowadays, heavy metals pollution is considered a major issue in many countries causing environmental damage due to industrial effluents, influenced by factors like absorption, distribution, species, bioavailability, ubiquity, and use [1]. The highly persistent nature of these pollutants results in increased accumulation in soil and water [2]. However, prokaryotic intrinsic defense methods allow heavy metal ion uptake and use as nutrients via absorptive and accumulative capacities. Similarly, it has been found that eukaryotic microbial organisms, such as fungi, have enough mechanisms to tolerate metal stress [3] The fungal species employs many strategies to endure in the presence of metals, such as attaching metal ions to the cellular membrane functional groups with high affinity, accumulating metals, complexing with various microbial biomolecules, and actively effluxing or excluding metals [4]. The ability of a wide range of microorganisms, including autotrophic and heterotrophic bacteria and fungi, to extract metal ions from solid wastes and transfer them

into a solution is used in bio-hydrometallurgy or bioleaching processes [5].

Heterotrophic fungi and bacteria need organic matter as their carbon source. These organic carbon sources play an essential part in the metabolism of these microorganisms, including the secreted organic acids (malic, oxalic, nitric, and citric acids) in the culture medium. Metals extraction from a solid matrix has been proven using these microbes, which frequently have acidic and chelating properties. Further, due to the protein catabolism in these heterotrophic bacteria, non-acidic compounds are formed, resulting in alkaline leaching systems [6,7]. Asghari and Mousavi [8] recovered metals from various wastes using several fungal strains such as *Aspergillus niger*, *Penicillium simplicissimum*, *Penicillium chrysogenum*, and bacteria such as *Gluconobacter oxydansseudomonas* strains, *Bacillus* strains, and *Chromobacterium violaceum.*

The bioleaching mechanism in fungi is associated with the generation of metabolites of low molecular weight such as organic acids. The following mechanisms are used in fungal bioleaching: acidolysis, complexolysis, and redoxolysis. Protons from the production of organic acids are involved in the process of metals solubilization by the acidolysis mechanism, whereas the protonation of oxygen

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atoms coats the surface of solid waste. Acid leaching is a similar mechanism. The carboxyl and hydroxyl groups of organic acids formed an organic acid-metal complex via the complexolysis mechanism [9]. Consequently, the bioleaching process is based on the creation of organic and inorganic acids (proton formation) or the excretion of complexing agents (i.e., ligand formation). The metal leaching that occurs by heterotrophic microorganisms is typically an indirect process, including the microbial generation of organic acids (e.g., lactic, oxalic, citric, and gluconic), amino acids, and other metabolites. These metabolites typically have a dual function of dissolving insoluble metal compounds from minerals by one or both of the following: (i) enhancing metal dissolution by changing the pH value towards acidity (pH decreases), and (ii) raising the concentration of soluble metals by complexing/chelating with soluble organometallic complexes. Microbial cell adhesion to mineral surfaces has been recognized for many years, and it is critical to determine the aspects of the interaction between microbes and metal ions involved in bioleaching processes [10]. The aim of this study is reaching to the optimal conditions (pH, ore concentration, incubation time, incubation temperature) that improve the fungal bioleaching activity of uranium. As well as estimating and evaluating the role of total protein and organic acid fungi excretion during the bioleaching process.

2 Experimental Sections

2.1 Sampling and Samples Preparation

Three radioactive geologically gripped samples were collected from the three members of the Um Bogma Formation in Gabal Um Hamd, southwestern Sinai, Egypt. The samples are stored in sterile polyethylene packets and coded as W1, W2, and W3 according to sampling area and layer, then crushed, quartered, and ground for investigations.

2.2 Chemical Analysis of Samples

2.2.1 Sample Digestion

After samples were crushed and grinded, each one was digested by an acid attack mechanism. In a Teflon beaker, a mixture of 10 ml conc. hydrofluoric acid (HF), 5 ml of conc. nitric acid (HNO_3), and 5 ml of perchloric acid were added to 0.5g of sample and then heated at 220˚C till complete dryness followed by adding 5 ml of 1:1diluted hydrochloric acid (HCl) then up to volume by adding distilled water to 50 ml [11]. All the chemical analyses were conducted in the laboratories of the Nuclear Materials Authority.

2.2.3 Sample analysis

The major elements, in the oxide form, were measured by the wet analysis technique, in which the SiO_2, Al_2O_3, TiO_2, and P_2O_5 were determined by colorimetric spectrophotometric (LABOMED: model- SPECTRO UVD, USA) [12]. While the total oxides as Fe_2O_3, MgO, and CaO were evaluated by the traditional titration techniques. Also, the contents of Na and K were determined using a flame photometric technique (JENWAY: model-PEP7, UK). The loss on ignition (L.O.I) was calculated gravimetrically. The estimated error for the major constituents is less than ±1% [13] and the analytical precision was ±3%. On the other hand, the uranium content of the studied samples was measured [14], as well as the titration method was utilized for both ore samples and bioleaching liquors against standard ammonium metavanadate (NH_4VO_3) where the endpoint will be obtained at the appearance of a purplish color, after that the uranium concentration will be calculated according to the following equation (Eq. 1):

$$U\ (mg/\ L) = T \times V_1 \times 103/\ V \quad ppm \quad (1)$$

where T is the titration intensity of NH_4VO_3 solution, V_1 is the volume of NH_4VO_3 solution consumed and V is the volume of the measured sample.

2.3 Microbiological Studies

2.3.1 Media Preparation

Sabouraud dextrose was the selected medium for the fungi cultivation. This medium was prepared by dissolving 10 g of peptone, 40 g of dextrose, and 20 g of agar in 1000 ml of bi-distilled water, then sterilized by autoclaving at 15 lbs. pressure (120°C) for 15 minutes [15-17].

2.3.2 Fungal Growth, Isolation, Purification, and Identification

The direct plating technique was used by spreading the fine powder of each sample on sabouraud agar plates and incubating for 7 days for fungi growth [18, 19]. Then from each separate colony, hyphal tips were removed and plated upon the surface of another sabouraud agar plate to get a pure fungal isolate. Each pure isolated colony was morphologically identified at the Regional Center for Mycology and Biotechnology (RCMB) Al-Azhar-University, Cairo, Egypt [20].

2.3.3 Determination of organic acids concentration

The tested isolated fungi were cultivated on sabouraud liquid media for 7 days at 35°C. The fungal organic acids were estimated using high-performance liquid chromatography (HPLC / UV) at the Fungal Center, Al-Azhar-University, Cairo, Egypt.

2.3.4 Investigation of factors affecting the uranium bioleaching and protein assay

Several factors were investigated to obtain the optimum conditions for uranium solubilization. i.e., the activity of different fungal strains, the influence of ore concentrations upon the growth of the isolated fungal strains, incubation periods, and incubation temperatures. These factors were assessed as follows: i) Preparation of the leach liquor: 25 ml of sabouraud liquid media was placed in 250 ml measuring flasks. The flasks were supplemented with different ore concentrations and autoclaved at 1.5 atm. for 20 min. After cooling, the flasks were inoculated with 0.5 ml of spore suspension and finally incubated at room temperature for 7 days in an orbital shaker at 100 rpm. Triplicate sets of flasks for each ore and organism were prepared. At the end of an incubation period, the mycelial mats were harvested and washed several times with distilled water. Each growing fungus was dried at 85°C for 24 hours. The culture filtrate of each treatment was filtered, centrifuged, and kept for uranium determination. ii) Determination of the total protein using a biuret solution that reacts with the peptide bonds producing a violet color because of compound formation, can be detected by the spectrophotometer at 540 nm [21].

3 Results and Discussion

The data tabulated data in Table 1 showed the description of the three samples under investigation which are Compact siltstone, Shale grey, and Sandy dolostone.

