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Effect of crude Fatty acid extract of *Streptomyces sps* on biofilm forming *Candida albicans* MTCC 3017

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Abstract

The effect of crude fatty acid extract of Streptomyces sps isolated from soil on the biofilm formation by Candida albicans MTCC 3017 was investigated. Totally, 25 Streptomyces sps were isolated and identified from the soil samples collected at Nilgiris hills. All the isolates were subjected to hydrogen peroxide assay to identify fatty acid production. Crude fatty acid extracts of all the positive isolates were analyzed for inhibition of biofilm formed by Candida albicans. The extracts of five isolates AP1, AP8, AP9, AP11 and AP23 showed inhibition percentage of 80.56%, 93.25%, 79.53%, 85.39% and 73.29% at 50 µg/ml. Furthermore the β-galactosidase activity of extracts indicated capability of inhibiting the production of enzymes and reducing the hyphal growth of C. albicans. This study suggests that the crude fatty acid extracts of Streptomyces sps may be useful in preventing biofilm formation by the pathogen.

Keywords: *Candida albicans* MTCC 3017, *Streptomyces sps*, fatty acid, biofilm inhibition.

Introduction

Candida albicans, is the most common human pathogen and a member of the indigenous human micro flora. In healthy individuals, *C. albicans* causes superficial mycoses of the skin, nails, and mucous membranes (thrush and vulvovaginitis). Individuals with immune deficiencies caused by chemotherapy treatment, or immunosuppression following transplantation are vulnerable to severe, life-threatening invasive candidiasis. Being the fourth leading cause of nosocomial infections, it is the most common fungal species causing bloodstream infections, with associated mortality rates of 38 to 49%[1-4]. The biofilms of *C. albicans* are defined as microbial communities, encased within a matrix of extracellular polymers and associate with a substrate[5]. The presence of a complex three-dimensional structure with extensive spatial heterogeneity, consisting of a dense network of yeasts, hyphae, and pseudohyphae encased within a matrix of exopolymeric material provide an excellent virulence capability for the pathogen. Biofilm formation occurs in three distinct stages: (i) attachment of yeast cells to a surface accompanied with colonization, (ii) germ tube formation and multiplication of yeast and filamentous cells, which allows the basal layer of cells to anchor with the surface, and (iii) growth of pseudohyphae, hyphae and secretion of a carbohydrate- and protein-rich extracellular matrix[6-8]. *C. albicans* biofilms have important clinical consequences. They are involved in mucosal candidiasis (thrush, vulvovaginitis), form at the surface of implanted medical instruments, such as prostheses, stents, shunts, implants, and different types of catheters, and can cause their failure, have increased antifungal drug resistance, resist host immune defenses, and constitute a sustained reservoir of infecting cells[9,10].

Drugs such as polyenes, azoles, echinocandins, allyamines, and flucytosine and their derivatives as currently used for antifungal treatment against Candida infections. The fungicidal and fungistatic activity of the drugs are due to their interference in the essential metabolic process of the pathogen[11]. Due to the extensive use of these antifungal drugs; the pathogens develop drug resistance and strains with multidrug resistance are prominent[12-14]. Antifungal drugs also lead to severe side effects in patients on par with the emergence of species refractory to conventionally used agents[2]. New and more effective antifungal drugs capable of inhibiting different morphological forms of Candida such as true hyphae, pseudohyphae and budding yeast cells, are need to be developed[15].

A recent study revealed that fatty acids such as major n-3 PUFA, EPA, DHA, α-linolenic acid (ALA), and their ester derivatives have strong antimicrobial activity against oral pathogens[16]. Fatty acids belonging to n-6, n-7, and n-9 families have been reported to exhibit antimicrobial activity against various pathogens[17,18]. Fatty acids such as capric acid (10:0) and lauric acid (12:0) are known to have antibacterial and antifungal properties. Lauric acid (12:0) are known to inhibit Candida by inhibiting growth of planktonic cells and hyphal forms[19-21]. Actinomycetes, a well known microbial biomass of soil have the capacity to produce a wide variety of antibiotics, extracellular enzyme, and antibiotics[22-23]. They produce antibiotics as secondary metabolites which have a high pharmacological and commercial interest including control of infectious diseases and

