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THE EFFECT OF CARBON SOURCE CONCENTRATION ON TOXIGENESIS AND LIPASE ACTIVITY OF *Penicillium aurantiogriseum*

SUMMARY

Penicillium aurantiogriseum (P. aurantiogriseum) is a species frequently contaminates olives and other foodstuffs including cereals and their derivatives. Because of its secondary metabolites, *P. aurantiogriseum* is used in the food and pharmaceutical industries. The aim of this work is to study the influence of carbon source concentration on the secondary metabolites production of *P. aurantiogriseum* in order to fight against this species and also to improve the production of its lipases. Lipase activity was measured by the titrimetric method with olive oil as a substrate. Mycotoxins production was determined by TLC technique. Our study revealed that *P. aurantiogriseum* secretes three mycotoxins (terrestric acid, penicillic acid, and aurantiamine) as well as two types of lipase, namely lipases I and II. The production of penicillic acid was detected in CYA, BM, and YES media, while aurantiamine was produced only in BM medium and terrestric acid in YES. Terrestric acid production increased proportionally to the sucrose concentration in YES. However, the penicillic acid production is inversely proportional to the concentration of sucrose in YES. On the other hand, a maximum of lipase activity was obtained in the CYA* medium under agitation condition.

Keywords: *P. aurantiogriseum*, carbon source, mycotoxins, lipases.

INTRODUCTION

Penicillium has long been used in food and pharmaceutical industry. However, some species are toxigenic and, thus, could be very harmful to human health. Numerous studies have reported that some mycotoxins can be secreted by a huge number of *Penicillium* species (Lu et al., 1994) including those used in food processing and pharmaceutical industry, whenever favorable physicochemical and trophic conditions are present. *P. aurantiogriseum* is frequently isolated from food (Yu et al. 2010; Khaddor et al., 2007; Maouni et al., 2002)**,** it secretes numerous mycotoxins namely terrestric acid, penicillic acid,

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and aurantiamine. *P. aurantiogriseum* is the interesting source of lipase (Li and Zong, 2010), which are typically extracellular and therefore relatively easy to recover after fermentation (Li and Zong, 2010; Lima et al., 2004). Lipases of *P. aurantiogriseum* are widely used in the agri-food industry as they play a major role in the development of characteristic flavors in ripened cheeses (Chopra et al., 1980; Salihu et al., 2012), pesticide production, and also their role in the pharmaceutical industry through the synthesis of drugs (Mase et al., 1995; Torre et al., 1996). Although several methods have been developed for the determination of lipase activity and mycotoxins production, our study was limited by the use of the titrimetric method using olive oil as a substrate. Mycotoxins production was determined by TLC technique. The aim of this work is to study the influence of carbon source concentration on the toxigenesis of *P. aurantiogriseum* and to improve lipases productivity.

MATERIAL AND METHODS

The strain

P. aurantiogriseum used in this study belongs to the collection of our EFBRT (Environmental and Food Biotechnology Research Team) which was used in previous studies (Khaddor et al., 2007), (Maouni et al., 2002; Sebti and Tantaoui-Elaraki, 1994). The strain was first cultivated on MEA (malt extract agar) at 25°C for 7 days, and the spores were suspended in 0,1 % of tween 80. The suspension density was adjusted to 10^7 spores/ml.

The purification was then made on three different media MEA (Samson and Gams, 1984), CYA (czapeck yeast extract agar) (Samson, 1981) and G25N (glycerol 25% nitrate agar) (Pitt, 1973) (Table 1).

Table 1: Composition of the media used for growth and metabolites production of *P. aurantiogriseum*

Medium	Composition per 1 liter of distilled water
G25N	Concentrated Czapeck**: 10ml; K_2HPO_4: 1g; Yeast Extract: 5g; Glycerol: 250 g; Agar: 16g.
MEA	Malt extract agar: 30 g; Glucose: 20 g; Agar: 10 g.
CYA	Concentrated Czapeck**: 10ml; K_2HPO_4: 1 g; Yeast extract: 5g; Sucrose : 30g; Agar: 15 g.
YES	Yeast Extract: 20g; Sucrose: 100g.
BM	Malt Extract: 130g; Agar : 15g.
** Czapek concentrate: $NaNO_3$: 30g ; KCl : 5g ; $MgSO_4$:5g ; $FeSO_4$: 0,1g ; $ZnSO_4$: 0,1g ; $CuSO_4$: 0,05g; Distilled water: 100ml	

Determination of mycelial dry weight

The growth of *P. aurantiogriseum* colonies was estimated by determination of cell dry weight and radial growth according to Zain et al. (2009). For cell dry weight determination, the whole of culture plate agar was mixed with 100 ml of distilled water and boiled to dissolve the agar. The agar

solution containing the fungal biomass was filtered through Whatman dry filter paper number 5, and the filter paper with the retained fungal cells was then dried in an oven at 80°C until a weight was reached constant dry. Radial growth was estimated by measuring the diameter of each colony with a ruler. All the experiments were performed in triplicate.

