

Divergence and Expression Levels of Beauvericin Synthetase Gene from *Beauveria bassiana* Bb-882.5 in Solid State Fermentation

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Abstract

Beauvericin (BEA) is a mycotoxin produced by *Beauveria bassiana*, through a non-ribosomal peptide synthase, known as beauvericin synthetase (*bbBeas*). BEA has a wide variety of biological activities, including insecticide, antimicrobial, antifungal, and antitumor. It cannot be produced by chemical synthesis, so solid-state fermentation (SSF) is an adequate strategy for its production. SSF has demonstrated high productivity of bioactive compounds (*i.e.*, secondary metabolites) that exceed production in submerged fermentation. This study evaluates the expression levels of the *bbBeas* gene in solid-state fermentation using shrimp shells as a substrate. Four *B. bassiana* strains were employed and molecularly identified. A fragment of *bbBeas* gene was sequenced and analyzed to examine expression levels on SSF. Only amplified in two of four strains identified as *B. bassiana* due to variability in the gene. The highest *bbBeas* gene expression occurred on day 9 of SSF compared with days 6 and 12. Based on these results, the expression levels of the beauvericin synthetase gene in the SSF allow us to identify the moment when there is the highest production of beauvericin in the solid culture and serve as a parameter for its subsequent scaling up of its production, as well as of other secondary metabolites.

Keywords

Beauvericin Synthetase, Solid-State Fermentation, Beauvericin, Expression Levels

1. Introduction

Entomopathogenic fungi produce a myriad of bioactive metabolites such as beauvericin (BEA), which has a great variety of biological activities [1]. It is an insecticide that is toxic to many insect species [2] [3], exerts an antimicrobial effect against gram-positive and gram-negative bacteria [4]-[6], has a synergistic antifungal effect in combination with antifungal agents [7], and has *antiviral activity as a selective inhibitor* of human immunodeficiency virus *type 1 (HIV-1) integrase* [8]. *In silico* studies, it has shown an antiviral effect against severe acute respiratory coronavirus 2 (SARS-CoV-2), binding to essential proteins for virus replication [9]. BEA also exerts anticancer activity by causing apoptosis in different cancer cell lines [10]-[12] and decreasing the size and weight of tumors *in vivo* studies [13]. BEA biosynthesis is carried out by a non-ribosomal peptide synthetase (BbBEAS in *Beauveria bassiana*) [14] [15]. Due to the high added value of BEA and the fact that its production by chemical synthesis is very difficult [16], it is important to determine *bbBeas* gene expression levels during the growth of *B. bassiana* to optimize its production. Although BEA has been produced in liquid cultures [4] [17], it's in solid-state cultures where there are recent achievements in the production of this metabolite [18] [19], the expression levels of *bbBeas* have not been determined in solid-state fermentation (SSF). The SSF has been used to produce metabolites such as hydrolytic enzymes, antibiotics, pigments, amino acids, organic acids, surfactants, and other bioactive compounds [20]-[24]. Several studies have shown the advantages of SSF compared with submerged fermentation (SmF) in terms of the productivity of bioactive compounds: culture volume is lower, energy consumption is reduced, aseptic conditions can be maintained, and there is a reduced or null affluent [25]-[27]. BEA production in SSF was recently shown to be higher compared with SmF when using the same medium [19]. Therefore, it is important to determine in SSF time where the highest gene expression is obtained, which can be an indirect measure of BEA production. In this study, we evaluated the expression levels of *bbBeas* from *B. bassiana* strain Bb 882.5 at different times of a SSF to determine the highest BEA production. We also performed a phylogenetic analysis including *bbBeas* and peptide synthetases from other fungi.

