Rice Recycling: A Simple Strategy to Improve Conidia Production in Solid-State Cultures

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Abstract

The aim of this work was to propose a strategy to revalorize the residual rice remaining at the end of a conventional conidia production process in solid-state culture. The proposal involved the reuse of rice in successive cycles of conidia production. As a result, the reuse of rice in successive cycles significantly increased the production and productivity parameters of the fungal strains *Trichoderma asperellum* Th-Th4 (3) and *Metarhizium robertsii* Xoch-8.1. Conidia production in *T. asperellum* Th-Th4 (3) increased from 1.0×10^9 to 4.9×10^9 con/gds, while in *M. robertsii* Xoch-8.1, this parameter increased form 5.7×10^8 to 3.8×10^9 con/gds. In addition, this strategy did not affect the viability of the conidia produced using recycled rice relative to the conidia produced using fresh substrate (unrecycled rice).

The proposed recycling approach was simple, and completely free of extra -drying, -sterilization, and -reinoculation steps. The technique required minimal operational intervention and thus can be easily adopted by small bio-factories or self-supply units.

1. Introduction

The economic losses caused by pests can be as high as 40 % of the total annual yield worldwide, which has led to an excessive dependence on chemical pesticides [1]. As part of the initiative to reduce the problems associated with the use of chemical pesticides, in recent years the use of biopesticides has been promoted [2].

Biopesticides are produced from plants, bacteria, and fungi [1]. The most widely used fungal biopesticides are made from infectious propagules of the genera *Beauveria, Metarhizium*, and *Trichoderma* [3, 4]. The production of these biopesticides can be carried out by submerged fermentation (SmF) or solid-state cultures (SSC) [5].

Cereal grains are the most common substrates for SSC; among them, rice has been extensively used due to its wide availability, good nutritional balance, and suitable structure [6, 7]. Interestingly, at the end of a conidia production cycle, around 80 % of the initial substrate that was added remains unused [7]. The reuse of this substrate has been proposed in previous works [8, 9, 10]; however, the suggested methodologies comprise extra drying, washing, and sterilization steps, which increase the cost of the process.

Therefore, in this work, we propose a substrate recycling strategy completely free of extra –drying, -sterilization, and –reinoculation steps. The proposed strategy was tested with two fungal strains belonging to different taxonomic families (*Trichoderma asperellum* Th-Th4 (3) and *Metarhizium robertsii* Xoch-8.1). A complete conidia production scheme was set up for each fungus. The production schemes started with the use of fresh rice (unrecycled rice) as the substrate, and continued with the use of recycled rice in successive cycles of conidia production. At the end of each cycle, the conidia production yield, productivity, conidia viability, and substrate consumption were determined and compared with those obtained in a conventional single-cycle process.

2. Methods

2.1 Microorganisms

Two fungal strains were studied, *Trichoderma asperellum* Th-Th4 (3) (Culture Collection of the Benemérita Universidad Autónoma de Puebla, Mexico) and *Metarhizium robertsii* Xoch-8.1 (Culture Collection of the Universidad Autónoma Metropolitana, Mexico).

2.2 Solid-state cultures for conidia production

2.2.1 First production cycle using unrecycled rice

The first cycle was carried out using fresh rice as the substrate (Parboiled rice, Verde Valle™, Uruguay). Solid-state cultures were carried out in 120 mL serological bottles containing 2.5 g of rice. The bottles were sterilized (121°C, 15 min) and inoculated with 1×10^5 and 1×10^6 conidia per gram of dry substrate (con/gds) for *M. robertsii* Xoch-8.1 and *T. asperellum* Th-Th4 (3), respectively. The substrate moisture was adjusted to 32 ± 2 % by adding sterile distilled water. All bottles were closed with cotton stoppers and incubated at 28 ºC with a light-dark photoperiod of 12 h : 12 h. When rice grains were covered with a heavy layer of greenish powder, the incubation was stopped and conidia were harvested and counted. To this end, Tween 80 (0.05 %) was added to each bottle and stirred for 10 min at 150 rpm using a magnetic stirrer; then, the contents of each bottle were carefully decanted, ensuring the removal of as much Tween 80 as possible and taking care that all rice grains remained inside the bottle. If necessary, this operation was repeated. The liquid phase containing the conidia was filtered through three layers of cheese cloth, and after appropriate dilution, conidia were counted in a hemocytometer. Results were expressed in conidia amount per gram of initial dry substrate (con/gds).

