EFFECT OF ZINC METAL AT HIGH CONCENETRATION ON SECONDARY METABOLIC PATHWAYS IN *PENICILLIUM CHRYSOGENUM* STRAIN

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ABSTRACT

Environmental pollution by heavy metals is a recent global problem threatening human life through the food chain. The used of fungi provides an alternative solution to eliminate these heavy metals. The current study aims to study the change of secondary metabolic pathways of fungal strain tolerant of Zinc metal. *Penicillium chrysogenum* selected strain was isolated from the sand of beaches polluted with Zinc at East Algerian area. The optimization of *Penicillium chrysogenum* strain was carried out on Czapek Yeast Agar medium (CYA) at different temperature and pH values. The effect of Zinc on the production of secondary metabolites by *Penicillium chrysogenum* strain was determined on solid medium, Thin Layer Chromatography (TLC) plate and High Performance Liquid Chromatography (HPLC). The results revealed that *Penicillium chrysogenum* strain can develop in a wide range of temperature and pH with an optimum of 28 ° C and 6. It is capable to tolerate high concentrations of Zinc (up to 1400 mg.l⁻¹).This study showed that high concentrations of Zinc (1200 and 1400 mg.l⁻¹) have a positive effect on antibiotic production, but they have a negative effect on the mycotoxin production except to neoxaline.

Keywords: antibiotic, fungi, heavy metal, mycotoxin, production, Zinc

INTRODUCTION

Soil and wastewater, particularly from industries contain high concentration of heavy metals which pass into human and animals through food chain (**LópezErrasquín and Vázquez, 2003**). Heavy metals are difficult to remediate. Therefore, environmental compartments (soil and water) are not able to eliminate these toxic metals (**Krämer and Chardonnens, 2001**). Chemical treatment methods have disadvantages which include high costs, high reagents and/or energy requirements and formation of toxic complex products that require disposal. Restoring metals in an efficient and economical procedure have necessitated the use of different options in metal-separating methods. Research shows that bioaccumulation of metals by organisms has been successful to some extent (**Salinas et al., 2000**). However, a long-term exposure of organisms mainly microorganisms at high metal concentration develops a resistance in them. Thus, the microorganisms isolated from contaminated wastewater and soil by metals with high concentrations represent a biological source for this metal removal (**Parameswari et al., 2010**).

A number of metals are required in minute amounts by most living organisms for normal maintenance. It is now known that Zinc at low concentration is essential for the activity of more than 25 enzymes, nucleic acid metabolism, wound healing and the synthesis of many industrially and medically significant microbial secondary metabolites (**Valee and Wacker, 1970; Saxena et al., 2006**). Some heavy metals particularly have been found recently in relatively high levels. The presence of these metals at high concentration causes lethal effect on living systems including fungi. The toxicity of these metals was affected by different factors like pH and the composition of growth medium. The ability of metals tolerance and uptake is varied with fungi (**Saxena et al., 2006**).

Among the more fascinating properties of fungi is their ability to synthesize a wide variety of secondary metabolites typically defined as small molecules that are not necessary for normal growth or development (**Fox and Howlett, 2008**). *Penicillium* is known as a genus of Ascomycetous fungi tolerant of various heavy metals at high concentrations. It is particularly known for its ability to produce important bioactive compounds in the field of industry and pharmaceuticals due to highly diversified spectrum of secondary metabolites, including antibacterial (**Gloer, 2007**), and potent mycotoxins (**Keating and Figgitt, 2003**). Production

of these secondary metabolites from *Penicillium* strains is intensely being examined particularly from the strains of unexplored habitats. The subgenus *Penicillium chrysogenum* has been commercially exploited for many decades due to its high production of β-lactam antibiotics such as penicillin G (**Weber et al., 2012**). Next to penicillins, other secondary metabolites such as roquefortines and glandicolines were isolated from liquid cultures of *P.chrysogenum*, which show pharmaceutically interesting properties, such as neurotoxic (**Scott et al., 1976**), antimicrobial (**Clark et al., 2005; Koolen et al., 2012**) and antitumor (**Du et al., 2010**) activities.

Some heavy metals at high concentration affect the growth of fungi and their metabolic pathway (**Yasmin et al., 2009**). But, only relatively few studies have been carried out with heavy metal-metabolites relation including secondary metabolites. Thus, the current study aims to study the effect of Zinc at high concentrations on the pathways of natural secondary metabolites in tolerant *Penicillium chrysogenum* strain.

MATERIALS AND METHODS

Isolation and purification of fungal strain

Isolation and purification of fungal strain from sand at East Algerian polluted beaches (with 7.8 mg.Kg⁻¹ of Zinc) were performed on Potatoes Dextrose Agar medium (PDA) at 25°C for 7 days (**Ezzouhri et al., 2009**). This strain was screened on 1000 mg.l⁻¹ of Zinc for its ability to produce secondary metabolites.

