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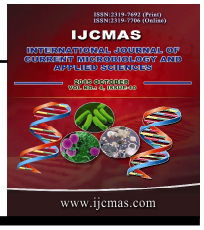

Original Research Article

Amplification of *laeA* Gene in *Aspergillus terreus*: A Strategy to Generate Lovastatin-Overproducing Strains for Solid-State Fermentation

Pérez Teresa, Mejía Armando and Barrios-González Javier*

Departamento de Biotecnología, Universidad Autónoma Metropolitana –Iztapalapa, Av. San Rafael Atlixco 186, Col. Vicentina, Iztapalapa, 09340 México D.F., Mexico
**Corresponding author*

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A B S T R A C T

Solid-state fermentation (SSF) has become an alternative industrial production system for secondary metabolites, but strains for submerged fermentation (SmF) do not perform well in SSF, so there is a need for methods to generate improved strains for SSF. In this work we evaluated the strategy of overexpressing the global regulator *leaA* in *Aspergillus terreus* to generate lovastatin hyper producing strains for SSF. Expressing this gene from a constitutive promoter was best, due to the abundance of overproducers for SSF in the transformant population (100%), and to the high lovastatin production increase obtained (104%). Overexpressing *laeA* from its own promoter generated a little lower abundance of overproducers for SSF (90%) and lower production increase (76%). The best transformants for SSF were not the best in SmF and vice versa. But *laeA* overexpression also generated overproducers for SmF, although in lower numbers, and own promoter was clearly the strategy of choice. In SSF the constitutive promoter-containing T2laeAcons reached 30.6 mg of lovastatin/g dry culture. In SmF the own-promoter transformant T1laeA produced 0.89 mg/ml. This molecular improvement method provides the additional advantage of improved sporulation rate. This strategy could be applied to other fungal secondary metabolites to generate overproducing strains for SSF.

Introduction

Lovastatin is a commercially valuable secondary metabolite produced by *Aspergillus terreus* and *Monascus purpureus*, although the former is used for industrial production. This compound, as well as its immediate derivative simvastatin, lower cholesterol levels in blood (Endo 2004; Barrios-Gonzalez and Miranda, 2010).

Lovastatin industrial production is conventionally performed with strains of *Aspergillus terreus* by liquid submerged fermentation (SmF); however, solid-state fermentation (SSF) has become an alternative industrial production system (Suryanarayan, 2003). One of the major positive aspects of SSF is that enzymes, and secondary metabolites, are in many cases, produced at much higher yields than in SmF (Barrios-González *et al.*, 1988; Barrios-González *et al.*, 1993; Balakrishnan and Pandey, 1996; Robinson *et al.*, 2001;

Barrios-González and Mejia, 2007). It is considered that this is part of the particular physiology shown by fungi in SSF, often called "physiology of solid medium" (Barrios-Gonzalez, 2012).

Another consequence of this different physiology, displayed by fungi in SSF, is that particular strains tend to show very different performance in SSF in relation to the one displayed in SmF. Reports on enzymes production indicate that high producing strains for SmF are generally poor producers in SSF, or at least cannot be relied upon to perform well in SSF (Shankaranand *et al.,* 1992). Similarly, studies on secondary metabolites production, using different strains, showed discrepancies in the individual performances in one or the other culture system. In this way, good producers in SmF were medium or low producers in SSF and vice versa. Hence, overproducing strains developed for SmF cannot be relied upon to perform well in SSF (Barrios-González *et al.,* 1993; Barrios-González and Mejía, 1996).

This is particularly relevant since, in both culture systems, the strain is a key factor in the development of a competitive commercial production process. However, little attention has been directed towards developing methods and strategies to generate this kind of strains (Barrios-Gonzalez and Mejía, 2009).

This means that there is a need for new strategies and genetic improvement methods to obtain special strains, particularly suited for SSF. These methods will contribute to obtain the full potential of SSF, so that this culture system can compete favorably with SmF processes.

An advanced strategy to generate secondary metabolites overproducing strains is the overexpression of positive regulatory genes. LaeA, a putative S-adenosylmethionine-dependent methyltransferase, was originally described as a global positive regulator of secondary metabolism in several *Aspergillus* spp. (Bok *et al.,* 2006), and later on shown to be also required for the biosynthesis of secondary metabolites in other fungi (Bayram *et al.,* 2008; Lodeiro *et al.,* 2009, Hoff *et al.,* 2010). Moreover, there is evidence that LaeA is associated with the cAMP-PKA signaling cascade, initiated with the heterotrimeric G protein signaling. This pathway senses environmental and nutritional signals from the environment. It is possible then that, if gene *laeA* is over expressed, different effects could be observed in SSF.

In this work we designed and tested two strategies, based on *laeA* overexpression, to develop high-producing strains for SSF.

Materials and Methods

Strains, culture media and culture conditions

Aspergillus terreus TUB F-514, was kindly provided by Dr. G. Szakács (from the collection of the Technical University of Budapest), was grown on Power Agar (Fierro *et al.,* 1996) for 5 days at 30°C for sporulation. Spores were collected and used to inoculate lovastatin fermentations.

Neurospora crassa FGSC 4200 (Fungal Genetics Stock Center) was used to quantify lovastatin by bioassay, and was grown on PDA medium (Potato Dextrose Agar from Bioxon) for 10 days at 30ºC for sporulation. This strain is sensitive to β-hydroxy acid lovastatin form (Kumar *et al.,* 2000).

Lovastatin production medium (40) was used for SmF. This medium contained 0.6%

glucose, 3.4% lactose, 0.3% soybean meal, 0.2% KNO_3, 0.3% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% NaCl, with final pH of 6.5. This medium was dispensed into 250 ml Erlenmeyer flasks. The cultures were inoculated to a concentration of $2x10^6$ spores/ml and incubated at 30°C in a rotary shaker (at 200 rpm) for different times.

For solid-state fermentation (SSF), a 2.5-fold concentrated version of lovastatin production medium (2.5X) was inoculated with spores of *A. terreus* ($2x10^6$ spores/ml), and used to impregnate the solid support to moisture content of 85%. High-density polyurethane was used as solid support, which was pre-treated, as described by Tomasini *et al.* (1997). One-centimeter polyurethane cubes were impregnated with the inoculated medium, and 6 g of the resulting solid medium were used in each 250 ml Erlenmeyer flasks, which were covered with aluminum foil and parafilm (Pechiney Plastic Packaging, Menasha, WI, USA). For both types of fermentation, samples were taken by triplicate at different times.