From the studied location, Gabal Um Hamd, Sinai, Egypt, the analyzed samples were found to have high contents of SiO_2, Al_2O_3, and Fe_2O_3 and low contents of TiO_2, CaO, MgO, P_2O_5, Na_2O, and K_2O, with a higher percentage in loss of ignition (L.O.I.) of 24.15 % (Table 2). As well as the chemical measurements of uranium in the studied samples W1, W2, and W3, they exhibited 250, 391, and 1161 ppm, respectively.

Table .1 Description of collected samples from the Gabal Um Hamd location.

Sample no.	Description
W1	Compact siltstone, medium hard highly ferruginous.
W2	Shale grey, fissile ferruginous, and gypsiferrous.
W3	Sandy dolostone, jointed and fractured, dark gray.

3.1 Microorganisms Isolation and Identification

Two fungal species were isolated from the tested samples. The most dominant fungal strains belong to two species, known as *Asperigillus* and *Penicillium*, identified according to their morphological features, meanwhile, the first one of *Aspergillus species* was identified as *Aspergillus hollandicus* (*A. hollandicus*) and the second was the *Penicillium species* known as *Penicillium citrinum* (*P. citrinum*). They were grown in the presence of sabrouad agar medium at room temperature for 7 days.

Table 2. Results of major oxides (wt%) of the studied samples.

Sample Oxides%	W1	W3	W5
SiO_2	44.33	50.45	28.80
TiO_2	00.75	00.95	00.13
Al_2O_3	13.90	12.71	15.90
Fe_2O_3	23.90	20.40	04.80
CaO	03.80	02.18	20.02
MgO	00.25	00.32	13.70
P_2O_5	00.30	00.24	00.17
Na_2O	01.99	02.12	00.76
K_2O	00.98	01.06	00.12
L.O. I	09.80	08.17	10.90
Total	99.91	98.58	100.1

3.2 Factors Affect the Bioleaching Process

Several factors play an important role in the bioleaching process as pH, ore concentration, incubation period, and incubation temperature These factors were determined as follows:

3.2.1 Bioleaching Efficiency and Initial pH Values

One of the most efficient factors in the bioleaching process is the pH value of the leaching solution. By applying the fungal strains, which have the highest leachability with 1% ore concentration from the studied samples W1, W2, and W3 at room temperature for 7 days using sabouraud as a growth medium, the optimum pH value was determined. The applied fungal strains were adapted to survive in the leaching solution by changing the initial pHs to their proper final pH value. Table (3) and Fig. (1) indicated that the percent of released uranium was inversely proportion with the initial pH values of the growth medium towards alkalinity. The highest bioleaching efficiency of uranium from the samples was obtained at a pH equal to 3 using *A.hollandicus*, followed by *P.citrinum*. The uranium best bioleaching efficiency firstly for *A.hollandicus* was achieved at 43.1, 51, and 48.7% with the studied samples W1, W2, and W3, respectively, as well as the secreted levels of total protein were 18.32, 17.7, and 11.63 mg/ml, transforming the pH value from 5.6 to 2.69, 2.95, and 4.81, respectively. Finally, for *P. citrinum* was attained 65.7, 60.3, and 54.2 % with W1, W2, and W3, respectively. The maximum T.P secretion value of *P. citrinum* after exposure to W3 was 27.4 mg/ml with an initial pH of 5.6 and a final pH was 3.39 since the minimum values of T.P were 6.8 and 6.7 mg/ml with W1 and W2 at final pHs 3.05 and 4.74, respectively. Amin [22] revealed that the maximum

solubilization of uranium was reached at the initial pH value of 4. The fluctuating chemistry of the metal complexation may be the reason for the variation in the ideal starting pH value of metal solubilization. The fungal cells' ability to bind was enhanced and the uranium's leaching efficiency was reduced when the pH of the growth medium was raised [23,24]. The pH of a culture medium has an immediate impact on microbial growth and chemical reactions, influencing soil microorganisms involved in activities such as organic matter decomposition, mineralization, and biological nitrogen fixation [25].

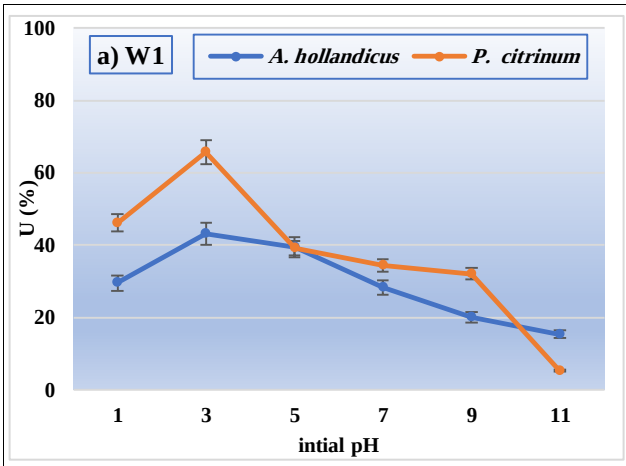
The obtained results of T.P. were in agreement with Ali et al.,[26], who revealed that the fungi may grow at different pHs (3.0–8.0), with a maximum production of dry weight and sporulation at pH 5.5 and pH 6.5. Higher or lower pH values showed inferior results; metabolic processes are highly susceptible to even slight changes in pH [27].

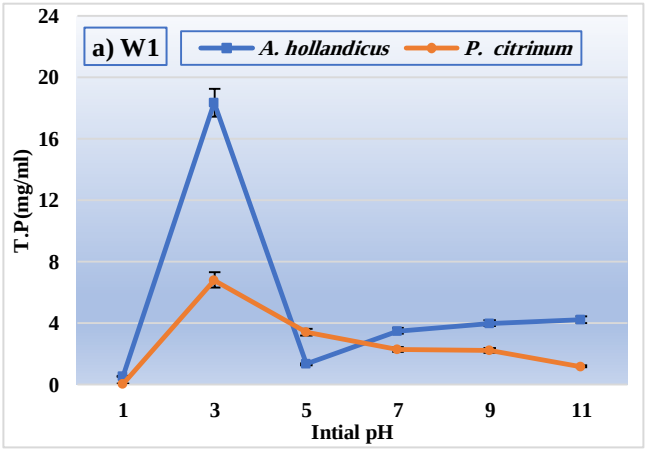
3.2.2 Bioleaching Efficiency and Ore Concentration

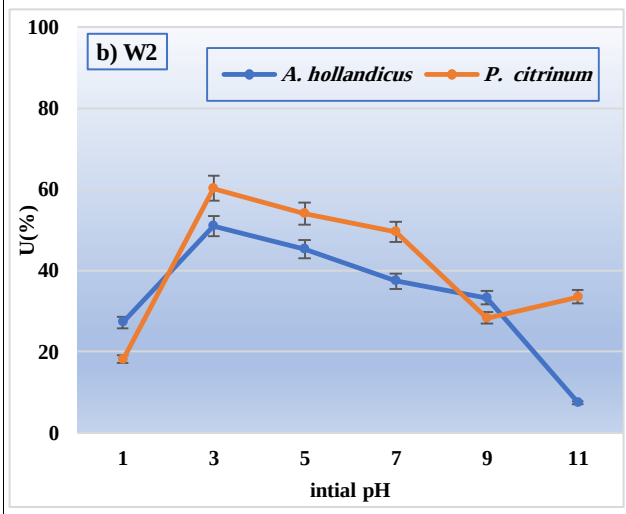
The ore concentration factor is one of several factors that affect the biological activities of isolated fungal strains, whether by activation or inhibition of their vital processes. From the obtained results of the bioleaching process (Table 4 and Fig.3), the highest bioleaching activity of *A. hollandicus* for uranium efficiency in the presence of 3% W1, W2, and W3 was 65, 45, and 60 %**,** respectively. On the other hand, the use of *P. citrinum* achieved 71, 38, and 62 % with W1, W2, and W3, respectively. The solubilization efficiency of uranium using the fungal strains *A. hollandicus* and *P. citrinum* decreased by increasing ore concentrations in the growth medium up to 11% in the three different grades of the studied samples (W1, W2, and W3). This may be due to insufficient oxygen transfer, limited nutrition provided to the microbes, or deformation of cells in the high solid concentrations [28]. As well as the decrease in bioleaching efficiency seen with rising sample concentrations, may be explained by the possibility that fungal mycelium binding capacity increases at higher concentrations [29,30]. Therefore, it led to a decrease in metal concentrations in the actual leach liquor, which may reduce the effectiveness of solubilization [31]. Furthermore, the secretion of the T.P. by the two fungal strains was studied, and the fungal ability to change the final pH value according to the change in the sample's concentration was observed (Fig.4).

