

# An Asarone Producing *Aspergillus Niger* From *Asarum Heterotropoides* With Whole Genome Sequencing

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## Research Article

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# Abstract

Asarone could decrease A $\beta$ , APP, Beclin-1 level and clearing A $\beta$  accumulation, which makes it to be one potential therapeutic agent for AD treatment. This study investigated the endophytic fungus got from *Asarum heterotropoides* Fr. Schmidt var. *mandshuricum* (Maxim.) Kitag. The phylogenetic tree based on single copy orthologues at protein level got from genome sequence analysis showed that the endophytic fungus we got was *Aspergillus niger*. The total length of nonredundant sequences was nearly 34.7 Mb, comprising approximately 61 contigs sequences. In total, 8,755 genes were predicted to encoding proteins, and 18,611,392 high-quality clean reads were generated using the Illumina NovaSeq platform with paired-end sequencing mode. In this study, the theoretical yield of cis-asarone was higher than the traditional ways extracted from essential oil, which was evaluated according to the reverse-phase high performance liquid chromatography (HPLC). The highest yield appeared from the slant cultivation instead of liquid fermentation, and the mature black spore were the final supporter of cis-asarone. This strain was capable of producing cis-asarone and could be a fascinating reservoir of cis-asarone for further industrial manufacture.

## Introduction

*Asarum heterotropoides* Fr. Schmidt var. *mandshuricum* (Maxim.) Kitag, whose root and rhizome are widely used to cure a broad range of diseases, is described as *Asari Radix et Rhizoma* (Xixin) in the Chinese Pharmacopoeia (page 230, 2015 edition). *Asarum* is usually used to extract essential oil (Wang et al. 2018). Different components possess different activity, such as kakuol for antifungal ability (Lee et al. 2005), asarinin for anticancer ability in human ovarian cancer cells (Jeong et al. 2018).

Asarone (2,4,5-trimethoxy-propenylbenzenes) has been classified into two main isomers,  $\alpha$ -(trans) and  $\beta$ -(cis). Different type isomers have been identified to possess a variety of biological functions in vivo and in vitro. Such as, neuroprotective (Limón et al. 2009; Yang et al. 2017), antioxidant (Manikandan and Devi 2005), anti-inflammatory (Chang et al. 2018), anxiety disorders resistance (Tian et al. 2017) and decreased the memory impairment, that are confirmed by bunches of experiments in vivo or in vitro.  $\beta$ -asarone could also accelerate proliferation of RSC96 cells (Xu et al. 2019). However, asarone possesses certain toxicity, which allows it to act as pesticide (Chan et al. 2003). Traditional ways to produce asarones are essential oil (EO) extraction and chemical synthesis. The former way is extracting  $\beta$ -asarone mainly from *Acorus tatarinowii* (Yang et al. 2013) or *Acorus calamus* L. rhizome (Marongiu et al. 2005) or *Acorus gramineus* (Lim et al. 2014),  $\alpha$ -asarone will be abstracted from other herb (Hwang et al. 2017). While the disadvantage of this method is that the essential oil yield is extremely low and the excessive cost will cause resources waste. After the purification, different asarone isomers are gathered. The chemical synthesis method to produce asarone with a series of reactions contains Grignard reaction or Wittig reaction with numerous intermediate products forming till the final products generate.

*Aspergillus niger* has been famous for over 100 years after the first discovery of its high yield of citric acid and was sequenced in 2007 (Pel et al. 2007) for the first time. As the development of DNA sequencing

technology and bioactive molecule discovered, up to 57 secondary metabolite clusters (de Vries et al. 2017) have been predicted. And the researches have more interest on encoding genes verification than products.

Nowadays, there is a growing trend showing that people start to focus on plant endophytes to produce natural products, including polyketides (Shi et al. 2020) from *Artemisia argyi*, dihydroisocoumarin derivative from *Diaporthe* sp. (Guo et al. 2020), hispidulones from *Chaetosphaeronema hispidulum* (Zhang et al. 2020). As plants have been the potential candidates to produce best sources of natural bioactive compounds which can be applied in agriculture, medicine and food industry, so that people have focused on these plant endophytes. Overall, the endophytic fungi could provide us with sufficient raw material for producing biological ingredients.