Optimization of fungal strain's growth

The growth of *Penicillium chrysogenum* was studied at different temperature and pH values on CYA (**Iram et al., 2013**); pH values were 1, 6 and 12. While temperature was ranging from 5 to 45 °C.

Tolerance Index

The tolerance index (TI) is an indication of the organism response to metal stress (**Le et al., 2006**). It was calculated as the ratio of the extended radius of the

treated colony to that of the untreated colony strain on CYA at 25±2 °C after 7 days.

$TI = \dfrac{Dt}{Du}$, Where Dt: is the diameter of radial extension (cm) of treated colony and Du: is the radial extension (cm) of untreated colony (control) (**Iram *et al.*, 2013**).

Bioaccumulation of Zinc

The amount of Zinc absorbed by fungal strain was evaluated in CYA broth medium containing concentration from 200 mg.l^{-1} to 1400 mg.l^{-1} of Zinc with interval of 200 mg.l^{-1}. Five millimeters of agar plugs were inoculated in these flasks (200 ml) from young fungal culture (7 days) and incubated on shaker at 150 rpm, 25±2°C for 7 days with the control. Fungal biomass was harvested after 7 days through centrifugation at 9000×g then was washed and dried at 70°C for 3 h, then was digested with nitric and perchloric acid (3:1 ratio). The digested fungal biomass was filtered using Whatman filter paper (N°1), then the volume was completed to 50 ml. Heavy metal uptake was estimated using Atomic Absorption Spectrophotometer (**Deepa *et al.*, 2006; Ezeonuegbu *et al.*, 2015**).

Microscopic observation of fungal mycelium at high concentration of Zinc

The samples were mounted on a glass slide with sterilized drops of distilled water, and observed under light microscope. Microscopic observation was performed in order to distinguish any form of Zinc storage and demonstrate the modifications shown of hyphal tips (**Lanfranco *et al.*, 2002**).

Effect of Zinc on antibiotic production

The identification of the secondary metabolites mainly antibiotics on a solid medium was achieved by the direct observation of the medium pigmentation and the outline of the fungal colonies (**Sajjad *et al.*, 2012**). The presence of β-lactam antibiotics at high concentrations of Zinc was assessed using qualitative disc test assay of the extract of *P. chrysogenum* grown in the presence of Zinc at high concentrations (1200 and 1400 mg.l^{-1}) against *Escherichia coli* strain (ATCC *25312)* (**Ali *et al.*, 2011**).

Effect of Zinc on mycotoxin production

On Thin Layer Chromatography (TLC)

The basal aqueous CYA medium of the *Penicillium chrysogenum* culture was subjected to a liquid-liquid extraction with ethyl-acetate thrice (99.5% purity) after 14 days old culture. The whole *P. chrysogenum* biomass was removed through centrifugation (9000×g) followed by filtration of liquid medium through filter paper Whatman (N°1). 10μl of obtained extracts were applied on sheet of silica gel layer as stationary phase. The compounds were separated for the mycotoxin detection on TLC plates using a mixture of solvent systems: Butanol, acetic acid, water (1/ 1/ 1) and chloroform, methanol (90 /10). The plates were dried using hair-dryer then were examined under long UV light (365 nm). The characteristics of all spots, that is, colour and retention factor (R$_f$) value were recorded (**Paterson *et al.*, 1994; Vinokurova *et al.*, 2001; Zain *et al*., 2011**).

On High Performance Liquid Chromatography (HPLC)

The TLC's positive samples were analyzed by HPLC system. The HPLC system "JASCO" have reversed phase is equipped with C18 column (ODS 5 μm. 25 × 0.46 cm) and mass spectrometry (MS) fragmentation spectra; the mobile phase was methanol. Analysis of mycotoxin from 14 days old culture of *P. chrysogenum* was carried in CYA medium using Zinc concentrations of the TLC's positive. The extract was filtered through filter paper and left at 4°C for 1 hour, Then was further filtered through a Whatman (N°1), 0.45 micron and 0.22 micron filters. This filtrate was divided into 50 ml aliquots and stored in the dark at 4°C until use with the control sample. A C18 gravity column was wetted with 100 ml of methanol and then washed with 50 ml of distilled water. A 50 ml aliquot of extract was added to the column and allowed to adsorb. The flow rate was 1.0 ml.min^{-1}, and the running conditions were as follows: 1-3 min, buffer A (50 mM sodium acetate); 3- 15 min, gradient buffer B (0 to 60% acetonitrile); 15-18 min, gradient buffer B (60 to 80% acetonitrile); 18-20 min, buffer B (80% acetonitrile); 20-22 min, gradient buffer B (80%to 0% acetonitrile); and 22- 25 min, buffer A (50 mM sodium acetate) 18, 19, 20 (**Onyegeme-Okerenta *et al.*, 2013**).

