*Desalination and Water Treatment*
www.deswater.com

doi: 10.1080/19443994.2015.1070293

57 (2016) 15200–15207
July

Taylor & Francis
Taylor & Francis Group

# *Aspergillus niger*-mediated bioremediation of Triton X-100-contaminated resources

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Received 23 February 2015; Accepted 30 June 2015

ABSTRACT

Water shortage, water pollution and climate changes are highly interrelated global issues. These have raised immense concerns about possible serious adverse effects on the quality, treatment and reuse of wastewater. This research aims to investigate the potential impact of Triton X-100 on the soilborne fungus *Aspergillus niger* (*A. niger*) as well as the ability of this organism to remove Triton X-100 from contaminated wastewater. We found that 2,250–6,000 mg/L Triton is toxic toward *A. niger* with irreversible effects, whereas 23–2,000 mg/L is inhibitory toward *A. niger* with reversible effects. These concentration ranges vary with initial concentrations of Triton and *A. niger* as well as with the contact time. At an initial concentration of 228 and 2,152 mg/L, *A. niger* can remove 20 and 28%, respectively, of Triton by day 1, which increase to 28 and 38%, respectively, by day 3. Another experiment showed that at an initial concentration of 50 and 100 mg/L, *A. niger* can biodegrade 29 and 15%, respectively, of Triton by day 1, which further increases to 87 and 34% by day 15. Adsorption and biodegradation are the mechanisms employed by the organism for Triton removal. Thus, *A. niger* can enhance biological treatment of wastewater.

*Keywords:* Triton X-100; Pollution; Water; *Aspergillus niger*; Toxicity; Biodegradability

## 1. Introduction

Increasing consumer awareness about water shortage and water pollution is necessary to reduce water consumption and wastage; however, this might have an adverse impact on wastewater quality by increasing the concentration of pollutants in wastewater. A major source of synthetic organic pollutants is surfactants [1], one of the main constituents of domestic wastewater. An example is the non-ionic surfactant group octylphenol ethoxylate, which is present in some household and industrial detergents. Metcalf and Eddy [2] classified surfactants as refractory organics that are resistant to conventional wastewater treatment. Surfactant-polluted water poses a major problem in countries with severe water scarcity, such as Jordan, where the use of reclaimed wastewater, for example, in irrigation, becomes a necessity [3]. Therefore, treating wastewater to remove polluting surfactants is a pre-requisite to reuse this water.

It is well accepted that decentralized sanitation and onsite treatment of wastewater [4] and [5] holds a promising future, in particular, when it is accompanied by reuse options available onsite [6] within the frame

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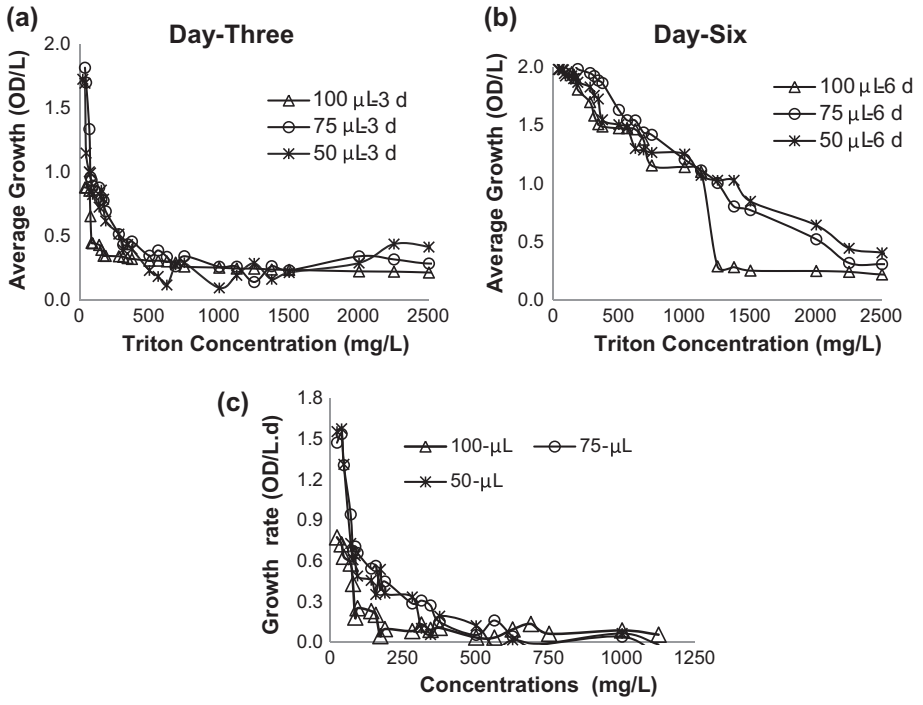