In the context of the pharmacological profile and biological characteristic of  $\beta$ -asarone, here, we isolated a new *Aspergillus niger* strain from fresh *asarum* root. Meanwhile, genome information will offer not only genome size, but also encoding protein information and functional protein annotation, from which the specific phylogenetic tree is inferred. The quantitative yield of  $\beta$ -asarone was assessed by high performance liquid chromatography and putative biosynthesis of  $\beta$ -asarone was inferred.

## Materials And Methods

### Preparation, isolation and characterization of target endophytic fungus

Standard substances including  $\beta$ -asarone were purchased from Must Biotechnology Co., Ltd., Chengdu, Sichuan, China. DNA Extraction Kit and Purified Kit and PCR related reagents were purchased from Solarbio Co., Ltd., Beijing, China. The chromatographic pure methanol used for high-performance liquid chromatography (HPLC) was purchased from Merck (Darmstadt, Germany).

*Asarum heterotropoides* Fr. Schmidt var. *mandshuricum* (Maxim.) Kitag was got from Heilongjiang University of Chinese Medicine, Heilongjiang, China, and maintained in our lab (45.75°N, 126.63°E). Over seven years old field-grown *A. heterotropoides* was used for isolation of endophytic microorganisms following previously established procedures. Healthy tissues of roots of *A. heterotropoides* were collected, washed under running tap water to remove dirt before surface sterilization. Tissues were surface sterilized using modified method, by dipping into 75% (v/v) ethanol for 3 min, sterile distilled water for 1 min, again 75% (v/v) ethanol for 1 min, then sterile distilled water for 1 min, and tapped dry with sterile filter paper. The root was cut into 0.5 cm segments by sterile scissor, then were immediately placed on the potato dextrose agar (PDA) media for 7 days. Streak plate method was applied every day when new fungus occurred, until each fungus was isolated and purified. Liquid culture medium was potato dextrose broth medium (PDB), solid culture medium was potato dextrose agar medium (PDA).

The hyphae of the target fungus colony were scraped from the slant cultivation, then transferred to a slide, stained by the lacto-phenol cotton blue dyeing method after slide cultivation for 4 days and observed under a light microscope (Olympus CX23, Japan). Observation about the spores was conducted

during the liquid vegetative stage. This strain was kept in China General Microbiological Culture Collection Center (CGMCC), CGMCC No. 20242.

### **Comparative genomics phylogenetic analysis**

Genome DNA sequencing, assembly and annotation were provided in *SI Materials and Methods*. In order to investigate the phylogenetic status of this strain within the *Aspergillus* genera, we constructed a phylogenetic tree of 16 species (achieved similar black appearance) based on protein sequence (fasta format) downloaded from NCBI database(<https://blast.ncbi.nlm.nih.gov>)(Table S2, *Penicillium expansum* was used as outgroup), which was built with SNP sites detected in all single-copied orthologs from genomes that are in scaffold and chromosome status. The whole genome phylogenetic analysis of the single copy orthologues was conducted by OrthoFinder(Auber et al. 2020; Emms and Kelly 2019) with some modifications: Individual protein sequences were firstly operated in OrthoFinder to get Single Copy Orthologue Sequences with all protein-coding genes alignment based on DIOMAND ( mentioned in SI Materials and methods) .The best-fit models of evolution determination and the maximum likelihood (ML) phylogeny was constructed using IQ-TREE version 2.0.3(Kalyaanamoorthy et al. 2017; Minh et al. 2020), and ultrafast bootstrapping analyses with 5,000 replicates each. The gene tree was modified using iTOL website (<https://itol.embl.de/>)(Letunic and Bork 2019). All the operation was based on Linux system. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JACCEF000000000.

### **Determination of $\beta$ -asarone producing ability**

The liquid fermentation mycelia at different fermentation periods and the pure spore grown on the slant cultivation were collected to conduct freeze drying and ultrasonic treatment (50 mL methanol added at room temperature for 30 min), respectively. The mixtures were centrifuged and the supernatant were condensed to dryness by rotating evaporator with a vacuumizer. The dryness residues were dissolved with 2 mL of 100% methanol and determined by Thermo Fisher series minUlimitate 3000 HPLC (Thermo Fisher Scientific Corporation, U.S.A.), using an Acclaim™ 120 C18 Reversed-Phase column (C18-4.6×150mm, 5 $\mu$ m, Thermo Fisher Scientific, U.S.A), with the temperature maintained at 30°C. After investigating various mobile phases in different combinations, we selected a gradient elute system at a flow rate of 0.2 mL/min. The mobile phase was starting with 15% methanol and 85% water. Each gradient was maintained for 10 min until the final phase turned to pure methanol within 100 mins. The ultraviolet (UV) detection was achieved at 313 nm. The mobile phases and the samples were filtered by using a 0.45 $\mu$ m filter and degassed by sonication for 10 min before use.

