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Research Paper

A Review On chemical and Molecular Characterization of Keratinophilic Fungi

Microbiology

KEYWORDS : Keratinophilic fungi, mycoses, keratinase, RAPD, RFLP, AFLP, restriction enzyme. MALDITOF-MS.

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ABSTRACT

Keratinophilic fungi is a group of fungi which can degrade keratinous materials and cause mycoses in both humans and animals. For identification keratinophilic fungi are isolated from soil using hair baiting technique. Keratinophilic fungi can be identified by chemical or molecular methods. In chemical identification keratinophilic fungi are identified by degradation of keratinous materials, screening of dyes and production of keratinase or amylase. Nucleic acid amplification or PCR based procedure such as RFLP, RAPD and AFLP require the use of specific primer and restriction enzyme. Specific primer sequence is designed and annealed for identification of keratinophilic fungi. MALDITOF-MS is reported as the rapid and revolutionary technique that candetect highly abundant proteins in a mass range between 2 and 20 kDa. These identification techniques more specific and less time consuming than morphological identification.c

INTRODUCTION

Soil is the main reservoir and source for occurrence of different types of pathogenic microorganism such as fungi and bacteria. The fungi and bacteria may be pathogenic to both humans and animals. Soil is also natural habitat of keratinophilic fungi known to degrade keratinous material. Keratinophilic fungi degrade hard keratin into components of low molecular weight. In humans and animals,many keratinophilic fungi frequently parasitize keratinous tissues, such as skin, nails and hair. Recently, many researchers are paying considerable attention on dermatophytes and related keratinophilic fungi.Keratinophilic fungi are reported in different countries such as India, Saudi Arabia, Pakistan, Australia, Korea, Iran, Egypt, Iraq and Nigeria.Prevalence of keratinophilic fungi in soil require suitable climatic condition of soil and environment. Soil is a good source for occurrence of keratinophilic fungi and the probability of the incidence of such fungi increases manifold if it is rich in keratinous materials**. (Marchisio VF 2000)**

Keratinophilic fungi are present in the environment with variable distribution patterns which may depend on presence of human or animal. Keratinophilic fungi cycle one of the most abundant and highly stable animal proteins on earth**(Gugnani*et al*2012, Sharma and Rajak 2003).**Some of keratinophilic fungi share some morphological features to dermatophytes. The dermatophytes and keratinophilic fungi belong to three genera: ***Microsporum, Trichophyton*and *Epidermophyton*.**The dermatophytesspecies are divided into three categories according to their natural habitats. Anthropophilic, in which human beings are the natural hosts: Zoophilic, who use variety of animals as natural hosts; Geophilic, whose natural habitat is soil.

Keratins are insoluble proteins found in hair, hooves, feathers and nails which are composed of tightly packed α –helix or β -sheet and are poorly biodegradable**(Cohlberg 1993).**The stability of keratin also depends on amino acid composition and molecular conformation their intermolecular disulphide bond cross-linking**(Karthikeyan*et al* 2007).**Keratinases are a group of enzymes which has proteolytic ability to hydrolyze insoluble protein keratin more efficiently than other proteases**.**Keratinases have also been defined as proteolytic enzymes that catalyse the cleavage and hydrolysis of keratins **(The American Heritage Medical Dictionary2007).**

The prevalence of keratinophilic fungi depends on different factors, such as the presence of animals, humans, organic matters, dissolved oxygen concentrations,creatinine contents in the soil and alsoenvironmental factors such as temperature, pH, and geographical location**(Deshmukh and Verekar 2006).**

Keratinophilic fungi cause some mycoses in animals and humans beings. For diagnosis, Identification of keratinophilic fungi is a necessary task for treatment of mycoses. Chemical and Biochemical identification techniques for keratinophilic fungi are mainly based on keratin destruction. For identification,keratinophilic fungi are cultured on Sabouraud's dextrose agar mediumcontaining keratinous materials such as keratin powder, hair, nails and feathers with keratin meal medium. If keratin material is degraded, fungi can be identified as keratinophilic fungi. Sensitivity to different environmental conditions and production or utilization of enzymes can be a key for identification of kerainophilic fungi.

The application of nucleic acid amplification procedures such as PCR in recent years has greatly enhanced phylogenic determination and identification ofdermatophyticfungi. The nucleic acid identification is based on the identification of a particular sequence in the gene of the organism to be tested.

In molecular identification a particular location in the genome or enzyme producing gene such as amylase producing gene are identified by comparing to the standard or marker gene sequence. Some additional manipulations such as polymorphism, sequence amplification,restriction endonuclease digestion, hybridization, and electrophoresis are also required in molecular identification. The different identification techniques include PCR and its types, RNA sequencing, MALDI-TOF, RAPD, RFLP and several other molecular identification techniques.