Agar plug cultures were performed with 2% agar production-medium cylinders and lovastatin was evaluated by bioassay, using *N. crassa* as indicator organism, all performed according to Kumar *et al.* (2000).

Analysis

pH determination. For SSF and SmF. pH was measured as described before Miranda *et al.* (2013).

Biomass determination

In SmF biomass was determined by filtering the whole content of the flask, and drying it to constant weight at 80°C. In SSF, biomass was determined indirectly, from glucosamine content (Tomasselli *et al.*, 2001). This is an indirect method to estimate biomass that takes advantage of the presence of chitin in fungal cell walls. Chitin is a poly-N-acetylglucosamine, which is hydrolyzed in this assay to quantify glucosamine by a colorimetric method. In SSF the moisture content was calculated by drying 1 g of the solid culture at 80ºC until constant weight: sample wet weight – sample dry weight/sample wet weight x 100.

Lovastatin extraction and quantification

The conditions for lovastatin extraction in SSF and SmF, as well as lovastatin determination, were performed as described by Miranda *et al.* (2013).

Plasmids

Plasmid pULC43 (25) contains the phleomycin fungal marker gene under the control of the promoter of gene pcbC from *P. chrysogenum* and terminator *CYC1* from *Saccharomyces cerevisiae*. Plasmid pAN52.1 (26) contains the promoter of gen *gpd* (glyceraldehyde-3-phosphate dehydrogenase) from *A. nidulans* and the terminator of gen *trpC*. These plasmids were used to clone different DNA fragments as indicated below. pGEM vector was used to clone cDNA of *laeA* gene for standard curve for real time PCR (Table 1).

Construction of transformation vectors

Plasmids were generated by standard techniques (Sambrook *et al.*, 1989), and the primers used to this end are listed in table 1. Polimerases Vent (BioLabs) and Taq were used in the PCRs performed.

Plasmid pULC43 was used as a base to overexpress *laeA* from its own promoter, and pAN52.1 to express it from the constitutive promoter of *gpdA.*

Complete *laeA* gene (with promoter and terminator) in a 2.6 kbp fragment was amplified with primers laeAF and laeAR and ligated as a BamHI fragment to pULC43 to generate plasmid pUAMTPlaeA. On the other hand, the cds sequence of *laeA* (1.25 kbp) was amplified with primers laeAconsF and laeAconsR and ligated directionally as a NcoI-BamHI fragment to pAN52.1 to generate PUAMTPlaeAcons.

Nucleotide sequences access numbers

Gene sequence analysis was done in NCBI, with the accession numbers for *A. terreus: laeA*, *lovE* and *H4*: XM_001210764.1, ATLOVBSGC2 and XM_001210673, respectively.

Transformantion of *A. terreus*

Protoplasts of *A. terreus* were obtained as in Fierro *et al.* (1993), and transformation was performed as in Cantoral *et al.* (1987) and Díez *et al.* (1987), using phleomycin (Sigma). In the case of pUAMTPlaeAcons, a co-transformation, with plasmid pULC43 was performed. Since the latter contains a phleomycin resistant marker, transformants were selected by their resistant to phleomycin (120 µg/ml) on Czapek-Sorbitol (1 M) medium.

Integration of the plasmids in the transformants was checked by Southern blot hybridization according to Barrios-Gonzalez *et al.* (2008), and by PCR.

Control transformants were transformed with pULC43, or with pAN52.1 and pULC43, to confirm that no positive effect on production was generated with the empty vectors.

Nucleic acids analysis

For genomic DNA isolation, *A. terreus* was grown in supplemented minimal-nitrate medium (Käfer, 1977), and genomic DNA was isolated as described by Timberlake *et al.* (1980). For Southern blot, digestions with restriction enzymes, electrophoresis on agarose gel, blotting to Hybond-Nylon membranes (Amersham), hybridization and *laeA* probe preparation was performed by standard methods (Sambrook, 1989; Church and Gilbert, 1984). *laeA* probe was amplified by PCR and radioactive labeling, hybridization and autoradiography were performed as described by Barrios-Gonzalez *et al.* (2008).

RNA extraction and expression analysis

RNA from N2 frozen mycelium samples was extracted with Trizol (Invitrogene), following the manufacturer's instructions. RNA was treated with RQ1 RNAse-Free DNAse (Promega), and concentration quantified by Nanodrop 2000, standardizing samples to 10 ng/µl.

Reverse transcription and real-time PCR were performed with an Express One-Step SYBR® GreenER™ qRT-PCR Universal kit from Invitrogene, using 25 ng of total RNA. *laeA* gene sequence for qRT-PCR was amplified with primers laeArtpcrF and laeArtpcrR (Table 1). Thermal cycling was performed with the fluorescence detector system Rotor-Gene 3000 (Corbett Research). Absolute quantification was performed by means of a laeA cDNA standard curve.

Gene H4 (histone) was used as endogenous control, and normalized values were expressed as a fraction of *laeA* concentration and the concentration of the housekeeping gene in the time point. The difference of *laeA* expression levels in the transformants was calculated as a fraction of the normalized sample and the normalized calibrator (parental strain).

Lovastatin extraction and quantification

The conditions for lovastatin extraction in SSF and SmF, as well as lovastatin determination, were performed as previously described (Miranda *et al.*, 2013).

For lovastatin quantification in agar plug cultures, the method described by Kumar *et al.* (2000) was used. Briefly, lovastatin extraction from the agar plugs was performed by macerating it in a test tube containing 5 ml of ethyl acetate (JT Baker), and 20 µl (supernatant) were impregnated on a sterile circular Whatmann filter paper, dried and quantified, against a standard curve, by a bioassay in agar medium inoculated with *Neurospora crass*.

Other physiological parameters

For sporulation index determination, petri dishes with 25 ml Potato Dextrose Agar (PDA) medium were inoculated with 10^5 spores in 300 µl, which was spread and incubated at 30°C for 4 days in triplicates. Conidia were collected with 5 ml of 0.05% Tween 80, and its concentration was calculated by means of a Neubauer chamber. Total spores number from a flask was divided by the agar area to obtain spores/cm^2. To determine radial growth rate, Petri dishes with PDA were inoculated in the center with 5 µl, containing 10^3 spores, by triplicate and incubated at 30°C for 7 days. Radial growth rate was determined by measuring colonies diameter, and dividing it by 7 (days).

Results and Discussion

Construction of recombinant strains over-expressing *laeA* (with increased *laeA* copy numbers).