Statistical Analysis

Each treatment of all above experiments was conducted in triplicate and the average values were used in the data analysis. The optimization of fungal growth and the TI results of Zinc were expressed as mean ± standard deviation (SD) using SPSS20.

RESULTS AND DISCUSSION

Optimization of fungal strain's growth

Penicillium chrysogenum strain showed rapid growth at pH = 6 rate with 90%. At pH = 12, the growth rate was estimated about 66%. Under the same conditions, there is a slow growth (24%) at pH = 1 (Figure 1). The optimum pH value of growth was 5.5-6. Therefore, this pH may be used for further study. Similar observations were made by **Abdul Rasol et al. (2009)**. The ability of *P. chrysogenum* to grow on extreme pH can be explained effectively by the intra-cellular changes to maintain the membrane potential in relation to the external environment, these changes involve control of proton movement intra and extra-cellular. It is also linked to control of osmotic pressure due to the involvement of cations and anions (**Magan, 2007**). *Penicillium chrysogenum* showed a slow growth at 5 °C (45%). Good growth with rate 150% was observed at 37 °C, when at 45 °C and 5 °C temperature, growth rates were 90% and 42% respectively (Figure 2).

The optimal temperature was found 27-30°C, but it was a thermotolerant strain (at 45 °C). Similar results were reported by **Kubicek and Druzhinina (2007)** who recorded that *P. chrysogenum* is a thermotolerant strain up at 45 °C, at the same time it is psychrotolerant. Several hypotheses have been proposed to explain the resistance to high temperature: 1) solubilization of lipids; 2) rapid resynthesis of essential metabolites; 3) molecular thermo-stability.
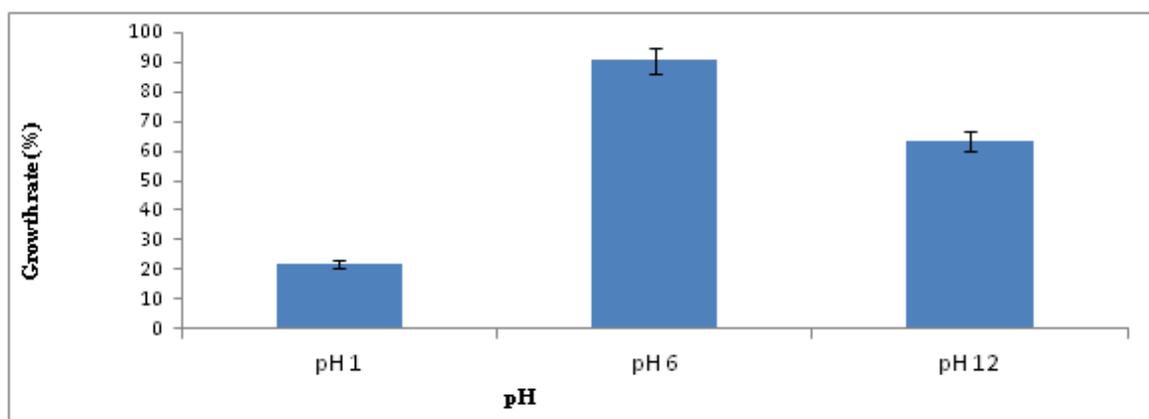

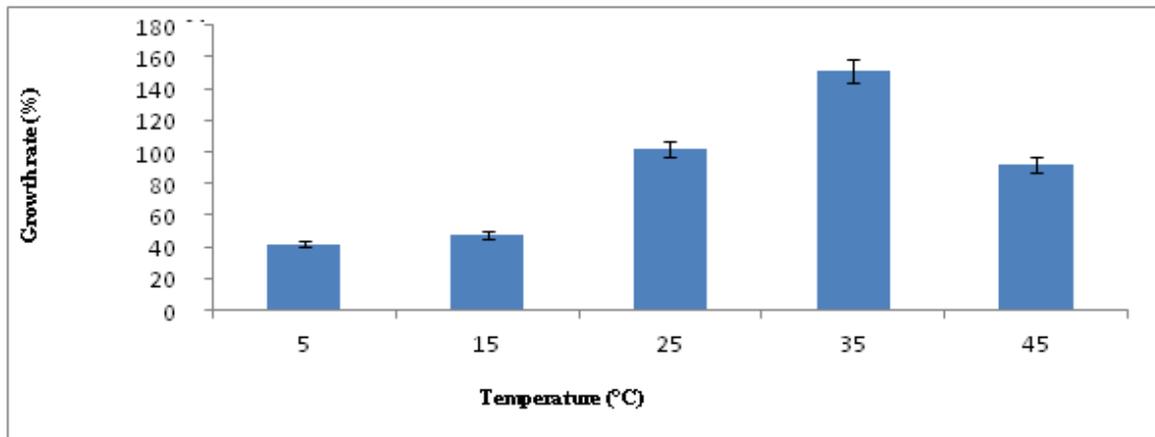**Figure 1** Effect of pH on growth of *Penicillium chrysogenum* at 5±2°C after 7 days on CYA

