Research Article

Medium Optimization of the endophytic fungus *Eupenicillium* SP. isolated from *Acacia nilotica* L. for its Antimicrobial Activity against oral Pathogens

Meenambiga.S.S*, Rajagopal.K
*Department of Biotechnology, Vels University, Pallavaram, Chennai, India.
***Corresponding author's E-mail:** meena.bt@gmail.com

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ABSTRACT

The present study is aimed to optimize the growth and antimicrobial activity of the endophytic fungus *Eupenicillium* sp. isolated from the medicinal plant *Acacia nilotica* against the major oral pathogens *Streptococcus mutans* and *Candida albicans*. The effect of various cultural conditions like different media, utilization of carbon and nitrogen sources, incubation period, pH and temperature on the growth and bioactivity against oral pathogens were studied by one factor at a time method. The optimum growth and antimicrobial activity of *Eupenicillium* sp. strain was achieved with malt extract broth as the basal medium with dextrose and peptone as the carbon and nitrogen sources respectively. The optimum pH and temperature of the strain was found to be 5.0 and 35 °C with 14th day of incubation under stationary conditions as optimum for the enhanced growth and antimicrobial secondary metabolite production. Ethyl acetate extract was found to be optimum for production of antibacterial compounds against *S.mutans* whereas chloroform extract was optimum against *C.albicans*. Thus, the intensive study on antimicrobials from *Eupenicillium* sp. leads to the discovery of pharmaceutically important products against oral pathogens.

Keywords: *Eupenicillium* sp., Biomass, Antimicrobial activity, *Streptococcus mutans*, *Candida albicans*, Optimization.

INTRODUCTION

Living organisms in nature are in constant relationship with each other. Fungal association with plants either as mycorrhizal or as endophytes exists from than 400 million years which plays an important role in understanding the evolution of life on land.[1]1.1 million Species of fungal endophytes have been estimated so far which represents an important component of fungal diversity. Tan and Zou (2001) reported that almost all plant species harbor one or more endophytic species.[2]Endophytic fungi synthesize a large number of antimicrobial secondary metabolites which depends on the nutritional and environmental factors. They are considered as an outstanding source of pharmaceutically important products for their ability to inhabit unique higher plants growing in unusual environments.[3]

The oral micro flora comprises of more than 700 bacterial species which makes it one of the most complex microbial flora of the human body. Over representation of pathogenic species contribute to the onset and progression of many oral diseases such as oral candidiasis, dental caries and periodontal diseases.[4] It has become very difficult to control these organisms because of their tolerance towards various antimicrobial agents in routine use during the course of therapy. Natural products make excellent leads for new drug development which are safer and biodegradable.

The traditional use of the medicinal plant *Acacia nilotica* for oral problems implies its antimicrobial activity against potential oral pathogens. The tender twigs of *Acacia nilotica* are used as toothbrushes for its germicidal property.[5] A remarkable antibacterial activity against the dental caries pathogen *Streptococcus mutans* was seen in the stem bark extract of the plant.[6] Dried fruits of *Acacia* species inhibit *Candida albicans* and are used in traditional medicine for oral candidiasis. Endophytic fungi are able to produce secondary metabolites with activities similar to or more than that of their respective hosts. Endophytic fungi studies on Acacia species have been studied and the endophytes isolated have various pharmaceutical uses as its host plant.[7,8] The anti-diabetic potential of a peptide from an endophytic *Aspergillus awamori* isolated from *Acacia nilotica* was exploited.[9]*Eupenicillium* sp., the teleomorphic state of *Penicillium* species has been isolated as endophyte from medicinal plants and their antimicrobial property was studied.[10]The endophytic fungus *Eupenicillium parvum* isolated from neem plant produces azadirachtin similar to its host plant.[11]

