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Influence of Culture Conditions on Production of NGPs by *Aspergillus tubingensis*

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The filamentous fungus *Aspergillus tubingensis* that belongs to the black *Aspergillus* section has the capacity to produce high-value metabolites, for instance, naphtho-gamma-pyrones (NGPs). For these fungal secondary metabolites, numerous biological properties of industrial interest have been demonstrated, such as antimicrobial, antioxidant and anti-cancer capacities. It has been observed that production of these secondary metabolites is linked with fungal sporulation. The aim of this research was to apply osmotic and oxidative environmental stresses to trigger the production of NGPs in liquid cultures with CYB (Czapek Dox Broth). In addition, numerous parameters were tested during the experiments, such as pH value, incubation time, container geometry, and static and agitation conditions. Results demonstrate that the produced amount of NGPs can be enhanced by decreasing the water activity (a_w) or by adding an oxidative stress factor. In conclusion, this study can contribute to our knowledge regarding *A. tubingensis* to present an effective method to increase NGP production, which may support the development of current industrial processes.

Keywords: *Aspergillus tubingensis*, naphtho-gamma-pyrones (NGPs), osmotic stress, oxidative stress, liquid static culture

Introduction

The demand for new natural molecules with biological activities of industrial interest (antimicrobial, antioxidant, anti-cancer) is a major concern in the fields of agronomy, food and health. Nowadays, bacteria, yeasts, and fungi are cultivated on a large scale to obtain those types of products with commercial value [1].

Several species of *Aspergillus* genus have great importance in multiple industrial research projects (food, cosmetic and pharmaceutical) due to their ability to produce metabolites with commercial interest. As an example, the genus *Aspergillus* section *Nigri*, discovered by Raoul Mosseray in 1934 [2], is performing a major role in metabolite production [3, 4]. It commonly consists of ten *Aspergillus* species with the capacity to produce around 145 secondary metabolites (*A. tubingensis; A. carbonarius; A. niger; A. acidus; A. brasiliensis; A. fonsecaus*; *A. awamori; A.* ibericus*; A. lacticoffeatus* and *A. sclerotionige)* [3, 4]. Thus, *Aspergillus niger* produces enzymes currently used in the food industry such as amylase, lipase, glucose oxidase, phytase, xylanase, acid phosphatase and xylosidase [5].

However, some species of the *Nigri* section had been described as capable also to produce mycotoxins such as ochratoxin A (OTA): *A. niger* [6], *A. carbonarius* [7], *A. lacticoffeatus* and *A. sclerotioniger* [8-9]. Some years ago, it was demonstrated that a fungal extract, composed mainly of naphtho-gamma-pyrones (NGPs), produced by *A. niger* isolated from grapes [10, 11], possessed great antioxidant activity. A few years later, this fungus was finally identified as *A. tubingensis* [12] whose extract could be valuable in several industrial applications as an antioxidant substitute [13]. Fortunately, that strain does not produce any mycotoxin due to the absence of the related genes thanks to its genome sequencing [12].

A. tubingensis is a black fungus that can be found in warm climates and tropical areas; it is commonly isolated from soil or agricultural crop residues [14]. It has the

capacity to grow in a wide range of temperatures; the optimal conditions being between 21°C to 36°C [14]. In reference to the pH, this fungus has a large range of tolerance between 2 to 7 [14].

In the literature, it is known that *A. tubingensis* can produce many metabolites such as citric acid [15–17], ascorbic acid [15] and NGPs [13]. Nevertheless, NGPs can also be found in other microorganisms; hence, they are produced not only by a wide variety of filamentous fungi but also by lichen [18], higher plants [19–21] and by echinoderms [22, 23].

The fungal NGPs are produced intracellularly and are linked to the sporulation of the fungus; they can be produced by *Aspergillus* and *Fusarium* genus [24]. The NGPs synthesized by *Aspergillus* from the *Nigri* section may be found in species such as *A. fonsecaus* [25], *A. awamori* [26], *A. tubingensis* [10] and *A. niger* [24, 27]. Otherwise the NGPs synthesized by *Fusarium* species can be found in the species *F. culmorum* and *F. gramineum* [28, 29]. Due to their chemical structure, they have many benefits, being endowed with antibacterial [30], antifungal [31], antioxidant [32], antiallergic, antidiabetic [33], antitumor [34], anticancer [35] and antiviral capabilities [29]. Concerning their chemical structure, the C13 (C6-C4-C3) basic skeleton consists of a naphthalene core and a pyrone core; although the naphthopyrone group comprises 18 isomeric forms, only three can exist as NGPs (Fig. 1A). The Greek letters α, β, γ indicate the position of the oxygen atom relative to the carbonyl group on the pyrone core [36]. The NGPs can appear in the form of monomers (flavosperones, rubrofusarines and fonscines) or dimers (aurasperones and asperpyrones) [35] (Fig. 1B). Owing to their structure, the NGPs have hydrophobic properties, allowing their extraction by a large number of organic solvents [19], such as ethyl acetate [36–38], acetone [38] and methanol [10, 11] [24], along with a few different combinations of solvents also mentioned in the literature: chloroform/methanol (2:1, v/v) [3, 31], acetone/ethyl acetate (1:1, v/v) [3], and methanol-dichloromethane-ethyl acetate (1:2:3 v/v) [3]. Besides, they can be co-extracted with other hydrophobic compounds and the ethanol solvent allowing accreditation for the "nonorganic" label, making them attractive for industrial applications [19, 33, 39].

