Mycopathologia (2010) 169:387–394
DOI 10.1007/s11046-009-9268-7

Medium Selection and Effect of Higher Oxygen Concentration Pulses on *Metarhizium anisopliae* var. *lepidiotum* Conidial Production and Quality

Saul Tlecuitl-Beristain · Gustavo Viniegra-González · Gerardo Díaz-Godínez · Octavio Loera

Received: 31 July 2009 / Accepted: 15 December 2009 / Published online: 29 December 2009

Abstract Rice and oat flours were analyzed as media for the production of conidia by *M. anisopliae* var. *lepidiotum*. The presence of peptone increased conidia yield regardless of the substrate used; however, the highest yield was achieved on oat flour media. The effect of oxygen on conidia production using oat-peptone medium was also studied at two levels: Normal atmosphere (21% O_2) and Oxygen-rich pulses (26% O_2). Maximum conidia production (4.25×10^7 conidia cm^{-2}) was achieved using 26% O_2 pulses after 156 h of culture, which was higher than 100% relative to conidial levels under normal atmosphere. Conidia yield per gram of biomass was 2.6 times higher with 26% O_2 (1.12×10^7 conidia mg^{-1}). Conidia quality parameters, such as germination and hydrophobicity, did not show significant differences ($P < 0.05$) between those treatments. Bioassays parameters, using *Tenebrio molitor* adults, were analyzed for conidia obtained in both atmospheres and data were fitted to an exponential model. The specific mortality rates were 2.22 and 1.26 $days^{-1}$, whereas lethal times for 50% mortality were 3.90 and 4.31 days, for 26% O_2 pulses and 21% O_2 atmosphere, respectively. These results are relevant for production processes since an oxygen increase allowed superior levels of conidia by *M. anisopliae* without altering quality parameters and virulence toward *Tenebrio molitor* adults.

Keywords *Metarhizium anisopliae* · Oxidative stress · Virulence · *Tenebrio molitor* · Conidial quality

S. Tlecuitl-Beristain · G. Viniegra-González · O. Loera (✉)
Departamento de Biotecnología, Universidad Autónoma Metropolitana-Iztapalapa, CP 09340 Iztapalapa, Mexico, DF, Mexico
e-mail: loera@xanum.uam.mx

G. Díaz-Godínez
Universidad Autónoma de Tlaxcala, Center for Biological Science Research, Tlaxcala, Tlaxcala, Mexico

Introduction

The fungus *Metarhizium anisopliae* is considered as an alternative for agricultural pest control. One limiting factor for its use as a biocontrol agent is the production of biomass and conidia in large-scale on artificial substrates [1]. Recently, cultivation methods have been optimized for the production of *M. anisopliae* conidia on natural substrates such as sugar cane bagasse [2], rice, sorghum and barley [3], and even insect cadavers like *Coptotermes formosanus* [4]. Artificial substrates have also been used including yeast extract-enriched Potato Dextrose Agar (PDA) [5–7], yeast extract-enriched Sabouraud Dextrose Agar (SDA) [4], and Malt

Extract Agar (MEA) [5]. Commonly, culture conditions such as substrate humidity and pH are manipulated for the production of conidia in solid media [2, 3]; moreover, some conditions have also been studied for the production of blastospores in liquid culture [8]. These works have contributed to obtaining greater yields of conidia and blastospores; nonetheless, parallel studies are necessary in order to determine the effect on quality parameters of those infective units.

The effect of aeration on *M. anisopliae* var. *acridum* cultures has been analyzed; nevertheless, conidia production did not show an effect as a result of the increase in aeration [2]. However, the increase in dissolved oxygen during the production of blastospores in a liquid culture resulted in a greater production level when compared to a non-regulated media [8], using a strain of *M. flavoviride* which was reassigned as *M. anisopliae* var. *acridum* as suggested previously [9]. In this context, aerobic organisms such as *M. anisopliae* use oxygen as a final electron acceptor in respiration for obtaining energy, facing the toxicity of the reactive oxygen species or ROS [10]. ROS are generated also under the influence of both internal and external factors, such as light and high temperature, ionizing radiation, changes in the environment atmospheric composition, and osmotic pressure [11].