2.2.2 Successive cycles of conidia production using recycled rice

The bottles containing the rice that remained at the end of the first production cycle were reused in the subsequent production cycles. The rice was re-distributed in the bottom of the bottle using a sterilized microspatula and left to stand for around 30 min at room temperature in order to allow excess water to evaporate. After that, the bottles were capped with cotton stoppers and re-incubated at 28 ºC with a light-dark photoperiod of 12 h : 12 h. It is important to emphasize that the bottles were not inoculated with new conidia; instead, the conidia that remained attached to the rice after the extraction with Tween 80 acted as the inoculum for the next production cycle. After incubation, the conidia produced were extracted and quantified, as previously described. The bottles containing the rice used in the second cycle were reused successively in the next cycles.

2.3 Moisture determination

The initial moisture content of the rice was determined at the beginning of the first production cycle and at the end of each of the subsequent production cycles. The evaluation was performed using an infrared

moisture analyzer (MA 35 Sartorius) and the results were presented in percentage relative to total mass in every sample.

2.4 Substrate consumption

The substrate consumed in each cycle was estimated based on the dry-weight of the rice residues after conidia harvest. Results were referred to the initial dry-substrate and were expressed as a percentage. Maximum substrate consumption and consumption rates were estimated by adjusting the experimental data to the following modified Gompertz equation:

$$M(t) = P exp\left\{-exp\left[\frac{R_s e}{P}(\lambda - t) + 1\right]\right\}$$

where *M(t)* is the substrate consumed (g) at an incubation time *t* (d); *P* is the maximum dry substrate consumed at the end of the whole production scheme (g); R_s is the maximum dry substrate consumption rate (g/d); and λ is the lag-phase time (d) [11]. The unknown parameters (P, R_s, and λ) were estimated by a nonlinear regression analysis using the Number Cruncher Statistical System (NCSS) software (Kaysville, Utah, USA). The experimental and fitted data were plotted using the SigmaPlot software (Version 12.5).

2.5 Conidia viability

In order to assess the quality of the conidia produced in each cycle, their viability was determined. For this purpose, 100 µL of a conidia suspension at a concentration of 1×10^4 con/mL for *T. asperellum* Th-Th4 (3), and 1×10^5 con/mL for *M. robertsii* Xoch-8.1, were plated on a thin layer of water-agar and distributed over the entire surface of the medium using a sterile Drigalsky spatula. After an incubation period of 18 h at 28°C, the proportion of germinated conidia was recorded using a light microscope at 400x magnification. Conidia were considered germinated when the length of the germ tube was greater than twice the diameter of the conidia [12].

2.6 Statistical analysis

Each experiment was carried in two independent times using four replicates at each time. One-way analysis of variance (ANOVA) or *t*-test were used to compare means. Post hoc multiple comparisons were performed using the Tukey method. In all cases, the set point of significance was $\alpha = 0.05$. Average values are presented with standard deviations. Statistical analyses were conducted using the Number Cruncher Statistical System (NCSS) software (Kaysville, Utah, USA).

3. Results And Discussion

3.1 Conidia production of *T. asperellum* Th-Th4 (3)

3.1.1 First production cycle using fresh rice as the substrate

In cycle 1, *T. asperellum* Th-Th4 (3) was cultivated in SSC using fresh rice as the substrate (i.e., sterile parboiled rice that had not been previously used). During this cycle, fungal mycelia quickly covered the rice grains and a uniform conidia layer was observed 3 days after inoculation (Fig. 1b). This point was considered the end of cycle 1, and therefore, the incubation was shortly interrupted in order to achieve the harvesting and counting of the conidia.

The conidia production yield was 1.0×10^9 con/gds, with a productivity of 3.5×10^8 con/gds·d (Table 1). Although *T. asperellum* Th-Th4 (3) is not a commercial strain, this production was higher than the optimum reported for *Trichoderma harzianum* growing in rice (5.8×10^8 con/gds; [6]), and in the same order of magnitude as the one obtained with *Trichoderma asperellum* TF1 growing in mixtures of agricultural residues (8.6×10^9 con/gds; [13]). In addition, the conidia of *T. asperellum* Th-Th4 (3) presented a viability higher than 98 %, which agreed with values previously reported for other *Trichoderma* strains [14].

Table 1
Production of *T. asperellum* Th-Th4 (3) conidia in successive production cycles.

