Conditions to optimize mass production of *Metarhizium anisopliae* (Metschn.) Sorokin 1883 in different substrates

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ABSTRACT

One of the best alternatives to reduce the amount of chemical insecticides released into the environment is biological agents. *Metarhizium anisopliae* (Metschn.) Sorokin 1883 (Hypocreales: Clavicipitaceae) is an entomopathogenic fungus with great potential as a biological pesticide to biologically control pests. However, the relatively high cost of the substrate needed for its mass production system increases product price and discourages its use. The objective of this study was to optimize the mass production conditions of *M. anisopliae* for use as a biological control agent using two solid substrates, new parboiled rice (NPR) and recycled parboiled rice (RPR). Conidial production was optimized by the response surface methodology (RSM). The effects of the temperature, time, and molasses variables and the interactions between them (conidia g^{-1}) were determined. For the NPR substrate, it was determined that the significant variables were time and temperature, and the interactions were temperature × molasses and temperature × time. For the RPR substrate, the significant variables were temperature and time, and the interactions were time × molasses and temperature × time. Both substrates obtained the highest industrial yields at 25 °C for a period of 20 d. Given that the percentage of molasses was not critical for yields, it is recommended that it be set at 5% to reduce costs. Finally, it was possible to use the RPR substrate from the *M. anisopliae* produ9ction itself as an alternative to solid substrate; mean industrial performance (conidia g^{-1}) was higher than values obtained with NPR and at a lower cost.

Key words: Biological control, entomopathogenic fungi, mass production, optimization, response surface methodology.

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INTRODUCTION

Biological insecticides are becoming increasingly relevant for safe, effective, and environmentally friendly pest control because of the harmful effects caused by chemical pesticides on the environment and human health. *Metarhizium anisopliae* (Metschn.) Sorokin 1883 is one of the best known entomopathogenic fungi; it is pathogenic to more than 200 species from different insect orders (Freimoser et al., 2005; Samson et al., 2013) and appropriate for commercial development. This fungus has the ability to directly penetrate the insect cuticle (Schneider et al., 2013) through combinations of mechanical pressure and cuticle-degrading enzymes (Beys-da-Silva et al., 2014). When attaching themselves to the body of a suitable host, conidia produce a germ tube, which through extension and growth give rise to hyphae that penetrate into and grow within the insect and causing its death.

Metarhizium anisopliae is commercially produced in solid substrates, but this type of production complicates process automation; it relies on batch production and does not provide a satisfactory economy of scale (Wraight et al., 2001). The two-phase culture (liquid and solid) is the most commonly used technique to mass produce *Metarhizium*. Liquid fermentation is used to produce blastospore (Riaz et al., 2013) and mycelium forms (Pereira and Roberts, 1990; Kruger et al., 2014). The solid phase is carried out in a solid substrate, which has a large surface area for aeration and physically supports the fungus to produce conidia, and it is also used as a source of nutrients (Jenkins et al., 1998). Different substrates of vegetable origin can be used to mass produce conidia, such as different forms of potato, wheat, soy, rice, and bran. Studies by Dorta and Arcas (1998) show that rice is a good medium to mass multiply *M. anisopliae* because it provides nutrients and a large surface area on which conidia can be produced. Conidial production using rice as a substrate is approximately 1×10^9 conidia g^{-1} (Barajas et al., 2010). The most used solid substrate is parboiled rice (pre-cooked); it is very expensive (Kruger et al., 2014) and thus increases the final selling price. It is recommended that the objective of the production process be low cost and high yield of viable, virulent, and persistent propagules (Kassa et al., 2008).

The most important environmental factors that affect the mass production of *Metarhizium anisopliae* are temperature (Li and Feng, 2009; Chen et al., 2014), which is considered as a critical factor during the incubation stage (Elósegui, 2006),

humidity of the solid substrate, which noticeably affects the sporulation process and is optimal between 57% and 58% RH (Arzumanov et al., 2005), pH, which needs to be slightly acidic in both phases to facilitate fungal growth and inhibit the growth of other microorganisms, and time (Kleespies and Zimmermann, 1992). In the mass production process, conidia are harvested 21 d after inoculation in the substrate; there have also been good results 14 d after inoculation (Rezende, 2009). One of the optimization methodologies that has been used in industrial processes is the response surface methodology (RSM); it combines mathematical and statistical techniques to build empirical models (Hanrahan and Lu, 2006). This methodology is advantageous because it allows identifying the effect of factors that generate a basis for additional experiments and setting values to factors that improve performance; this leads to savings in time, materials, and labor (Gohel et al., 2006). Therefore, the aim of this study was to optimize the mass production of *Metarhizium anisopliae* in different substrates.