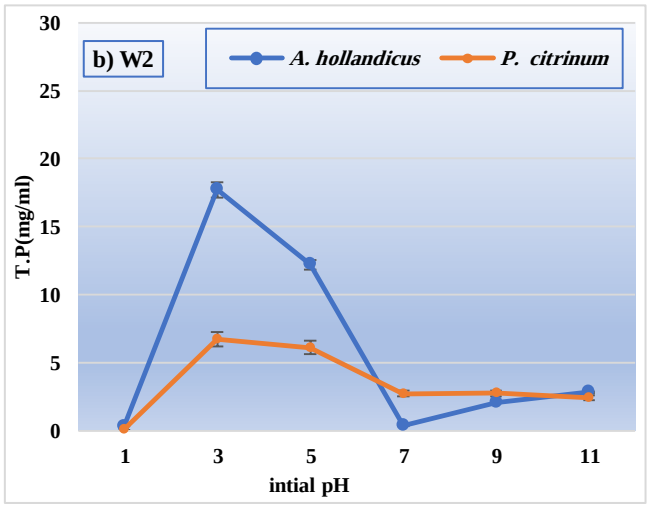
Table 3. Effect of initial pHs on uranium solubilization by *A. hollandicus* and *P. citrinum* at room temperature for 7 days.

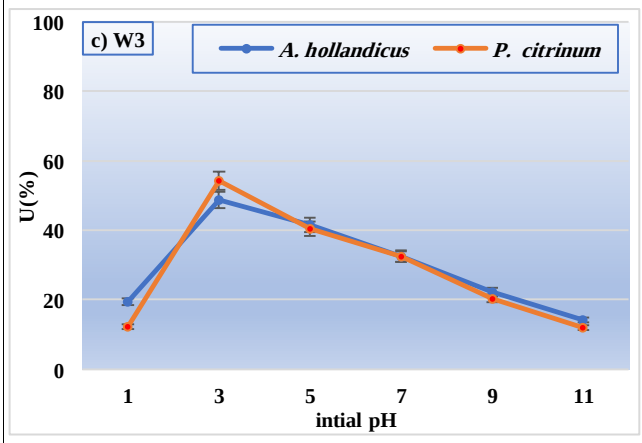
Fungal Sp.		Samples	Initial pH values					
			1	3	5	7	9	11
A. hollandicus	U%	W1	29.5±0.44	43.1±0.66	39.4±0.61	28.3±0.43	20.1±0.31	15.4±0.23
		W2	27.2±0.41	51±0.78	45.2±0.69	37.4±0.57	33.3±0.50	7.4±0.10
		W3	19.4±0.29	48.7±0.74	41.5±0.63	32.4±0.49	22.2±0.33	14.3±0.21
	T.P (mg/ml)	W1	0.5±0.007	18.32±0.28	1.3±0.02	3.47±0.0535	3.98±0.06	4.2±0.06
		W2	0.3±0.004	17.7±0.273	12.2±0.188	0.33±0.005	2.07±0.031	2.8±0.043
		W3	0.93±0.04	11.63±0.179	8.72±0.134	5.37±0.082	2.54±0.039	2.9±0.044
	Final pH	W1	0.74	2.69	3.86	4	4.38	5.49
		W2	0.68	2.95	3.56	3.98	5.38	6.18
		W3	0.61	4.19	4.81	5.64	5.45	5.12
P. citrinum	U %	W1	46.2±0.70	65.7±1.01	39.2±0.61	34.4±0.57	32.1±0.49	5.3±0.07
		W2	18.1±0.27	60.3±0.92	54.1±0.83	49.5±0.75	28.3±0.43	33.5±0.50
		W3	12.3±0.18	54.2±0.83	40.4±0.62	32.5±0.49	20.4±0.30	12±0.18
	T.P (mg/ml)	W1	0.02±0.001	6.8±0.104	3.4±0.052	2.26±0.03	2.18±0.03	1.17±0.01
		W2	0.05±0.002	6.7±0.103	6.1±0.09	2.7±0.041	2.74±0.04	2.4±0.03
		W3	0.12±0.012	27.4±0.42	12.5±0.34	7.3±0.11	5.6±0.08	5.5±0.08
	Final pH	W1	1.23	2.92	3.48	3.15	3.21	3.89
		W2	1.25	3.05	3.34	3.41	3.59	3.74
		W3	1.22	3.39	4.74	5.11	5.43	6.9











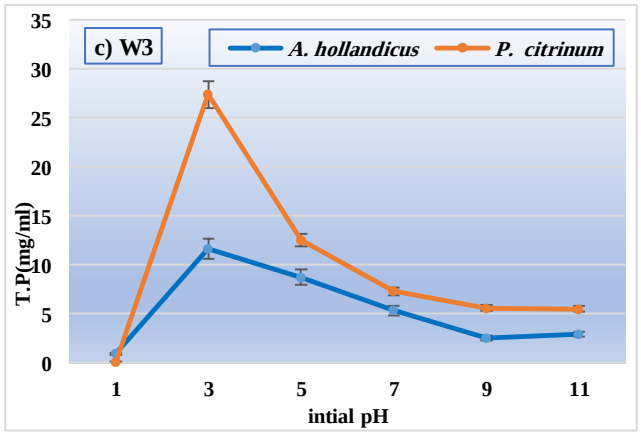


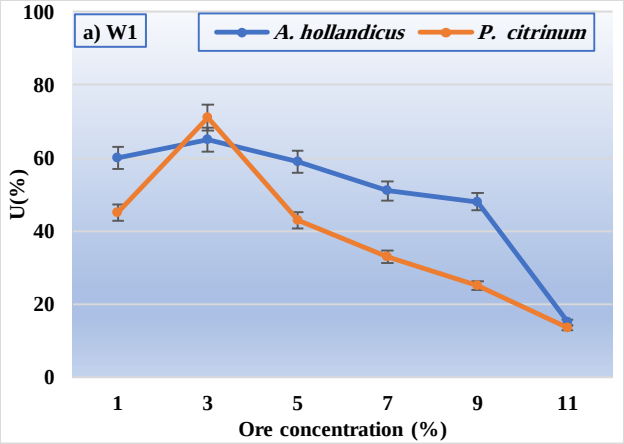
Fig. 1. Relation between different initial pHs and uranium (%) by *A.hollandicus* and *P. citrinum* at room temperature for 7 Days: (a) W1, (b) W2, and (C) W3

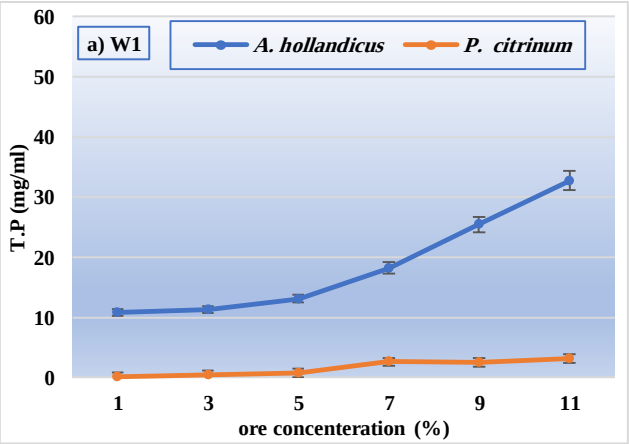
Fig. 2. Relation Between Different Initial pH and T.P (mg/ml) by *A. hollandicus* and *P. citrinum* at room temperature for 7 Days:(a) W1, (b) W2, and (C) W3