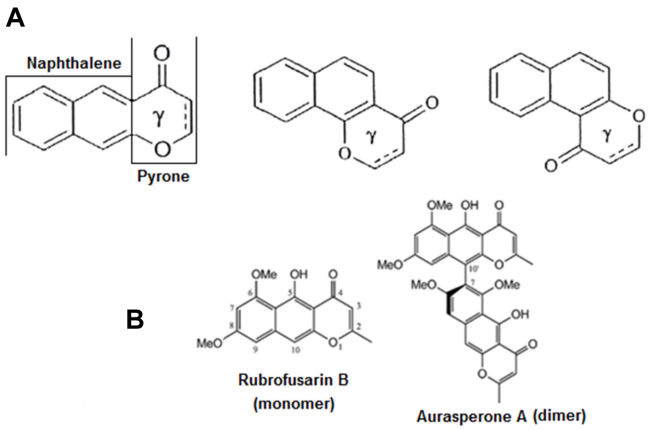


Fig. 1. Chemical structures of the NGPs.
(**A**) Basic skeleton of the NGPs with its three isomers forms. (**B**) Chemical structure of a monomer form (rubrofusarin) and a dimer form (aurasperone A).

The secondary metabolites produced by members of the *Aspergillus* genus have been produced, according to the literature, using different culture medium compositions but mainly in solid media; this has been the case with yeast extract sucrose (YES) agar, Sigma YES agar, oatmeal agar, malt extract agar and the potato sucrose agar [3], Czapek yeast autolysate agar (CYA) and CYA with 5% salt (CYAS) [3]. In the literature, the culture conditions vary from 25°C to 30°C for the temperature criteria, from 6 to 7 regarding the pH level and from 7 to 10 days of incubation depending on the author [10, 11]. Solid-State Fermentation (SSF) has been used during the last years in the industry and the research, in order to optimize the secondary metabolite production [40]; however, in solid culture medium the separation of the *Aspergillus* biomass from the medium is difficult (because the biomass sticks and degrades the solid medium), causing contamination in the biomass extract.

Otherwise, a few authors have proved that some secondary metabolites in filamentous fungi can be regulated by stress triggers [39], as is the case with oxidative stress by the addition of ROS (reactive oxygen species) [41]. Concerning the activity of water (a_w), the addition of substances including glycerol or certain types of salt and sugar can decrease the a_w and consequently cause an osmotic stress and the morphology and the metabolite production of the microorganism can be affected [42, 43].

Several studies demonstrated that the use of containers with different geometry and distribution of the liquid medium gave different results, due to the different amounts of oxygen available but also due to the diameter of the inlet air and the different surface area [36, 44, 45].

Thereby, in this study *A. tubingensis* was used to produce NGPs, using liquid culture media, with the intention to implement bioreactors in the future. The objective of this study was to compare culture performance using different container types, *i.e.* Fernbach flask, Erlenmeyer flask and Bioassay plate, under different conditions, as inducing

stress (osmotic and oxidative) can scale up the NGP production for industrial purposes.

Materials and Methods

The path that was followed during this research is summarized in Fig. 2.

Strain

All experiments were performed with the *A. tubingensis* G131 strain, isolated from a French Mediterranean vineyard and stocked as a spore suspension in a Tween 0.5% (v/v) solution and glycerol 20% (v/v) at −20°C [14]. Spore production for stock or inoculum was carried out using Czapek Yeast extract Agar (CYA) composed of (g/l): sucrose (30); yeast extract (5); agar (15); 50 ml of solution A (in g/0.5 L distilled water): $NaNO_3$ (20); KCl (5); $MgSO_4{\cdot}7H_2O$ (5) and $FeSO_4{\cdot}7H_2O$ (0.1); 50 ml of solution B (in g/0.5 L distilled water): K_2HPO_4 (10) and 1 ml of a mineral solution composed of (g/0.1 L): $ZnSO_4{\cdot}7H_2O$ (1) and $CuSO_4{\cdot}5H_2O$ (0.5) [10], in Petri dishes (94mm x15mm) and incubated at 28°C in an a Votsch chamber (Memmert CTC256) at 90% of initial humidity during 7 days. The fungal spore suspension was performed with 5 ml of 0.5% Tween (v/v) solution in each Petri dish.