MATERIALS AND METHODS

Strain and culture conditions

The selection of *Metarhizium anisopliae* was based on work by France et al. (2000) in which the inoculum was obtained by growing the fungus in potato dextrose agar (PDA) and incubating it at 25 ± 2 °C for 15 d. Microscopic examination of fungal isolates resulted in the preliminary identification of *Metarhizium* sp., and it was confirmed as *M. anisopliae* var. *anisopliae* by sequencing the ITS region (Internal Transcribed Spacers, ITS-5.8S rDNA). The two-phase culture was used for mass production. During the liquid phase, the isolated sample was taken from tubes and deposited in Petri dishes with an agar and sucrose medium enriched with *Galleria mellonella* Linnaeus 1758 (Riaz et al., 2013). The fungus was placed in the dishes and kept for 4 d in the incubation chamber at 25 °C until fungus sporulation occurred. Conidial concentrations were determined by direct count with a Neubauer hemocytometer, and conidial viability tests were carried out (mean 97%) using the methodology described by Moore et al. (1995). The conidial suspension was adjusted to 1×10^6 conidia mL^{-1} by diluting it with Tween 80 (0.1% v/v) (Garcia et al., 2005). A suspension with 1 L sterile distilled water, 1% Tween 80 (0.1% v/v), 25 g yeast, and 20 g commercial sucrose was then prepared. It was deposited in 2 L jars that were autoclaved at 120 °C and 120 psi for 20 min. Once the jars were cold, the inoculum was added and jars were connected to a ventilation system that oxygenated and agitated the suspension to form small mycelium pellets. After 3 d, the solid phase began with substrate preparation.

Two substrates were used for the trials, new parboiled rice (NPR) and recycled parboiled rice (RPR). The RPR was recycled from a previous production of *M. anisopliae*, and it was harvested dry and washed with water three times. Both substrates were submerged and drained to achieve a 40% moisture level. Polyethylene bags (325 × 435 mm) with 500 g each of the substrates and two different levels of beet (*Beta vulgaris* L. subsp. *vulgaris*) molasses (Industria Azucarera Nacional S.A.) were sterilized (Kruger et al., 2014) and taken to a laminar flow chamber where they were inoculated with 10 mL liquid inoculum prepared as mentioned above. Bags were plugged with a ventilated cap to minimize contamination and allow passive aeration during growth and conidiogenesis. After inoculation, the bags were put in different production rooms. Room 1 was kept at 20 ± 1 °C while Room 2 was kept at 25 ± 1 °C. Both rooms had air extraction systems to allow appropritate ventilation. When the conidium production process ended, bags were removed from the chambers and emptied onto trays that were placed in the drying room where they were kept at 25 ± 1 °C and 50 ± 5% RH for 10 d. To determine the production level, 1 g rice with conidia was taken and a suspension of 100 mL sterile distilled water and Tween 80 (0.05% v/v) was prepared. Conidia obtained from the suspension were counted and dilutions were prepared when necessary.

Experimental design

A completely randomized experimental design with a factorial model was used; this allows the study of three factors at two levels. The experimental unit was a 500 g bag of substrate inoculated with the fungus. Two solid substrates were used to mass produce the conidia, NPR and RPR (Table 1).

The information was collected with a replicated two-level factorial design. First, it was modeled by estimating the effective dispersion coefficients using the least squares method and then obtaining the model for central tendency. Both were contrasted by the half-normal probability chart (Daniel, 1959) to construct the global model, which allows process optimization. To use the two-level factorial design and generate an orthogonal design matrix, it was necessary to codify variables according to the following transformation (Vergara et al., 2013):

$$X_i = \frac{unit\ variable - average\ variable}{(width\ of\ interval)/2} \quad [1]$$

These variables were obtained through a 2^3 factorial design with three replicates of each experiment for a total of 24 experiments by measuring each production.