Screening of organisms for potential bioactive products is followed by optimization of process conditions such as physical and chemical conditions. Optimization of process parameters has a significant role in designing an appropriate production media and conditions which have an impact on yield and the product concentration. Nutritional factors such as carbon and nitrogen sources and environmental factors such as pH, temperature, aeration and other physical factors have to be optimized to enhance the production of desired secondary metabolite. The physical and chemical factors differ for each fungus and depend on the genera they belong.[12] Medium optimization by conventional method is the initial step which processes one factor at a time keeping other factors constant. The endophytic fungi *Eupenicillium* sp. isolated from the leaves of *Acacia*

nilotica plant showed good antimicrobial activity against the oral pathogens *S.mutans* and *C.albicans*.[13]

The present study was done to optimize the media composition of endophytic fungi *Eupenicillium* sp.from the medicinal plant *Acacia nilotica* for its growth and bioactive metabolites production against the major oral pathogens *S.mutans* and *C.albicans*.

MATERIALS AND METHODS

Isolation, identification and screening

The endophytic fungus *Eupenicillium* sp. was isolated from the leaves of *Acacia nilotica*. The strain was identified based on morphological analysis and authenticated as *Eupenicillium* sp. by National Fungal Culture Collection of India, Agharkar research Institute, Pune, India. *Eupenicillium* sp. has potent antimicrobial activity against the major oral pathogens *S.mutans* and *C.albicans* as we described earlier.[14]

Culture medium and extraction

Extraction procedure was carried out as described by Radji *et al*., 2011.[15] The endophytic strain isolated was grown on potato dextrose broth inoculated with a mycelial agar block of actively growing colony in 100 ml flask. The flask was incubated under alternate light and dark conditions at 27°C for 21 days. After the incubation period, the fungal culture broth was filtered and the crude filtrate was used for optimization studies.

Test organisms used

The test organisms used were *S.mutans* and *C.albicans*, the major oral pathogens which were isolated from dental caries of the affected patients from the laboratory of Billroth hospitals, Chennai. The pathogens were identified and authenticated by Dr. Kayalvizhi, Microbiologist, Billroth Hospitals,Chennai. *S.mutans* was cultured and maintained on brain heart infusion broth (HiMedia Pvt. Ltd. Mumbai, India) whereas Sabouraud's Dextrose broth (HiMedia Pvt. Ltd. Mumbai, India) was used for *C.albicans.* Slant cultures of both the pathogens were maintained at 4°C for further use.

Antimicrobial assay by agar well diffusion method

Antimicrobial assay was done by agar well diffusion method in Muller Hinton agar (Hi Media Pvt. Ltd. Mumbai, India)as described by NCCLS 172056.[16] Inoculum was standardized by picking six colonies of *S.mutans* and *C.albicans* in to their respective media and incubated at 37°C for 18-24 hours. Inoculum of the test microorganism was adjusted to 0.5 McFarl and standard (10^8 Cfu/ml) and 100 µl of the standardized inoculum was swabbed on to Muller-Hinton agar for antimicrobial assay by agar well diffusion method. The wells were punched using sterile cork borer and 100 µl of 100 µg/ml concentration of fungal crude extracts were loaded on to the agar wells. 2% chlorhexidine was used as positive control. The plates were incubated at 37°C for 24 hours. The diameter of inhibition zone around the well was measured in millimeter. (Antibiotic zone scale, Hi Media Laboratories, Mumbai). The experiments were carried out in triplicates and the results were expressed as their mean values.

Selection of culture media

Standardization of basal media for optimal growth and bioactive metabolites production was done using different media such as potato dextrose broth (PDB) (Potato 200g, Dextrose 20 g), Czapek Dox broth (Sucrose 30g, Sodium nitrate 2g, Dipotassium phosphate 1g, Magnesium sulphate 0.5g, Potassium chloride 0.5g), Malt extract broth (Malt extract 20g, peptone 1g, dextrose 20 g), Yeast extract peptone dextrose broth (Yeast extract 10g, Peptone 20g, Glucose 20 g) and Sabouraud's dextrose broth (Dextrose 40g, Peptone 10g) were used. All the media components were purchased from HiMedia Pvt. Ltd, Mumbai, India. After 21 days of incubation at 25°C, secondary metabolites were extracted as described above and the antimicrobial assay was performed against *S.mutans* and *C.albicans*. The medium in which the isolate *Eupenicillium* sp.showed maximum antimicrobial activity expressed as zone of inhibition and maximum biomass was chosen as the basal medium.