Figure 2 Effect of temperature on growth of *Penicillium chrysogenum* after 7 days on CYA

Tolerance index

The tolerance index (TI) is an indication of the *Penicillium chrysogenum* response to metal stress (**Le** *et al.***, 2006**). It was observed that *Penicillium chrysogenum* used more *adaptive* mechanisms of resistance at the higher concentration (1200 mg.l^{-1}) with rates of TI; 35% (Figure 3). It is able to develop and continue its growth in presence of Zinc in the medium at high concentration (1400 mg.l^{-1}) with rates of TI; 18%. This is in agreement with results reported by

Ezzouhri et al. (2009) who founded that this subgenus was able to tolerate up to 1000 mg.l^{-1} Zinc, and **Sharma et al. (2014)** who demonstrated that *P. chrysogenum* can tolerate Zinc up to 1200 mg.l^{-1}. Different types of tolerance processes or resistance mechanisms were exposed by different fungal species, the used of culture medium can have a great influence on the maximum tolerated values by this fungal strain (**Shivakumar** *et al.***, 2014**).
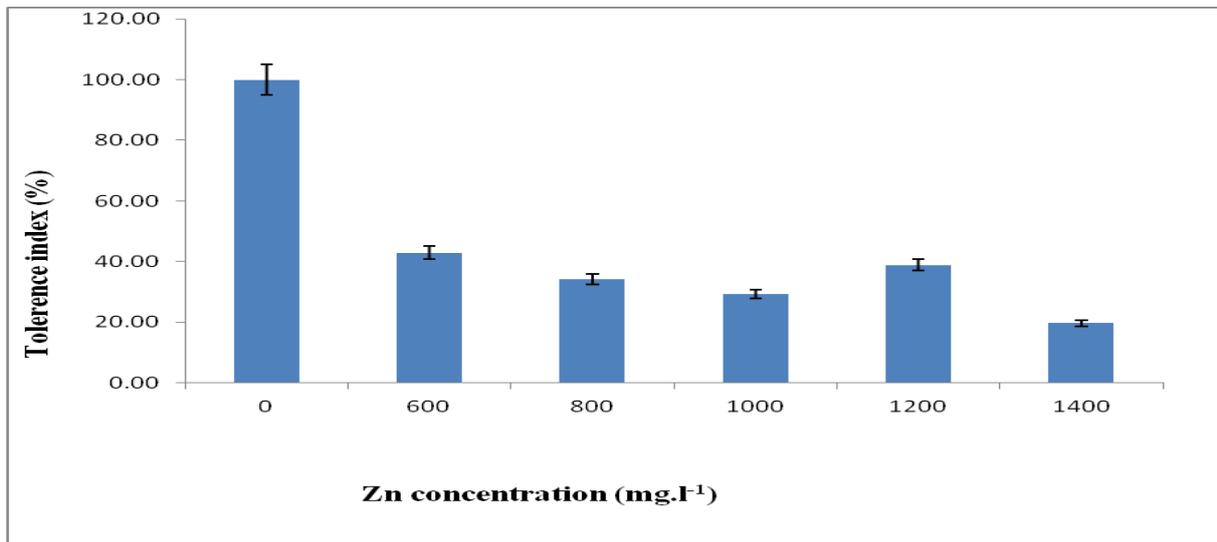

Figure 3 Tolerance Index of fungal strain *Penicillium chrysogenum* at different concentrations of Zinc on CYA at 25±2°C after 7 days

Zinc bioaccumulation

This test only allows us to estimate the amount of the metal uptaken using the screened fungal strain at different concentrations against the control without addition of Zinc (Figure 4). The ability of the strain to accumulate this metal increased rapidly with increasing the initial concentrations of Zinc to a maximum value (248 mg.g^{-1}) at 1200 mg.l^{-1}. This accumulation capacity decreased until reaching a value of 155 mg.g^{-1} at 1400 mg.l^{-1}. These results are correlated with the observations obtained from the tolerance index (Figure 3). In the same context, the capacity of *Penicillium chrysogenum* on Zinc uptake is explained by the biosorption of extra-cellular mechanisms through cell wall which exhibits excellent metal binding properties as it is negatively charged due to the presence of various anionic structures, such as glucan and chitin, this gives fungus the

ability to bind metal cations (**Anahid** *et al.***, 2011**), or by the intra-cellularly accumulation with transport proteins of metal compounds that may be involved in metal tolerance: efflux out of the metal ion cell or by compartmentalization in vacuoles or additionally, by complication with cytoplasmic proteins called metallothioneins (**Siddiquee** *et al.***, 2015**).