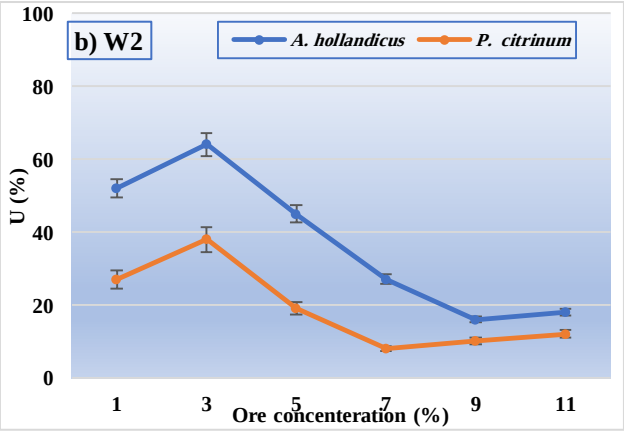
The ore concentrations of the studied samples in the growth medium affected the production of T.P., which was increased by increasing the ore sample concentration. Whereas the maximum significant level of T.P secreted by *A. hollandicus* was 32.75, 23.8, and 45.1 mg/ml as exposed to W1, W2, and W3 with an ore concentration of 11%, respectively, may be due to the fungal defense mechanism against toxicity, while the minimum level was 10.91, 11.5, and 12.3 mg/ml, respectively, with an ore concentration of 3 %. Meanwhile, a noticeable change in the pH values occurred with a great increase in the final pH value of 3.63, 3.81, and 4.9 at 11% ore concentration, respectively, and the final pH values of the leach liquor were 2.75, 2.73, and 4.25 at 3% ore concentration, respectively. Also, the same behavior was noticed by the second fungal strain *P. citrinum* with an increase in the T.P. by increasing the ore concentration. The measured T.P after exposure to W1, W2, and W3 with 1% ore concertation was 0.15, 1.05, and 0.07 mg/ml as the lowest values, causing the final pH values to partially increase from 3 to 3.26, 3.62, and 4.55, respectively. Since the largest values of T.P 3.15, 2.85, and 4.01mg/ml were carried out at 11% ore concentration, there was a significant increase in the value of the final pHs of 4.3, 4.76, and 6.62, respectively. The obtained results demonstrate that the T.P. of *A .hollandicus* was excreted in higher amounts than that of *P. citrinum*, which is in agreement with previous research's conclusion that the most fungal species able to tolerate heavy metal toxicity were *Aspergillus, Penicillium, and Fusarium* **[**32,33**].** However, the decreased attitude in solubilization efficiency may be due to an increase in the medium's final pH **[**34,35**].** Likewise, the maximum solubilization of uranium and alumina occurred at 4% (w/v) from gibbsite, dolostone, and mudstone ores by *A. niger* [36], as well as Attia et al.,[37] recorded that the most significant bioleachability value of uranium was observed at 3% ore concentration, which exceeded 82% by *A. nidulans*.

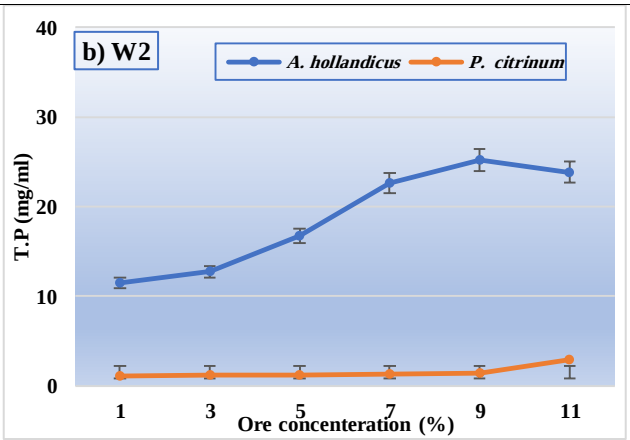
Table 4. Effect of different ore concentrations (W1, W2, and W3) on uranium solubilization by *A. hollandicus* and *P. citrinum* at room temperature for 7 days.

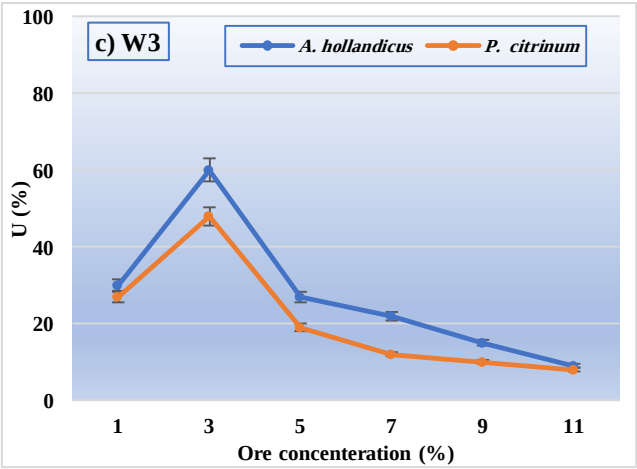
Fungal Sp.		*Ore Samples*	Ore concentration %					
			1	**3**	**5**	**7**	**9**	**11**
A. hollandicus	**U%**	**W1**	60±2.4	65 ±2.7	59±2.4	51±2.1	48±2.0	15±0.6
		W2	27 ±1.1	45±1.8	64 ±2.6	45 ±1.8	16 ±0.7	18 ±0.7
		W3	22 ±0.9	60 ±2.4	30 ±1.2	22 ±0.9	27±1.1	11 ±0.4
	T.P (mg/ml)	**W1**	10.91±0.16	11.29±0.17	13.14±0.202	18.25±0.28	25.5±0.39	32.75±0.5
		W2	11.5±0.177	12.7±0.19	16.7±0.257	22.6±0.348	25.17±0.38	23.8±0.367
		W3	12.3±0.18	22.45±0.346	33.6±0.518	34.2±0.52	38.8±0.351	45.1±0.695
	Final pH	**W1**	2.75	2.97	3.15	4	4.48	4.63
		W2	2.73	3.09	3.19	3.53	3.53	3.81
		W3	4.25	4.6	4.63	4.61	4.72	4.9
P. citrinum	**U%**	**W1**	45 ±1.8	71 ±2.9	43 ±1.8	33±1.3	25.1 ±1.0	13.5 ±0.6
		W2	27 ±1.1	38 ±1.6	19 ±0.8	8 ±0.3	10 ±0.4	12 ±0.5
		W3	47 ±1.9	62 ±2.5	47 ±1.9	42 ±1.7	35 ±1.4	33 ±1.3
	T.P (mg/ml)	**W1**	0.15±0.002	0.48 ±0.007	0.78±0.01	2.64 ±0.04	2.53±0.03	3.15±0.04
		W2	1.05 ±0.16	1.15 ± 0.017	1.16±0.01	1.32±0.02	1.4±0.02	2.85 ±0.043
		W3	0.07±0.001	0.23±0.003	0.35±0.005	0.34±0.005	1.54 ± 0.023	4.01 ±0.061
	Final pH	**W1**	3.26	3.8	4.17	4.4	4.5	4.3
		W2	3.62	3.73	4.6	4.09	4.11	4.76
		W3	4.14	4.55	5.52	6.43	6.5	6.62