Control Culture Conditions

The basal culture conditions for the production of NGPs are the following: Czapek Yeast extract Broth (CYB) composed as the CYA mentioned in section 2.1 but without the agar [10]. The pH was adjusted at 5 using HCl solution (1N) and this pH was defined after a preliminary test of production of NGPs at pH 3, 5 and 7. The medium was inoculated with a spore suspension obtained as described in section 2.1; the inoculum rate was 5.10^4 spores/ml of liquid medium. The static condition and temperature at 28°C were set based on previous unpublished results. The optimum incubation time, 9 days, was established by a kinetic that monitored the production of metabolites and assimilation of nutrients.

Influence of Other Parameters

Under static conditions in Erlenmeyer flasks, the production of NGPs occurs on the fungal mycelium, which develops on the surface of the medium that is in contact with oxygen. For this reason, the culture surface seems to have a direct impact on fungal biomass production. However, as NGPs are secondary metabolites, a higher production of biomass does not guarantee a higher production of NGPs. Based on this information, tests were carried out in different containers, with the objective of knowing the impact of the geometry, medium distribution and oxygen availability on the production of NGPs. Moreover, in previous researches the production of biomass and secondary metabolites has been linked with stress conditions such as oxidative and osmotic stress.

Containers. After determining the control culture conditions in Erlenmeyer flasks, the objective was to study the influence of the container geometry (Fig. 2) (surface area in contact with air,

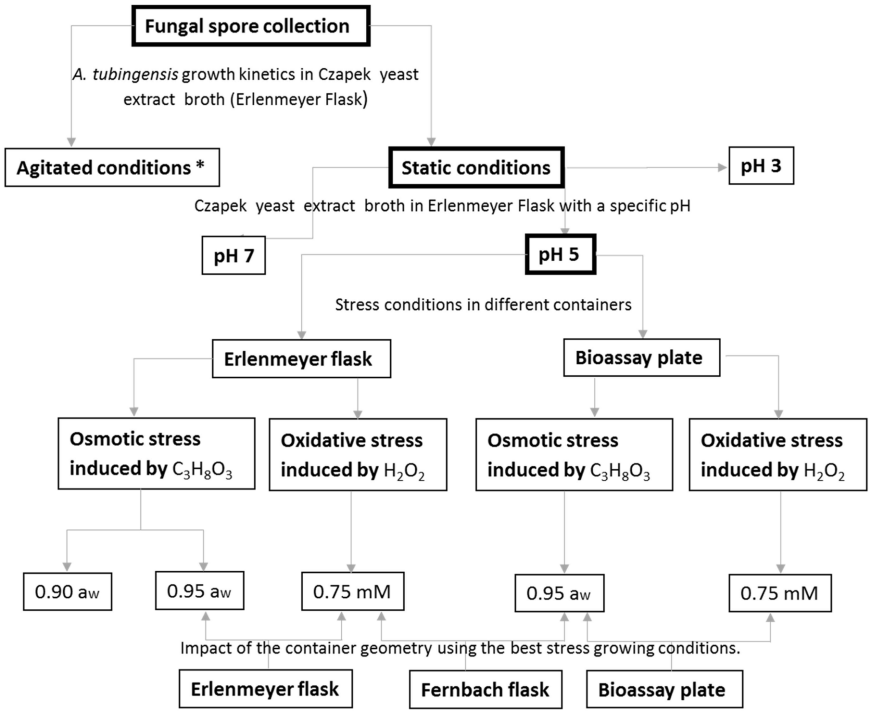


Fig. 2. General schema of the methodology used during the research.
*The agitated conditions were carried out on an orbital shaker (New Brunswick, Innova 44/44R) at 120 rpm in flasks without baffles.

height of the culture medium, air inlet and amount of oxygen available) with the goal of increasing the production of the metabolites of interest.

Osmotic stress. Osmotic stress was induced by adding pure glycerol ($C_3H_8O_3$) to the medium, and based on literature four different values of activity of water (a_w) were tested: 0.80, 0.85, 0.90, 0.95 in comparison to the control at 0.99 [39]. In previous studies, it has been reported that the addition of some substances such as glycerol, sodium chloride, sorbitol or potassium chloride can influence the mycelial growth and sporulation [39].

Oxidative stress. Oxidative stress conditions were performed by adding hydrogen peroxide 30% (H_2O_2) to the culture medium, in order to obtain eight different concentrations: 0.05, 0.10, 0.25, 0.50, 0.75, 1, 5, and 10 mM [41].

Dry Fungal Weight

At the end of the incubation time (9 days), the liquid medium was removed, the fungal mycelium was recovered and the wet biomass was weighed (wet biomass). Subsequently, the NGP extraction was performed. At the end of the NGP extraction, the biomass was placed in an aluminum tray and dried in an oven at 105°C for 24 h in order to obtain the dry weight. It is important to mention that the dry biomass cannot be measured before the NGP extraction, and it is the only manner to obtain the dry fungal weight.