Fig. 1. *A. niger* average growth (a) on day 3 and (b) day 6, in microtiter plates filled with 100, 75, or 50 µL of initial seed volume, nutrient broth, and different concentrations of Triton X-100 and (c) *A. niger* growth rate on day 3 in the test wells in microtiter plates filled with 100, 75, or 50 µL of seed in the presence of nutrient broth and different concentrations of Triton X-100. Growth was measured as OD using the ELISA reader.

of sustainability concept [3] and [7]. Climate change has caused a decrease in the level and an increase in the variability of precipitation, which increases the potential negative effects of using reclaimed wastewater for irrigation, such as surfactant accumulation in soil that can ultimately pollute groundwater. Furthermore, Triton X-100, which is one of the octylphenol ethoxylate non-ionic surfactant groups, is an effective ingredient in many household and heavy industry detergents [8]. In fact, Triton X-100 is extensively used in biomedical applications such separation of proteins [9]. A primary source of concern surrounding Triton X-100 is that its toxicity and biodegradation depend on external conditions such concentration, contact time and the organism being treated [10].

Although many technologies can be used to treat wastewater [11–15], biological treatment is recognized as one of the cost-effective methods [14] and [16]. This treatment method relies on microbial consortia, with bacterial cells being the chief component [11] and [17]. Nevertheless, fungi possess quite a few characteristics that render them good candidates for use in biological treatment, especially in the treatment of refractory pollutants. Therefore, *Aspergillus niger* (*A. niger*), an aerobic filamentous fungus abundantly found in the environment in soil, litter, compost and decaying plant material [18] can be used to remediate the aquatic pollution mediated by Triton X-100. *A. niger* is one of the organisms found in the activated sludge system of the wastewater treatment plants investigated by Cooke [19]. The optimal growth conditions for *A. niger* include warm humid places [20], where it can grow within a temperature range of 6–47˚C and at pH range of 1.4–9.8 [21]. In addition, *A. niger* is a rich source of enzymes such as pectinase, protease, amyloglucosidase, cellulase, and hemicellulase [18] and [22]. One of these enzymes, cellulase, is used in the detergent industry because of its ability to degrade cellulose [23], in the pulp industry [24] and [25], and in the food and feed industry [26].

In this study, the aim was to evaluate the potential of using *A. niger* to improve the biological methods of wastewater treatment to enhance the removal of surfactants and similar materials in conventional treatment plants. To meet this aim, we studied the ability of *A. niger* to tolerate, remove and degrade Triton X-100.

## 2. Materials and methods

### 2.1. Preparation of solutions

Autoclaved distilled water was used to prepare solutions; autoclaving was performed using Tuttnauer

Table 1
Final concentrations of Triton X-100, with the substrate

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 6,000 | 5,500 | 5,000 | 4,500 | 4,000 | 3,500 | 3,000 | 2,500 | 2000 | 1,500 | 1,000 |
| B | 3,000 | 2,750 | 2,500 | 2,250 | 2000 | 1,750 | 1,500 | 1,250 | 1,000 | 750 | 500 |
| C | 1,500 | 1,375 | 1,250 | 1,125 | 1,000 | 875 | 750 | 625 | 500 | 375 | 250 |
| D | 750 | 688 | 625 | 563 | 500 | 438 | 375 | 313 | 250 | 188 | 125 |
| E | 375 | 344 | 313 | 281 | 250 | 219 | 188 | 156 | 125 | 94 | 63 |
| F | 188 | 172 | 156 | 141 | 125 | 109 | 94 | 78 | 63 | 47 | 31 |
| G | 94 | 86 | 78 | 70 | 63 | 55 | 47 | 39 | 31 | 23 | 16 |
| H | Control | Control | Control | Control | Control | Control | Control | Control | Control | Control | Control |

3850_CE for 15 min at 121˚C and 60 Psi. Potato dextrose broth and agar were prepared according to the manufacturer's instructions (Biolab ZRT. 1141 Bp. Ov U. 43).