Two constructions were designed, one expressing *laeA* from its own promoter (pUAMTPlaeA), and another one expressing this gene from a constitutive promoter (pUAMTPlaeAcons). As can be observed in figure 1A, pUAMTPlaeA was obtained by cloning the complete gene l*aeA* from *A. terreus* with its own promoter and terminator sequences (2.6 kbp) in the BamHI site of plasmid pULC43. Panel B shows the construction of pUAMTPlaeAcons, that was obtained by directional cloning of the coding sequence of l*aeA* (1.25 kpb) in the NcoI and BamHI sites of plasmid pAN52.1. This vector contains a strong constitutive promoter from gene *gpdA* of *A. nidulans*, and the terminator *trpC* from the same fungus. Protoplasts of *Aspergillus terreus* TUB-514 were transformed with pUAMTPlaeA or pUAMTPlaeAcons, to obtain 2 sets of transformants that will overexpress *laeA* from its own promoter or from a constitutive one.

Pre-selection and selection stages: Lovastatin production by transformants, in agar plugs, SmF and SSF.

All transformants were initially evaluated by their production of lovastatin on agar plugs cultures (APC) to decrease the population. That is, to eliminate false transformants or transformants that had integrated the vector in inconvenient sites in the genome.

The first strategy (or own promoter-), was based on transformation with pUAMTPlaeA, and generated 21 transformants resistant to phleomycin (120 µg/ml of agar culture). In APC, 10 transformants showed higher lovastatin production than the parental strain.

The second strategy (or constitutive promoter-), was based on transformation with pUAMTPlaeAcons, and was more laborious, due to the co-transformation procedure with plasmid pULC43, since the

former vector does not contain a fungal marker. Only 12 transformants resistant to phleomycin were obtained, but 10 showed higher lovastatin titers than the parental in APC.

The best 10 producers in APC, of each population (strategy), were pre-selected to continue its characterization.

In the selection stage, these preselected transformants were further characterized by measuring lovastatin production (at day 7) in SSF and in SmF.

Own-promoter *laeA* transformants: As can be seen in table 2, 90% of the transformants of this group showed higher lovastatin production than the parental strain (overproducers) in this SSF test. The production increase found in these transformants ranged from 11 to 47%, and T9laeA was the best producer in these SSF 7 days-cultures, so it was selected for the next characterization stage.

On the other hand, 50% of these transformants were lovastatin overproducers in SmF, with production increases varying from 5 to 82%. T1laeA was the highest yielding mutant in SmF (Table 2), so it was selected.

Constitutive-promoter *laeA* transformants: In this case, all selected transformants turned out to be lovastatin overproducers in SSF (Table 3), with increases ranging from 11 to 60%. T2laeAcons was the best producer of this group, so it was selected for next stage. Contrasting, T5laeAcons was the only overproducer in SmF (62% production increase) in this group, and it was selected for next characterization stage.

Selected transformants: Lovastatin production kinetics and *laeA* expression Southern Blot and PCR analysis (data not shown) confirmed integration of the corresponding constructions in the selected transformants (SSF: T9laA and T2laeAcons; and SmF: T1laeA and T5laeAcons). After this, more detailed kinetic studies were performed on these strains.

In SSF, T2laeAcons and T9laeA, showed very high lovastatin yields, reaching peak productions of 30.6 and 26.4 mg of lovastatin/gdc (respectively). These titers compare very well with the parental production level (15.3 mg/gdc). In the culture with T9laeAcon, the time of maximum production shifted to day 9, showing high production rates until then. T2laeA reached a slightly lower titre, displacing peak production to day 7. It is important to note that peak production was reached at day 5 by the parental strain (Fig. 2). In this way, T2laAcons was the best transformant for SSF showing 104% lovastatin production increase. Biomass determination data confirmed that specific production was much higher in transformants: T2laeAcons 392 µg lovastatin/ mg of biomass, T9laeA 372µg/ mgb and 159.27 µg/mgb in the parental.

In SmF, the selected transformants, T5laeAcons and T1laeA, also extended the period of the metabolite's biosynthesis from day 3 to day 9, and displayed lovastatin production increases of 97.7 and 102 % respectively (Fig. 3). The transformant expressing *laeA* from a constitutive promoter (T5laeAcons) displayed a notoriously constant production rate throughout idiophase in SmF. This same feature was observed in the transformant with constitutive promoter selected for SSF (described above). In this way, both transformants were superior producers, reaching lovastatin production levels of 0.87 mg/ml for T5laeAcons and 0.89 mg/ml for T1laeA. In this case too, transformants

showed higher specific production: T1laeA = 83.11, T5laAcons = 73.38 and parental = 31.1 µg lovastatin/ mg of biomass.

To confirm the overexpression of gene *laeA* in the transformants selected for each culture system, an expression profile of this gene was performed on the parental and the transformant strains. As expected, in this experiment the 4 transformants showed higher *laeA* expression than the parental strain. Results showed that *laeA* transcripts were present since trophophase (18 and 21 h) in both, the parental and the transformant strains. In SSF T2laeAcons y T9laeA showed higher *laeA* expression levels than the parental strain, in trophophase. In fact T2laeAcons showed higher transcript levels throughout lovastatin culture, (up to 18-fold higher in trophophase and 5-fold on day 5), while in T9laeA *laeA* expression was only clearly superior in trophophase (Fig. 4). In SmF, T5laeAcons and T1laeA showed higher levels of *laeA* transcripts accumulation throughout the culture (except T1laeA at day 7, which was similar to the parental's) (Fig. 5).

The same experiment was used to look for a correlation between *laeA* expression profile and the shape of the lovastatin production kinetics of these transformants. In both culture systems, the selected transformants showed higher *laeA* expression than the parental in the last part of idiophase (days 5 and 7). However, a reasonable expression level was also evident in the parental strain at those times.

In SmF, lovastatin production stopped at day 3 in the parental strain culture (Fig. 3). However, *laeA* gene still showed a relatively high expression at days 5 and 7 (Fig. 5). A similar situation was observed in SSF, where lovastatin production with the parental lasted for 5 days (Fig. 2), while *laeA* expression was still evident at days 5 and 7 (Fig. 4).

OE: laeA as a genetic improvement method to generate overproducers for SSF or for SmF

In general terms, production increases shown by the selected transformants were very high with both promoter strategies. To perform a more detailed comparison of the strengths and limitations of both strategies, results of this section were used to calculate some production-related parameters (Table 4). These parameters include the proportion of transformants with production levels lower than the parental in a particular culture system.