Secondary Metabolite Extraction

NGP extraction was performed by adding 30 ml of pure ethanol (C_2H_5OH) per each gram of dry fungal biomass. After adding the solvent, the sample was incubated for 20 min at room temperature and subsequently sonicated for 20 min at 50 Hz in a Mechanical Ultrasonic Cleaning Bath (Fisher FB 15051).

The next step consists of a filtration of the solution using a 113 V Whatman qualitative filter paper grade (GE Healthcare) then a centrifugation at 5,000 RPM = 4,696 ×*g* (Thermo Scientific Heraeus Megafuge 16R Centrifuge) for 20 min, was conducted to eliminate the rest of the fungal biomass.

NGP Quantification

In order to eliminate other contaminants from the obtained crude extract, a supplementary filtration was conducted using a PTFE 0.45 µm filter (VWR international). Total quantification of the NGPs was performed by High-Performance Liquid Chromatography (HPLC) (Dionex Ultimate 3000RS/Column C18-SH inverse phase (prontosil 120-5C18-SH), 5.0 µm, 150 × 4.0 mm) coupled with a DAD. HPLC conditions were performed as follows: 1 ml/min flow rate, column temperature at 30°C and acidified water/acetonitrile eluents (70:30, *v/v initial ratio, gradient elution*). Quantification of the NGPs was made at 280 nm using a calibration curve (25 to 1,000 mg/l) of the commercial standard rubrofusarin. NGPs concentration is reported in mg/l of crude extract.

Antioxidant Activity

The antioxidant properties were analyzed by the Trolox Equivalent Antioxidant Capacity method using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and ABTS (2.2′-azino-bis-3-etilbenzothiazoline-6-sulfuric acid) [10, 11], the spectrophotometer Anthelie UV/Visible Light Advanced (SECOMAM Anthelie Advanced) at λ(em) = 734 nm were utilized to measure the absorbance. Microsoft Excel software 2016 (Microsoft, USA) was used as a statistical tool.

Culture Medium Analysis

The culture medium composition was analyzed, in terms of nitrogen and sucrose sources, using colorimetric methods, such as YAN (Yeast Assimilable Nitrogen), a method to measure the primary organic (free amino acids) and inorganic (ammonia and ammonium) sources of nitrogen that can be assimilated [46], DNS (3,5-Dinitrosalicylic acid), a method to measure the presence of free carbonyl group (c=o) (in this case a hydrolysis with HCl was required) [47], and Brucine-nitrogen colorimetric, a method to determine the nitrate-nitrogen in an aqueous solution (method based on the reactions involving sulfanilic acid and the nitrate-nitrogen present in a sample) [48, 49].

Results and Discussion

Kinetics of Growth under Static Conditions

The dry fungal biomass, NGP production and the TEAC in control conditions are shown in Fig. 3. The kinetics initiate from day 4 due to the day being when the fungal biomass weight can be measured. The highest NGP production is reached at day 10, the moment when the dry fungal weight began to decrease, perhaps due to a degradation of the fungal biomass [50]. It is well documented that the hyphal growth stops after a scarcity of nutrients [51].

The highest dry fungal biomass production was reached at days 8 to 11 (more than 2 g), in relation with NGP production, where the maximum level was obtained between days 9 to 11 (more than 0.16 mg/ml). Moreover, the antioxidant capacity reached maximum value at the same time (days 9 to 11, more than 0.5 mM). It could be assumed that considering the dry fungal weight, NGP production and antioxidant capacity in the Erlenmeyer flask, the maximum production was observed between days 8 to 11 because from day 12 we noticed the beginning of a degradation of the biomass and a potential loss of NGP production.

The culture medium composition was analyzed in terms of nitrogen and sucrose sources. According to this information, the carbon-nitrogen ratio (C:N) was determined in order to know if there is any relationship between this rate

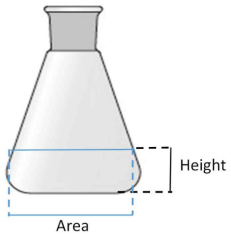
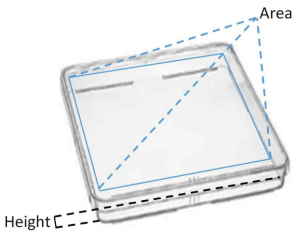
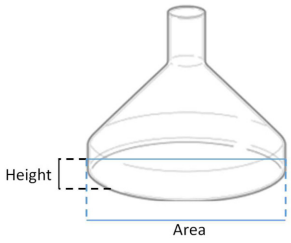
Erlenmeyer Flask (Fisher) Area : 56 cm^2 Volume of medium : 200 ml	Bioassay plate (Nunc) Area : 500 cm^2 Volume of medium : 300 ml	Fern Bach Flask (Duran) Area : 299 cm^2 Volume of medium : 300 ml
		
Medium Height : 3.2 cm	**Medium Height :** 1 cm	**Medium Height :** 1.8 cm
Ratio Surface area / volume: 0.28	**Ratio Surface area / volume:** 1.66	**Ratio Surface area / Volume:** 0.99
Ratio Surface area / height: 18	**Ratio Surface area / height:** 500	**Ratio Surface area / height:** 166