	Conidia production cycles		
	Cycle 1 (fresh rice)	Cycle 2 (recycled rice)	Cycle 3 (recycled rice)
Cycle length (d)	3	2	3
Production yield (con/gds)	$1.0\times10^9 \pm 2.1\times10^8$ a	$2.9\times10^9 \pm 4.4\times10^8$ b	$9.9\times10^8 \pm 1.7\times10^8$ a
Productivity (con/gds·d)	$3.5\times10^8 \pm 7.2\times10^7$ a	$1.4\times10^9 \pm 2.2\times10^8$ b	$3.2\times10^8 \pm 5.9\times10^7$ a
Conidia viability (%)	98.1 ± 1.7 a	97.2 ± 1.4 a	94.3 ± 4.9 a
Initial moisture content (%)	34.2 ± 0.74 a	46.8 ± 2.96 b	58.1 ± 2.74 c
Cumulative substrate consumption (%)	18.3 ± 6.1	53.2 ± 9.8	63.8 ± 8.9

d: days; con/gds: conidia per gram of dry substrate; con/gds·d: conidia per gram of dry substrate per day. This experiment was performed in two independent times using four replicates at each time. Each individual determination was carried out two times. Means (± SD) followed by the same letter within the same line are not significantly different according to Tukey ($p < 0.05$).

Regarding substrate consumption, at the end of cycle 1, around 80 % of the initial substrate that was added remained unused. The visual inspection of the residual rice revealed that most grains maintained their initial geometry and consistency, and only a slight change in color, from orange to white, was detected. Considering these observations, the residual substrate was reused in a second production cycle.

3.1.2 Second production cycle using recycled rice as the substrate

In the second cycle, the residual rice generated in cycle 1 was used as the substrate. This residual rice was not re-sterilized or re-inoculated; consequently, the conidia that remained attached to the substrate after the first harvest acted as the inoculum to start the second cycle. Also, it is important to highlight that the residual rice was not subjected to a drying process, so the moisture content at the beginning of cycle 2 corresponded to the moisture content recorded after the conidia harvesting in cycle 1.

During the second cycle *T. asperellum* Th-Th4 (3) grew faster and sporulated earlier than in cycle 1. The rice grains were completely covered with a dense layer of conidia after only 2 days of incubation (Fig. 1c). Therefore, the second cycle was ended at this point and the conidia were harvested and counted. Interestingly, the production yield was 2.9×10^9 con/gds, which represented a 3-fold increase in comparison with cycle 1. In addition, due to early sporulation, the productivity increased 4-fold, going from 3.5×10^8 to 1.4×10^9 con/gds·d. No significant differences were found between the viability of the conidia produced in cycles 1 and 2 (Table 1).

Regarding substrate consumption, a conspicuous increase in the substrate consumption was detected in cycle 2 compared to cycle 1. At the end of the cycle 2, the fungus had consumed 53.2 % of the initial substrate that was added. Considering that the substrate consumption in cycle 1 was 18.3 %, the fungus consumed 34.9 % of the initial substrate in only the second cycle. This behavior can be clearly observed in Fig. 2, which graphically illustrates the substrate consumption profile.

For *T. asperellum* Th-Th4 (3), the amount of substrate consumed between days 3 and 5 (cycle 2) was significantly higher than that registered between days 0 and 3 (cycle 1). These results support the proposed recycling strategy, since the substrate consumption, sporulation yield, and productivity were significantly improved by using recycled rice in comparison with fresh rice. Furthermore, it was not necessary to subject the residual substrate to a drying process. Table 1 shows that the initial moisture content in cycle 1 was 34.2 %, while in cycle 2, it was almost 47 %. According to [6], the optimum moisture content for the sporulation of *Trichoderma* strains growing in rice is around 35 %; however, as shown here, *T. asperellum* Th-Th4 (3) sporulated more profusely when it was cultivated in recycled rice, even when this substrate presented a moisture content of 47 %.

At the end of the conidia harvesting, the residual substrate was visually inspected again. At this time, the rice grains were turned light brown, some of them appeared broken, and, in general, the substrate began to show a slightly pasty appearance. The moisture content of this rice was 58.1 %.