As above-mentioned, chemical changes methods could increase/activate the binding sites on the biomass surface, they include pretreatment, binding site enhancement, binding site change and polymerization, e.g the grafting of long polymer chains onto the biomass surface through direct grafting or polymerization of a monomer could introduce functional groups onto the surface of biomass (**Vijayaraghavan** *et al.***, 2008**).
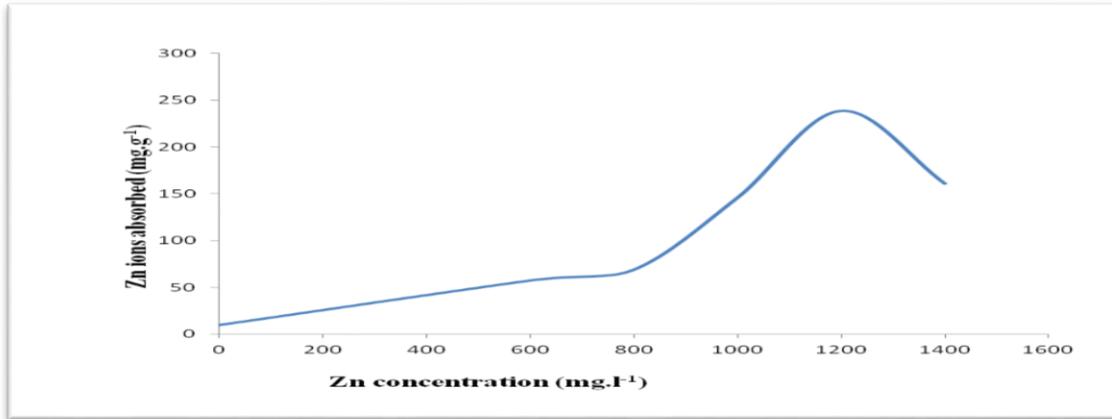
Figure 4 Bioaccumulation of Zinc by *Penicillium chrysogenum* in CYA broth at 25±2 °C after 7 days

Microscopic observation of fungal cell

Here, investigated microscopic observation was unexpected (Figure 5). The microscopic observation contradicts all the theories mentioned before (**Avery *et al.*, 2007**), excepting those that were previously proposed which said that efflux of Zinc back into compartmentalization in the organelles or in vacuoles (**Blaudez *et al.*, 2000;** Eide *et al.*, 2003).

In the absence of Zinc in the medium, fungal hyphae grew using apical growth with a typical tapered apex and absence of branching from the tip. The presence of high concentrations of Zinc did modify the hyphal morphology, and the most characteristic features of Zinc-treated hyphae, when compared to control samples, were the overall increase in hyphal branching, swelling and septation. At high concentration of Zinc in the medium, fungal hyphae grew with pattern resulted in parallel-oriented hyphae that were loose at the edge of the colony and organized in bundles that sometimes coiled in the inner part. Therefore, hyphae became increasingly vacuolated with rounded refractive vacuoles (**Lanfranco *et al.*, 2002**) as indicated in Figure 5.
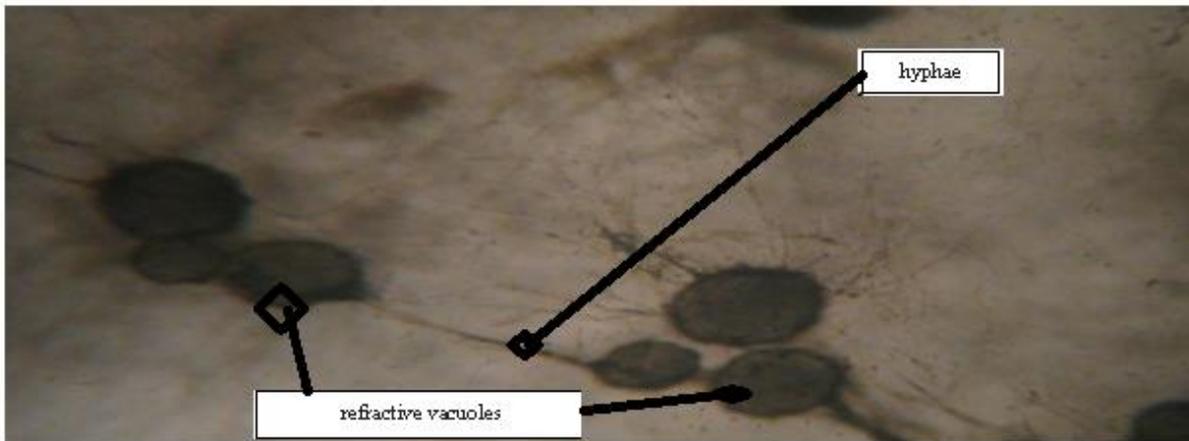