2. Materials and Methods

2.1. Fungal Strains

This study used four strains of *B. bassiana*: Strains 11 and 12 belong to the culture fungal collection of the Universidad Autónoma Metropolitana-Xochimilco, strain UAMII belongs to the culture fungal collection of the Universidad Autónoma Metropolitana-Iztapalapa, and strain 882.5 belongs to the culture fungal collection of the Institute of Phytosanity of the Colegio de Posgraduados, in Montecillo, Mexico. The fungi were maintained on potato dextrose agar (PDA) at 25 °C for 15 days. The conidia were harvested from the surface by adding 0.05% (v/v) Tween

80 solution. The conidia suspension obtained was adjusted to 1×10^7 conidia mL^{-1} and stored at 4°C until further use.

2.2. Molecular Identification

To confirm the species identity of the strains, fungal cells were grown on PDA for 15 days. The mycelia were lysed by grinding in liquid nitrogen. Then, the commercial DNeasy Plant Mini Kit (Qiagen) was used to extract DNA, following the manufacturer's instructions. The extracted DNA was then used for polymerase chain reaction (PCR). The universal primers used for amplification and sequencing targeted the internal transcribed spacer (ITS), specifically ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCG-TAACAAGG-3'), corresponding to the ITS1 and ITS2 regions as well as the ribosomal gene 5.8 [28]. Each reaction contained 20 ng of template DNA, 20 μmol of each primer, 2 mM dNTP mix, 5 μL of $10 \times$ PCR buffer, 0.2 U of Taq polymerase (Thermo Scientific), and sterile distilled water added to a final volume of 50 μL . Amplification was performed using a MAXIGENE II[®] PCR system (Axygen). The thermal cycling parameters were 94°C for 10 min; 31 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s; and a final extension step at 72°C for 7 min. The PCR products were examined by gel electrophoresis using a 1% agarose gel run at 100 V in $0.5 \times$ Tris-acetate-EDTA (TAE) running buffer. The gel was stained with Redgel[®] and visualized with a UV light transilluminator (Ultraviolet Products). PCR amplicon sizes were estimated by comparison to a molecular size marker (Promega), purified, and finally submitted to Sanger capillary sequencing by the Molecular Biology Unit of the Institute of Cellular Physiology of National Autonomous University of Mexico (UNAM). The sequences were compared with the GenBank database to identify the *B. bassiana* strains.

2.3. Solid State Fermentation (SSF)

SSF was performed in 250-mL Erlenmeyer flasks. The culture medium was prepared as described by [29]. Shrimp shells (60 g L^{-1}) were used for the induction of metabolite production. A concentration of 1×10^6 conidia per gram of wet matter ($\text{g} \cdot \text{WM}^{-1}$) was inoculated. The culture conditions were 75% relative humidity, pH 5.0, and 25°C .

2.4. Partial Amplification, Identification, and Sequencing of *bbBeas*

The oligonucleotides used for the partial amplification of the *bbBeas* gene were Beau_synFW (5'-ATTGGATGATGGCTACACCG-3') and Beau_syn2 (5'-CTTTCCGGTGGCAGTGCG-3'). They were designed against the adenylation domain based on the alignment of the DNA and messenger RNA (mRNA) sequences of the *Beas* genes with the following accession codes: JX975482.1, HQ141932.1, JQ617289.1, and XM_008604824.1. The PCR reaction mix contained the same ingredients previously described, except for the primers. The

thermal cycling parameters were 94°C for 10 min; 31 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s; and a final extension step at 72°C for 7 min. The PCR amplicon sizes were estimated by comparison to a molecular size marker (Promega) and then purified and sequenced as previously described. The sequences were compared with the GenBank database.

2.5. Phylogenetic Analysis

Phylogenetic analysis and alignment were done using MEGA 11 software, where genes of beauvericin synthetase (BEAS), enniatin synthetase (ES), bassianolide synthetase (BS), peptide synthetase (PS) and other hypothetical proteins (HP) of different origins were analyzed. The statistical method of phylogenetic reconstruction used was that of maximum likelihood; the robustness of the nodes was evaluated with 1000 bootstrap repeats; the nucleotide substitution model was Jukes-Cantor. The reference sequences were taken from GenBank, and all have a length of 722 bp.