Pepió and Polo (1999) estimated the effects of scattering and the variances associated with each treatment of the

Table 1. Variables and their levels for new parboiled rice and recycled parboiled rice.

Variable	Low level (-1)	High level (+1)
A. Temperature, °C	22	25
B. Time, d	14	20
C. Molasses, %	5	10

model. With the minimum quadratic estimators of the dispersion coefficients, estimation efficiency can be increased by a credible maximum for the estimators. The analysis of the impact location is not verified when the assumption of equal treatment variance is expressed by the linear model:

$$Y_{ij} = m_i + \sigma_i \varepsilon_{ij} = \sum_{k=1}^{n} a_{ik} \beta_k + \sigma_i \varepsilon_{ij} \ , \ j = 1,2,...,r \qquad [2]$$

The theoretical development by Pepió and Polo (1999) improved the work done by Nair and Pregibon (1988) to model joint variability and central tendency of an industrial production process using a type 2^p factorial design, which allows variables to consider two levels with a total of $n = 2^p$ treatments or samples codified in the lines specified in the matrix design and replicated *r* times.

Given that m_i is the mean and σ^2_i is the variance of observations for the i^{th} treatment (i^{th} line of the array design), the model connects the mean and the variance with the β_k factors and their interactions through the location coefficients β_k and dispersion θ_k. This model allows expressing the responses and the sums of squared differences in terms of the coefficients. Inasmuch as the estimated location coefficients β_k differ depending on whether the variances σ^2_i can be considered to be statistically the same or not, it is first necessary to estimate the dispersion coefficients and explore their significance.

RESULTS AND DISCUSSION

The predicted response along with the experimental data of both substrates that are shown in Tables 2 and 3 reveal a close relationship between values. The industrial yield of the fungus in all the treatments was greater than 1×10^9 conidia g^{-1} and this coincides with results reported by Barajas et al. (2010) for *M. anisopliae* and substrate (parboiled rice). However, Prakash et al. (2008) used an optimized fermentation process and harvested 5.275×10^{10} conidia g^{-1} in rice substrate. Temperature plays a major role in conidial production of *M. anisopliae* in rice substrates, and a higher temperature (25 °C) allows obtaining more conidia than a lower temperature (20 °C).

The effects and interactions, with their respective standard error, were calculated for NPR. Calculations of effects and standard error for NPR and RPR are shown in Tables 2 and 3, respectively. By using the half-normal probability plot method (Daniel, 1959), it can be seen that there was no significant variable or interaction, $p > 0.05$; the variance of the treatments was therefore established as being constant (Figure 1).

Since the variance of the treatments in both trials was accepted as being constant, the behavior of the mean production of replicates was modeled. The effects and interactions were estimated with the results from the trials; by the half-normal probability plot method for NPR, it was observed that the temperature and time variables, and the temperature × molasses and temperature × time interactions were significant, $p < 0.05$ (Figure 2). For RPR, the significant variables were temperature and time whereas the time × molasses and temperature × time interactions were significant, $p < 0.05$ (Table 4).

The multiple regression model permits the estimation of industrial performance (conidial yield) based on the significant variables and interactions for NPR:

$$Y = 1729286603.62 + 206182122.98 \text{ temperature} + 204774854.92 \text{ time} + 87404648.45 \text{ temperature} \times \text{time} - 140184355.10 \text{ temperature} \times \text{molasses} \qquad [3]$$

Since the molasses variable was not significant in the studied variation range, it was fixed at a low level (5%)

Table 2. Mean production values for each treatment and lnXi values for new parboiled rice.