Figure 5 Microscopic observation of *Penicillium chrysogenum* mycelium at 1200 mg.l^{-1} of Zinc (60×)

Effect of Zinc on antibiotic production

The growth of *Penicillium chrysogenum* on CYA medium appeared in yellowish brown and bluish-colored, powdery and velvety-textured colonies with a white outline (Figure 6 a, b, c). Highlighted of secondary metabolite production by *P. chrysogenum* on CYA revealed that there is antibiotic production at the highest concentration of Zinc (1400 mg.l^{-1}). On the same medium, bluish-colored with a white outline colour indicated that observed colonies represent *P. chrysogenum* species. Their mycelium becomes thick in comparison to the control. *Additionally*, growth of the *Penicillium chrysogenum* on CYA medium at high concentrations of Zinc ions was accompanied by a yellow substance production which is a response to the stress induced in the presence of Zinc in the medium.

The efforts of researchers are still focused on *Penicillium* species as a continuous source of antibiotic production. The routine disc assay of the antibiotic production showed production of β-lactam antibiotics, at least the penicillin in the medium. **Sajjad et al. (2012)** isolated penicillin from a pure culture of the *P. chrysogenum* strain on CYA medium. Thus, the colored substance indicates the production of secondary metabolites by *P. chrysogenum* strain which may be an antibiotic such as β-lactam (penicillin ... Etc.). As shown in Table 1, it is interesting to note that there is a remarkable inhibition of extracts of *P. chrysogenum* against *E. coli* strain. Optimum production of antibiotic was obtained at Zinc concentration of 1400 mg.l^{-1} which gave the maximum zone of inhibition during disc test with diameter ; 3.8±0.7 cm.

Figure 6 Colony aspect of *Penicillium chrysogenum* after 7 days on CYA at 25°C at different concentrations of Zinc (a: control, b: 1200 mg.l^{-1}, c: 1400 mg.l^{-1})

Table 1 Diameter of zone inhibition of extracts of *P. chrysogenum* against *E.coli* strain ATCC *25312* (mean ± SD)

Diameter	control	1200 mg.l⁻¹	1400 mg.l⁻¹
Extracts of *P. chrysogenum*	2.4 ± 0.06	2.9 ± 0.6	3.8 ± 0.2

Effect of Zinc on mycotoxin production

On TLC

The mycotoxin were detected as fluorescent spots in UV-chamber at 365 nm. The revelation of the plate (TLC) allowed us to separate two spots corresponding to the mycotoxins produced by the *Penicillium chrysogenum* strain. The two spots 1, 2 have a retention factor $R_f = 0.65$, $R_f = 0.64$, respectively. Figure 7 demonstrated that there is a spot 1 corresponding to the production of a metabolite in the control and a spot 2 corresponding also to the production this metabolite at 800 mg.l⁻¹ of Zinc. The retention factor of the control (0.65) is almost the same at concentration of 800 mg.l⁻¹, indicating that both spots correspond to the same mycotoxin. At the higher concentrations (1200 and 1400 mg. l⁻¹), there is no migration of the molecules (spots 3 and 4). Thus, there is no production of these mycotoxins at the high concentrations. Compared to the results obtained in Figure 39b and 40c, the growth of *Penicillium chrysogenum* at the highest concentration of Zinc ion (1400 mg.l⁻¹) showed production of yellow substance that is strongly an antibiotic. Thus, the present investigation indicates that the highest concentration of Zinc (1400 mg.l⁻¹) has a positive effect on antibiotic production, but there is a negative effect on the production of mycotoxin (TLC). *Penicillium chrysogenum* synthesizes mycotoxins from the alkaloid family and certain mycotoxins from the sterol (**Sunesson *et al.*, 1996**; **Lugauskas *et al.*, 2005**). From TLC plate result, it is evident that the mycotoxin production was inhibited at the highest concentration of Zinc (1400 mg. l⁻¹).
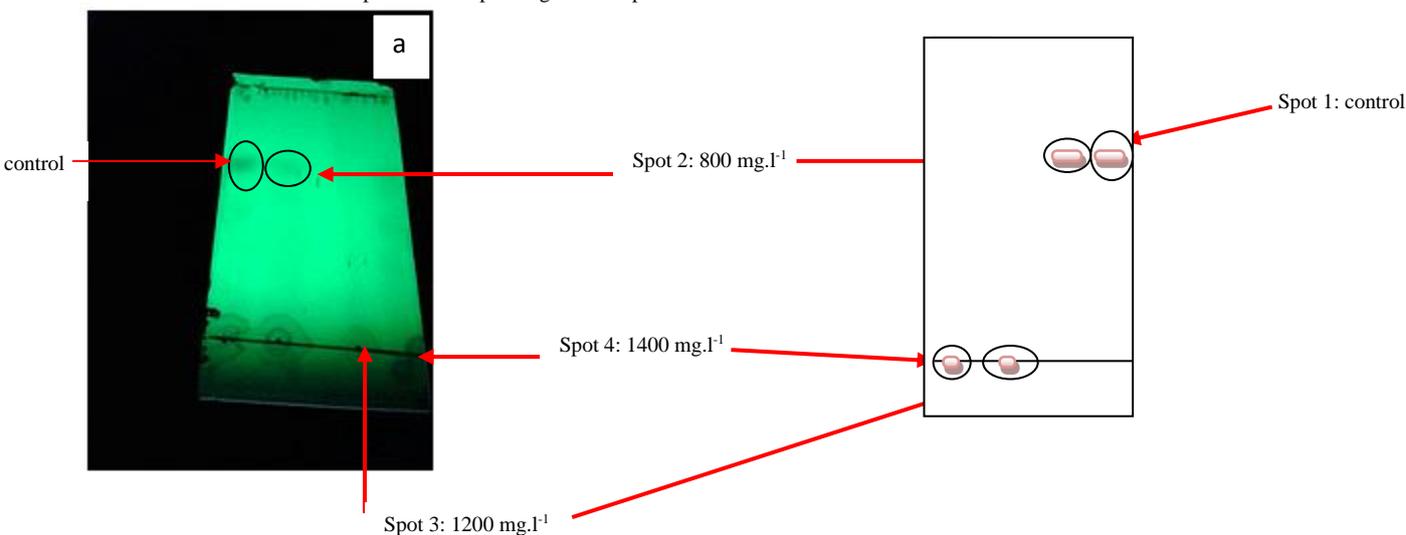