To determine which culture or which chemical would accelerate the yield, different liquid cultures (Czapek–Dox Medium, Matin and malt), carbon sources (lactose, sucrose, galactose) and some chemicals (phenylalanine, cumaric acid, felurate, isoeugenol, anethole, tyrosine ) which were inferred the possible precursors of asarone were added in the liquid cultures, respectively.

### **Statistical Analyses**

All analyses were performed with Microsoft Excel Professional Plus 2016. Error bars represent standard error for three replicates.

## Results

### Determination of fermentation conditions

#### Fermentation period

HPLC analysis was conducted to validate what time was suitable for the fermentation. The flasks were grouped by the different fermentation periods (every three days), then transferred to -20°C refrigerator to terminate the reaction. Under HPLC conditions (same as the pilot experiment), we analyzed the extracts of the fungal  $\beta$ -asarone produced in different periods by external reference method with standard  $\beta$ -asarone. Final fungal fermentation products were analyzed by HPLC (Fig. 1 and Fig. 2 A). The results showed the peak positions and peak shapes were identical or very close to that of the chemical reference.

#### $\beta$ -asarone producing position

Liquid fermentation supernatant, white mycelia and mycelia with black spores (as the fermentation proceeded, the black spores gradually formed at the surface of the mycelia) were centrifuged (Fig. 1 B) to test which part of them was the  $\beta$ -asarone producing position. The results showed that  $\beta$ -asarone only appeared in the mycelia with black spores instead of the other two parts. Furthermore, the total yield of  $\beta$ -asarone might increase as the fermentation period lasted.

#### NaCl/CaCl<sub>2</sub> and pH stability

To determine the range of pHs in which the yield of cis-asarone is the most, we measured the content of cis-asarone under different pH conditions PDA plate and cultured for 3 weeks. The peak areas of cis-asarone were quite stable at pH 6 and 7 (Fig.1 C), the minimum and maximum relative content came across at pH 5 and pH 9. While the *A. niger* had difficulty on growth at pH 4 and pH 9 with more time to grow and the product abundance was higher at pH 6 and pH 7 (data not shown) which meant the neutral culture was good to fermentation.

To determine whether the addition of Na<sup>+</sup> and Ca<sup>2+</sup> will increase the cis-asarone yield (Fig.1 D), different concentration of NaCl or CaCl<sub>2</sub> was added into the PDA plate and cultured for 3 weeks. The peak areas appeared the most at 1% either NaCl or CaCl<sub>2</sub>, and generally decreased from 2% and both remained to 0 when the concentration was over 10%, and the plates had no *A.niger* when the concentration was over 25%.

#### Evaluation of the yield of $\beta$ -asarone

The third column (Table1) presented that  $\beta$ -asarone was the major component in some species (Dan et al. 2010; Deng et al. 2004; Govinden-Soulange et al. 2004; Perumalsamy et al. 2010), while considering the

essential oil yield was often the minor part showing in the second column. Hence, when estimating the yield of  $\beta$ -asarone (C) of the plant, the yield of essential oil (A) was multiplied by the yield of  $\beta$ -asarone (B). As not every study showed all these data at the same time, and based on the reference we could get, the total EO yield was no more than 5%, and the  $\beta$ -asarone yield from any essential oil was no more than 50%, when came across data deficiency, 5% and 50% were used to substitute the yield of EO and  $\beta$ -asarone, respectively. It was obvious that the theoretical yield of  $\beta$ -asarone obtained from this study was the highest, which provided a further evidence that this strain could be the potential  $\beta$ -asarone supplier.

#### Evaluation of the culture with extra additions

According to the KEGG pathway, some potential chemicals were inferred to be the precursor for asarone, and added into the PDB liquid cultures to evaluate whether the yield of  $\beta$ -asarone would be improved. From Fig.S1, isoeugenol and anethole inhibited the growth obviously, especially at the first 7 days. This strain would grow with almost all the chemicals at first, and No. 4 was the only one to have black spores first, while when the days lasted, the cultures changed hugely, and 3 of them had no black spores appeared in the end (No.8, 9 and 10).