On the other hand, discrepancies were detected in the production level of individual transformants in SSF and in SmF. This can be observed in tables 2 and 3. In this sense, T9laeA was an exceptional since it showed good performance in both culture systems. Possibly, exceptions can include T6laeA with medium level production increase in both culture systems.

Besides these, the rest showed contrasting performances, like T3laeA and T2laeA with very good production levels in SSF but very bad yields in SmF. Similarly, T2laeA and T4laeA, with medium-level productions in SSF and very bad performances in SmF.

On the other hand, T5laeAcons showed excellent performance in SmF and med-level production in SSF. Moreover, most of the transformants with constitutive promoter showed good improvements for production in SSF, and disastrous reductions in SmF. Interestingly, a certain proportion of the transformants showed lower production than the parental in SmF, while none showed a production decrease in SSF (Table 4).

Effect of *laeA* overexpression on conidiation and growth

The effect of *laeA* overexpression (OE:*laeA*) on sporulation, growth and other physiological parameters were also studied in all pre-selected transformants. Although our results did not show a clear effect on sensitivity to oxidative stress, OE:*laeA* did show interesting effects on conidiation and on growth rate.

All transformants expressing *laeA* from its own promoter exhibited important sporulation increases (Fig. 6). Although particular values differed, 240% average sporulation increase was calculated for this population. Moreover, the selected transformants, T1laA and T9laeA, displayed sporulation increases of 75% and 135% respectively. On the other hand, transformants OE:*laeA* from a constitutive promoter presented a comparatively smaller sporulation increase (65% average increase in this group). In this case, selected transformants, T2laeAcons and T5laeAcons, exhibited discrete sporulation-index increases of only 24 and 9%.

Results (Table 5) also showed that OE:*laeA* also had a positive effect on growth. All (except one) of the own-promoter transformants and all (except one) of the transformants with constitutive promoter, showed increased radial-growth rates (13.7% average). This includes the selected transformants for SmF: T1laeA and T5laeA, and for SSF: only T5laeAcons, although T9laeA displayed a smaller increase.

Although the positive effect of laeA overexpression on lovastatin production has been reported before (Bok *et al.,* 2004), that study had a different purpose, so the medium used was not a lovastatin production medium (glucose minimal medium) nor the metabolite was quantified with precision (TLC was used).

On the other hand, LaeA is associated with the cAMP-PKA signaling cascade that senses environmental signals, so its overexpression could generate different effects in SSF. Moreover, we have obtained evidence that some components of this pathway behave differently in SSF (manuscript in preparation). Hence, overexpression of gene *laeA* was proposed here as a molecular-genetic improvement approach to generate lovastatin-overproducing strains of *Aspergillus terreus*, particularly suited for SSF. Two alternative strategies were tested: a) overexpressing gene *laeA* from its own promoter; and b) overexpressing it from a constitutive promoter.

In general terms, our results showed that OE:leaA is an excellent method to generate (lovastatin) overproducing strains for SSF. Under certain circumstances, the approach was also successful to obtain overproducing strains for SmF. It is important to note that, presumably because of the integration of *laeA* gene in different loci, the transformants showed differences in production level and other characteristics. In this way, most of the improved transformants showed good performance in SSF or SmF. That is, the best producers in SSF were not the best in SmF and vice versa. Although the reason for this is unclear, this agrees with the conclusions of other studies on enzymes or secondary metabolites production mentioned earlier. However, good performers in both culture systems, although not common, can be obtained. This was the case of T9laeA that was the best own-promoter transformant for SSF and the second best for SmF in this group.

From a practical standpoint, the proposed methods are formed by one pre-selection stage where all transformants were

evaluated by their metabolite production in a rapid APC (followed by a bioassay), and a selection stage, where the top 10 transformants were evaluated again by their lovastatin production in a one-time point (7 days) SSF and SmF.

From the pre-selection stage, it became apparent that the own-promoter and constitutive-promoter transformant populations showed different characteristics. Transformants overproducing lovastatin in APC were more abundant in the constitutive- promoter transformant population: 10 of 12 vs. 10 of 21 in the own-promoter population.

These APC overproducers (preselected populations) were further tested in the selection stage, where lovastatin production data indicated that high-yielding strains for SSF were easier to obtain than overproducer for SmF, from both transformant populations. That is, improved strains for SSF were more abundant, that strains for SmF. It was found that 100% of the constitutive-promoter transformants, and 90% of the own-promoter transformants were lovastatin overproducers in SSF. In contrast, 50% of the own-promoter transformants, and only 10% of the constitutive-promoter transformants, turned out to be overproducers in SmF. The different response (to OE:laeA) in SSF and in SmF, suggests that cAMP-PKA signaling pathway does contributes to sensing these different environments.

The best 2 transformants (one of each strategy) for each culture system were selected: SSF: T9laA and T2laeAcons; and SmF: T1laeA and T5laeAcons. These were further characterized in kinetic studies, where lovastatin yields reached were much higher than the ones found in the previous stage. The reason was that the time of peak production was displaced towards later culture times. Results confirmed that the best transformant in SSF was the constitutive promoter-containing T2laeAcons, which extended production stage to day 9 (parental produced until day 5), reaching 30.6 mg of lovastatin/gdc. This represents a 104% production increase.

This is not only a high production increase, but this lovastatin concentration is the highest reported in SSF. Baños *et al.* (2009) reported a peak lovastatin production of 19.95 mg/gdc in artificial inert support SSF, while Valera *et al.* (2005) reported the production of 16.78 mg/gdc by an improved strain in wheat bran SSF.

Transformants selected for SmF T5laeAcons and T1laeA, also extended the period of the metabolite's biosynthesis from day 3 to day 9, reaching T1laeA the highest lovastatina production with 0.89 mg/ml (102% production increase). This is an interesting production level, when compared with other reports on improved strains (Vilchis-Ferrón *et al.*, 2004; Sreedevi *et al.*, 2011; Li *et al.*, 2011).

In the present work, an expression analysis confirmed that *laeA* was over expressed in the selected transformants. All the transformants showed a markedly higher *laeA* expression than the parental, in both culture systems and particularly in trophophase. Although it is clear that production improvement in the transformants was due to OE:laeA, and that higher production yields were due to a longer production period in the transformants, we were unable to find a correlation between *laeA* expression in the last part of idiophase and the extension of lovastatin production period. Hence, it is at the present not clear how could *laeA* be involved in this.