The presence of ROS in an oxidant state has been described as a trigger of cellular differentiation and conidiation in *Neurospora crassa* [12, 13]. In the case of *M. anisopliae,* a recent report correlated oxidative stress with the production of mycotoxins against *Spodoptera litura* larvae [14]. In addition, Wang et al. [15] correlated an osmosensor with the response to oxidative stress caused by hydrogen peroxide. Nevertheless, there are no reports analyzing the effect of an oxygen-rich atmosphere on *M. anisopliae* conidiation, since this could be related to oxidative stress causing a faster cellular differentiation as mentioned earlier.

In this study, the effect of oxygen was determined on conidia production by *M. anisopliae* var. *lepidiotum*. The evaluation also comprised the quality of conidia harvested from two different atmospheres, as well as their virulence against adults of the yellow mealworm beetle *Tenebrio molitor*.

Materials and Methods

Microorganism

The isolate *Metarhizium anisopliae* CP-Oax belongs to the fungal collection of the *Colegio de Posgraduados* in Texcoco, Mexico. A monosporic culture was obtained on Potato Dextrose Agar (PDA) after 7 days at 28°C. PDA was used as propagation medium for obtaining inocula for all experiments.

Molecular Identification of Strain CP-Oax

Fungal DNA was extracted according to a protocol DNA Purification Kit (Wizard® Genomic Promega: num. Cat. A1120). Polymerase chain reaction (PCR) amplification was carried out using ITS5 and ITS4b primers with PCR conditions reported by [16]. Primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4b (5′-TCCTCCGCTTATTGATATGC-3′) have been designed from fungal 18S-rDNA and 28S-rDNA conserved sequences, respectively [16]. The nucleotide sequence from the amplified fragment was compared with published fungal sequences through a standard nucleotide–nucleotide BLAST homology search (http://www.ncbi.nlm.nih.gov/BLAST), which allowed to assign this isolate as *Metarhizium anisopliae* var. *lepidiotum* strain CP-Oax (GenBank accession number is FJ876298). A recent report by Bischoff et al. [17] suggests *Metarhizium lepidiotae* as a current name for this organism.

Culture Media

Culture media containing rice or oat flour (particle sizes under 0.25 mm) as main carbon sources were analyzed. The effect of meat peptone (Bioxon) on conidia production in these media was evaluated. The four media contained (g l^{-1}): (a) rice flour: 33.3; (b) oat flour: 33.3; (c) rice flour: 33.3, and meat peptone: 10; and (d) oat flour: 33.3, and meat peptone: 10. The theoretic C/N ratios for each culture medium were 20.46, 15.71, 6.85, and 5.73, respectively. All these media contained bacteriological agar (Bioxon) at a final concentration of 15 g l^{-1}, and initial pH value was adjusted to seven. The experimental units were 120-ml serological bottles (Wheaton) containing

10 ml of culture medium in each bottle, which were inoculated with 1×10^7 conidia per bottle (6.9×10^5 conidia cm^{-2}). These bottles were then incubated at 28°C for 7 days and conidia were harvested using 10 ml of a Tween 80 solution (0.01%) by agitation with a magnetic stirrer. The conidia count was performed in a Neubauer chamber.