Temperature	Time	Molasses	R1	R2	R3	Y	Xi	InXi
			conidia g^{-1}				(conidia g^{-1})^2	
-1	-1	-1	1.29×10^9	1.34×10^9	1.34×10^9	1.33×10^9	1.59×10^{15}	35.00
1	-1	-1	1.93×10^9	1.85×10^9	1.85×10^9	1.88×10^9	4.39×10^{15}	36.02
-1	1	-1	1.54×10^9	1.59×10^9	1.59×10^9	1.58×10^9	2.05×10^{15}	35.26
1	1	-1	2.56×10^9	2.33×10^9	2.33×10^9	2.41×10^9	3.38×10^{16}	38.06
-1	-1	1	1.37×10^9	1.54×10^9	1.54×10^9	1.48×10^9	2.11×10^{16}	37.59
1	-1	1	1.41×10^9	1.40×10^9	1.40×10^9	1.41×10^9	5.78×10^{13}	31.69
-1	1	1	1.71×10^9	1.70×10^9	1.70×10^9	1.70×10^9	6.74×10^{13}	31.84
1	1	1	1.98×10^9	2.08×10^9	2.08×10^9	2.05×10^9	6.44×10^{15}	36.40

R1-R3: Conidial yield for each replicate, Y: mean conidial yield, Xi: variance numerator ($(n - 1)S^2$).

Table 3. Mean production values for each treatment and lnXi values for recycled parboiled rice.

Temperature	Time	Molasses	R1	R2	R3	Y	Xi	InXi
			conidia g^{-1}				(conidia g^{-1})^2	
-1	-1	-1	1.68×10^9	1.05×10^9	1.45×10^9	1.40×10^9	2.07×10^{17}	39.87
1	-1	-1	1.53×10^9	2.02×10^9	2.21×10^9	1.92×10^9	2.45×10^{17}	40.04
-1	1	-1	1.58×10^9	1.90×10^9	1.88×10^9	1.79×10^9	6.54×10^{16}	38.72
1	1	-1	2.57×10^9	2.67×10^9	2.77×10^9	2.67×10^9	1.90×10^{16}	37.48
-1	-1	1	2.42×10^9	1.65×10^9	2.28×10^9	2.11×10^9	3.33×10^{17}	40.35
1	-1	1	2.08×10^9	2.27×10^9	2.09×10^9	2.15×10^9	2.24×10^{16}	37.65
-1	1	1	1.51×10^9	1.67×10^9	1.50×10^9	1.56×10^9	1.97×10^{16}	37.52
1	1	1	2.20×10^9	2.61×10^9	2.20×10^9	2.34×10^9	1.11×10^{17}	39.25

R1-R3: Conidial yield for each replicate, Y: mean conidial yield, Xi: variance numerator ($(n - 1)S^2$).

Figure 1. Half-normal chart of LnXi effects for new parboiled rice (NPR) (a) and recycled parboiled rice (RPR) (b).

