



Optimization of submerged culture conditions for mycelial biomass production with enhanced antibacterial activity of the medicinal macro fungus *Xylaria* sp. Strain R006 against drug resistant bacterial pathogens

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Abstract

In the present study the fruiting bodies of macro fungus was isolated from courtallum hills and identified as *Xylaria* sp. R006 based on morphological characteristics and it was reinforced by 18S rRNA gene sequence analysis. Due to the insufficient wild fruiting bodies of *Xylaria* sp., submerged culture conditions and nutritional requirements for the production of mycelial biomass with improved antibacterial activity were studied in shaker flask culture. Among the various culture media, maximum biomass production of 1.5, g/L was observed on potato dextrose broth over a period of 40 d incubation at pH of 5.5 in 25°C -30°C. Among the carbon sources, glucose supplemented basal medium showed an effective biomass production of 2.3 g/L. whereas in nitrogen sources, yeast extract had significant biomass production of 1.3 g/L. The optimized ethyl acetate extract of cultural filtrate showed a maximum inhibition zone of 28.4 mm and 26.3 mm against multidrug resistant *P. aeruginosa* strain 1 and *S. aureus* strain 5, respectively.

Key words – environmental parameter – improved bioactivity – *Xylaria* sp. multi drug resistant human bacterial pathogens

Introduction

Macro fungi have been proved to be one of the most productive sources for producing a large and diverse variety of secondary metabolites with significant bioactivities (Mugdha et al. 2010). Several nutraceutical products have been isolated from medicinal macro fungi and three of these, which are carcinostatic polysaccharide drugs. These are krestin from the cultured mycelium of *Trametes versicolor*, lentinan from the fruiting bodies of *Lentinus edodes* and schizophyllan from the culture fluid of *Schizophyllum commune* (Mizuno 1993). Their previous successes in yielding useful natural products, their extensive habitat range and number of species

yet to be discovered imply that fungi will continue to be a promising source of novel natural products.

Xylaria is a first described genus of the family xylariaceae (Martin 1970). Xylariaceae is a large family comprising of around 40 genera and although it has representatives in most countries of the world the xylariaceae exhibits its greatest diversity in the tropics (Laessoe 1994, Ju & Rogers 1996). They are saprobic or sometimes weakly to strongly parasitic on woody plants. Although they are found mostly on wood, some species are found on sawdust, leaf and dung or soil. The genus *Xylaria* has been proven to be a valuable source of bioactive agents such as chemokine receptor (CCR5) antagonist 19, 20-epoxycytochalasin Q (Jayasuriya et al. 2004), the antifungal multiplolides A and B (Boonphong et al. 2001), the NPY Y5 receptor antagonists xylarenals A and B (Smith et al. 2002) and the cytotoxic eremophilanolides (Isaka et al. 2010). Recently, we have isolated bioactive metabolites producing *Xylaria* spp. against drug resistant bacterial human pathogens from courtallum hills, Western Ghats of southern Tamil Nadu (Ramesh et al. 2012a & 2012b). Since there are still many unexplored resources in species of *Xylaria*, the potential for finding new bioactive compounds producing macro fungi and thereby new metabolic pathway is also enormous.

The potential application of the macro fungal species of *Xylaria*, particularly in the prospect of bioactive compounds production, makes it more noteworthy to study the different cultural conditions that affect the growth of these microorganisms. In the present study, attempt was made to investigate the cultural conditions in *Xylaria* sp. for improved biomass production and antibacterial activity.

Materials & Methods

Source of macro fungus

Fruiting body of the macro fungus was collected from tropical evergreen forest of Courtallum Hills, Western Ghats, Tamil Nadu, India. At the time of collection, it was identified as xylariaceae based on typical characteristics of this family. Therefore, this macro fungal isolate was designated as *Xylaria* R006 and used for this investigation. The fruiting bodies were placed in paper bags after removal of excess moisture. The identification of the macro fungus was confirmed by 18S rRNA gene sequence comparisons (Altschul 1990). The fruiting bodies of this macro fungus were tissue cultured to obtain mycelia, which was maintained on the potato dextrose agar.