Triton X-100 solution was prepared in 2 batches; in batch 1, the following solutions were prepared by dissolving weighed Triton X-100 in 1,000 mL of distilled water: 24,000; 22,000; 20,000; 1,800; 16,000; 14,000; 12,000; 10,000; 8,000; 6,000; and 4,000 mg/L, and in batch 2, the following solutions were prepared: 4,000; 3,500; 3,000; 2,500; 2,000; 1,750; 1,500; 1,250; 1,000; 500 and 250 mg/L.

*A. niger* (ATCC 6275; Tech. Supplies Co./Lab Division) seed solution was prepared by cultivating the cells in potato dextrose broth for 30 d.

### 2.2. Experimental setup

Three experimental setups were used in this study: (1) growth experiments using microtiter plates and ELISA reader, (2) oxygen consumption experiments using OxiTop devices, and (3) Triton X-100 utilization experiment using spectrophotometry. These setups served the dual function of estimating the toxicity and elimination of Triton X-100.

#### 2.2.1. Growth experiments using microtitre plates and ELISA reader

Each 96-U-shaped-well microtitre plate used in this experiment consisted of 8 rows and 12 columns. Of these, 88 wells were used to study 4 variables: initial Triton concentration, initial *A. niger* seed volume with and without potato dextrose broth and time. Tables 1 and 2 show the 2 sets of final Triton concentrations. Table 1 represents the concentrations used in batch 1, which were used for 3 microtitre plates containing 100, 75, and 50 μL of seed volumes with potato dextrose broth. On the basis of the results of this batch, batch 2 was prepared with the Triton concentrations shown in Table 2, where 2 plates were inoculated with 100 μL of seed volume with and without potato dextrose broth. The growth of *A. niger* was monitored by measuring the optical density (OD) at 650 nm using the ELISA reader (ASYS/Hitech-Expert Plus Biochrom). Each batch was repeated thrice.

Batch 1: Each of the 88 wells, in all 3 plates, was filled with 100 μL of potato dextrose broth. Then, each of the 11 wells in row A was filled with 100 μL of each of the 11 Triton stock solutions. The content of each well was mixed properly, and then, 100 μL of this mixture was withdrawn and added to the adjacent row. This step was repeated up to row G, and the last aliquot was discarded. Finally, 100, 75, and 50 μL of *A. niger* seed solution was added to each of the 88 wells in plates 100, 75, and 50 μL, respectively. Row H represents the blank/control wells that contained 100 μL of the broth, 100, 75 or 50 μL of seed solution and 0, 25, and 50 μL of distilled water, respectively. The final Triton concentration in each well of the prepared plates, as mentioned above, is listed in Table 1. All 3 plates were incubated for 6 d at 37˚C. Sterilized distilled water was added, as required to maintain the volume in each well at 200 μL.

On the basis of the results of *A. niger* growth, 3 wells at 3,000, 4,000, and 6,000 mg/L from the microtitre plate containing 100 μL of seed volume and one of the control wells were double-checked for the presence of *A. niger*, by inoculating 100 μL from each well into a test tube filled with 9.9 mL of broth. The growth was monitored for 3 d, followed by enumeration of cells in each tube.

Batch 2: Two plates with and without broth, where sterilized distilled water was used in place of the broth. The first row of each plate was filled with each of the 11 stock solutions. About 100 μL of seed volume was used. Experiments similar to those performed in batch 1 were performed on both microtitre plates. The final Triton concentration in each well of the plates is listed in Table 2.