Effect of the Atmosphere on Conidia Production

Metarhizium anisopliae var. *lepidiotum* was grown in selected culture medium based on oat flour and peptone (medium "**d**"). For every sampling period, three replicates were carried out for each treatment: normal atmosphere (21% O_2) and oxygen-enriched (26%) pulses. The gaseous mix containing 26% O_2 was manufactured by the Praxair Company (Mexico). In the first treatment, the serological bottles were loosely closed with cotton caps in order to facilitate continuous gas exchange with external atmosphere. The second treatment began similarly in a normal 21% O_2 atmosphere (with cotton caps), and after 60 h of culture (pre-stationary phase), cotton caps were aseptically replaced for rubber hermetic seals. Then, atmosphere was periodically flushed with 26% O_2 pulses until the end of cultures as follows: two needles were simultaneously placed in the rubber seals, and a modified atmospheric (26% O_2) flow of 20 cm^3 s^{-1} was passed through one needle into the experimental unit for one min; the second needle worked as a constant outlet during flushing, thus a complete atmospheric replacement was achieved. Both needles were removed after this process, and these atmospheric exchanges were repeated every 24 h for the second treatment. Samples from both treatments were taken every 12 h for biomass and conidial production estimations. Conidia were harvested by rinsing the surface with 10 ml of a 0.01% Tween 80 solution, and production was reported as conidia for unit area of agar surface (conidia cm^{-2}).

Determination of Biomass

Biomass (X) was determined by dry weight measurement. Following conidia collection, 20 ml of distilled water were added to the bottles and then heated briefly in a microwave oven (Mabe, Litton) for 30 s in order to ease liquefaction of agar media, which was filtered through a Whatman No. 1 filter paper. This harvested material was then rinsed with 40 ml of boiling water in order to eliminate agar residues. Filter paper with the biomass was dried at 60°C for 24 h in order to determine dry weight of biomass. Results show mean values for three replicas.

Germination

Germination percentage (G) was determined for the conidia freshly harvested at the time of maximum production (156 h). Under aseptic conditions, one drop of 1.5% water-agar solution (Bioxon) was placed on sterile slides [18]. These were allowed to cool for approximately five min, and then 100 µl of a 1×10^6 conidia ml^{-1} dilution was placed on the surface and a cover slip was used for every preparation. Then, slides were placed on a damp filter paper disc inside a Petri dish and incubated at 28°C for 18 h. Germination percentage was determined under the microscope, this included a count of at least fifty conidia. One conidium was considered to be germinated if germination tube was greater than diameter of the ungerminated conidia [19].

Conidial Hydrophobicity

Conidial surface hydrophobicity (H) was assessed using an aqueous-solvent partitioning assay with *n*-hexadecane (Sigma) as the organic phase [20]. *Metarhizium anisopliae* conidia from each treatment were suspended in 0.1 mol l^{-1} KNO_3 solution and adjusted to a final concentration of 1×10^7 conidia ml^{-1}. Optic density (OD) of the conidial suspension, after a 30 s agitation in a vortex, was determined at 660 nm using a spectrophotometer (Beckman DU-640, USA) and this was referred to as OD_{total}. The conidial suspension (3 ml) was then transferred to a tube containing one ml of *n*-hexadecane and agitated for 20 s. After 30 min, the organic phase was removed using a Pasteur pipette, the aqueous phase (aq) was collected, and OD was recorded after a brief agitation on a vortex as mentioned earlier since conidia tend to settle down. Relative hydrophobicity was then determined as follows:

$$\text{Hydrophobicity} = 100\left(1 - \frac{\text{OD}_{\text{aq}}}{\text{OD}_{\text{total}}}\right) \quad (1)$$

where: OD_{total} and OD_{aq} represent values for initial sample and post-treatment aqueous phase, respectively [20].

Kinetic Parameters

The evolution of dry biomass $X = X(t)$ was followed by the Velhurst-Pearl or logistic equation

$$\frac{dX}{dt} = \mu\left[1 - \frac{X}{X_{\max}}\right]X \quad (2)$$

where: μ is the maximal specific growth rate (h^{-1}) and $X_{\max}$ is the maximum (or equilibrium) biomass level, reached when $\frac{dX}{dt} = 0$ for $X > 0$. The solution for Eq. 2 is the following:

$$X = \frac{X_{\max}}{1 + Ce^{-\mu t}} \quad (3)$$

where: $C = \frac{X_{\max} - X_0}{X_0}$; and X_0 is the initial biomass value.