Effect of carbon sources on biomass and bioactive metabolites production

Different carbon sources such as glucose, dextrose, glycerol and starch were used for optimization. Based on the concentrations of carbon source in basal media selected by optimization, concentration of carbon sources were fixed and added to the basal medium. Basal media containing different carbon sources were inoculated with 5 mm disc of seven days old culture and incubated. After the incubation period, crude extract was tested for its antimicrobial activity. Mycelial biomass and the zone of inhibition were recorded.

Effect of nitrogen sourceson biomass and bioactive metabolites production

Different nitrogen sources such as peptone, sodium nitrate, yeast extract and beef extract were used for optimization. Based on the concentrations of nitrogen source in basal media selected by optimization, concentration of nitrogen sources were fixed and added to the basal medium. Dextrose was used as the sole source of carbon for *Eupenicillium* sp. Basal media containing different nitrogen sources were inoculated with 5 mm disc of seven days old culture and incubated. After the incubation period, the antimicrobial assay was performed for both the oral pathogens. Mycelial biomass and the zone of inhibition were recorded.

Effect of incubation days on biomass and bioactive metabolites production

25 ml of basal medium containing optimized carbon and nitrogen sources inoculated with actively growing mycelial disk was kept at stationary conditions at 25°C. The growth of the endophytes was recorded from 4th day till 20th day of incubation. The antimicrobial assay was

carried out on all the consecutive days starting from 4th to 20th day. The incubation day on which the maximum zone of inhibition recorded was noted down.

Effect of pH on biomass and bioactive metabolites production

The effect of pH on the growth and antimicrobial metabolite production was recorded in liquid basal media with different pH levels (4 – 7). 25 ml of the basal media was transferred aseptically in to a 100 ml flask and adjusted to the desired pH level using 0.1 N NaCl or 0.1 N HCl. After sterilization, the flasks were inoculated with mycelial disks and incubated. The antimicrobial assay was performed for both the oral pathogens. The pH at which maximum zone of inhibition and fungal biomass obtained were recorded.

Effect of temperature on biomass and bioactive metabolites production

The basal media inoculated with endophytes in a 100 ml flask were grown in different temperatures from 15°C to 45°C at a difference of 10°C under stationary conditions. After the incubation period, the antimicrobial assay was performed using the crude extract for both the oral pathogens. The temperature at which maximum zone of inhibition and fungal biomass obtained were recorded.

Effect of different solvents used for extraction

Eupenicillium sp. was grown in its optimized basal media supplemented with carbon and nitrogen sources under stationary conditions. After the incubation period, the fungal biomass was extracted with four different solvents and tested for their activity against *S.mutans* and *C.albicans*. Solvents such as ethyl acetate, chloroform, hexane and methanol were used for the extraction of secondary metabolites. Equal volume of solvent was added to the culture filtrate and mixed vigorously for 10 minutes. They were kept still till two immiscible clear layers were formed. The upper layer with the extracted compounds was separated using separating funnel. The mycelium was grinded using ethyl acetate and filtered using cheese cloth. Both culture filtrate and mycelia extracts were pooled and used for antimicrobial assay. The solvent extracts which produced maximum zone of inhibition against both the oral pathogens were recorded.

RESULTS AND DISCUSSION

Standardization of basal media

Eupenicillium sp. was grown in five different media and the ethyl acetate extract of the endophyte grown in different media were used for antimicrobial studies against *S.mutans* and *C.albicans*. Secondary metabolites that were produced in malt extract broth have better activity than those produced in other media with a zone of inhibition of about 18.8 mm and 16.4 mm against *S.mutans* and *C.albicans* respectively (Fig.1). The dry weight of the mycelium was also higher in malt extract broth (35.4 mg/25ml) followed by the dry weight of about 32.5 mg/25ml in Potato Dextrose broth. The least antimicrobial activity and the biomass production were observed in yeast extract broth.