Table 2
Final Concentrations of Triton X-100, without the substrate

| | 1.00 | 2.00 | 3.00 | 4.00 | 5.00 | 6.00 | 7.00 | 8.00 | 9.00 | 10.00 | 11.00 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1,000.0 | 875.00 | 750.00 | 625.00 | 500.00 | 437.50 | 375.00 | 312.50 | 250.00 | 125.00 | 62.50 |
| B | 500.00 | 437.50 | 375.00 | 312.50 | 250.00 | 218.75 | 187.50 | 156.25 | 125.00 | 62.50 | 31.25 |
| C | 250.00 | 218.75 | 187.50 | 156.25 | 125.00 | 109.38 | 93.75 | 78.13 | 62.50 | 31.25 | 15.63 |
| D | 125.00 | 109.38 | 93.75 | 78.13 | 62.50 | 54.69 | 46.88 | 39.06 | 31.25 | 15.63 | 7.81 |
| E | 62.50 | 54.69 | 46.88 | 39.06 | 31.25 | 27.34 | 23.44 | 19.53 | 15.63 | 7.81 | 3.91 |
| F | 31.25 | 27.34 | 23.44 | 19.53 | 15.63 | 13.67 | 11.72 | 9.77 | 7.81 | 3.91 | 1.95 |
| G | 15.63 | 13.67 | 11.72 | 9.77 | 7.81 | 6.84 | 5.86 | 4.88 | 3.91 | 1.95 | 0.98 |
| H | Control | Control | Control | Control | Control | Control | Control | Control | Control | Control | Control |

#### 2.2.2. Triton X-100 utilization experiment using spectrophotometry

The second experiment was conducted in 2 Erlenmeyer flasks (250 mL). Each flask was filled with 30 mL of *A. niger* seed solution, and then 1.0 and 9.5 mL of 24,000 mg/L Triton solution was added to flask 1 and 2, respectively. Autoclaved distilled water was then added to make the total volume to 105 mL. The flasks were incubated for 30 d at 37˚C. Samples were withdrawn starting from time 0, at irregular intervals until day 30. Subsequently, the samples were analyzed by spectrophotometry at 200 nm. This experiment was repeated thrice.

#### 2.2.3. Oxygen consumption experiments using OxiTop devices

The third experiment was conducted in 4 dark bottles (550 mL) was filled with 30 mL of *A. niger* seed solution. Then, the bottles were filled with 0, 1, 2, and 10 mL of 2,500 mg/L Triton stock solution. Autoclaved distilled water was then added to each bottle to make the total volume to 50 mL. Next, a sleeve filled with 3 soda pellets was inserted in each bottle to absorb $CO_2$. The bottles were capped with OxiTop heads to measure the change in internal pressure, which represents oxygen consumption. The bottles were incubated for 15 d at 37˚C. The experiment was repeated thrice.

## 3. Results

### 3.1. Toxicity of Triton X-100

The growth of *A. niger* in the control wells containing broth started on day 2 and continued up to day 6. On day 2, the average growth of *A. niger* in the control wells with 50, 75, and 100 µL of seed volume was 0.85, 1.28, and 1.38 OD/L, respectively, indicating the substrate was available in excess of that required by *A. niger* for growth, at least in the first 2 plates. The results obtained for the test wells, which were incubated for 6 d in the presence of the substrate, showed that Triton X-100 prevented *A. niger* growth at concentrations ranging from 2,250 to 6,000 mg/L. In the wells with 23–500, 23–313, and 23–188 mg/L Triton X-100, *A. niger* started growing on day 3, that is a day after the control wells, in the plates containing 50, 75, and 100 µL, respectively (Fig. 1(a)). During the next 3 d, growth was observed in the rest of the test wells up to 2,000, 2,000, and 1,250 mg/L in the plates containing 50, 75, and 100 µL of seed volume, respectively (Fig. 1(b)). Thus, the initial seed volume and contact

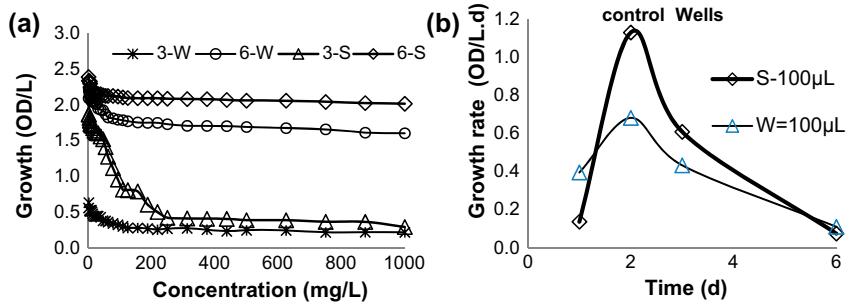


Fig. 2. (a) *A. niger* average growth in the test wells in microtiter plates filled with 100 µL of seed and different concentrations of Triton with substrate (S) and without substrate (W) and (b) *A. niger* growth rate in the control wells in microtiter plates filled with 100 µL of seed with and without substrate for the 6-d period; The growth was measured as OD using the ELISA reader.