2.6. Extraction and Purification of Total RNA

Total RNA was extracted at three different times of SSF: days 6, 9, and 12. One gram of mycelium grown in SSF was ground in liquid nitrogen and transferred to a 1.5-mL microtube. The RNA was extracted with the TRIzol Reagent® (Invitrogen) according to the manufacturer's instructions. Total RNA was resuspended in 50 µL diethyl pyrocarbonate (DEPC)-treated water. The Nanodrop® 2000c (Thermo Scientific) was used to determine the RNA purity, based on the absorbance at 260/280 nm, and the concentration. RNA integrity was verified by electrophoresis with 1% agarose gel. The gel was stained with RedGel® and visualized with a UV light transilluminator (Ultraviolet Products). Residual DNA was removed by treating RNA with RNase-free DNase® (Invitrogen) according to the manufacturer's instructions. RNA was stored at -80°C until further use.

2.7. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Relative *bbBeas* gene expression was evaluated with RT-PCR, with OneStep RT-PCR® (Qiagen) performed according to the manufacturer's instructions. Each reaction included 0.5 µg of total RNA and the oligonucleotides Beau_synFW (5'-ATTGGATGATGGCTACACCG-3') and Beau_syn2 (5'-CTTTCGGTGGCAGTGCG-3'). The 18S ribosomal RNA gene was used as a reference gene due to its constitutive expression; it was amplified with the forward primer 18S RNAr L (5'-CCATCCAATCGGTAGTAGCG-3') and the reverse primer 18S RNAr R (5'-GTAACCCGTTGAACCCATT-3'). The amplification protocol involved 30 min at 50°C for reverse transcription; 15 min at 95°C for initial activation; and 40 cycles of 1 min at 94°C, 30 s at 55°C, and 1 min at 72°C. After amplification, the products were run on a 1% agarose gel, which was stained with RedGel® and visualized with a UV light transilluminator (Ultraviolet Products). Each RT-PCR for the *bbBeas* gene and the ribosomal controls was performed in triplicate.

2.8. Quantification of *bbBeas* Expression by Gel Densitometry

Days 6, 9, and 12 of culture were selected to quantify expression levels because they are critical time points in the production and degradation of BEA in *in vivo* studies. For example, Jiang *et al.* (2013) [30] determined in an *in vivo* model that the maximum BEA production occurred around day 10 of infection, with a significant decrease in BEA concentration by day 12. Similarly, Vásquez-Bonilla *et al.* (2022) [19] found in SSF that the highest BEA concentration was observed on day 9. In both studies, BEA concentration continued to increase on day 6. To quantify *bbBeas* gene expression, 3 μ L of each RT-PCR product was separated by electrophoresis in a 1% agarose gel, which was then stained with ethidium bromide and visualized with a UV light transilluminator (Ultraviolet Products). The Kodak EDAS 290 System was used to obtain images of the gels. Densitometric analysis was performed with the Kodak Digital Science 1D 3.6 software to determine the band intensity of the amplicons as the integrated area (pixels). The obtained band intensity and area were multiplied by each other for each of the bands, taking as a reference the concentration of the 800 bp band of the Axygen molecular size marker (M-DNA-100BP, Cat. PID0173430). The concentration was determined in triplicate for each time point. The data were compared with analysis of variance (ANOVA).

3. Results

3.1. Molecular Identification

The ITS fragments were amplified successfully with the ITS1–ITS4 primer pair. The PCR products of *B. bassiana* strains 11, 12, Bb 882.5 and UAMII were submitted to agarose gel electrophoresis and produced products of 561, 559, 562, and 568 base pairs (bp), respectively (Figure 1). DNA sequence similarities were identified using BLASTn (<http://www.ncbi.nlm.nih.gov/BLAST>) of GenBank. *B. bassiana* 11, 12, 882.5 and UAMII showed 100% identity with *B. bassiana* isolates NBAIL-Bb-69 (gi|380743568|JQ434752.1), PB Percevejo Bronz SC (gi|364524467|JN195743.1), F. Carrap. (gi|364524465|JN195741.1), and Percevejo Bronz (I) (gi|364524464|JN195740.1).

