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SHAROF RASHIDOV NOMIDAGI SAMARQAND DAVLAT UNIVERSITETI

САМАРКАНДСКИЙ ГОСУДАРСТВЕННЫЙ УНИВЕРСИТЕТ ИМЕНИ ШАРОФА РАШИДОВА

SAMARKAND STATE UNIVERSITY NAMED AFTER SHAROF RASHIDOV

# ILMIY AXBOROTNOMA

## НАУЧНЫЙ ВЕСТНИК SCIENTIFIC JOURNAL

## ANIQ VA TABIIY FANLAR SERIYASI

Matematika, Mexanika, Informatika
Fizika, Kimyo, Biologiya, Geografiya

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# OPTIMIZATION OF CULTURE CONDITION FOR GROWTH AND PRODUCTION OF BIOACTIVE SECONDARY METABOLITES BY ENDOPHYTIC FUNGUS THERMOTHELOMYCES THERMOPHILES XJF-9

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**Abstract.** In this work aimed to optimize the culture condition of the fungal strain *Thermothelomyces thermophiles* XJF-9 to increase the growth and production of pharmacological active secondary metabolites. The potent fungus is subjected to optimization in various culture medium and incubation time. The optimized culture medium was Potato Dextrose Broth (PDB) using dextrose as carbon source and incubation time 14 days for the maximum growth and production of bioactive natural compounds by *T. thermophiles* XJF-9. Moreover, secondary metabolites of crude ethyl acetate extract were investigation melanin content assay and tyrosinase activity. These secondary metabolites are effectively utilized in medicinal and pharmaceutical industry.

**Keywords:** thermothelomyces thermophiles XJF-9, optimization, melanin synthesis.

## Endofitik qo‘ziqorin Thermothelomyces thermophiles XJF-9 tomonidan biologik faol ikkilamchi metabolitlarning o‘sishi va ishlab chiqarilishi uchun madaniyat holatini optimallashtirish

**Annotatsiya.** Ushbu ishning asosiy maqsadi endofit zamburug`i *Thermothelomyces thermophiles* XJF-9ning usishi va u tomonidan ishlab chiqariladigan farmakologik faol ikkilamchi metabolitlar miqdorining oshishi uchun optimal ozuqa muhiti va inkubatsion vaqtning topishga qaratilgan. *T. thermophiles* XJF-9 ning maksimal o'sishi va biofaol tabiiy birikmalarning ishlab chiqarilishi uchun uglerod manbai sifatida kartoshka dekstros hamda unkubatsiya vaqti 14 kun optimal muhit hisoblanadi. Bundan tashqari umumiy etil asetat ekstraksiyasining ikkilamchi metabolitlari melanin pigmentining sintezlanishiga va tirozinas faolligiga ta`siri o`rganildi. Ushbu ikkilamchi metabolitlar tibbiyot va farmatsevtika sanoatida samarali qo'llanilishi mumkin.

**Kalit so‘zlar:** *thermothelomyces thermophiles* XJF-9, optimallashtirish, melanin sintezi.

## Оптимизация условий культивирования для роста и продукции биоактивных вторичных метаболитов эндофитным грибком Thermothelomyces thermophiles XJF-9

**Аннотация.** В этой работе стремились оптимизировать условия культивирования грибкового штамма *Thermothelomyces thermophiles* XJF-9 для увеличения роста и продукции фармакологически активных вторичных метаболитов. Сильнодействующий гриб подвергается оптимизации в различных культуральных средах и времени инкубации. Оптимизированной культуральной средой был картофельно-декстрозный бульон (PDB) с использованием декстрозы, в качестве источника углерода и временем инкубации 14 дней для максимального роста и продукции биоактивных природных соединений *T. thermophiles* XJF-9. Кроме того, вторичные метаболиты сырого этилацетатного экстракта исследовали на содержание меланина и активность тирозиназы. Эти вторичные метаболиты эффективно используются в медицинской и фармацевтической промышленности.

**Ключевые слова:** *thermothelomyces thermophiles* XJF-9, оптимизация, синтез меланина.

**Introduction.** Fungal endophytes which reside asymptomatically living inside plants tissue [1-3] and have been isolated from marine, mangrove, mosses, fern and medicinal plants[1, 4, 5]. Endophytic fungi play an important role in protecting their host from attack by phytopathogens and also facilitate the fixation of atmospheric nitrogen; and the production of various hydrolytic enzymes, and biosynthesis antibiotics [3, 6]. Culture condition strongly influence in production of secondary metabolites and to be regulated by carbon and nitrogen sources, pH, temperature, incubation time. We previously isolated an endophytic fungus *T. thermophiles* XJF-9 from the healthy leaf of *Vernonia anthelmintica.* Fungal strain exhibited promising results towards antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli* and the yeast *Candida albicans*, displayed cytotoxic and anti-diabetic activities [7-11]. In the present study, the optimal medium composition and culture conditions for *T. thermophiles* XJF-9 were determined. Addition investigated its effect of melanin synthesis and tyrosinase activity.