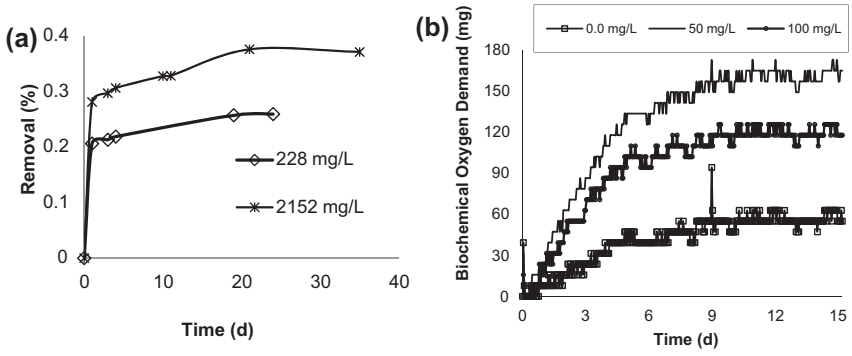


Fig. 3. (a) Percentage of Triton X-100 removal by *A. niger*at an initial Triton concentration of 228 and 2,152 mg/L over the 30-d period and (b) oxygen consumption by *A. niger*at an initial Triton concentrations of 0.0, 50, and 100 mg/L over the 15-d period.

period have significant impact on *A. niger* growth. Furthermore, we found that the average growth of *A. niger* in test wells decreased with increasing initial Triton concentration (Fig. 1(a) and (b)). Additionally, the growth rates in the test wells, on day 3, decreased not only with increasing Triton concentration but also with increasing initial seed concentration (Fig. 1(c)). The maximum growth rate in the plate containing 100 µL was 0.77 OD/d on day 3 at 23 and 39 mg/L (Fig. 1(c)), which was less than the growth rate in the plates containing 75 and 50 µL (1.55 OD/d) on day 3 at 23 and 39 mg/L (Fig. 2(c)).

On comparing the growth trend in the test wells with that in the control wells, we found that on day 3, *A. niger* growth in the test wells with 141–500, 70–313, and 23–188 mg/L Triton X-100 in plates containing 50, 75, and 100 µL of seed volume, respectively, was less than that observed in the corresponding control wells. However, on the following 3 d, *A. niger* recovered its growth ability in the rest of the test wells with concentrations up to 2,000, 2,000, and 750 mg/L in the plates containing 50, 75, and 100 µL of seed volume, respectively. It can be concluded that on one hand, the maximum non-inhibitory concentrations (NICs) of Triton to *A. niger*, as noted on day 3, were 94, 47, and not detectable mg/L, in the plates containing 50, 75, and 100 µL, respectively. These NICs increased to 2,000, 2,000, and 750 mg/L, respectively, on day 6. On the other hand, the minimum inhibitory concentrations (MICs) of Triton to *A. niger*, as noted on day 3, were 536, 344, and 281 mg/L in the plates containing 50, 75, and 100 µL of seed volume, respectively. These MICs increased to 2,250, 2,250, and 1,250 mg/L, respectively, on day 6. The inhibitory effect of Triton X-100 on *A. niger* was confirmed by the *A. niger* count conducted at the end, which showed a colony count of 0 in the test wells with 3,000, 4,000, and 6,000 mg/L Triton.

### 3.2. Biodegradability of Triton X-100

The second and equally important objective of this study was to investigate the biodegradability of Triton X-100 by *A. niger* The results of batch 2 of the first

experimental setup confirmed batch 1 results where the growth of *A. niger* was detected on day 2 in the control wells with and without substrate, but the presence of the substrate increased *A. niger* growth. However, the growth in all test wells with and without substrate was detected on day 3 (Fig. 2(a)). The maximum growth rate, in the control wells without the substrate was found to be 0.68 OD/L d, on day 2, which was less than the maximum growth rate observed in the presence of the substrate (1.13 OD/L d; Fig. 2(b)). The results also showed that the growth in the test wells without the substrate containing Triton X-100 at concentrations ranging from 0.98 to 188 mg/L was found to be 1.3-folds of the growth observed in the corresponding control wells. Similar results were observed on day 6 for wells containing Triton X-100 at concentrations up to 250 mg/L.