LaeA is still an enigmatic protein: although it was originally identified as a regulator of secondary metabolites biosynthesis, it was subsequently found to have important functions in other physiological aspects like development and parasitism among other traits (Bok *et al.*, 2005). For example, studies on *Penicillium chrysogenum*, have shown that, PcLaeA, also possesses a function in hyphal differentiation, finding a hyperbranching phenotype in ΔPclaeA mutants (Hoff *et al.*, 2010). Deletion of the *laeA* gene in *P. chrysogenum*, but also in *A. fumigatus* and *A. flavus*, caused reduced conidiation (Bok *et al.*, 2005; Chang *et al.*, 2012).

In this way, it was interesting to determine if *laeA* overexpression in *A. terreus* affected other physiological aspects, like conidiation and growth. Transformants O:E laeA from its own promoter presented very high increases in sporulation index (240% average), while constitutive-promoter transformants showed a comparatively smaller increase (65% average). The effect of laeA:OE on conidiation, observed in this work, could be explained by the role of LaeA in directing the formation of velvet family complexes. It has been reported that, in *A. nidulans*, LaeA negatively controls the concentration of the dimmer VosA-VelB, which represses asexual spores formation (Ni and Yu, 2007; Sarikaya-Bayram *et al.*, 2010).

On the other hand, results indicated that LaeA overexpression also had a positive effect on growth (RGR). In contrast with sporulation, this increase was similar in both populations, i.e. own-promoter and constitutive promoter. Apparently, conidiation is a more delicate or sophisticated physiological process than growth.

In general, industrial over-producing strains, which have been subjected to several mutation and selection programs, have reduced vigor, expressed in their lower growth rate and poorer conidiation. In this sense, higher sporulation and growth rate increase, obtained as collateral benefits in our transformants, are additional advantages of the genetic improvement methods proposed here.

To evaluate which strategy is more convenient to obtain overproducing strains for SSF, two factors were mainly taken into account: ease with which these mutants can be obtained (abundance in transformant population), and production increase (Table 4). With this perspective, expressing *laeA* from a constitutive promoter was more suitable to obtain lovastatin overproducing strains for SSF, due to the very high abundance of these mutants within the transformant population (100%), and to the high lovastatin production increase obtained with the best transformant (104%). Contrasting, the own-promoter population contained 90% overproducers; and the best mutant showed a 76% production increase.

Overexpressing *laeA* from its own promoter can also work to obtain overproducers for SmF. However, overproducers for this culture system were relatively scarce. This population contained 50% overproducers in SmF; and the best mutant showed 102% production increase. An intriguing aspect was that an important proportion of the transformants showed strongly decreased production levels in SmF (lower than the parental): Forty % of the transformants with own promoter, and a surprising 90% for constitutive promoter. This phenomenon was not observed in SSF. The reason for this is not clear, but it seems that physiology in SmF is more vulnerable to inconvenient integration sites.

Table.1 Primers design

Primers	Sequence[a]	Restriction site design or	Amplified fragment (bp)
laeAF	CTCCAGGATCCGAGTTCACGGTGC	BamHI in *laeA* promoter	2,624 bp
laeAR	AGGCGGATCCGTCTCAACTACAGC	BamHI in *laeA* terminator	
laeAconsF	ACCTATCACCATGGCCCCTCCG	NcoI in l *laeA* coding sequence initiation	1,250 bp
laeAconsR	GGACTCCAGGATCCACTCTTGG	BamHI in *laeA* coding sequence end	
plaeAF	CCAAGAGTGGATAATGGAGTCCC	Terminator *laeA*	1,250 bp
plaeAR	CGACAGTTGACAGAGCCAATGGC	Promoter pcbC (ble marker)	
plaeAconsF	CCAGAGGGTCATGACTTGAGC	Promoter gpdA	1,444 bp
plaeAconsR	CCACGTCAATTGCCCAGATCC	coding sequence laeA	
laeAsonF	GGACGGCTGTACCATGCGTATCG	coding sequence laeA	364 bp
laeAsonR	CGCTTCCGCAGCCCATTTGGAGG	coding sequence laeA	
laeArtpcrF	CGAGAACATAATCCTGGCTACAGC	coding sequence laeA	122 bp with RNA 214 bp with DNA
laeArtpcrR	CCTTTTCGATACGCATGGTACAGC	coding sequence laeA	
lovErtpcrF	CGGACCAAGAACCTCTTTACGGC	coding sequence lovE	84 bp with RNA or DNA
lovErtpcrR	CGACAGGAGCAACTCCGATATGG	coding sequence lovE	
H4rtpcrF	CGTGACAACATCCAGGGTATCACC	coding sequence histone H4	98 bp
H4rtpcrR	CGGGTCTCCTCGTAGATCATGGC	coding sequence histone H4	

[a] The underlined sequence show the placement of the restriction site.

Table.2 Lovastatin production by transformants with pUAMTPlaeA (own promoter), in 7 days SSF and SmF. Transformants were ordered according to their production level in SSF

pUAMTPlaeA transformants	SSF: Lovastatin production (mg/gdc)	SmF: Lovastatin production (mg/mL)
Parental	$15.3\pm0.75^{a,b}$	$0.44\pm0.05^{b,c}$
T9laeA	$\mathbf{22.5\pm0.84^{e}}$	$0.73\pm0.03^{e,f}$
T13laeA	$20.6\pm0.20^{d,e}$	0.33 ± 0.01^{b}
T12laeA	$18.8\pm0.06^{c,d}$	0.37 ± 0.01^{b}
T1laeA	$18.4\pm0.74^{c,d}$	$\mathbf{0.81\pm0.01^{f}}$
T15laeA	$18\pm0.45^{c,d}$	$0.46\pm0.05^{b,c,d}$
T16laeA	$17.9\pm0.48^{b,c,d}$	$0.56\pm0.05^{c,d}$
T2laeA	$17.6\pm0.86^{a,b,c}$	0.15 ± 0.03^{a}
T7laeA	$17.1\pm1.17^{a,b,c}$	$0.44\pm0.04^{b,c}$
T4laeA	$16.9\pm0.62^{a,b,c}$	0.35 ± 0.01^{b}
T14laeA	14.9 ± 0.67^{a}	$0.59\pm0.05^{d,e}$