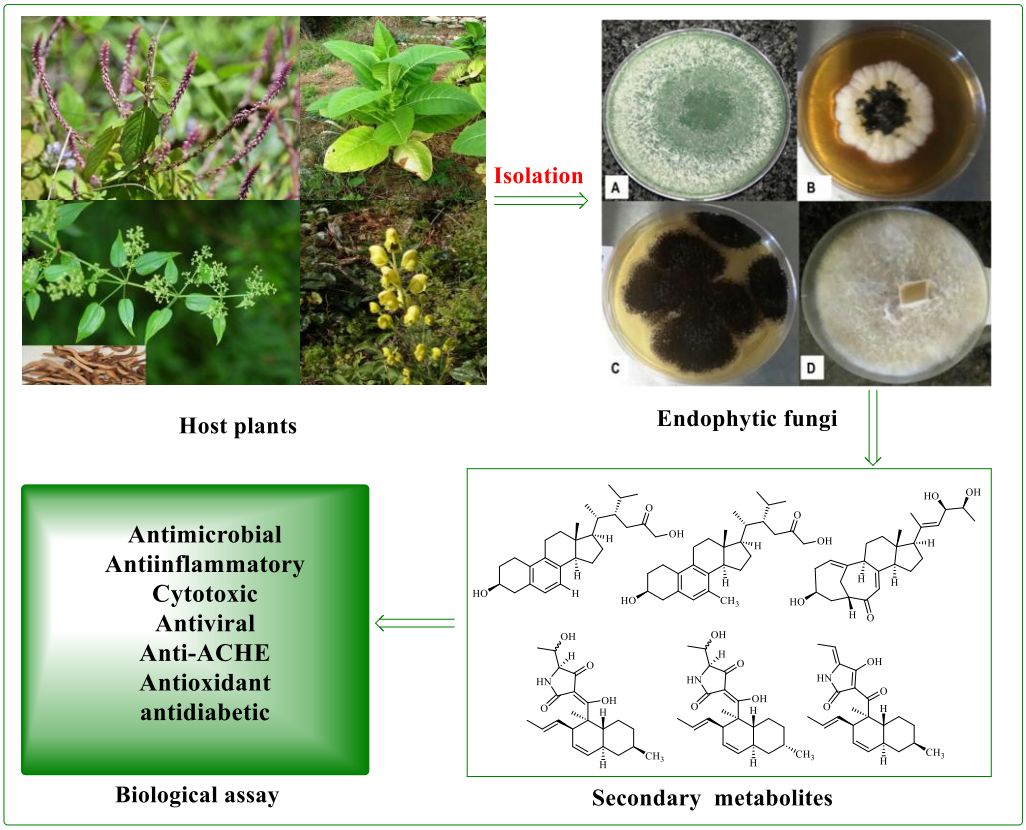



**Figure 1.** Secondary metabolites isolated from the endophytes and their biological activities.

**The effect of culture medium on secondary metabolites production.** The culture conditions are played important role for growth and production bioactive secondary metabolites by endophytes. The fungal strain *T. thermophiles* XJF-9 was growth in different media: yeast extract peptone dextrose (YPD) (yeast extract, 1 g/L; dextrose, 20 g/L; peptone, 20 g/L), malt extract broth (MEB) (malt extract 30 g/L), Czapeck- Dox medium (CDM) (sucrose, 30 g/L; $NaNO_3$, 3 g/L; $K_2HPO_4$, 1 g/L; $MgSO_4$, 0.5 g/L; KCl, 0.5 g/L; $FeSO_4$, 0.01 g/L), beef extract broth (BEB) (beef extract, 3 g/L; NaCl, 5 g/L), peptone beef extract glycogen medium (PBG; peptone, 10 g/L; beef, 10 g/L; glycogen, 4 g/L; NaCl, 5 g/L; $NaC_{12}H_{25}SO_4$, 0.1 g/L) and potato dextrose broth (PDB) (potato extract 4 g/L, dextrose 20 g/L) have been used for selection of suitable growth medium for the accumulation of biomass and production of secondary metabolites. The media in which the endophytic fungus exhibits maximum growth and production pharmacological bioactive natural products have

been selected as optimized medium for further study. After incubation on the different liquid media, crude extracts were extracted with ethyl acetate. The total extract of the endophytic fungi were tested for biological activity and analyzed by HPLC.