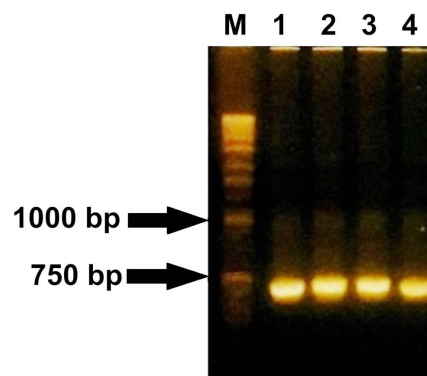


Figure 1. Molecular identification by agarose gel electrophoresis (1%) of ITS fragments: M: molecular weight marker (Thermo Scientific®). 1) *B. bassiana* strain 882.5. 2) *B. bassiana* strain UAMII. 3) *B. bassiana* strain 11. 4) *B. bassiana* strain 12.

The *bbBeas* gene was amplified with the primers Beau_synFW and Beau_syn2 in *B. bassiana* Bb 882.5 and UAMII. However, it was not amplified in *B. bassiana* 11 and 12 (Figure 2). Thus, we performed PCR with a gradient annealing temperature (data not shown). We only obtained non-specific bands that did not correspond to the expected size. The amplified fragments obtained from *B. bassiana* Bb 882.5 and UAMII were 778 and 787 bp, respectively. The amplified complementary DNA (cDNA) was sequenced and compared with other sequences of *bbBeas* gene using BLASTn. They showed 97% and 96% homology with *B. bassiana* isolate Bb0062 (JQ617289.1) and clone BbBVRC (HQ141932.1), respectively. The strains had 96% homology to *B. bassiana* ARSEF 2860 (XM_008604824.1) and 95% homology to *B. bassiana* strain ATCC 7159 (EU886196.1). Due to the presence of partial gene amplified and high homology with the *bbBeas* gene, strain Bb 882.5 was selected to perform gene expression levels. The alignment of the deduced amino acid sequences (Figure 3) of the isolated strains showed that both sequences possess high homology with these related enzymes. *B. bassiana* Bb 882.5 and UAMII have 96% and 92% homology, respectively, with the enniatin synthetase of *B. bassiana* D1-5 (KGQ11754.1), respectively; 96% and 91% homology, respectively, with *bbBeas* gene of *B. bassiana* (AFJ44691.1); 95% and 90% homology, respectively, with *bbBeas* gene of *B. bassiana* ARSEF 2860 (XP_008603046.1); 73% and 69% homology, respectively, with enniatin synthetase of *Hirsutella minnesotensis* 3608 (KJZ71937.1); 69% and 65% homology, respectively, with Beas of *Cordyceps fumosorosea* ARSEF 2679 (XP_018708236.1); and 70% and 66% homology, respectively, with enniatin synthetase of *Fusarium oxysporum* (EXL90988.1).