Cultivation and identification of macro fungal isolate

The fruiting bodies of the macro fungus were washed thoroughly with sterile distilled water and were thereafter aseptically broken with aid of a sterile forceps. A small piece of 2 × 2 mm of the fruiting body was aseptically transferred onto plates containing potato dextrose agar with 50 µg/mL of streptomycin to suppress bacterial growth. The plates were incubated at 30°C for three weeks for the development of macro fungus. The fungus growing out from the fruiting bodies was subsequently transferred onto fresh potato dextrose agar plates without antibiotics. After that, the DNA extraction and isolation from cultured mycelia were done by Synergy Scientific Services, Chennai. The macro fungal isolate was identified to genus level at Mycological Lab, Department of Botany VHNSN College, Virudhunagar, Tamil Nadu, India. Then it was reinforced by the gene sequence analysis of its 18S rRNA gene.

Effect of culture media

The media requirements of macro fungus were determined by mycelial dry weight method (Jonathan 2002). For effect of culture media on biomass production studies, four different culture media such as malt extract broth, Potato dextrose yeast extract broth, Potato dextrose broth and basal growth medium consisted of peptone (2.0 g), yeast extract (2.0 g), KH₂PO₄ (1.0 g), MgSO₄·7H₂O (0.6 g) and 1000 mL distilled water of pH 5.5 at 30°C for a period of 10, 20, 30 and 40 d were used. The mycelial growth was studied according to Kadiri & Fasidi (1994).

Effect of temperature and pH

The temperature and pH requirements of macro fungus were determined by mycelial dry weight method (Jonathan 2002). For temperature, potato dextrose broth medium was dispensed into 500 ml conical flasks (200 ml per flask) and the mouth was sealed with aluminium foil. The flasks were autoclaved at 121°C for 20 min. After cooling, each conical flask was inoculated with mycelia disc (5.0 mm diameter) from 6 day old culture and incubated at different temperatures (15-35°C) for 35 d. Each treatment was replicated three times. The mycelia were harvested using the method of Jonathan (2002). For pH, the same potato dextrose broth medium was employed but the medium was adjusted to pH values of 3.5–7.0. 200 ml of each treatment was dispensed into 500 ml conical flask and replicated three times. They were autoclaved, inoculated and incubated as described in the temperature experiment.

Effect of carbon and nitrogen sources

The macro fungus was grown in basal liquid medium that was described by Jin-zhong et al. (2003). The basal medium was supplemented separately with carbon sources (15.0 g/L) such as glucose, fructose, maltose, cellulose, starch & xylan and in the case of nitrogen (10 g/L), various nitrogen sources such as ammonium nitrate, ammonium sulphate, potassium nitrate, sodium nitrate, calcium nitrate and yeast extract. The media were autoclaved at 121°C for 15 min. After cooling, each flask was inoculated with 5 agar discs (5.0 mm size) of 6 d old mycelium and incubated at 30°C for 40 d. The mycelial mats were harvested and dried at 40°C and weighed. Assessment of mycelial weight was carried out using the procedure described by Kadiri & Fasidi (1994).

Extraction of bioactive compounds

Discs were cut from the edge of an actively growing colony on PDA with a flamed cork borer (5 mm diameter) and transferred aseptically into 500 mL flasks containing optimized basal media containing glucose as carbon source (15 g/mL) and yeast extract as nitrogen source (10 g/mL). The culture was incubated for 40 d at 30°C and pH 5.5. After the incubation period, fungal mycelium was separated from the culture filtrate by cheesecloth. The filtrate and dried mycelium were extracted three times with ethyl acetate. The culture filtrate extract was concentrated by evaporation under reduced pressure at 45°C using rotary vacuum evaporator. The dried extract was dissolved in 10% dimethyl sulfoxide (DMSO) and stored at 4°C for further study.

Antimicrobial activity

Test microorganisms

The clinical strains of *S. aureus* were obtained from skin infections and wounds of some patients, whereas strains of *P. aeruginosa* were obtained from lung infections of cystic fibrosis patients from Bose Clinical Laboratory (Madurai, Tamil Nadu, India). All the clinical strains were stored in screw cap tube at –20°C in deep freezer. *Staphylococcus aureus* and *P. aeruginosa* strains were identified by standard biochemical methods (Essers & Radebold 1980, Pourshadi & Klaas 1984).