#### Bio-informatic analysis

##### Annotation

The target strain (DFY1) was primarily classified to be *A. niger*. After we isolated the *A. niger* stain (DFY1) which could produce  $\beta$ -asarone, whole genome sequencing was conducted, and the results showed the *A. niger* strain (DFY1) genome was assembled approximately 2.668 Gb with 61 contigs and 47 scaffolds, containing 8,875 genes encoded proteins. The results and evaluation of genome assembly completeness showed in Table S2. Complete BUSCOs occupied nearly 99.7% and the purified GC content was around 50.04%, which was similar to the existing data(de Vries et al. 2017). Quality of sequence results showed in Table S2.

CAZy prediction (Fig.S2A) presented that glycoside hydrolases group was the majority that had 247 genes annotated in this class. Glycosyl transferases, auxillary activities and carbohydrate esterases groups had similar genes from around 100. Polysaccharide lyases and carbohydrate-binding modules were the minor parts that could be ignored. From this CAZy prediction, some maltases were found (Table S3) which might explain why this strain performed better in malt liquid culture than other cultures. All the functional annotation gene numbers showed in the Table S2. In total, 39,560 GO terms were assigned (Fig.S2B), from which 11,283 were GO terms related to Cellular component class, 12,641 – to Molecular function class, and 15,636 – to Biological process class. KEGG classification (Fig.S2C) revealed that genetic information (belonged to Brite hierarchies), signaling and cell (belonged to Brite hierarchies) and signal transduction (belonged to Environmental information processing) matched over 500 genes. EggNOG annotation (Fig.S2D) of protein coding genes was conducted by eggNOG-mapper. Carbohydrate transport and metabolism (G)and secondary metabolites biosynthesis, transport and catabolism(Q) possessed the most major functions except for the unknown part (S).

## Phylogenetic tree

As the fungi has difficulty on species identification, and there are too many *Aspergillus* genera presented black, single copy orthologues got from genomes were used to analyze the genetic relationship by using OrthoFinder. According to BIC score in IQ-Tree, the best-fit model was JTT+F+R6, the phylogenetic tree (Fig.3 A) showed that strain DFY1 was closer to *A.niger* rather than other *Aspergillus* genera or species with similar black morphological characteristics, which provided further identification that strain DFY1 was *A.niger*.

Isolation and morphological characteristics of culturable endophytic fungi in *A. heterotropoides*. The 6 endophytic fungal strains were isolated from the bark tissues of *A. heterotropoides* based on the morphological characteristics, and only one strain possessed the ability to produce  $\beta$ -asarone after 18 days fermentation during the pilot experiments (data not shown). The target fungus strain (named as DFY1) was stained by cotton-blue dyeing method (Fig. 3B and C). Under the light microscope, it was clear to see the hypha had black round conidium formed, which had extremely similar characteristic with *A. niger* (oil immersion, 1000X, Fig.3.C). The mycelia were white at the beginning, and the black spores generally appeared in the liquid medium.

## Discussion

A cliffy increasing research trend shows that endophytic fungi deserve more attention, especially those plants who achieve outstanding disease-curing features (Ibrahim et al. 2018; Li et al. 2018), so that people may find potential medicine or new chemicals to (Liu and Liu 2018) replace the plants on the edge of extinction. Besides, the mechanism why these plants can produce that kind of chemicals or how the endophytic fungi create the second metabolisms still remains discussions and verifications. According to the PDB liquid culture, the minimum period for *A. niger* fermentation was 18 days and the black spores gradually appeared since from day 18, which suggested that the formation of the black spores induced the production of  $\beta$ -asarone. The ability of this strain that could produce  $\beta$ -asarone catches our eyes. To unambiguously clarify the fungus vigor, we turned our efforts to utilize HPLC method to quantify the  $\beta$ -asarone production in different conditions. This strain could produce more  $\beta$ -asarone in malt liquid and more mycelia with less time than other cultures (Fig.S1), which was identified by CAZy analysis that this stain had different types of maltase that might permit this strain grow better in this culture.

$\beta$ -asarone could serve as potential candidates for drug development in neurodegenerative diseases (Huang et al. 2013). The existing methods to prepare trans-asarone and cis-asarone, either essential oil (Gu et al. 2016; Lim et al. 2014; Zuo et al. 2012) or chemical synthesis will lead to the regent and plant wastes and low yield. Secondary metabolites always generate after a long period fermentation (Xia et al. 2020). From Table 1, it could be easily inferred that the composition and yield of the essential oil will be extremely affected by the plant species and the method they used (Strelke et al. 1989). The increase of the  $\beta$ -asarone content might be associated with the appearance of the spores.