Research of mycotoxins

The experiment was done in two steps; we first used three growth media (CYA, MB, and YES) to identify mycotoxins produced by *P. aurantiogriseum* in each medium. After that, we have used only the medium that gave more than one mycotoxin and we tried to change the concentration of its basic carbon source to determine the maximum production of each mycotoxin. For the second step, the medium chosen for mycotoxins production was the YES (Yeast Extract Sucrose) (Frisvad, 1983). For that, 25 ml of YES, with different concentrations of sucrose, ranging from 0 to 300 g/l, was distributed in 100 ml Erlenmeyer flasks and inoculated with 2 ml of spore suspension (10^7 spores/ml). All cultures were incubated for 10 days at 25°C. The experiment was done three times.

The toxigenesis study was made according to the method reported by (Khaddor et al., 2007). The TLC plates used are 60 Kieselguhr F254. Mycotoxins standards used by the reference of migration forehead (Rf) were patulin (P), citrinin (C), ochratoxin A (OTA), penicillic acid (PA), and griseofulvin (Gi). Ten ml each of ethanol extract and of standard solutions (1 mg/ml) were spotted on TLC plates. Elution systems used are: toluene-ethyl acetate - formic acid (5/4/1, v/v/v) and chloroform - acetone - 2-propanol (85/15/20, v/v/v). The plates were examined in daylight and by ultraviolet 365 and 254 nm after spraying the spots by ANIS (p-anisaldehyde solution) and 8 min heating to 120°C. The ratio (Rf), color and fluorescence intensity of the extracts were compared with different reference concentrations of P, C, Gi, PA and OTA (Cunniff and Association of Official Analytical Chemists, 1995). Fluorescence intensity was expressed by a variable number of "+" signs (Hameed et al., 2012)

Lipase production
Detection of enzymatic activity in the solid medium

The determination of lipase production by *P. aurantiogriseum* was carried out according to Beisson et al. (2000)**.** The growth medium contained the following elements: 5 g of peptone, 2 g of NaCl; 1 g of NO_3NH_4, 15 g of agar; 25 g of the olive oil and 20 ml of rhodamine B 0.5%. 10^8 spores are cultured in the medium for 4 days at 25 °C. Rhodamine B is used as an indicator of the enzymatic activity.

Determination of lipase I and II in liquid medium

In this study, we used the media of *P. aurantiogriseum* growth and toxinogenesis as media for lipase production with modification of the carbon sources that have been substituted by their values in olive oil. The purpose of this study is to improve the production of P.A lipase while maintaining the same

basic culture conditions. It is for this reason that we think of just replacing the carbon sources with their value in olive oil instead of changing the whole culture medium.

The extracellular lipase was measured by free fatty acids assay in the supernatant of growth medium (Ginalska et al., 2004; Sarda et al., 1958). A volume of 100 ml, of each medium, was placed in Erlenmeyer flask (1000 ml) and added with 1 ml of 10^8/ml of spore suspension. The medium, thus seeded, was incubated for one week at 25°C. This incubation was carried out without agitation (for lipase I production) and with agitation on shaker-incubator at 120 rpm (for lipase II production). After incubation, the media were filtered to determine the lipolytic activity which depends on the number of fatty acids released by the hydrolysis of the triglycerides contained in the buffered emulsion.

A volume of 200 μl of the filtrate was incubated for 20 minutes at room temperature with 2.5 ml of 50 mM Tris buffer pH 7 and 2.5 ml of an emulsion prepared by homogenizing by ultraturax (the emulsion is made with Ultra-Turrax, with stirring at maximum speed for 2 minutes) 2 g of tributyrin in 100 ml of an aqueous solution of 3% gum arabic. After 20 minutes of incubation, 10 ml of alcohol was added to block lipolysis. The same volume of the filtrate, in this case, 200 μl, was used as a control by incubating it for 10 min at a temperature of 100 °C to inactivate the lipases secreted by *P. aurantiogriseum*. The released fatty acids were titrated with a 0.05 M NaOH solution and phenolphthalein as an indicator. One unit of lipase was defined as the number of enzymes that releases 1 μmol of fatty acid/min. The experiment was done three times.