3.3. Phylogeny

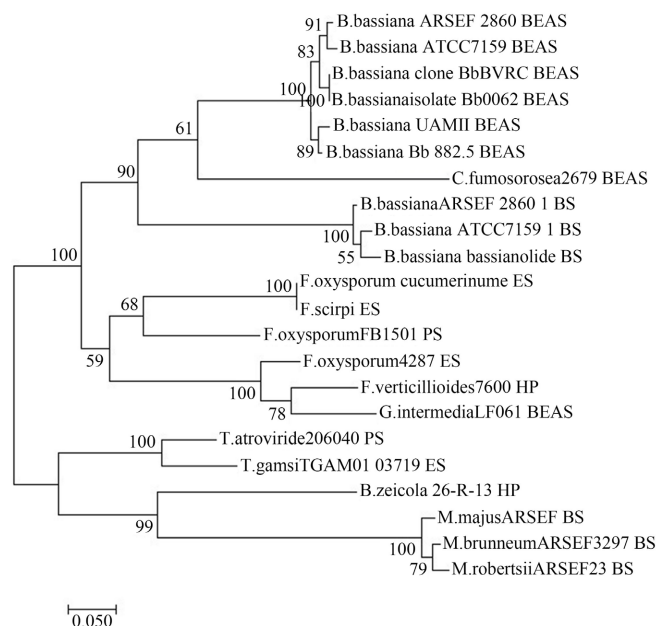


Figure 4. Phylogenetic analysis of the genes of beauvericin synthetase (BEAS), enniatin synthetase (ES), bassianolide synthetase (BS), peptide synthetase (PS) and other hypothetical proteins (HP) of different origins. The bar represents 5 substitutions per 100 nucleotides.

The phylogenetic tree of *bbBeas* gene and other peptide synthetases (Figure 4) shows two main clades. The first clade includes *B. bassiana*, *C. fumosorosea*, *Fusarium* spp. and *Gibberella intermedia*; *bbBeas* gene of *B. bassiana* and *C. fumosorosea* have the same common ancestor. The second clade includes the peptide synthetases of *Metarhizium*, *Trichoderma*, and *Bipolaris*.

3.4. *bbBeas* Gene Expressions Levels in Solid State Fermentation

Hence, we carried out SSF cultures of *B. bassiana* BB 882.5 to evaluate *bbBeas* gene at three time points, namely days 6, 9, and 12. Figure 5 clearly shows that gene was expressed at days 6, 9, and 12 of the SSF. At each time, there is an ~800 bp fragment corresponding to the cDNA generated by RT-PCR. The expression of the gene was variable at each time compared with the 18S ribosomal RNA control. The quantity of cDNA was determined by densitometry and is expressed in $\text{ng}\cdot\mu\text{L}^{-1}$ (Figure 6). The highest expression level was $10.62 \text{ ng}\cdot\mu\text{L}^{-1}$ on day 9; it was significantly higher than at days 6 and 12 ($F = 54.26$, $P \leq 0.0001$), at which time the expression level was 25.59% and 12.22% lower, respectively. The 18S ribosomal control expression level did not differ between the time points ($F = 0.5445$, $P = 0.6063$).

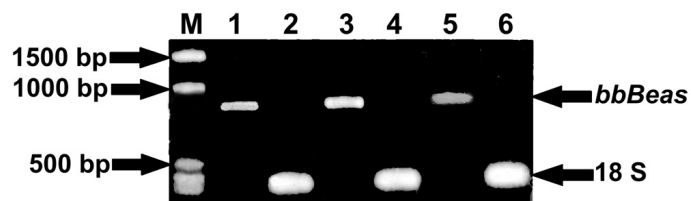


Figure 5. Agarose gel 1% electrophoresis with the RT-PCR products of *bbBeas* gene at different culture times, staining with ethidium bromide; M: molecular weight marker (Thermo Scientific®), 1: *bbBeas* gene to seven days of culture, 2: 18S control to seven days of culture, 3: *bbBeas* gene to nine days of culture, 4: 18S control to nine days of culture, 5: *bbBeas* gene to nine days of culture, 6: 18S control to twelve days of culture.

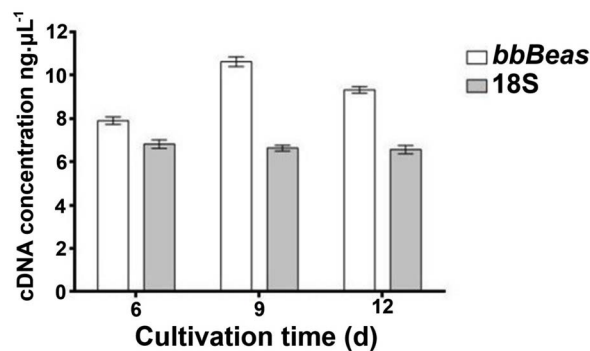


Figure 6. Quantification of the expression of *bbBeas* gene by densitometry, represented in $\text{ng of cDNA}\cdot\mu\text{L}^{-1}$ (Tukey's test $p < 0.05$).