Susceptibility test

The Kirby–Bauer disk diffusion test was used to determine the antibiotic resistance of *S. aureus* strains (1–10) and *P. aeruginosa* strains (1–8). The isolated colonies of the above strains from mother culture plates were inoculated into nutrient broth. The broth was incubated at 37°C until it equals 0.5 McFarland standards. A McFarland 0.5 turbidity standard corresponds to an inoculum of 1×10^8 CFU ml/L (Acar & Goldstein 1991). The antibiotic disks of methicillin (5 µg/disk), penicillin (10 units/disk), and vancomycin (30 µg/disk) were used for clinical strains of *S. aureus*. Ciprofloxacin (5 µg/disk), cefotaxime (30 µg/disk), ofloxacin (5 µg/disk), and amikacin (30 µg/disk) were used for clinical strains of *P. aeruginosa*. Inoculated plates were inverted and incubated at 37°C for 18 h. After the incubation period, the diameter of zone of inhibition was

measured, and results were interpreted according to the standards of Clinical and Laboratory Standards Institute (NCCLS 2008).

Disk diffusion method

The paper disk diffusion method was used to determine the antibacterial activity of endophytic fungal extract (Acar & Goldstein 1996). Sterile disks (6 mm) were impregnated with 10 ml of extract at a concentration of 1mg/mL. For bacteria, microorganisms were swabbed on the surface of muller hinton agar was used. Paper disks treated with 10% DMSO were used as negative controls. The plates were incubated at 37°C for 18 h for bacteria. The diameter of the inhibition zone around each disk was measured at the end of the incubation time. Experiments were performed in triplicate and the antimicrobial activity was expressed as the average of inhibition zone diameters (in mm) produced by the fungal extract.

Statistical analysis

The triplicate data are expressed as the mean value \pm standard error and presented in the form of figures. The error bars are depicted at 5% limit. The overlapping and non-overlapping bars show non-significant and significant respectively differences among different treatments.

Results

Morphological characteristics of *Xylaria* sp. Strain R006

Fruiting bodies are 3–9 cm in length, 0.5–1.5 cm in broad (Fig.1), growing either singly or in groups which are emerging from partially decomposing wood with soil. The fruiting bodies arise during spring season. Ascospores are dark brown to black, with straight germ slit running almost trough the full length of the spore, 19.5 – 21.8 μ m broad, 30.5 – 34.3 μ m in length. Growth rate is 5.8 – 7.5 cm/week, covering petriplate in 15 – 20 d. The morphological characteristic of the macro fungal isolate was observed on PDA after 2 weeks of growth at 30°C. Colonies on PDA was circular, raised, at first white, later it was brown to black colored. Hyphae were thin walled and branched. Morphological characteristics such as size, shape, and color of the fruiting bodies allow the identification of the macro fungus as *Xylaria* sp. strain R006, which is reinforced by the sequence of its 18S rRNA gene that gives a 91% sequence (GenBank No. KC405623) similarity to those accessible at the BLASTN of *Xylaria* sp. (GenBank No. FN868478).



Fig. 1 – Morphological characteristics of *Xylaria* sp. Strain R006. a, Cultural morphology of *Xylaria* sp. Strain R006 on PDA plate, b, Development of fruiting bodies on PDB medium, c, Natural fruiting bodies and d, Ascospores.

Effect of various culture media on fungal biomass production

To study the effect of culture media on biomass production, four different culture media were used. The fungal isolates was grown in malt extract broth, Potato dextrose yeast extract broth, Potato dextrose broth and basal growth medium of pH 5.5 at 30°C for a period of 10, 20, 30 and 40 d. The maximum biomass production of 1.5 g/L was observed on potato dextrose broth over a period of 40 d in *Xylaria* sp. Strain R006, whereas significant mycelial biomass was also observed on basal medium and Potato dextrose yeast extract broth (Fig. 2).