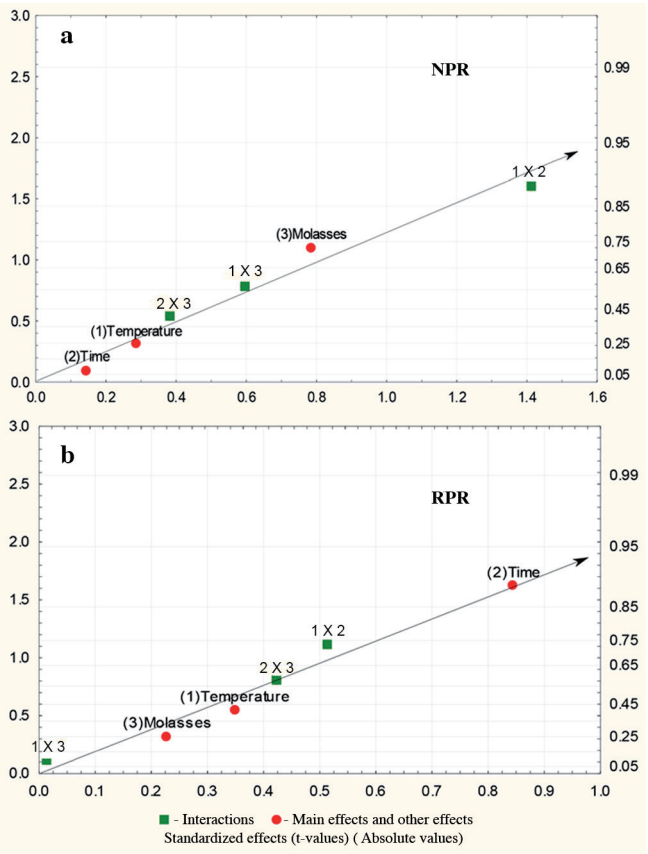
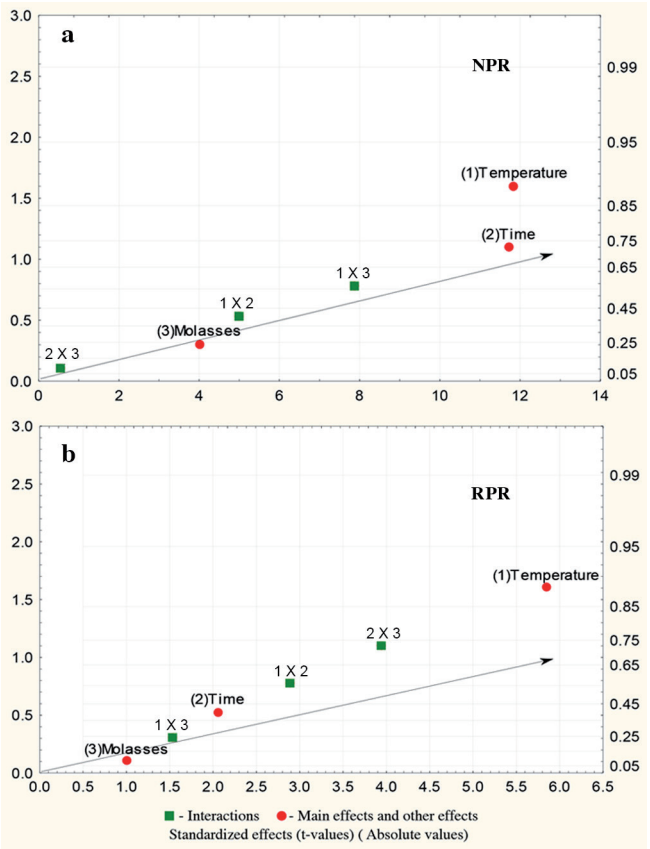


Figure 2. Half-normal chart of effects for new parboiled rice (NPR) (a) and recycled parboiled rice (RPR) (b).



which reduces costs. The reduced model for NPR can be expressed as:

$$Y = 1729286603.62 + 206182122.98\ \text{temperature} + 204774854.92\ \text{time} + 87404648.45\ \text{temperature} \times \text{time} \quad [4]$$

The chart for the NPR response surface in Figure 3 shows that most of the estimated *M. anisopliae* production with a low level of molasses (-1), 5%, can be obtained by setting temperature and time to a high level with values close to 2.4×10^9 conidia g^{-1}; these results were higher than those obtained by Kruger et al. (2014) and Latifian et al. (2014), thus demonstrating process efficiency. In the corresponding contour plot, each line represents the same production at different levels of the significant variables. When setting the concentration of molasses to a high level (+1), 10%, most of the estimated conidial production was produced when temperature and time were set to a high level with values close to 2.1×10^9 conidia g^{-1} (Figure 4). Setting the amount of molasses to a low level decreased the process costs (-1). Results partly agree with observations by Karanja et al. (2010), who demonstrated that maximum yield was achieved when fungi were grown on rice at 23 °C for 3 wk.

The multiple regression model permits the estimation of the industrial performance of *M. anisopliae* based on the significant variables and interactions for RPR:

$$Y = 1991978528.77 + 277452488.97\ \text{temperature} + 97160190.83\ \text{time} + 137180650.84\ \text{temperature} \times \text{time} - 188641698.72\ \text{time} \times \text{molasses} \quad [5]$$

Since the molasses variable was not significant in the studied variation range, it was fixed at a low level (5%) which reduces costs. The reduced model for RPR can be expressed as:

$$Y = 1991978528.77 + 277452488.97\ \text{temperature} + 97160190.83\ \text{time} + 137180650.84\ \text{temperature} \times \text{time} \quad [6]$$

For RPR, the results of the response surfaces (Figures 5 and 6) revealed the best industrial performance at 2.7×10^9 conidia g^{-1}; the highest levels of the temperature (25 °C) and time (20 d) variables must be used while the nonrelevant molasses variable can be set at the lowest level to decrease process costs. These results were higher than those obtained by Babu et al. (2008). The higher production, compared with NPR, could be explained by the fact that RPR was more fragmented and had a larger surface area for conidial formation; this was indicated by Kruger et al. (2014)

Table 4. Regression coefficients and significant variables and interactions of new parboiled rice (NPR) and recycled parboiled rice (RPR).