Estimation of kinetic parameters in the previous equation was performed using a non-linear least square-fitting by the Solver routine (Excel, Microsoft). Conidia yield in terms of biomass ($Y_{C/X}$) was estimated as conidia production divided by dry biomass weight. Productivity (P) was calculated dividing conidia production by harvesting time.

Virulence Bioassay

Conidia obtained at 156 h of culture in 21 and 26% oxygen atmospheres were assayed separately against adult *Tenebrio molitor* insects. The insects were immersed for three s in a 10-ml conidial suspension (1×10^8 conidia ml^{-1}). A 0.01% Tween 80, conidia-free solution was used as a control. Experimental units consisted of Petri dishes containing 12 insects with oat flakes as food. These plates were kept at 28°C with a 12:12-h photoperiod. Bioassay was monitored for 7 days. Four replicas were made for each treatment. Mortality was recorded every 12 h, and dead insects were transferred to damp chambers in order to facilitate external sporulation and confirm death by fungal infection [6]. Time to reach 50% mortality (LT_{50}) was estimated from plotting accumulated mortality *versus* time. Data were modeled by an exponential decay function, as follows:

$$Y = (100 - S)e^{-k(t-t_0)} + S \quad (4)$$

$Y = 100$; if $0 \leq t \leq t_0$where: Y is the survival percentage at time t; k is the specific death rate ($days^{-1}$); t_0 is the time delay for the first death to occur (d), and S is the estimated asymptotic survival level (%). This model corresponds to the solution of a first-order differential equation with the indicated time delay, the aforementioned initial condition, and the asymptotic value $Y \to S$ for $t \to \infty$ [21].

Statistical Analysis

The results obtained in this study were expressed as mean values ± SD. Values were compared by a standard Student's test. Data for culture medium selection were analyzed using one-way analysis of variance (ANOVA), using the Tukey least significant difference test at $P < 0.05$. Statistical analysis was done with the software NCSS.

Results

Conidia production levels in different culture media are shown in Fig. 1. Production levels were different for every substrate after 7 days of incubation; however, oat flour media were the best substrates for conidia production. On the other hand, the addition of peptone as a nitrogen source favored a threefold

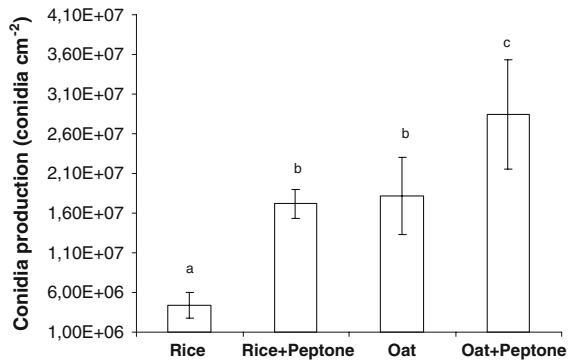

Fig. 1 Conidia production by *Metarhizium anisopliae* var. *lepidiotum* on simple rice or oat-based media and with addition of peptone. Columns with the same letter did not show significant differences ($P < 0.05$)

increase in conidia yield obtained on rice media; similarly, peptone enhanced 56% conidial yield on oat-based media.

The growth profile of *M. anisopliae* var. *lepidiotum* is shown in Fig. 2. In the atmosphere containing 21% O_2, the pre-stationary phase occurred after 60 h, corresponding to the point where normal atmosphere was modified in the parallel treatment, using 26% oxygen pulses. Logistical equation adjustments (2) were analyzed only for the normal atmosphere, since the 26% oxygen atmosphere was modified in the pre-stationary phase of growth (see Materials and Methods). Specific growth rate (μ) for the 21% O_2 atmosphere was 0.04 $h^{-1} \pm 0.002$.