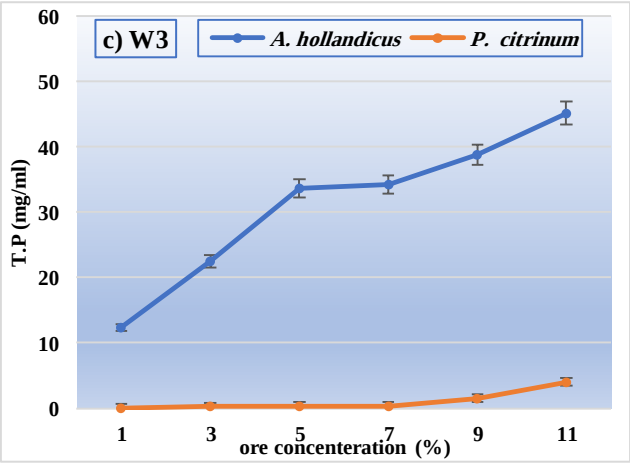


Fig. 3. Relation between different ore concentrations % and uranium solubilization by *A. hollandicus* and *P. citrinum* at room temperature for 7 Days: (a)W1, (b) W2 and (C) W3

Fig. 4. Relation Between Different Ore concentrations % and Total protein concentration by *A. hollandicus* and *P. citrinum* at room temperature for 7 Days: (a) W1, (b) W2 And (C)W3

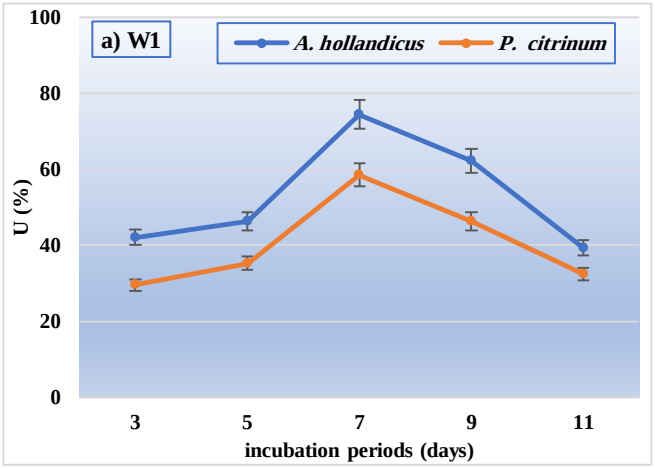
3.2.3Bioleaching Efficiency and Incubation Periods

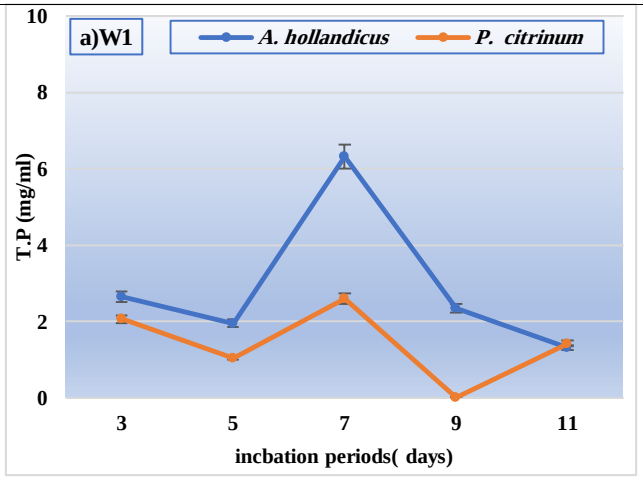
The incubation period of fungal strains is considered the most important factor that affects the solubilization process, which supports the full growth of the fungal spores/hyphae that yield maximum efficiency through the bioleaching process. The best bioleaching activity of the uranium concentration occurred after 7days of incubation. However, after 7 days of incubation, the uranium solubilized efficiency was slightly decreased with increasing incubation periods up to 11 days. The efficiency of uranium dissolved by *A. hollandicus* in the samples W1, W2, and W3 was 74.4%, 64%, and 78%, respectively (Table 5 and Fig. 5). Since the uranium leachability was 58, 69.6, and 64.5 % by applying *P.citrinum* on W1, W2, and W3, respectively. The amount of uranium solubilized in the growth medium slightly decreased with increasing the incubation period above 7 days. These results agreed with those obtained by **El Sayed [**38**],** where eight and six days were the optimum incubation periods for *A. niger* and *A. fumigatus* to solubilize uranium and REEs from gibbsite-bearing shale samples. Otherwise, the most preferable leachability happened during the 7 days, whereas the solubilized amount of uranium from the sample was 80% [37].

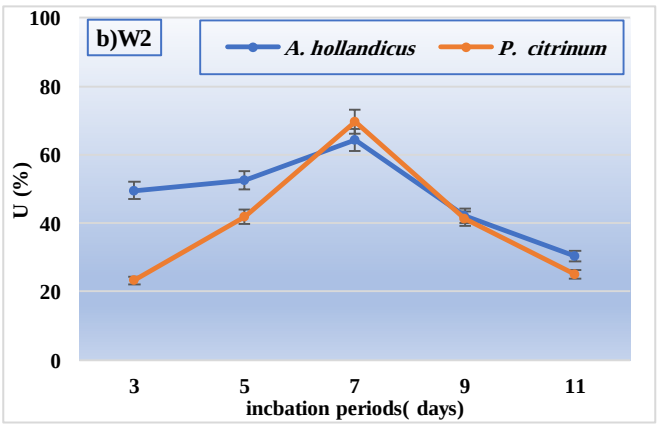
From the other point of view, the incubation periods of the studied samples have affected the level of extracellular T.P secreted by *A. hollandicus* and *P. citrinum* grown upon sabouraud medium at room temperature with an ore concentration of 3%, and the initial pH equals 3. The amount of total protein secreted was slightly decreased with increasing incubation periods up to 7 days (Fig. 6); the maximum significant level of T. P secreted by *A. hollandicus* was 6.33, 5.8, and 10.75 mg/ml as exposed to W1, W2, and W3 for a7 days incubation period, respectively, causing slight changes in the final pH values to 2.73, 3.02, and 4.68, respectively. The measured T.P for *P. citrinum* after exposure to W1 and W3 was 2.6 and 4.86 mg/ml at 7 days and the final pH changed to 4.4 and 6.5, respectively. Furthermore, the largest value was 13.3 mg/ml achieved with W3 at 5 days. These results are similar to those obtained by **Ali et al., [**26**]** who reported that all fungal isolates showed initial growth after 4 days and the consequence of incubation time on the growth of the fungi growing on sabouraud dextrose agar was between 6-10 days since all fungal isolates showed approached growth compared with control, as well as specific periods required during 3-7 days varying according to species and variety[39]**.**

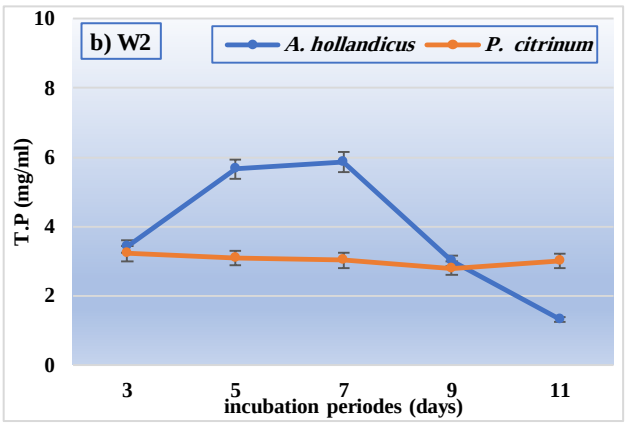
Table 5. Effect of different incubation periods on uranium solubilization of W1, W2, and W3 by *A. hollandicus* and *P. citrinum* at room temperature and pH 3 with an ore concentration of 3%.