For this work, a special *A. niger* strain DFY1 that could produce  $\beta$ -asarone was isolated and the yield was attractive. Other *A. niger* strains showed great ability to produce citric acid (Amato et al. 2020) and hydrolases (Cairns et al. 2018) for industrial production. The eggNOG annotation results showed that there were more unknown functions haven't been annotated, which meant this strain deserved more researches on the function classification, and deeper understanding about how to regulate secondary metabolisms. And we do really want to have further analysis on this strain to see which gene clusters regulate  $\beta$ -asarone production and asarone metabolism pathway. The further verification of the pathway and the controlling gene clusters are needed in the next researches.

Taken together, this  $\beta$ -asarone-producing fungus *A. niger* strain DFY1 showed great potential in the secondary metabolism development and deserves deeper research on the mechanism and biosynthesis pathway.

## Declarations

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**Conflict of Interest:** The authors declare that they have no conflicts of interest.

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## Abstract·Important notes

- A new *Endophytic fungus* strain that can produce asarone.
- The theoretical yield of  $\beta$ -asarone is higher than the existing methods.
- Prediction of the presumable pathway of  $\beta$ -asarone in *Aspergillus niger* strain based on the genome sequence.

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## Table

**Table 1** Theoretical yield of  $\beta$ -asarone from different resources

Resource	EO yield%	$\beta$ -asarone yield%	$\beta$ -asarone yield%
	A	B	C
Dried <i>Asarum heterotropoides</i> (Perumalsamy et al. 2009)	2.98	No data	0.0149
Dried <i>Acorus tatarinowii</i> Schott. (Deng et al. 2004b)	No data	47.76 or 49.16	0.02388 or 0.02458
<i>Psiadia terebinthina</i> (Joyce 2004)	No data	2.1	0.00105
Dried <i>Asarum heterotropoides</i> (Dan et al. 2010a)	1.15	No data	0.00575
<b><i>A. niger</i> (This study)</b>	<b>No data</b>	<b>No data</b>	<b>0.045</b>

Except for the last line, where came across no data, we used 5% and 50% to substitute yield of EO or  $\beta$ -asarone, respectively. The 2<sup>nd</sup> column (A) multiplied by the 3<sup>rd</sup> column (B) to obtain the 4<sup>th</sup> column(C) data. B:  $\beta$ -asarone yield% (relative to essential oil); C:  $\beta$ -asarone yield% (relative to dried raw materials)