Conidia production in both atmospheres is shown in Fig. 3. In the atmosphere at 26% O_2, maximum production (4.25×10^7 conidia cm^{-2}) was achieved after 156 h of culture, thus 96 h after oxygen pulses initiated. In terms of conidial production, this values represented a 100% increase compared to that obtained with normal atmosphere, and 10 times higher than production level achieved on rice flour medium (Fig. 1).

Table 1 shows growth parameters for *M. anisopliae* var. *lepidiotum* as well as conidia productivity at 156 h of cultivation. Maximum biomass was obtained in the normal atmosphere, while conidia production was greater as a result of oxygen-enriched pulses. Conidia yield in relation to biomass ($Y_{C/X}$) was 2.6 times greater (1.12×10^7 conidia mg^{-1} of dry biomass) in the 26% O_2 pulses, compared to the 21% O_2 atmosphere (0.43×10^7 conidia mg^{-1} of dry

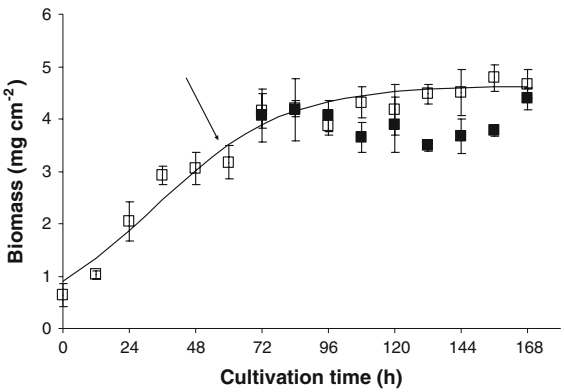

Fig. 2 *Metarhizium anisopliae* var. *lepidiotum* biomass grown under normal atmosphere (*open square*) or modified atmosphere by pulses (26% O_2) from the pre-stationary phase (*filled square*). *Arrow* shows initial time for atmosphere modification

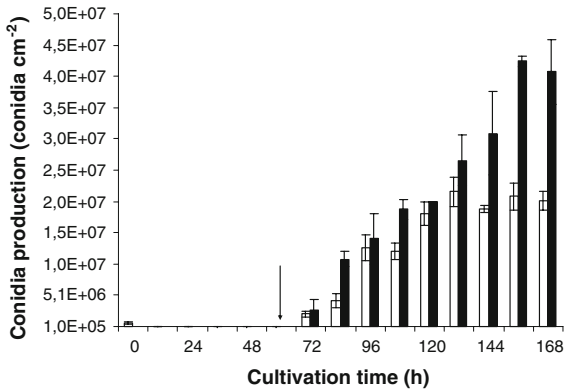

Fig. 3 *Metarhizium anisopliae* var. *lepidiotum* conidia production under normal (*open square*) or modified atmosphere by pulses (26% O_2) from the pre-stationary phase (*filled square*). *Arrow* shows initial time for atmosphere modification

biomass). Similarly, conidia productivity (P) was almost double in the oxygen-rich atmosphere at the time of maximum production.

Table 2 shows conidial quality values such as germination (G) and relative hydrophobicity (H) for conidia harvested in the every atmospheric treatment. These parameters did not show significant difference ($P < 0.05$) between the two atmospheres. Hydrophobicity of the conidial surface is other quality parameter linked to virulence of entomopathogenic fungi [20]. In our work, relative hydrophobicity was around 77%, independently from the atmosphere in which conidia were produced.