3.1.3 Third production cycle using recycled rice as substrate

In the third cycle, the residual rice generated after the conidia harvesting in cycle 2 was used as the substrate. As previously mentioned, the moisture content of this residue (58.1 %) was out of the optimal range for the growth and conidia production of *Trichoderma* strains. However, since the objective of this work was to study a recycling strategy free of extra processing steps (drying, sterilization, etc.), this residual substrate was reused without any further treatment.

Interestingly, *T. asperellum* Th-Th4 (3) was able to grow and sporulate under these conditions, and after three days of incubation, the conidia production reached 9.9×10^8 con/gds. Figure 3a shows that the excess humidity caused a decline in the sporulation yield, probably because of the substrate compaction and lack of oxygen diffusion [15, 10]. However, no significant differences were observed between the production yield obtained in cycle 1 (fresh rice) and cycle 3 (two times recycled rice), and the viability of these conidia was as high as in the two previous cycles (Table 1). These results showed that, under the conditions tested, the rice can be reused at least two times, obtaining, at each time, production yields equal or greater than those obtained using fresh rice.

At the end of cycle 3, most rice grains had lost their original structure and presented a pasty appearance (Fig. 1d). Besides that, the moisture content in the residual rice was 63 %. Consequently, the production scheme was stopped at this point, and the residual substrate was not reused in another production cycle.

Considering the 3 cycles, the production scheme took 8 days and produced 4.9×10^9 con/gds, which was almost 5-fold more conidia than the conventional single-cycle process (only cycle 1). In addition, this production yield was 2-fold higher than that reported by [16] in a single production cycle of 15 days (2.5×10^9 con/gds), and 8-fold higher than what was reported in other optimized processes (5.8×10^8 con/gds; [6]).

3.2 Conidia production of *M. robertsii* Xoch-8.1

In order to evaluate the effectiveness of the proposed recycling strategy with another biological control agent, a similar set of assays was performed using the entomopathogenic fungus *M. robertsii* Xoch-8.1. *Metarhizium* strains have been used for the production of several commercial bioinsecticides [5].

3.2.1 First production cycle using fresh rice as the substrate

As previously described for *T. asperellum* Th-Th4 (3), in the first cycle, *M. robertsii* Xoch-8.1 was cultivated in SSC using fresh rice as the substrate. This first cycle lasted 10 days and produced 5.7×10^8 con/gds with a productivity of 5.7×10^7 con/gds·d (Table 2). The viability of these conidia was 63.2 %.

Table 2
Production of *M. robertsii* Xoch 8.1 conidia in successive production cycles.

	Conidia production cycles			
	Cycle 1	Cycle 2	Cycle 3	Cycle 4
Cycle length (d)	10	6	6	6
Production yield (con/gds)	5.7×10^8 ± 6.7×10^7 a	1.4×10^9 ± 3×10^8 b	1.2×10^9 ± 1.3×10^8 b	6.0×10^8 ± 6.2×10^7 a
Productivity (con/gds·d)	5.7×10^7 ± 6.7×10^6 a	2.4×10^8 ± 5×10^7 b	2.1×10^8 ± 2.1×10^7 b	1.0×10^8 ± 1×10^7 a
Conidia viability (%)	62.3 ± 2.6 a	71.2 ± 23 ab	88.1 ± 1.2 b	89.4 ± 3.3 b
Initial moisture content (%)	32.0 ± 1.2 a	43.1 ± 2.8 b	50.4 ± 0.8 c	57.0 ± 3.1 d
Cumulative substrate consumption (%)	15.1 ± 6.8	37.3 ± 6.7	46.4 ± 8.7	48.2 ± 5.5

d: days; con/gds: conidia per gram of dry substrate; con/gds·d: conidia per gram of dry substrate per day. This experiment was performed in two independent times using four replicates at each time. Each individual determination was carried out two times. Means (± SD) followed by the same letter within the same line are not significantly different according to Tukey ($p < 0.05$).

During this cycle, the fungus consumed 15.1 % of the added substrate, so almost 85 % of the initial rice remained unused at the end of the cycle. The residual substrate practically conserved its original structure, consistency, and color (Fig. 4c), so it was reused in a second production cycle.

3.2.2 Second production cycle using recycled rice.

In a similar way to *T. asperellum* Th-Th4 (3), *M. robertsii* Xoch-8.1 grew faster and sporulated earlier in cycle 2 than in cycle 1. The second cycle lasted 6 days and produced 1.4×10^9 con/gds. This production yield was 2.5-fold higher than that registered in the first cycle (5.7×10^8 con/gds). Also, the productivity improved from 5.7×10^7 to 2.3×10^8 con/gds·d, a 4-fold increase in comparison with cycle 1. In addition, no significant differences were found between the viability of these conidia and that of the conidia produced in the first cycle (Table 2).