**The effect of incubation time on secondary metabolites production.** The fungal strain *T. thermophiles* XJF-9 was subjected to different incubation time ranging between 7 days to 42 days for determining the optimum time required for the growth and bioactive natural compounds yield [12]. The basal media was sterilized and incubated with fungal endophytes, every 7 days the fungal culture was adjusted to 7, 14, 21, 28, 35 and 42 days respectively.

**Scanning Electron Microscopy analysis of endophytic fungi.** The morphological characteristics of endophytic fungi were placed in a petri dish on the surface of a PDA medium as mycelial plug and cultured for 14 days. The mycelia of fungi were di-rectly coated with gold by an iron sputter coater (SuPro, ISC 150, Germany), and the appearance of surface of the fungi such as shape, color, the diameter of the colony and colony reversal was observed under a scanning electron microscope (Carl Zeiss Jena, SUPRA 55VP, Germany).

**HPLC analysis.** The bioactive secondary metabolites (15 mg were dissolved in 1 mL) production endophytic fungus *T. thermophiles* XJF-9 in PDB media was analysis by HPLC grade methanol, and HPLC was carried out with the DIONEX Ultimate 3000 HPLC system (Thermo-Fisher, USA) combined with a Sunfire C18 column (4.6×250 mm, 5 µm) (Wa-ters, USA) and a Sunfire C18 guard column (4.6×20mm, 5 µm) (Waters, USA). Mobile phase A was 0.2% (v/v) formic acid (HCOOH) in water; Mobile Phase B was acetoni-trile (ACN). The elution profile was: 0-10 min 100% B (isocratic), 1-86 min 10-100% B (acetonitrile). The flow rate was 1 mL/min and the injection volume was 10 µL. UV chromatograms were recorded at 210, 254 and 330 nm; this analysis enabled the char-acterization of alkaloids, terpenoids and phenolic compounds on the basis of their retention time and UV spectra

**Melanin content assay.** ***Cell Culture:*** Murine B16 melanoma cell lines (B16F10) were obtained from CAS (Chinese Academy of Sciences, China). Cells were cultured in Dulbecco`s modified Eagle's medium (DMEM, Gibco Life Technologies, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin G (100 U/mL), and streptomycin (100 mg/mL) (Gibco-BRL, Grand Island, NY, USA) at 37 °C in a humidified atmosphere of 5% $CO_2$.

***Melanin Measurement:*** The melanin content assay of ethyl acetate extracts of the endophytic fungi associated with *V. anthelmintica* flowers was determined in accordance with the procedure described previously [7, 13]. Exponentially growing B16 cells were seeded at a density of 2 × $10^5$ cells per well in a 6-well plate and incubated for 24 h. After that, test samples were added to the individual wells, and the cells were incubated for 48 h, then washed twice with ice-cold PBS. After that, the cells were lysed, and the assembled cells were centrifuged at 10,000 g for 20 min. Each lysate (150 µL) was placed in a 96-well microplate and read spectrophotometrically at 405 nm using a multi-plate reader. The amount of protein of each sample was determined by a BCA Protein Assay Kit (Biomed, Beijing, China). The melanin content was normalized to the cellular protein concentration. Melanin content was calculated according to the formula:

$$Melanin\ content\ (\%) = \frac{S}{B} \times 100 \qquad 1)$$

where, S is the absorbance of the cells treated by the samples and B is the absorbance of the wells containing untreated cells. Each measurement was carried out in triplicate.

***Tyrosinase Activity Assay:*** Tyrosinase activity was determined by the method of Chao et al. [14]. First, the B16 cells were seeded in a 6-well plate at a concentration of 2 × $10^5$ cells per well and incubated for 24 h. Test samples were then added to each well and incubated for 24 h. Cells were then

washed twice with ice-cold PBS and lysed using 1% Triton X-100 solution containing 1% sodium deoxycholate for 30 min at −80 °C. The lysates were centrifuged at 12,000 × g for 15 min. After quantification and adjustment of the protein concentration of the supernatants, 90 µL of the supernatant was mixed and incubated with 10 µL of a freshly prepared substrate solution (10 µM 8-MOP) in duplicate in a 96-well plate, followed by incubation at 37 °C. Samples were then read at 490 nm. The results were calculated by the formula used for calculating the melanin content.