many other pathogens[24]. The ability of Actinobacteria to grow in aerated fermentation vessels and the vast amount of knowledge obtained from antibiotic production is useful in formulating commercially viable and more valuable products on large scale. Despite the presence of a variety of gene clusters, these groups of bacteria are underexplored in the area of lipids, which indicates that many types of potentially valuable compounds are yet to be discovered from this Phylum that could have tremendous commercial potential in the medical sector[25]. Actinomycetes are reported for lipid biosynthesis and are relevant, not only for the generation of cell membrane phospholipids but also for the synthesis of poly ketide compounds with useful pharmaceutical properties. The objective of this study is to extend the research to lipids, in order to find an alternative source of biofilm inhibiting compound; using soil isolates of actinomycetes.

Material and Methods

Bacterial strain: *Candida albicans* MTCC 3017 was used for antibiofilm activity. YPD (1% yeast extract, 2% peptone and 2% dextrose) medium was used for culturing the pathogen. For the present study crude fatty acid extracts of *Streptomyces sps* were evaluated for their role on fungal biofilm.

Screening of PUFA producing actinomycetes: Screening of the *Streptomyces sps* isolate for fatty acid production was performed by following the method of Ashwini Tilay and Uday Annapure[26]. Briefly, Starch casein agar (SCA) medium plates, supplemented with 1 mM Sodium azide (NaN_3) (catalase inhibitor), were freshly prepared. 48 hrs culture of *Streptomyces sps* (OD = 1.0 at 600 nm) isolated and identified using conventional methods; from rhizosphere soil obtained from, The Nilgiris foot hills, Tamilnadu, was swabbed on the agar surface uniformly. Hydrogen peroxide solution was prepared at different concentrations of 0.1 %, 0.5 % and 1.0 % from a stock of 30 % solution. Whatmann no.1 filter paper was cut into small pieces of 5 mm sized disc and was placed on the agar surface. Then the filter paper discs were incorporated with different concentration of hydrogen peroxide solution and the plates were incubated at 37°C for 48 h. The plates were observed for the zone of inhibition after incubation period. Absence of inhibition zone correlates to the resistance exhibited by *Streptomyces sps* against hydrogen peroxide due to fatty acid synthesis.

Extraction of fatty acids from Actinomycetes: About 40mg of Actinomycetes cells were harvested from Starch Casein broth. The cells were placed in 10 ml screw cap culture tubes. To this 1 ml of 1 ml of Reagent A (Sodium hydroxide, Methanol, and Distilled water) was added and the tubes were sealed with Teflon lined caps.Then the tubes were vortexed and kept in boiling water bath for 5 min. Next, the tubes were vigorously vortexed for 5-10 seconds and heated again in a boiling water bath for 30 min. The tubes were cooled and 2 ml of Reagent B (Hydrochloric acid and Methyl alcohol) was added and vortexed. Then, the tubes were heated at 80°C for 10 min and 1.25 ml of Reagent C (Hexane and Methyl tert-butyl ether) was added to the cooled tubes followed by gentle tumble on a clinical rotator for 10 min. The aquesous phase was pipette out and discarded. Then 3 ml of Reagent D (Sodium hydroxide and Distilled water) was added to the organic phase and tumbled for 5 min.About two third portion of the organic phase was taken for analysis[27].

Minimum inhibitory concentration assay: Anti-biofilm activity was tested by using various concentrations of crude fatty acid extract (10, 20,30,40,50 µg/ml). 1 µl of the overnight culture of *C.albicans* (MTCC 3017) with the optical density of 0.1 at 600 nm was added to each wells of 96-well titre plate. Different concentration of the lipid extract was added to each wells of the titre plate. The 96-well plate was then incubated at 37 °C for 16 h. The incubated plate was later observed for the growth of *C. albicans*[28]. After incubation the growth was recorded spectrophotometrically at 600 nm.

Biofilm inhibition assay: Overnight culture of *C.albicans* was incubated on 24 well microtitre plate containing 1 ml of YPD (1% yeast extract, 2% peptone and 2% dextrose) with and without lipid extracts[28]. Plate was incubated without agitation at 37 °C for 18 h. After incubation, planktonic cells were discarded and the adherent cells on the slide were gently rinsed twice with deionized water and air dried. The biofilm was stained with 0.4% crystal violet solution for 5 min and then rinsed twice with deionized water. Finally it was resuspended in 1 ml absolute ethanol and the absorbance was observed at 570 nm.