RESULTS

Mycotoxin production
Media effect on radial growth rate and cell mass production

The effect of medium composition on growth and toxigenesis of *P. aurantiogriseum* are summarized in Table 2. The radial growth rate was significantly high in CYA medium compared to YES and BM media. The highest cell production was obtained from CYA, while the lowest was obtained from BM. A slight difference in cell production was observed between CYA and YES.

Media effect on mycotoxins production

The chromatographic analysis of the extracts obtained from different media of *P. aurantiogriseum* (YES, CYA and BM) allowed highlighting three mycotoxins: penicillic acid, terrestric acid, and aurantiamine (Table 2)**.** The production of penicillic acid was detected in CYA, BM and YES media, while aurantiamine was produced only in the BM medium and terrestric acid in the YES.

Effect of sucrose concentration on mycotoxins production

Terrestric acid production was observed in the YES medium. This production increased proportionally to the sucrose concentration in YES.

However, the penicillic acid production is inversely proportional to the concentration of sucrose in YES (Figure 1 and Table 3).

Table 2: Growth and toxigenesis of *P. aurantiogriseum* on different media

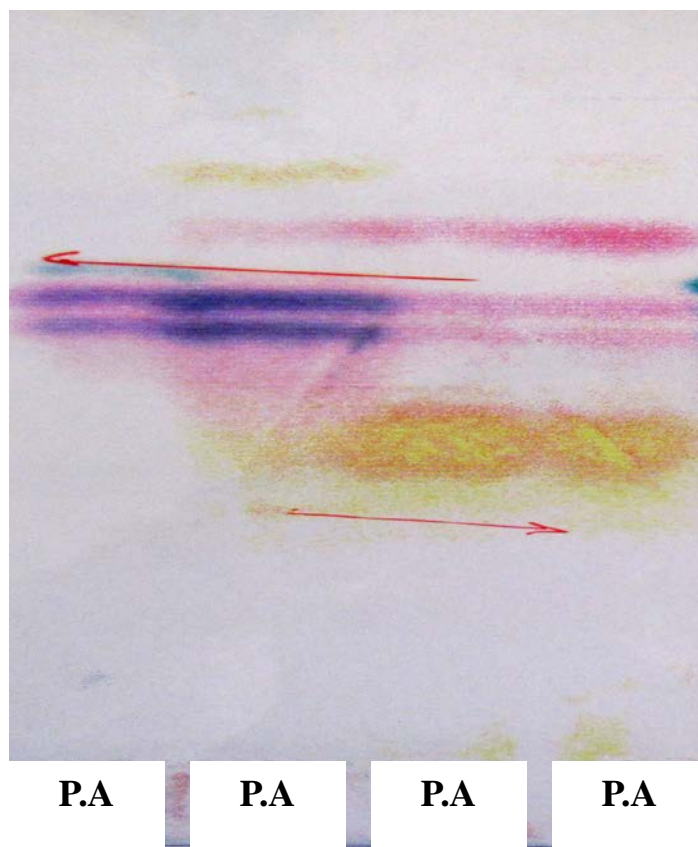| Medium	Mycotoxins intensity on chromatogram			Dry weight (mg)	Colony diameter (cm)
	Penicillic acid	Terrestric acid	aurantiamine		
CYA	+	-	-	1.1	3.5
BM	+	-	+	0.5	2.5
YES	+	+	-	0.8	3.0

Figure 1: Chromatogram showing the mycotoxins produced by *P. aurantiogriseum* in different sucrose concentrations on YES medium.

P.A: *P. aurantiogriseum*; **S**: the concentration of sucrose in the YES medium expressed in g/l. The arrows indicate the degree of mycotoxins production; from left to right: the increase in the production of terrestric acid (yellow spot). From right to left: increased production of penicillic acid (blue lilac spot).