Variables	Regression coefficient NPR	Regression coefficient RPR
Interaction mean	1.73×10^9	1.99×10^9
Temperature	2.06×10^8	2.77×10^8
Time	2.05×10^8	9.72×10^7
Temperature × Time	8.74×10^7	1.37×10^8
Temperature × Molasses	-1.40×10^8	-
Time × Molasses	-	-1.89×10^8

Figure 3. 3-D response surface plot (a) and contour plot (b) by monitoring temperature, time, and setting molasses to level -1 (5%) for *Metarhizium anisopliae* conidial production in solid-state fermentation using new parboiled rice as substrate.

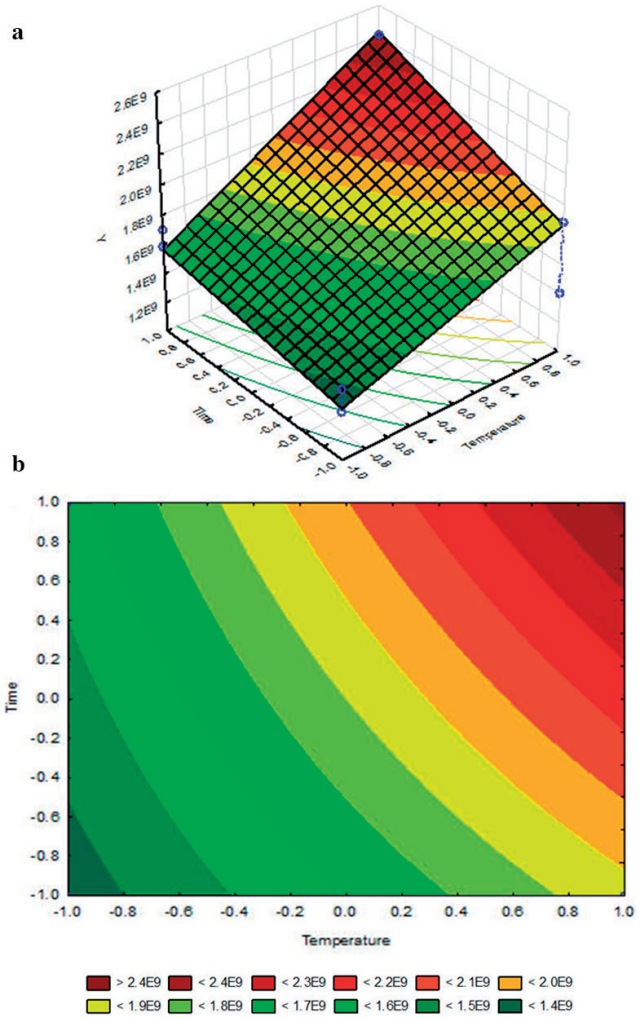
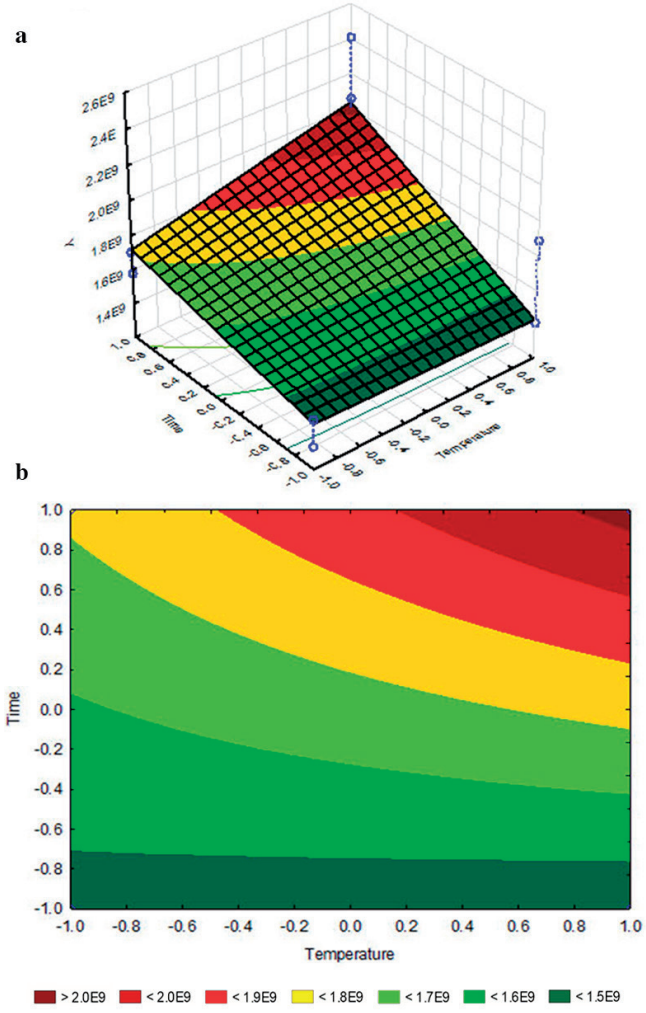