Microscopic observation of biofilm: The biofilms were allowed to grow on glass pieces (1x1 cm) placed in 24-well polystyrene plates supplemented with crude fatty acid methyl esters (10, 20, 30, 40, 50 µg/ml) and incubated for 24 h at 37 ºC. The slides were stained using crystal violet and were placed on slides with biofilm pointing upwards. The slides were observed under light microscopy at magnification of ×40. Visible biofilms were documented with an attached digital camera (Kozo Optics Model: XJS900T)[28].

β- Galactosidase assay: Overnight culture of *C.albicans* were washed in sterile distilled water and serially diluted to 1×10^{-6} cells ml^{-1} in prewarmed media. 1 ml of spider medium was added to the Twenty-four-well polystyrene microplates. 50µl (50 µg/ml) of Crude fatty acids extract were added to wells. Microplates were incubated at 37°C for 4 h. Then, 150 µl of ONPG solution (4 mg/ml) were added to the tube and mixed well. The reaction was stopped by addition of 400 µl of 1.5M Na_2CO_3. The sample was centrifuged for 30 seconds at 16000 X g and the absorbance of the supernatant read at 420 nm[29].

Hyphal growth assays: Overnight culture of *C.albicans* was diluted to 1×10^{-6} cells ml^{-1} in Spider medium supplemented with Crude fatty acids extract (50 µg). Flasks were incubated with orbital shaking at 30 °C. A_{600} was measured every hour for 12 h[30].

Statistical analysis: Statistical analysis was performed using SPSS version 16 (Chicago, USA). Data are expressed as mean and standard deviation of results from duplicate biological samples.

Results and Discussion

Screening of fatty acid synthesizing *Streptomyces sps:* In H_2O_2 - plate assay (figure 1), the cells which were susceptible to externally-added H_2O_2 were not able to grow suitably and thus showed an inhibition zone, which was proportional to the added concentration of H_2O_2 on Whattman filter paper disc. Growth was observed on the agar plate without the zone of inhibition, which indicated that the actinobacterial cells produced fatty acid and were able to grow in presence exogenous H_2O_2, due to the membrane-shielding effect of fatty acids[19]. The concentration of NaN_3 was opted from report studied by Teixeira and Mota[31]. If microorganism produced catalase enzyme, NaN_3 inhibited catalase enzyme which helped to infer and promote the actual interpretation of plate assay. Out of 25 *Streptomyces sps* isolates used in this study, 20 isolates were found to be non producer of fatty acid under primary screening. In this assay, 5 isolates did not show, zone of inhibition and these were selected for further studies[32] (table 1) (figure1).