**Results and discussion. The effect of culture medium and incubation time on secondary metabolites production.** In our previously work we have reported isolation, identification and several biological activities of endophytic fungus *T. thermophiles* XJF-9 associated with *V. anthelmintica* leaf [12]. In this study is to optimize the culture condition of *T. thermophiles* XJF-9 to enhance the growth and synthesis biological active secondary metabolites (Figure 2). As shown in the Figure 2 A on growth and production secondary metabolites was found maximum in PDB and MEB media. Fungal endophyte on PDB and MEB medium induces higher growth and produced 350-340 mg/L total biomass respectively. The effect of different time on growth and synthesis bioactive metabolites by *T. thermophiles* XJF-9 is shown in Figure 2 B, maximum growth and production pharmacological products were observed when incubated at 14 days.

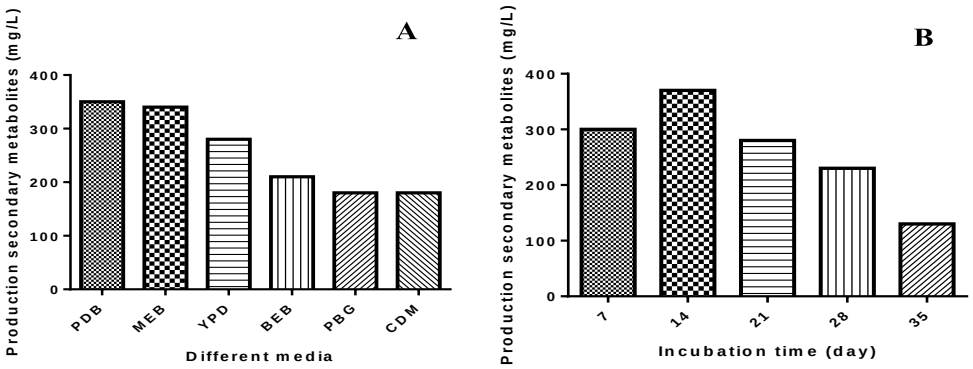



**Figure 2.** Effect of different culture medium (A) and incubation time for production secondary metabolites (B) on biomass and bioactive metabolite production by *T. thermophiles* XJF-9.




**Figure 2.** Extraction secondary metabolites production on different media

**SEM analysis of endophytic fungi.** A scanning electron microscope (SEM) observations were carried out to determine the morphological characteristics of endophytic fungal strain. This revealed the distinct conidiophore, hyphae, sporangia and mycelium of the endophytic fungi. (Figure 3).



**Figure 3.** Appearance of endophytic fungus of *T. thermophiles* XJF-9. of mycelium and conidiophore of SEM.

**Melanin synthesis and Tyrosinase activity evaluation in B16 cells.** The crude extract, as well as effects of the pure isolated secondary metabolites of endophytic bacteria and fungi isolated from the different parts of *V. anthelmintica* strongly increased melanin synthesis and tyrosinase activity in murine B16 cells [3, 7, 9, 15]. In the present study according to the result, the crude extract of *T. thermophiles* XJF-9 increased melanin synthesis (5 µg/mL, 106.31%; 10 µg/mL, 122.17% and 50 µg/mL, 134.98%). and tyrosinase activity dose dependent manner (5 µg/mL, 99.12%; 10 µg/mL, 111.02% and 50 µg/mL, 119.63%). All results compared with positive control 8-MOP (129.94 and 124.59%).

**Table 1.** Effect of crude extracts of endophytic fungus *T. thermophiles* XJF-9 on melanin production and tyrosinase activity in B16 cells.

| Sample | Concentration | Intracellular melanin content (% of control) | Tyrosinase activity |
|---|---|---|---|
| **Control** | **DMSO** | 100.00 | 100.00 |
| **8-MOP** | 50 µM | 129.9 | 124.59 |
| *T. thermophiles* XJF-9 | 5 µM | 106.31 | 99.12 |
| | 10 µM | 122.17 | 111.02 |
| | 50 µM | 134.98 | 119.63 |