Fig. 3. Different containers used for the current study.

and the NGP production (Table 1). In the literature, there is a large number of studies conducted with the purpose of determining the effect of the nutrients on the growth and sporulation of fungal species. Accordingly, we evaluated the effects of carbon concentration and carbon-to-nitrogen ratio on the biomass and NGP production [52, 53]. On one hand, the nitrogen source (tryptone, urea, soy peptone and yeast extract) is one of the nutrients with more influence in the sporulation [54, 55]. On the other hand, a higher C:N ratio can also influence the growth and sporulation because this ratio affected the number of conidia produced [56–59].

We determined the C:N ratio throughout the kinetic period; the first nine days, the C:N ratio ranged from 40:1 to 50:1. Moreover, the C:N ratio reached its maximum point at day 10 (53:1), at the same time the NGPs reached the highest production (Fig. 3). In previous publication, a higher C:N ratio (160:1) affected the sporulation, but by contrast, the optimal C:N ratio varied from 10:1 to 80:1 depending on the fungal isolation [60, 61]; however, it was not the case in our work.

After 11 days of incubation, the C:N ratio had fallen dramatically, maybe because the source of nutrients was exhausted.

Table 1. Kinetics of nutrient assimilation in static condition, during 13 days.

							Ratio C:N	
Time (Day)	Sucrose (g/l)	NaNO3 (g/l)	mgC (mg/l)	mol C (mol/l)	mgN (mg/l)	mol N (mol/l)	Ratio (mgC:mgN)	Ratio (molC:molN)
0	30.00	2.00	12642	1.053	330	0.024	38 : 1	45 : 1
4	24.46	0.70	10306	0.858	278	0.020	37 : 1	43 : 1
5	24.95	0.59	10512	0.875	228	0.016	46 : 1	54 : 1
6	15.42	0.48	6497	0.541	161	0.012	40 : 1	47 : 1
7	14.24	0.28	6002	0.500	121	0.009	50 : 1	58 : 1
8	11.19	0.18	4714	0.393	99	0.006	48 : 1	56 : 1
9	8.88	0.16	3743	0.312	84	0.006	45 : 1	52 : 1
10	4.77	0.07	2010	0.167	38	0.003	53 : 1	62 : 1
11	1.74	0.10	734	0.061	42	0.003	17 : 1	20 : 1
12	1.06	0.08	445	0.037	30	0.002	15 : 1	17 : 1
13	0.08	0.06	32	0.003	23	0.002	1 : 1	1 : 1

Cultures performed in Erlenmeyer flask with CYB, Czapek yeast broth, at pH 5 and 28°C in static conditions (control conditions).

Impact of pH on Fungal Growth and NGP Production

Although *A. tubigensis* has a wide pH range of tolerance [3], in the literature it is known that the pH influences the fungal growth and metabolite production. In the case of sporulation, most of the *Aspergillus* species have an optimal sporulation pH between 5 to 7 [62]. Besides, in some research the acid pH of 2 to 3 has been studied due to its industrial advantage of favoring the sterility of the culture medium [63]. Because of this, concerning the pH level, three different initial pH levels 3, 5, and 7 were tested in control conditions. The pH behavior in all the cases was similar; it decreased to 3 rapidly after 48 h of incubation. The results were compared considering the parameters of TEAC, NGP and biomass production.

Based on the concentration of NGPs quantified by HPLC, at the initial pH 5, *A. tubingensis* produced the highest concentration of NGPs (0.18 mg NGPs/ml of liquid medium), compared to pH 3 (0.16 mg NGPs/ml of liquid medium), and also to pH 7 (0.16 mg NGPs/ml of liquid medium). With respect to the antioxidant activity, the variation between the three conditions was minimal with a value of 0.5 mM (TEAC) in all cases. Regarding the production of dry biomass, a similar level in all three cases was also observed (2.0 ± 0.5 g). Although in the literature, some fungal strains have significant differences depending on the pH, such as inhibition of sporulation at acid pH (2 to 3) [62], in the case of *A. tubingensis*, the differences concerning the NGP production varied between 10% in relation to the three pH levels.

Biomass Production

Effects of the agitation. In industrial applications, submerged agitated cultures are widely used in the commercial production of secreted secondary metabolites [64]. Experiments with *A. tubingensis* under agitation conditions have shown a different morphology comparing with static conditions. Under agitated conditions, the fungal morphologies observed were, pellets and free mycelium production without sporulation; the same behavior has been previously reported in literature on *A. niger* [64, 65]. However, although the biomass production was higher comparing with static conditions, the sporulation was limited and consequently NGP production was not observed.

Effects of container geometry. In some areas of research, many projects are conducted using a variety of container sizes and shapes in order to analyze the culture medium distribution and the volume of air space [64]. The size of the air entry and the material used to cover the entry also have an impact in the oxygen transfer [44, 45].