At the end of cycle 2, the residual substrate practically maintained its initial structure and consistency (Fig. 4e). Besides, at this point, 60 % of the initial substrate that was added was still unused, and therefore available for setting up another production cycle.

3.2.3 Third production cycle using recycled rice

In the third cycle, conidia production yields were as high as in the second cycle, so once again, the use of recycled rice resulted in an average conidia production that was 2-fold higher than that obtained using fresh rice (Table 2). The conidia productivity was 3.7-fold higher in cycle 3 in comparison with cycle 1, and the conidia viability was unaffected.

At the end of this cycle, the residual substrate showed more significant changes in its macroscopic structure. Some rice grains appeared broken and the whole substrate presented a pasty appearance. Nevertheless, since *T. asperellum* Th-Th4 (3) was shown to be able to grow and sporulate in a substrate with these characteristics, it was decided to reuse the residual substrate again and evaluate the behavior of *M. robertsii* Xoch-8.1 under these conditions.

3.2.4 Fourth production cycle using recycled rice

Metarhizium robertsii Xoch-8.1 was able to grow and sporulate using 3-times recycled rice. More interesting was the finding that the conidia yield reached in cycle 4 was statistically equal to that obtained in cycle 1 (Table 2). In addition, the conidia viability was unaffected, so the use of 3-times recycled rice did not reduce the conidia quality or conidia yields in comparison with the use of fresh rice.

At the end of the fourth cycle, 50 % of the initial substrate that was added was still unused (Table 2), but this residual substrate presented a highly deteriorated appearance, so it was decided to end the assay and not to reuse the residual rice in a fifth cycle.

It is clear from Fig. 3a-b that for both fungi (*M. robertsii* Xoch-8.1 and *T. asperellum* Th-Th4 (3)), the excess moisture content negatively affected the conidia yields. The relation between these two variables was described by 3-parameter quadratic models defining curves where *y (conidia production yield)* rises to a maximum, but declines with further increases in *x (substrate moisture content)*. According to this, it is likely that a reduction in the substrate moisture at the beginning of cycles 3 and 4, for *T. asperellum* Th-Th4 (3) and *M. robertsii* Xoch-8.1, respectively, could have resulted in higher conidia yields in these particular cycles. However, as is shown in Tables 1 and 2, the cumulative conidia production obtained without drying processes or humidity adjustments were highly competitive in comparison with a single-cycle process using fresh rice, and also in comparison with the results reported in previous works. Therefore, the employment of drying processes or humidity adjustments is not recommended.

On the other hand, the use of cheap texturizers mixed with the initial dry-substrate in cycle 1 could be an interesting alternative. The use of water hyacinth or sugar cane bagasse improve the porosity of rice, which in turn, increase oxygen and heat transfer across the substrate bed [17, 8]. Therefore, it is probable that the use of these kinds of materials from the beginning of the first cycle could allow the substrate to be reused for at least one more cycle without needing extra drying or sterilization steps. However, this should be studied in future works.

3.3 Kinetic analysis of substrate consumption

The substrate consumption data obtained for each fungus were adjusted to the modified Gompertz model in order to estimate the kinetic parameters: P (maximum dry substrate consumed at the end of the whole production scheme) and R_s (maximum dry substrate consumption rate). As can be observed in Table 3, the P value for both fungi corresponded to a final substrate consumption close to 50 %. This showed that the proposed recycling strategy significantly increased the substrate usage in comparison with a single-cycle scheme where the substrate consumption is 20 % or less [7].

Table 3
Parameters of substrate consumption estimated by the modified Gompertz model.

	P (g)	R_s (g/d)	R^2
T. asperellum Th-Th4 (3)	1.57 ± 0.20 a	0.56 ± 0.07 a	0.944
M. robertsii Xoch-8.1	1.12 ± 0.13 b	0.08 ± 0.02 b	0.927

P: Maximum dry substrate consumed at the end of the whole production scheme (2.5 g of substrate was added at the beginning of the production scheme), R_s: Maximum dry substrate consumption rate. Means with different lowercase letters within the same column are significantly different ($p < 0.05$).

