ISOLATION OF BIOACTIVE METABOLITES PRODUCING FUNGAL STRAINS FROM MILK PRODUCTS AND MILK INDUSTRIES OF RAIPUR DISTRICT

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ABSTRACT

For the discovery of new therapeutic candidates, natural products continue to play an important role. Over the past 3 decades, natural products or their derivatives have been reported to produce about 60% of new anticancer agents and almost 75% of all new antimicrobial molecules which account for antibacterial, antifungal, antiprotozoal and antiviral molecules. More than a hundred natural products and natural product-derived substances were being evaluated in clinical trials or were being registered at the end of 2015. Bioactive or secondary metabolites have been isolated from a variety of terrestrial and marine organisms, including plants, marine invertebrates and microorganisms. Microorganisms (traditionally bacteria and filamentous fungi, but the recent advances include cyanobacteria, microalgae and myxobacteria) are one of the most prevalent sources for the production of bioactive molecules. Exploitation of their specialized (or secondary) metabolism has guaranteed for decades the discovery of novel antibiotics and other compounds with unprecedented chemical characteristics and biological properties not existing in screening. The current study is based on isolation and exploitation of the secondary metabolism of such fungal strains that prove potential source of such bioactive molecules and also for pharmaceutical drug discovery programs.

KEYWORDS: Bioactive molecules, fungi, drugs, secondary metabolites.
INTRODUCTION
Traditional microorganisms such as bacteria, cyanobacteria, certain algae and innumerable fungal species are known to produce certain compounds popularly known as secondary metabolites that contain some degree of bioactivity.\cite{1} These compounds found profound use as antibiotics against a wide variety of infectious diseases such as Diabetes, HIV-1, Hepatitis A, B, etc. Drugs such as cephalosporin, penicillin, vancomycin and streptomycin, etc are extremely effective against multiple bacterial infections.\cite{2} Some other drugs such as dactinomycin, bleomycin, etc are useful against carcinomas. These also can act as immune suppressant and can lower the risks of coronary heart diseases. Since the 1920s when the first antibiotic Penicillin was discovered, it was believed that soil microorganisms are the largest source of novel drugs. It is their biological and chemical diversity, coupled with the underlying competence to produce novel secondary metabolites with antimicrobial and nutritive effects that has led to the widespread use of microorganisms in the economic, industrial scale production of drugs.\cite{3,4}

Fungi are widely recognized to produce such secondary metabolites that include many life saving compounds and also highly toxic mycotoxins. For example, the fungus Curvularia sp. FH01 was found to be an efficient producer of phytotoxic and antifungal compounds.\cite{6} Daldinia eschscholzii, isolated from the gut of the mantis species Tenodera aridifolia, was reported to simultaneously generate four novel skeletons and seven structurally unique metabolites that have the potential to become immune inhibitors.\cite{7,8} These bioactive molecules or microbial products continue to represent till today as the most interesting sources for the discovery of novel drugs. Researches made in this field are being benefitted from discoveries made in several other related fields viz. metabolomics, microbial ecology, or synthetic biology. These are the fields that have provided a thorough understanding of the microbiome or microbial genome and leading to the development of faster tools facilitating the discovery of novel druggable compounds. Gene clusters of microorganisms that encode for novel biosynthetic pathway producing bioactive products are being unveiled through the ever increasing knowledge of newly sequenced microbial genomes.

Milk of all mammalian species contains a heterogeneous mixture of lacteal secretion which contains numerous components which exhibit a wide variety of chemical and functional activities. Milk and colostrum of bovine and other dairy species are considered as the most important source of natural bioactive components. Over the past a few decades, major
advances and developments have been achieved on the science, technology and commercial applications of bioactive components which are present naturally in the milk. Also recent studies have revealed that casein phosphatides drastically increase the production of extracellular proteins in some species of Aspergillus.[5]

MATERIALS AND METHODS

Sample Collection, Storage and Transportation: Soil samples were collected from five milk industries of Raipur district during pre monsoon season of 2016. Samples were collected in sterile polythene bags, sealed and were brought to the laboratory for testing.

Sample collection from milk products: Homemade cheese (“paneer”) and condensed milk (“khova”) were allowed to stand at room temperature for about 3-10 days. Fungal growth were observed in both milk products and colonies were inoculated through point inoculation in Potato Dextrose Agar (PDA) with 50 gm Amoxicillin powder (Lupin, India) to eliminate bacterial contamination.