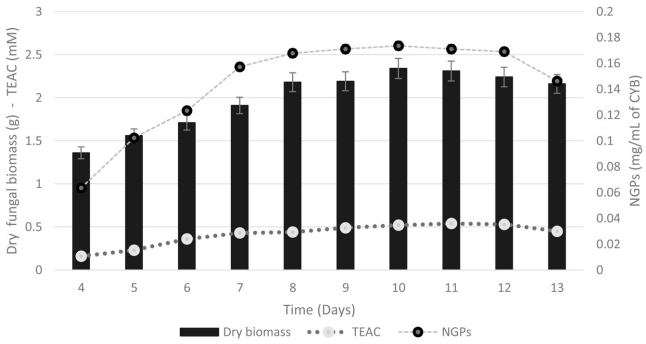


Fig. 4. Kinetics of fungal biomass, TEAC and NGPs production in static conditions, Cultures performed in Erlenmeyer flasks with CYB, Czapek Yeast Broth, at pH 5 and 28°C in static conditions (control conditions).

Based on this information, different containers, *i.e.* Erlenmeyer flask, bioassay plate, and Fernbach flask were tested. In Fig. 4, the biomass production using different containers is shown. Except in control conditions where the geometry of containers has no impact on the dry fungal weight (less than 10 g/l in each container), in the other conditions (stresses conditions) the type of container has an impact on the fungal biomass production. Thus, in Fernbach flask at 0.95 a_w, the maximum fungal biomass reached 20 g/l against 15 g/l in Erlenmeyer flask.

Effects of stress conditions. Other critical factors that are mentioned in the literature during the production of metabolites are the stress conditions [41]; because the objective in this study is to increase the production of NGPs, two different stress conditions that have been reported in previous studies, osmotic and oxidative stress, were chosen [39, 41].

In all cases of stress conditions applied, the fungal biomass production is higher than in control conditions. The higher fungal biomass yield was obtained in Fernbach flask under 0.95 a_w, as it reached more than 20 g/l. Besides, the addition of oxidative stress (0.75 H_2O_2) also has the capacity to increase the fungal biomass production but in less quantity (13 g/l). Additionally the mix of both stresses conditions (0.95 a_w + 0.75 Mm H_2O_2) gave interesting results in Erlenmeyer flask with the yield reaching more than 15 g/l.

In the literature, another point to consider is that the biomass production is not intimately linked with the production of metabolites because in some conditions the biomass begins to grow conically and thick towards the bottom of the container [41, 66].

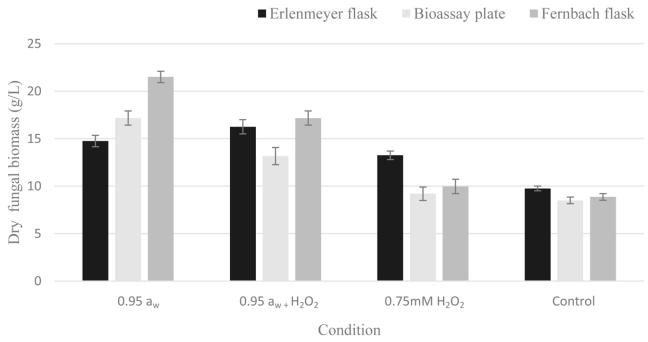


Fig. 5. Biomass fungal production (g/l) in different containers. Cultures performed with CYB, Czapek Yeast Broth, at pH 5 and 28°C, during 9 days in static conditions (control conditions).

NGP Production under Stress Conditions

NGP production under osmotic stress. The role of the a_w is considered as important an environmental parameter as the pH, temperature, CO_2, or nutrients. For example, a low value of a_w is a parameter documented in the literature linked with a decrease of the microorganism growth [67]. The decrease of the fungal growth was observed at the macroscopic level in the bioassay plate in the conditions 0.80, 0.85, and 0.90 a_w where the growth was considerably reduced comparing with the control (Fig. 5).

In the Fig. 5 the changes in the morphology induced by the decrease of the a_w is shown. At 0.95 a_w, the fungal biomass changes color from pale yellow to bright orange; in addition, it changes from a thin and fragile fungal biomass to one that is thick and dense. Some authors have mentioned that osmotic stress is capable of affecting hyphal morphology [68]. In some research, the evidence provided that some fungal species clearly indicate a connection between the osmotic stress regulatory system and the fungal development; it can affect the conidia viability and feasibility of spores [69].

In the case of *A. tubingensis*, the addition of glycerol to achieve the value of 0.95 a_w in bioassay plate and Erlenmeyer flask increased the spore quantity (in control conditions 1.5×10^9 spores/ml of liquid medium are counted against 2.4×10^9 spores/ml of liquid medium at 0.95 a_w). Moreover, the NGP production and antioxidant capacity increased. It is well known that the addition of some substances as glycerol, sodium chloride, sorbitol or potassium chloride can positively affect vegetative growth; in the case of *A. flavus* and *A. nidulans* it increased the conidiation [70]. However, the quantity of glycerol added needs to be controlled, because the fungal growth can decrease, as was observed in the conditions 0.80, 0.85, and 0.90 a_w (Table 2).