Table 3: Growth and mycotoxins production at different sucrose concentrations

Sucrose concentration in YES medium (g/l)	Colony diameter (cm)	Mycelial dry weight (mg)	Mycotoxins intensity	
			Terrestric acid	Penicillic acid
0	3.8	1.3	-	++
100	3.0	0.8	+	>+++
200	2.5	0.7	>+++	>+
300	3.2	1	++	+

Lipase activity of P. aurantiogriseum
Rhodamine B assay

P. aurantiogriseum showed rapid growth after 24 h. Positive results were obtained using rhodamine B as indicators. After 24 hours of incubation, we observed a lysis zone in *P. aurantiogriseum* colonies. The enzymatic concentration is related to the diameter of the intensification zone. This diameter increases with the incubation time (Figure 2). The intense development of color can be observed around lipase production in colonies. Therefore, we propose that this technique is a simple and reliable technique for detection of lipolytic activity in *P. aurantiogriseum.*
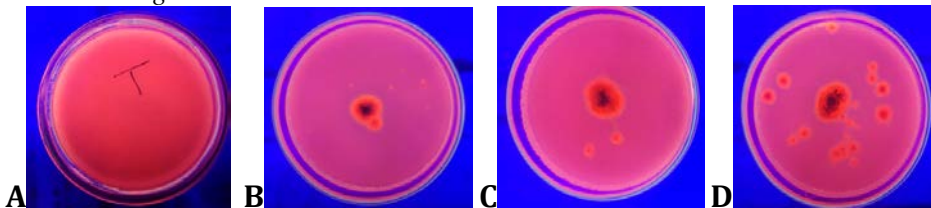
Figure 2: Detection of enzymatic activity in *P. aurantiogriseum*.

Rhodamine B was complexed with the fatty acids released during lipolysis. This complex gave an orange coloration which is read at 350 nm. **A:** control, **B:** after 24 h of incubation, **C:** after 3 days, **D:** after 4 days.

Determination of lipase activity

The lipase activity of *P. aurantiogriseum* on different media was detected after 20 min of the substrate hydrolysis reaction. The results showed a maximum lipase activity of 4.25 U/ml of fatty acid obtained in the CYA* medium. The activity was low in MEA* and YES* media with 3.04 U/ml and 1.6 U/ml respectively (Figure 3).

P. aurantiogriseum recorded an activity of 2.0125 U/ml of fatty acid in the non-agitated medium and 4.25 U/ml in the agitated medium indicating that lipase II is the most dominant in our strain (Figure 4).
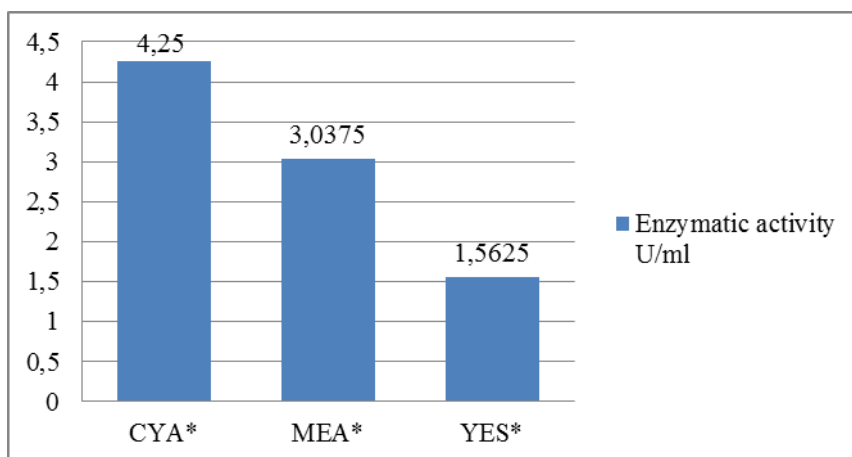
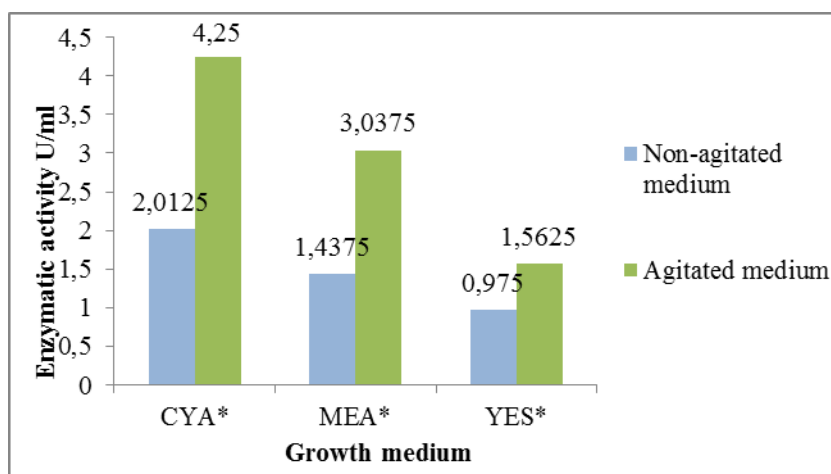Figure 3: Enzymatic activity on different culture media.