Bioassay parameters are also shown in Table 2, these include delay time (t_0), specific death rate (k), and time to reach 50% mortality (LT_{50}). Data analysis confirmed that there were no significant differences between treatments for these parameters predicted by the model described earlier. Additionally, after 7 days of infection, a 100% mortality value ($S = 0$) was obtained for *Tenebrio molitor* adults. The utility of the model presented in Eq. 4 was verified on data obtained by Wang et al. [15], even with *M. anisopliae* bioassays on *Manduca sexta* infected either by immersion or injection. These authors reported that when infected by immersion, larvae showed a $LT_{50} = 4.8$ days; whereas, LT_{50} value was 4.6 days when infection was via injection. These results were reproducible when data were adjusted using the model described, with a correlation coefficient of $R^2 = 0.98$ (data not shown). Additionally, from the same set of data, values for t_o (4.06 and

Table 1 Growth and conidia production parameters of *Metarhizium anisopliae* var. *lepidiotum* after 156 h of cultivation

Treatment	X (mg cm^{-2})	C ($\times 10^7$conidia cm^{-2})	$Y_{C/X}$ ($\times 10^7$conidia mg^{-1} X)	P ($\times 10^5$ conidia cm^{-2} h^{-1})
21% oxygen	4.78* (±0.25)	2.08 (±0.21)	0.43 (±0.05)	1.33 (±0.13)
26% oxygen	3.78 (±0.18)	4.25* (±0.13)	1.12* (±0.03)	2.72* (±0.08)

Values with the asterisk mark in each column show significant differences ($P < 0.05$)

X = Dry biomass (mg cm^{-2})

C = Conidial production (conidia cm^{-2})

$Y_{C/X}$ = Conidia yield in relation to biomass (conidia mg^{-1} X)

P = Productivity (conidia h^{-1})

Table 2 Conidial quality parameters of *Metarhizium anisopliae* var. *lepidiotum*. Bioassays were assayed on *Tenebrio molitor*

Treatment	G (%)	H (%)	t_0 (days)	k ($days^{-1}$)	LT_{50} (days)
21% oxygen	79.57 (±3.08)	77.18 (±2.70)	3.75 (±0.09)	1.26 (±0.22)	4.31 (±0.11)
26% oxygen	83.03 (±1.86)	77.21 (±2.57)	3.56 (±0.45)	2.20 (±0.76)	3.90 (±0.35)

Values in each column did not show significant differences ($P < 0.05$)

G = Germination percentage

H = Conidial surface hydrophobicity

t_o = Delay time

k = Specific death rate

LT_{50} = Time to reach 50% mortality

4.24 days) and k (0.959 and 1.59 $days^{-1}$) were estimated, numbers in parenthesis represent values after infection through immersion or injection, respectively.

Discussions

In a recent study by Prakash et al. [3], the use of yeast extract at low concentrations (0–0.5%) improved conidia production levels; however, optimal concentrations for *M. anisopliae* conidia production in different substrates such as rice, barley, and sorghum were 1.45, 2.21, and 1.54%, respectively. On the other hand, SDA (C/N = 35) was optimal for conidia production on superficial cultures of *M. anisopliae* V245 and V275 [6]. Issaly et al. [8] observed that at C/N = 1.6, high levels of *M. flavoviride* Mf189 (reassigned as *M. anisopliae* var. *acridum* as suggested previously [9]) blastospores were obtained in liquid cultures, using sucrose and yeast extract as carbon and nitrogen sources, respectively. In our results, conidia yields enhanced at low C/N values after adding peptone to media based on rice and oat flour (C/N: 6.85 and 5.73, respectively), relative to the same carbon sources without peptone and higher C/N values (20.46 and 15.71, respectively).

Conidia production observed in this research agreed with a report [8], describing that in a submerged culture, the increase in dissolved oxygen caused a 20 times augment in blastospore production by *M. anisopliae* var. *acridum* (previously referred to as *M. flavoviride*). However, forced aeration did not favor *M. anisopliae* var. *acridum* conidia production [2] suggesting that air itself (21% O_2) is not a determining factor in conidia overproduction. From our results, high oxygen concentrations (26%) favored conidia production in *M. anisopliae*, probably as a response to reactive oxygen species present under this atmosphere.