Among fungal genera, the osmotic stress response is quite diverse. For example, in the filamentous fungus *Fusarium graminearum*, a significant relationship between osmotic stress and the fungal morphology and secondary metabolism has been reported [71]. Furthermore, some studies have also shown that in some cases the response to osmotic and oxidative stress by fungi is linked to the biosynthesis of natural products, including mycotoxins [41]. Consequently, the osmotic stress affects not only fungal growth and development, but also the production of other secondary metabolites in *aspergilli* [72]. In Table 2 the results obtained under osmotic stress in Erlenmeyer flask and bioassay plate are shown, the higher production of NGPs was produced under condition 0.95 a_w in both containers.

NGP production under oxidative stress. In earlier studies, the addition of a reactive oxygen species (ROS) has been shown to influence secondary metabolite production [73].

In the Fig. 6 we observed that adding high quantities of H_2O_2 (5.00 to 10.00 mM), the fungal growth can be inhibited; we also observed the same reaction occurring under osmotic stress. Less tolerance of osmotic stress generally reduces the mycelial growth and inhibits metabolite production [74].

In the samples with less addition of ROS (1.00 mM to 0.0 5 mM), the fungal growth and sporulation appear but the morphology changes, in color and shape, compared to the control; it means that the fungal cells can adapt to low

Table 2. NGP production under osmotic stress in Erlenmeyer flask and bioassay plate.

Condition	Bioassay plate				Erlenmeyer flask			
	NGPs (mg)	CYB (ml)	NGPs/ml CYB	TEAC (mM)	NGPs (mg)	CYB (ml)	NGPs/ml CYB	TEAC (mM)
Control	41 ± 3	300	0.13	0.52	38 ± 3	200	0.18	0.59
0,95 a_w	83 ± 5	300	0.27	0.59	66 ± 4	200	0.30	0.89
0,90 a_w	9 ± 2	300	0.03	0.43	64 ± 6	200	0.32	0.95
0,85 a_w	0.9 ± 0.5	300	0.002	0.01	51 ± 5	200	0.25	0.69
0,80 a_w	19 ± 2	300	0.06	0.44	37 ± 4	200	0.18	0.66

Cultures performed with CYB, Czapek yeast broth, at pH 5 and 28°C during 9 days in static conditions (control conditions).

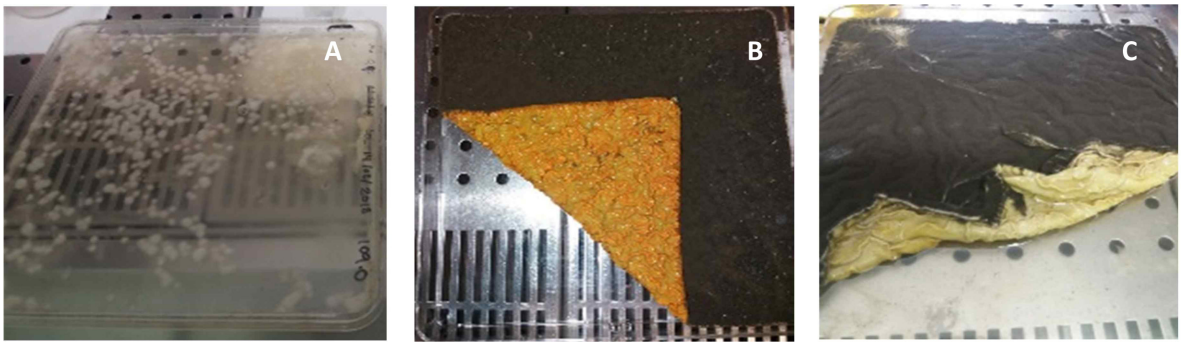


Fig. 6. *A. tubingensis* morphologies under different values of a_w in bioassay plate at pH 5 and 28°C during 9 days, static conditions. (**A**) Culture at 0.80 a_w (**B**) Culture at 0.95 a_w (**C**) Culture under control conditions (0.98-0.99 a_w).

concentrations of ROS but it causes a modification in its morphology.

In addition to morphology differences, Fountain *et al*. has reported that the ability to produce secondary metabolites was detected maybe as a consequence to maintain and regulate its metabolism under oxidative stress; it was already observed in previous studies where the aflatoxin production from *A. flavus* isolates were correlated with the concentration of H_2O_2 added [75].

In Table 3, it was observed that the NGP production increased comparing with the control, and consequently the antioxidant activity. However, comparing with the samples under osmotic stress the NGP production was lower (Tables 2 and 3).