Figure 4: Determination of *P. aurantiogriseum* enzyme activity in two media states.

DISCUSSION
Mycotoxin production in P. aurantiogriseum
Effect of media on mycotoxins production

In this study, three different media were used to obtain a basal medium favorable to improve *P. aurantiogriseum* toxigenesis. The results demonstrated that mycotoxin production in *P. aurantiogriseum* was greatly influenced by the carbon source. The strain was able to grow and produce mycotoxins greatly when sucrose has been used. The YES medium was preferred to *P. aurantiogriseum* for growth and toxigenesis.

Chromatographic profile of extracts obtained from *P. aurantiogriseum* on different media (YES, CYA and BM) revealed three mycotoxins: penicillic acid, terrestric acid, and aurantiamine. These mycotoxins have been reported to be

produced by this species in previous works (Colombo et al., 2003; Frisvad and Samson, 2004; Khaddor et al., 2007).

In this study, we showed a great diversity regarding mycotoxin production by *P. aurantiogriseum* on different media. Penicillic acid production was detected in CYA, BM and YES media, while aurantiamine was produced only in the BM medium and terrestric acid in the YES medium. The comparison between media (CYA, BM and YES) indicates that the variation in mycotoxin production was related to different carbon sources used in different media (Davis et al., 1969; Mills et al., 1995). *P. aurantiogriseum* growth was increased when sucrose was present in YES as a carbon source. The production of mycotoxins was observed in the BM and YES media as it was demonstrated in similar studies (Davis et al., 1969; Mills et al., 1995a; Zain et al., 2009).

Effect of sucrose concentration on mycotoxin production

As it was mentioned in the Material and Methods section, we chose the medium which the mycotoxin production was clearer and contains more than one mycotoxin. This medium was the YES on which we changed the concentration of sucrose from 0 to 300 g/l to determine the maximum production of terrestric acid and penicillic acid.

We have observed, on the one hand, the terrestric acid production in YES medium. This production increased proportionally to the sucrose concentration in the medium. On the other hand, penicillic acid production is inversely proportional to the sucrose concentration in the YES. It appears that the production of terrestric acid is induced by sucrose. Therefore, we suggest that the terrestric acid has a strong affinity towards sucrose since it is produced only in the YES medium. This is in agreement with the results of Mills et al. (1995) which showed that the terrestric acid is produced in YES medium in which sucrose is the sole carbon source. Unlike the terrestric acid, penicillic acid is produced in three media (CYA, MB and YES), so it rhymed with several types of carbon source. However, when we increased the sucrose concentration in the medium, penicillic acid production decreased and this could be due to the difficulty of assimilation of sucrose by the strain even in the presence of the invertase enzyme which appeared to be little in the intracellular medium.

P. aurantiogriseum develops on various carbon sources, including sucrose. Sucrose growth resulted in high levels of biomass, but mycotoxin production decreased as carbohydrate concentration increased. Therefore, the maximum product concentrations were not reached under conditions supporting the highest biomass level. Our results suggest some control of the carbon catabolism of mycotoxin production in *P. aurantiogriseum*. To achieve the highest volumetric title, it is not sufficient to provide conditions for a high specific output. A critical level of mycotoxin production is also needed. Sucrose at the concentration between 100 and 200 g/l provided the critical degree of production of penicillic acid and terrestric acid, respectively. We postulate that this is due to the slow sucrose hydrolysis. This created a situation of carbon limitation and releases the culture to the carbon source regulation (Aharonowitz and Demain, 1978). It has

been shown in several works (Chander et al., 1981; Flipphi et al., 2009; Zain et al., 2011) that the production of antibiotics and other secondary metabolites is negatively affected when the carbon source is rapidly used for growth; however, when the carbon source is slowly introduced into the culture, the secondary metabolite is efficiently produced.