ISOLATION OF KERATINOPHILIC FUNGI

Keratinophilicfungi are isolated by Hair Baiting technique and spread plate method on Saboraud's Dextrose Agar media.

Vanbreuseghem's Hair Bait Technique -The kratinophilicfungi can be isolated from soil by implanting hair due to their keratinolytic nature. The'hair baiting' technique initially developed by Belgian mycologist **R Vanbreuseghem**in1952. Sincethen, number of studies have been conducted in different countries for isolation of keratinophilic fungi from soil.

Hair Baiting: Soil samples from different places is collected.For baiting sterile petri dishes are half filled with soil samples. Then sterilized defatted human or animal hair is spreaded over the surface of soil. Sterile distilled water is then added to soil and incubated at room temperature for about 4 weeks in the dark. The growth found after incubation is cultured on Sabouraud's dextrose agar medium.

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CHEMICAL IDENTIFICATION OF KERATINOPHILIC FUNGI

Identification of keratinophilic fungi is needed for isolation of particular fungal species. Chemical identification techniquesare easy to perform and are used when morphological identification is not sufficient. Several researchers prefer chemical identification due to low cost and good results. Keratinophilic fungi are identified by analyzing the degradation of keratinous material (feather or hair). During degradation process keratinase enzyme is produced. **Ugohand Ijigbade(2010)** studied on production and characterization of keratinase produced during degradation of feather.Keratinophilic fungi can also be screened for keratinase activity by inoculating in milk agar plates containing peptones, yeast extract, sterile nonfat milk.The clear zone in the colony show the positive result of proteolytic activity**(Savitha*et al* 2007)**. Several substrates other than skim milk such as casein, hair, peptone, skim milk powder and woolcan also be used as carbon or nitrogen source for keratinase production.

Iyer and Rao (2001) observed amino acid profile of keratinophilic fungi for identification.Chromatography techniques can be used for screening of proteinase enzymes. Chemical identification of keratinophilic fungi is also based on effect of different conditions such as pH, and thermotolerance. Chemical identification is perfect where molecular identification is not possible.

FUNGAL IDENTIFICATION BY KERATIN DEGRADATION TEST

Detection of Keratinolytic Activity

Identifying whether a fungus is keratinolytic is quite easy if one is employing hair bait method for isolation. This method of assessing keratin utilization is based on procedures for the assessment of cellulolytic activitydemonstrated by **Thorn (1993)** and chitin degradation of **UntereinerandMalloch(1999)**.In this method Keratin azure tubes are inoculated from actively growingcultures on kerratinophilic fungi and incubated at 21^0C in darkness and for 6 weeks. Degradation of keratin is inferred from the release ofazure dye into Broth Medium at lower layer. Assays are scored by direct visual examination inartificial daylight by comparison of an inoculated tubewith anuninoculated reference tube **(Scott and. Untereiner 2004)**

Screening for Keratinolytic activity

The isolates are screened for keratinase production based on the method of **Wawrzkiewicz*et al*(1987)**usingsolid mineral media. For the preparation of the media, standard keratin powder as a keratin source isadded to the sterile agar medium with fungal culture. Keratinolytic activity of the isolates is detected as a clear zone around the colony afterincubation at 25^0C for 5 days. The diameter of the clear zone is measured to quantify the enzymeactivity**(Narula and Sareen 2011)**

Keratin degradation test using feather

In this method sterilized feathersare incubated with loop full of fungal spores onSabouraud dextrose agar plate for 7 days. For detection of feather degradation the tubes are observed regularly. Control tubes can also be maintained to see the difference in results (**Riffel and Brandelli 2002)**

Keratinase production Test

Keratinase activity is measured by the method described by **Dozie*et al*(1994).**The reaction mixture for feather degradation is incubated for 2 hour and after incubation, Feather and other insoluble residues are then removed by filtration. For comparison of results one control tube is prepared. The Proteolytic products in the supernatant are determined by reading at 280nm against controls using UV-Visible spectrophotometer. **Kumar and Kushwaha (2014)** analyzedthe results in such a way that increase of 0.01 in the absorbance is considered as equivalents to 1 unit of enzyme activity per ml.The protein concentration is measured by theFolin phenol reagent method.

Identification by Production of Amylase

Amylase potentiality of fungal isolates is determined by inoculating in medium containingsoluble starch, peptone, ammonium sulphate,magnesium sulphateheptahydrate, potassium dihydrogen phosphate, and calcium chloride and employed as the substrate for fungalisolates. Spore suspension is asepticallyintroduced into each tube of fermentation medium. Amylase is then extracted and amylase production is assayed by reading absorbance using spectrophotometer **(Sakthi*et al* 2012)**.