## Figures

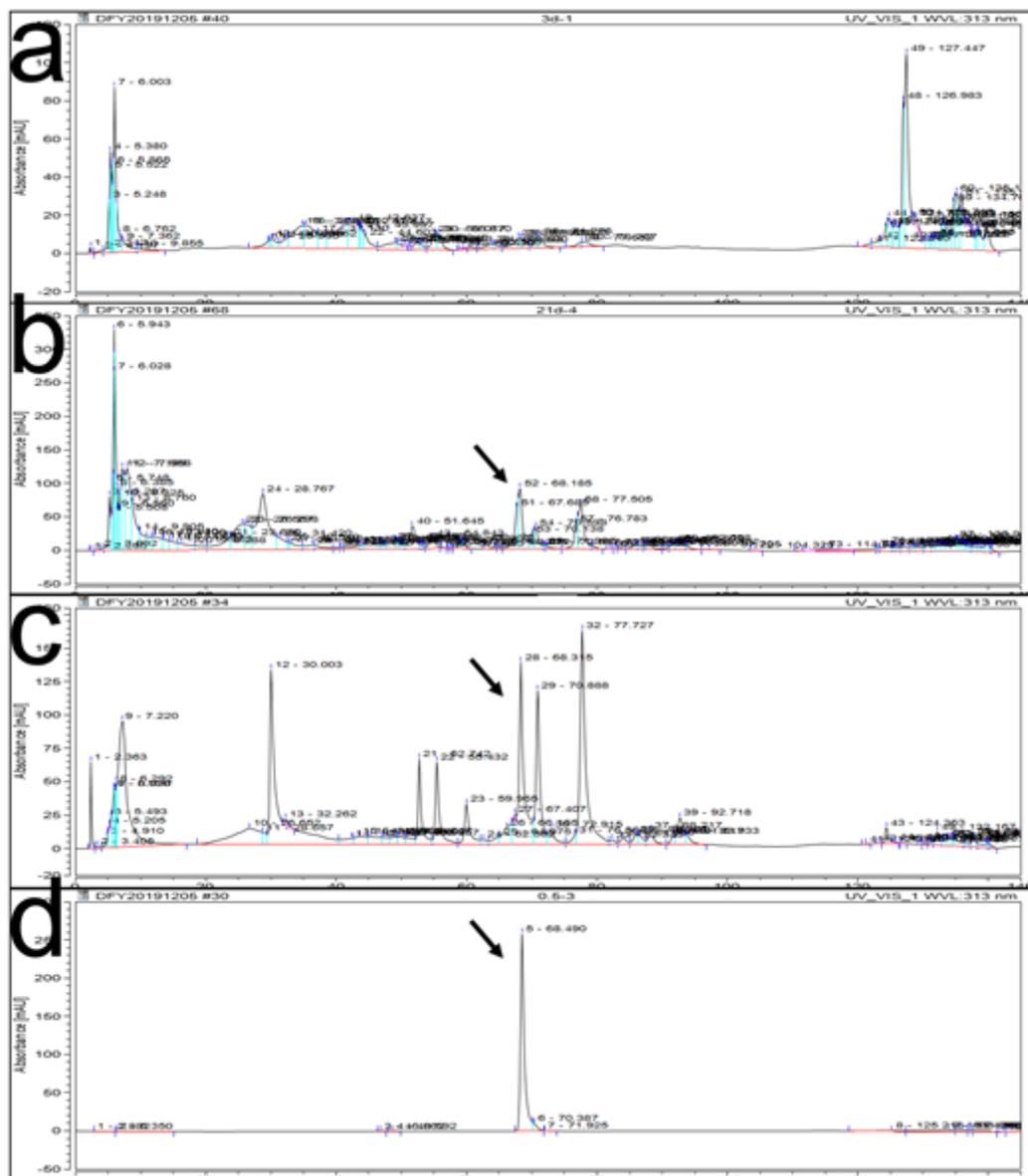
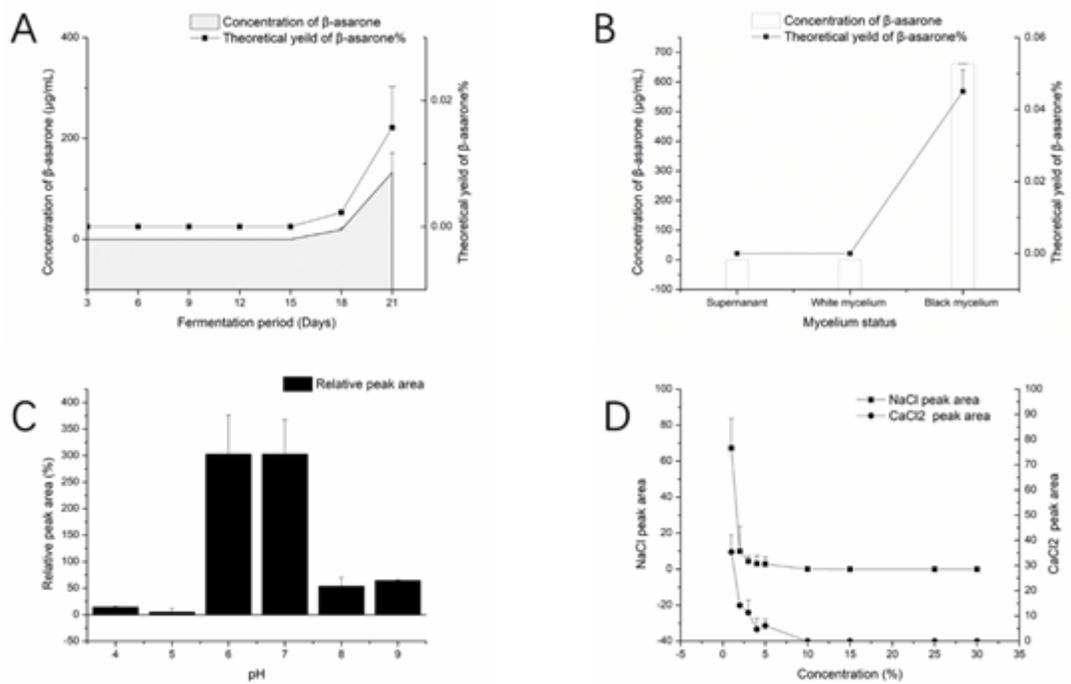