Even though PDB was used for the isolation and antimicrobial studies of *Eupenicillium* sp., further optimization proved that the strain requires malt extract for its active growth and antimicrobial metabolites production. Hence, malt extract dextrose broth was further used for the optimization of various other factors that affect the growth and secondary metabolite production of *Eupenicillium* sp. The result also suggests that malt extract and peptone are the essential components for *Eupenicillium* sp. required for the growth and active secondary metabolite production against the oral pathogens.

Effect of different carbon and nitrogen sources

Figure 2 shows the effect of different carbon and nitrogen sources on biomass and antimicrobial metabolite production. Different carbon sources such as sucrose, dextrose, glycerol and starch were used for optimization. Dextrose provided better results when compared to other carbon sources. A higher biomass (28.6 mg/25 ml) and higher zone of inhibition (20.2 mm for *S.mutans* and 18.8 mm for *C.albicans*) were obtained when dextrose was used as carbon source. Sucrose served as the second best carbon source of higher biomass (22.5 mg/25 ml) when compared to control (32.5 mg/25 ml). The results show that simple sugars such as dextrose, fructose, sucrose enhance the biomass and secondary metabolites production when compared to complex sugars such as starch, galactose etc.[17,18]. The effect of dextrose on the antimicrobial metabolite production by the endophytic *Fusarium* sp. has been studied.[19]

Similarly, nitrogen source was optimized keeping dextrose as carbon source and peptone proved to be the best source for *Eupenicillium* sp. Of all the nitrogen sources tested, peptone produced maximum biomass concentration of about 35.2 mg/25 ml. The antimicrobial activity was also higher with the zone of inhibition of about 18.4 mm and 17.2 mm for *S.mutans* and *C.albicans* respectively when peptone was used as the nitrogen source. Sodium nitrate also produced better antimicrobial activity when compared to other nitrogen sources but the biomass concentration of*Eupenicillium* sp. was lesser. Sodium nitrate promoted antimicrobial metabolite production by the endophytic fungus *Apiospora montagnei*.[20]

Effect of incubation period

The antimicrobial activity was maximum at the 14th day of incubation after which the zone of inhibition starts decreasing as shown in figure. The maximum zone of inhibition of about 18.4 mm (*S.mutans*) and 16.8 mm (*C.albicans*) was observed at the 14th day of incubation and the biomass concentration was about 35.2 mg/25 ml. As shown in figure 3, the biomass concentration increased from 12th day and remained constant up to 15

days and then starts decreasing. After 15 days, the biomass concentration and the antimicrobial secondary metabolite production have reduced.