Table.3 Lovastatin production by transformants with pUAMTPlaeAcons (constitutive promoter), in 7 days SSF and SmF. Transformants were ordered according to their production level in SSF

pUAMTPlaeAcons transformants	SSF: Lovastatin production (mg/gdc)	SmF: Lovastatin production (mg/mL)
Parental	15.3±0.75	0.44±0.05
T2laeAcons	**24.8±0.61**	0.30±0.03
T7laeAcons	21.3±0.66	0.39±0.02
T9laeAcons	20.7±0.30	0.14±0.01
T4laeAcons	20.3±1.44	0.15±0.02
T8laeAcons	19.0±0.01	0.36±0.02
T5laeAcons	19.0±0.41	**0.72±0.04**
T10laeAcons	18.6±0.14	0.41±0.02
T1laeAcons	17.9±1.28	0.38±0.10
T6laeAcons	17.8±0.17	0.32±0.01
T3laeAcons	16.9±0.62	0.43±0.05

Table.4 Efficiency of strategies (type of promoter) to generate high lovastatin producing strains for SSF or SmF

Strategy	% Oveproducer Transformants		Best Transformant Prodution Increase (%)		% Transformants Showing Decreased Production	
	SSF	SmF	SSF	SmF	SSF	SmF
Own Promoter	90	50	76	102	0 *	42.8
Constitutive Promoter	100	10	104	97.7	0	85.7

* One transformant did show lower production, but only 2%, so it was not considered.

Table.5 Radial growth rates in pUAMTPlaeAcons and pUAMTPlaeA transformants

	Transformants	Radial growth rate (cm/day)	Radial growth rate increase (%)*	Strain selected for
	Parental	0.614±0.01	0	
Transformants expressing gene *laeA* from own promoter	T9laeA	0.629±0.02	2.44	SSF
	T13laeA	0.541±0.04	-11.88	
	T12laeA	0.786±0.02	28	
	T1laeA	0.736±0.05	19.87	SmF
	T15laeA	0.727±0.02	18.4	
	T16laeA	0.652±0.003	6.19	
	T4laeA	0.704±0.01	14.65	
	T14laeA	0.656±0.002	6.84	
Transformants expressing gene *laeA* from constitutive promoter	T2laeAcons	0.730±0.002	18.9	SSF
	T7laeAcons	0.782±0.005	27.36	
	T9laeAcons	0.689±0.005	12.21	
	T8laeAcons	0.696±0.005	13.35	
	T5laeAcons	0.673±0.002	9.6	SmF
	T10laeAcons	0.707±0.01	15.14	
	T6laeAcons	0.589±0.005	-4.07	

* Transformants were ordered according to their lovastatin production increase in SSF

Fig.1 Construction of vectors expressing *laeA* from different promoters. A) Cloning of the complete *laeA* gene (with its promoter and terminator) in plasmid pULC43 to obtain vector pUAMTPlaeA. B) Cloning of *laeA* coding sequence in plasmid pAN52.1, to obtain construction pUAMTPlaeAcons

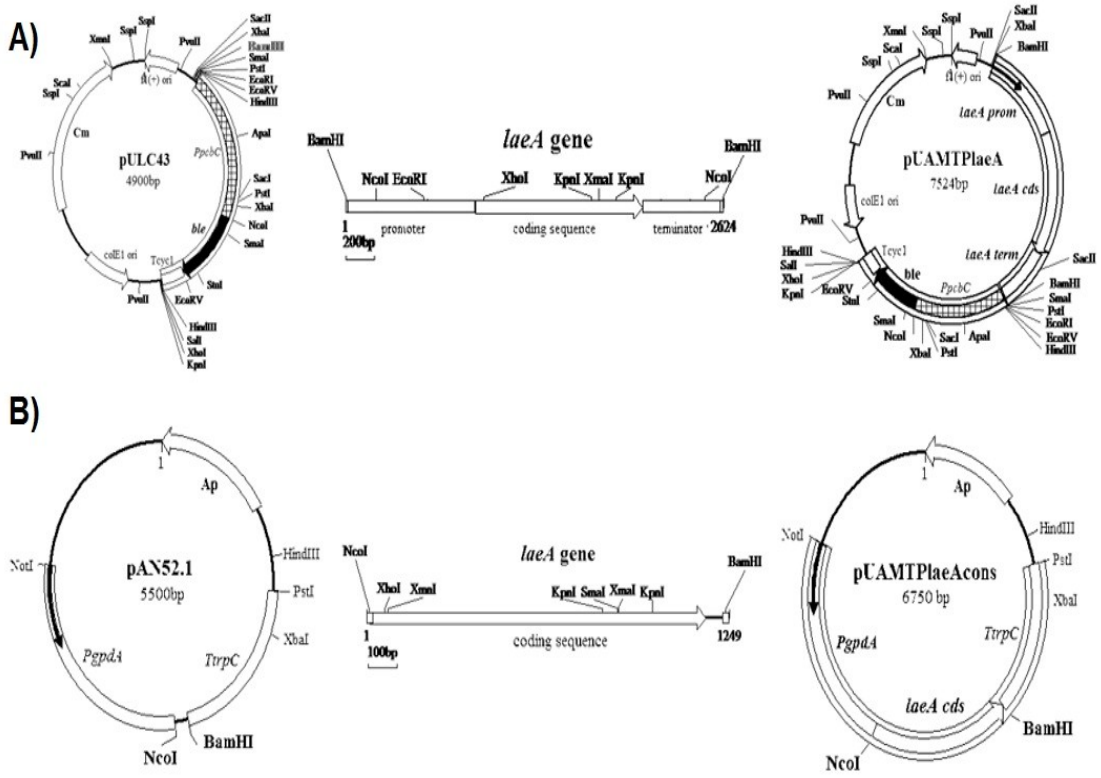


Fig.2 SSF: Time course of lovastatin production by *A. terreus*. Parental strain (■) and selected transformants: T2laeAcons (▲) and T9laeA (●). *Lovastatin concentrations with different letters are significantly different from the rest, according to Analysis of Variance (ANOVA) contrast Low Significance Difference (LSD) test ($p < 0.05$)