In the second experimental setup, spectrophotometric analysis of Triton-contaminated water showed that *A. niger* decreased the concentration of Triton present in water over time. On day 1, *A. niger* removed 47 and 605 mg/L Triton, which is about 20 and 28% of the initial Triton concentration, respectively (Fig. 3(a)). This percentage increased to 28 and 38%, respectively, over 3 d. However, extending the experiment duration to 30 d did not show any significant impact on Triton concentration (Fig. 3(a)). Furthermore, the results showed that the ability of *A. niger* to remove Triton X-100 increased with the increase in the initial concentration of Triton. The results of the third experimental setup contradicted these findings (Fig. 3(b)). Oxygen consumption is an indicator of the biodegradability of Triton. We found that oxygen consumption decreased with increasing Triton concentration. *A. niger*-mediated biodegradation of 50 mg/L Triton was faster than that of 100 mg/L Triton; oxygen consumption in both cases was higher than that in the control wells (Fig. 3(b)). In addition, we found that *A. niger* consumed less than 30 mg/L oxygen at Triton concentrations of 50 and 100 mg/L on day 1; extending the experiment duration to 15 d significantly increased oxygen consumption to 150 and 110 mg/L, respectively.

## 4. Discussion

The growth of *A. niger* in the presence of Triton occurred in 3 phases, namely, no growth, inhibited growth, and growth. The findings about growth rate supported the hypothesis that the growth on day 3 was limited by the initial seed volume, as the growth decreases with the increasing initial seed volume. The growth rate on day 6 was limited by substrate and space availability. The cut-off points between these 3 phases, i.e., the MIC and NIC values, were found to increase with decreasing initial seed volume and increasing incubation time. The inhibitory effect of 2,250–6,000 mg/L Triton was irreversible, while that of 23–2,000 mg/L Triton was reversible, assuming the presence of an acclimatization period. The inhibitory effect of Triton X-100 toward fungi has been documented even at lower concentrations, for example, 1–13 mg/L Triton is inhibitory toward the filamentous fungus *Thermomyces lanuginosus* [27]. Moreover, 1,793 mg/L Triton is found to have an inhibitory effect depending on the fungal species [28] and [29]; the most tolerant species are *Beauveria alba*, *Verticillium lecanii*, *Dichotomomyces cejpii*, *Penicillium italicum*, and *Rhodotorula rubra*, which experience only 10% reduction in size, and the least tolerant species include *Cladosporium herbarum*, *Doratomyces stemoniti*, and *Coniothyrium sporulosum*, experience more than 30% reduction in size. Other species that show 10–30% reduction in size include *Coniophora arida*, *Phanerochaete chrysosporium*, *Cylindrocarpon destructans*, *Fusarium moniliforme* var. *subglutinans*, *Penicillium chrysogenum*, *Cryptococcus albidus*, *and Agaricus bitorquis*. Thus, our results indicate that *A. niger* is more tolerant to Triton X-100 than all other species tested and confirm the findings of Benoit-Guyod et al. [30] that the type of fungus is the key to effective handling of pentachlorophenol as well as the findings of Abu-Ghunmi et al. [10] that correct selection of the biodegrading organism is important in effective handling of Triton X-100.

On one hand, the irreversible inhibitory effect of Triton X-100 is supported by the findings of the presence of no growth phase, zero oxygen consumption, and reduction in viable cell count. On the other hand, the reversible inhibitory effect is supported by the findings of the presence of inhibited growth as well as growth phases, potential recovery of the growth ability of *A. niger*, and improved oxygen consumption over time. Therefore, the mechanism employed by Triton to inhibit *A. niger* can be attributed to the interactions between Triton, *A. niger* and the substrate. These interactions function as barriers that prevent *A. niger* from accessing the substrate, and thus stop, delay, or slow down its growth because of food deficit. Furthermore, the interactions between Triton X-100 and *A. niger* can cause the erosion of the cell membrane of *A. niger* Another possible mechanism employed for growth suppression is the toxicity of the intermediate products produced during the primary biodegradation of Triton [31]. Koley and Bard [32] reported irreversible damage to HeLa cells, in less than 1 min, in the presence of 130 mg/L Triton X-100;

this damage can be attributed to the solubilization of the cellular lipid bilayers because of their interactions with Triton.