Identification by Ink Blue

In this Method Ink Blue is incorporatedinto a medium containing glucose Mycological Peptoneand Agar and incubated with keratinophilic and non-keratinophilic fungi.After one week of incubation at room temperature the presence or absenceof discoloration in the agar is recorded as a halo around the colony and measurements made, over an illuminated source. The dye used need to be completely non-toxic to both fungal growth and spore production**(M. Baxter 1965)**.

Thermo tolerance studies

Thermo tolerance of the test isolates was determined by incubating them onSDA and PDA media (supplemented with chloramphenicol in SDA and PDA media) at 28°C, 37°C, 40°C &45°C.

MOLECULAR CHARACTERIZATION OF KERATINOPHILIC FUNGI

Often different dermatophytes species produce similar symptoms which are difficult to distinguish through clinical examination. Consequently characterization by microscopy and in-vitro culture is required for appropriate diagnosis and treatment as well as epidemiological prevention of the infection. Microscopy is a rapid screening for mycoticidentification, but it is non-specific and show low detection frequency of fungal elements. In-vitro culture is sensitive and specific; however, the long incubation period represents a significant drawback for rapid diagnosis and treatment **(Liu *et al 2002)***.

The PCR based nucleic acid amplification procedures such arbitrary primed PCR (AP-PCR) amplification techniques can rapidly distinguish dermatophytes and keratinophilic fungi species examined through the generation of characteristic band patterns. Development of species-specific primers and probes for individual dermatophytes are more practical and precise method for molecular detection**(Pakshir*et al*2013).**

Molecular markers play important role for identification of particular specie. Molecular markers are either PCR based or non PCR based methods. Molecular markers include Restriction Fragment length Polymorphism (RFLP), Random Amplified Polymorphic DNA

(RAPD), Amplified Fragment length Polymorphism (AFLP), Simple Sequence Repeats (SSR), Inter Simple Sequence Repeats (ISSR), and Single Nucleotide Polymorphism (SNP) etc.Molecular markers are also known as DNA markers**.** DNA markers are sequence that is readily detected and whose inheritance can be easily monitored. **(Singh *et al* 2013)**

For molecular identification, similarity and phylogeny of internal transcribed spacer (ITS) sequences of two related species is also studied**(Woodgyer 2004).** These ITS regions of ribosomal DNA are used as primer and amplified.For isolation of a particular ITS regions specific restriction enzymes are used.Location of a specific gene in the genome is also used for molecular identification. For example *Squaleneepoxidase* is responsible for ergosterol synthesis which influences permeability, activation of enzymes and phothooxidation . The ergosterol enzyme is also detected in *T. rubrum***(Obsorne*et al* 2005).** For PCR reaction specific primers are designed and the primers used for PCR are ITS1, ITS2

and ITS4 which amplify the variable sequence surrounding the small or large coding subunits. For confirmation of results nucleotide sequence of DNA are compared with the counter parts in gene data bank (NCBI, NIH).

Molecular identification of keratinophilic fungi can also be done by purification of keratinase. Keratinase is separated by SDS-PAGE technique. The prtotein separation by SDS-PAGE is based on molecular weight of the compounds. In this technique polyacrylamide gel is used for separation. When electric field is applied, keratinase is separted according to molecular weight. For identification, the gel is stained with coomassie blue for 2 hour. **(El-Gayar*et al* 2012)**

Keratinophilic fungi is isolated from soil using hair baiting technique and cultured on SDA agar medium. High molecular weight DNA from fungiisisolated by method given by **Choi *et al*(1990*)*.**For extraction of genomic DNA from keratinophilic fungi, colony is isolated from Sabouraud Dextrose agar using a sterile tooth pick, to this colony cell lysis buffer, containing: Tris-HCl, EDTA, SDS, and proteinase, is added which results in disruption of mycelia. The suspension is then incubated at 65°C for 1 hour and the cellular debris is removed by centrifugation. DNA is extracted by adding phenol-chloroform-isoamyl alcohol and then chloroform-isoamyl alcohol.The DNA is then precipitated by addition of an equal volume of ethanol and resuspended in distilled water.

Identification by PCR Using Molecular Techniques:

The genomic DNA is analyzed by PCR with standard protocol using synthetic oligonucleotide primers. In PCR reaction mixture Primer,10X PCR buffer + MgCl2, dNTP, Taq DNA polymerase and template (genomic DNA) are added to a fix volume.Amplification of a appropriate part of gene with the PCR program is employed by denaturation, annealing and renaturation, while the program is repeated 36 cycles. Then electrophoresis is performed for analysis of PCR products through agarose gel matrix. Gel electrophoresis is used in clinical chemistry to separate proteins by charge and/or size to separate a mixed population of DNA and RNA fragments by length.**(Kryndushkin*et al* 2003)**. After electrophoresis the product is isolated and purification for screening. For screening the product or band is compared to the original strain as a marker. After comparing the isolated strain can be identified **(Motavaze*et al*.2008)**