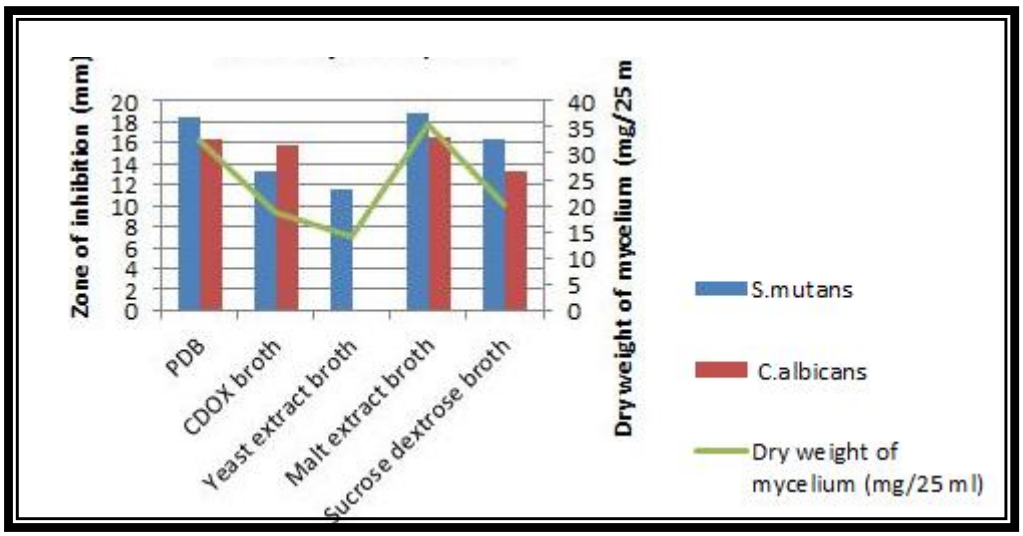

Figure1: Effect of different media on antimicrobial activity of *Eupenicillium* sp.

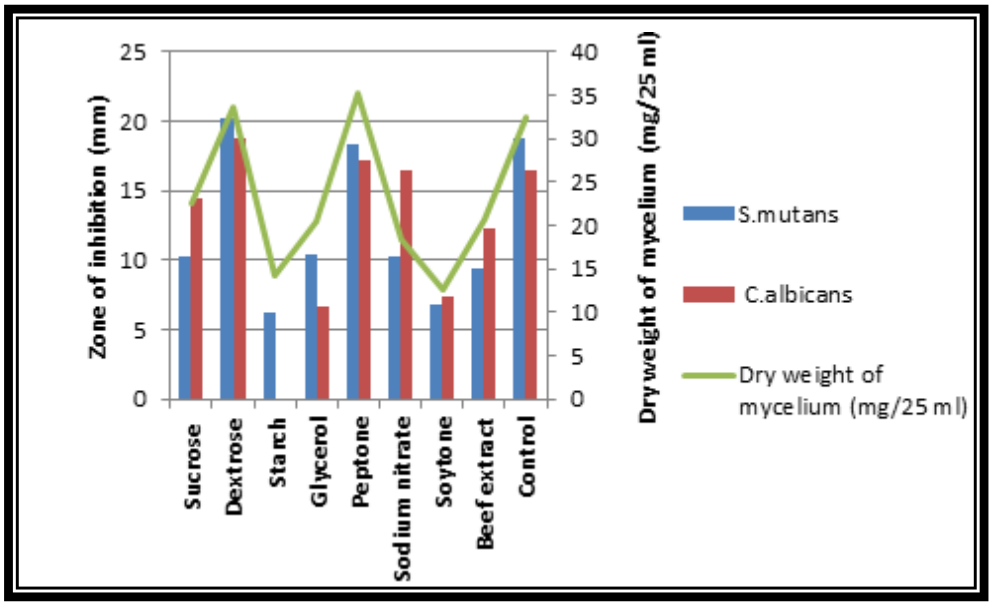

Figure 2: Effect of different carbon and nitrogen sources on antimicrobial activity of *Eupenicillium* sp.