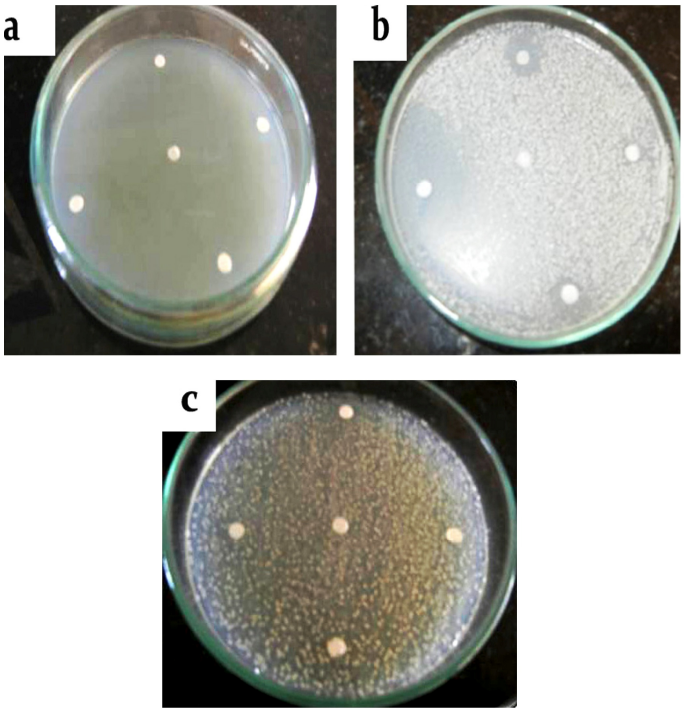

Figure-1
Screening of PUFA producing Streptomyces sps using Hydrogen peroxide assay. 1(a) Control, 1(b) PUFA non producer – the isolate AP5 was inhibited by H_2O_2 on all concentrations, 1(c) PUFA producer – the AP8 isolate produced PUFA and so it sustained the presence of hydrogen peroxide

Table-1
Hydrogen peroxide plate assay for determination of PUFA synthesizing actinomycetes

Strain	H_2O_2[†] Concentration (%)			PUFA[*] +ve/-ve
	0.1	0.5	1	
AP1	**+**	**++**	**+++**	**+ ve**
AP2 to AP7	**-**	**-**	**-**	**- ve**
AP8 to AP9	**+**	**++**	**+++**	**+ ve**
AP10	**-**	**-**	**-**	**- ve**
AP11	**+**	**++**	**+++**	**+ ve**
AP12to AP22	**-**	**-**	**-**	**- ve**
AP23	**+**	**+**	**+**	**+ ve**
AP24 to 25	**-**	**-**	**-**	**- ve**

[†]describes zone of inhibition due to presence H_2O_2; +/++ describes growth of microorganisms or no zone of inhibition due to presence of PUFA. [*]PUFA +ve denotes PUFA producer and –ve denotes PUFA non-producer.

Extraction of total lipid and esterification: Total lipid content was extracted from the selected isolates AP1, AP8, AP9, AP11, and AP23 using the protocol followed by Sherlock Microbial Identification System[27] and their dry weight were found to be 40, 48, 42, 37 and 34 µg/ml respectively. According to the EPA- and DHA-expressing bacteria were reported to be more resistant to exogenous H_2O_2[31]. The membrane-shielding effects of *n*-3 LC-PUFAs have been shown only for bacterial cells producing EPA[33, 34]. An experiment study conducted by Ashwini Tilay and Uday Annapure[26] revealed that the long chain fatty acids of marine bacteria also responsible for the protecting effect against exogenous H_2O_2 which was also well supported with the experiment carried out by Okuyama *et al.,*[19]. These results support our hypothesis that fatty acid synthesis by actinobacteria might also be responsible for protection against exogenous H_2O_2.

Minimum inhibitory concentration assay: There was a gradual decline in the turbidity of isolates treated with increasing concentration of crude fatty acid extract. The entire five crude fatty acid lipids extract of AP1, AP8, AP9, AP11 and AP23 isolates inhibited the pathogen at a minimal concentration of 10 µg/ml. Synthetic compounds such as fluoroquinolone failed to inhibit clinical isolates of *Streptococcus pyogenes* at a minimal concentration which proved that biofilms are developing resistance strategies against the metabolic drug[35]. The effectiveness of the fatty acid against the biofilm forming *S. pyogenes* has not been assessed previously. Long chain fatty acids from marine sources have been extensively studied for their antibiofilm property against *Candida albicans* and *Candida dubliniensis*[36]. The present study elucidated that natural crude fatty acid extract from *Streptomyces sps* could inhibit the biofilm forming *C.albicans* at a much lower concentration thus enabling us to confer its effectiveness.

Biofilm inhibition assay: The crude fatty acid extracts of AP1, AP8, AP9, AP11 and AP23 inhibited the biofilm formation of

the *C.albicans* MTCC 3017. The concentration of the fatty acid extracts used to assess the biofilm inhibition ranged from 10 µg - 50 µg /ml. From figure-3, it was observed that all the concentrations of crude fatty acid extracts of the isolates showed good biofilm inhibition. AP1, AP8, AP9, AP11 and AP23 extracts showed the highest percentage inhibition of 80.5%, 93.25%, 79.53%, 85.39% and 73.29% at 50µg/ml and hence it was observed to be the biofilm inhibitory (BIC) concentration.