Figure 7 Separation of mycotoxin from *Penicillium chrysogenum* grown in the presence of Zinc with the high concentrations on TLC sheet under long UV light with 365nm (a: *real* photo, b: *schematic illustration*)

On HPLC

In present study, we described the identification of various abundant metabolites. As illustrated in Table 2, the identification of four compounds was confirmed by comparing retention time as was described by **Ries et al. (2013)**. HPLC elution profiles of the control indicated the presence of several clearly peaks (around 7). Comparing retention time and MS fragmentation spectra allowed us to identify four compounds: Peak (12), at 15.1 min as roquefortine N. Peak (11) at 16.5 min as roquefortine C. Peak (10) at 17.8 min such as neoxaline, peak (9) at 22.2 min as roquefortine F. The others were not identified: peak (13) at 13.5 min, peak (8) at 25.7 min and peak (7) at 28.5 min (Figure 8A).

HPLC elution profiles of *P.chrysogenum* extract at 1200 mg.l⁻¹ of Zinc suggested the production of three mycotoxins: non-identified peak at 13.5 min. neoxaline as peak (10) at 17.8 min. roquefortine F as peak (9) at 22.8 min (Figure 8B). The extract of *P.chrysogenum* at 1400 mg.l⁻¹ of Zinc indicated that a single mycotoxin was produced which is neoxaline showed in peak (10) at 17.8 min (Figure 8C). The reported results demonstrated that the high concentrations of Zinc (1200 and 