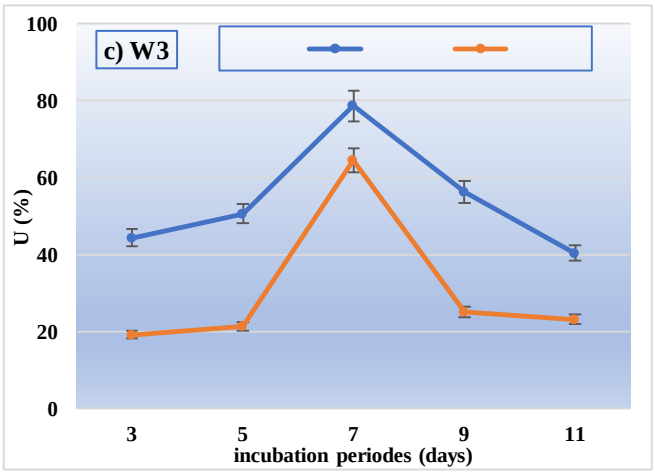
Fungal Sp.		Samples	Incubation Periods (days)				
			3	**5**	**7**	**9**	**11**
A. hollandicus	**U%**	**W1**	42.1± 0.63	46.3±0.70	74.4±1.14	62.2±0.95	39.3±0.61
		W2	49.5±0.77	52.5±0.80	64.3±0.98	42.1±0.65	30.4±0.46
		W3	44.4±0.67	50.6±0.75	78.6±1.19	56.3±0.82	40.4±0.61
	T.P (mg/ml)	**W1**	2.65±0.04	1.96±0.03	6.33±0.097	2.35±0.032	1.32±0.02
		W2	3.42±0.052	5.66±0.087	5.87±0.09	3.01±0.046	1.32±0.02
		W3	6.35±0.097	6.44±0.099	10.75±0.16	2.15±0.033	4.01
	Final pH	**W1**	3.27	3.15	2.73	2.95	2.84
		W2	3.22	3.09	3.02	2.79	3
		W3	5.27	4.27	4.68	4.94	4.52
P. citrinum	**U %**	**W1**	29.5±0.44	35.2±0.54	58.5±0.89	46.3±0.71	32.3±0.49
		W2	23.3±0.37	42.0±0.63	69.6±1.06	41.3±0.63	25.1±0.38
		W3	19.2±0.29	21.3±0.32	64.5±0.98	25.1±0.37	23.2±0.35
	T.P (mg/ml)	**W1**	2.07±0.031	1.05±0.016	2.6±0.04	0.02±0.0003	1.43±0.02
		W2	2.29±0.035	13.3±0.205	3.04±0.04	0.11±0.001	2.85±0.04
		W3	2.68±0.041	2.46±0.037	4.86±0.07	3.58±0.055	3.99±0.06
	Final pH	**W1**	4.17	3.8	4.4	4.3	4.3
		W2	4.09	4.6	3.73	4.11	4.76
		W3	4.14	6.43	6.5	5.52	6.62











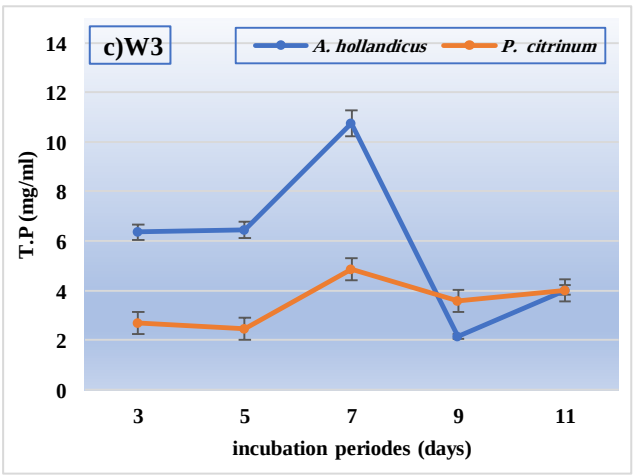


Fig. 6. Relation between different incubation periods and uranium (%) by *A. hollandicus* and *P. citrinum* at room temperature for 7 Days: (a)W1, (b) W2 and (C) W3

Fig. 7. Relation between different incubation period and total protein concentration by *A.hollandicus* and *P. citrinum* at room temperature for 7 Days: (a)W1, (b) W2 and (C) W3

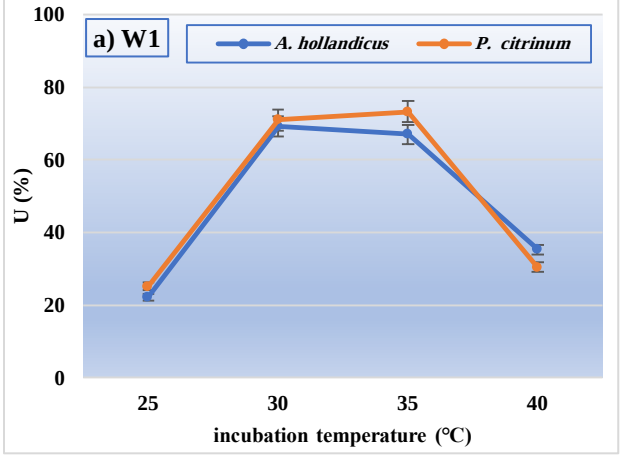
3.2.4 Bioleaching Efficiency and Incubation Temperatures

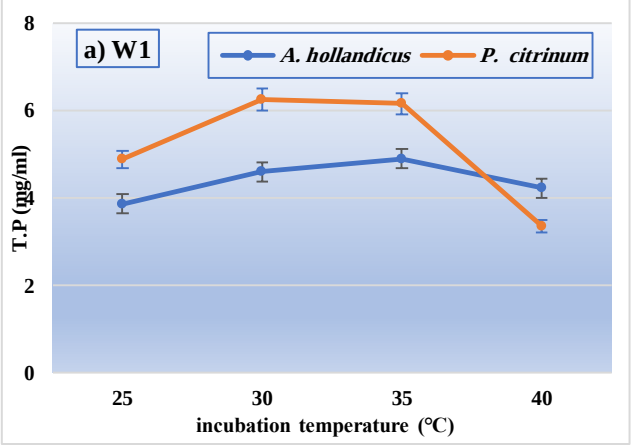
The temperature factor has an economic effect on any experiment, as the less energy used, the more economical it is. The solubilization of uranium in the tested samples was found to be highly affected by the incubation temperature (Table 6 and Fig. 7). The maximum leachability was exhibited at 30 ºC and 35ºC. *A. hollandicus* solubilized 69.1%, 56.6%, and 74.7% of uranium at 30 ºC and 67, 57.8, and 73 % at 35 ºC found in the samples W1, W2 and W3, respectively. Whereas the maximum leachability of uranium using *P. citrinum* at 30ºC revealed 71.3%, 63.5%, and 53% as well as 73 %,65.5 %, and 51.8% at 35 ºC found in the samples W1, W2, and W3, respectively. Most fungi are mesophilic, i.e., they can grow at temperatures within the range of 10 -35℃ while the optimum temperatures for growth may range between 15 and 30℃ [40]. The effects of heat on fungi are related to the chemical reactions within the fungal cells [41]. The temperatures must be in a range that allows the most efficient progression of the chemical reactions necessary for growth. As temperatures progress above the optimum temperature, chemical reactions occur less efficiently, and growth slows [42]. By studying the cell viability parameters and temperature, Schaefer et al., [43] concluded that *P. simplicissimum* KS1 removes U(VI) actively from solution at 30 ℃, via extracellular biomineralization, aside from minor biosorption and bioaccumulation.