Regarding the consumption rates, *T. asperellum* Th-Th4 (3) showed the higher R_s value; however, it was interesting to observe that both fungi reached the maximum substrate consumption rate in the second cycle (i.e., when they grew on recycled rice) (Table 3 and Fig. 2). This observation and the high conidia production yields obtained for both fungi in their corresponding cycle 2 were clear indications that the use of recycled rice did not negatively affect the growth and sporulation physiology of these fungi, on the contrary, it increased it. Previous works have shown that the geometry, shape and size of the substrates used in SSC significantly affect the production of extracellular enzymes and the fungal growth rates [18]. Therefore, it is likely that the changes in rice geometry during the course of each of the production cycles have favored the growth and sporulation of the tested fungi. On the other hand, it has been reported that sporulating fungal colonies produce volatile organic compounds (VOCs) (e.g. 1-octen-3-ol, 3-octanol and 3-octanone) that can act as chemical elicitors of conidiation [19]. According to this, the accumulation of VOCs in the residual rice, could have caused some induction of the conidiation process, which could explain the increase in the conidia yields observed when recycled rice was used as the substrate. The analysis of this hypothesis will be the subject of further study.

Finally, since *T. asperellum* Th-Th4 (3) and *M. robertsii* Xoch-8.1 belong to different taxonomic families (Hypocreaceae and Clavicipitaceae, respectively), it is likely that the proposed recycling strategy will be useful for other commercially important fungi (e.g., *Beauveria bassiana, Isaria fumosorosea, Trichoderma harzianum*, etc.).

4. Conclusion

The proposed recycling methodology was efficient to revalorize the residual substrate remaining at the end of a conventional conidia production process in SSC. The reuse of this residual substrate in successive cycles of conidia production significantly increased the production and productivity parameters of the fungal strains *T. asperellum* Th-Th4 (3) and *M. robertsii* Xoch-8.1. In addition, this strategy did not affect the viability of the conidia produced using recycled rice relative to the conidia produced using fresh rice.

The proposed recycling approach is very simple, and is completely free of extra -drying, -sterilization, and -reinoculation steps, as the residual substrate obtained at the end of each conidia production cycle is reused in the next cycle without any further treatment. Thus, this method requires minimal operational intervention and can be easily adopted by small bio-factories or self-supply units. In addition, the savings due to substrate recycling and the elimination of extra processing steps would help reduce processing costs.

5. Declarations

-Ethical Approval. No human experiments were performed in this study.

-Consent to Participate. As no human experiments were performed, no written consent from participants was required.

-Consent to Publish. As no human experiments were performed, no written consent to publish from participants was required.

-Authors Contributions: ACN: Investigation and formal analysis, JEMH: Conceptualization, formal analysis, writing-original draft, visualization. OL: Conceptualization, funding acquisition, resources, project administration, reviewing and editing.

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-Competing Interests. The authors declare that they have no conflict of interest.

-Availability of data and materials. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

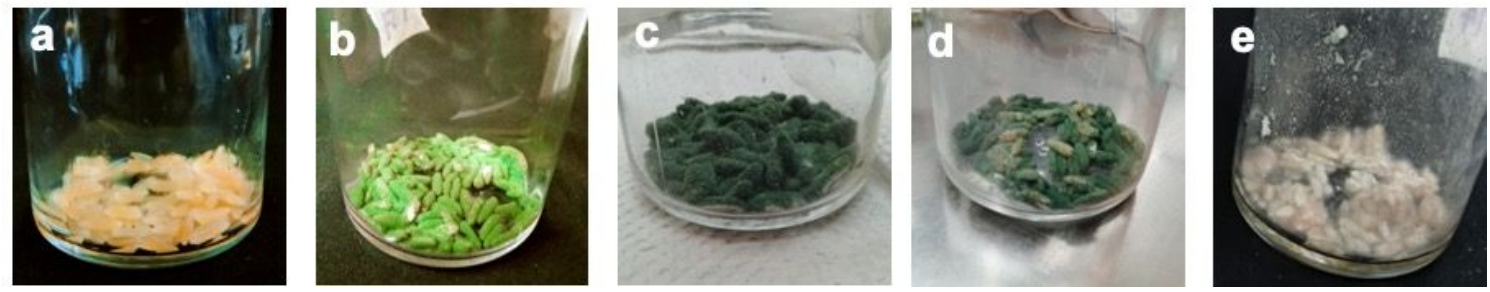

Figure 1

Solid-state cultures of T. asperellum Th-Th 4 (3). a) beginning of cycle 1; b) end of cycle 1; c) end of cycle 2; d) end of cycle 3; e) substrate remaining after harvesting the conidia produced in cycle 3