4. Discussion

Although we molecularly identified the four strains as *B. bassiana*, we could only

amplify a portion of the *bbBeas* gene, corresponding to the adenylation domain, in two of the four *B. bassiana* strains. The amplified fragment of the *bbBeas* gene encodes a part of adenylation domain in non-ribosomal peptide synthetases such as BEAS and enniatin synthetase. Given that we could not amplify *bbBeas* in all four strains, we recommend designing a pair of degenerate oligonucleotides using more sequences of different species based on the conserved sites in BEAS, due to the variability of this gene is very high, the *bbBeas* amplification is difficult [15]. In addition, we suggest that another gene is needed to identify closely related species.

The fragments obtained from the *bbBeas* gene of *B. bassiana* BB 882.5 and UAMII share common ancestors and important conserved regions with BEAS, enniatin synthetases, bassianolide synthetases, and other peptide synthetases from different fungal species. This finding suggests a divergent evolutionary process in which important regions have been preserved in the adenylation domain of all these enzymes. We noted the presence of three important regions in all the sequences (including *B. bassiana* 882.5) (Figure 3). The first of these regions includes 15 amino acids and is semi-preserved in the eight sequences: Only three (XP_018708236.1, KJZ71937.1, and EXL90988.1) of them differ specifically in amino acids at positions 4, 10, and 13 with respect to this region. The second and third important regions in the adenylation domain have 12 amino acids each and are conserved in the eight compared sequences. These three sequences contain important sites in the adenylation domain previously described by [31] in a bassianolide synthetase from *Xylaria* spp. The first region has 15 amino acids (GELVIESPGIARGYI), which are found in the first region (semi-preserved), as shown in the alignment (Figure 3). The second site comprises six amino acids (YRTGDL) located in the second conserved region indicated in the alignment. The third site with 11 amino acids (DSQVKIRGQRV) is located in the third conserved region indicated in the alignment.

The presence of conserved regions in the alignment and their homology with the regions reported by [31] show the presence of conserved sites, specifically in the adenylation domain, in BEAS, enniatin synthetase and bassianolide synthetase of different fungal species, including *B. bassiana*, *F. oxysporum*, *C. fumosorosea*, *H. minnesotensis*, and *Xylaria* spp. The homology between these enzymes in different domains has been [32]-[34]. Hence, these regions could be conserved catalytic sites in all these species, because BEA, enniatins, and bassianolide have a similar molecular structure—they are cyclic depsipeptides—and are synthesized by multifunctional enzymes (*i.e.*, enzymes with several catalytic sites). However, the amino acid alignment also shows important differences in the enzyme sequences due to insertions and deletions that occur at the sites of critical points that cause conformational changes, as described previously by [15] [35]. These alterations could explain the difference in *bbBeas* expression gene and BEA production among fungal species or even among different strains of the same species, as described previously [18]. The close evolutionary relationship of the *bbBeas* gene

with other genes means that some of the sequences have ancestors in common even though they are different enzymes in different fungal species. However, there is also the presence of evolutionary clades within the phylogenetic tree, which may be due to the similarity of *bbBeas* with other depsipeptide synthetases [15] [32] [35]. Despite these variations in the different peptide-producing enzymes and the presence of evolutionary clades in the phylogenetic tree, it has also been reported that these enzymes, which are responsible for non-ribosomal synthesis of peptides, can contain more than just adenylation, peptidyl transporter, and condensation domains: They can present extra domains according to the peptide that is synthesized. The following domains stand out: a second adenylation domain, an epimerization domain, a heterocyclic domain, an oxidation domain, an *N*-methylation domain, and a thiolation domain, among others, that are activators of intermediates in the synthesis of peptides [17] [31] [32] [35]. Because the aligned sequences belong to the adenylation domain, which has conserved regions present in all the enzymes, it is possible to determine the phylogenetic relationship among the compared sequences, thus explaining the evolutionary divergence of this gene in different species.

