FUNGAL-FUNGAL INTERACTION FOR SCREENING OF ANTIMICROBIAL AGENTS AGAINST HUMAN PATHOGENS

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Abstract

Microorganisms are very rich sources of chemical compounds. Amongst them Fungi have proven their resourcefulness as producers of various novel metabolites which can be used as lead drug molecules. In nature, they are known to grow together and interact. Cocultivation is one such method emulated from nature where two organisms from different species or even different kingdoms can be grown together in the same environment to obtain novel bioactive compound or boost the action of existing molecules showing antimicrobial activity. The production of these compounds is credited to the biotic stress induced due to competition for nutrients, space and other resources which subsequently leads to the activation of previously silent metabolic pathways that normally would have been suppressed under normal conditions. Fungal strains were isolated from city effluent and forest soil samples. Cross-Streak method was used to identify the symbiotic combinations among the fungi and then, the interactions between those Fungi were studied using Mixed Fermentation. The cell free extract of the fermentation broth was analysed for its antimicrobial activity by using agar well diffusion against different bacterial test strains. For further study the compound from positive combination shall be isolated, extracted and analyzed for its properties using HPLC analysis.

Index Terms: Fungal-Fungal interactions, Antimicrobial agents, Bioactive compounds, Co-cultivation.

I. INTRODUCTION

Fungi are rich and prolific sources of chemical compounds that can be used to the benefit of Mankind, be it secondary metabolites like Antibiotics or other compounds like Enzymes and Acids [1]. These compounds are a product of their monoculture, which they produce at some stage of their life cycle. But, considering the possibility of variety that can be obtained if they were to grow together. This very thought brought into existence a method called cocultivation. Cocultivation is a creative method of growing two organisms either of same species or different species in the same media and making them compete for resources and space which as a stress can lead to production of new metabolites. These molecules might have not been produced previously at all or could have been produced in very low concentrations. This production of new compounds can be a result of defense mechanism or inter-species talk and can be acknowledged to the expression of cryptic biosynthetic pathways previously observed to remain silent in their monocultures [1].

A. Types of interactions observed between microbes:

Cocultivation is possible because of various interaction mechanisms that are observed in nature between microbes:

1) Co-operation or Mutualism
2) Competition or Antagonism
3) Symbiosis or Commensalism
4) Synergism
5) Neutralism [1, 2]

B. Fungal-Fungal Interactions:

Among the microbial communities, Fungi have distinctive ways of communication which in turn can result in the subsequent action of antimicrobial agent production. The interactions mostly observed between two fungal mycelia can be mutualistic, neutralistic or competitive and can be contact based or can be mediated by chemical molecules
[2].

The underlying molecular mechanisms pertaining to these known interactions still remain obscure. Still with what has been researched Fungi have been found to interact with other Fungi as well as bacteria via the above two mentioned methods

1. Direct contact (cellular contact)
2. Mediated via molecules

Direct contact is when there is cell to cell contact between interacting organisms, surface proteins and surface saccharides the key elements participating. These are lectin like non-enzymatic proteins, which often act as recognition molecules in mixed-species interaction [3].

Interactions mediated via molecules. At times microbes do not associate via direct surface contact, instead choose the alternative route of communication i.e. via excreted molecules like enzymes, metabolites like acids, saccharides, quorum sensing molecules (alcohols), toxins or other type of messengers like peptides, lipids, volatile gases and small molecules like acetaldehyde [3,4,5].

II. MATERIALS AND METHODS

A. Sources of soil sample:

Samples of soil from forest and city effluent were collected for isolating fungi. Forest sample was collected from Taljai forest, Pune and soil from city effluent was collected from Vadgaon canal, Pune.

B. Isolation of Fungi:

Isolation of fungi from the soil samples collected from two different sites was done using cumulative dilution followed by spreading it on the media plates. Initially, the samples were diluted with ratio of 1:10 in saline solution (0.9% NaCl). The 0.1 ml samples from tubes having dilution coefficient of 7, 8, 9, 10, 11 were spread on the YPD agar plates (1% Yeast extract powder, 2% Peptone, 2% Dextrose, 1.5% agar, pH 6.2$\pm$0.2; HiMedia Chemicals). These agar plates were incubated for 5 days at room temperature. Distinct colonies of fungi were picked and sub cultured. In all 09 fungal strains were isolated. These isolated fungi were stored using YPD slants at 4^0 C.