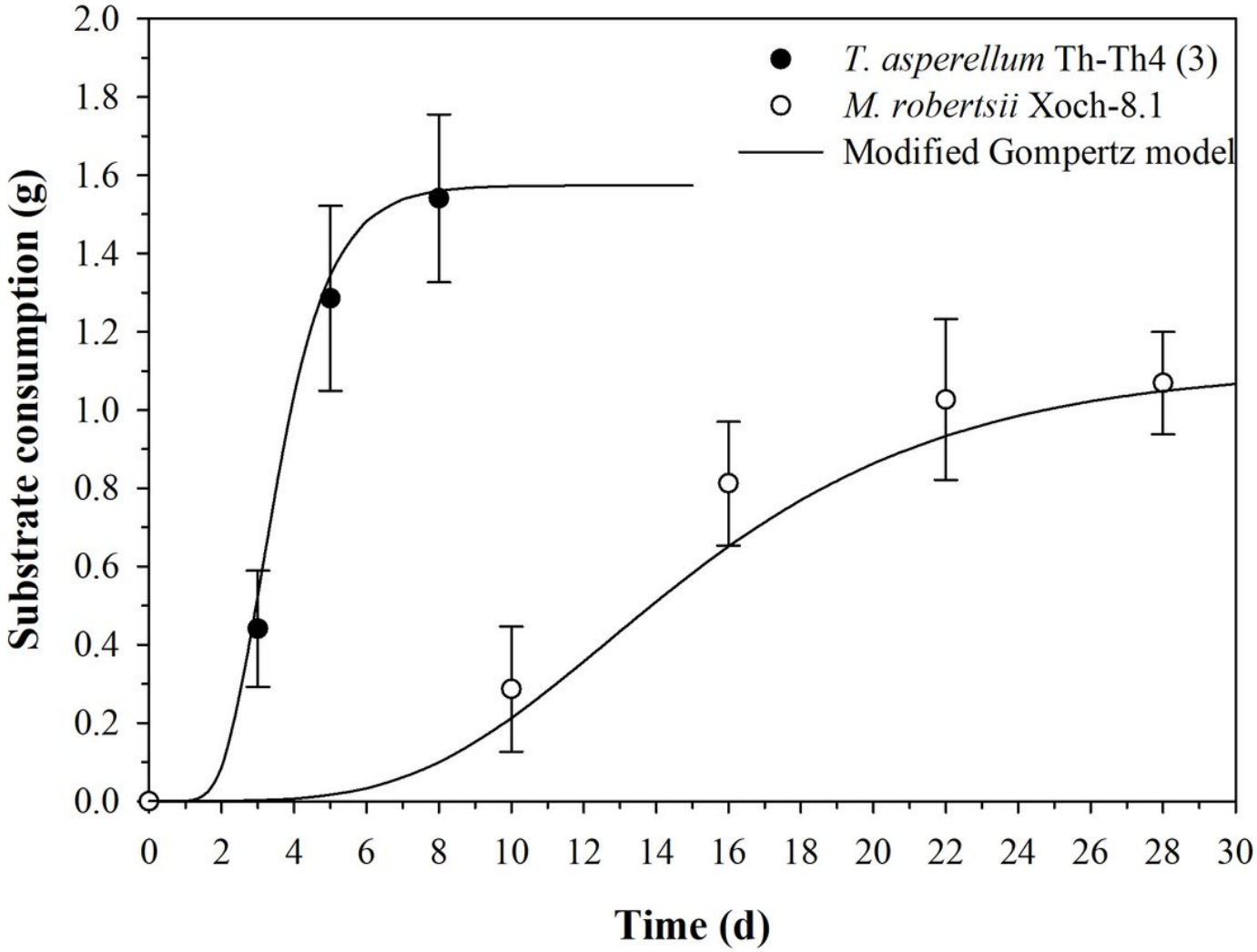

Figure 2

Substrate consumption curves obtained after adjusting the experimental data to the modified Gompertz model. The open and solid circles represent the accumulated grams of dry substrate consumed at the end of each conidia production cycle (3 cycles for T. asperellum Th-Th4 (3) and 4 cycles for M. robertsii Xoch-8.1)