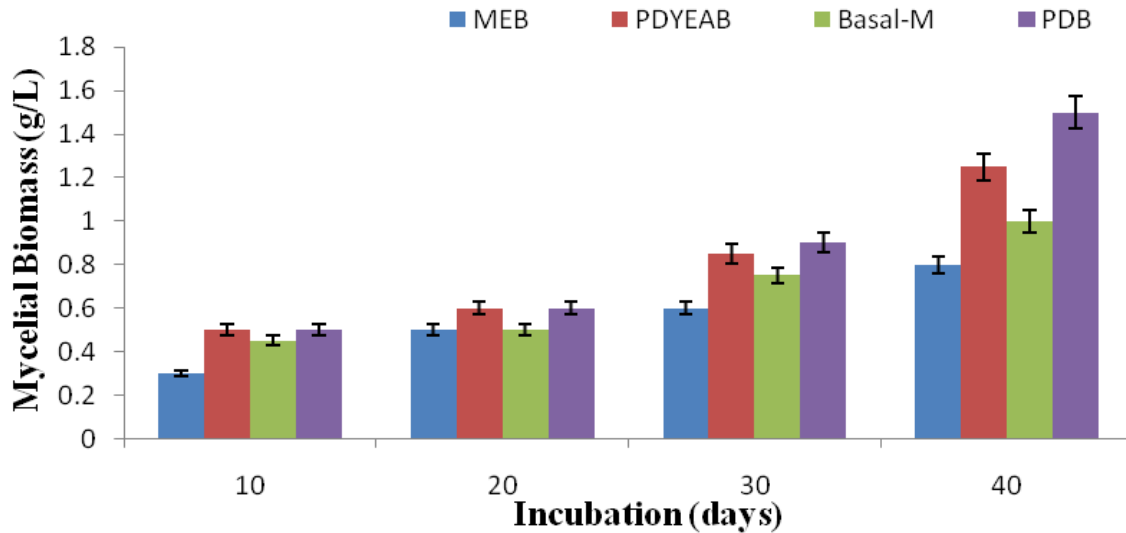


Fig. 2 – Effect of various culture media on biomass production of *Xylaria* sp. Strain R006.

Effect of temperature and pH on biomass production

The mycelia biomass of *Xylaria* sp. Strain R006 was investigated at various temperatures (15°C to 40°C) in potato dextrose broth at pH 5.5. The influences of temperature on the mycelia biomass of fungus was presented in Fig. 3. The maximum biomass of 1.5 g/L was observed at 25°C and pH 5.5, whereas moderate mycelial biomass of 1.4 g/L was observed at temperature 30°C in pH 5.5. At 15°C there was no fungal growth was observed. The results indicated that the optimum temperature for maximal biomass production was 25°C to 30°C and the pH was 5.5 in Fig. 4.

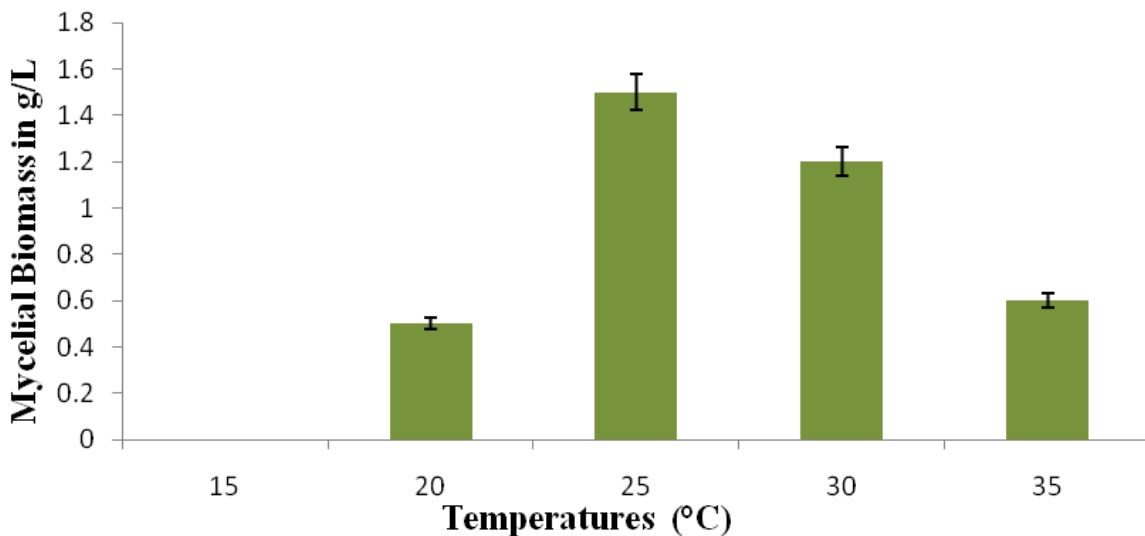


Fig. 3 – Effect of various temperature on biomass production of *Xylaria* sp. Strain R006.