1400 mg.l⁻¹) inhibit some branches of the secondary metabolite pathway yielding variety of intermediates as explained by **Ries et al. (2013)** in roquefortine / Meleagrin pathway via inhibition of *enzyme activity*. The concentration of 1200 mg.l⁻¹ has an influence on yielding of variety intermediates between the precursor and roquefortine F. While, 1400 mg.l⁻¹ of Zinc affects the metabolic pathway between the precursor and the final product which was identified as neoxaline.
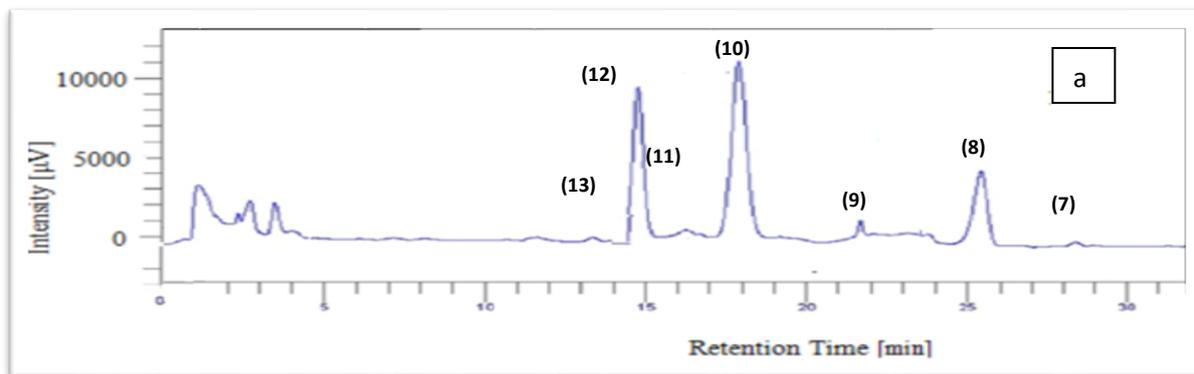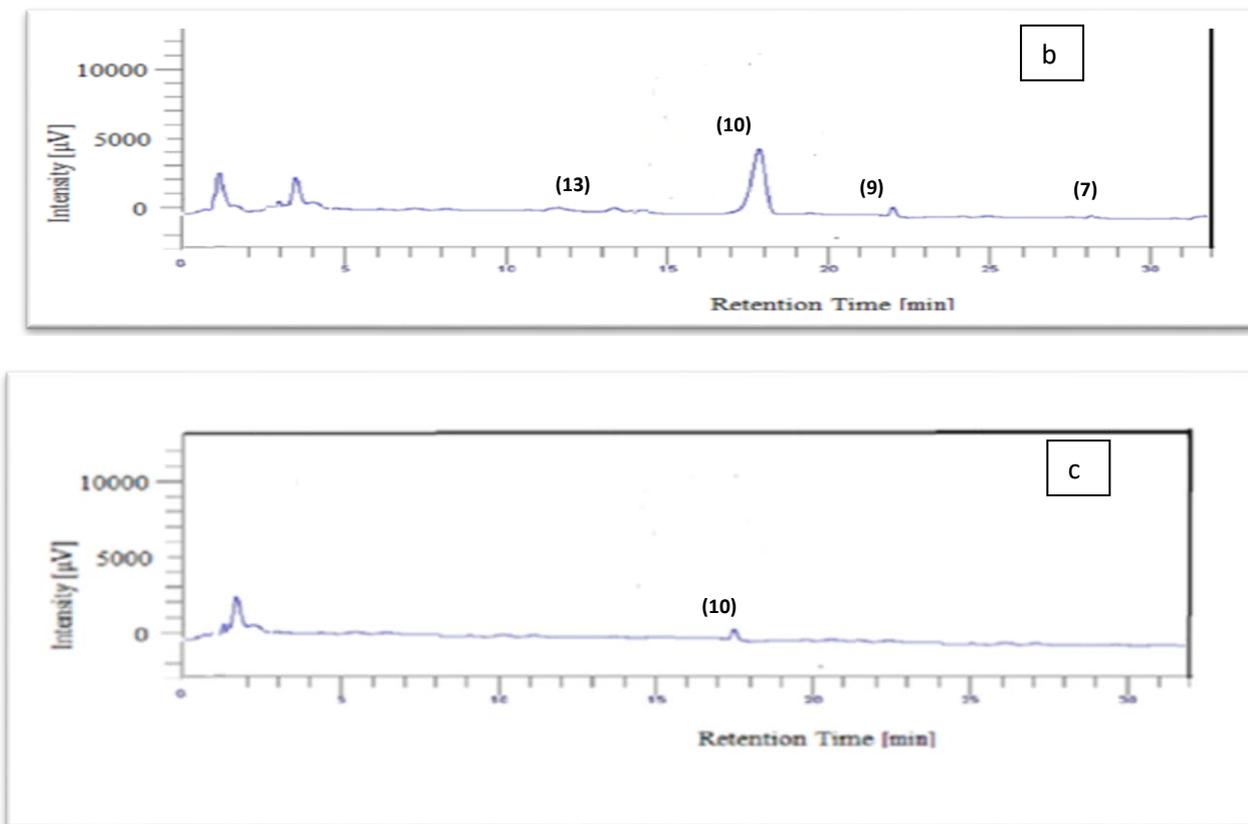
Figure 8 HPLC elution profiles of *P. chrysogenum* mycotoxins (a: control, b: at 1200 mg.l^{-1} and c: at 1400 mg.l^{-1} of Zinc)

Table 2 Description of the molecules and the Rt of HPLC peaks

Molecule	Peak	Retention time (Rt) / min
non identified	7	28.5
non identified	8	25.7
roquefortine F	9	22.8
neoxaline	10	17.8
roquefortine C	11	16.5
roquefortine N	12	15.1
non identified	13	13.5

CONCLUSION

In conclusion, the addition of Zinc with the highest concentration (1400 mg.l^{-1}) into the incubation medium *Penicillium chrysogenum* affects positively antibiotics production and negatively mycotoxin production except to neoxaline. Finally, much remains to be understood about the influence of high concentration of heavy metals (as Zinc) on metabolic pathways of tolerant fungal strains.

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