Microscopic observation of biofilm assay: Analysis of growth kinetics and the architecture of biofilms are important to understand and interpret their behaviour. Therefore evaluation of crude fatty acid against *C.albicans* MTCC 3017 biofilm was performed using conventional and novel quantification techniques. The crude lipid extracts (AP1, AP8, AP9, AP11, and AP23) showed effective antibiofilm activity against the *C.albicans* MTCC 3017 biofilm formation even at minimal concentration of 10 µg/ml. figure-2 depicts the efficient disintegration of *C.albicans* MTCC 3017 biofilm by compounds even at a minimal concentration which is visualized using light microscopy, as the concentration of the fatty acid extract increased the biofilm formation decreased. The effect of crude lipid extracts of *Streptomycetes sps* on biofilm formation was observed (figure-2). The decrease in the biofilm formation and the reduction in their surface area after the treatment of crude lipid extract, were assessed by comparing the control biofilm formed on the solid matrix.

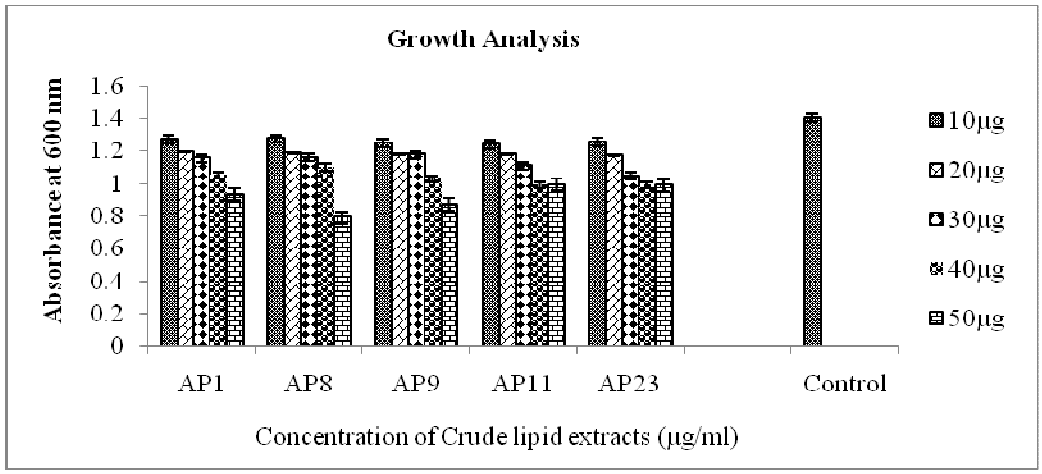

Figure-1

Represents the gradual inhibition of *C.albicans* MTCC 3017 exposed to the varying concentrations of the crude lipid extracts after 16 h incubation. The MIC of the AP1, AP8, AP9, AP11 and AP23 crude lipid extract against *C.albicans* MTCC 3017 was found to be in range of 10 to 50 µg/ml concentrations, above which complete inhibition was observed