Our results suggest that *bbBeas* expression levels can be used as an indicator of BEA production and are related to the studies [19] [30], which determined BEA production in an *in vivo* infection model of *B. bassiana* and in an SSF model using *Fusarium oxysporum*, finding that in both cases, the highest BEA production occurred on day 9 of infection or culture. This finding is consistent with our study: we observed the highest *bbBeas* gene expression on day 9 of SSF with shrimp shells. In addition, [36] reported that the lowest BEA concentration occurred during the first days of infection because the expression of genes involved in secondary metabolite synthesis remains low in the early stages of *B. bassiana* infection in an *in vivo* model, which is related to the lower *bbBeas* gene expression observed in our study on day 6. This directly links *bbBeas* gene expression to BEA production. However, to our knowledge, the correlation between *bbBeas* gene expression and actual BEA production has not yet been verified through direct experimental evidence.

It has been reported that the biosynthesis of secondary metabolites is regulated by different genetic, epigenetic, and environmental factors [35]. Regulation of *B. bassiana* genes involved in the infection process has been classified based on the stage of the infection where their expression changes significantly: 1) when *B. bassiana* penetrates the cuticle of the insect, 2) when it invades the hemolymph of the insect, 3) when the hyphal body begins to grow within the insect, and 4) when the mycelium grows on the body of the insect [36] [37]. The variability in *bbBeas* gene expression in SSF and the similarity to its expression in *in vivo* infection models indicate that SSF with shrimp shells stimulates *B. bassiana* infection because it can activate the signaling pathway responsible for its expression. According to [37], *B. bassiana* activates a *G-protein-coupled receptor* (GPCR), leading to the expression of genes related to the metabolites expressed and thus explaining the

evolutionary divergence of this gene in different species. The results of this study indicate that this or another signaling pathway is being activated in SSF at a cultivation time like when the highest BEA production has been observed. This reinforces the conclusions of various studies, confirming that SSF is an efficient and cost-effective cultivation system compared to current methods like SmF [19]-[21] [27], which, in the specific case of BEA, may require up to 21 days to reach maximum production [38].

It has been noted that the production of secondary metabolites is regulated by mitogen-activated protein kinase (MAPK) and PKA, which is dependent on cAMP [39]. Some genes can be expressed in two or even three stages of *in vivo* *B. bassiana* infection [39]-[41], so *bbBeas* gene expression may depend on more than one factor. The shrimp shells present in the SSF may activate signal transduction pathways analogous to those involved in *B. bassiana* *in vivo* infection models, as these shells closely resemble the insect cuticle [42]. Additionally, SSF simulates the natural infection conditions of *B. bassiana*, since the fungus invades and grows on a solid support, providing greater oxygen availability and limiting free water. These environmental factors, including humidity and temperature variations inherent to the SSF process [43] [44], may influence the expression of the *bbBeas* gene and, consequently, the production of beauvericin by modulating stress responses and fungal adaptation mechanisms. Our findings provide a guideline for the research on the signaling pathways responsible for the expression of the *bbBeas* gene that is activated in the SSF with shrimp shells.

5. Conclusion

SSF has proved to be a successful way to produce value-added bioactive compounds at a low cost and with advantages over other types of culture. Therefore, it is important to determine the times in which metabolites are obtained. It is important to determine the solid culture time where the highest expression of the gene is obtained as an indirect measure of BEA production. Our results show the potential of SSF as a useful tool for the production of secondary metabolites such as BEA by simulating the expression of the *bbBeas* gene in *in vivo* models using inert solid supports such as sugarcane bagasse.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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