C. Screening of the Combinations-cross streak method:

These fungi were grown in the following combinations by using cross streak method for checking their symbiotic nature. The table of combinations is provided below. YPD agar plates were used for cross streak method. In this method, one microorganism is streaked horizontally in the center and the other microorganism is streaked perpendicular to the previous microorganism from the edges of the plate. [6]

Table I: Combinations for cross-streak methods

F1	F2	F3	F4	F5	F6	C1	C2	C3
F2	F3	F4	F5	F6	C1	C2	C3	
F3	F4	F5	F6	C1	C2	C3		
F4	F5	F6	C1	C2	C3			
F5	F6	C1	C2	C3				
F6	C1	C2	C3					
C1	C2	C3						
C2	C3							

D. Mixed Fermentation:

The co-culture combinations found positive for their symbiotic nature were selected fermented together. Mixed fermentation was also used as a confirmatory test to prove their ability to survive and grow together. 150ml of YPD broth was used for the fermentation purpose. Totally three sets of cultures of each combination were maintained as follows:

1) One flask inoculated with 5 ml of 48 hours old culture of 1st strain. (monoculture)
2) One flask inoculated with 5 ml of 48 hours old culture of 2nd strain. (monoculture)

3) One flask inoculated with 5 ml of 48 hours old cultures of 1st and 2nd strains together. (co-culture)

These 3 flasks, the 2 monoculture flasks and the one having mixed culture (or co-culture) were incubated at room temperature for 15 days and spent media was collected by filtering it. The spent media was centrifuged and was analyzed for antimicrobial activity against different test strains of bacteria by agar well diffusion method.

Selected combinations that were co-cultivated in liquid media:

Table II: Combinations for co-cultivations in liquid media

Monocultures		Co-cultures
F1	F3	F1-F3
F1	C3	F1-C3
F2	F3	F2-F3
F2	C1	F2-C1
F2	C2	F2-C2
F2	C3	F2-C3
F3	C2	F3-C2
F4	C2	F4-C2
F4	C3	F4-C3
F5	C3	F5-C3
F6	C2	F6-C2
C2	C3	C2-C3

E. Screening for antimicrobial activity:

The antimicrobial activity of freshly fermented media was checked using agar well diffusion method. The fermented media was filtered and the cell free extract was collected and centrifuged at 10,000 rpm for 15 min for precipitating any debris if remaining even after filtration. This cell-free supernatant obtained was then analyzed for its antimicrobial activity by agar well diffusion technique. For agar well diffusion, initially the Luria Bertini Agar plates (2% LB powder, 2% agar; HiMedia chemicals) were streaked with 24 hours old cultures of indicator strains. The wells for pouring the supernatant were bored using the cork borer. Each plate contained three wells, two for monocultures of fungi and one for the combination of both fungi co-cultivated together. The indicator strains used were Staphylococcus aureus (Gram positive), Klebsiella pneumoniae (Gram negative) and Pseudomonas aeruginosa (Gram negative).

III. RESULTS

A. Isolation:

Total of 09 strains of distinct fungi were isolated. Out off, those 09, 06 of them were isolated from forest sample (Taljai forest, Pune) and 03 from city effluent sample (Vadgaon canal, Pune). For convenience, these isolates were named as:

Forest sample isolates: F1, F2, F3, F4, F5, F6.

City effluent sample isolates: C1, C2, C3.

B. Screening of combinations- cross streak method:

The combinations obtained that can grow together are:

Table III: Positive cross-streak combinations

F1-F3	F6-C2	F2-F3
F1-C3	C2-C3	F2-C1
F4-C2	F3-C2	F2-C2
F4-C3	F5-C3	F2-C3

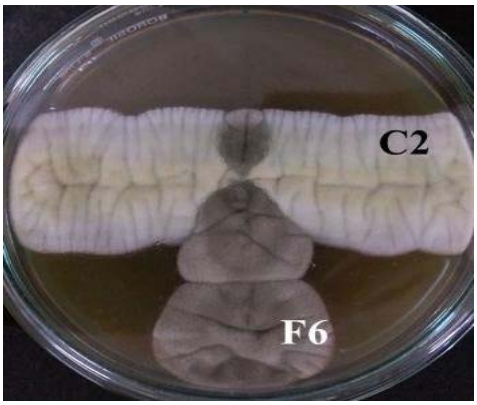

Fig. 1: Cross-streak for F6-C2

These combinations obtained were then co-cultivated in liquid media to obtain their supernatant which was then used to check their antimicrobial activity.

C. Screening for antimicrobial activity:

The antimicrobial activity of the product obtained was assayed using the agar well diffusion method. The combinations that were showing zone of inhibition (antimicrobial activity) for indicator strains are

Table IV: Strains and combinations giving zone of inhibition after agar well diffusion

Staphylococcus aureus		
F1 +	F3 +	F1-F3 +
F2 -	F3 +	F2-F3 +
F2 -	C1 -	F2-C1 +
F2 -	C3 +	F2-C3 +
F3 +	C2 +	F3-C2 +
F4 -	C2 +	F4-C2 +
F4 -	C3 +	F4-C3 +
F5 -	C3 +	F5-C3 +
F6 +	C2 +	F6-C2 +
K lebsiella pneumoniae		
F4 -	C2 +	F4-C2 +
F4 -	C3 +	F4-C3 +
F5 -	C3 +	F5-C3 +
F6 +	C2 +	F6-C2 +
Ps eudomonas aeruginosa		
F2 -	F3 +	F2-F3 +
F6 +	C2 +	F6-C2 +