Lipase activity in P. aurantiogriseum

Our results showed a maximum lipase activity of 4.25 U/ml of the fatty acid obtained in the CYA* medium. The activity was low in the MEA* and the YES* medium with 3.04 U/ml and 1.56 U/ml respectively. These results are in agreement with those reported by Singh et al. (2012) where the enzymatic activity of *P. aurantiogriseum* in YES was very low, whereas, in a medium supplemented with another source of carbon (like maltose on MEA) or other trace elements (like microelements on CYA), the production was remarkable.

Salihu et al. (2012) have shown that the presence of olive oil in the medium induces lipase production during the growth phase. The triglycerides of olive oil are the best carbon sources for lipase production. It is possible that the consumption of fatty acids leads to the immediate induction of lipases. Cordova et al (1998) had suggested that when *P. aurantiogriseum* is grown in the presence of an inducer, the lipase that produced is derived from a primary metabolism.

In this work, the concentration of olive oil was 3 % in CYA* medium and the corresponding lipase activity was 4.25 U/ml. This is not coherent with the results obtained by Lima et al (2003) where the concentration of 2 % of olive oil gave an activity of 3 U/ml. On the other hand, in MEA* medium (2 % olive oil) and YES* (10 %), the activity was much lower compared to CYA* with values of 3.04 and 1.56 U/ml respectively. Lima (2003) explains that the inhibition of lipase synthesis at high concentrations of olive oil may be due to the difficulty of transferring oxygen to the environment. Low oxygen stores can alter fungal metabolism and consequently lipase production. However, in our study, the high concentrations of olive oil did not affect lipase activity. This could be explained that the strain can grow both in anaerobic and in aerobic conditions. Moreover, the concentration of olive oil, the sole carbon source, in the medium could be assimilated by our strain by using it in the growth, survival, and production of metabolites.

It should be noted that the production of lipase is influenced by certain factors, such as aeration. Chahinian et al**.** (2000) reported that when *Penicillium cyclopium* (nearest equivalent description of *P. aurantiogriseum* according to Raper and Thom (1949)) was cultured in a non-agitated liquid medium, it produced lipase I specific for triacylglycerols. But in the agitated medium, the strain produced the lipase II which is active only on mono- and diacylglycerols. In the present study, *P. aurantiogriseum* recorded an activity of 2.01 U/ml of fatty acid in non-agitated medium and 4.25 U/ml in the agitated medium, indicating that lipase II is the most dominant in the strain.

In our study, the non-agitated medium showed a difference in the enzymatic activity of *P. aurantiogriseum* in the three media with the values of

2.01 U/ml of fatty acids on CYA*, 1.44 U/ml on MEA* and 0.97 U/ml on YES*. These media contained an inducer of lipase production, which is olive oil, with different concentrations. This induction caused the lipase to be produced in the exponential phase of the strain as a primary metabolite. That explains the high activity in CYA* which olive oil is the sole carbon source. In the MEA* medium, which contained maltose in addition to olive oil, the strain first used maltose as a carbon source and consequently the production of lipase was delayed by giving a small amount of lipase compared to CYA*. The high concentration of olive oil in the YES* medium makes oxygen uptake very slow and the strain has no other source of carbon which influences its growth and, therefore, its lipase production.

These results have a low value compared to that obtained in agitated medium, suggesting that in the non-agitated medium, the growth and production of lipase was limited by the lack of oxygen used during respiration. The agitation created a kind of aeration in the colony to release CO2 and use the maximum of O_2 in the medium (Börjesson et al., 1990; Chander et al., 1981).

CONCLUSIONS

The mode of secondary metabolites production has long been very useful for the identification and classification of fungi. However, production of these compounds can be affected by environmental conditions. Our results showed that the growth parameters and secondary metabolites production of *P. aurantiogriseum* were affected by the nature and concentration of carbon source. Results from this study revealed that mycelium weight, colony diameter, and mycotoxins production of *P. aurantiogriseum* were significantly affected by increased sucrose concentrations. As a result, secondary metabolites are sensitive to environmental conditions and their production is limited under certain conditions, therefore the use of secondary metabolites in fungal taxonomy should be reconsidered. The current study showed that olive oil significantly increased lipase production in *P. aurantiogriseum*. This is the first time that higher lipolytic activity and shorter production times were achieved for this species.

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