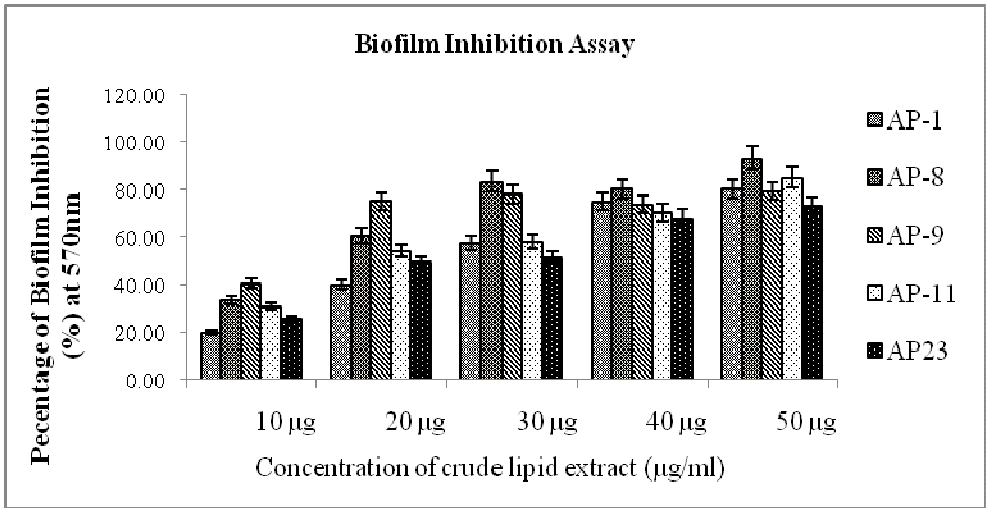

Figure-3

Representation of the percentage inhibition of the lipid extracts of actinobacterial isolates AP1, AP8, AP9, AP11, and AP23 against the biofilm forming *C.albicans* MTCC 3017

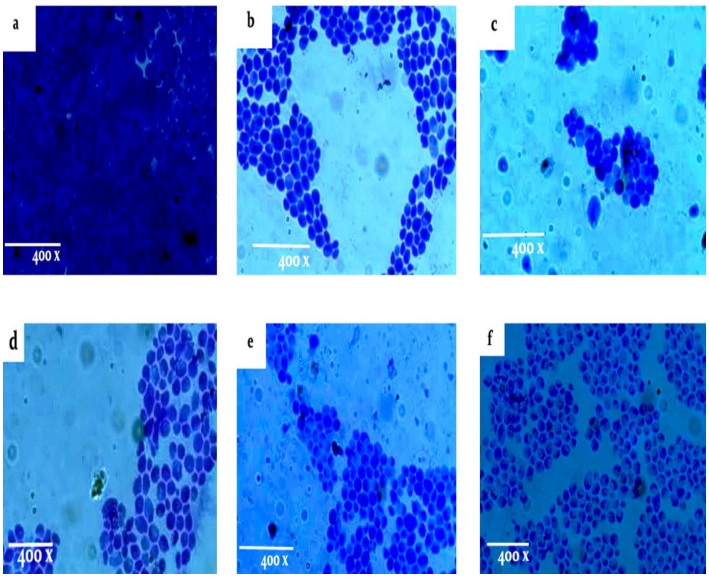

Figure-4
Light Microscopic observation (x40) of *C.albicans* MTCC 3017 adhesion phases on the glass surfaces at (50 µg/ml) of (a) Control, (b) AP1, (c) AP8,(d) AP9, (e) AP11, and (f)AP 23, crude *Streptomyces sps* lipid extracts against *Candida albicans* MTCC 3017

β- Galactosidase assay: Crude lipid extracts of *Streptomyces sps* isolates inhibited the hyphal growth in *C. albicans* grown in hyphal-inducing conditions. Several fatty acids, including conjugated linoleic acid were recently shown to inhibit *Candida albicans* germ tube formation in various hypha-inducing liquid media[30]. This assay was performed by subjecting the *C. albicans* against different concentration of crude lipid extract in which the production of β- Galactosidase is correlated with the percentage of hyphal growth in the culture media. Among the concentrations of lipid extract used, 50 µg/ml aliquot showed the highest reduction in the β- Galactosidase enzyme activity (figure-3). Fatty acids, such as oleic, linoleic, α- and γ-linolenic acids have been reported to modulate the hyphal growth in *C. albicans*. The β-galactosidase enzyme activity, cellular and colony morphology after exposure with fatty acids, indicated that all fatty acids interfered with the *C. albicans* hyphal growth at different extent[36].

Hyphal growth assays: The yeast-to-hypha transition was induced in cells inoculated in Spider medium at 37°C. Spider media contained the hypha-inducing substance which acted as a source of fermentable carbon, various amino acids, and salts which may explain the hyphal growth. However, by incorporating the crude fatty acid extract of AP1, AP8, AP9, AP11 and AP23 at 50 µg/ml concentration, showed hyphal inhibition (figure-4). The hyphal inhibition decreased with the increase in incubation time and was high after 12 h of incubation. According to the experiment conducted by Toenjes *et al.,*[37] the filaments of *C. albicans* was inhibited by cytotoxic or cytostatic molecules. Experimental growth conditions, at 37°C and conjugate linoleic acid treatment, are less favorable than control growth conditions and also resulted in a metabolic decrease[38].