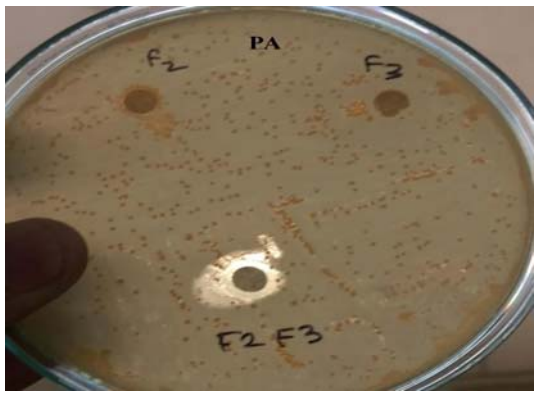

Fig. 2: Disk diffusion for *P. aeruginosa* for F2-F3

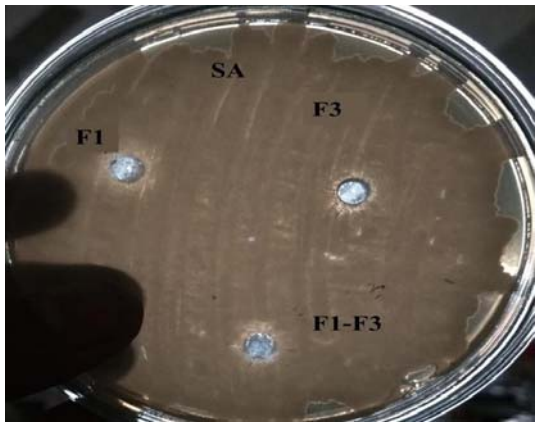

Fig. 3: Agar well diffusion for *S. aureus* for F1-F3

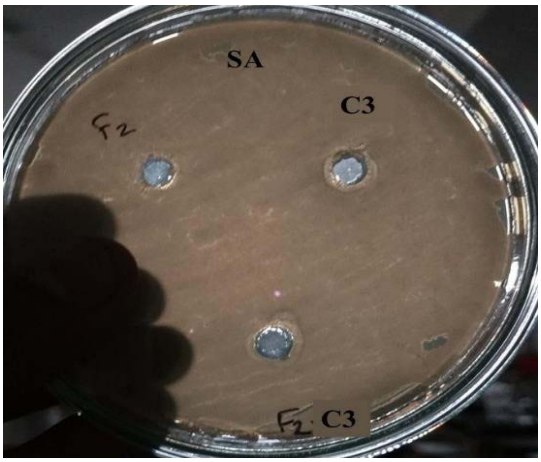

Figure 4: Agar well diffusion for *S. aureus* for F2-C3

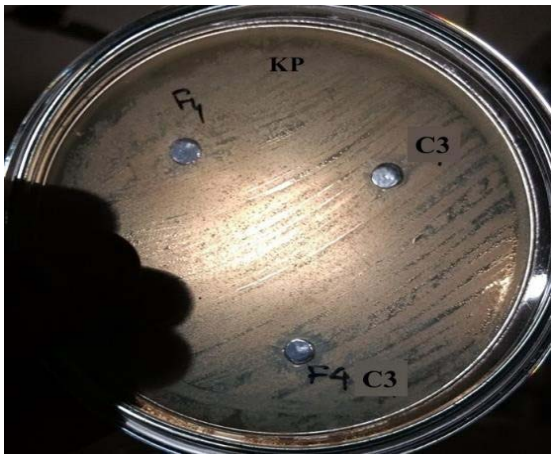

Figure 5: Agar well diffusion for *K. pneumoniae* for F4-C3

Figure 6: Agar well diffusion for *K. pneumoniae* for F5-C3

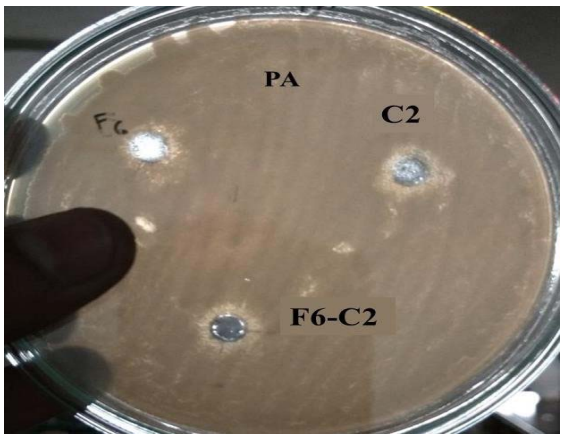

Figure 6: Agar well diffusion for *P. aeruginosa* for F6-C2Units 1)

IV. DISCUSSION

Using agar well diffusion the combinations showing antimicrobial activity were identified. These show potentials for being used as future antimicrobial agents. The HPLC analysis for promising combinations will be carried out following extraction. Characterization of the Fungi showing antimicrobial activity in combination will be done. Further tests shall be performed to check the novelty of compound.

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