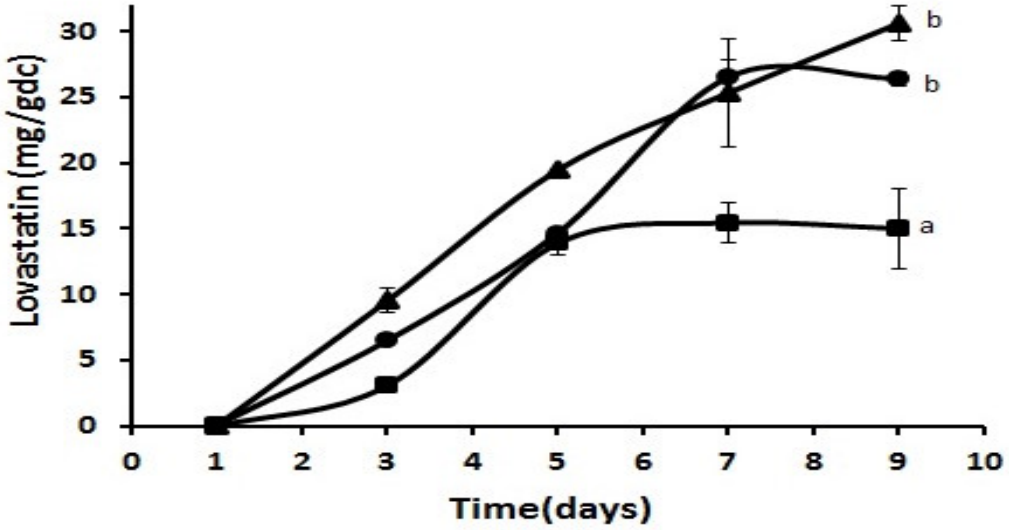


Fig.3 SmF: Time course of lovastatin production by *A. terreus*. Parental strain (■) and selected transformants: T5laeAcons (▲) and T1laeA (●). *Lovastatin concentrations with different letters are significantly different from the rest, according to Analysis of Variance (ANOVA) contrast Low Significance Difference (LSD) test ($p < 0.05$)

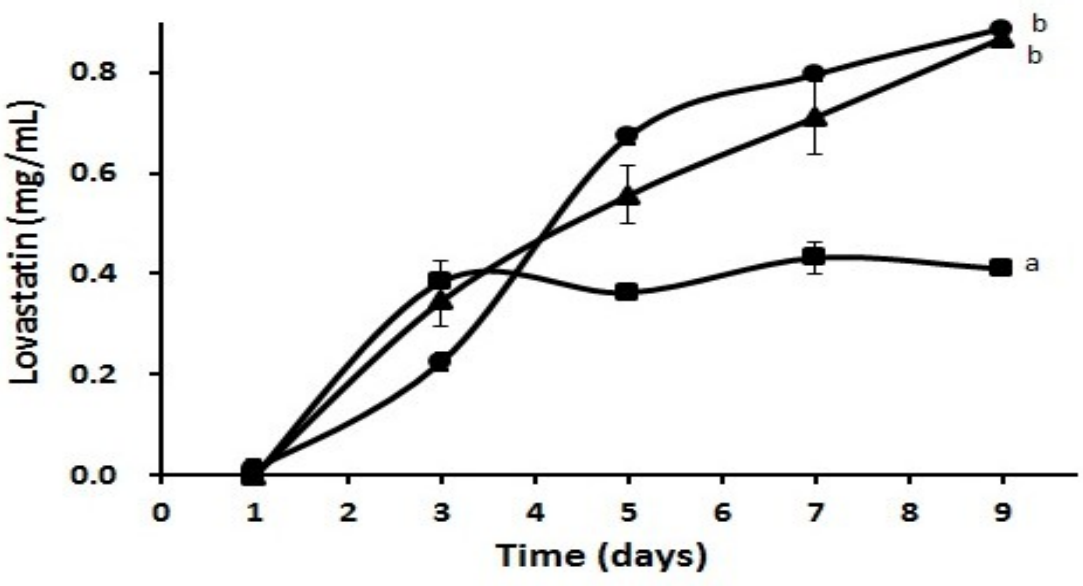


Fig.4 SSF: laeA expression levels in *Aspergillus terreus* TUB F-514 during lovastatin fermentation. Parental strain (■) and transformants selected for this culture system: T2laeAcons (▩) and T9laeA (□)

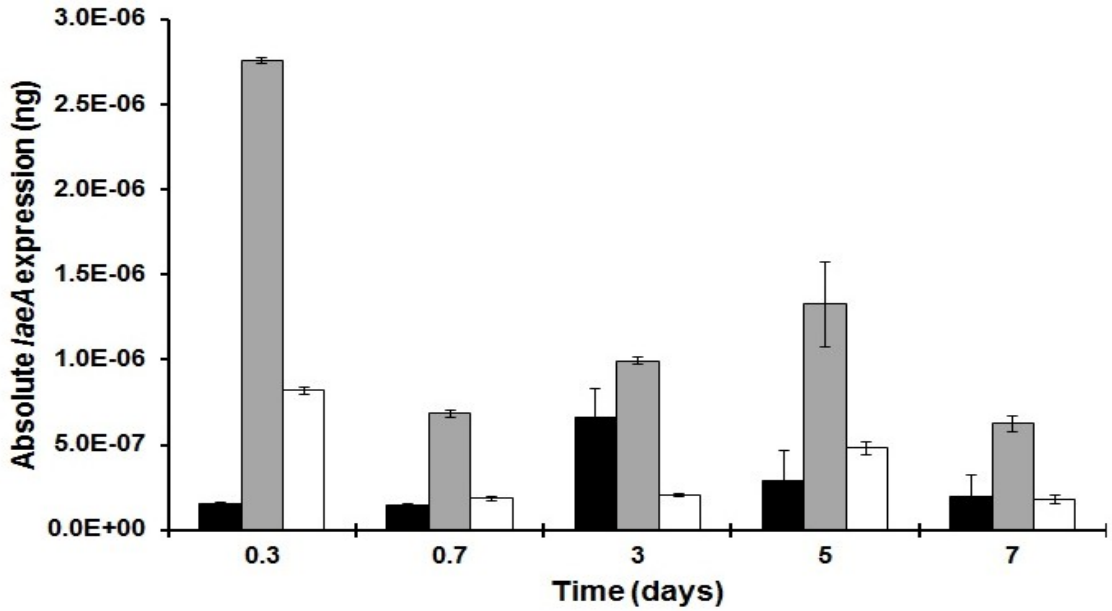


Fig.5 SmF: laeA expression levels in *Aspergillus terreus* TUB F-514 during lovastatin fermentation. Parental strain (■) and transformants selected for this culture system: T5laeAcons (▩) and T1laeA (□).