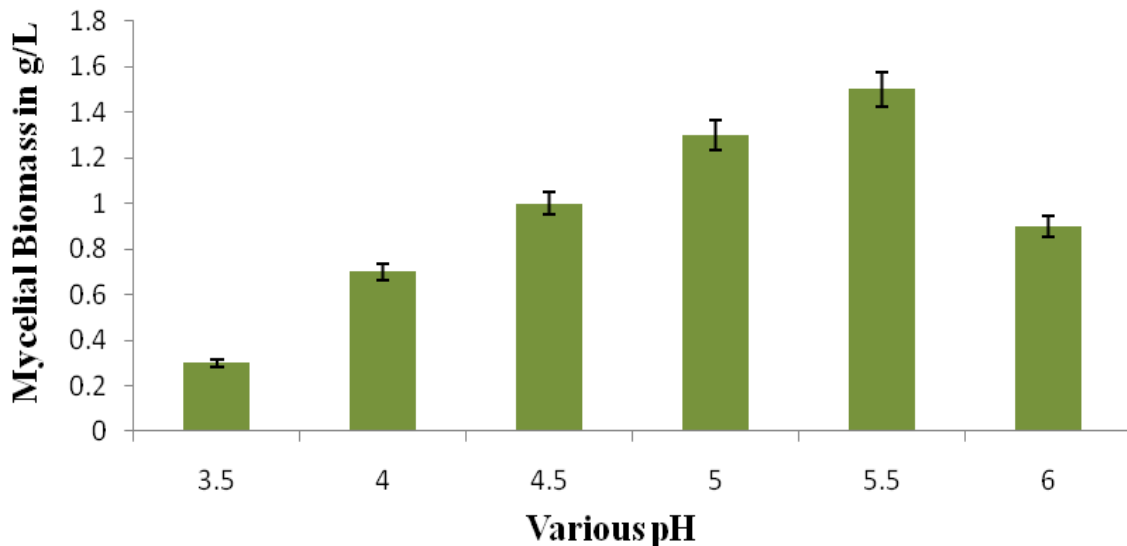


Fig. 4 – Effect of various pH on biomass production of *Xylaria* sp. Strain R006.

Effect of carbon and nitrogen sources on production of fungal biomass

The *Xylaria* sp. Strain R006 was grown in basal growth medium of pH 5.5 at 30°C for 40 d. To find out the suitable carbon sources for the maximum mycelial biomass production, various carbon sources were separately provided instead of glucose (15 g/L) in the basal medium. Among the various carbon sources, glucose supplemented medium produced maximum biomass of 2.3 g/L. The other carbon sources produced the moderate mycelial biomass (Fig. 5).

To determine the effect of nitrogen sources on biomass production, various nitrogen sources were used. Among the nitrogen sources, yeast extract was found to be the best nitrogen source for the maximum production of biomass. Ammonium nitrate and ammonium sulphate were also produced an effective biomass. The maximum fungal biomass of 1.3 was observed on yeast extract supplemented medium in (Fig. 6). After 40 d of incubation, the final pH of the medium was found to be 3.3 to 5.2.