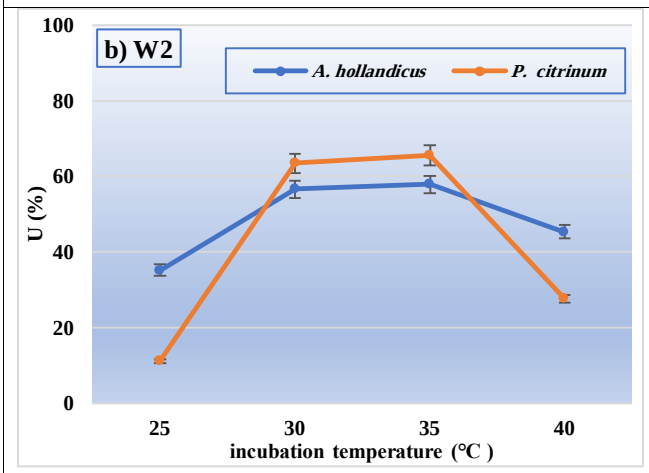
Table 6. Effect of different incubation temperatures on uranium solubilization from 3% of samples (W1, W2, and W3) by *A. hollandicus* and *P. citrinum* for 7 days**.**

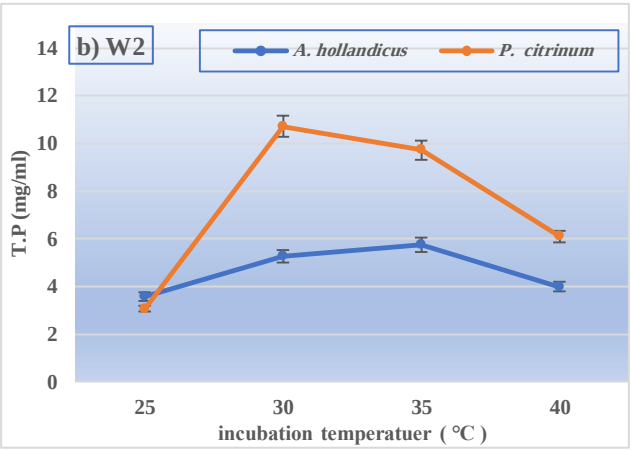
Fungal Sp.		Samples	Incubation Temperatures			
			25 ℃	**30** ℃	**35** ℃	**40** ℃
A. hollandicus	**U %**	**W1**	22.2±0.33	69.1±1.06	67±1.01	35.3±0.83
		W2	35.2±0.54	56.6±0.86	57.8±0.88	45.3± 0.84
		W3	46.1±0.71	74.7±1.14	73.1±1.11	50.2±0.65
	T.P (mg/ml)	**W1**	3.87±0.05	4.6±0.07	4.9 ±0.06	4.23±0.08
		W2	3.56±0.05	5.27±0.08	6.75±0.09	4.99±0.05
		W3	3.11±0.06	3.69±0.05	3.9±0.06	3.45±0.10
	Final pH	**W1**	3.24	3.47	3.46	3.06
		W2	3.43	3.03	3.04	3.34
		W3	3.56	3.18	3.13	3.33
P. citrinum	**U %**	**W1**	25.2±0.34	71.3±1.09	73.3±1.1.10	30.5±0.47
		W2	11.1±0.16	63.5±0.97	65.5±0.97	27.6±0.41
		W3	42.1±0.71	53.8±0.61	51.8±0.61	36.2±0.55
	T.P (mg/ml)	**W1**	4.89±0.07	6.26±0.09	6.16±0.13	3.36±0.05
		W2	3.06±0.04	10.72±0.16	9.72±0.18	2.67±0.04
		W3	4.81±0.07	6.15±0.09	6.10 ±0.11	5.24±0.14
	Final pH	**W1**	2.52	3.55	3.51	3.45
		W2	3.25	3.11	3.00	3.63
		W3	4.78	4.64	4.60	3.53

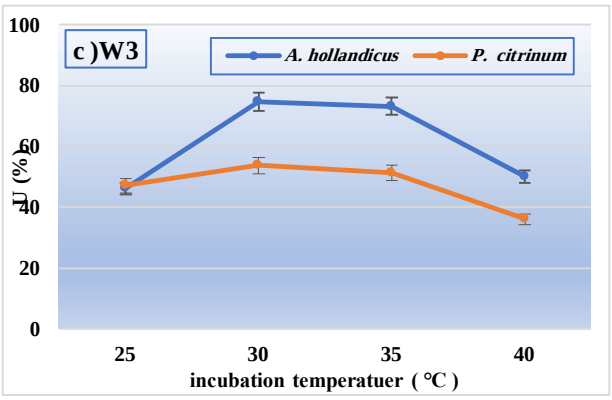
The levels of extracellular T.P secreted by *A. hollandicus* and *P. citrinum* grown upon sabouraud media for 7 days with the studied samples have been affected by the incubation temperatures shown in Fig. (8), whereas the level of T.P secreted by *A. hollandicus* reached its maximum values at 30℃ as 4.6, 5.27, and 3.45 mg/ml and at 35℃ reached (4.9, 6.75, and 3.9 mg/ml) after being exposed to W1, W2, and W3 for 7 days incubation period, respectively, changing the final pH values to 3.47, 3.03, and 3.18 at 30℃ respectively. The maximum T.P for *P. citrinum* after exposure to W1 and W3 was 6.26, and 10.72 at incubation temperature 30℃, causing a slight increase in the final pH to 3.55 and 3.11 respectively. Otherwise, the largest value was 9.24 mg/ml achieved with the sample W3 at 25℃ and the final pH was 4.78. **El Sayed [**38**]** mentioned that the maximum bioleaching of heavy metals occurred at 30 ℃ for *A. niger* and 35 ℃ for *A. fumigatus*. At higher temperatures (40 ℃ and 45 ℃), the metabolic rate of the organism decreases, and the organism might even not survive **[28].** Eventually, the temperature can reach a point where growth stops, and cell components begin to be damaged by the heat as proteins that structurally change when heated to their limit of tolerance **[**44**].**











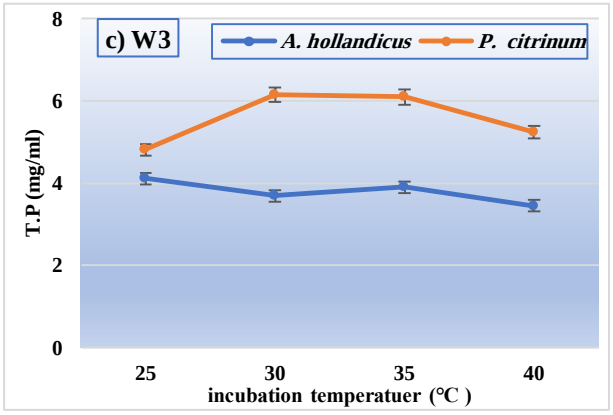


Fig. 7. Relation between different incubation temperatures and uranium solubilization by *A. hollandicus* and *P. citrinum* for 7 Days: (a) W1, (b) W2 and (C) W3.

Fig. 8. Relation between different incubation temperatures and total protein solubilization by *A. hollandicus* and *P. citrinum* for 7 Days: (a) W1, (b) W2 And (C) W3

From the above results, it can be summarized that by applying *A. hollandicus* and *P. citrinum* grown upon sabouraud media on the studied samples W1, W2, and W3, the optimization occurred at a pH value equals 3, 3% (w/v) ore concentration, and 7 days as an incubation period at 30℃-35℃.