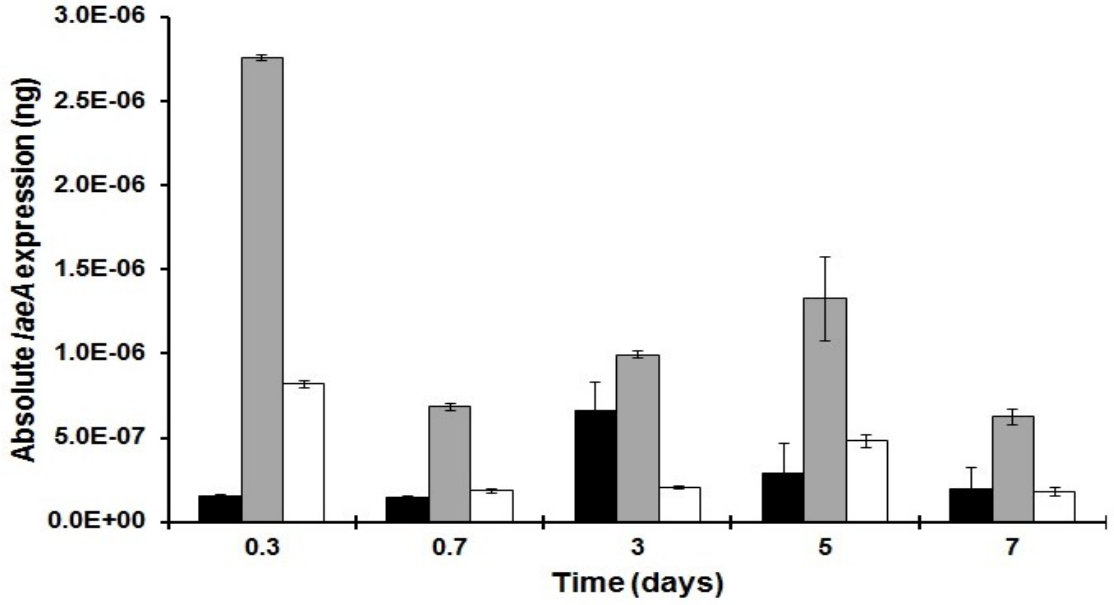
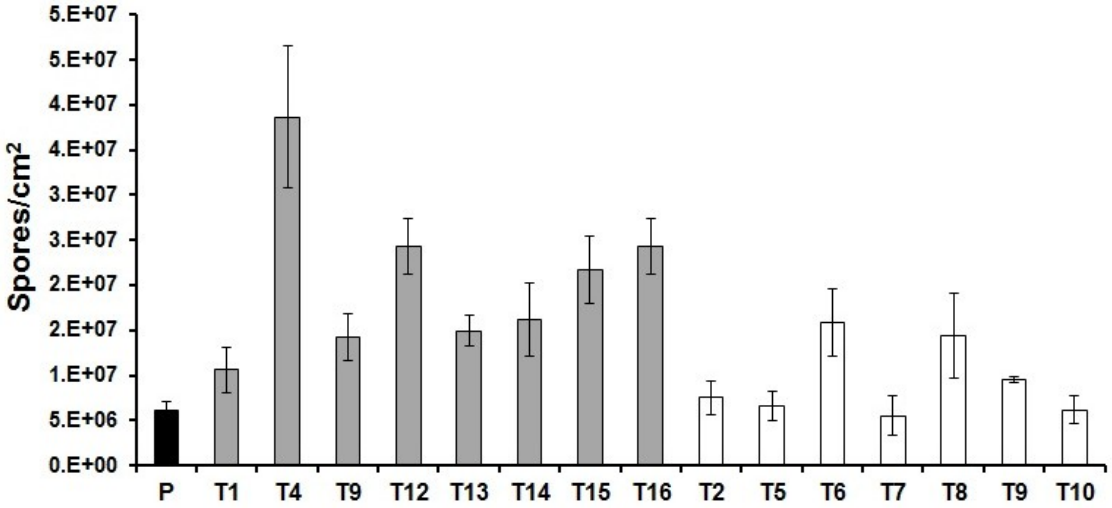


Fig.6 Sporulation index of *A. terreus*. Parental strain (■) and transformants pUAMTPlaeA (□) and pUAMTPlaeAcons (▩)



Very few works have explored molecular genetic improvement strategies to obtain strains for SSF. In relation to enzyme production, Te Biesebeke *et al.* (2006) expressed a fungal hemoglobin gene from a constitutive promoter (*gpdA*) *in Aspergillus oryzae* and obtained higher growth and enzyme production. While Ishida *et al.*

(2006) used an improved *glaB* (SSF-specific glucoamylase) gene promoter to obtain increased recombinant glucoamylase production (*glaA*) in SSF.

In the field of secondary metabolites production, Campos et al (2008) increased the dosage of the whole penicillin gene cluster in 2 strains of *P. chrysogenum*, and evaluated penicillin production in SSF and in SmF. Strains were the low-producing strain Wis 54-1255 (closer to the wild type) and the high-producing strain P2-32, developed for SmF. The authors screened the transformants in ACP, and only tested the best transformant of each strain in SSF and in SmF, so it is not known if in that study overproducers for SSF were more abundant than overproducer for SmF in these populations. In any case, transformants from the Wisconsin (TW) and from P2 strain (TP) showed important increases in penicillin titers in both culture systems. However, these increases were higher in the case of transformant derived from the high-producing strain, probably because it already contained other mutations to process and export high amounts of the antibiotic. In SSF, production increase shown by TP was 93% vs 67% of TW. Although both strains showed a higher production increase in SSF, in relation to the one obtained in SmF, this difference was greater in the case of TW (2.4-fold vs 1.7-fold of TP). The authors discuss that apparently the "genes allowing good performance in SSF", are better conserved in these strains closer to the wild.

In the present work, the production of lovastatin was used as a model of secondary metabolite production in SSF. It was shown that overexpressing the global regulator *leaA* in *Aspergillus terreus* is an excellent method to generate lovastatin-overproducing strains for SSF. The method is more conducive to generate overproducers for SSF, since this kind of mutants were much more abundant, in the transformants population, than over producers for SmF.

Expressing *laeA* from a constitutive promoter was more convenient due to the very high abundance of overproducers for SSF within the transformant population (100%), and to the high lovastatin production increase obtained with the best transformant (104%). Higher sporulation and growth rate increase, observed in *laeA* overexpressing transformants, represent additional advantages of the genetic improvement methods proposed here.

Since LaeA is a global regulator of secondary metabolism, it is very probable that this method can be applied to other fungal secondary metabolites to generate overproducing strains for SSF.

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