The growth of *A. niger* in control wells without the substrate can be attributed to the utilization of internal nutrients, while its growth in the presence of substrate can be attributed to the utilization of potato dextrose. The growth of *A. niger* in test wells wherein it grows 1.3 times and 1.27 times more than the growth in the corresponding control wells can be attributed to the utilization of Triton. The higher growth rate in the presence of the substrate can be attributed to the utilization of Triton as the carbon source and the increase in *A. niger* cell permeability to available substrates. Increase in cell permeability is consistent with the finding reported by Koley and Bard [32] about the ability of Triton in increasing the permeability of HeLa cells. However, it should be noted that this study showed that smaller concentrations of Triton, namely, 23 and 39 mg/L, can possibly enhance the permeability of *A. niger* Koley and Bard [32] found that 110 mg/L Triton X-100 increases the permeability of hydrophilic molecules and causes reversible damage to the cell membrane, which can be eliminated by removing Triton. Furthermore, the higher growth rate in the presence of the substrate indicates that the addition of the external substrate can enhance the removal of Triton from contaminated sources by *A. niger*

Consistent with the above findings, *A. niger* was found to consume 30 mg/L oxygen on day 1 in the purification of water contaminated with 50 and 100 mg/L Triton by the oxygen consumption experiment. The oxygen consumption increased on day 15 and was found to be 90 and 70 mg/L for water contaminated with 50 and 100 mg/L Triton, respectively. This means that *A. niger* biodegrades 29 and 14% of Triton by day 1 and 87 and 34% by day 15 in water polluted with 50 and 100 mg/L Triton, respectively. Thus, *A. niger* according to the classification described by Garon et al. [28] is efficient in removing Triton X-100 from polluted water. Furthermore, the results of spectrophotometry showed that *A. niger* was able to remove 47 and 605 mg/L Triton, equivalent to 98 and 1,257 mg/L oxygen, respectively. It is clear that the amount of Triton eliminated and measured by spectrophotometry is larger than that eliminated and measured by oxygen consumption. This indicates that *A. niger* uses 2 mechanisms to purify Triton-contaminated water, namely, adsorption and biodegradation [10] and that adsorption is faster than biodegradation. The mechanism underlying adsorption could be attributed to the hydrophobic nature of both Triton and the cell wall of *A. niger* [33].

Consequently, Triton has 2 opposing effects on *A. niger*, and the net effect depends on the initial concentration and contact time of Triton. This finding suggests many useful applications of Triton X-100, such as production of insecticides and herbicides to protect trees and fruits from the rotting caused by *A. niger* Furthermore, it suggests that *A. niger* is a potential degrader of Triton and can be used in the bioremediation of Triton-contaminated water and soil. One of the useful implications of this ability to degrade is the use of *A. niger* in conventional wastewater treatment plants to improve the extent of surfactant removal from wastewater.

## 5. Conclusions

Triton X-100, a water pollutant, can serve as both food and fungicide to *A. niger* The effect of Triton depends on initial Triton concentration, initial *A. niger* concentration, and contact time. Triton exerts a toxic effect on *A. niger* at concentrations ranging from 2,250 to 6,000 mg/L, while it exerts a reversible inhibitory effect on *A. niger* at concentrations ranging from 23 to 2,000 mg/L.

*A. niger*-mediated purification of Triton-contaminated water occurs through adsorption and biodegradation. Adsorption is faster than biodegradation; increasing the initial concentration of Triton accelerates adsorption, but it decelerates biodegradation. Thus, *A. niger* has good potential to improve the performance of conventional wastewater treatment plants toward removal of surfactants from wastewater.

## Acknowledgments

This research was funded by The Jordanian Scientific Research Support Fund (Project Number EWE/2/06/2011).

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