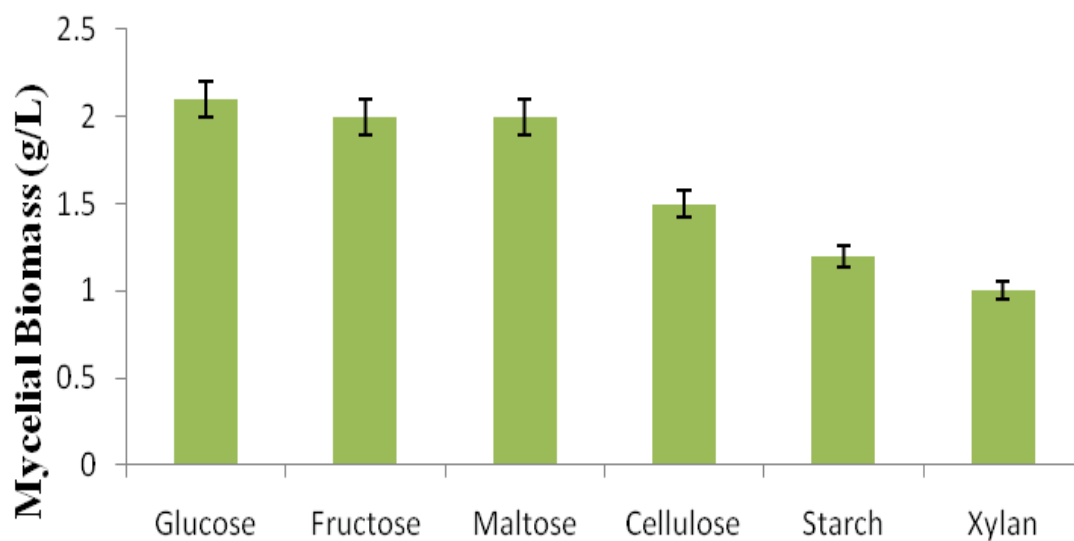


Fig. 5 – Effect of various carbon sources on biomass production of *Xylaria* sp. Strain R006.

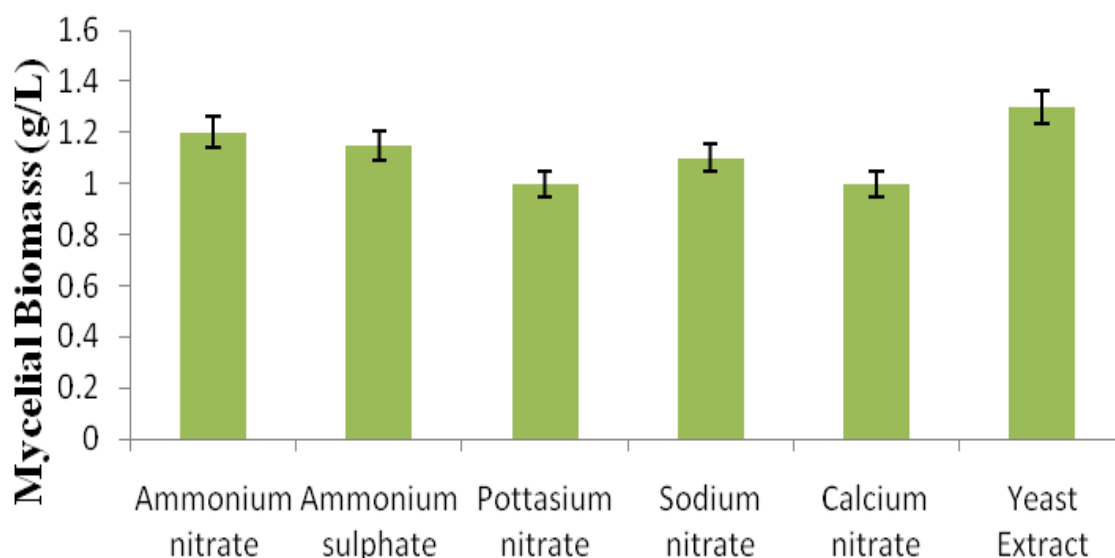


Fig. 6 – Effect of various nitrogen sources on biomass production of *Xylaria* sp. Strain R006.

Antibacterial Activity

The clinical strains of *S. aureus* (1–10) were found to be positive for various biochemical tests such as the coagulase test, mannitol utilization test, DNase test, and catalase activity. The antibiotic resistance profile of *S. aureus* strains (1–10) was determined using commercial antibiotics such as methicillin, penicillin, and vancomycin. In contrast, *P. aeruginosa* (1–8) were identified by colony morphology, growth on cephaloridine fucidin centrimide agar, a positive oxidase test, and growth at 42°C. Antibiotics such as ciprofloxacin, cephotaxime, ofloxacin, and amikacin were used to determine the resistance profile of *P. aeruginosa* strains (1–8).

The optimized ethyl acetate extract of cultural filtrate showed a maximum inhibition zone of 28.4 mm and 26.3 mm against multidrug resistant *P. aeruginosa* strain 1, and *S. aureus* strain 5, respectively (Table 1).

Discussion

Identification macro fungal species of *Xylaria* sp. Strain R006

The macro fungal isolate of Strain R006 could not be identified to a species level using available sequence data in *Xylaria* database (BLASTN) and morphological characteristics. Because, Xylariaceae is a large family of 40 genera and although it has representatives in most countries of the world the xylariaceae exhibits its greatest diversity in the tropics (Laessoe 1994, Ju & Rogers 1996). However, based on the morphological and sequence analysis, these isolates are found to be different species of *Xylaria*, hence we have named as *Xylaria* sp. Strain R006.