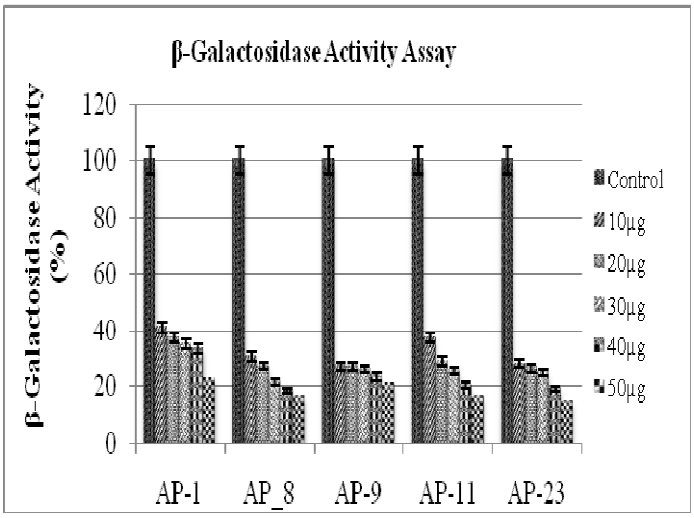

Figure-5
β- Galactosidase assay *C. albicans* MTCC3017 treated with varying concentration of crude fatty acid extracts of *Streptomyces sps* isolates

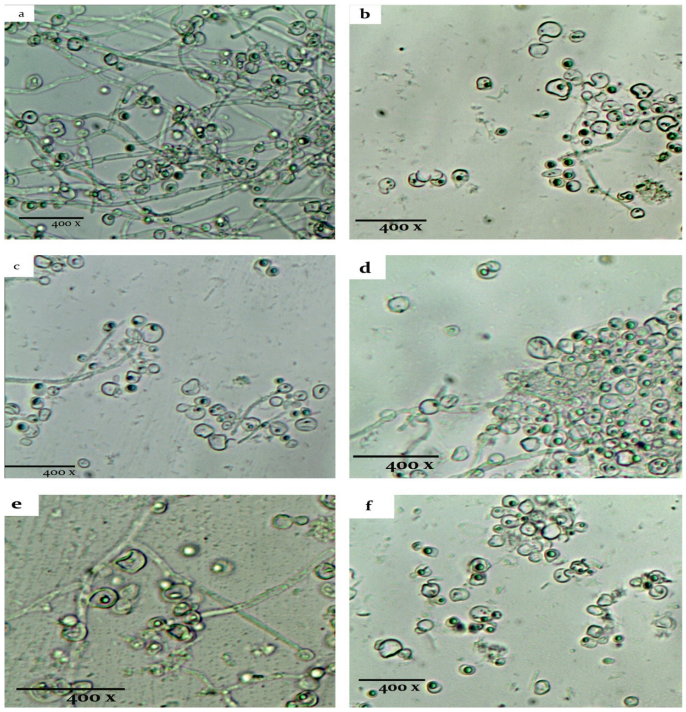

Figure-3
Hyphal growth assay showing the inhibition of yeast to hyphal transition by the crude lipid extracts of different *Streptomyces sps.* (a) Control, (b) AP1 treated, (c) AP8 treated, (d) AP9 treated, (e) AP11 treated, (f) AP23 treated.