For *N. crassa*, it has been reported that glutation and NADPH oxidation occur at the beginning of conidiation [13]. There are also reports showing that enzymes such as catalases occur differentially at the start of conidiation [22]. Results of the present study are in agreement with the hypothesis describing that

hyperoxidant states, such a higher oxygen concentration, stimulate cellular differentiation and conidiation in *N. crassa* [12].

In a study by Kamp and Bidochka [5], conidia production on the PDA was studied for the *M. anisopliae* isolates 2575, 54 A-1b, MAA1-2iii, and HAA2-2b. Productivities for the isolates were 4.57×10^5, 1.62×10^5, 1.01×10^5, and 0.68×10^5 (conidia cm^{-2} h^{-1}), respectively. In our results, comparable values were obtained in the oat medium containing peptone: 1.33×10^5 and 2.72×10^5 (conidia cm^{-2} h^{-1}) in the 21 and 26% O_2 atmospheres, respectively.

Some authors show that nutritional factors affect conidia germination by *M. anisopliae* strains [6, 7]. For *M. anisopliae* grown at C/N = 35, even when high levels of conidia production are achieved, germination was lower than 47% [6]. Rangel et al. [7] found that on preferred carbon sources, high conidia levels are also achieved; however, these conidia showed low germination after being exposed to radiation by UV-B light, when compared with conidia obtained from non-preferred carbon sources, even when conidial yields were lower for the latter media. These arguments show that massive conidia production may diminish quality of infective units.

In terms of hydrophobicity, values ranging from 77.9 to 93.9% for *M. anisopliae* have been reported, although this variability was related to repeat culturing processes in artificial media [6]. Another factor affecting conidial hydrophobicity is the type of culture medium where conidia are collected from; for instance, for *Penicillium oxalicum*, the greatest hydrophobicity was observed when conidia were harvested from superficial cultures, compared to submerged cultures [23]. Culture environment affects the adhesion capacity of *M. anisopliae* conidia to their hosts, since modification of conidial carbohydrates may occur [19]. In this study, we succeeded in increasing conidia production through changes in atmosphere composition without affecting germination and hydrophobicity values in relation to the normal atmosphere.

As final remarks, the type of substrate affects conidia production, and presence of peptone in the culture media favors conidial yields. This report describes for the first time that an oxygen-enriched atmosphere enhanced conidia production by *Metarhizium anisopliae* var. *lepidiotum* with no effect on quality parameters such as germination and hydrophobicity. Additionally, production of conidia in these 26% O_2 atmosphere did not affect virulence on *Tenebrio molitor* adults.

Acknowledgments Tlecuitl-Beristain, S. (Reg. No. 117823) thanks the Mexican National Council for Science and Technology (CONACyT) for the scholarship. This study was financed by the Universidad Autónoma Metropolitana-Iztapalapa and Red PROMEP-Mexico. Authors wish to thank Dr. FJ. Fernández for his technical help in the molecular identification of the strain.