Optimal culture conditions

The growth profile of *Xylaria* sp. Strain R006 included studies on mycelial biomass. The maximum growth in terms of mycelia production occurred on Potato dextrose broth followed by potato dextrose yeast extract and basal medium, while it was minimum on malt extract broth. On the contrary, Bilay et al. (2000) reported that *Ganoderma lucidum* had a slow growth rate in PDB medium, whereas there was a significant fungal biomass production in basal medium. Similarly, the maximum fungal biomass production of *Ganoderma* species was observed in basal medium (Roberts 2004). The study of mycelial characteristics showed white, thick mat of fluffy growth on Potato dextrose broth while it was white thick mat of strand growth on malt extract broth. At the same time, slow growth of slighty strandy mycelium was observed on malt extract broth.

Table 1 Antibacterial activity of optimized cultural filtrate extract of *Xylaria* sp. R006 against multidrug resistant *S. aureus* strains (1– 10) and *P. aeruginosa* strains (1– 8)

Zone of inhibition (mm)				
<i>S. aureus</i>	Penicillin (10 uits/mL)	Methicillin (10 µg/mL)	Vancomycin (30 µg/mL)	Ethyl acetate extract
<i>P. aeruginosa</i>	CF (5 µg/mL)	CE (30 µg/mL)	OF (5 µg/mL)	
Strain 1	12.8 ± 0.15 (R)	10.8 ± 0.14 (R)	16.5 ± 0.08 (S)	21.3±0.12
	14.5 ± 0.4 (R)	12.4 ± 0.4 (R)	12.5 ± 0.35 (R)	28.4±0.33
Strain 2	12.8 ± 0.14 (R)	11.2 ± 0.31 (R)	15.4 ± 0.28 (S)	24.6±0.44
	21.6 ± 0.1 (S)	23.5 ± 0.14 (S)	20.3 ± 0.28 (S)	14.3±0.15
Strain 3	14.2 ± 0.28 (R)	12.1 ± 0.16 (R)	16.0 ± 0.22 (S)	25.2±0.22
	15.4 ± 0.28 (R)	14.9 ± 0.14 (R)	13.5 ± 0.4 (R)	27.6±0.33
Strain 4	11.5 ± 0.35 (R)	10.2 ± 0.31 (R)	17.2 ± 0.14 (S)	21.1±0.11
	23.7 ± 0.23 (S)	16.8 ± 0.1 (R)	18.6 ± 0.31 (S)	22.7±0.30
Strain 5	10.8 ± 0.14 (R)	8.9 ± 0.22 (R)	18.5 ± 0.28 (S)	26.3±0.01
	24.3 ± 0.04 (S)	24.8 ± 0.15 (S)	19.5 ± 0.14 (S)	22.4±0.22
Strain 6	10.3 ± 0.15 (R)	9.5 ± 0.35 (R)	12.4 ± 0.35 (R)	21.0±0.21
	22.3 ± 0.15 (S)	25.4 ± 0.07 (S)	18.4 ± 0.28 (S)	19.3±0.31
Strain 7	13.8 ± 0.16 (R)	15.5 ± 0.07 (S)	16.9 ± 0.14 (S)	23.4±0.10
	16.5 ± 0.22 (R)	23.7 ± 0.18 (S)	13.9 ± 0.4 (R)	23.0±0.17
Strain 8	10.5 ± 0.23 (R)	8.8 ± 0.14 (R)	13.5 ± 0.21 (R)	22.7±0.21
	18.7 ± 0.14 (R)	16.5 ± 0.35 (R)	16.5 ± 0.14 (S)	22.8±0.15
Strain 9	14.0 ± 0.21 (R)	12.2 ± 0.10 (R)	14.8 ± 0.22 (R)	20.4±0.13
Strain 10	14.9 ± 0.1 (R)	8.9 ± 0.18 (R)	17.4 ± 0.35 (S)	22.6±0.22

R– Resistant and **S** – Sensitive, **CF**– Ciprofloxacin, **CE**– Cefotaxime, **OF** – Ofloxacin

The initial medium pH is a critical factor associated with the growth of fungi because it will affect the cell membrane function, cell morphology and structure, the solubility of salts, the ionic state of substrates, the uptake of various nutrients and product biosynthesis (Qing & Jian 2002). In this study, *Xylaria* sp. Strain R006 was cultivated in the PDB and basal medium with different initial pHs 4 - 7 and different initial temperatures 15-40°C, in culture conditions to investigate the effects of pH and temperature on mycelial biomass and antibacterial activity.