3.3 Role of organic acids and mineral constituents

By applying the previous optimum conditions to the studied samples, the uranium leachability percent in the three samples (W1, W2, and W3) recorded was 66, 42, and 49% using *A. hollandicus*, and 60.8, 35, and 68% by *P. citrinum* respectively [45]. Besides that, the organic acid experiments were carried out by the two isolated fungal strains. Production of several organic acids like oxalic, lactic, citric, quinic, and maleic were observed by the two types of fungi except butyric acid, which did not appear in the case of *A. hollandicus* (Table 7). Firstly, *A. hollandicus* produced an amount of oxalic acid that was higher than the control, whereas quinic and lactic acid slightly increased compared to the control. This observation agreed with **Sukla and Panchanadikar [46],** who suggested that oxalic acid was the most effective acid in the leaching of laterite minerals. Otherwise, the citric acid was produced in tiny amounts with sample W1, and the appearance of maleic acid in samples W2 and W3 was observed. Secondly, the organic acids secreted by *P. citrinum* showed an increase in oxalic and citric acids, whereas an appearance of quinic and butyric with the disappearance of lactic acid was observed with sample W1, while with ore samples W2 and W3, lactic and maleic acids appeared since oxalic, citric, and butyric amounts decreased. Tzeferis et al., [47] found that the tested fungal strains *Penicillium* sp. and *Aspergillus* sp. have an effective role in nickel recoveries depending on their ability to produce hydroxycarboxylic acids, especially citric acid. Metabolites of both isolated fungal strains were subjected to proper chromatographic analysis (Fig.9). The analysis proved that *A. hollandicus* and *P. citrinum* secreted organic acids as secondary metabolites, which were effective in uranium solubility. The role of carboxylic acids that were secreted by the free-living and symbiotic fungi, such as oxalic, citric, and gluconic acids, was to decrease the pH of the media and act as metal chelators in transforming insoluble uranium into soluble uranium species [48]. The common observations by Hefnawy et al.,[23]; and Abhilash and Pandey[49] clarified that a greater quantity of uranium is leached by *Aspergillus terreus* and *Penicillium spinulosumthese* fungal species may be attributed to the production of carboxylic acids in the media that shift the pH to a lower acidity while forming soluble complexes. On the other hand, quartz sand, kaolin, and clays, which have lower-quality minerals can be removed by these microorganisms. The best results were achieved when oxalic and citric acids were the main components in the leaching solution. Consequently, *Aspergillus* species can synthesize oxalic acid as a metabolite, which could leach an appreciable amount of uranium from geological rocks [50].

Table 7. Concentrations of organic acids (µg/ml) secreted by the isolated fungal strains during the uranium leaching process

Organic Acids Conc. (µg/ml)	*Retention time (min)*	*A. hollandicus*				*P. citrnium*			
		Control	**W1**	**W2**	**W3**	**Control**	**W1**	**W2**	W3
Oxalic acid	**3**	0.046	10.22	0.49	1.33	6.44	8.22	4.33	1.99
Maleic acid	**4**	6.23	---	---	0.86	---	---	7.45	3.55
Quinic acid	**5**	5.14	---	8.69	8.24	---	3.56	---	---
Lactic acid	**6.8**	8.46	9.45	0.74	0.78	11.29	---	2.14	6.54
Citric acid	**8**	---	0.63	0.83	0.69	2.36	2.45	9.63	---
Butyric acid	**9.7**	---	8.47	---	---	---	10.86	10.22	8.69
U Leaching Efficiency (%)	---	---	66	42	49	---	60.8	35	68

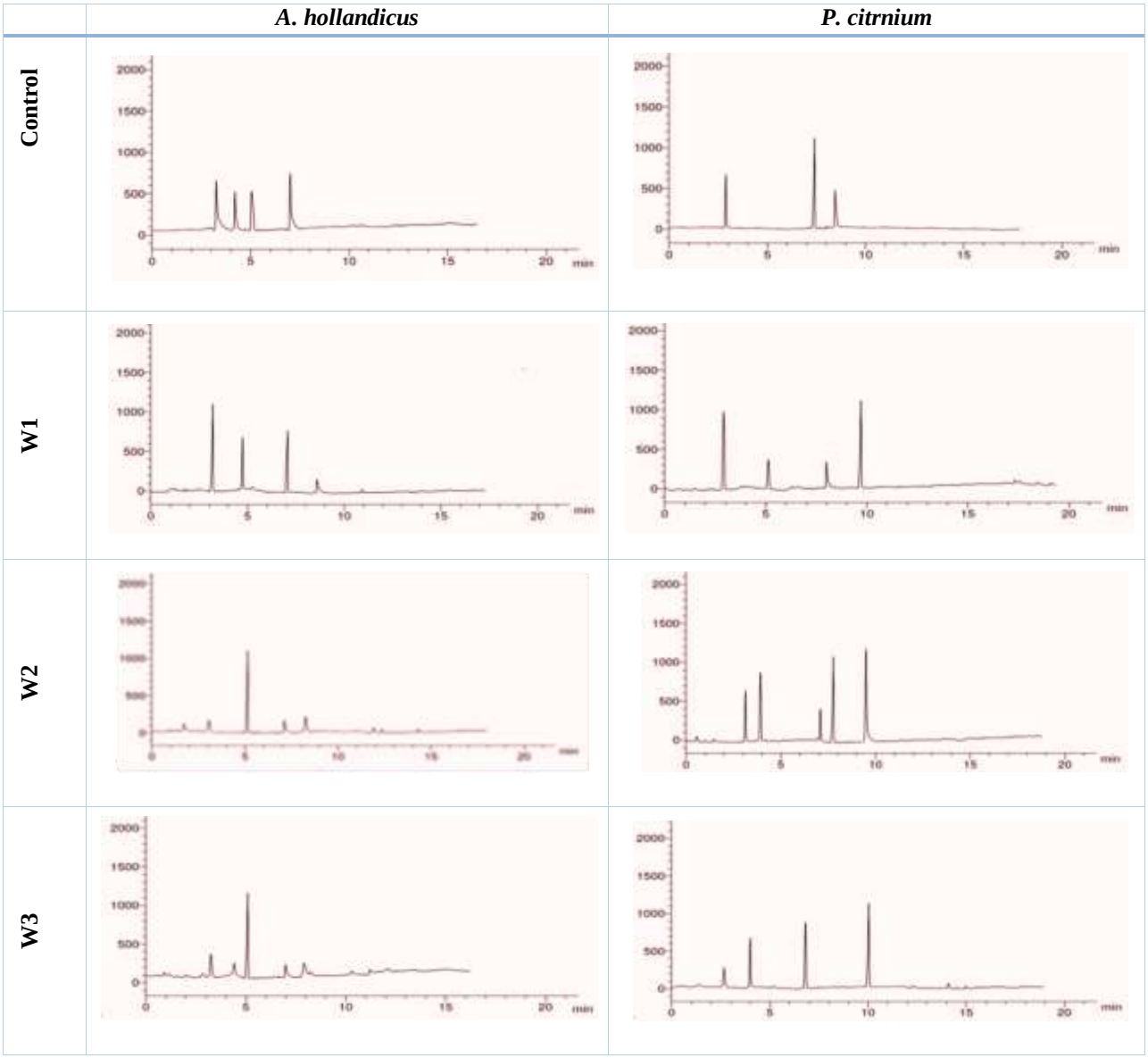


Fig.9. Typical organic acids chromatograph in the culture filtrate secreted by *A. hollandicus* and *P. citrinum* (oxalic, maleic, quinic, lactic, citric, and butyric acids) with studied samples W1, W2, and W3, respectively.

Conclusions

The studied optimum conditions of the uranium bioleaching controlling factors from three samples (W1, W2, W3) using two fungal strains **(***A. hollandicus* and *P. citrinum)* were achieved at 7 days as the incubation period, 30-35 ºC as the incubation temperature, pH 3, and ore concentration 3 % pulp intensity of the core samples. *A. hollandicus* and *P. citrinum* exhibit appreciable potential for generating a variety of proteins and organic acids, which play an important and effective role in the uranium bioleaching process. The protein has a significant role in the fungal bioleaching process by modulation of the medium pH value which facilitates the metal removal process (metal sequestration) by increasing the binding of proteins /legends to grow under stress. Where organic acids have a major role in increasing uranium solubilization. Also, protein is considered a protective factor for fungi to survive against exposure to different environmental stress.

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