References

1. Sun MH, Liu XZ. Carbon requirements of some nematophagous, entomopathogenic and mycoparasitic Hyphomycetes as fungal biocontrol agents. Mycopathologia. 2006;161:295–305.
2. Arzumanov T, Jenkins N, Roussos S. Effect of aeration and substrate moisture content on sporulation of *Metarhizium anisopliae* var. *acridum*. Process Biochem. 2005;40:1037–42.
3. Prakash BGVS, Padmaja V, Kiran SRR. Statistical optimization of process variables for the large-scale production of *Metarhizium anisopliae* conidiospores in solid- state fermentation. Bioresour Technol. 2008;99:1530–7.
4. Sun J, Fuxa JR, Henderson G. Sporulation of *Metarhizium anisopliae* and *Beauveria bassiana* on *Coptotermes formosanus* and in vitro. J Invertebr Pathol. 2002;81:78–85.
5. Kamp AM, Bidochka MJ. Conidium production by insect pathogenic fungi on commercial available agars. Lett Appl Microbiol. 2002;35:74–7.
6. Shah FA, Wang CS, Butt TM. Nutrition influence growth and virulence of the insect-pathogenic fungus *Metarhizium anisopliae*. FEMS Microbiol Lett. 2005;251:259–66.
7. Rangel DEN, Anderson AJ, Roberts DW. Growth of *Metarhizium anisopliae* on non-preferred carbon sources yields conidia with increased UV-B tolerance. J Invertebr Pathol. 2006;93:127–34.
8. Issaly N, Chauveau H, Aglevor F, Fargues J, Durand A. Influence of nutrient, pH and dissolved oxygen on the production of *Metarhizium flavoviride* Mf189 blastospores in submerged batch culture. Process Biochem. 2005;40:1425–31.
9. Driver F, Milner RJ, Trueman JWH. A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. Mycol Res. 2000;104:134–50.
10. Angelova BM, Pashova BS, Spasova KB, Vassilev VS, Slokoska SL. Oxidative stress response of filamentous fungi induced by hydrogen peroxide and paraquat. Mycol Res. 2005;109:150–8.
11. Belozerskaya TA, Gessler NN. Oxidative stress and differentiation in *Neurospora crassa*. Microbiology. 2006;75:427–31.
12. Hansberg W, Aguirre J. Hyperoxidant states cause microbial cell differentiation by cell isolation from dioxygen. J Theor Biol. 1990;142:287–93.

13. Toledo I, Rangel P, Hansberg W. Redox imbalance at the start of each morphogenetic step of *Neurospora crassa* conidiation. Arch Biochem Biophys. 1995;319:519–24.
14. Sowjanya Sree K, Padmaja V. Oxidative stress induced by destruxin from *Metarhizium anisopliae* (Metch.) involves changes in glutathione and ascorbate metabolism and instigates ultrastructural changes in the salivary glands of *Spodoptera litura* (Fab.) larvae. Toxicon. 2008;51: 1140–50.
15. Wang C, Duan Z, St Leger RJ. MOS1 osmosensor of *Metarhizium anisopliae* is required for adaptation to insect hemolymph. Eukaryot Cell. 2008;7:302–9.
16. León-Santiesteban H, Bernal R, Fernández FJ, Tomasini A. Tyrosinase and peroxidase production by *Rhizopus oryzae* strain ENHE obtained from pentachlorophenol-contaminated soil. J Chem Technol Biotechnol. 2008;83: 1394–400.
17. Bischoff JF, Rehner SA, Humber RA. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. Mycologia. 2009;101:512–30.
18. Samuels KDZ, Heale JB, Llewellyn M. Characteristics relating to the pathogenicity of *Metarhizium anisopliae* toward *Nilaparvata lugens*. J Invertebr Pathol. 1989;53:25–31.
19. Ibrahim L, Butt MT, Jenkinson P. Effect of artificial culture media on germination, growth, virulence and surface properties of the entomopathogenic hyphomycete *Metarhizium anisopliae*. Mycol Res. 2002;106:705–15.
20. Shah AF, Allen N, Wright JC, Butt MT. Repeated in vitro subculturing alters spore surface properties and virulence of *Metarhizium anisopliae*. FEMS Microbiol Lett. 2007;276:60–6.
21. Rodríguez-Gómez D, Loera O, Saucedo-Castañeda G, Viniegra-González G. Substrate influence on physiology and virulence of *Beauveria bassiana* acting on larvae and adults of *Tenebrio molitor*. World J Microbiol Biotechnol. 2009;25:513–8.
22. Michán S, Lledas F, Baldwin JD, Natvig DO, Hansberg W. Regulation and oxidation of the large monofunctional catalases. Free Radical Bio Med. 2002;33:521–32.
23. Pascual S, De Cal A, Magan N, Melgarejo P. Surface hydrophobicity, viability and efficacy in biological control of *Penicillium oxalicum* spores produced in aerial and submerged culture. J Appl Microbiol. 2000;89:847–57.