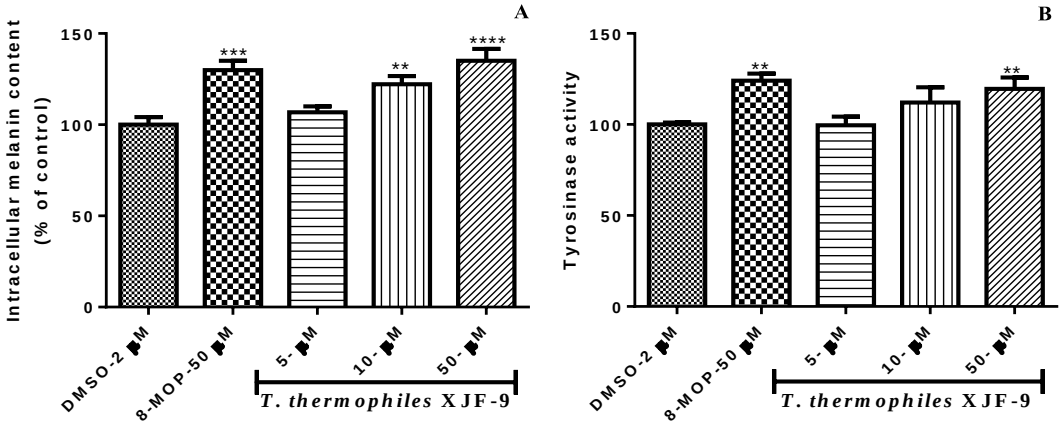



**Figure 4.** Stimulation of melanin content of B16 cells by secondary metabolites of crude extract of the fungi; **A:** Melanin content activity of different concentration; **B:** Tyrosinase activity of different concentration: Note: * compared to the blank control group (DMSO), P <0.05; ** compared to the blank control group (DMSO), *** P <0.001 compared with the blank control group (DMSO); P <0.01 **** compared to the blank control group (DMSO), P <0.0001.

**HPLC.** HPLC analyses was performed to identify and confirm secondary metabolites from the ethyl acetate extract and compared by their retention times (Figure 5). The results showed fungal extract have six main peaks in its chromatogram at retention time $R^t$ (min) 19.530, 23.290, 26.087, 30.157, 34.917 and 41.833.

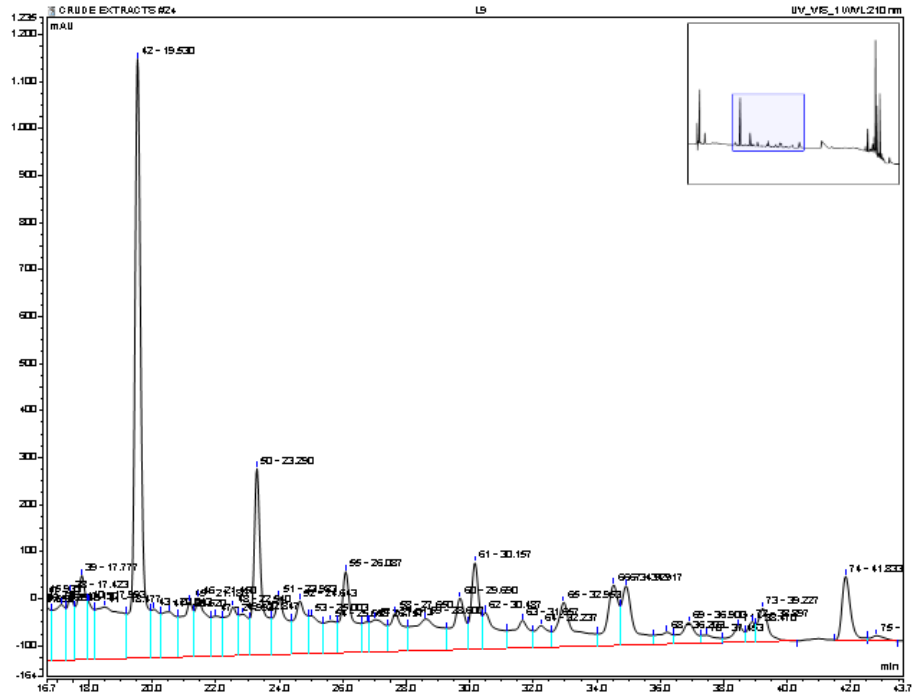



**Figure 5.** Analytical HPLC chromatograms of the crude ethyl acetate extract of *T. thermophiles* XJF-9. Recorded at 210 nm and performed using a waters column C18 and acetonitrile, 0.1 % acidified water with formic acid as mobile phase.

**Conclusions.** In conclusions, the crude ethyl acetate extract of *T. thermophiles* XJF-9 possessed cytotoxic, antimicrobial, anti-diabetic and melanin production and tyrosinase activity in B16

cells. Therefore, has been found optimal culture conditions. According PDB and MEB media, incubation time14 days are optimal ambience for growth and synthesis pharmacological active compounds by *T. thermophiles* XJF-9.

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