Figure 4. 3-D response surface plot (a) and contour plot (b) by monitoring temperature, time, and setting molasses to level +1 (10%) for *Metarhizium anisopliae* conidial production in solid-state fermentation using new parboiled rice as substrate.



in a study where broken white rice exhibited the highest production (3.7×10^9 conidia g^{-1}). It can also be that broken rice has better aeration along with this increased surface area or that rice was internally softer, which provides a better supply of nutrients.

The substrates used in the present study had the highest production of conidia per gram; this was better than results obtained by Ibrahim et al. (2015) with a shorter drying time (1 wk less) and demonstrates that the process was more efficient and could significantly reduce production costs. Although the 20 d cycle resulted in higher production than the 14 d cycle, short cycles would allow more production runs in a year, 26 and 18 cycles per year, respectively; if capital costs are taken into account, the economic results might be better. Temperatures proved to be a significant variable in both substrates with 25 °C being the optimum temperature to achieve high levels of conidial production; and this result agrees with Lu et al. (2004).

CONCLUSIONS

Based on the results, it was concluded that the response surface methodology allows optimizing the mass production of *Metarhizium anisopliae*. The optimal combination of the studied variables was 25 °C and 20 d, regardless of the level of molasses. Therefore, it is recommended that 5% molasses should be used to decrease production costs. This combination was the same for both substrates in the present study. The mean industrial performance for the new parboiled rice substrate and for the recycled parboiled rice substrate were 2.41×10^9 and 2.67×10^9 conidia g^{-1}, respectively, which is higher than the mean obtained for *M. anisopliae* (1×10^9 conidia g^{-1}). Finally, it is possible to use the recycled rice substrate from the *M. anisopliae* production itself as an alternative for solid substrate, thus obtaining a higher mean industrial performance (conidia g^{-1}) than when using new parboiled rice, and this will reduce the production costs of this entomopathogenic fungus.

Figure 5. 3-D response surface plot (a) and contour plot (b) by monitoring temperature, time, and setting molasses to level -1 (5%) for *Metarhizium anisopliae* conidial production in solid-state fermentation using recycled parboiled rice as substrate.