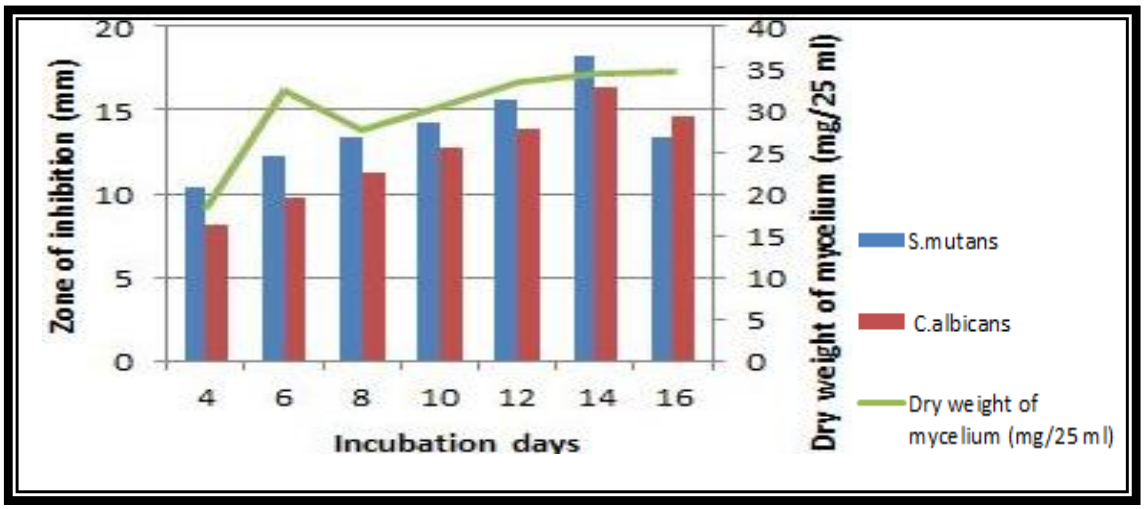

Figure 3: Effect of incubation days on antimicrobial activity of *Eupenicillium* sp.

Effect of pH

The different pH of the basal media selected did not show much difference in the antimicrobial activity against both the pathogens but at pH 5, the antimicrobial activity was higher when compared with other pH values. The zone of inhibition against *S.mutans* was 20.2 mm and against *C.albicans* it was 16.8 mm at pH 5. The biomass concentration (21.8 mg/25 ml) increases rapidly at pH 5 and at higher pH values of 6 and 7, the amount of biomass decreases. The pH of the culture medium is one of the major factors that determine the secondary metabolite production of microorganisms as it influences the concentration of ion in the medium. The effect of ion uptake or loss is related to the permeability of cell wall and membrane which is related to its pH. The biomass and antimicrobial production of *Eupenicillium* sp.at different pH (4-7) is shown (Fig.4). Antimicrobial compounds are synthesized by most of the organisms at pH ranging from 5.5 to 8.5.[21] In our study, maximum growth and antimicrobial metabolites production was obtained at pH 5 which shows the acidophilic nature of *Eupenicillium* sp.

Effect of temperature

As shown in figure 5, the optimal temperature was 35°C for enhanced antimicrobial activity of *Eupenicillium* sp. and at the higher temperature of 45°C the zone of inhibition is reduced for both the pathogens. Then biomass concentration was about 35.2 mg/25 ml at 35°C and the zone of inhibition was 21.8 mm and 19.2 mm against *S.mutans* and *C.albicans* respectively. Very less antimicrobial activity and biomass concentration was found at lesser temperature of 15°C. The figure shows the exponential growth pattern at temperature between 15°C to 45°C since at lower temperatures, the metabolic activity of the endophytic fungi is stopped. The optimum temperature of the growth of *Eupenicillium parvum*was reported to be 37°C.[22]

Effect of different solvents used for extraction

The solvents used for extraction played a major role in optimization. The ethyl acetate extract of *Eupenicillium* sp. produced maximum activity against *S.mutans* whereas the non-polar chloroform extract inhibited *C.albicans* growth at higher rate when compared to other solvent extracts (Fig.6). This shows that more polar compounds were produced in ethyl acetate extract which are active against caries causing *S.mutans*. The ethyl acetate extract produced a zone of inhibition of about 21.2 mm against *S.mutans* and 14.8 mm zone of inhibition against *C.albicans*. The antimicrobial activity against *C.albicans* (18.2 mm) was higher in chloroform extract than in ethyl acetate extract. Thus, chloroform extract produces maximum antimicrobial metabolite against *C.albicans*. The ethyl acetate extract of various endophytic fungi isolated from *Artemesia Annua* have shown activity against various bacterial pathogens including *S.mutans*.[23] Pranay Jain and Tarun Kumar have shown that ethyl acetate extract of endophytic fungi from *Azadirachta Indica* inhibit the growth of *S.mutans*.[24] The chloroform extracts of Datura leaf has inhibitory activity against *S.mutans* and *C.albicans.*[25]