NGP production under stress conditions in different containers. Finally, the impact of the container geometry under the condition 0.95 a_w was tested. Table 4 reports a summary of the results obtained in three different containers, with the highest NGP production obtained in the Fernbach flask under 0.95 a_w. It is important to mention that the surface area is lower comparing with the bioassay plate area; it means that a long surface area is not always correlated with an increase in the production of NGPs.

Results demonstrated that the NGP production can be enhanced by reducing the value of a_w (0.95). In the Erlenmeyer flask the production reached 0.30 mg/ml of liquid medium, and in the bioassay plate it reached 0.27 mg/ml of liquid medium and in the Fernbach flask 0.36 mg/ml of liquid medium. The oxidative stress leads to an increase in the NGP production comparing to the control conditions, but the results are less interesting comparing with osmotic stress conditions. Findings also show that agitation conditions favor the production of fungal biomass, while static conditions favor fungal sporulation. In addition, a significant alteration of fungal morphology was observed which depended on the culture conditions.

In conclusion, we have demonstrated that the induction of an osmotic stress (0.95 a_w) by glycerol or an oxidative stress (0.75 mM) by H_2O_2 may increase the NGP production, the biomass production and the antioxidant activity in the

Table 3. NGP production under oxidative stress in Erlenmeyer flask and bioassay plate.

Condition	Bioassay plate				Erlenmeyer flask			
	NGPs (mg)	CYB (ml)	NGPs (mg)/ml CYB	TEAC (mM)	NGPs (mg)	CYB (ml)	NGPs (mg)/ml CYB	TEAC (mM)
Control	40 ± 2	300	0.12	0.54	38 ± 2	200	0.18	0.59
0.05 mM H_2O_2	46 ± 5	300	0.15	0.67	68 ± 4	200	0.34	0.85
0.10 mM H_2O_2	42 ± 5	300	0.14	0.61	42 ± 5	200	0.20	0.78
0.25 mM H_2O_2	37 ± 7	300	0.12	0.52	43 ± 5	200	0.21	0.77
0.50 mM H_2O_2	49 ± 8	300	0.16	0.68	50 ± 2	200	0.24	0.81
0.75 mM H_2O_2	60 ± 8	300	0.19	0.75	44 ± 9	200	0.21	0.75
1.00 mM H_2O_2	55 ± 3	300	0.18	0.71	42 ± 5	200	0.21	0.73
5.00 mM H_2O_2	0	300	0	0	0	200	0	0
10.00 mM H_2O_2	0	300	0	0	0	200	0	0

Cultures performed with CYB, Czapek yeast broth, at pH 5 and 28°C during 9 days in static conditions (control conditions).

Table 4. Effect of the geometry container under osmotic stress.

Condition	CYB (ml)	0.95 a_w NGPs (mg)	NGPs (mg)/CYB (ml)	Control NGPs (mg)	NGPs (mg)/CYB (ml)	Surface area (cm^2)
Erlenmeyer flask	200	62 ± 6	0,30	38±3	0,18	56
Bioassay plate	300	83 ± 5	0,27	41±5	0,13	500
Fernbach flask	300	111 ± 15	0,36	50±3	0,16	299

Cultures performed with CYB, Czapek yeast broth, and pH 5 at 28°C during 9 days in static conditions (control conditions).

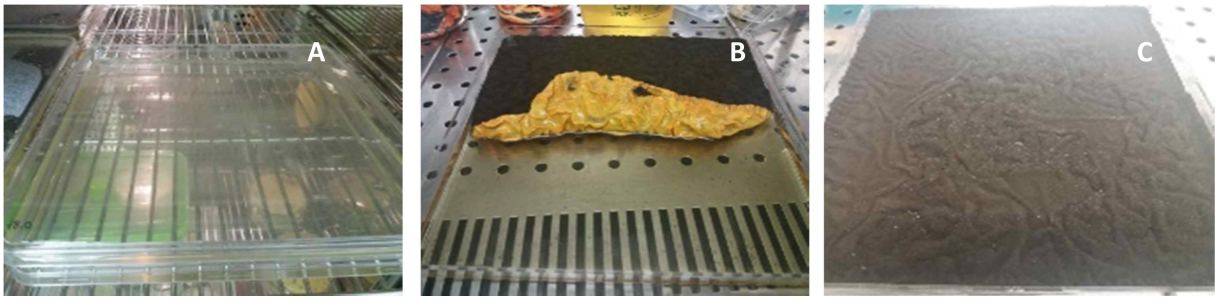


Fig. 7. *A. tubingensis* morphologies under different oxidative stress in bioassay plate at pH 5 and 28°C during 9 days, static conditions.
(**A**) Culture with 10.00 mM of H_2O_2 (**B**) Culture with 0.75 mM of H_2O_2. (**C**) Culture under control conditions.

A. tubigensis strain, in addition, the container can also influence the NGP production depending on the geometry and the medium distribution on it. This research represents a relevant step to improve the knowledge about the production of NGPs from fungal cultures and optimize the bioprocess, due to the prospective commercial value of the molecule produced.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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