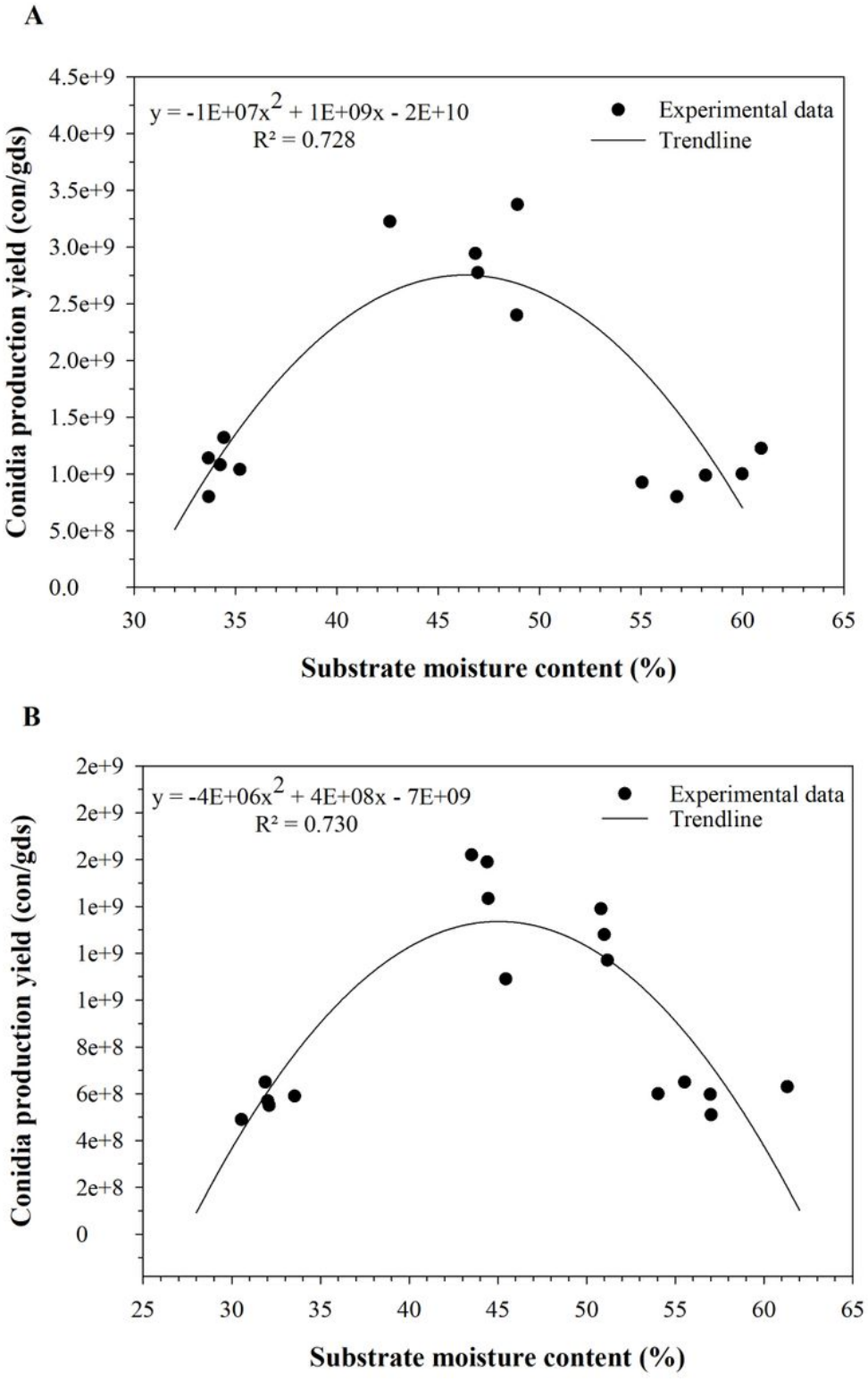

Figure 3

Relation between substrate moisture and conidia production yield of A) T. asperellum Th-Th4 (3) and B) M. robertsii Xoch-8.1, during successive cycles of conidia production

Figure 4

Solid-state cultures of M. robertsii Xoch-8.1. a) beginning of cycle 1; b) end of cycle 1; c) end of cycle 2; d) end of cycle 3; e) end of cycle 4