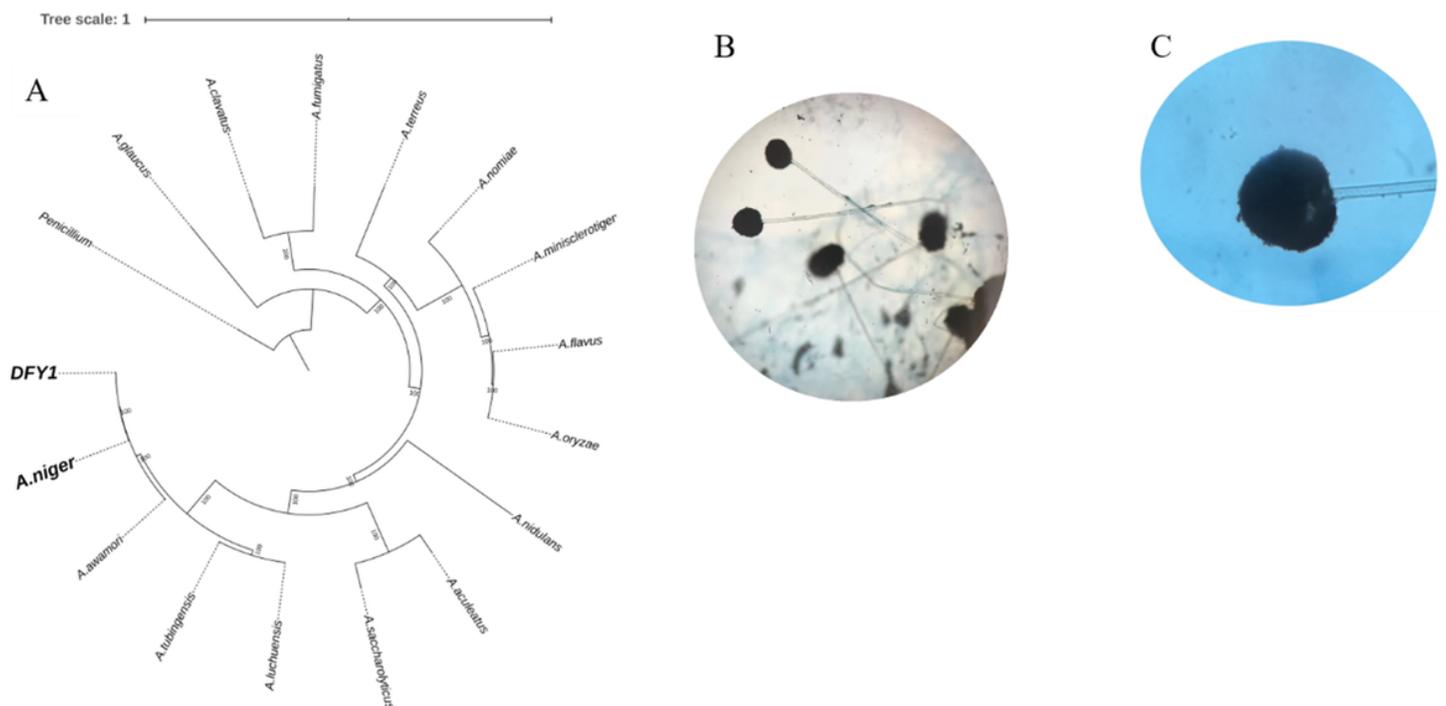
Figure 1

HPLC conditions with different fermentation periods (a,b,c represented fermentation with 3,21,28 days, respectively; d was the standard  $\beta$ -asarone reagent).



**Figure 2**

Concentration of  $\beta$ -asarone with different fermentation conditions (a: the concentration and theoretical yield of  $\beta$ -asaone with different periods; b: the concentration and theoretical yield of  $\beta$ -asaone with different liquid fermentation parts; c: the concentration of  $\beta$ -asaone with different pH; d: the concentration of  $\beta$ -asaone with different Na<sup>+</sup> and Ca<sup>2+</sup>).



**Figure 3**

Phylogenetic tree based on comparative genome analysis (a: phylogenetic tree; b, c : the hypha stained by cotton-blue dyeing method under the light microscope 400X and oil immersion1000X, respectively).

## Supplementary Files

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