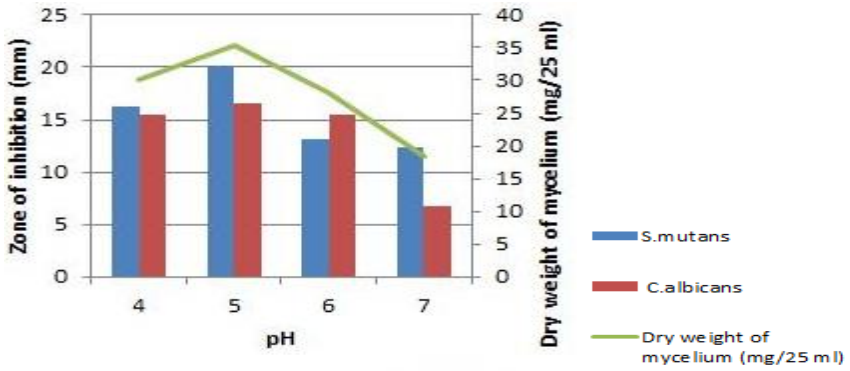

Figure 4: Effect of different pH on antimicrobial activity of *Eupenicillium* sp.

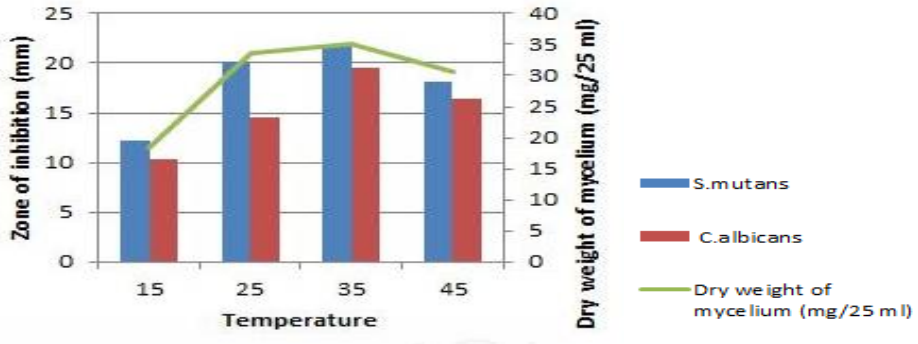

Figure 5: Effect of temperature on antimicrobial activity of *Eupenicillium* sp.

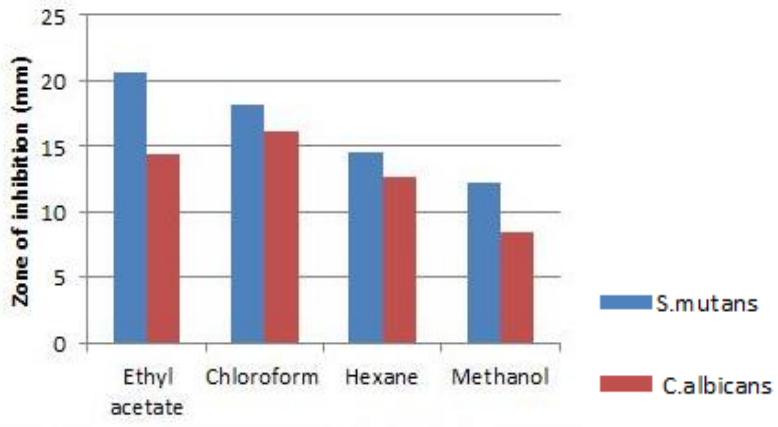

Figure 6: Effect of different solvents on antimicrobial activity of *Eupenicillium* sp.

CONCLUSION

Endophytic fungi are still relatively little explored group of microorganisms and they form plethora of secondary metabolites which have pharmaceutical uses.

The present study explored the optimization of culture conditions of the endophyte *Eupenicillium* sp.under stationary conditions for its use as antimicrobials against oral pathogens.The medium enriched with malt extract, dextrose and peptone at pH 5.0 and at temperature 35°C with an incubation period of 14 days was found to be optimum for the enhanced biomass and antimicrobial activity. These findings thus suggest suitable criteria for active secondary metabolite production from *Eupenicillium* sp.against *S.mutans* and *C.albicans*.

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