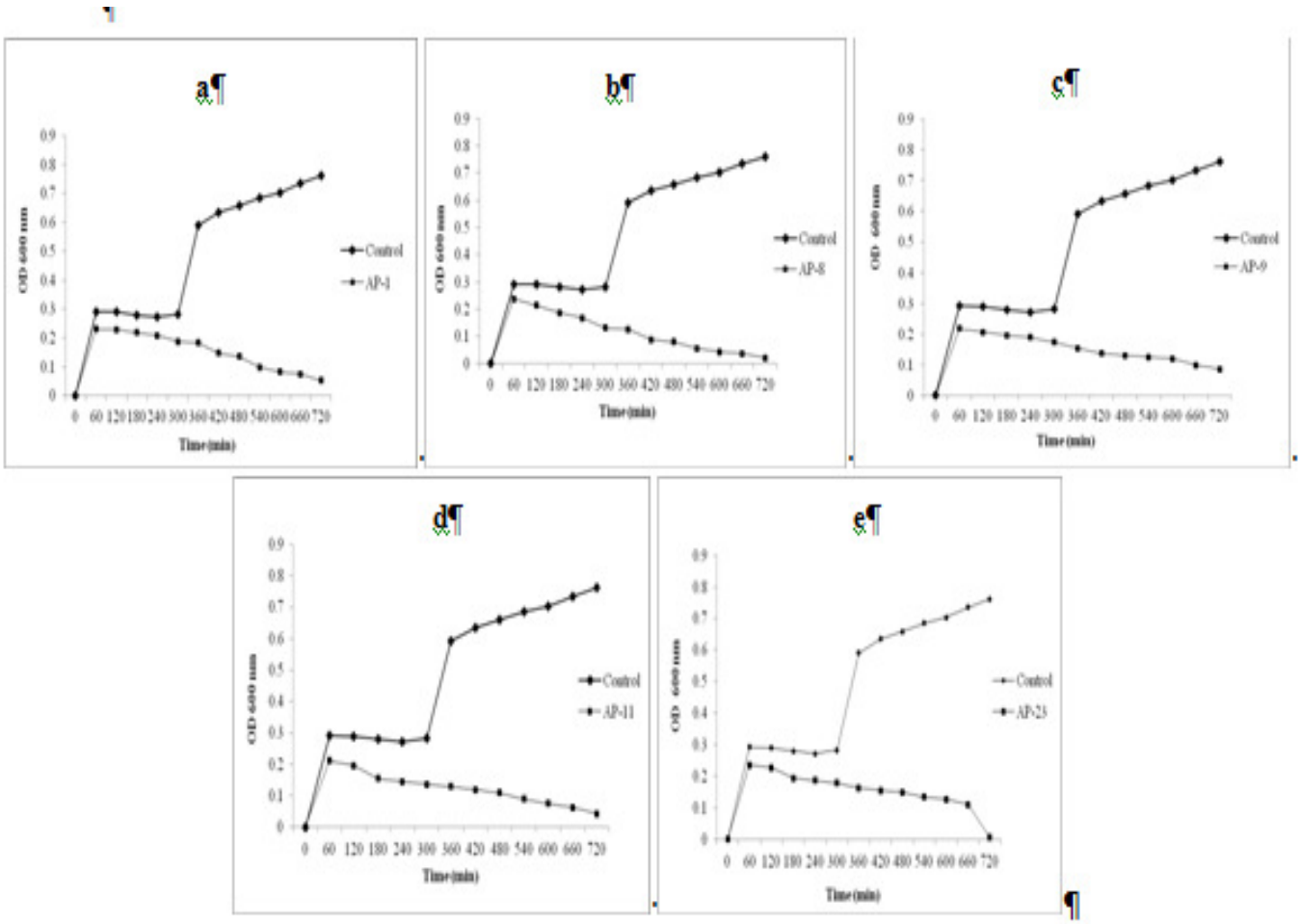

Figure-7
Representation of hyphal growth inhibition assay in liquid media by crude fatty acid extracts of a) AP1,b) AP8, c) AP9, d) AP11, and e) AP23

Conclusion

The results emphasize the role of *Streptomyces sps* crude fatty acid extract, on inhibiting the biofilm formation of *C.albicans* MTCC 3017. Among the 25 isolates, the crude lipid extract of the *Streptomyces sps* isolates AP1, AP8, AP9, AP11 and AP23 isolated from the rhizosphere soil of Nilgiris foot hills, Tamilnadu have significant characteristics to inhibit the biofilm formation. Light Microscopic images showed the disruption and inhibition of biofilm at a concentration of 50 µg/ml. All the five extracts inhibited the biofilm at a minimal inhibitory concentration of 10 µg/ml. The result of the β Galactosidase Activity of extracts indicated capability of inhibiting the production of enzymes. The hyphal growth assays also showed the inhibition of yeast to hyphal transition after 12 h of incubation. In conclusion this study reveals the preliminary result on the anti biofilm activity of actinomycete isolates against *C. albicans* biofilm. Further studies on the crude fatty acid extract using GC/MS, FTIR, and NMR would lead to better understanding on the mechanism of biofilm inhibition.

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