The results indicated that the optimum temperature and pH for maximal biomass production were 5.5 and 25°C to 30°C respectively. Earlier reports revealed that many kinds of macro fungi grow at acidic pH optima (Kim et al. 2003, Lee et al. 1999, Shu & Lung 2004). Similarly, Lee et al. (2004) reported that the optimum pH was 5.5 for the maximum production of mycelial biomass of the macro fungus *Grifola frondosa*. The effect of temperature on mycelial production is comparable to the growth of many kinds of macro fungal species (Bae et al. 2000, Kim et al. 2003). Similarly, present results seem to be consistent with other reports in which the optimal temperature for macro fungal growth was 20°C to 30°C (Boddy 1985, Lee et al. 2004, Lai et al. 2012).

Optimal nutrient sources

In general, mycelial cells of macro fungi grow over a broad range of carbon source (Burns et al. 1994). To determine the suitable carbon source for the production mycelial biomass with enhanced antibacterial compound in *Xylaria* sp. Strain R006, six carbon sources were separately provided at the concentration of 15 g/L instead of glucose employed in the basal medium. Among the carbon sources tested, glucose yielded the highest mycelial production.

Medium containing glucose was significant in yielding the highest mycelia growth when compared to the other carbon sources. Similarly, Xiaobo et al. (2006) reported that glucose, sucrose, yeast and peptone were supported more fungal biomass production in *Xylaria* sp. 2508. Glucose was found to be the best source for maximum biomass production in *Lentinus edodes* (Song & Cho 1987).

Nitrogen plays an important role in fungal growth and metabolite production (Kim et al. 2005). Thus, the effect of nitrogen on mycelial biomass production of *Xylaria* sp. Strain R006 in the form of ammonium nitrate, ammonium sulphate, potassium nitrate, sodium nitrate, calcium nitrate and yeast extract were studied. Among the six nitrogen sources that have been tested in this study, yeast extract was most suitable for the growth of mycelial biomass (1.3 g/L). However, current findings are contrary to those of Shih et al. (2006) that suggested most basidiomycetes prefer complex organic nitrogen sources for their favorable submerged cultures. The final pHs of the medium remain in acidic level. It is generally known that the byproduct of fungal metabolic process was acidic in nature (Fang & Zhong 2002).

Antibacterial Activity

Many medicinal macro fungi have a great potential for the production of useful bioactive metabolites and they are a prolific resource for drugs. The spectrum of pharmacological activities of macro fungi is fascinating. Discovery and evaluation of new bioactive compounds from various fungi as new safe compounds for the treatment of various diseases has become a hot research spot. In present study, the antibacterial activity of the optimized ethyl acetate extract of culture filtrate was investigated against drug resistant bacterial pathogens. The antibiotic resistant profiles of *S. aureus* and *P. aeruginosa* strains were reported earlier (Ramesh et al. 2012). Maximum inhibition zone of 28.4 mm and 26.3 mm was observed against multidrug resistant *P. aeruginosa* strain 1, and *S. aureus* strain 5, respectively. Similarly, Iwalokun et al. (2007) reported that the petroleum ether and acetone extract of macro fungal species of *Pleurotus ostreatus* showed significant antibacterial activity against multidrug resistant *P. aeruginosa*.

Therefore, from the above results it can be concluded that it was possible to develop an optimized medium with regard to cultural conditions and nutritional sources to produce biomass of *Xylaria* sp. Strain R006 with enhanced antibacterial activity against drug resistant human bacterial pathogens. High biomass was obtained in culture filtrate using the optimized medium with 15 g/L of glucose and 10 g/L of yeast extract as carbon and nitrogen sources respectively. To the best of our knowledge the nutritional requirements for a large scale submerged fermentation of *Xylaria* sp. have not been demonstrated so far. Moreover, this is the first report concerning the highest biomass production obtained in submerged fermentation by the same species.

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