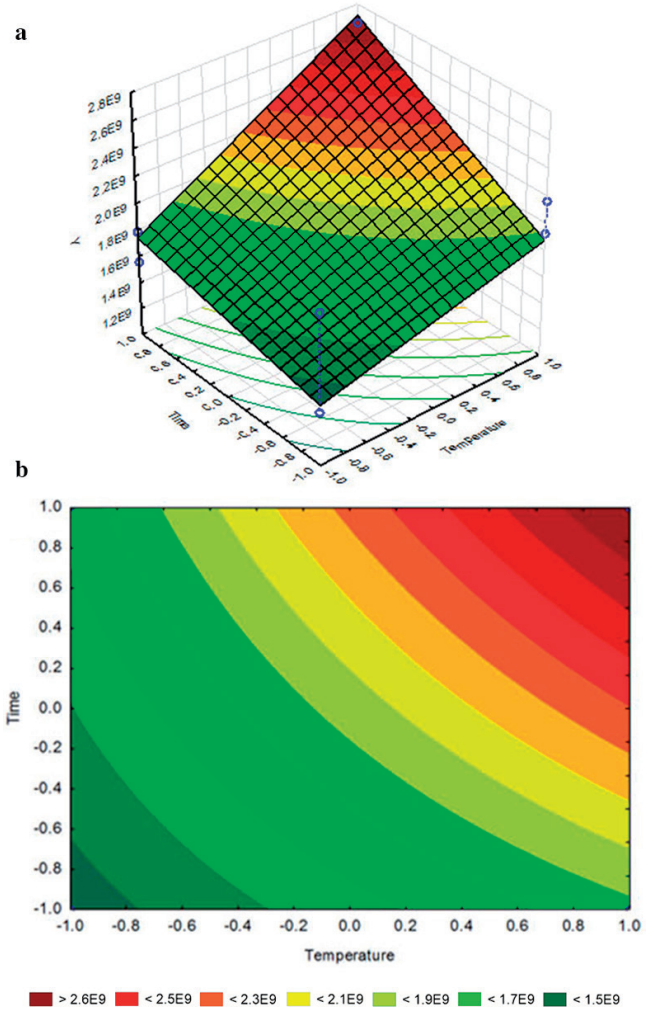
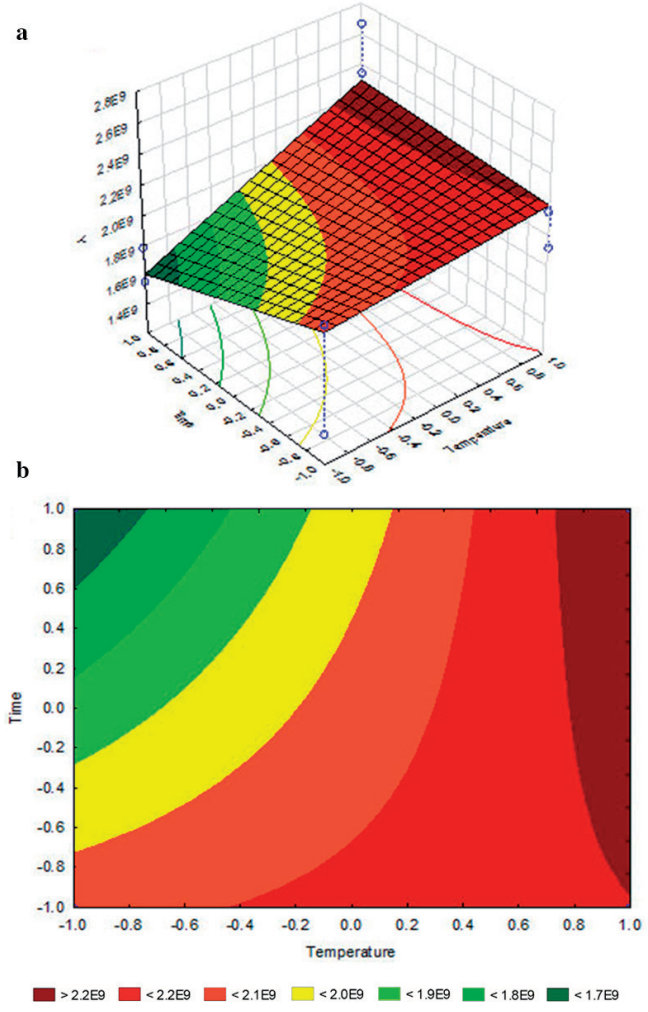


Figure 6. 3-D response surface plot (a) and contour plot (b) by monitoring temperature, time, and setting molasses to level +1 (10%) for *Metarhizium anisopliae* conidial production in solid-state fermentation using recycled parboiled rice as substrate.



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