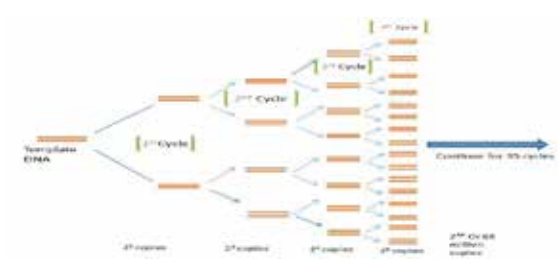

Fig. 1: PCR amplification Process

Random amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP) are PCR based method. For Identification by RAPDspecific primer is selected and mixed with PCR reaction mixture in a thermocycler for amplicfication. After amplification, PCR product is electrophoresed and purified. The purified product is then seen under UV light. In RFLP analysis specific primers are constructed and amplified by PCR in PCR reaction mixture. After amplification, the DNA products are analysed by gel electrophoresis and DNA is purified and centrifuged. The pellet contain the DNA sample, is digested with restriction endonuclease enzyme. The DNA fragment is electrophoresed and purified. The purified DNA sample is stained with dye and seen under UV light for identification of sample DNA **(Pereira *et al* 2005)**.

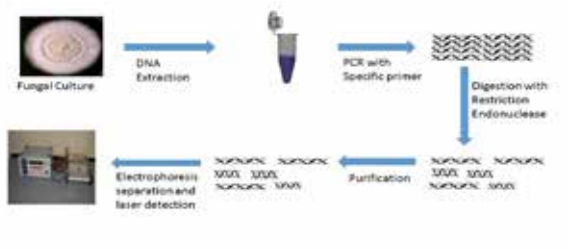

Fig. 2: Molecular Characterization of fungal DNA

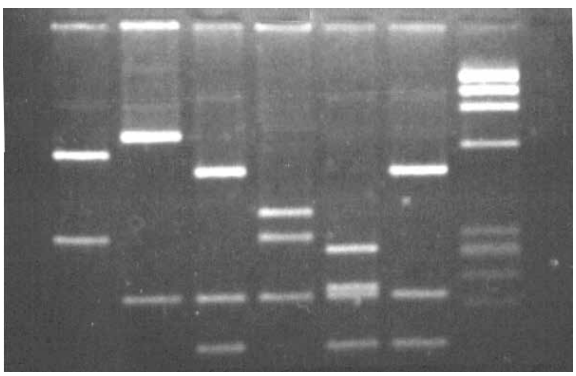

Fig. 3: Gel Electrophoresis

MALDI-TOF-mass spectrometry

Matrix Assisted laser desorption ionization time-of-flight was introduced in 1980 by Karas and Coworkers. Identification of dermatophtes by MALDI-TOF is reported by **Erhard *et al*(2008)**.MALDI-TOF is generally used for rapid identification of microorganisms. This technique is used to detect highly abundant proteins in a mass range between 2 and 20 kDa. The majoradvantage of mass spectral over genetical or morphological procedures is that it is rapid and sample preparation procedure is very simple and straightforward required for analysis.For identification of microorganisms by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDITOF-MS) is a revolutionary technique,that is increasingly used in molecular and microbiology laboratories. Microorganisms can be identified based on their proteins. Identification of keratinophilic fungi is based on morphological structure and also supported by genomic sequence comparison by MALDITOF-MS. Using a software programme the spectra is then compared to a database data of the reference strain. Then all the related fungal strains are identified. By testing colonies, it only takes a few minutes to obtain a precise identification, which results in species, subspecies and strain levels identificationof microorganisms.

In MALDI-MS, the sample to be analysed is mixed with a matrix compound solutionin crystalline structure and deposited on a sample support. The cocrystals are irradiatedwith a nanosecond laser beam such as ultraviolet (UV) laser which causesstructural decomposition of the irradiated crystaland generates a particle cloud (the plume) fromwhich ions are extracted by an electric field. This results in the disintegration of the crystal. The ions drift through afield-free path after accelerationthrough the electric field, and then reach the detector. Ion masses (mass-to-charge ratios [m/z]) are calculated by measuring their TOF. Ion masses are used as numerical data for directprocessing and subsequent analysis.

Volume : 4 | Issue : 1 | January 2015 • ISSN No 2277 - 8179

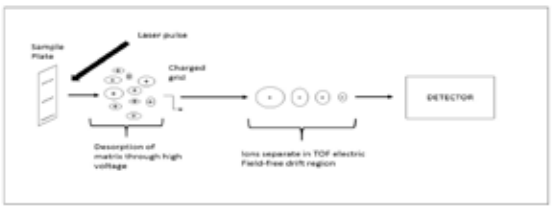

Fig. 4: MALDI-TOF Detection

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