Sample inoculation and fungal growth: 1 gm soil was dissolved in 10 ml autoclaved, sterile distilled water, shaken gently, allowed standing for 2-3 minutes and serially diluted for 4 dilutions. For fungal growth observation 1 ml supernettant was inoculated in Potato Dextrose Agar (PDA) media. The media was supplemented with 50 gm Amoxicillin (Lupin, India) powder to put bacterial contamination under control.

The plates were then sealed with sterile parafilm and incubated for 5-7 days at 27°C. After 7 days the plates were observed for fungal growth. The identification of fungal strains was done according to the cultural and microscopic characteristics such as shape, size, color, arrangement and pattern of mycelium, shape and size of conidiophores, etc. All identifications were done consulting relevant literature.[9,10] The pure cultures of isolated strains were maintained in PDA slant with Amoxicillin at 4°C.

Study of influence of milk products on fungal growth: Fungal colony plugs were cut from PDA plates using a cork borer of 4mm diameter and were inoculated into a combination of 50 ml Potato Dextrose Broth and 25 ml milk and into a combination of 50 ml Potato Dextrose Broth and 25 ml whey and were inoculated at 27°C for 5 days in a BOD incubator in static condition. After 5 days of mycelial growth the flasks were placed in a rotary shaker for 48 hrs
at 180 rpm. The growth of fungal colonies were observed after 7 days and then screened for secondary metabolite production.

**Calculation of wet weight and dry weight of fungal biomass:** After 7 days the flasks were filtered and filtrate and biomass were separated. The weight of fungal biomass was determined for all the colonies. It was then placed in watch glass and then dried in hot air oven overnight at 60°C. The weight was again determined after drying.

**Extraction of secondary metabolites from fungal cultures:** After filtration the supernatant were treated with Tween 80 for spore destruction and then filtered again. The resultant liquid was then treated with Ethyl acetate, Chloroform and Methanol in the ratio 3:2:1 and then concentrated till it remained about half of its original volume. The crude extracts were then collected stored at 4°C for further analysis.

**Screening for production of secondary metabolites:** The filtrates were then placed in a waterbath at 60°C for about 8-10 hours to concentrate and extract about half the volume of filtrates. 5 ml of each filtrate was then pipetted in test tubes. 5ml of chloroform was then poured into the filtrates and 3 ml of conc. H$_2$SO$_4$ was poured from the sides of test tubes. A brown ring formation at the conjunction of both the layer of liquids prove the presence of terpenoids in the filtrate.\[^{[11]}\]

**RESULTS**

The soil samples were collected from milk industries so that the fungal strains isolated from soil have the ability to grow in accordance with *Lactobacillus* sps. All the isolated strains of fungi were then inoculated in media supplemented with milk and whey. Also the fungal strains isolated from cheese and khova were inoculated in the same.

![Fig. 1: Cheese culture from which fungal colonies were isolated.](image1)

![Fig. 2: Plate showing fungal colony isolated from soil.](image2)

![Fig. 3: Microscopic view of fungal mycelium isolated from soil.](image3)
The secondary metabolite producing fungal colonies isolated from soil were identified to be *Aspergillus ochraceous and Aspergillus niger*. From cheese culture two such colonies were isolated and were identified as *Aspergillus parasiticus and Aspergillus niger*. Fungal colony isolated from khova was identified as *Penicillium citrinum*.

When the fungal colonies were inoculated into milk and whey containing media some colonies showed immense growth while the others did not. Only such colonies were selected for terpenoid detection which showed flourished growth in the presence of *Lactobacillus* sps.
The terpenoid detection test was done for all the filtrates but only two strains namely *Aspergillus ochraceous* and *Penicillium citinum* showed the presence of terpenoids.

**DISCUSSION**

Antagonism amongst soil microflora is a common phenomenon. This is supposedly one of the very few works done about the concept of using antagonism amongst microorganisms for the production of secondary metabolites. Presence of terpenes in any filtrate demonstrate the ability of any fungal strain to produce metabolites that can inhibit certain microorganisms to some extent and can be used as possible drug targets.

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**REFERENCES**


